Scientia Horticulturae 244 (2019) 1–9

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Cryopreservation of viroid-infected chrysanthemum shoot tips

Jing-Wei Li[a,b], Munetaka Hosokawa[b,c,*], Tomoyuki Nabeshima[b], Ko Motoki[b], Haruka Yamada[b], Qiao-Chun Wang[a,**]

[a] State Key Laboratory of Crop Stress Biology for Arid Areas, Key Laboratory of Genetic Improvement of Horticultural Crops of Northwest China, College of Horticulture, Northwest A&F University, Yangling, 712100, Shaanxi, People's Republic of China
[b] Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto, 606-8502, Japan
[c] Faculty of Agriculture, Kindai University, 3327-204, Naka machi, Nara, 631-8505, Japan

ARTICLE INFO

Keywords:
Chrysanthemum
CChMVd
Cryopreservation
CSVd
In situ hybridization
Shoot tips

ABSTRACT

Chrysanthemum stunt viroid (CSVd) and chrysanthemum chlorotic mottle viroid (CChMVd) are the two viroids most frequently infecting chrysanthemum. This study attempted to cryopreserve *in vitro* shoot tips of *Chrysanthemum morifolium* 'Piato' infected with high or low titers of CSVd, and co-infected with CSVd and CChMVd. By optimizing several key factors including the time and light regimes during cold-hardening of stock shoots, and the size of shoot tips, an encapsulation-vitrification procedure was established for cryopreservation of viroid-infected shoot tips. Viroid-infected stock shoots were cold-hardened *in vitro* at 4 °C in a 16-h photoperiod for 6 weeks. Shoot tips (1.5 mm in size) containing 3-4 LPs were excised from the cold-hardened stock shoots and subjected to encapsulation-vitrification cryopreservation. With this protocol, about 65%, 45% and 42% of shoot regrowth levels were obtained in cryopreserved shoot tips derived from *in vitro* stock shoots infected with low or high titers of CSVd, and co-infected with CSVd and CChMVd, respectively. All regenerants from cryopreservation maintained their viroid-infected status, identical to those of their *in vitro* stock shoots. Histological observations and *in situ* hybridization elucidated why cryopreservation could maintain viroids in cryopreserved shoot tips. Cryopreservation of viroid-infected plant materials has potential applications to all types of viroid-related basic and applied studies.

1. Introduction

Chrysanthemum (*Chrysanthemum morifolium*), the second most economically important ornamental crop worldwide, is mainly used as cut and potted flowers (Anderson, 2006), as medicine (China Pharmacopoeia Commission, 2010), edible flowers and important additives to many beverages (Wang et al., 2014a).

Availability of and easy access to diverse genetic resources are necessary for genetic improvements to obtain novel cultivars in both traditional and biotechnological programs (Wang et al., 2014b). Cryopreservation is at present time considered an ideal mean for the long-term conservation of plant genetic resources (Engelmann, 2011; Wang et al., 2014b; Li et al., 2017a). Since 1990s, various cryogenic procedures have been developed for cryopreservation of chrysanthemum shoot tips, including two-step cooling (Fukai, 1990), preculture-desiccation (Hitmi et al., 1999, 2000) and dimethyl sulfoxide (DMSO) droplet (Halmagyi et al., 2004), and vitrification-based methods such as encapsulation–dehydration (Sakai et al., 2000; Halmagyi et al., 2004; Martín and González-Benito, 2005; Martín et al., 2011), vitrification (Martín and González-Benito, 2005; Jeon et al., 2015) and droplet-vitrification (Halmagyi et al., 2004; Lee et al., 2011; Wang et al., 2014c; Bi et al., 2017). Plant growth, flower production, genetic stability and biochemical compounds have been assessed in the regenerants of chrysanthemum following cryopreservation (Martín and González-Benito, 2005; Martín et al., 2011; Lee et al., 2011; Wang et al., 2014c; Bi et al., 2017), and the results were quite promising. These studies made chrysanthemum one of ornamental crops most amenable to cryopreservation.

