

Combined thermotherapy and cryotherapy: an effective method for the eradication of three common viruses in cultivated apples

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Research

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Abstract

Background

Apple virus disease is a serious problem affecting apple quality and crop production efficiency, and solutions are being actively and urgently explored. In particular, the cultivation of virus-free seedlings is being increasingly explored as an important technical method for facilitating the virus-free breeding of excellent apple cultivars. In this study, the apple cultivars 'Ruiyang', 'Ruixue', 'Rui Xianghong', and 'Qinyue' were used as source materials to compare the effectiveness of thermotherapy and combined thermo- and cryotherapy in eradicating apple chlorotic leaf spot virus (ACLSV), apple stem grooving virus (ASGV), and apple stem pitting virus (ASPV).

Results

Viral presence in the in vitro shoots of the four apple cultivars was detected using a reverse transcription-polymerase chain reaction. All four cultivars were found to carry ASGV and ACLSV, and 'Rui Xianghong' and 'Qinyue' additionally carried ASPV. In vitro shoots of the four cultivars were subjected to varying periods of thermotherapy (0, 2, 4, 6, and 8 weeks). Shoot tips were then removed and half were cultured directly, and the remainder were cultured after cryotherapy treatment; their survival and regeneration rates were then evaluated to determine the efficiency of virus eradication after 8 months of subculture. We showed that the eradication of viruses in each of the cultivars and their survival rate are both the highest after 4 weeks of thermotherapy. When combined with cryotherapy, application of this period of thermotherapy achieved a 100% eradication of ASPV and ACLSV, and over 50% of ASGV.

Conclusions

This study demonstrated an effective methodology for developing both new virus-free plant materials and new apple cultivars that will support sustainability of the apple production industry in China and globally.

Background

Apple (*Malus × domestica* Borkh.) is a globally important cash crop. It is particularly valuable in China, which is both the largest producer and consumer of apples in the world. A total of 2.07 million hectares in China is dedicated to apple cultivation; the output of 39.23 million tons accounts for nearly 50% of global output [1]. In this context, apple viral diseases, which are among the main factors restricting healthy and sustainable development in apple cultivation [2], have received much attention. Viral infection of an apple tree damages normal cell proliferation, therefore seriously restricting the growth potential and other physiological mechanisms; it also reduces apple quality and yield, with consequent adverse implications for the apple industry [3–5]. Viruses can spread from infected plants to their offspring through asexual

reproduction, or to healthy plants via insect vectors [6]. Pesticide spraying is ineffective once a plant has become infected [7, 8]. The focus has therefore been on cultivating virus-free apple seedlings as an effective and core technology in the global control of apple viral diseases [9–12].

Viruses in fruit trees, such as apple stem pitting virus (ASPV), apple stem grooving virus (ASGV), and apple chlorotic leaf spot virus (ACLSV) are often present as complexes [13]; this presents a challenge to researchers working on the development of effective ways to produce virus-free apple plants. For example, ASGV is more difficult to eradicate than ASPV or ACLSV [14–19]. Methods of producing virus-free plants that have been considered include chemotherapy [20], cryotherapy [21, 22], micrografting [8, 20, 23], shoot tip culture (STC) [24, 25], and thermotherapy [10, 25, 26].

Cryotherapy, which uses liquid nitrogen as a coolant, has proved promising as a technique, playing an important role in developing virus-free plant production [27, 28]. In some research, a small number of virus-free cells regenerated as healthy, detoxified plants after liquid nitrogen was used to kill stem tip cells [29, 30]. In garlic (*Allium sativum* L.), cryotherapy has been shown to eradicate viral complexes successfully [31] and, in combination with STC, to eradicate ASPV and ASGV from M9 and M26 apple rootstocks [32]. The principle behind thermotherapy, which was reported in 1998 as an application suitable for virus eradication, is exposing plant tissue to a high temperature to inhibit viral spread; this minimises the extent to which meristem tissue in the stem tip carries viral particles, thus yielding virus-free seedlings [10]. However, there is growing interest in the effects of combined thermo- and cryotherapy. For example, it is more difficult to eradicate raspberry bushy dwarf virus from the in vitro shoots of raspberry when using only cryotherapy, compared with its use in combination with thermotherapy [29].

In this study, our objective was to compare and evaluate the effectiveness of thermotherapy alone and combined thermo- and cryotherapy in successfully eradicating ASPV, ASGV, and ACLSV from apple plants, using the in vitro shoots of four apple cultivars.

