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

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1    **Long-term preservation of Potato leafroll virus, Potato virus S and Potato**  
2    **spindle tuber viroid in cryopreserved shoot tips**

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20    Running title: Cryopreservation of viruses and viroids  
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## Abstract

Availability of and easy access to diverse viruses and viroids are a prerequisite in applied and basic studies related with virus and viroids. Plant viruses and viroids are obligate intracellular parasites that colonize only inside the living cells of the hosts, and long-term preservation of the virus and viroids is difficult. A protocol was described for long-term preservation of Potato leafroll virus, Potato virus S and Potato spindle tuber viroids in cryopreserved shoot tips of potato. Shoot regrowth levels following cryopreservation were higher (58-60%) in 1.5 mm-shoot tips than those (30-38%) in 0.5 mm-ones. 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 during after 4 times (12 weeks) of subculture following cryopreservation, similar efficiencies were obtained after 6 times (16 weeks) of subculture in pathogen-preserved shoots recovered from cryopreservation, compared with the diseased *in vitro* stock shoots (the control). Similar patterns of the concentrations of the three pathogens-preserved shoots by RT-qPCR were similar to those of shoot micropropagation. The three pathogens cryopreserved in shoot tips were readily transmitted by grafting and mechanical inoculation to the potato hosts. PLRV, PVS and PSTVD represent a diverse range of plant viruses and viroids in terms of taxonomy and infectious ability. Therefore, shoot tip cryopreservation opens a new avenue for long-term preservation of the virus and viroids.

**Keywords:** cryopreservation; potato; shoot tips; virus, viroids

## Introduction

Serological methods have long been used for plant virus detection (Hull, 2002). Antigen preparation is necessary in virus detection by serological methods.

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4 52 Pathogen-derived resistance (PDR) has been used in genetic transformation to  
5 53 produce virus-resistant plants (Sudarshana et al., 2007). In the past several decades,  
6 54 plant-based production of vaccines provided a new strategy for the manufacture of  
7 55 vaccines for the prevention and treatment of human diseases (Salazar-González et al.,  
8 56 2015; Loh et al., 2017). Recent studies have shown plant viruses have potential  
9 57 applications to nanotechnology to produce nanodrugs (Lomonossoff and Evans, 2011;  
10 58 Yang et al., 2018). Availability of and easy access to diverse viruses is prerequisite in  
11 59 these applied studies and basic researches such as origin and evolution of viruses and  
12 60 viroids (Hull, 2002; Di Serio et al., 2017).

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20 61 Plant viruses and viroids are obligate intracellular parasites that replicate only  
21 62 inside the living cells of the hosts by using the host's biochemical machinery (Hull,  
22 63 2002; Flores et al., 2017). Since viruses and viroids do not capture or store free energy  
23 64 and therefore cannot live without living tissues (Hull, 2002; Flores et al., 2017).  
24 65 Preservation of viruses and viroids has long received interests of scientists working on  
25 66 virus- and viroids-related fields. So far, no information is available on long-term  
26 67 preservation of viroids. Over the several past decades, various strategies have been  
27 68 developed for preservation of plant viruses, including freeze (Fukumoto and  
28 69 Tochinaru, 1998), freeze-drying (Hollings and Stone, 1970; Purcifull, 1975;  
29 70 Fukumoto and Tochinaru, 1998; Yordanova et al., 2000), dehydration by physical  
30 71 drying (Grivell et al., 1971) and chemical drying (Mckinney et al., 1965), and *in vitro*  
31 72 culture (Chen et al., 2003; Infante et al., 2008), among which freeze-drying was the  
32 73 most widely and reliable method. With this method, although Cucumber mosaic virus  
33 74 could be preserved for up to 240 days, their infection efficiency rapidly decreased as  
34 75 preservation time increased, with only 7% infection frequency maintained after 240  
35 76 day of preservation (Yordanova et al., 2000).

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50 77 De and Suda-Bachmann (1979) reported Potato virus Y (PVY) and Watermelon  
51 78 mosaic virus (WMMV) contained in leaf powder could be cryopreserved in LN for 22  
52 79 months for the former and 32 months for the latter, without any decreases in  
53 80 infectivity of the viruses. Recently, Fan et al. (2014) reported preservation of viral  
54 81 genomes in 700-y-old caribou feces from a subarctic ice patch. These data indicate

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4 82 cryopreservation of virus seems a very promising long-term preservation method for  
5 83 plant viruses and viroids.

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7 84 Cryopreservation, i.e. storage of living cells, tissues and organs in extra low  
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9 85 temperatures, usually that of the liquid nitrogen (LN), is at present time considered an  
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11 86 ideal means for long-term preservation of plant genetic resources. Since Sakai (1960)  
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13 87 reported for the first time successful plant cryopreservation, this technique has been  
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15 88 widely applied to almost all economically important agricultural crops (Wang et al.,  
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17 89 2009a; Feng et al., 2011; Vollmer et al., 2017), horticultural plants (Höfera 2015;  
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19 90 Wang et al., 2018a) and forest trees (Li et al., 2017). Recently, cryobanks have been  
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21 91 established for some vegetatively propagated crops such as potato at International  
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23 92 Potato Center (CIP) in Peru (Vollmer et al., 2017), apple at the Julius Kühn-Institute  
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25 93 for Breeding Research on Fruit Crops in Germany (Höfer 2015) and garlic at National  
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27 94 Agrobiodiversity Center in South Korea (Kim et al., 2012).

28 95 Shoot tip cryopreservation has been shown to efficiently eradicate plant pathogens  
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30 96 including viruses (Wang and Valkonen, 2009a; Wang et al., 2009b; 2014a). Although  
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32 97 shoot tip cryopreservation produced much higher frequencies of pathogen eradication  
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34 98 than the traditional methods like meristem culture, pathogen eradication frequencies  
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36 99 varied with types of pathogens and plants (Brison et al., 1997; Helliott et al., 2002;  
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38 100 Wang et al., 2003, 2006; Li et al., 2016), as well as infection status and combinations  
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40 101 of viruses and hosts (Wang and Valkonen, 2008; Li et al., 2016; Kushnarenko et al.,  
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42 102 2017). Frequently, not all plants recovered from shoot tip cryopreservation were  
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44 103 pathogen-free and a certain proportions of the recovered plants were still pathogen-  
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46 104 infected, i.e. pathogen-preserved (Wang et al., 2009b, 2014a). Furthermore, shoot tip  
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48 105 cryopreservation completely failed to eradicate viruses and viroids that can infect  
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50 106 meristematic cells of the shoot tips, such as Raspberry bushy dwarf virus (RBDV,  
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52 107 Wang et al., 2008), Apple stem grooving virus (ASGV, Li et al., 2016), Potato spindle  
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54 108 tuber viroids (PSTVd, Bai et al., 2012) and Chrysanthemum stunt viroids (CSVd, Zhang  
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56 109 et al., 2014). These data indicate that shoot tip cryopreservation may be used for long-  
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111 110 term preservation of the viruses and viroids.

57 The present study attempted to cryopreserve Potato leafroll virus (PLRV),  
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4 112 Potato virus S (PVS) and PSTVd, three major pathogens attacking potato and widely  
5 113 present in potato-growing regions of the world, in shoot tips of potato 'Zihuabai'.  
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7 114 Concentrations of the cryopreserved pathogens in the pathogen-preserved shoots were  
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9 115 quantitatively analyzed by RT-pPCR. The cryopreserved pathogens were tested for  
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11 116 their infectious ability to infect the healthy potato hosts by grafting and mechanical  
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13 117 inoculation.

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## 15 16 119 **Results**

### 17 18 120 **Effects of size of shoot tips on shoot regrowth and pathogen preservation**

#### 19 20 121 **following cryopreservation**

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22 122 Sizes of shoot tips significantly affected shoot regrowth levels in cryopreserved shoot  
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24 123 tips. Larger shoot tips (1.5 mm) produced significantly higher shoot regrowth levels (52-  
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26 124 60%) than small ones (0.5 mm, 30-38%) among the three pathogen-infected shoots  
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28 125 (Table 2). No significant differences were found in shoot regrowth levels produced in  
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30 126 the same sizes of shoot tips of shoots infected with different pathogens (Table 1). For  
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32 127 PLRV, cryopreservation of 0.5 mm shoot tips produced no virus-preserved shoots and  
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34 128 1.5 mm shoot tips resulted in production of 35% of virus-preserved shoots (Table 2). For  
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36 129 PVS and PSTVd, 100% of shoots recovered from cryopreservation were pathogen-  
37  
38 130 preserved, regardless of their sizes of shoot tips (Table 2).