Viroids are small pathogens consisting of a small (250–400 nucleotides), nonprotein-coding, single-stranded, circular RNA that cause infectious diseases in various crops including chrysanthemum (Flores et al., 2005). Chrysanthemum stunt viroid (CSVd) and chrysanthemum chlorotic mottle viroid (CChMVd) are two viroids that infect chrysanthemum widely distributed in many of the chrysanthemum-growing countries (Liu et al., 2014; Flores et al., 2017; Palukaitis et al., 2017). CSVd is a member of the genus *Pospiviroid* in the family *Pospiviroidae*

* Corresponding author at: Faculty of Agriculture, Kindai University, 3327-204, Naka machi, Nara, 631-8505, Japan.
** Corresponding author.
E-mail addresses: mune@nara.kindai.ac.jp (M. Hosokawa), qiaochunwang@nwsuaf.edu.cn (Q.-C. Wang).

https://doi.org/10.1016/j.scienta.2018.09.004
Received 3 May 2018; Received in revised form 20 July 2018; Accepted 4 September 2018

(Palukaitis et al., 2017), while CChMVd belongs to the genus *Pelamoviroid* in the family *Avsunviroidae* (Navarro and Flores, 1997; Flores et al., 2017). CSVd and CChMVd can be transmitted by mechanical inoculation, vegetative propagation and seeds, making chrysanthemum prone to viroid infection and accumulations from generation to generation by vegetative propagation (Palukaitis et al., 2017). Field-grown chrysanthemum plants are frequently infected by viroids, often in mixed infection (Liu et al., 2014). Surveys conducted in field-grown chrysanthemum showed CChMVd infection on 20% of cultivars in Akita, Japan (Yamamoto and Sano, 2006) and 10% in Karnataka, India (Adkar-Purushothama et al., 2015a), while those of CSVd infection were 11.5% in China (Zhang et al., 2011), 9.7–66.8% in South Korea (Chung et al., 2005), and 70% in India (Singh et al., 2010). Like other plants, in shoot tip cryopreservation of chrysanthemum, the source plants are collected from the field and maintained in greenhouse conditions. Then, explants are taken from the greenhouse-maintained source plants and introduced into *in vitro* cultures to establish stock shoots, from which shoot tips are excised and used for cryopreservation (Fukai, 1990; Fukai et al., 1994; Jeon et al., 2015). In all previous studies on cryopreservation of chrysanthemum, the sanitary status of *in vitro* stock cultures was never clarified, except in the study of Jeon et al. (2016), in which cryopreservation was tested for viroid eradication. Cryopreservation of viroid-infected plant materials has potential applications to all types of viroid-related basic and applied studies (Gómez et al., 2009; Adkar-Purushothama et al., 2015b).

The present study attempted to cryopreserve shoot tips of *in vitro*-grown chrysanthemum shoots infected with low or high titers of CSVd, and co-infected with CSVd and CChMVd. Histological observations and *in situ* hybridization were conducted to investigate the mechanism making possible viroids that could be maintained in the regenerants recovered from cryopreserved shoot tips.

2. Materials and methods

2.1. Plant materials

In vitro stock shoots of *Chrysanthemum morifolium* 'Piato' single-infected with low titer of CSVd (low), high titer of CSVd (high) and co-infected with CSVd and CChMVd, established by Hosokawa et al., 2004, 2005, were used. The viroid-infected status of the *in vitro* stock shoots was confirmed using reverse transcription PCR (RT-PCR), *in situ* hybridization and real-time RT-qPCR (supplementary material 1), as described below. Viroid-free 'Piato' *in vitro* stock shoots were not available. The cultures were maintained on a basic medium (BM) composed of Murashige and Skoog (1962) medium (MS) supplemented with 20 g/l sucrose and 8 g/l agar (pH 5.8), and placed at 23 ± 3 °C under a 16-h photoperiod of light intensity of 40.5 μmol $m^{-2}s^{-1}$ provided by white fluorescent tubes, according to Hosokawa et al. (2004). Subculture was done every 3 weeks.

2.2. Cold-hardening of in vitro stock shoots

Terminal shoot segments (3 cm in length) with two fully-opened leaves (Fig. 1A) were excised from 3-weeks old *in vitro* stock shoots and cultured on MS medium. The cultures were cold-hardened for up to 6 weeks at 4 °C under two light regimes: 16-h photoperiod with 40.5 μmol $m^{-2}s^{-1}$ intensity (light) and consistent darkness (dark). Shoot growth and morphologies were recorded after 4 and 6 weeks of the cold-hardening treatments.

