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ORIGINAL ARTICLE

Culture of shoot tips from adventitious shoots can eradicate *Apple stem pitting virus* but fails in *Apple stem grooving virus*

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Abstract This study attempted to eradicate *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV) from 'Gala' apple using shoot tips from of adventitious shoots derived from leaf segments. Leaf segments were excised from in vitro 4-week-old stock shoots and cultured on shoot regeneration medium containing 9.1 μM thidiazuron and 0.25 μM indole-3-butyric acid (IBA) to induce adventitious shoot formation. Shoot tips of different size and different developmental stage were excised from adventitious shoots and cultured on shoot tip culture medium containing 1.1 μM 6-benzyladenine and 0.05 μM IBA. Results showed size and developmental stage of shoot tips excised from adventitious shoots did not influence survival rate, but significantly affected shoot regrowth rate and ASPV-free frequency. Shoot regrowth rates increased from 10 to 15 % in 0.3 mm shoot tips containing two leaf primordia (LP) excised after 2–3 weeks of shoot regeneration, to 53–55 % in those containing three LP excised after 3–4 weeks. The highest shoot regrowth rate (82 %) was obtained in shoot tips of 0.4 mm shoot tips containing four LP excised after 4 weeks. ASPV-free frequencies (95–100 %) were high in 0.2–0.4 mm shoot tips containing two to three LP excised after 2–4 weeks, but low (20 %) in 0.4 mm shoot tips containing four LP excised after 4 weeks. None of the shoots regenerated from the shoot tips were ASGV-free, regardless of the size and developmental stage at which shoot tips were excised. Histological studies and virus localization provided explanations to the varying frequencies of the virus eradication using different size of shoot tips that were excised from adventitious shoots at different developmental stages. The protocol described here was characteristic of high frequency ASPV eradication and high efficiency of production of shoot tips that were used for virus eradication, and thus may have potential applications to virus eradication in other plant species.

Keywords Apple · ASGV · ASPV · Shoot regeneration · Shoot tips · Virus-free

Abbreviations

AD	Apical dome
ApMV	*Apple mosaic virus*
ASGV	*Apple stem grooving virus*
ASPV	*Apple stem pitting virus*
BA	6-Benzyladenine
CMV	*Cucumber mosaic virus*
GCLV	*Garlic common latent virus*
GLV	*Garlic latent virus*
IBA	Indole-3-butyric acid
ISSR	Inter-simple sequence repeat
LP	Leaf primordia
LSV	*Lily symptomless virus*
LYSV	*Leek yellow stripe virus*
MD	Meristematic dome
MS	Murashige and Skoog medium
NAA	Naphthalene acetic acid
OYDV	*Onion yellow dwarf virus*
RAPD	Random amplified polymorphic DNA
RT-PCR	Reverse transcription-polymerase chain reaction
SLV	*Shallot latent virus*

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SRM Shoot regeneration medium
STCM Shoot tip culture medium
TDZ Thidiazuron

Introduction

Apple is one of the most economically important fruit crops worldwide. Virus diseases constitute a major constraint on sustainable development of apple production (Hadidi and Barba 2011). Virus infection causes graft incompatibility, reduces bud break and growth vigor, induces lethal decline, leads to drop of premature leaves, and eventually results in about 15–50 % yield loss, depending on the type of virus and apple cultivar (Koike et al. 1993; Cieslinska and Rutkowski 2008; Hadidi and Barba 2011). *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV), two latent viruses, are among the serious viruses attacking apple (Jelkmann and Paunovic 2011; Massart et al. 2011). Mixed infections of ASGV, ASPV and *Apple chlorotic leaf spot virus* (ACLSV) usually occurred in the field (Wang et al. 2010; Ji et al. 2013).

Apple is vegetatively propagated by grafting, thus resulting in virus transmission from generation to generation. Unlike other diseases caused by fungi and bacteria, virus diseases are difficult to control by application of chemicals, once the trees are infected. In practice, use of virus-free planting material is an effective strategy to prevent apple viral diseases (Faccioli and Marani 1998; Mink et al. 1998; Laimer and Barba 2011). Virus-free apple plants have been and are being widely used in major apple production countries including Europe (EPPO 1998) and North America (Mink et al. 1998). Use of virus-free plants proved to promote plant growth vigor, and increase fruit yield and quality (Koike et al. 1993; Cieslinska and Rutkowski 2008), thus bringing great benefits to the apple industry. Over the last decades, great efforts have been exerted to develop methods of apple virus eradication, with various methods established, such as shoot tip culture (also called meristem culture) (Laimer and Barba 2011; Plopa and Preda 2013; Li et al. 2016), thermotherapy followed by shoot tip culture (Wang et al. 2006; Paprstein et al. 2008; Tan et al. 2010; Laimer and Barba 2011), micrografting (Huang and Millikan, 1980; Dobránszki and Teixeira da Silva 2010; Laimer and Barba 2011; Conejero et al. 2013), chemotherapy followed by shoot tip culture (Hansen and Lane 1985; James et al. 1997; O'Herlihy et al. 2003; Sedlak et al. 2011), combination of chemotherapy and thermotherapy (Hu et al. 2012, 2015) and cryotherapy (Li et al. 2016).