Results

Virus detection in plant materials

The results for the detection of ASPV, ASGV, and ACLSV in the in vitro shoots of four apple cultivars ('Ruiyang', 'Ruixue', 'Rui Xianghong', and 'Qinyue') are shown in Fig. 1. Two latent viruses, ASGV and ACLSV, but not ASPV, were detected in 'Ruiyang' and 'Ruixue'. However, in vitro shoots of 'Rui Xianghong' and 'Qinyue' carried all three latent viruses. This therefore clarified the viruses potentially present in each cultivar, thus making it possible to focus eradication efforts appropriately.

Effects of thermotherapy timings on shoot tip survival and regrowth

Shoot tips were obtained from in vitro shoots that been subjected to thermotherapy but not cryotherapy treatment (treatment -LN), for different periods; the resulting growth is shown in Fig. 2a. Regrowth mainly presented as normal (Fig. 2a-) with a few vitrified shoots (Fig. 2a-, ). Normally formed shoots regrew faster; the shoot tips were therefore cultured for 1 (Fig. 2a-) and 4 (Fig. 2a-) weeks, respectively. Shoot tip growth after the treatment combining thermo- and cryotherapy (treatment + LN) is shown in Fig. 2b; growth was observed to be slower with than without cryotherapy treatment (Fig. 3k), and the size of shoot tip cultured to 1 weeks (Fig. 2b-, ) and 8 weeks (Fig. 2b-, ). Regrowth presented normal shoots (Fig. 2b-, ), leaves (Fig. 2b-, ), vitrified shoots (Fig. 2b-, ), and a callus mass (Fig. 2b-, ).

Survival and regrowth rates after the STC treatment are shown in Fig. 2c. In the -LN treatment, the survival rate of all four cultivars decreased with increasing thermotherapy period. When in vitro shoots were subjected to thermotherapy for up to 8 weeks, the shoot tip regrowth rate for 'Qinyue', 'Rui Xianghong', 'Ruixue', and 'Ruiyang' gradually decreased from 72.5%, 59.6%, 69.4%, and 75.6–19.8%, 26.3%, 21.3%, and 35.9%, respectively. In the + LN treatment, the shoot tip survival and regrowth rate decreased gradually with increasing thermotherapy period; the regrowth rate for 'Qinyue', 'Ruixue', and 'Ruiyang' decreased to zero by week 8. However, the important point is that, for any length of thermotherapy treatment, the shoot tip survival and regeneration rates are higher without than with cryotherapy treatment.

Effectiveness of different treatments in virus eradication

The results of reverse transcription-polymerase chain reaction (RT-PCR) detection of in vitro shoots subcultured 10 times are shown in Fig. 4b. Cultivars 'Qinyue' and 'Rui Xianghong' were subjected to the -LN treatment for 8 weeks, and eradication of ASPV increased from nil to 25.6% and 30%, respectively. There was no eradication of ASGV from 'Qinyue', 'Rui Xianghong', 'Ruixue', and 'Ruiyang' at 2 weeks, but the value then increased to 10%, 12.5%, 10.9% and 10%. Eradication of ACLSV gradually increased with prolonged thermotherapy, up to 38.6% ('Ruixue').

In the + LN treatment, eradication of ASPV and ACLSV in the cultivars 'Qinyue', 'Rui Xianghong', 'Ruixue', and 'Ruiyang' increased rapidly with increasing thermotherapy period. After 4 weeks of thermotherapy, ASPV (Fig. 4a-) and ACLSV (Fig. 4a-, ) were eradicated successfully. ASGV eradication was slightly more difficult, however, increasing gradually from 0 to 8 weeks. Nonetheless, at 6 weeks, it was completely eradicated in 'Ruixue' and 'Ruiyang' and by over 70% in 'Qinyue' and 'Rui Xianghong'. After thermotherapy for 4 weeks combined with cryotherapy, ASGV eradication in vitro shoots had achieved a value of over 50%; the results are shown in Fig. 4a-, , .

Discussion

In this study, we assessed two effective detoxification methods: thermotherapy and combined thermo- and cryotherapy. These two techniques were applied to in vitro shoots of four apple cultivars subcultured at the age of 2 weeks. Our results determined that combined thermo- and hydrotherapy was more effective in virus eradication than thermotherapy alone, which is in agreement with previous studies [31, 33].

