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Combining Thermotherapy with Cryotherapy for Efficient Eradication of *Apple stem grooving virus* from Infected In-vitro-cultured Apple Shoots

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Abstract

Apple stem grooving virus (ASGV), a difficult-to-eradicate virus from apple propagative materials, causes serious damage to apple production. The use of virus-free plants has been and is an effective strategy for control of plant viral diseases. This study aimed to eradicate ASGV from virus-infected in-vitro-cultured shoots of four apple cultivars and one rootstock by combining thermotherapy with cryotherapy. In vitro stock shoots infected with ASGV were thermo-treated using an alternating temperature of 36°C (day) and 32°C (night). Shoot tips were excised from the treated stock shoots and subjected to cryotherapy. Results showed that, although thermotherapy did not influence shoot survival rates, it reduced shoot growth and proliferation of in vitro shoots. Shoot regrowth rates decreased while virus eradication frequencies increased in cryo-treated shoot tips as time durations of thermotherapy increased from 0 to 6 weeks. Shoot regrowth and frequency of virus eradication were positively and negatively correlated, respectively, with the size of shoot tips. The protocol established here yielded shoot regrowth rates and virus eradication frequencies of 33 to 76% and 30 to 100%, respectively, in the four apple cultivars and one rootstock. Thermotherapy altered virus distribution patterns, subsequently resulting in production of a larger virus-free area in the thermo-treated shoot tips. Many cells in the top layers of apical dome and some cells in the youngest leaf primordia survived in cryo-treated shoot tips; these cells were most likely free of virus infection. Thus, plants regenerated from the procedure of combining thermotherapy with cryotherapy were free of ASGV, as judged by reverse-transcription polymerase chain reaction. To the best of our knowledge, this is the widest-spectrum technique reported thus far for the production of ASGV-free plants and provides a novel biotechnology for the production of virus-free plants in *Malus* spp.

Virus diseases have long been a threat to sustainability of apple production (Hadidi and Barba 2011). For a long time, certified virus-tested propagation materials have been used for efficient control of plant virus diseases (Faccioli and Marani 1998; Laimer and Barba 2011; Massart et al. 2011; Mink et al. 1998). Over the past four decades, great efforts have been exerted to develop reliable techniques for the production of apple virus-free propagative materials. Various traditional methods have been developed, including shoot tip culture (STC) (Laimer and Barba 2011; Plopa and Preda 2013), thermotherapy followed by STC (Laimer and Barba 2011; Paprstein et al. 2008; Tan et al. 2010; Wang et al. 2006), micrografting (Conejero et al. 2013; Dobránszki and Da 2010; Huang and Millikan 1980; Laimer and Barba 2011), chemotherapy followed by STC (Hansen and Lane 1985; James et al. 1997; O'Herlihy et al. 2003; Sedlak et al. 2011), and a combination of chemotherapy and thermotherapy (Hu et al. 2012, 2015).

Apple stem grooving virus (ASGV), the type member of the genus *Capillovirus,* has been reported in many apple-growing regions all over the world (Massart et al. 2011). A recent survey in China showed that ASGV was widely spread in all apple-growing regions and its infection frequency was 73.7% (Ji et al. 2013). ASGV is one of the most important latent viruses that infect *Malus* plants and other fruit crops, including *Pyrus, Citrus*, and *Actinidia* spp., as well as a number of important ornamental crops (Massart et al. 2011). ASGV infection caused graft incompatibility, reduced growth vigor, and decreased fruit yield (Massart et al. 2011). Previous studies showed that ASGV was the most difficult-to-eradicate virus from apple propagative materials among the other apple viruses such as *Apple chlorotic leafspot virus* (ACLSV), *Apple stem pitting virus* (ASPV), and *Apple mosaic virus* (ApMV) (Campbell 1968; Cropley 1968; Knapp et al. 1995; Li et al. 2016; Wang et al. 2006; Wang et al. 2016).

Cryotherapy has proven to be an efficient strategy for the production of virus-free plants (Romadanova et al. 2016; Vieira et al. 2015; Wang and Valkonen 2009; Wang et al. 2008). Romadanova et al. (2016) reported that cryotherapy could eradicate ASGV from in-vitro-cultured shoots but the virus-free frequency was lower than for other viruses such as ACLSV, ASPV, and ApMV. Li et al. (2016) found cryotherapy resulted in 80 to 85% frequencies of ASPV-free plantlets in two apple rootstocks (M9 and M26) but completely failed in producing ASGV-free plantlets. Combining thermotherapy with cryotherapy was proven to be more efficient than cryotherapy alone to eradicate *Raspberry bushy dwarf virus* (RBDV) that is able to infect meristematic tissues from in-vitro-cultured raspberry shoots (Wang et al. 2008).

The objective of this study was to test the efficacy of combining thermotherapy with cryotherapy in eradicating ASGV from in-vitro-cultured apple shoots. Possible mechanisms for successful virus eradication were discussed using data generated from virus localization in the infected shoot tips and histological observations on cell survival patterns in cryo-treated shoot tips.