In vitro shoot regeneration has been extensively studied and leaf segments were the most frequently used explant for efficient shoot regeneration of *Malus* species (Dobránszki and Teixeira da Silva 2010; Magyar-Tábori et al. 2010). Shoot regeneration has been widely used in genetic transformation (Aldwinckle and Malnoy 2009), and has potential applications in micropropagation (Dobránszki and Teixeira da Silva 2010), production of artificial seeds (Brischia et al. 2002) and cryopreservation of *Malus* species (Li et al. 2014).

In our preliminary studies, we observed that when leaf segments of apple were cultured for adventitious shoot regeneration, the first cell divisions occurred in sub-epidermal cells of the explants after 3–4 days of culture, leading to formation of meristematic domes and eventually regeneration into adventitious buds in about 2 weeks. We hypothesize that the origin and fast growth of adventitious buds may allow shoot tips to escape the virus infection, consequently resulting in production of virus-free shoots.

The objectives of the present study were to test if culture of shoot tips from adventitious shoots regenerated from leaf segments can eradicate ASPV and ASGV. Histological studies and virus immunolocalization were performed to provide possible explanations to why culture of shoot tips can eradicate ASPV, but fails in ASGV.

Materials and methods

Plant material

'Gala' apple (*Malus* × *domestica*), a major cultivar grown worldwide, was used. Healthy and co-infected shoots with ASPV and ASGV were collected from the plants grown in greenhouse conditions of the Experimental Station of Northwest A&F University, Shaanxi, China. In vitro stock shoots were established according to Li et al. (2014). Since ACLSV frequently occurred in mixed infections with ASPV and ASGV in the field (Wang et al. 2010; Ji et al. 2013), diseased in vitro stock shoots were tested for ACLSV by reverse transcription-polymerase chain reaction (RT-PCR) according to Ji et al. (2013) after 6 months of establishment, to verify absence of ACLSV in diseased in vitro stock shoots. Healthy in vitro stock shoots and co-infected shoots with ASPV and ASGV were maintained on shoot tip culture medium (STCM) composed of Murashige and Skoog (MS 1962) medium supplemented with 1.1 μM 6-benzyladenine (BA), 0.05 μM indole-3-butyric acid (IBA), 30 g/l sucrose and 8 g/l agar (Sigma-A7921, MO, USA) (Li et al. 2014). The pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. The stock shoots were cultured in 200 ml jars containing 25 ml medium and were maintained at 22 ± 2 °C under a 16-h photoperiod at 50 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Sub-culturing to fresh medium was performed every 4 weeks.

Shoot regeneration

Shoot regeneration was conducted as described by Li et al. (2014). In brief, the first three fully opened leaves were excised from 4-week-old diseased in vitro stock shoots. Leaf segments were trimmed to 0.8 × 0.6 cm, and four cuts at 1 mm intervals were made transversally to the midrib. The explants were cultured with adaxial surface in contact with shoot regeneration medium (SRM) composed of MS medium supplemented with 9.1 μM thidiazuron (TDZ) and 0.25 μM IBA and cultured at 23 ± 2 °C in the dark for 3 weeks before transfer to the light conditions as used for in vitro shoot stock cultures. Sub-culturing to fresh medium was done every 4 weeks.

Shoot tip culture

Shoot tips of different sizes (0.3 mm containing two or three LP and 0.4 mm containing four LP) and at different developmental stages (2–4 weeks) (Table 1) were excised from adventitious shoots and cultured on STCM in the dark for 3 days, and then placed under the light conditions as used for the in vitro stock shoots. Survival was expressed as the percentage of shoot tips showing green color 1 weeks after culture, whereas shoot regrowth was expressed as the percentage of surviving shoot tips regenerating shoots ≥5 mm in length with at least two fully-opened leaves after 4 weeks of culture. Shoots (≥5 mm) regenerated from shoot tips were transferred to fresh STCM for further shoot elongation. Sub-culturing was conducted every 4 weeks. After 3 months of culture on STCM, shoots ≥2.0 cm in length with five to six leaves were transferred to rooting medium composed of half-strength MS medium supplemented with 2.7 μM naphthalene acetic acid (NAA), 30 g/l sucrose and 8 g/l agar, and kept in the dark for 5 days, and then transferred to half-strength MS medium containing 30 g/l sucrose and 8 g/l agar without any plant growth regulators under the light conditions (Li et al. 2014). Roots formed after 4 weeks of culture. Sub-culturing to fresh medium was conducted every 4 weeks.