The thermotherapy temperature was alternated between 38 °C in the day and 30 °C at night, rather than imposing a constant high-temperature environment. This benefited the in vitro shoots by relieving the discomfort of the higher temperature at night while not lowering the temperature for too long [34–36].

The viral inhibition under thermotherapy treatment potentially reduces the transport of viral particles to the apical meristem [36, 37], and expands the non-infected area at the stem tip, which is therefore virus-free [10]. The length of the thermotherapy treatment is key to the successful establishment of a detoxification system. When thermotherapy treatment is too short, a large number of virus particles will remain in the shoot tips, therefore preventing full detoxification. However, if thermotherapy is administered for too long, the heat-resistant function of the shoots will begin to decline. Tan et al. [35] found that a combined treatment of thermotherapy for 35 days (at 37 °C) and STC could produce ASGV- and ACLSV-free pear plants from shoot tips of 1.0 mm in length; however, shoot tips longer than 2.0 mm could not produce virus-free plants. In our study, we were able to produce ASPV- and ACLSV-free plants using thermotherapy, a result in agreement with previous reports [35, 38].

The effects of droplet-vitrification and encapsulation-dehydration procedures on post-cryotherapy recovery patterns in apple shoot tips have been compared [39, 40]. These studies have shown that shoot tip regrowth rates obtained by droplet-vitrification is more stable than rates yielded by encapsulation-dehydration throughout the year, i.e. there was no seasonal effect. In our study, we used droplet-vitrification as our cryotherapy programme.

ASGV, ACLSV, and ASPV are found in most apple cultivars in commercial orchards [41]. A previous study suggested that using cryotherapy alone could not eradicate ASGV [18]. Some studies have clearly indicated serious ASGV infection and localisation in the shoot tips and meristematic cells, which may explain why it was difficult to eradicate [18, 33].

Previous reports have shown that shoot tips (about 1 mm in length) can produce virus-free plants after thermotherapy [36], but the survival rate of shoot tips of this length under cryotherapy treatment is very low [19, 29, 33]. In our study, we cut shoot tips to a length of 1.5 mm after thermotherapy; when then combining this treatment with cryotherapy, we showed a strong improvement in virus eradication from the cultivars 'Ruiyang' and 'Ruixue', which increased with increasing thermotherapy period, consistent with the previous studies [29, 33]. We found that a combined thermo- and cryotherapy treatment of shoot tips (1 mm) in 'Ruiyang', 'Ruixue', 'Qinyue', and 'Rui Xianghong' cultivars showed an improved effectiveness in virus eradication, the rate of which increased with increasing thermotherapy period. This provides a basis for promoting healthy and sustainable development of the apple industry, so long as the development and performance of virus-free plants are strictly tested and supervised in the production process.

Conclusions

In conclusion, our study explored two effective methods of eradicating viral infections from apple plants, that is, thermotherapy and combined thermo- and cryotherapy. We demonstrated that combining the

application of thermotherapy for 4 weeks with cryotherapy can effectively eradicate ASPV and ACLSV, and is effective in minimising ASGV, which is harder to eradicate completely. This study therefore provides a guaranteed technical method that supports the development of new virus-free apple plant materials and the application and promotion of new cultivars.

Materials And Methods

Plant material

We selected four apple cultivars based on their strong developmental potential as crops. They were: 'Ruiyang' (Fuji × Qinguan, S-SV-MD-009-2018) and 'Ruixue' (Fuji × Cripp's Pink, S-SV-MD-010-2018), both of which are registered with the National Forestry and Grassland Administration in China; and 'Qinyue' (Fuji × Gala, GPD-2018-610058) and 'Rui Xianghong' (Fuji × Cripp's Pink, S-SC-MR-002-2019), which are medium and late ripening cultivars, respectively, with crisp flesh, a rich fragrance, and strong disease resistance. Trees of the four cultivars from which samples originated were all grafted onto 11-year-old M26 rootstock. In March 2017, branches (15 branches per cultivar; approximate length, 30 cm) were removed from the trees in the field and transported to the laboratory. There, they were soaked in a solution of 1% NaClO for 10 min, followed by hydroponic culture at 25 °C (with no further additional nutrient); water was changed daily. Shoot tip samples (length, approximately 1.5 cm) (Fig. 3a) per apple cultivar were extracted from the branches, rinsed under tap water for 30 min, treated with 75% alcohol for 30 s and then with 0.1% HgCl₂ (adding 2–3 drops of surfactant [Tween 20]) for 10 min (Fig. 3b). The samples were finally rinsed in sterile water 5–7 times. Shoot tip surfaces in contact with the disinfectant solution were removed and inoculated on to the culture medium (Fig. 3c).