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#### 40 132 **Detection of PLRV, PVS and PSTVd by RT-PCR**

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42 133 At the beginning of the cryopreservation experiments, specific bands of 155 bp for  
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44 134 PLRV, 137 bp for PVS and 224 bp for PSTVd were detected in all *in vitro* stock  
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46 135 shoots infected with the corresponding pathogens, whereas no such bands were found  
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48 136 in the healthy ones (Fig. 1A), thus ensuring sanitary status of the *in vitro* stock shoots  
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50 137 used in this study. When RT-PCR was applied to detection of sanitary status in shoot  
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52 138 recovered from cryopreservation after 6 times (18 weeks) of subculture, specific  
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54 139 bands of about 155 bp for PLRV, 137 bp for PVS and 224 bp for SPTVd were  
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56 140 detected in PLRV-, PSV and PSTVd-preserved shoots, respectively (Fig. 1A). For

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4 142 PLRV, the results of virus detection were identical in shoots recovered from  
5 143 cryopreservation after 2 times (6 weeks) of post-culture and in plants grown in soil in  
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7 144 the net-proof greenhouse for 3 months  
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11 146 **Quantitative analysis by qRT-PCR of relative mRNA expression of PLRV, PVS**  
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13 147 **and PSTVd in pathogen-preserved shoots recovered from cryopreservation**

14 148 Stable and similar values were obtained in the virus- and viroid-infected shoots  
15  
16 149 recovered from cryopreservation when the reference gene *EF-1a* was used (Table 3),  
17  
18 150 indicating the RT-qPCR method used here was reliable. Patterns of relative mRNA  
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20 151 expression levels of virus and viroid were similar in the three pathogen-preserved  
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22 152 shoots recovered from cryopreservation and subcultured for different times (Table 3).  
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24 153 The relative mRNA expression levels were low in the pathogen-preserved shoots after  
25 154 the 2 times (6 weeks) of subculture, significantly increased as subculture times  
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27 155 increased and reached similar levels after 6 times (18 weeks) of subculture, compared  
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29 156 with those of the *in vitro* diseased stock shoots without cryopreservation (Table 3).  
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33 158 **Cell survival patterns**  
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35 159 Living cells in the positive control showed dense TB-stained and well-preserved  
36 160 cytoplasm, and clearly visible nucleolus enclosed in the nucleus (Fig. 2A). Damaged  
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38 161 or dead cells in the negative control showed reduced levels of TB-stained cytoplasm  
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40 162 and the nuclei were heavily condensed (Fig. 2B). In cryopreserved shoot tips of PLRV-  
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42 163 infected shoots, surviving cells were found in the upper part of apical dome (AD) (Fig.  
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44 164 2C) and leaf primordia (LPs) 1–3 (Fig. 2D-F). Surviving cells were occasionally found  
45 165 in LP 4 (Fig. 2G). Among 20 shoot tips tested, 6 shoot tips showed this survival  
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47 166 patterns, accounting for 30%. Cells in LPs 5-6 (Fig. 2H and I), and other older tissues  
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49 167 were damaged or killed.  
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53 169 **Virus localization**  
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55 170 With the histoimmunological virus localization protocol used in the present study,  
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57 171 tissue infected with PLRV showed specific purple color reaction, while the healthy



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4 172 tissue did not show such color reaction (Fig. 3A). PLRV was not detected in AD (Fig.  
5 173 3B) and LPs 1-3 (Fig. 3B), while it was found in LP 4 and older tissues (Fig. 3B).  
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7 174 Close reviews showed PLRV was phloem-limited (Fig. 3C and D).  
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11 176 **Micropropagation of pathogen-preserved shoots recovered from**  
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13 177 **cryopreservation**

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15 178 Patterns of micropropagation of the three pathogen-preserved shoots recovered from  
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17 179 cryopreservation were similar (Table 4). Shoot length and number of nodes were  
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19 180 significantly short and few in pathogen-preserved shoots recovered from  
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21 181 cryopreservation after 4 times (12 weeks) of subculture, significantly increased as the  
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23 182 subculture times increased and reached the similar levels after 6 times of subculture  
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25 183 (18 weeks) of subculture, compared with pathogen-infected *in vitro* stock shoots  
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27 184 (Table 4).  
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31 186 Detection by RT-PCR of PLRV, PVS and PSTVd in grafting and mechanically  
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33 187 inoculated plants

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35 188 Specific bands of 155 bp for PLRV, 137 bp for PVS and 224 bp for SPTVd were  
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37 189 readily detected in the healthy rootstocks grafted with the corresponding pathogen-  
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39 190 preserved scions after 4 weeks of grafting (Fig. 1B). The same was true in the healthy  
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41 191 plants mechanically inoculated with cryopreserved PVS and PSTVd after 4 weeks of  
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43 192 mechanical inoculation (Fig. 1C).  
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46 194 **Discussion**

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48 195 Most of methods reported so far for preservation of plant viruses used dried materials  
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50 196 (Mckinney et al., 1965; Grivell et al., 1971; Hollings and Stone, 1970; Purcifull, 1975;  
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52 197 Fukumoto and Tochinara, 1998; Yordanova et al., 2000), and *in vitro* tissue culture  
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54 198 preserved viruses in living tissue (Chen et al., 2003; Infante et al., 2008). When  
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56 199 preserved in dried materials, some viruses were not stable and their infection ability  
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201 200 decreased as time durations of preservation increased (Hollings and Stone, 1970;  
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203 201 Grivell et al., 1971; Yordanova et al., 2000). For example, infection frequencies of

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4 202 CMV preserved by freeze-drying were 95% and only 7% after 15 and 240 days of  
5 203 preservation, respectively (Yordanova et al., 2000). Following preservation, the virus  
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7 204 can be transmitted only by mechanical inoculation to the target hosts (Mckinney et al.,  
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9 205 1965; Hollings and Stone, 1970; Grivell et al., 1971; De and Suda-Bachmann, 1979;  
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11 206 Yordanova et al., 2000). Since a number of plant viruses cannot be transmitted by  
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13 207 mechanical inoculation (Hull, 2002), such preservation methods largely limited  
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15 208 applications of the virus preservation. In *in vitro* culture for virus preservation, virus-  
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17 209 infected tissues have to be periodically subcultured (Chen et al., 2003; Infante et al.,  
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19 210 2008). Subculture has risks of contamination, which may result in total loss of the stored  
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21 211 materials. In addition, *in vitro culture* can be used only for medium-term virus  
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23 212 preservation.

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25 213 In the present study, PLRV, PVS and PSTVd were successfully cryopreserved in  
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27 214 living shoot tips of potato. PLRV and PVS are a type number of the genus *Polerovirus*  
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29 215 and *Potyvirus*, respectively, and attack a wide range of plant species (Valkonen, 2007).  
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31 216 PSTVd belongs to the genus *Pospiviroid* and the family Pospiviroidae (Owens et al.,  
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33 217 2012), and infects *Solanum* plants and a diverse array of ornamental species such as  
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35 218 *Chrysanthmum* and *Argyranthemum* (Owens et al., 2017). PLRV is a phloem-limited  
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37 219 virus and does not invade AD (Valkonen, 2007), which is also proven in the present  
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39 220 study, while PSTVd is present in AD of plants (Zhang et al., 2015). PVS is a difficult-to-  
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41 221 eradicate virus (Kushnarenko et al., 2017), indicating its infectious ability of shoot tips  
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43 222 is stronger than PLVS. Thus, the three pathogens studied in the present study represent a  
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45 223 wide range of viruses and viroids in terms of taxonomy and infectious ability. We  
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47 224 previously reported successful preservation of ASGV in cryopreserved shoot tips of  
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49 225 apple ‘Gala’ (Wang et al., 2018b). Gene sequencing of coat protein (CP) and  
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51 226 movement protein (MP) of ASGV genome showed that cryopreserved ASGV shared  
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53 227 99.87% nucleotide identities with shoot tip culture-preserved virus, indicating  
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55 228 cryopreserved virus is genetically stable. In addition, using the same potato cultivar  
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57 229 and the cryogenic protocol, Wang et al. (2014b) reported no polymorphic bands were  
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59 230 detected by genetic markers in the  
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61 231 plants recovered from cryopreservation, indicating the plants recovered from

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4 232 cryopreservation were genetically stable, as already proven in many plant species  
5 233 (Wang et al., 2014a, 2018a; Li et al., 2017).