Virus detection

Virus status was assessed by RT-PCR in the diseased in vitro stock shoots used for adventitious shoot regeneration, in shoots regenerated from shoot tips after 10 months of in vitro regeneration and in plantlets after 6 months of establishment in soil in greenhouse conditions, according to Li et al. (2016). In brief, total RNA was extracted from fresh leaf tissue (0.5 g) using the Trizol Reagent (Invitrogen Ltd., Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNA was synthesized on 35 μg of total RNA using recombinant Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. Forward and reverse primers used by Menzel et al. (2002) for ASPV and MacKenzie et al. (1997) for ASGV were included in the PCR reaction, to amplify specific bands of 524 bp for ASPV and 370 bp for ASGV. The PCR products were separated by electrophoresis in 2 % (w/v) agarose gel in Tris–acetate (TAE) buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0), stained with 0.1 % (w/v) ethidium bromide and visualized and photographed under ultraviolet light.

Histological observations

Samples were taken at different developmental stage of adventitious shoot regeneration from leaf segments and used for histological observations, as described by Feng et al. (2013). In brief, samples were fixed in FAA (50 % ethanol:formalin:acetic acid; 18:1:1), dehydrated, and embedded. Sections (5 μm) were cut with a microtome (Leica RM 2235, Germany) and stained with 0.1 % toluidine blue (Sakai 1973). The stained sections were observed under

Table 1 Survival and shoot regrowth rates, and frequency of ASPV- and ASGV- free shoots using shoot tip culture of 'Gala' apple

Size and excision time of shoot tips	Survival (%)	Shoot regrowth (%)	Frequency of virus eradication (%)	
			ASPV	ASGV
0.3 mm, two LP, 2 weeks	100a	10 ± 2a	100 (6/6)	0 (0/6)
0.3 mm, two LP, 3 weeks	100a	15 ± 2a	100 (9/9)	0 (0/9)
0.3 mm, three LP, 3 weeks	100a	53 ± 5b	100 (20/20)	0 (0/20)
0.3 mm, three LP, 4 weeks	100a	55 ± 5b	95 (19/20)	0 (0/20)
0.4 mm, four LP, 4 weeks	100a	82 ± 8c	20 (4/20)	0 (0/20)

Data of survival and shoot regrowth rates were presented as mean ± SE with different letters in the same column indicating significant differences at $P \leq 0.05$ by Student's t test. Virus eradication frequency was calculated based on data of RT-PCR analysis in shoots regenerated from shoot tips after 10 months of in vitro regeneration. Numbers in parentheses indicate positive reactions to ASPV and ASGV by RT-PCR/total samples tested

LP leaf primordia, *ASPV Apple stem pitting virus*, *ASGV Apple Grooving virus*, *RT-PCR* reverse transcription-polymerase chain reaction

a light microscope (Leica DM 2000, Germany). Serial consecutive sections obtained from the same samples were used for both histological observations and virus localization.

Virus localization

Samples were prepared as for histological observations. The healthy in vitro shoots were used as negative controls. Protocols described by Li et al. (2016) for immunolocalization of ASPV and ASGV were used. Briefly, the sections were first treated with phosphate buffered saline (PBS) containing 4 % bovine serum albumin for 30 min, followed by overnight incubation at 5 °C with the rabbit polyclonal antibodies to ASPV or ASGV (Adgen Biotechnology Ltd, Beijing, China) (dilution 1:500 with PBS) coat protein. After raising with PBS three times, the samples were incubated with mouse anti-rabbit monoclonal antibodies conjugated with alkaline phosphatase (Sigma Chemical Co., USA) (dilution 1:500 with PBS) for 30 min at room temperature. After raising again three times with PBS, samples were stained using a freshly prepared Fuchsin substrate solution. The sections were observed using a light microscope (Leica DM 2235, Germany).

Experimental design and data statistical analysis

In experiments of adventitious shoot formation and culture of shoot tips, ten samples were included in each of three replicates. All experiments were conducted twice. The data were presented as means with standard error. Thirty samples were collected from two independent experiments of adventitious shoot regeneration and subject to histological studies and virus localization.