In vitro shoots of the four cultivars were cultured in a shoot maintenance medium [42] comprising Murashige and Skoog basal medium (MS) [43], supplemented by 6-benzyladenine (0.5 mg L⁻¹), indole-3-butyric acid (IBA; 0.03 mg L⁻¹), sucrose (30 mg L⁻¹), and agar (8 mg L⁻¹). The medium was sterilised by autoclaving for 20 min at 121 °C after adjusting the pH to 5.8. The culture experiment was performed at 25 ± 2 °C and with a 16/8 h photoperiod (light intensity, 50 μEs⁻¹ m⁻²) (Fig. 3d); the subculture period was 4 weeks.

Virus detection

All plant materials were tested twice using RT-PCR. The in vitro shoots for each apple cultivar (Fig. 3e) were analysed to determine viral presence. The regenerated plants were then investigated to assess the virus eradication frequency. After the subculture process had been repeated 10 times (from October 2017 to June 2018), the samples were analysed again to detect the presence of viruses (Fig. 3l).

RNA was extracted from 0.1 g of fresh in vitro shoots and purified, using a TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China). The cDNA was synthesised from 2 mg of total RNA using a PrimeScript RT Master Mix Kit (TaKaRa, Dalian, China). The products of each virus were amplified using the following primer pairs: ASPV (370 bp), (5'-ATGTCTGGAACCTCATGCTGCAA-3') and (5'-

TTGGGATCAACTTTACTAAAAAGCATAA-3'); ASGV (524 bp), (5'-CTGCAAGACCGCGACCAAGTTT-3') and (5'-CCCGCTGTTGGATTTGATACACCTC-3'); ACLSV (794 bp), (5'-GAGARTTTCAGTTTGCTMGA-3') and (5'-AGTCTACAGGCTATTTATTATAAGT-3') [44–46]. The PCR reaction volume was 25 μ L, incorporating 2 \times Taq Master Mix (12.5 μ L) (Kangwei, Beijing, China), 0.8 μ L of each primer, 1 μ L of cDNA, and 9.9 μ L of RNase-free water. The PCR reaction procedure was as follows: 2 min at 94 $^{\circ}$ C; 30 s at 94 $^{\circ}$ C; 45 s at 53 $^{\circ}$ C (ASPV), 57 $^{\circ}$ C (ASGV), and 54.5 $^{\circ}$ C (ACLSV); 50 s at 72 $^{\circ}$ C; 35 cycles of amplification; and a final finally extension of 10 min. The PCR products were validated using agarose (1%) gel electrophoresis, and then visualised under ultraviolet light.

Thermotherapy and treatment

In vitro shoots of the four cultivars were subcultured for 2 weeks, and then moved into a growth chamber (RXZ-500D-LED, Ningbo, China) under light conditions of 50 μ E s⁻¹ m⁻² (Fig. 3f). The temperature in the chamber was set to alternate between 38 $^{\circ}$ C during the day (6:00 am to 22:00 pm) and 30 $^{\circ}$ C at night (22:00 pm to 6:00 am). At 0, 2, 4, 6, and 8 weeks of thermotherapy, 100 shoot tips of each cultivar were cut under an anatomical microscope to a length of approximately 1.5 mm (four to five leaf primordia) (Fig. 3g). The shoot tips were divided into two groups: 50 shoot tips for direct STC without cryotherapy (-LN) (Fig. 3h), and 50 for cryotherapy treatment (+LN) (Fig. 3j). All experiments were repeated in triplicate.

Cryotherapy

Feng et al. [39] describe the procedure for droplet-vitrification. First, shoot tips were cultured under stable conditions on a basic medium (BM) for 1 day, then transferred to the MS solution (0.8 M sucrose, 2 M glycerol), also for 1 day. The shoot tips were transferred into droplets containing 6 μ L plant vitrification solution 2 (PVS2) [47] on aluminium foil (5 \times 1.5 cm) (Fig. 3i), and frozen in liquid nitrogen for 1 h. The PVS2 solution was composed of 30% glycerol (wt/vol), 15% dimethylsulfoxide (wt/vol), 15% ethylene glycol (wt/vol), and sucrose (0.4 M) in MS (pH 5.8). The shoot tips were then removed quickly from the liquid nitrogen and placed into an MS unloading solution (containing 1.2 M sucrose) for 20 min at a temperature of 20 \pm 2 $^{\circ}$ C. Finally, the shoot tips were placed into a BM medium in dark culture conditions for 3 days, after which the shoot tips were transferred to light culture conditions. Within 3 days of the dark culture treatment, the shoot tips were transferred to the new medium twice, after being frozen for 12 h and 36 h, to reduce browning. After regenerating into normal shoots, they were subcultured every 4 weeks.