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7 234 In this study, shoot tip cryopreservation produced 100% of PVS- and PSTVd-  
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9 235 preserved plants, regardless of the shoot tip sizes. However, cryopreservation of 0.5  
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11 236 mm-shoot tips completely failed to preserve PLRV, and of 1.5-mm shoot tips resulted in  
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13 237 35% of virus-preserved plants and 65% of virus-free plants. In order to understand why  
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15 238 frequencies of PLRV preservation varied with sizes of shoot tips, cell survival patterns  
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17 239 and virus localization were conducted. Results showed PLRV was not present in AD  
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19 240 and LPs 1-3, but it was readily found in LP 4 and older tissues. The majority of cells in  
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21 241 AD and some cells in LPs 1-3 survived following cryopreservation. A few cells in LP 4  
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23 242 survived in about 30% of cryopreserved shoot tips tested. This figure is almost equally  
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25 243 to 35% of the virus-preserved shoots. All the data generated above provided explanations  
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27 244 to varying frequencies of PLRV-preserved shoots recovered from cryopreservation  
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29 245 when 0.5 mm- and 1.5 mm-shoot tips were used for cryopreservation.

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31 246 When viruses and viroids are cryopreserved in shoot tips, cryopreservation  
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33 247 durations of shoot tips, shoot regeneration of cryopreserved shoot tips and  
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35 248 propagation efficiency of the recovered shoots are important factors determining  
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37 249 pathogen preservation efficiency. Once samples are stored in LN, cellular divisions  
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39 250 and metabolic processes are arrested, and theoretically, plant materials can be stored  
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41 251 for a definitive period of time (Benson, 2008). In plant preservation, a sample is  
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43 252 considered as successfully cryopreserved if it has a minimum recovery rate of  $\geq 30\%$   
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45 253 (Vollmer et al., 2017). Potato is a plant that is more vulnerable to cryopreservation,  
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47 254 and shoot regrowth levels were generally high ( $>50\%$ ) in most of the previous studies  
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49 255 (Wang et al., 2009a; Vollmer et al., 2017). Shoot regrowth levels of 58-60% were  
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51 256 obtained in the present study, which can be considered high enough for  
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53 257 cryopreservation (Vollmer et al., 2017). Previous studies showed that shoot regrowth  
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55 258 levels maintained unchanged in potato shoot tips that had been cryopreserved for up  
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57 259 to 10 years (Keller et al., 2006). In the present study, although shoot proliferation levels  
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59 260 of the pathogen-cryopreserved shoots were lower than that of the control after 4 times  
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4 262 (12 weeks) times of subculture, they reached similar levels as the control after 6 times  
5 263 (18 weeks) time of subculture. These data demonstrate pathogen-preserved shoots  
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7 264 recovered from cryopreservation can be efficiently micropropagated. In addition,  
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9 265 infectious abilities of virus- and viroids-preserved plants recovered from  
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11 266 cryopreservation were verified by grafting and mechanical inoculation to the potato  
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13 267 hosts.

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15 268 In conclusion, PLRV, PVS and PSTVd were for the first time successfully  
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17 269 cryopreserved in shoot tips. These pathogens represent a diverse range of viruses and  
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19 270 viroids in term of the infectious ability and taxonomy. Shoot tip cryopreservation  
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21 271 opens a new avenue for long-term preservation of viruses and viroids, and has  
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23 272 potential applications to studies in fields related.

## 24 25 26 274 **Materials and methods**

### 27 275 **Plant materials**

28  
29 276 Potato 'Zihuabai', a cultivar susceptible to the pathogens studied, was used in the  
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31 277 present study. *In vitro* certified healthy (virus-free) shoots and diseased shoots single-  
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33 278 infected with PLRV, PVS and PSTVd, respectively, were maintained on a basic medium  
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35 279 (BM) composed of solid half-strength Murashige and Skoog (1962) medium (MS)  
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37 280 supplemented with 30 g L<sup>-1</sup> sucrose and 7 g L<sup>-1</sup> agar (pH=5.8), according to Li et al.  
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39 281 (2013, 2018). Sanitary status of all the *in vitro* stock shoots was confirmed before  
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41 282 cryopreservation using reverse transcription-polymerase chain reaction  
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43 283 (RT-PCR), as described below. The cultures were grown at 22±2 C under a 16-h  
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45 284 photoperiod at 50 μmol m<sup>-2</sup> s<sup>-1</sup> provided by cool-white fluorescent tubes. Subculturing  
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47 285 was conducted every 3 weeks.

### 48 49 287 **Virus cryopreservation**

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51 288 Shoot tips were cryopreserved by droplet-vitrification, as described by Wang et al  
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(2013, 2014b).  
Nodal segments  
(1 cm in length),  
each containing  
an axillary bud,

ock cultures and cultured on BM under the same conditions as described for the *in vitro* stock cultures. Shoots (1–1.5 cm in length)

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4 292 developed from axillary buds after 7 days of culture and transferred to a growth  
5 293 chamber for cold-hardening in the dark at 5 °C for three weeks. Two sizes of shoot  
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7 294 tips: 0.5 mm and 1.5 mm in length containing 2-3 and 5-6 leaf primordia (LPs),  
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9 295 respectively, were excised from the cold-hardened stock shoots and precultured on  
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11 296 BM containing 0.3 M sucrose in the dark at 5 C for 3 days. Precultured shoot tips  
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13 297 were treated for 30 min with a loading solution containing 2 M glycerol and 0.4 M  
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15 298 sucrose in MS medium and then dehydrated with PVS2 (Sakai et al., 1990) at 0 °C for  
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17 299 40 min. PVS2 consisted of 30% (w/v) glycerol, 15% (w/v) dimethyl sulfoxide  
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19 300 (DMSO), 15% (w/v) ethylene glycol and 0.4 M sucrose in MS medium. Dehydrated  
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21 301 shoot tips were transferred onto 3 µL PVS2 droplets on aluminum foils, followed by a  
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23 302 direct immersion in LN for 1 h. Frozen foil strips with shoot tips were removed out  
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25 303 from LN and rapidly transferred into an unloading solution composed of MS  
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27 304 supplemented with 1.2 M sucrose at 25 °C for 20 min.  
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#### 306 **Post-culture for shoot recovery of cryopreserved shoot tips**

307 Cryopreserved shoot tips were post-cultured on a shoot recovery medium composed  
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32 308 of BM supplemented with 0.05 mg L<sup>-1</sup> GA<sub>3</sub>, for shoot regrowth. The cultures were  
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34 309 grown kept in the dark at 22 ± 2 °C for 3 days and then transferred into the light  
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36 310 condition, as used for the *in vitro* stock shoots. Shoot regrowth was defined as  
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38 311 percentage of the total number of shoot tips regenerating into normal shoots (≥5 mm)  
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40 312 6 weeks after post-culture. Subculturing was conducted every 3 weeks.  
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#### 44 314 **Detection of PLRV, PVS and PSTVd by RT-PCR**

45 315 Detection of PLRV, PVS and PSTVd was conducted in the *in vitro* stock shoots before  
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47 316 cryopreservation, to confirm their sanitary status. Detection of PLRV, PVS and  
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49 317 PSTVd was conducted again in shoots recovered from cryopreservation after 2 times  
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51 318 (6 weeks) of post-culture. For PVS and PSTVd, since all samples tested this time  
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53 319 were positive responses, and they were considered to be pathogen-preserved and used  
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55 320 for micropropagation. For PLRV, samples showing positive responses were  
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57 321 considered to be virus-preserved and used for micropropagation. Samples showing

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322 negative responses were considered to be virus-free and cultured for further growth.  
323 After 6 times (18 weeks) of post-culture, they were transferred to soil in the net-proof  
324 greenhouse condition. Their virus status was tested again after 3 months of growth.