Results

Adventitious shoot regeneration

After 3–4 weeks of culture on SRM, approximately eight adventitious shoots developed on each of three leaf segments. Totally, approximately 24 shoot tips could be produced from one in vitro stock shoot in 3–4 weeks of adventitious shoot regeneration.

Virus detection

With RT-PCR, specific amplified segments at about 370 and 524 bp were detected for the ASPV- and ASGV-infected in vitro stock shoots, respectively (Fig. 1a, b). All samples of the diseased in vitro stock shoots subjected to RT-PCR detection showed these two bands, indicating co-

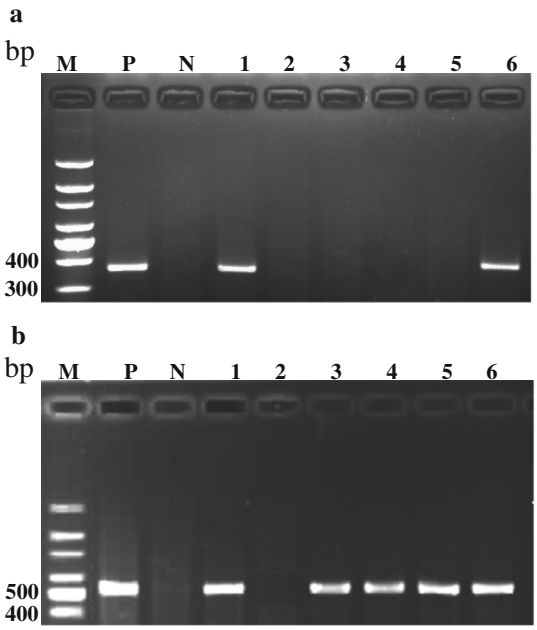

Fig. 1 Detection of ASPV (**a**) and ASGV (**b**) by RT-PCR in healthy and virus-infected in vitro stock shoots, and plantlets regenerated from shoot tips of leaf segment-derived adventitious shoots of 'Gala' apple. *M* molecular marker, *P* positive control (ASPV-infected in **a** and ASGV-infected in **b**), *N* negative control (healthy in **a** and **b**), *Lane 1* RNA from diseased in vitro stock shoots used for adventitious shoot regeneration; *Lane 2* RNA from healthy in vitro stock shoots; *Lanes 3–4* RNA from plantlets regenerated from 0.3 mm shoot tips containing two to three leaf primordia excised from adventitious buds after 2–3 weeks of shoot regeneration; *Lane 5* n RNA from plantlets regenerated from 0.3 mm shoot tips containing three leaf primordia excised from adventitious buds after 3 weeks of shoot regeneration; *Lane 6* RNA from plantlets regenerated from 0.4 mm shoot tips containing four leaf primordia excised from adventitious buds after 4 weeks of shoot regeneration

infection by ASPV and ASGV before adventitious shoot regeneration, while no such bands were detected in the healthy in vitro stock shoots (Fig. 1a, b). When virus status was detected in shoots regenerated from shoot tip culture after 10 months of in vitro regeneration and in plantlets after 6 months of establishment in soil in greenhouse conditions, samples showing either or both specific bands were considered infected, while those without these specific bands were considered healthy (Fig. 1a, b).

Survival, shoot regrowth, and virus eradication

All shoot tips survived, regardless of their size and developmental stage at which shoot tips were excised from adventitious shoots, but shoot regrowth rates varied considerably (Table 1). When 0.3 mm shoot tips containing two LP were excised after 2–3 weeks of adventitious shoot regeneration, four types of regenerants were produced: (1) only callus formation (Fig. 2a), (2) callus with roots