Materials and Methods

Plant material. *Malus* × *domestica* 'Gala' apple was used to establish a virus eradication procedure by combining thermotherapy with cryotherapy. Three other apple cultivars (*Malus* × *domestica* 'Fuji', 'Ruixue' and 'Nongguo 25') and a rootstock (*Malus paradisiaca* 'M9') were further tested for their virus eradication by combining thermotherapy with cryotherapy established in the present study. Gala and Ruixue were used in experiments of histological observations

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and virus localization. In vitro shoots infected with ASGV that had been obtained by STC, as described by Wang et al. (2016), were maintained on shoot maintenance medium (SMM) composed of Murashige and Skoog (1962) medium (MS) supplemented with sucrose at 30 g liter^{-1}, 6-benzyladenine at 0.25 mg liter^{-1}, indole-3-butyric acid at 0.01 mg liter^{-1}, and agar at 8 g liter^{-1}, as described by Feng et al. (2013). The pH of the medium was adjusted to 5.8, prior to autoclaving at 121°C for 20 min. The stock cultures were kept at a constant temperature of 24 ± 2°C under a 16-h photoperiod with a light intensity of 50 μE s^{-1} m^{-2} provided by cool-white fluorescent tubes. Subculture was performed once every 4 weeks.

Thermotherapy. Shoot segments (1 to 2 cm long) containing two to three nodes were excised from 4-week-old diseased stock shoots and cultured on SMM in the same light conditions used for maintenance of the in vitro stock cultures. After 2 weeks of culture, the shoot segments were moved into a growth chamber (PQX-400; Haixiang Equipment, Shanghai, China) with the same light conditions as used for the in vitro stock shoots. An alternating temperature was set at 36°C (day) and 32°C (night). Effects of thermotherapy on survival, growth, and proliferation of in vitro stock shoot were analyzed.

STC and cryotherapy. Two experiments were performed in combining thermotherapy with cryotherapy for virus eradication. In the first experiment, shoot tips (1.5 mm in length) containing 4 to 5 leaf primordia (LP) were excised from in vitro diseased stock shoots after 0, 2, 4, and 6 weeks of thermotherapy. These treatments were designated as Th_{0wk}, Th_{2wks}, Th_{4wks}, and Th_{6wks}, respectively. The excised shoot tips were divided into two groups: one for STC (designated as Th + STC) and another for cryotherapy (designated as Th + Cryo). In Th + STC, explants were cultured for shoot recovery on SMM in the same light conditions used for maintenance of the in vitro stock cultures. In Th + Cryo, three sizes of shoot tips (1.5 mm in length containing 2 to 3 LP, 1.5 mm containing 4 to 5 LP, and 2.0 mm containing 5 to 6 LP) were excised from in vitro diseased stock shoots receiving Th_{4wks} and subjected to cryotherapy, as described by Li et al. (2015). The excised shoot tips were incubated in SMM for 1 day, followed by preculture in liquid MS containing 2 M glycerol and 0.8 M sucrose for 1 day. The precultured shoot tips were exposed to plant vitrification solution 2 (PVS2) (Sakai et al. 1990) at room temperature for 40 min. PVS2 contained 30% (wt/vol) glycerol, 15% (wt/vol) ethylene glycol, 15% (wt/vol) dimethylsulfoxide, and 0.4 M sucrose in MS (pH 5.8). After dehydration, each shoot tip was transferred into 2.5-μl PVS2 droplets carried on sterile aluminum foil strips (1.8 by 0.4 cm) and then directly immersed in liquid nitrogen (LN) for 30 min. Cryo-treated shoot tips were rapidly rewarmed by removing the frozen aluminum foil strips from LN and immediately placing them in unloading solution containing 1.2 M sucrose in MS at room temperature for 20 min. The cryo-treated shoot tips were postcultured on SMM. The cultures were placed in the dark for 3 days, during which they were transferred to fresh SMM every 16 to 24 h to reduce browning (Li et al. 2015). After that, the shoot tips were transferred to light conditions for shoot regrowth, which was defined as percentage of the total number of shoot tips regenerating into normal shoots (≥5 mm) 4 weeks postculture.

In Th + STC and Th + Cryo, subculture was conducted once every 4 weeks until shoots (≥1.5 cm in length) with three to four fully opened leaves formed in about 3 months. Shoots (≥1.5 cm in length) were transferred onto rooting medium (RM) and placed under the light conditions. RM was composed of MS supplemented with sucrose at 30 g liter^{-1}, naphthalene acetic acid at 0.5 mg liter^{-1}, and agar at 8 g liter^{-1} (pH 5.8). After 4 weeks of rooting, plantlets with well-developed roots were transferred into soil and grown in screenhouse conditions, according to Feng et al. (2013).

Virus detection. The sanitary status of plant materials was tested twice in the present study using reverse-transcription polymerase chain reaction (RT-PCR). ASGV was detected in the in vitro stock shoots to ensure that all plant materials were virus infected before thermotherapy. ASGV was detected again in the regenerated plants to assess virus eradication frequency; these plants had been grown in a screenhouse for 10 months, a period that encompasses one dormant season (from August 2015 to June 2016).

RT-PCR was conducted according to MacKenzie et al. (1997), with some modifications. Total RNA was extracted from fresh tissue