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

### 337 **Quantitative analysis by RT-qPCR of relative mRNA expression levels of** 338 **pathogens in pathogen-preserved shoots recovered from cryopreservation**

339 Relative mRNA expression levels of the pathogens were quantitatively analyzed by RT-  
340 qPCR in the pathogen-preserved shoots recovered from cryopreservation during the 2  
341 times (6 weeks) to 6 times (18 weeks) of subculture. Total RNA extraction and cDNA  
342 reverse transcription was described as above. The RT-qPCR was performed using a  
343 CFX1000 (Bio-Rad, USA) instrument and a SYBR Premix ExTaq II Kit (Takara,  
344 Dalian, China) reagent. *EF1a* was used as reference gene (Wang et al. 2018b). All  
345 primers and the reference gene used for RT-qPCR are listed in Table 1. The relative  
346 expression levels of each gene were normalized to the expression of *EF1a*  
347 gene (Wang et al. 2018b). The relative mRNA expression levels of PLRV, PVS and  
348 PSTVd were expressed as Ct values (Chung et al., 2016).

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4 352 In order to understand why frequencies of PLRV cryopreservation varied with  
5 353 different sizes of shoot tips, cell survival patterns in cryopreserved shoot tips and virus  
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7 354 localization in the PLRV-infected *in vitro* stock shoots were conducted, according to  
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9 355 Wang et al. (2014b) and Li et al (2016), respectively. For histological observations on  
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11 356 cell survival patterns, cryopreserved shoot tips of PLRV-infected shoots were  
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13 357 collected 1 day after post-culture, fixed in formalin-acetic-alcohol (FAA) (ethanol :  
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15 358 formalin : acetic acid = 18:1:1) for 24 h, and dehydrated through an incremental  
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17 359 ethanol series (70, 85, 90, 95, and 100% ethanol). After embedding in paraffin,  
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19 360 sections (5 µm thick) were cut with a microtome (Leica 2235, Germany) and stained  
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21 361 with 0.01% toluidine blue (TB) (Sakai, 1973). The stained sections were observed  
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23 362 under a light microscope (Leica DM2000, Germany). Shoot-tips that were freshly  
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25 363 excised from stock shoots served as a positive control, while those that were freshly  
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27 364 excised, directly immersed in LN served as a negative control. Both positive and  
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29 365 negative controls received the same histological processes as described above.

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31 366 For PLRV localization, shoot tips were harvested from the *in vitro* PLRV-  
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33 367 infected stock shoots. Samples of the healthy *in vitro* shoots were used as negative  
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35 368 controls. Cross sections were obtained, as described above. Virus localization was  
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37 369 conducted, as described by Li et al. (2016). The sections were treated with phosphate  
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39 370 buffered saline (PBS) containing 4% bovine serum albumin (BSA) for  
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41 371 30 min, followed by overnight incubation at 5°C with coat protein (rabbit polyclonal  
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43 372 antibodies to PLRV) (dilution 1:500 with PBS). After washing with PBS three times,  
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45 373 the samples were incubated with alkaline phosphatase-conjugated antibodies (mouse  
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47 374 anti-rabbit monoclonal antibodies) (dilution 1:500 with PBS) for 30 min at room  
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49 375 temperature. After washing again three times with PBS, samples were stained using a  
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51 376 freshly prepared Fuchsin substrate solution. The sections were observed with a light  
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53 377 microscope (Leica DM 2235).

#### 52 53 379 **Micropropagation of pathogen-infected shoots recovered from cryopreservation**

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55 380 After the 2 times (6 weeks) of post-culture for shoot regrowth, shoots recovered from  
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57 381 cryopreservation were transferred onto BM and cultured under the same light

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4 382 conditions as used for *in vitro* stock shoots, for micropropagation. Pathogen-infected  
5 383 *in vitro* shoots without cryopreservation were used as controls. Subculturing was  
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7 384 conducted every 6 weeks. Shoot length and node number, the two major parameters  
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9 385 determining micropropagation efficiency in potato, were recorded every 2 times of  
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11 386 subculture.  
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### 16 388 **Establishment of the diseased plants recovered from cryopreservation in soil**

17 389 After 6 times (18 weeks) of subculture, plantlets with well-developed roots were  
18 390 transferred into soil and grown in the net-proof house, with regular irrigation and  
19 391 fertilization, according to practical managements. The plants were used for the  
20 392 pathogen transmission by grafting and mechanical inoculation, as described below.  
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### 27 395 **Transmission of the cryopreserved pathogens to potato hosts by grafting and** 28 396 **mechanical inoculation**

29 397 For grafting transmission, PLRV-, PVS- and PSTVd-preserved plants that recovered  
30 398 from cryopreservation and had grown for 6 weeks in soil in net-proof greenhouse  
31 399 were used as inocula materials. The healthy plants of potato ‘Zihuabai’ grown in the  
32 400 same net-proof greenhouse were used as rootstocks. Shoot segments (2.0-2.5 cm),  
33 401 each containing 2-3 well-developed leaves, were excised from middle to low parts of  
34 402 the pathogen-preserved plants and used as scions. A ‘V’ shape (approximately 0.5 cm  
35 403 in length) was cut at the base of the scions. The healthy rootstocks were decapitated  
36 404 approximately 5.0 cm above the soil. A vertical cut (approximately 0.6 cm in length)  
37 405 was made at the top of the rootstocks. Grafting was performed by inserting the ‘V’  
38 406 shape of scions into the vertical cut of rootstocks, and then parafilm was used to fix  
39 407 the graft union. Sanitary status of the rootstocks was tested by RT-PCR after 4 weeks  
40 408 of grafting, as described above.  
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51 409 For mechanical transmission, since PLRV cannot be mechanically transmitted, it  
52 410 was excluded in this experiment. PVS- and PSTVd-preserved plants that recovered  
53 411 from cryopreservation and had been grown in soil in net-proof greenhouse for 3  
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months were used  
for mechanical  
inoculation to  
transmit PVS and  
PSTVd to potato

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4 412 'Zihuabai' and *Solanum jasminoides* plants, respectively. All plants were grown in a  
5 413 net-proof greenhouse at  $22 \pm 2$  °C with 16-h photoperiod. Mechanical transmission of  
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7 414 PVS was conducted as described by Li et al. (2015). Briefly, leaves positioning  
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9 415 between 5 to 7 nodes (counting from shoot terminal downward) were taken from  
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11 416 PVS-preserved plants and ground with PBS (1 g/5 ml) contained in a plastic bag (Li  
12 417 et al., 2015). The freshly prepared virus inoculum was maintained on ice until use.  
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14 418 The first 2-3 fully-opened leaves (counting from shoot terminal downward) of the  
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16 419 inoculum plants that had been slightly dusted with carborundum were inoculated by  
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18 420 rubbing gently with a cotton-swab soaked in the virus solution. Plants inoculated with  
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20 421 the inoculation buffer served as negative controls. Inoculated plants were grown in a  
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22 422 growth chamber at 25 °C under the light conditions. Leaves were taken from the  
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24 423 inoculated plants after 4 weeks of inoculation and used for virus detection by RT-PCR,  
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26 424 as described above.

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28 425 Mechanical transformation of SPTVd was performed, according to Verhoeven et  
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30 426 al. (2010). Briefly, leaves positioning between 5 to 7 nodes (counting from shoot  
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32 427 terminal downward) were taken from PSTVd-preserved plants and ground with SPB  
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34 428 (1 g/10 ml), as described above. The freshly prepared viroid inocula were maintained  
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36 429 on ice until use. The first 2-3 fully-opened leaves (counting from shoot terminal  
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38 430 downward) of the inoculum plants were inoculated with PSTVd-contaminated razor  
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40 431 blades. Plants inoculated with PBS served as negative controls. Inoculated plants were  
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42 432 grown in a growth chamber at 25 °C under the light conditions. Leaves were taken  
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44 433 from the inoculated plants after 4 weeks of inoculation and used for viroid detection  
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46 434 by RT-PCR, as described above.

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#### 437 **Experimental design and data analysis**

438 For experiments of shoot tip cryopreservation and micropropagation, ten samples  
439 were included in each treatment of three replicates. All experiments were conducted  
440 twice. Data were presented as means with their standard errors and analyzed using one-  
441 directional ANOVA and Students' t-test. Significant differences were calculated at

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4 442 P<0.05. Twenty samples were included in histological observations on cell survival  
5 443 patterns, PLRV localization, and virus transmission by grafting and mechanical  
6 444 inoculation. Five biological replicates were used in analyses of RT-PCR and RT-  
7 445 qPCR.  
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#### **Author contribution statements:**

24 454

25  
26 455 J-W Li, M-R Wang and L Zhao: performance of experiments, data collection and  
27 456 analysis, and preparation of manuscript; H-Y Chen: assistance to performance of  
28 457 experiments; Z-H Cui: assistance to data collection and analysis; Z Zhang: valuable  
29 458 discussions; D-R Blystad: valuable discussions; Q-C Wang: chief scientist of the  
30 459 project, financial supports; experimental design and preparation of manuscript.  
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#### **Competing interests**

36 461

The authors declare no competing interests.