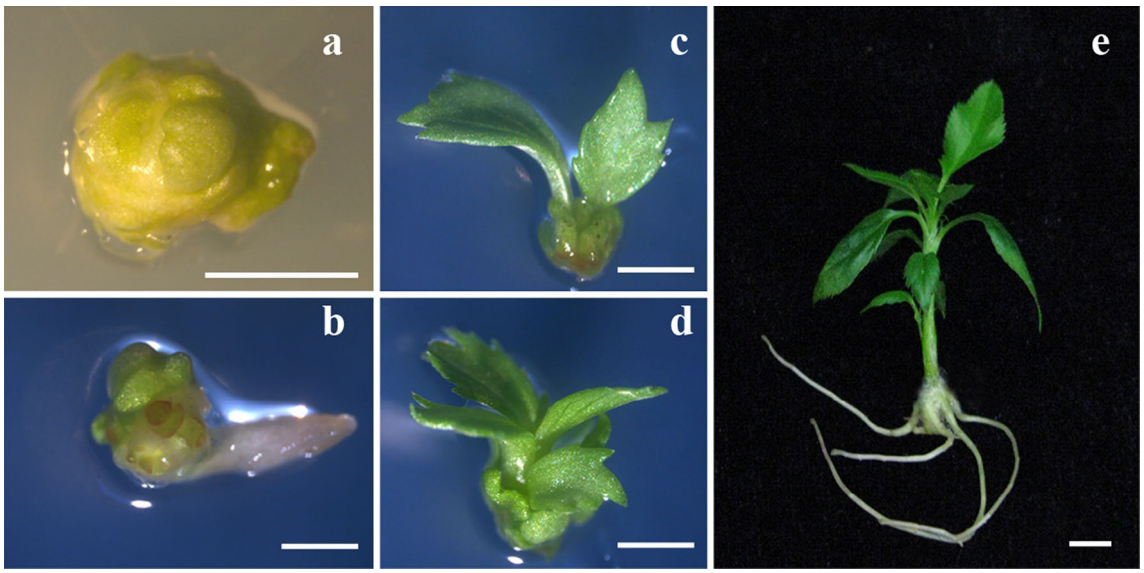

Fig. 2 Recovery types of shoot tips of adventitious shoots regenerated from leaf segments and whole plantlet development of 'Gala' apple. **a** Callus formation from shoot tips after 4 weeks of culture on shoot tip culture medium. **b** Callus formation with root from shoot tips after 4 weeks of culture on shoot tip culture medium. **c** Leaves developed from shoot tip after 4 weeks of culture on shoot tip culture medium. **d** Shoot formation from shoot tip after 4 weeks of culture on shoot tip culture medium. **e** Plantlet with roots after 4 weeks of culture on rooting medium. *Bars* 2 mm

(Fig. 2b), (3) leaves without shoot elongation (Fig. 2c), and (4) shoot regrowth (Fig. 2d), each accounting for approx. 30–40, 10–20, 30–45 and 10–15 %. Shoot regrowth rates significantly increased to 53 or 55 % in 0.3 mm shoot tips containing three LP that were excised after 3 or 4 weeks of adventitious shoot regeneration, respectively (Table 1). Approximately 10–17 and 30–35 % of said shoot tips developed into callus and leaves without shoot elongation, respectively. Approximately 82 % of 0.4 mm shoot tips containing four LP that were excised after 4 weeks of adventitious shoot regeneration resumed shoot growth (Table 1) and the rest (18 %) developed into leaves without shoot elongation. Rooted shoots were produced after 8 weeks of culture on rooting medium (Fig. 2e).

Results of virus status detected by RT-PCR were identical between shoots regenerated from shoot tip culture after 10 months of in vitro regeneration and plantlets after 6 months of establishment in soil in greenhouse conditions (data not shown). More than 95 % of shoots regenerated from 0.3 mm shoot tips containing two to three LP that were excised from adventitious buds after 2–4 weeks of shoot regeneration were ASPV-free (Table 1; Fig. 1a). About 20 % of ASPV-free frequencies were obtained in 0.4 mm shoot tips containing four LP that were excised from adventitious buds after 4 weeks of shoot regeneration (Table 1; Fig. 1a). None of the shoots regenerated from shoot tip culture were free of ASGV, regardless of the size or developmental stage at which shoot tips were excised from adventitious shoots (Table 1; Fig. 1b).

Histological observation and virus localization

Histological structure of leaf segments at day 0 of culture for adventitious shoot regeneration was shown in Fig. 3a. With immunohistochemical staining for virus localization, virus-infected tissues showed a purple-red color, while healthy tissues did not show any color reaction (Fig. 3u, v). ASPV was detected in vascular tissues and parenchyma cells, but not in epidermal and sub-epidermal cells (Fig. 3b). ASGV was detected broadly across the leaf tissues including sub-epidermal cells, vascular tissues and parenchyma cells, but not in epidermal cells (Fig. 3c). First cell divisions were observed in sub-epidermal cells of adaxial surface of the leaf segments in about 4 days of culture (Fig. 3d, e). ASPV was not included in the dividing cells (Fig. 3f), while ASGV was (Fig. 3g). These dividing cells differentiated into meristematic domes (MD) in about 10 days of culture (Fig. 3h). At this stage, ASPV was not detected in the MD (Fig. 3i), but ASGV was clearly seen in the MD (Fig. 3j). The MD further developed into adventitious buds, with apical dome (AD) and two LP clearly observed after 2 weeks of culture (Fig. 3k). At this stage, ASPV was not present in the adventitious buds (Fig. 3l), while ASGV was easily detected inside the buds (Fig. 3m). These adventitious buds further elongated and protruded from the leaf segment explants after 3 weeks of culture (Fig. 3n). At this stage, the adventitious buds were still not infected by ASPV (Fig. 3o), but heavily invaded by ASGV (Fig. 3p). Adventitious buds of 0.4–0.5 mm in length