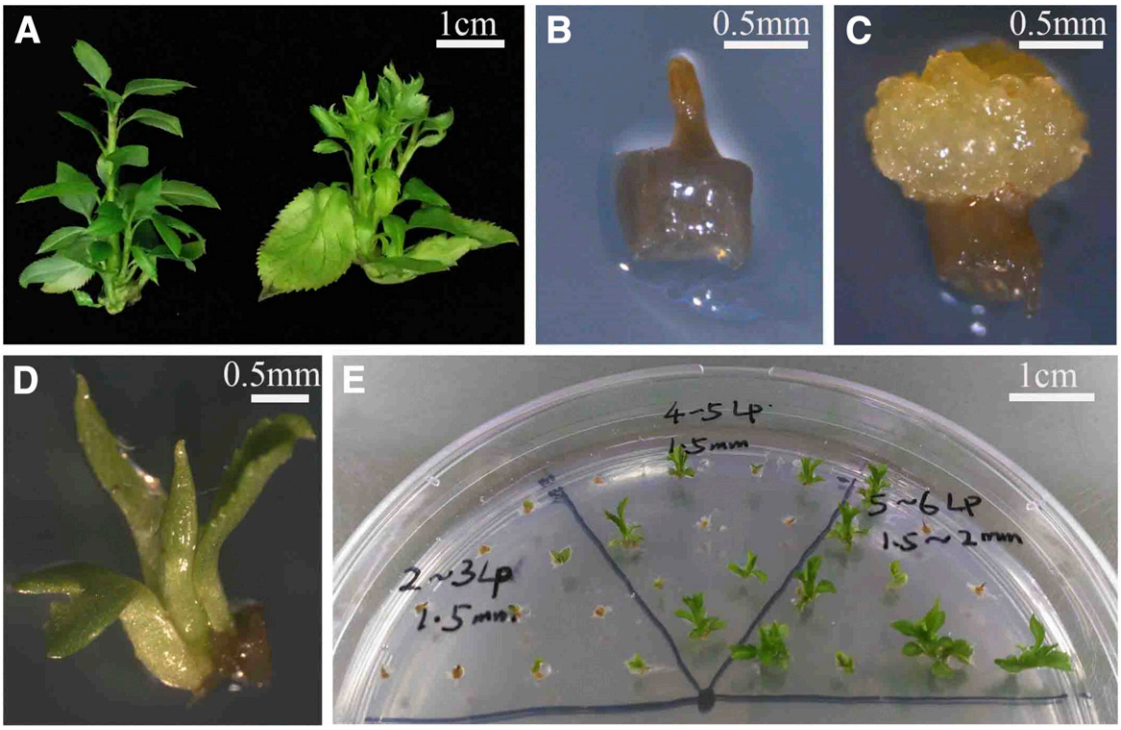

Fig. 1. *Apple stem grooving virus* (ASGV)-infected shoot of Gala following thermotherapy and shoot regeneration after thermotherapy combined with cryotherapy. **A,** ASGV-infected in vitro stock cultures without thermotherapy (in the left) and with thermotherapy at an alternating temperature of 36°C (day) and 32°C (night) for 4 weeks (on the right). **B,** Dead shoot tip after cryotherapy following thermotherapy. **C,** Callus formation from surviving shoot tip after cryotherapy following thermotherapy. **D,** Normal shoot regenerated 4 weeks postculture following thermotherapy and cryotherapy. **E,** Recovery of three sizes of shoot tips, as indicated on the Petri dish, following thermotherapy and cryotherapy after 4 weeks of postthaw culture. LP = leaf primordia.

(0.5 g) using Trizol Reagent (Invitrogen Ltd., Carlsbad, CA), according to the manufacturer's instructions. cDNA was synthesized on 2 μg of total RNA using recombinant Moloney murine leukemia virus reverse transcription (Promega Corp., Madison, WI), according to the manufacturer's instructions. The forward primer (5′-CTGCAAGACCGCGACCAAGTTT-3′) and reverse primer (5′-CCCGCTGTTGGATTTGATACACCTC-3′) were used to amplify a product of 524 bp (MacKenzie et al. 1997). The PCR was performed in a 25-μl reaction volume containing 1 μl of 0.5 μM each primer, 12.5 μl of 2× Taq DNA polymerase Mix (Kangwei, Beijing, China), 2 μl of template cDNA, and 8.5 μl of RNAase-free water. The PCR products were separated by electrophoresis in 2% agarose gels in Tris-acetate-EDTA buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0), stained with ethidium bromide, and visualized under an ultraviolet light.

Histological observations. The highest and lowest frequencies of virus-free plants were obtained in Gala and Ruixue following Th + Cryo. Therefore, these two cultivars were used for histological observations and virus localization, as described below, to elucidate a mechanism for efficient eradication of ASGV and understand why virus-free frequencies vary with host genotypes in the procedure combining thermotherapy with cryotherapy. Shoot tips were collected 3 days postculture following cryotherapy and Th_{4wks} + Cryo, and processed according to Feng et al. (2013). In brief, samples were fixed in 50% ethanol/formalin/acetic acid (18:1:1), dehydrated, and embedded. Thin sections (5 μm) were cut with a microtome (Leica RM 2235; Nussloch, Germany) and stained with 0.1% toluidine blue (TB) (Sakai 1973). The stained sections were observed under a light microscope (Leica DM 2000). Shoot tips without any treatments were used as positive controls to show characteristics of living cells, while those that were freshly excised, directly immersed in LN, and postcultured for 3 days served as negative controls to show characteristics of dead cells. Both positive and negative controls underwent the same histological processes as described above.

Table 1. Effects of thermotherapy on survival, regrowth, and proliferation of *Apple stem grooving virus* (ASGV)-infected in-vitro-cultured shoots of Gala apple[z]

Treatment	Survival (%)	Mean length of shoots (cm)	Number of proliferated shoots/explant	Leaf color
Thermotherapy	100 a	2.5 ± 0.3 b	4.7 ± 0.9 a	Yellowish
Nonthermotherapy (control)	100 a	3.5 ± 0.4 a	2.5 ± 0.6 b	Green

[z] ASGV-infected in-vitro-cultured shoots were thermo-treated at an alternating temperature of 36°C (day) and 32°C (night) for 4 weeks. Data were presented as means ± standard error; different letters in the same parameter indicate significant differences at $P < 0.05$ by Student's *t* test.