Virus-free plants higher than 2 cm (Fig. 3l) were selected from all four cultivars and observed to grow healthily. Prior to rooting, they were inoculated onto 1/2 MS medium with IBA concentrations of 0.9 mg L⁻¹, 0.75 mg L⁻¹, 0.9 mg L⁻¹, and 1.1 mg L⁻¹ for the 'Ruiyang', 'Ruixue', 'Rui Xianghong', and 'Qinyue' cultivars, respectively. When the root length of the in vitro shoots reached approximately 2 cm, plants were transferred to the seedling refinement room. The cap of each tissue culture bottle was opened for 1–2 days, until no fungus was visible in the bottle, prior to transplanting. After the seedlings were removed from the culture medium to be transplanted, culture medium attached to the roots of the seedlings was washed with clean water (Fig. 3m) and transferred to a nutrition bowl containing peat soil, perlite, and

vermiculite (volume ratio of 3:0.5:0.5). The transplanted seedlings were cultured for 7 days (Fig. 3n) in low light ($< 20 \mu\text{Es}^{-1} \text{m}^{-2}$), and then gradually transitioned to normal light levels ($50 \mu\text{Es}^{-1} \text{m}^{-2}$) (Fig. 3o).

Assessment of shoot survival and regrowth

The survival and regrowth rate of the shoot tips were evaluated after the -LN (Fig. 3h) and +LN treatments (Fig. 3j). Survival rate was expressed as the percentage of the total number of tips that were green after 7 days of STC in both treatments (-LN and +LN). Shoot tips can grow new leaves within 8 weeks, and elongation of the shoot segments is considered to indicate regeneration. The normal regenerated in vitro shoots were analysed to assess any content of ASGV, ASPV, or ACLSV after 10 months, and the level of virus eradication in each cultivar was calculated.

Data analysis

Data were sorted using Microsoft Office Excel software (2007) and plotted using the ggplot2 function in R software (version 3.6.3).

Abbreviations

ACLSV: Apple chlorotic leaf spot virus; ASGV: Apple stem grooving virus; ASPV: Apple stem pitting virus; IBA: Indole-3-butyric acid; MS: Murashige and Skoog medium; PVS2: Plant vitrification solution 2; RT-PCR: Reverse transcription-polymerase chain reaction.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets and materials used and (or) analysed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare they have no competing interests.

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Authors' contributions

LL, YY and ZZ conceived the research; LL designed and performed the experiments, analyzed data and drafted the manuscript; SL and WY participated in coordination of the study; CX, YL and JY managed the plant in the field; YY and ZZ designed and supervised of review; LL, YY and ZZ wrote the paper. All authors read and approved the final version of the manuscript.