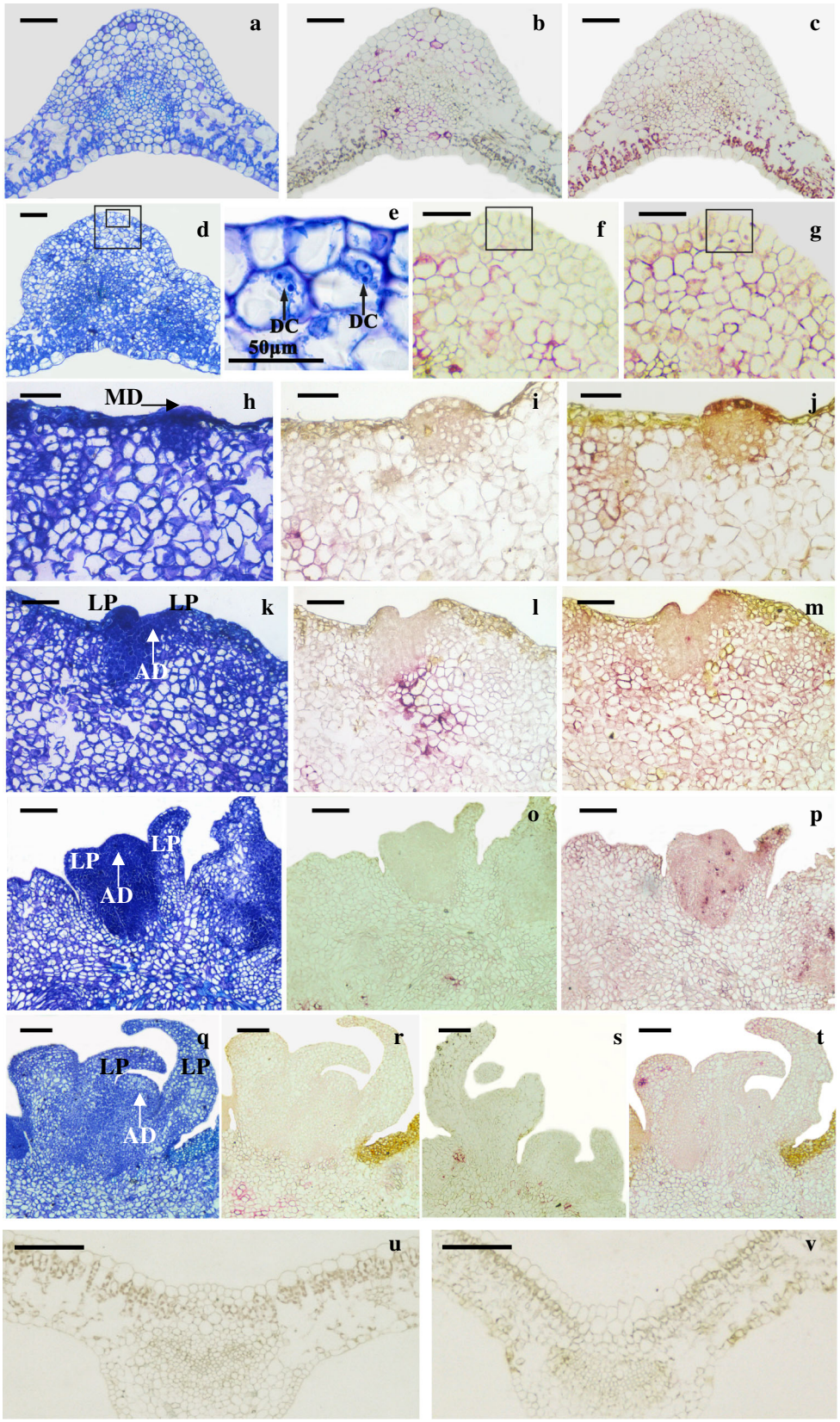

Fig. 3 Histological observation and virus localization in adventitious shoots regenerated from leaf segments of 'Gala' apple. Histological structures of adventitious shoot regeneration in leaf segments cultured for 0 day (**a**), 4 days (**d**, **e**), 10 days (**h**), 2 weeks (k), 3 weeks (**n**) and 4 weeks (**q**). (**e**) A close-up view from the *small square* of (**d**) showing the first cell division. ASPV localization in leaf segments and adventitious buds after 0 day (**b**), 4 days (**f**), 10 days (**i**), 2 weeks (**l**), 3 weeks (**o**) and 4 weeks (**r**, **s**) of culture. (**f**) A close-up view from the *big square* of (**d**) and *square* in (**f**) indicated an area in which the first cell divisions started. ASGV localization in leaf segments and adventitious shoots after 0 day (**c**), 4 days (**g**), 10 days (**j**), 2 weeks (**m**), 3 weeks (**p**) and 4 weeks (**t**) of culture. (**g**) A close-up view from the *big square* of (**d**) and *square* in (**g**) indicated an area in which the first cell divisions started. Healthy control for ASPV (**u**) and ASGV (**v**). With immunohistochemical staining for virus localization, virus-infected tissues showed purple-red color reactions, while healthy tissues did not show any color reaction. *AD* apical dome, *DC* dividing cells, *LP* leaf primordia, *MD* meristematic dome. Unless stated otherwise, *bars* 100 μm

containing more than four LP formed after 4 weeks of culture (Fig. 3q). At this stage, ASPV was not detected in the whole adventitious buds in eight out of thirty samples (27 %) (Fig. 3r), but observed in the basal parts of the buds in the remaining 22 samples (73 %) (Fig. 3 s). The distance of the infected tissue to the AD was estimated to be about 0.2–0.3 mm in length. Comparatively, ASGV widely spread inside the adventitious buds (Fig. 3t).

Discussion

To the best of our knowledge, this is the first report on apple virus eradication using shoot tips of adventitious shoots regenerated from leaf segments. With the protocol described here, ASPV could be completely eradicated, although ASGV was still difficult to eradicate.