Table 2. Shoot regrowth and *Apple stem grooving virus* (ASGV) eradication frequency by combining thermotherapy with shoot tip culture (STC) or cryotherapy (Cryo) in Gala apple[w]

	Shoot regrowth (%)[x]		Frequency of ASGV eradication (%)[y]	
Time (weeks)[z]	STC	Cryo	STC	Cryo
0	100.0 a	62.2 ± 3.2 a	0 (0/15)	0 (0/15)
2	88.9 ± 6.3 ab	55.5 ± 3.2 ab	0 (0/15)	20 (3/15)
4	77.8 ± 3.2 b	44.4 ± 6.3 b	20.0 (3/15)	93 (14/15)
6	64.5 ± 3.2 c	20.0 ± 5.5 c	40.0 (6/15)	100 (12/12)

[w] ASGV-infected stock shoots were treated at an alternating temperature of 36°C (day) and 32°C (night) for 4 weeks. Shoot tips (1.5 mm) containing 4 to 5 leaf primordia were used for STC and Cryo.

[x] Data of shoot regrowth rates are presented as means ± standard error; different letters in the same column indicate significant differences at $P < 0.05$ by Student's *t* test.

[y] Numbers in parentheses are plantlets negative reaction to ASGV/total samples analyzed by reverse-transcription polymerase chain reaction.

[z] Duration of thermotherapy.

Virus localization. Shoot tips were taken from virus-infected in vitro stock shoots of Gala and Ruixue after treatments of Th_{0wk}, Th_{2wks}, and Th_{4wks} and prepared for virus localization, as described by Li et al. (2016). The healthy in vitro shoots were used as negative controls. Briefly, the thin sections (5 μm), as produced for histological observations, were treated with phosphate-buffered saline (PBS) containing 4% bovine serum albumin for 30 min, followed by overnight incubation at 5°C with the polyclonal antibodies (dilution 1: 400 in PBS) (150812; Bioreba, Reinach, Switzerland) to ASGV coat protein. After washing in PBS three times, the samples were incubated with antirabbit antibodies conjugated with alkaline phosphatase (A3937; Sigma-Aldrich, St. Louis) (dilution 1:400 in PBS) for 30 min at room temperature. After rinsing three times with PBS, samples were stained using a freshly prepared Fuchsin substrate solution. The sections were observed using a light microscope (Leica DM 750).

Experimental design and data statistical analysis. Ten samples were included in each treatment of three replicates in experiments of Th + STC and Th + Cryo, and all experiments were conducted twice. The data were presented as means ± standard error and analyzed using one-directional analysis of variance. Least significant differences were calculated at $P < 0.05$ by Student's *t* test. Ten samples were used in each treatment of two replicates in virus localization and histological observations. In all, 15 plants recovered from Th + STC and Th + Cryo were used for virus detection, except in the treatment of Th_{6wks} + Cryo, where only 12 regenerated plants were available.

Results

Effect of thermotherapy on survival, growth, and proliferation. After Th_{4wk}, all in vitro shoots of Gala survived. Leaves of heat-treated

Table 3. Shoot tip size on shoot regrowth and *Apple stem grooving virus* (ASGV) eradication of Gala after combining thermotherapy with cryotherapy[w]

Size of shoot tips[x]	Shoot regrowth (%)[y]	Frequency of ASGV eradication (%)[z]
1.5 mm + 2 to 3 LP	11.1 ± 3.1 b	100 (15/15)
1.5 mm + 4 to 5 LP	46.7 ± 5.4 a	100 (15/15)
2.0 mm + 5 to 6 LP	49.5 ± 4.1 a	73 (11/15)

[w] ASGV-infected stock shoots were thermo-treated at an alternating temperature at 36°C (day) and 32°C (night) for 4 weeks, followed by cryotherapy.

[x] LP = leaf primordia.

[y] Data of shoot regrowth rates are presented as means ± standard error; different letters indicate significant differences at $P < 0.05$ by Student's *t* test.

[z] Numbers in parentheses are plantlets negative reactions to ASGV/total samples analyzed by reverse-transcription polymerase chain reaction.

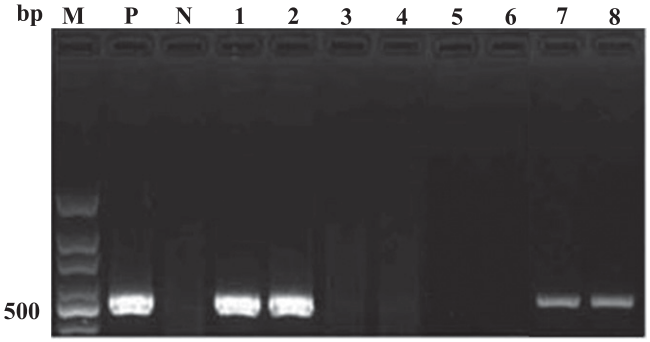

Fig. 2. Detection of *Apple stem grooving virus* (ASGV) by reverse-transcription polymerase chain reaction in Gala apple. Lane M = molecular marker, lane P = positive control (ASGV-infected Gala apple), lane N = negative control (virus-free Gala apple), lanes 1 and 2 = ASGV-infected in-vitro-cultured stock shoots before thermotherapy, lanes 3 and 4 = plants regenerated from the procedure combining thermotherapy for 4 weeks with cryotherapy, lanes 5 and 6 = plants regenerated from the procedure combining thermotherapy for 4 weeks with shoot tip culture, lane 7 = plants regenerated from the procedure combining thermotherapy for 2 weeks with cryotherapy, and lane 8 = plants regenerated from the procedure combining thermotherapy for 4 weeks with shoot tip culture.

shoots turned yellowish, particularly those at base of the shoots, compared with the nonthermo-treated control (Fig. 1A; Table 1). Although numbers of proliferating shoots per explant (4.7) were greater in thermo-treated shoots than those of the control (2.5), the latter produced much longer shoots (3.5 cm) than the former (2.5 cm) (Fig. 1A; Table 1).