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#### **References**

39 464

40 465 Bai, J., Chen, X., Lu, X., Guo, H., Xin, X. and Zhang, Z. (2012) Can  
41 466 Cryopreservation Eliminate the Potato Virus X (PVX) and Potato Spindle Tuber  
42 467 Viroid (PSTVd)? *Biosci. Meth.* 3, 34-40.

43 468

44 469 Benson, E.E. (2008) Cryopreservation of phytodiversity: a critical appraisal of theory  
45 470 & practice. *Crit. Rev. Plant Sci.* 27, 141–219.

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47  
48  
49  
50  
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52  
53  
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55  
56  
57  
58  
59  
60

Chen, P., Buss, G.R., Tolin, S.A. and Veilleux, R.E. (2003) A tissue culture method for

the  
preservation  
of Soybean  
mosaic virus.  
*Plant Cell  
Tiss. Org.  
Cult.* 74, 185–

- 1  
2  
3 472 192.  
4  
5 473 Chung, B.N., Canto, T., Tenllado, F., Choi, K.S., Joa, J.H., Ahn, J.J., Kim, C.H. and  
6  
7 474 Do, K.S. (2016) The effects of high temperature on infection by Potato virus Y,  
8  
9 475 Potato virus A, and Potato leafroll virus. *Plant Pathol. J.* 32, 321-328.  
10  
11 476 De, W. and Suda-Bachmann, F. (1979) The long-term preservation of Potato virus Y  
12 477 and Watermelon mosaic virus in liquid nitrogen in comparison to other  
13 478 preservation methods. *Neth J Pl Path.* 85, 23-29.  
14  
15  
16 479 Di Serio, F., Navarro, B. and Flores, R. (2017) Origin and evolution of viroids. In:  
17 480 Hadidi A, Flores R, Randles JW, Palukaitis P. *Viroids and Satellites*. Academic  
18 481 Press, London, UK. pp. 125-134.  
19  
20 482 Fan, N.T.F., Chen, L.F., Zhou, Y.C., Shapiro, B., Stiller, M., Heintzman, P.D., Varsani,  
21 483 A., Kondov, N.O., Wong, W., Deng, X., Andrews, T.D., Moorman, B.J.,  
22 484 Meulendyk, T., MacKay, G., Gilbertson, R.L. and Delwart, E. (2014)  
23 485 Preservation of viral genomes in 700-y-old caribou feces from a subarctic ice  
24 486 patch. *Proc. Natl. Acad. Sci. USA.* 111, 16842–16847.  
25  
26 487 Feng, C.-H., Yin, Z.-F., Ma, Y.-L., Zhang, Z.-B., Chen, L., Li, B.-Q., Huang, Y.-S.,  
27 488 Wang, B. and Wang, Q.-C. (2011) Cryopreservation of sweetpotato and its  
28 489 pathogen elimination by cryotherapy. *Biotechn. Adv.* 29, 84-93.  
29  
30 490 Flores, R., Minoia, S., López-Carrasco, A., Delgado, S., Martínez de Alba, Á.-E. and  
31 491 Kalantidis, K. (2017) Viroid replication. In: Hadidi A, Flores R, Randles JW,  
32 492 Palukaitis P. *Viroids and Satellites*. Academic Press, London, UK. pp. 71-81.  
33  
34  
35 493 Fukumoto, F. and Tochinara, H. (1998) Preservation of purified Peanut stripe and  
36 494 Turnip mosaic Potyviruses by freezing and freeze-drying. *J. Phytopath.* 146, 89-  
37 495 95.  
38  
39 496 Grivell, A.R., Grivell, C.J., Jackson, J.F. and Nicholas, D.J. (1971) Preservation of  
40 497 lettuce necrotic yellows and some other plant viruses by dehydration with silica  
41 498 gel. *J. Gen. Virol.* 12, 55-58.  
42  
43 499 Hollings, M. and Stone, O.M. (1970) The long-term survival of some plant viruses  
44 500 preserved by lyophilization. *Ann Appl Biol.* 65, 411-418.  
45  
46  
47 501 Höfer, M. (2015) Cryopreservation of winter-dormant apple buds: establishment of a  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3  
4 502 duplicate collection of *Malus*, germplasm. *Plant Cell Tiss. Org. Cult.* 121, 647–  
5 503 656.
- 6  
7 504 Hull, R. (2002) Mathews' Plant Virology. Academic Press, Harcourt Place, 32  
8  
9 505 Jamstown Road, London NW1 7BY, UK.
- 10  
11 506 Infante, R., Fiore, N. and Seibert, E. (2008) Preservation of Grape fanleaf virus on  
12 507 callus culture of *Vitis vinifera* cv. Cabernet Sauvignon. *Acta Phytopath. Entomol.*  
13 508 *Hungari.* 43, 101-108.
- 14  
15  
16 509 Keller, E.R.J., Senula, A., Leunufna, S. and Grübe, M. (2006) Slow growth storage  
17 510 and cryopreservation-tolls to facilitate germplasm maintenance of vegetatively  
18 511 propagated crops in living plant collection. *Inter. J. Refri.* 29, 411-417.
- 19  
20 512 Kim, H.H., Popova, E., Shin, D.J., Yi, J.Y., Kim, C.H. and Lee, J.S. (2012)  
21 513 Cryobanking of Korean *Allium* germplasm collections: results from 10 year  
22 514 experience. *CryoLett.* 33, 45-57.
- 23  
24 515 Kushnarenko, S., Romadanova, N., Aralbayeva, M., Zholamanova, S., Alexandrova,  
25 516 A. and Karpova, O. (2017) Combined ribavirin treatment and cryotherapy for  
26 517 efficient Potato virus M and Potato virus S eradication in potato (*Solanum*  
27 518 *tuberosum* L.) *in vitro* shoots. *In Vitro Cell. Dev. Biol.-Plant.* 53, 425-4322
- 28  
29 519 Li, J.-W., Wang, B., Song, X.-M., Wang, R.-R., Zhang, H., Zhang, Z. and Wang Q.-C.  
30 520 (2013) Potato leafroll virus (PLRV) and Potato virus Y (PVY) influence  
31 521 vegetative, physiological metabolism of *in vitro*-cultured shoots of potato  
32 522 (*Solanum tuberosum* L.). *Plant Cell, Tiss. Org. Cult.* 114, 313–324
- 33  
34 523 Li, R., Baysal-Gurel, F., Abdo, Z., Miller, S.A. and Ling, K.-S. (2015) Evaluation of  
35 524 disinfectants to prevent mechanical transmission of viruses and a viroid in  
36 525 greenhouse tomato production. *Viol. J.* 12, 5. DOI 10.1186/s12985-014-0237-5
- 37  
38 526 Li, B.-Q., Feng, C.-H., Hu, L.-Y., Wang, R.-R. and Wang, Q.-C. (2016) Shoot tip  
39 527 culture and cryopreservation for eradication of *Apple stem pitting virus* (ASPV)  
40 528 and *Apple stem grooving virus* (ASGV) from apple rootstocks 'M9' and 'M26'.  
41 529 *Ann. Appl. Biol.* 168, 142-150.
- 42  
43  
44 530 Li, J.-W., Ozudogru, E.A., Li, J., Wang, M.-R., Bi, W.-L., Lambardi, M. and Wang,  
45 531 Q.-C. (2017) Cryobiotechnology of forest trees: recent advances and future  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
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3  
4 532 prospects. *Biodiv. Cons.* 27, 795–814.
- 5 533 Li, J.-W., Chen, H.-Y., Li, J., Zhang, Z., Blystad, D.-R. and Wang, Q.-C. (2018)  
6 534 Growth, microtuber production and physiological metabolism in virus-free and  
7 535 virus-infected potato *in vitro* plantlets grown under NaCl-induced salt stress. *Eur.*  
8 536 *J. Plant Path.* doi.org/10.1007/s10658-018-1485-9.
- 9 537 Loh, H.S., Green, B.J. and Yusibov, V. (2017) Using transgenic plants and modified  
10 538 plant viruses for the development of treatments for human diseases. *Curr Opin*  
11 539 *Virology* 26, 81-89.
- 12 540 Lomonosoff, G.P. and Evans, D.J. (2011) Applications of plant viruses in  
13 541 bionanotechnology. *Curr. Top. Microbiol.* 375, 61-87.
- 14 542 Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays  
15 543 with tobacco cell cultures. *Physiol. Plant.* 15, 473-497.
- 16 544 Mckinney, H.H., Silver, G. and Greeley, L.W. (1965) Longevity of some plant viruses  
17 545 stored in chemically dehydrated tissues. *Phytopath.* 55, 1043-1044.
- 18 546 Owens, R.A., Flores, R., Di Serio, F., Li, S.F., Pallas, V. and Randles, J.W. (2012)  
19 547 Viroids. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds), *Virus*  
20 548 *Taxonomy, Ninth Reports of the International Committee on Taxonomy of Viruses.*  
21 549 Elsevier/Academic Press, London, UK, pp. 1221-1234.
- 22 550 Owens, R.A. and Verhoeven, J.Th.J. (2017) Potato spindle tuber viroids. In: Hadidi A,  
23 551 Flores R, Randles JW, Palukaitis P. *Viroids and Satellites.* Academic Press,  
24 552 London, UK. pp. 149-158.
- 25 553 Purcifull, D.E. (1975) Preservation of plant virus antigens by freeze-drying.  
26 554 *Phytopath.* 65, 1202-1205.
- 27 555 Sakai, A. (1960) Survival of the twigs of woody plants at -196°C. *Nature* 185, 392-  
28 556 394.
- 29 557 Sakai, W. (1973) Simple method for differential staining of paraffin embedded plant  
30 558 material using toluidine blue O. *Stain Technol.* 48, 247–249.
- 31 559 Sakai, A., Kobayash, S. and Oiyama, I. (1990) Cryopreservation of nucellar cells of  
32 560 navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. *Plant*  
33 561 *Cell Rep.* 9, 30-33.