When shoot tip-related techniques were applied for virus eradication, virus-free frequency was inversely proportional to the size of the excised shoot tip, while the regeneration ability was positively proportional to size (Faccioli and Marani 1998; Mink et al. 1998; Laimer and Barba 2011). Plopa and Preda (2013) reported that shoot recovery rate was 40–50 % in 0.3 mm shoot tips, much lower than 80–95 % of shoot recovery in 1.0 mm shoot tips of three apple cultivars. Frequencies of *Apple mosaic virus* (ApMV)-free plants resulted from shoot tip culture were 50–58 % in 0.3 mm shoot tips and 17–23 % in 1.0 mm (Plopa and Preda 2013). Our previous study (Li et al. 2016) also found that shoot regrowth rates significantly increased from 0 to 92 % as the size of shoot tips increased from 0.2 mm containing two LP to 1.0 mm containing four LP. While 0.5 mm shoot tips containing three to four LP could not eradicate ASGV and ASPV, 0.5 mm shoot tips containing two LP produced 100 % ASPV-free shoots, although these failed to produce any ASGV-free shoots in the two apple rootstocks 'M9' and 'M26'. Higher shoot regrowth in the larger shoot tips than in the smaller ones, and greater frequency of virus eradication in the latter than in the former were also noted in previous studies on other apple cultivars (Huang and Millikan 1980; Kaushal et al. 2005) and other plant species such as cassava (Kartha and Gamborg 1975), plum (Brison et al. 1997) and grapevine (Wang et al. 2003).

ASPV-free frequency obtained in this study was higher than or comparable with the previous studies on *Malus* plants using various methods (Paprstein et al. 2008; Tan et al. 2010; Sedlak et al. 2011; Hu et al. 2012, 2015; Plopa and Preda 2013; Li et al. 2016). In addition, production of shoot tips used for virus eradication in this study was much more efficient than those reported previously. In most of the previous studies on *Malus* species, meristems were excised from apical buds of 4 or 6 weeks-old stock cultures (O'Herlihy et al. 2003; Paprstein et al. 2008; Sedlak et al. 2011; Li et al. 2016), and only one shoot tip could be obtained from each stock shoot in that length of time. In our study, the first three fully-opened leaves from one stock shoot were used and produced at least 24 shoot tips (three leaves × eight shoots per leaf segment) within 7 weeks. Thus, this production rate of shoot tips is almost 12 times that of the previous studies.