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Figures

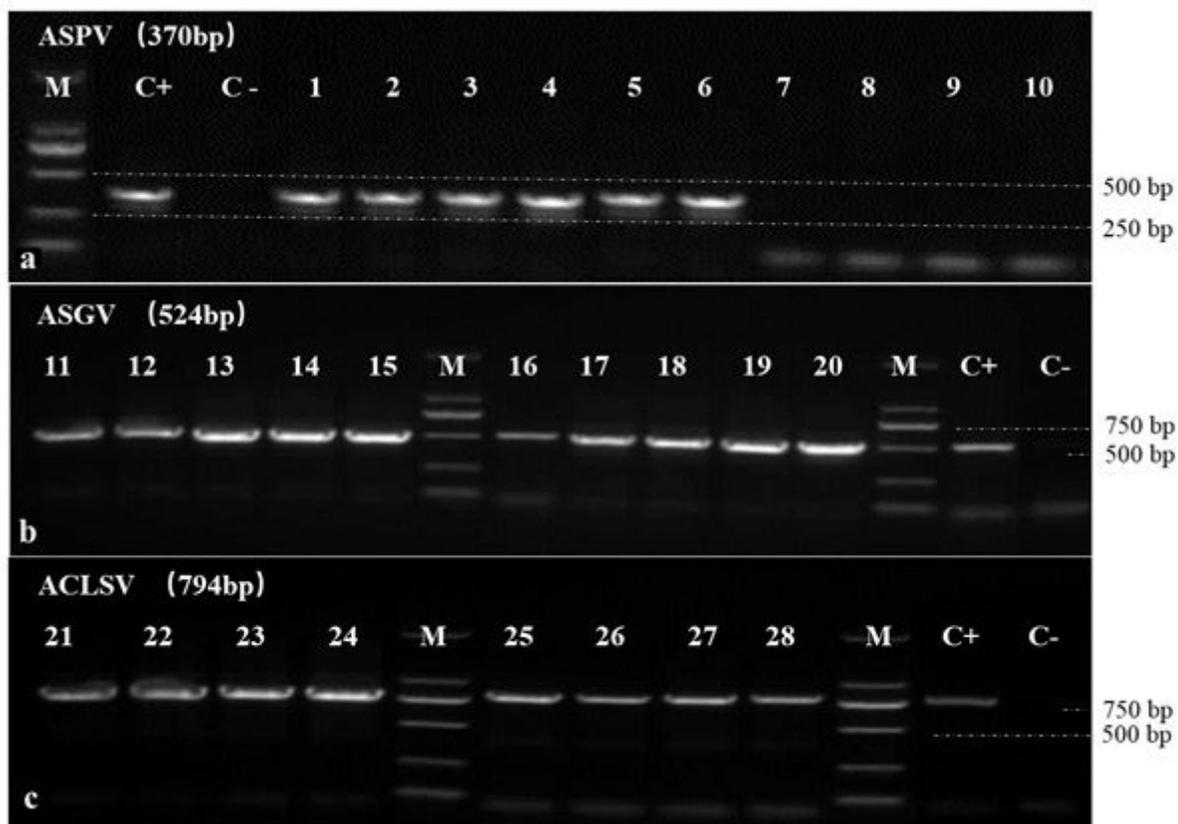


Figure 1

Virus detection by reverse transcription-polymerase chain reaction in four apple cultivars before thermotherapy treatment. a Molecular marker (M); positive control (C+), apple stem pitting virus (ASPV)-infected apple cultivar 'Gala'; negative control (C-), virus-free 'Gala'; cultivars 'Qinyue' (1-3), 'Rui Xianghong' (4-6), 'Ruixue' (7-8), and 'Ruiyang' (9-10). b Molecular marker (M); positive control (C+), apple stem grooving virus (ASGV)-infected apple cultivar 'Gala'; negative control (C-), virus-free 'Gala'; cultivars 'Qinyue' (11-13), 'Rui Xianghong' (14-16), 'Ruixue' (17-18), and 'Ruiyang' (19-20). c Molecular marker (M); positive control (C+), apple chlorotic leaf spot virus (ACLSV)-infected apple cultivar 'Gala'; negative control (C-), virus-free 'Gala'; cultivars 'Qinyue' (21-22), 'Rui Xianghong' (23-24), 'Ruixue' (25-26), and 'Ruiyang' (27-28).