- 1  
2  
3  
4 562 Salazar-González, J.A., Angulo, C. and Rosales-Mendoza, S. (2015) Chikungunya  
5 563 virus vaccines: Current strategies and prospects for developing plant-made  
6 564 vaccines. *Vaccine* 33, 3650-3658.
- 7  
8  
9 565 Sudarshana, M.R., Roy, G. and Falk, B.W. (2007) Methods for engineering resistance  
10 566 to plant viruses. In: Ronald, P.C. (eds), *Plant-Pathogen Interactions*, Methods in  
11 567 Molecular Biology, vol 354. Humana Press, pp. 183-195.
- 12  
13  
14 568 Valkonen, J.P.T. (2007) Viruses: Economical losses and biotechnological potential. In:  
15 569 Vreugdenhil, D. (ed), *Potato biology and biotechnology advances and perspectives*.  
16 570 Elsevier, Amsterdam, pp. 619–641.
- 17  
18  
19  
20 571 Verhoeven, J.Th.J., Hüner, L., Marn, M.V., Plesko, I.M. and Roenhorst, J.W. (2010)  
21 572 Mechanical transmission of Potato spindle tuber viroid between plants of  
22 573 *Brugmansia suaveoles*, *Solanum jasminoides* and potatoes and tomatoes. *Eur. J.*  
23 574 *Plant Pathol.* 128, 417–421.
- 24  
25  
26  
27 575 Vollmer, R., Villagaray, R., Cárdenas, J., Castro, M., Chávez, O., Anglin, N.L. and  
28  
29 576 56 Ellis, D. (2017) A large-scale viability assessment of the potato cryobank at the  
30 591  
31 577  
32  
33 578  
34 579  
35  
36 580  
37  
38 581  
39  
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43  
44 584  
45 585  
46  
47 586  
48  
49 587  
50  
51 588  
52  
53 589  
54  
55 590

.T. (2009) Cryotherapy of shoot tips: novel pathogen eradication method. *Trend.*

*Plant Sci.* 14,119-122.

International  
Potato Center  
(CIP). *In Vitro*  
*Cell. Dev.*  
*Biol.—Plant.*  
53, 309–317.

Wang, Q.-C., Mawassi, M., Li, P., Gafny, R., Sela, I. and Tanne, E. (2003) Elimination of *Grapevine virus A* (GVA) by cryopreservation of *in vitro*-grown shoot tips of *Vitis vinifera* L. *Plant Sci.* 165, 321-327.

Wang, Q.-C., Lui, Y., Xie, L.R. and You, M.S. (2006) Efficient elimination of *Potato leafroll virus* (PLV) and *Potato virus Y* (PVY) by cryopreservation of shoot tips. *Potato Res.* 49,119-129.

Wang, Q.-C., Cuellar, W.J., Rajamaki, M.-L., Hirata, Y. and Valkonen, J.P.T. (2008). Combined thermo-therapy and cryotherapy for efficient virus eradication: relation of virus distribution, subcellular changes, cell survival and viral RNA degradation in shoot tips. *Mol. Plant Pathol.* 9, 237-250.

Wang, B., Yin, Z.-F., Feng, C.-H., Shi, X., Li, Y.-P. and Wang, Q.C. (2009a) Cryopreservation of potato shoot tips. In: Benkeblia, N. and Tennant, P. (eds),

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- 592 *Potato I. Fruit, Vegetable and Cereal Science and Biotechnology 2* (Special Issue  
593 1), Global Science Book, London, pp. 46-53.
- 594 Wang, Q.-C., Panis, B., Engelmann, F., Lambardi, M. and Valkonen, J.P.T. (2009b)  
595 Cryotherapy of shoot tips: a technique for pathogen eradication to produce  
596 healthy planting materials and prepare healthy plant genetic resources for  
597 cryopreservation. *Ann. Appl. Biol.* 154, 351-363.
- 598 Wang, B., Zhang, Z., Wang, R.-R., Ma, L.-Y. and Wang, Q.-C. (2013) Development of  
599 three vitrification-based cryopreservations of shoot tips for China's potato.  
600 *CryoLett.* 34, 369-380.
- 601 Wang, B., Wang, R.-R., Cui, Z.-H., Li J.-W., Bi, W.-L., Li, B.-Q., Ozudogru, E.-A.,  
602 Volk, G.M. and Wang, Q.-C. (2014a). Potential applications of cryobiotechnology  
603 to plant genetic transformation and pathogen eradication. *Biotechn. Adv.* 32, 583-  
604 595.
- 605 Wang, B., Li, J.-W., Zhang, Z., Wang, R.-R., Ma, Y.-L., Blystad, D.-R., Keller, E.R.J.  
606 and Wang, Q.-C. (2014b) Three vitrification-based cryopreservation procedures  
607 cause different cryo-injury to potato shoot tips while all maintain genetic integrity  
608 in regenerants. *J. Biotechn.* 84, 47-55.
- 609 Wang, M.R., Chen, L., Teixeira da Silva, J.A., Volk, G.M. and Wang, Q.-C. (2018)  
610 Cryobiotechnology of apple (*Malus* spp.): development, progress and future  
611 prospects. *Plant Cell Rep.* 37, 689-709.
- 612 Wang, M.-R., Zhao, L., Li, J., Li, J.-W., Liu, K., Yu, J.-W., Wu, Y. and Wang, Q.-C.  
613 (2018b). Cryopreservation of virus: a novel biotechnology for long-term  
614 preservation of virus in shoot tips. *Plant Meth.* (accepted).
- 615 Yang, C., Zhang, M. and Merlin, D. (2018) Advances in plant-derived edible  
616 nanoparticle based lipid nano-drug delivery systems as therapeutic  
617 nanomedicines. *J. Mater. Chem. B.* 6, 1312. DOI: 10.1039/c7tb03207b.
- 618 Yordanova, A., Stoimenova, E. and Donev, T. (2000) Prediction of the preservation of  
619 freeze-dried cucumber mosaic virus. *Biotechn Lett.* 22, 1779-1782.
- 620 Zhang, Z., Haugslie, S., Clark Liu, J.H.L., Spetz, C., Lee, Y., Sivertsen, A., Skjeseth,  
621 G., Blystad, D.-R. and Wang, Q.-C. (2014) Cryotherapy could not eradicate

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infected *Argyranthemum maderense* 'Yellow Empire'. *Acta Hortic.* 1039, 201-

208.