Table 4. Shoot regrowth and *Apple stem grooving virus* (ASGV) eradication by combining thermotherapy with shoot tip culture (STC) or cryotherapy (Cryo) in three apple cultivars and one apple rootstock[y]

	Shoot regrowth (%)		Frequency of ASGV eradication (%)[z]	
Genotypes	**STC**	**Cryo**	**STC**	**Cryo**
M9 (rootstock)	76	38	36 (4/11)	83 (5/6)
Fuji (cultivar)	63	33	38 (5/13)	100 (5/5)
Ruixue (cultivar)	90	76	7 (1/14)	30 (4/13)
Nongguo 25 (cultivar)	83	66	8 (1/12)	40 (2/5)

[y] An alternating temperature of 36°C (day) 32°C (night) was used to treat the virus-infected stock shoots for 4 weeks. Shoot tips (1. 5 mm) containing 4 to 5 leaf primordia were excised from the heat-treated stock shoots and used for STC and Cryo.

[z] Numbers in parentheses are plantlets negative reaction to ASGV/total samples analyzed by reverse-transcription polymerase chain reaction.

Effects of Th + STC and Th + Cryo on shoot regrowth and virus eradication. Shoot regrowth rates in Th + STC and Th + Cryo significantly decreased as time durations of thermotherapy increased from 0 to 6 weeks (Table 2). STC and Th_{2wks}+ STC were not able to produce any ASGV-free plants (Table 2). Frequencies of ASGV eradication were 20 and 41% in shoot tips excised from in vitro shoots receiving treatments of Th_{4wks} and Th_{6wks}, respectively. Following Th + Cryo, three types of shoot tips formed. Dead shoot tips turned brown and black (Fig. 1B). About 10% of surviving shoot tips produced only callus, without shoot regeneration (Fig. 1C). Normal shoots directly regenerated without callus formation within 8 weeks postculture (Fig. 1D). Cryotherapy without thermotherapy did not produce any ASGV-free plants (Table 2). Frequencies of ASGV-free plants produced by Th + Cryo significantly increased from 20 to 100% as time durations of thermotherapy increased from 2 to 6 weeks (Table 2). In Th + Cryo, size of shoot tips affected shoot regrowth rate and virus eradication frequency (Fig. 1E; Table 3). Shoot regrowth rates increased as size of shoot tips increased from 1.5 mm containing 2 to 3 LP to 1.5 mm containing 4 to 5 LP and 1.5 to 2.0 mm containing 5 to 6 LP. However, virus eradication frequencies decreased with