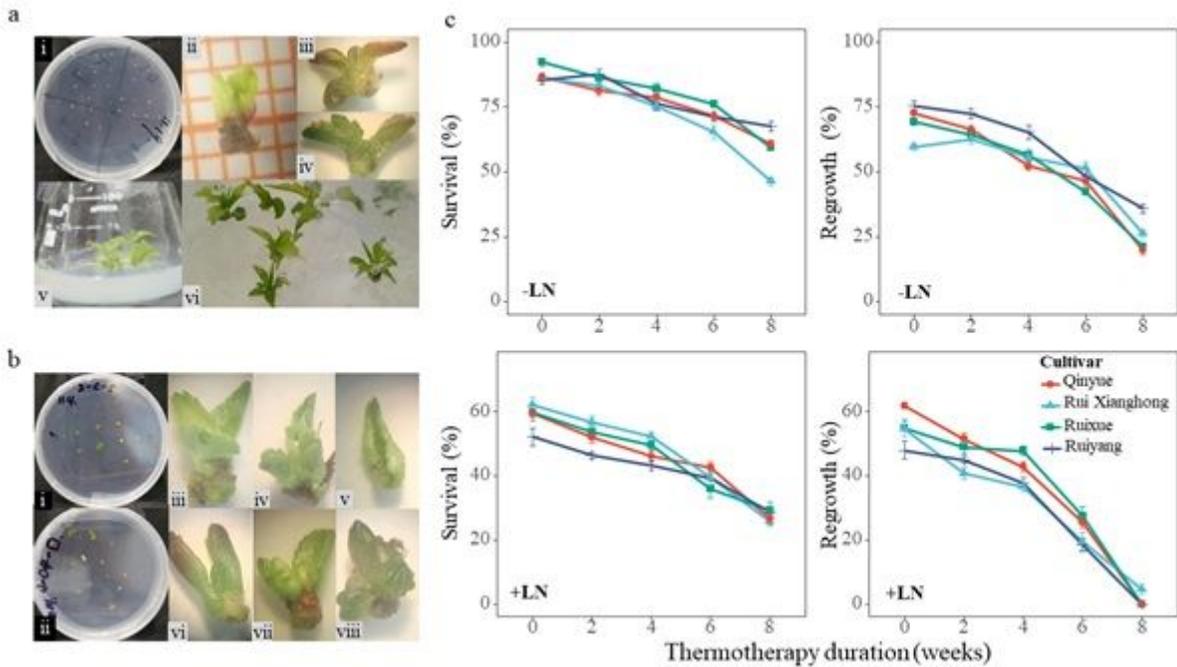


Figure 2

Different shoot regrowth and survival characteristics with (+LN) and without (-LN) liquid nitrogen (cryotherapy). a Regrowth after thermotherapy treatment (-LN): shoot tips were cultured for 1 week (i) and displayed different forms of regrowth after 4 weeks (ii-iv); plants with normal growth after eight weeks are shown in v and vi. b Regrowth after combined thermo- and cryotherapy treatment (+LN): shoot tips were cultured for 1 week (i and ii), showing different forms of shoot regrowth after 8 weeks (iii-viii). c Survival and regrowth rates between one and eight weeks in both treatments (-LN and +LN).



Figure 3

Illustrations of stages in production and detoxification of in vitro plants of apple cultivar 'Ruixue'. a Branches are cultivated in water; b sterilisation using HgCl₂; c shoot tips cultured for 2 weeks and d 4 weeks; e shoot tips subcultured for 2 weeks; f in vitro shoots subject to thermotherapy for 4 weeks; g shoot tips cut to length of 1.5 cm; h shoot tips cultured after thermotherapy; i shoot tips treated using droplet-vitrification; j shoot tips treated with cryotherapy for 4 weeks; k the size of stem tips in culture after 10 weeks ; l plants larger than 2 cm before rooting; m plants after rooting; plants n 1 week and o 10 weeks after transplantation.