Studies using in vitro organogenesis for virus eradication have been quite limited. Han et al. (2006) found that high rates of anther- and filament-dervied friable callus were infected by *Lily symptomless virus* (LSV) and *Cucumber mosaic virus* (CMV), and the majority of bulblets developed were also virus-infected, while anther-wall-derived nodular callus were rarely virus-infected and high frequencies (70 %) of regenerants were free of LSV and CMV. Ayabe and Sumi (2001) reported that four viruses including *Leek yellow stripe virus* (LYSV), *Onion yellow dwarf virus* (OYDV), *Shallot* (and garlic) *latent virus* (SLV, GLV), and *Garlic common latent virus* (GCLV) could be completely eradicated in shoots regenerated from dome-shaped structures that developed on the surface of stem-disc explants of garlic (*Allium sativum*) cloves. In their study, the dome-shaped structures were excised at different developmental stages, and only those (0.5–1.0 mm in size) excised within 7–10 days of culture produced virus-free regenerants, while those (>1.0 mm in size) excised after 2 weeks were still virus-infected. These results indicated that virus eradication using dome-shaped structures was correlated with size and developmental stage (Ayabe and Sumi 2001). Similar results were also noted in the present study.

Virus trafficking inside plants was believed to be through cell-to-cell movement by plasmodesmata for short distances and though vascular bundles for long distances (Hull 2002). Ayabe and Sumi (2001) found that no plasmadesmata and vascular tissues were observed in the dome-shaped structures, and primary buds developed on the stem-disc of garlic, and plantlets regenerated were virus-free. The pre-cambia were observed in the shoots regenerated from dome-shape structures after 2 weeks of culture, and plantlets regenerated were virus-infected. The study of Ayabe and Sumi (2001) did not localize the viruses, and could, therefore, not identify if dome-shaped structures originated from virus-infected tissues or from the healthy tissues. In the present study, we revealed that the meristematic domes were most likely to originate in healthy tissues and thus escape ASPV infection. During the first 3 weeks of adventitious bud development, ASPV movement from the infected tissues of the leaf segments could not keep up with the growth speed of the adventitious buds, thus resulting in production of virus-free shoot tips of 0.3 mm in size containing three LP. After 3 weeks of shoot regeneration, ASPV movement was fast enough to invade the basal parts of the shoot tips, leaving only 0.2–0.3 mm

in size free of ASPV. Certainly, the mechanism as to why ASPV failed to invade 0.2–0.3 shoot tips found in the present study was probably more complex than just a matter of virus movement speed. High auxin concentration, competition for nucleic acids and highly metabolic activity in fast-growing shoot tips may prevent virus from entering shoot tips, thus resulting in production of ASPV-free shoot tips (Faccioli and Marani 1998; Laimer and Barba 2011). For ASGV, the meristem domes initiated from the virus-infected tissues, thus resulting in infection of the shoot tips, regardless of size and developmental stage of the adventitious shoot regeneration.

Somaclonal variation, which may occur in in vitro-derived regenerants, is a major concerned issue on potential application of adventitious shoot regeneration including apple (Dobránszki and Teixeira da Silva 2010). Existing studies showed that no polymorphic bands were detected by inter-simple sequence repeat (ISSR) (Pathak and Dhawan 2010, 2012) and by random amplified polymorphic DNA (RAPD) (Caboni et al. 2000; Montecelli et al. 2000; Gupta et al. 2009) in plantlets regenerated from in vitro-derived cultures of apple rootstocks and cultivars including 'Gala'. Using the same protocol of adventitious shoot regeneration of 'Gala' apple, our previous study did not detect any polymorphism analyzed by ISSR in regenerants (Li et al. 2014). Indeed, somaclonal variations were detected in in vitro-derived regenerants by RAPD in apple cultivars 'Golden Delicious Bovey' and 'Goldspur' (Viršcek-Marn et al. (1998), and rootstock 'MM106' (Modgil et al. 2005). Although types of explants, culture conditions, and genotype were shown to be responsible for somaclonal variation in in vitro-derived apple plants, variation was governed more by the genotype than by culture conditions (Dobránszki and Teixeira da Silva 2010). Thus, the studies of Montecelli et al. (2000) and Li et al. (2014) demonstrated that 'Gala' may be more genetically stable than other apple cultivars and rootstocks.

In conclusion, ASPV can be completely eradicated, although ASGV cannot, if the proper size of shoot tips is excised at the proper developmental stage of adventitious shoot formation from leaf segments of 'Gala' apple. Frequencies of the virus eradication were higher than or comparable with those reported in the previous studies, and production of the shoot tips that were used for virus eradication was much more efficient than all previous studies. The protocol described here could have potential applications in other plant species.

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