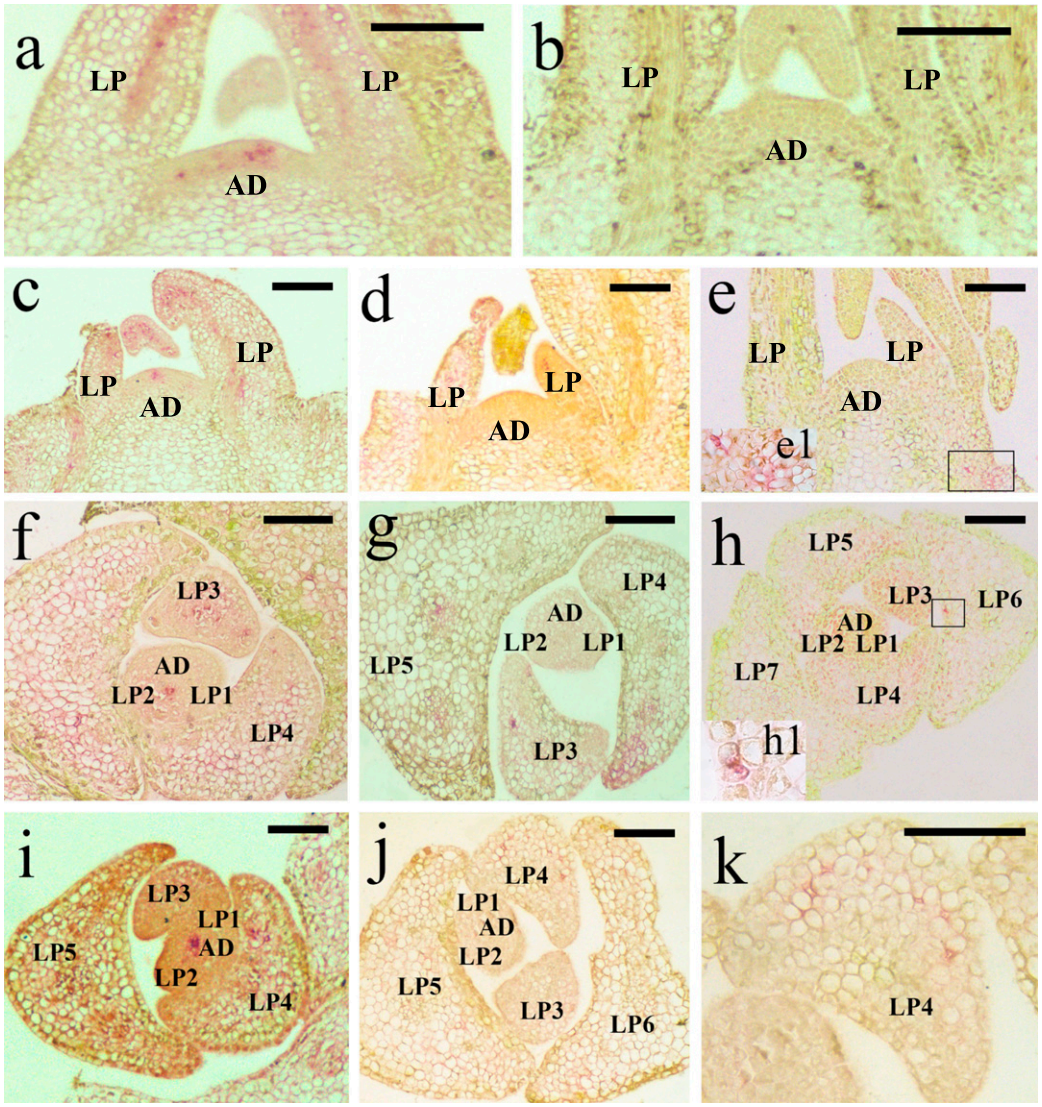

Fig. 3. Immunolocalization of *Apple stem grooving virus* (ASGV) in the virus-infected apple shoot tips of Gala and Ruixue after thermotherapy at an alternating temperature of 36°C (day) and 32°C (night). Virus-infected cells show a color reaction, while healthy cells do not. Longitudinal sections of **a,** ASGV-infected (positive control) and **b,** healthy (negative control) Gala shoot tips. Longitudinal sections of Gala shoot tips after **c,** 0; **d,** 2; and **e,** 4 weeks of thermotherapy. **e1,** A closer view showing virus-infected cells in the black square in e. Cross sections of Gala shoot tips after **f,** 0; **g,** 2; and **h,** 4 weeks of thermotherapy. **h1,** A closer view showing virus-infected cells in the black square in h. Cross sections of Ruixue shoot tips after **i,** 0 and **j,** 4 weeks of thermotherapy. **k,** A closer view of the leaf primordium 4 (LP4) in j. AD = apical dome. Bars = 100 μm.

increases in size of shoots from 1.5 mm containing 2 to 3 and 4 to 5 LP to 1.5 to 2.0 mm containing 5 to 6 LP (Table 3).

Virus detection. An ASGV-specific band of 524 bp produced by RT-PCR was detected in all diseased in vitro stock shoots that were used for virus eradication but was absent in the virus-free negative control Gala apple (Fig. 2). When RT-PCR was applied on plants recovered from Th + STC or Th + Cryo, samples showing such specific bands were considered to be ASGV infected, while those without such specific bands were considered to be ASGV free (Fig. 2).

Applications of Th + STC and Th + Cryo to other apple genotypes. Shoot regrowth rates and virus eradication frequencies varied with apple genotypes (Table 4). With Th + STC, the lowest (63%) and highest (90%) shoot regrowth rates were found in Fuji and Ruixue, and the lowest (7%) and highest (38%) virus eradication frequencies were produced in Ruixue and Fuji (Table 4). With Th + Cryo, shoot regrowth rates were the lowest (33%) in Fuji and the highest (76%) in Ruixue, and virus eradication frequencies were the lowest (30%) in Ruixue and the highest (100%) in Fuji (Table 4).

ASGV localization. A purple color reaction formed in the ASGV-infected cells of the positive control (Fig. 3a), whereas no such color reaction was found in the healthy cells (Fig. 3b). In diseased Gala stock shoots, strong purple color reactions were easily seen in the very top cells in the apical dome (AD) and the youngest LP in shoot tips before thermotherapy (Fig. 3c and f). After Th_{2wks}, virus was not found in the AD and LP1 but was still found in LP3 and older LP (Fig. 3d). Virus was also detected in LP2 in 6 of 12 samples tested, accounting for 40% (Fig. 3d and g). After Th_{4wks}, virus was not detected in the AD and LP1 to -5 but was still detected in LP6 and older LP (Fig. 3e, h). In Ruixue stock shoots, a similar pattern of virus distribution was found in the diseased shoot tips before thermotherapy (Fig. 3i). After Th_{4wks}, virus was not detected in the AD and LP1 to -3 but was found in LP5 and older LP (Fig. 3j). Virus was also detected in LP4 in 16 of 20 samples tested, accounting for 80% (Fig. 3j and k).

Histological observations. In the positive control, living cells showed TB-stained dense color with fully integrated cytoplasm and nucleolus enclosed in the nucleus (Fig. 4a and a1). In the negative control, dead cells did not show TB-stained cytoplasm, and cell membranes were ruptured and the nuclei were heavily condensed (Fig. 4b and b1). In Gala, the majority of the cells in the AD and some cells in LP1 to -4 survived in shoot tips following cryotherapy without thermotherapy (Fig. 4c, c1, and c2). Following Th_{4wks} + Cryo, many cells located in the top layers of the AD and some cells in LP1 to -3 survived, whereas other cells were killed in shoot tips (Fig. 4d,