Zhang, Z., Lee, Y., Spetz, C., Clarke Liu, J.H., Wang, Q.-C. and Blystad, D.-R. (2015)

Invasion of shoot apical meristems by *Chrysanthemum stunt viroid* differs among

*Argyranthemum* cultivars. *Front. Plant Sci.* 6, 53. doi:10.3389/fpls.2015.00053.

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3 628 Table 1 Names, sequences and amplified bands of primers and reference used for  
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5 629 detection of PLRV, PVS and PSTVd by RT-PCR and real time RT-qPCR in potato  
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7 630 'Zihuabai'.  
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Primer name	Sequence(5'-3')	Amplified bands (bp)	Ref
PLRV-F	CCCACTGGAAGAGGGATGTAAC	155	Designed in this study
PLRV-R	CTTCGGATGCTTCCCGCTCTA		
PVS-F	CAGATGTGCCAGAGCCAAGT	137	Designed in this study
PVS-R	GCCAGACCCAGATTACCAAAA		
PSTVd-F	ATCGATGAGGAGCGCTTCAGGGATC	224	Designed in this study
PSTVd-R	GTCGACGGAGCTTCAGTTGTTTCC		
<i>EF1a-F*</i>	ATTGGAAACGGATATGCTCCA	101	Wang et al. 2018
<i>EF1a-R</i>	TCCTTACCTGAACGCCTGTCA		

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28 632 \**EF1a-F* and *EF1a-R* were used only in RT-qPCR.  
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635 **Table 2** Effects of shoot tip sizes on shoot regrowth levels and frequencies of pathogen  
636 preservation in diseased *in vitro* shoots following cryopreservation in potato ‘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 (20/20)	0 (0/20)
	1.5 mm, 5-6 LPs	60±5a	65 (13/20)	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)

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

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

640 Data of shoot regrowth are presented as means ± SE and followed by different letters indicate  
641 significant differences at  $P < 0.05$  analyzed by Student's *t*-test.  
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643 **Table 3** Relative mRNA expressions levels (Ct values) of PLRV, PVS and PSTVd  
644 analyzed by RT-qPCR in pathogen-preserved shoots recovered from cryopreservation  
645 after different times of subculture in potato ‘Zihuabai’.

Types of shoots	Subculture times after shoot regrowth		
	3	6	9
PLRV			
Virus-preserved shoots	48.2±2.7Aa	36.1±1.8Ba	24.5±0.9Ca
Infected <i>in vitro</i> stock shoots	23.1±1.1Ab	22.5±1.0Ab	22.3±0.9Aa
PVS			
Virus-preserved shoots	42.5±2.6Aa	30.3±1.5Ba	20.4±0.8Ca
Infected <i>in vitro</i> stock shoots	20.5±0.7Ab	20.8±0.9Ab	20.0±0.8Aa
PSTVd			
Viroid-preserved shoots	38.6±2.1Aa	28.4±1.8Ba	21.5±0.7Ca
Infected <i>in vitro</i> stock shoots	21.4±0.8Ab	20.5±0.7Ab	20.8±0.8Aa
Ct value of reference gene <i>EF-1α</i>	18.2±0.8	18.5±0.8	17.8±0.7

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

650 **Table 4** Micropropagation of pathogen-preserved shoots recovered from cryopreservation after  
651 different times of subculture in potato ‘Zihuabai’.

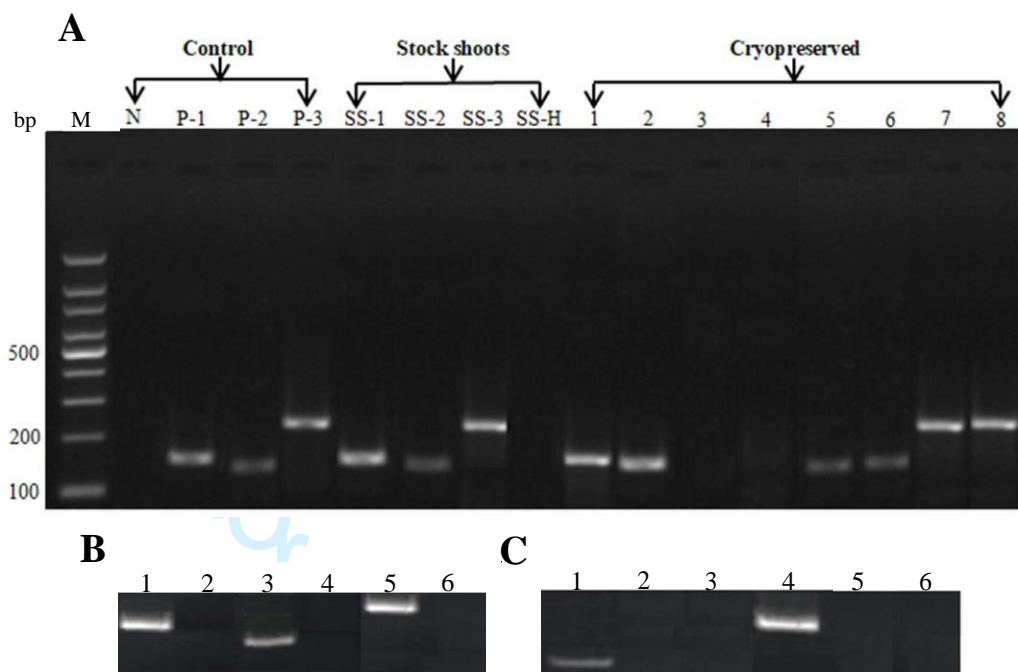
Types of shoots	Subculture times after shoot regrowth					
	2		4		6	
	Shoot length (cm)	Node number	Shoot length (cm)	Node number	Shoot length (cm)	Node number
PLRV						
Virus-preserved shoots	0.7±0.2Cb	1.5±0.2Cb	3.6±0.4Bb	4.5±0.5Bb	6.8±0.4Aa	7.4±0.7Aa
Virus-infected <i>in vitro</i> stock shoots	6.9±0.4Aa	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 <i>in vitro</i> stock shoots	6.6±0.5Aa	7.8±0.7Aa	7.2±0.7Aa	8.1±0.7Aa	7.0±0.5Aa	7.3±0.6Aa
PSTVd						
Viroid-preserved shoots	0.6±0.1Cb	1.4±0.2Cb	3.3±0.3Bb	4.0±0.4Bb	6.5±0.5Aa	7.1±0.6Aa
Viroid-infected <i>in vitro</i> 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

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

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**Figure 1** Detection by RT-PCR of potato leafroll virus (PLRV), Potato virus S (PVS) and Potato spindle tuber viroid in *in vitro* stock shoots before cryopreservation and shoots recovered after cryopreservation in potato ‘Zihuabai’ (A), in the healthy rootstocks grafted upon the virus- and virus-preserved rootstocks in potato ‘Zihuabai’ (B), and in the healthy potato ‘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;

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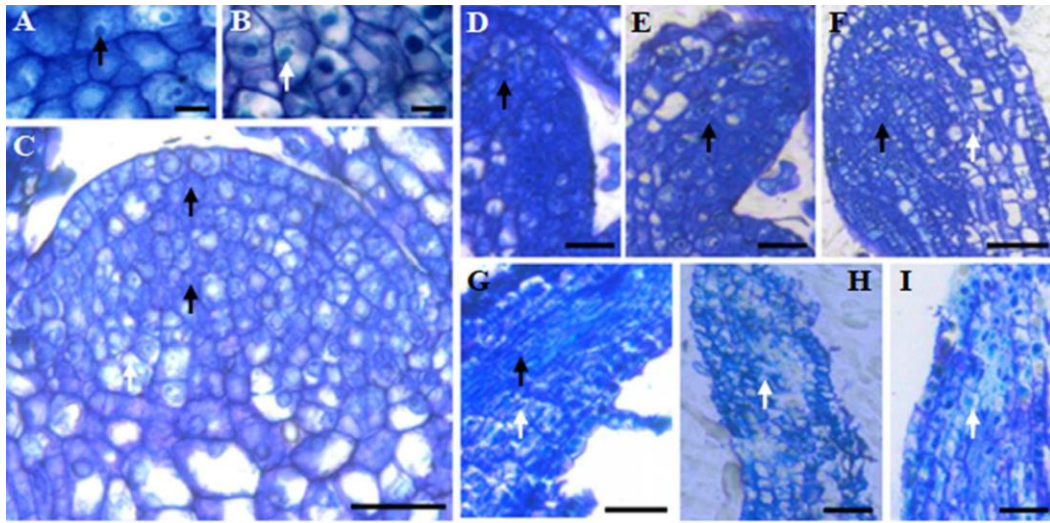
hy samples; lane 4=inoculated with PSTVd-preserved samples; lane 5=mock inoculation  
for PSTVd; lane 6=inoculated with the healthy samples.