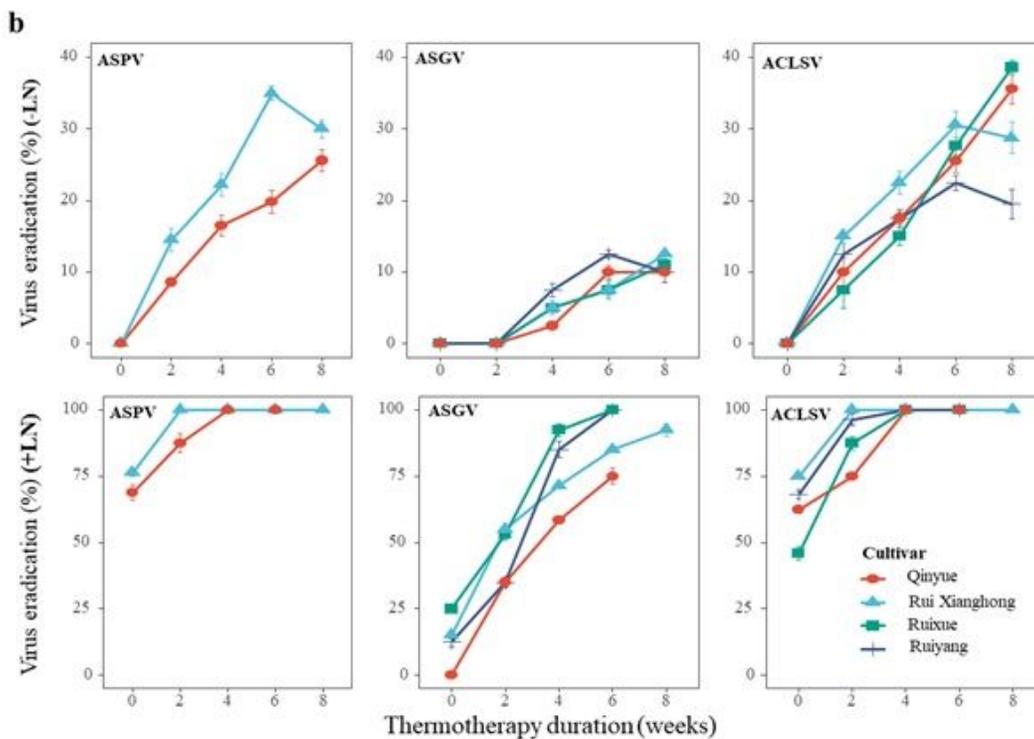
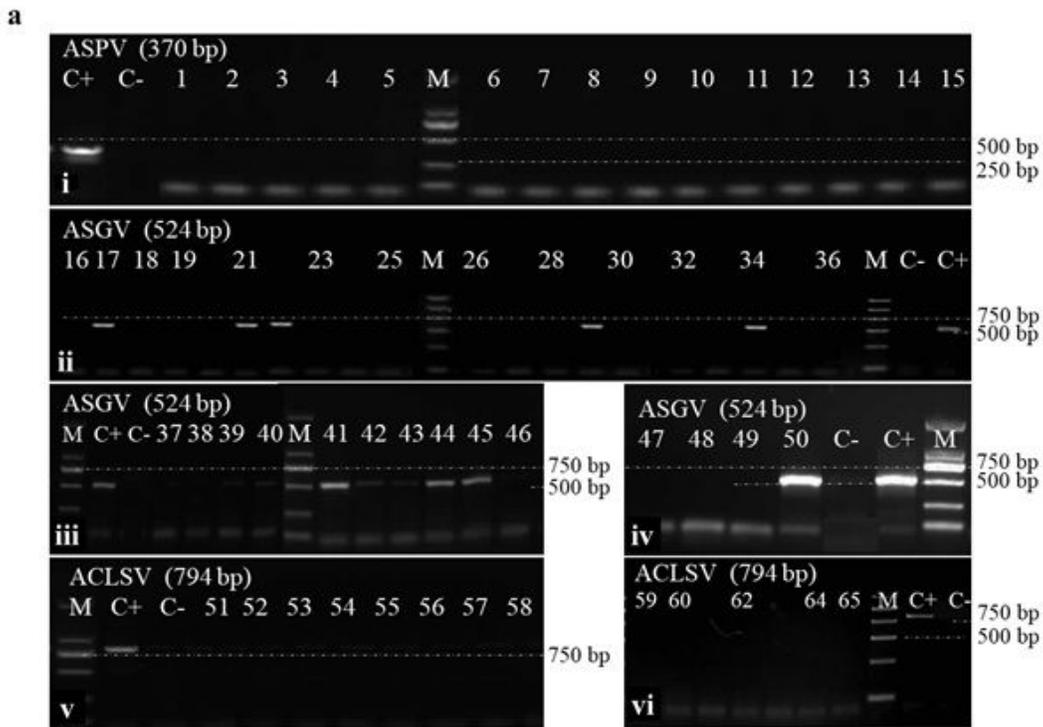


Figure 4

Virus detection using reverse transcription-polymerase chain reaction in in vitro shoots (subculture, eight months). a Results for plants regenerated during the procedure combining four weeks of thermotherapy with cryotherapy. i Molecular marker (M); positive control (C+), apple stem pitting virus (ASPV)-infected apple cultivar 'Gala'; negative control (C-), virus-free 'Gala'; cultivars 'Qinyue' (1-3), 'Rui Xianghong' (4-7), 'Ruixue' (8-11), and 'Ruiyang' (12-15). ii, iii, and iv Molecular marker (M); positive control (C+), apple stem

grooving virus (ASGV)-infected apple cultivar 'Gala'; negative control (C-), virus-free 'Gala'; cultivars 'Qinyue' (16–25), 'Rui Xianghong' (26-36), 'Ruixue' (37-46), and 'Ruiyang' (47-50). v and vi Molecular marker (M); positive control (C+), apple chlorotic leaf spot virus (ACLSV)-infected apple cultivar 'Gala'; negative control (C-), virus-free 'Gala'; cultivars 'Qinyue' (51-54), 'Rui Xianghong' (54-58); 'Ruixue' (59-62), and 'Ruiyang' (62-65). b Results of virus detection in shoot tips after ten months of subculture, for treatments with (+LN) and without (-LN) cryotherapy.

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