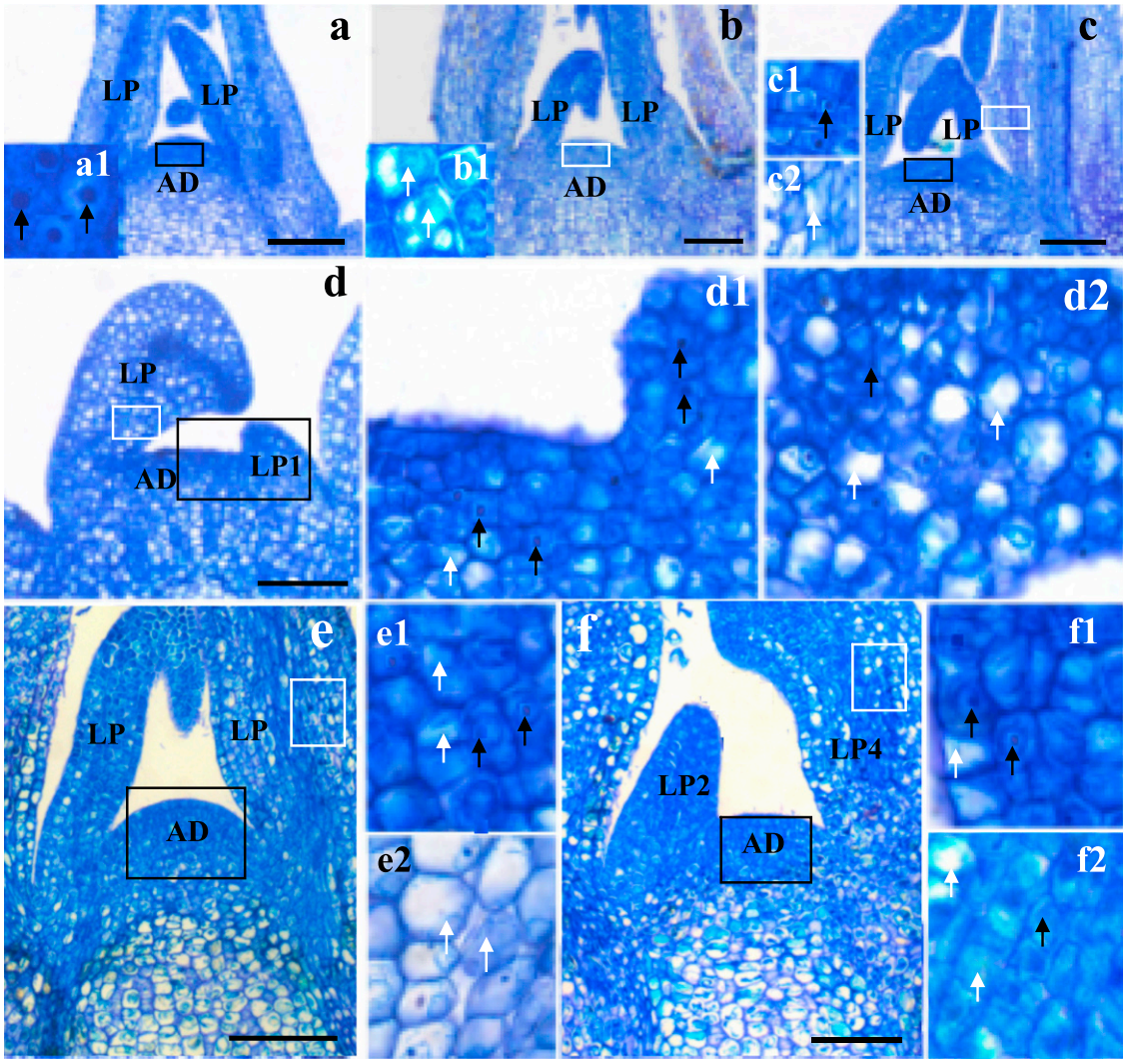

Fig. 4. Histological observations of cell survival patterns in cryo-treated shoot tips with and without thermotherapy in Gala and Ruixue apple. **a,** A Gala shoot tip that was freshly excised from 4-week-old stock shoots, serving as a positive control. **a1,** A closer view showing typical surviving cells in the black square of apical dome (AD) in a. **b,** A Gala shoot tip that was excised from 4-week-old stock culture and immediately immersed in liquid nitrogen for 1 h, serving as a negative control. **b1,** A closer view showing damaged or dead cells in the white square of AD in b. **c,** A Gala shoot tip following cryotherapy without thermotherapy. Closer views of **c1,** AD and **c2,** leaf primordium (LP) 5 in c. **d,** A Gala cryo-treated shoot tip following 4 weeks of thermotherapy. Closer views of the **d1,** black square of AD and **d2,** white square of LP in d. **e,** A Ruixue cryo-treated shoot tip following 4 weeks of thermotherapy. Closer views of the **e1,** black square of AD and **e2,** white square of LP in e. **f,** A Ruixue cryo-treated shoot tip following 4 weeks of thermotherapy. Closer views of the **f1,** black square of AD and **f2,** white square of LP in f. Black and white arrows represent surviving and damaged cells, respectively. Bars = 100 μm.

d1, and d2). In Ruixue, cell survival patterns in cryo-treated shoot tips without thermotherapy were similar to those of Gala (Fig. 4e, e1, and e2). However, many cells located in the top layers of the AD and in LP1 to -3 survived, and some cells in LP4 were also found alive in shoot tips following Th_{4wks} + Cryo (Fig. 4f, f1, and f2).

Discussion

STC and Th + STC are among the traditional approaches that have been the most frequently used for virus eradication in *Malus* propagative materials (Campbell 1968; Cropley 1968; Faccioli and Marani 1998; Knapp et al. 1995; Li et al. 2016; Mink et al. 1998; Paprstein et al. 2008; Wang et al. 2016). In STC, the size of shoot tips is critical for shoot regrowth and virus eradication (Faccioli and Marani 1998; Li et al. 2016; Mink et al. 1998; Wang et al. 2016). Working on apple rootstocks M9 and M26, Li et al. (2016) found that shoot recovery rates were much higher in 0.5- or 1.0-mm shoot tips containing 3 or 4 LP than in 0.5-mm shoot tips containing 2 LP. However, ASPV-free frequencies significantly decreased as size of shoot tips increased from 0.5 to 1.0 mm and LP number increased from 2 to 3 or 4. Similar results were also obtained in apple rootstocks M9 and M26 (Li et al. 2016) and cultivar Gala (Wang et al. 2016). The studies of Li et al. (2016) and Wang et al. (2016) further found that STC completely failed to eradicate ASGV from infected in-vitro-cultured apple shoots, regardless of the size of the shoot tips. These results and ours support the idea that ASGV is a virus that is difficult to eradicate from apple propagative materials.