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**Figure 2** Cell survival patterns in cryopreserved shoot tips of PLRV-infected shoots of potato '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  $\mu$ m, and in C-I=20  $\mu$ m

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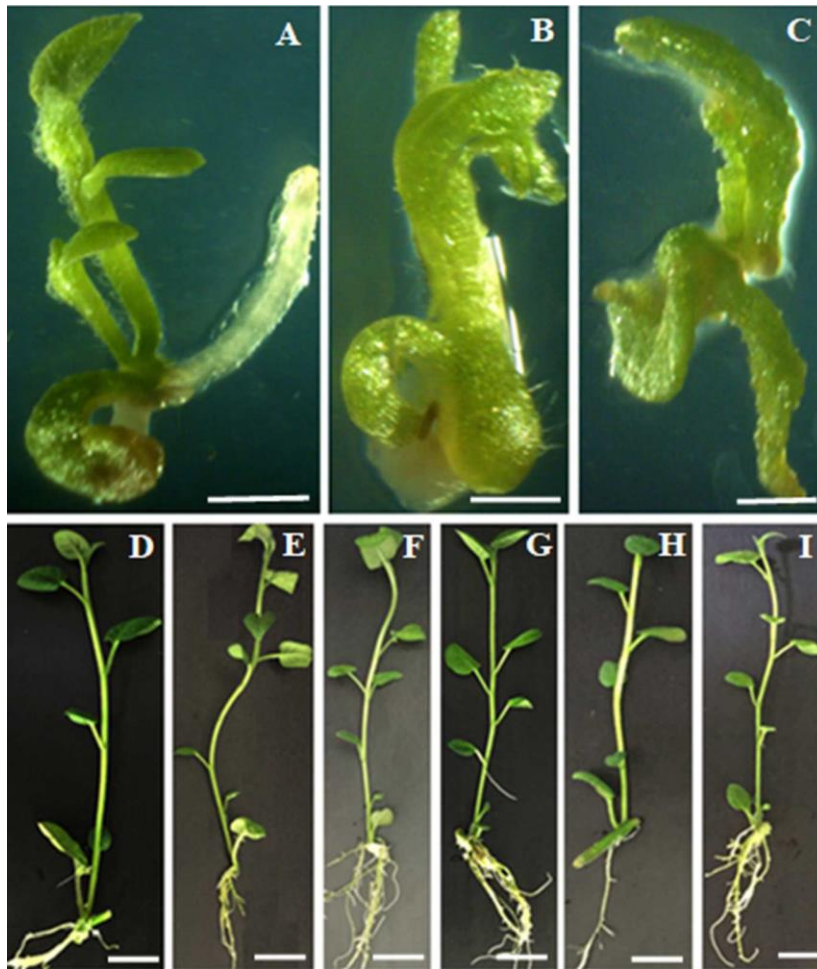
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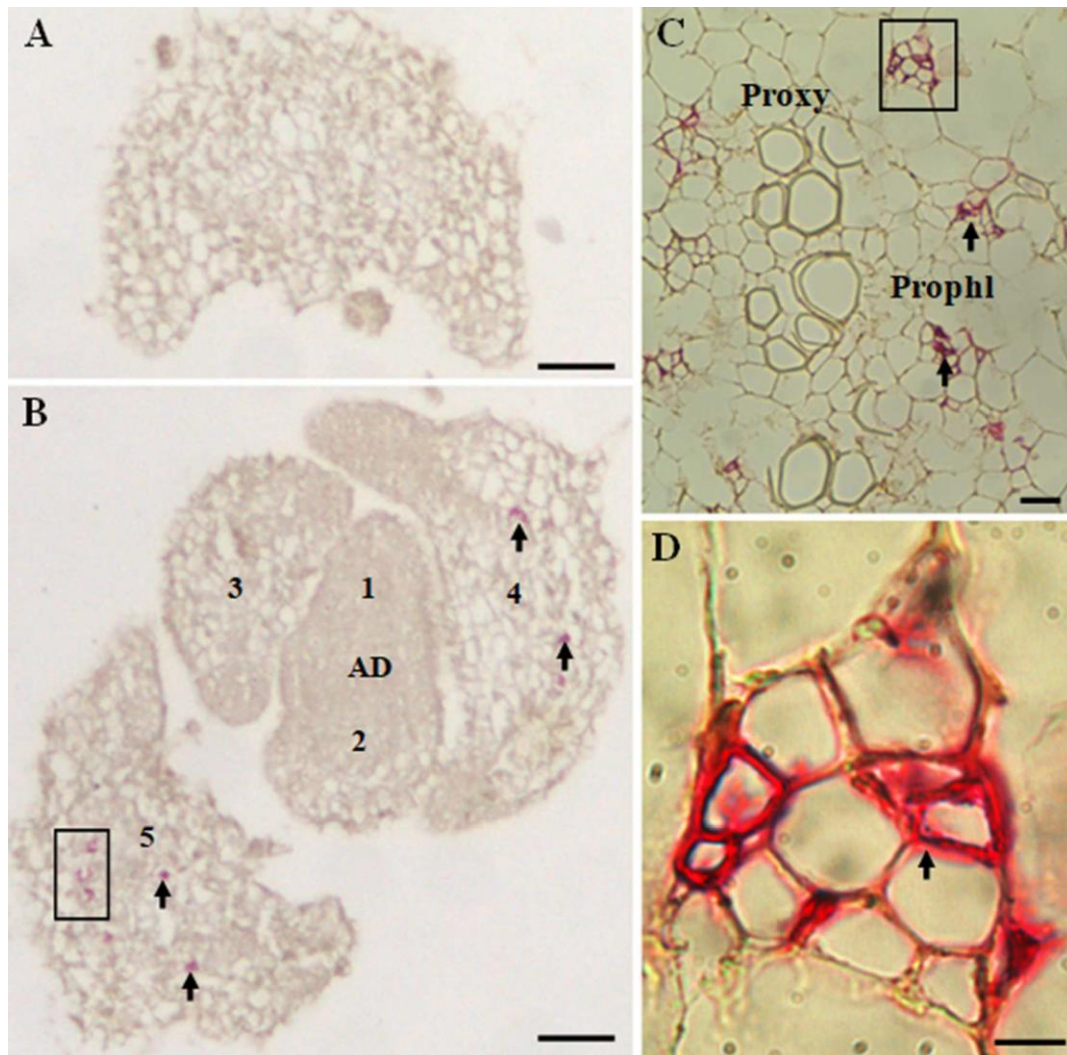
ropropagation of PLRV-, PVS- and PSTVd-cryopreserved shoots following cryopreservation in potato 'Zihuabai'. Shoot regrowth from cryopreserved shoot tips of



PLRV (A)-, PVS (B)- and PSTVd (C)-infected shoots after the 2 times (6 weeks) of subculture. Micropropagated shoots from PLRV (D)-, PVS (F)- and PSTVd (H)-cryopreserved shoots after 6 times (18 weeks) of subculture. Micropropagated shoots from *in vitro* stock shoot tips infected with PLRV(E)-, PVS (G)- and PSTVd (I)-infected shoots after 9 times (27 weeks) of subculture. Scale bars in A, B and C=0.5 cm, and in D-I=1.0 cm.



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72 **Fig 4** Histoimmunological localization of Potato leafroll virus (PLRV) in the diseased  
 73 *in vitro* shoot tips of potato 'Zihuabai'. Cross section of healthy tissues (A) Cross  
 74 section of virus-infected shoot tips (B). Close view of the black square in B (C). Close  
 75 view of the black square in C (D). PLRV-infected tissues gave purple color reaction, as  
 76 indicated by black arrows, while healthy tissues did not give such reaction. Virus  
 77 AP=apical dome; 1, 2, 3, 4 and 5=leaf primordia 1, 2, 3, 4 and 5, respectively;  
 78 Prophl=prophyloem; Proxy=proxyloem. Scale bars in A and B=50  $\mu$ m, and in C and D=  
 79 10  $\mu$ m.