2.3. Encapsulation-vitrification cryopreservation

Shoot tips excised from viroid-infected stock shoots that had been cold-hardened were used for cryopreservation, as described by Li et al. (2017b), with some modifications. Shoot tips were precultured on MS medium supplemented with 0.5 M sucrose at 25 °C in the dark for 16 h. Precultured shoot tips were encapsulated with 3% (w/v) Na-alginate solution to form beads, each being 4–5 mm in diameter and containing one shoot tip. The beads were treated with a loading solution composed of MS supplemented with 0.4 M sucrose and 2 M glycerol at 25 °C in the dark for 1.5 h, followed by exposure to plant vitrification solution 2 (PVS2, Sakai et al., 1990) on ice for 5 h. PVS2 is composed of MS supplemented with 0.4 M sucrose, 30% (w/v) glycerol, 15% (w/v) dimethyl sulfoxide (DMSO) and 15% (w/v) ethylene glycol (pH 5.8). Following PVS2 dehydration, 10 beads were transferred into each of 2-ml cryovials (Sarstedt, Nümbrecht, Germany) containing 1.0 ml PVS2, prior to direct immersion in liquid nitrogen (LN) for at least 1 h. Cryopreserved shoot tips were thawed by removing the frozen beads and immediately placing those at 38 °C for 2–3 min. Thawed beads were incubated in an unloading solution at room temperature for 20 min. Unloading solution is composed of MS containing 1.2 M sucrose. Cryopreserved, thawed shoot tips were extracted from the beads and post-thaw cultured on half-strength Knop medium (Knop, 1965) supplemented with 0.05 mg/l gibberellic acid (GA_3) in the dark for 1 day, and then transferred onto MS in the light conditions for shoot regrowth. Shoot regrowth was defined as the percentage of the total number of shoot tips regenerating into normal shoots (≥ 5 mm) 4 weeks after post-thaw culture.

Three experiments were conducted to optimize some key factors affecting shoot regrowth in cryopreserved shoot tips, including time durations and light regimes of cold-hardening, and shoot tip sizes.

2.4. Histological observations

Histological observations were performed on cryopreserved shoot tips derived from CSVd (low)-infected *in vitro* stock shoots cold-hardened in the light and dark for 6 weeks, as described by Li et al. (2017b), with some modifications. Shoot tips were collected 3 days after post-thaw culture and fixed overnight in FAA [3.7% paraformaldehyde (w/v), 5% acetic acid (v/v), and 50% ethanol (v/v)], dehydrated with ethanol series (50%, 70%, 90% and 95%, 2 h for each concentration) and stored in 100% ethanol. After embedding in paraffin, sections (5 μm) were cut with a microtome (RM2155, Leica, Germany) and stained with 0.05% toluidine blue (TB) (Sakai, 1973). Stained sections were observed under a microscope (BX53, Olympus, Japan). The total number of cells and the number of cells that appeared to be living were observed in cryopreserved shoot tips and recorded in each of the shoot tip cross sections by two operators and the resulting data were subjected to blind analysis. Shoot-tips that were freshly excised from *in vitro* stock shoots served as a positive control, while those that were freshly excised, directly immersed in LN were the negative control. Both positive and negative controls underwent the same histological processes as described above.

2.5. Viroid detection by RT-PCR

Viroid detection was performed twice in the present study. The first time was conducted in *in vitro* stock shoots, to confirm their viroid status. The second was performed in the regenerants after two months of post-thaw culture following cryopreservation.

CSVd and CChMVd detection by RT-PCR was conducted, according to Hosokawa et al. (2004) and Nabeshima et al. (2012), respectively. RNA was extracted from fresh leaves (0.1 mg) using sepasol-RNA I super G (Nakarai tesque, Kyoto, Japan), according to manufactory instructions. cDNA synthesis was conducted at 42 °C for 30 min in reaction solution containing 0.5 μL of 20 μM random primer (6–9 mer), 1 μg of total RNA, 1 μL of 10 mM dNTP mixture, 0.5 μL of 20 U RNase inhibitor (Toyobo Co., Ltd., Osaka, Japan), 0.5 μL of reverse transcriptase (Toyobo Co., Ltd., Osaka, Japan) and 2 μL of buffer, with RNase-free water added to reach a total volume of 10 μL. PCR reaction was done in 10 μL reaction volume containing 0.1 μL of each primer (20 μM each), 1 μL of 2 mM dNTPs, 0.1 μL of 2U Blend Taq DNA polymerase (Toyobo