Thermotherapy temperatures affected shoot survival, growth, and proliferation of the thermo-treated in-vitro-cultured stock shoots, as well as survival and shoot regeneration of shoot tips excised from the thermo-treated in vitro shoots (Hu et al. 2012, 2015; Knapp et al. 1995; Paprstein et al. 2008; Tan et al. 2010). Although constant or alternating temperatures can be applied to the stock cultures, the latter can alleviate negative effects of high constant temperature on the stock cultures and STC. For example, Knapp et al. (1995) reported that all in vitro apple shoots survived after they were thermotreated by an alternating temperature of 38°C (day) and 36°C (night) for 33 days. Beneficial effects of alternating temperature treatments on survival, growth, and proliferation were also reported by Tan et al. (2010) in pear and in the present study.

The extent of virus eradication was affected by the duration and temperature of the thermotherapy, as well as the size of the in vitro shoots. Thermotherapy of apple plants at 36°C for 20 days followed by the excision of 1-mm shoot tips produced higher frequencies of plants free of ACLSV (80 to 85%), ASPV (74 to 80%), and ASGV (70 to 86%) than when plants were treated at 34°C (Hu et al. 2015). Tan et al. (2010) found that thermotherapy of pear plants at 37°C for 35 days followed by culture of 1.0-mm shoot tips produced ASGV- and ACLSV-free pear plants, and that no virus-free plants were obtained in shoot tips larger than 2.0 mm. They also found that diseased in-vitro-cultured shoots that had been heat treated for more than 55 days with an alternating temperature of 42°C (day) and 34°C (night), followed by the excision of 1.0-mm shoot tips, resulted in 100% of the regenerated plants free of ACLSV, ASPV, and ASGV. The results obtained in the present study were consistent with those reported by Tan et al. (2010), Paprstein et al. (2008), and Hu et al. (2015).

Romadanova et al. (2016) reported that ASGV could be eradicated by cryotherapy alone. However, results from our previous study (Li et al. 2016) and the present study showed that cryotherapy alone completely failed to eradicate ASGV. In the studies of Li et al. (2016) and Wang et al. (2016) and this study, virus localization in shoot tips without thermotherapy clearly showed that meristematic cells were heavily infected by ASGV, while these cells survived following cryotherapy. Therefore, plants regenerated from cryo-treated shoot tips were still ASGV infected. Nevertheless, virus–host combinations and cryotherapy methods used in the study of Romadanova et al. (2016), and those of Li et al. (2016) and this study might be responsible for the differences in ASGV eradication. In addition, virus was detected in the plants regenerated only after 6 to 8 weeks following cryotherapy in the study of Romadanova et al. (2016), during which the virus numbers might be too low to be detected.

In the current ASGV study, we found that regrowth levels of cryo-treated shoot tips decreased and the frequency of virus eradication increased as the length of thermotherapy increased from 0 to 4 to 6 weeks. We also found that size of excised shoot tips after thermotherapy affected shoot regrowth and virus eradication. Similar results were also reported by Wang et al. (2008) for the eradication of RBDV from infected in vitro raspberry shoots. There are only a limited number of studies that have assessed the distribution of viruses in thermo-treated shoot tips. Wang et al. (2010) observed ACLSV and ASGV in 1-mm shoot tips without thermotherapy in diseased pear in vitro shoots. After a 50-day thermotherapy treatment, ACLSV and ASGV were not detected in shoot tips smaller than 2.0 and 0.5 mm, respectively. The virus-free area was enlarged in raspberry in vitro shoot tips infected with RBDV after thermotherapy for 28 days (Wang et al. 2008). In the present study, we found that the virus-free areas after thermotherapy varied among apple cultivars. Following Th_{4wks}, ASGV was not detected in the AD and LP1 to -5 in Gala but it was detected in LP4 in Ruixue. Following Th + Cryo, surviving cells were observed in the AD and LP1 to -4 in both Gala and Ruixue. These differences explained why there was a much higher frequency of ASGV eradication in Gala than in Ruixue, as well as why virus-free frequencies varied among other apple genotypes tested in the present study. Due to a limited number of plants available in other apple genotypes tested, statistical analysis was not done in virus-free frequencies among them in the present study.

High-temperature treatment was found to inhibit viral replication (Cooper and Walkey 1978), prevent virus movement toward the meristematic cells (Mink et al. 1998), cause virus RNA degradation (Wang et al. 2008), or induce gene silencing (Chellappan et al. 2005). More recently, Liu et al. (2015, 2016) found that thermotherapy (37°C) caused upregulation of the expression of several key genes involved in RNA silencing, induced the biogenesis of versus iRNA, and inhibited viral RNA accumulation in pear in vitro shoot tips infected with ASGV. These alternations may eventually contribute to production of larger virus-free areas in the heat-treated shoot tips than in nontreated ones (Wang et al. 2008; Wang et al. 2010).

In conclusion, a protocol combining thermotherapy with cryotherapy for efficient production of ASGV-free plants was developed in the present study. To the best of our knowledge, this is the most wide-spectrum technique thus far reported for ASGV eradication and provides a novel biotechnology for production of apple plants free of ASGV, the most difficult-to-eradicate virus in *Malus* spp.

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