ORIGINAL ARTICLE



Histological and endogenous plant growth regulators changes associated with adventitious shoot regeneration from in vitro leaf explants of strawberry (*Fragaria* × *ananassa* cv. 'Honeoye')

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Abstract Few studies have been conducted on the links between histological and hormonal variation during shoot regeneration. Therefore, we investigated this link in strawberry (Fragaria × ananassa cv. 'Honeoye'). Main plant growth regulators were measured using reverse-phase liquid chromatography-tandem mass spectrometry. Histological observations were conducted to understand the pattern of adventitious shoot regeneration from the leaf segments. After 14 days of dark culture, a mean shoot regeneration frequency of 94.7 % was obtained on MS medium supplemented with 2.0 mg L^{-1} thidiazuron, 30 g L^{-1} sucrose, and 6 g L^{-1} agar (pH 5.9). During shoot regeneration, indole acetic acid (IAA) concentrations increased, abscisic acid (ABA) decreased, and gibberellic acid (GA₃) and zeatin showed peaks. The results could be correlated with the cell division and differentiation that occurred during shoot regeneration. Histological observation showed that the adventitious shoots were derived from subepidermal cells and the epidermal cells of the midrib near the cut. The meristemoids, primordia and shoots were formed sequentially on day 6, day 12, and day 18 after culture. During the meristemoids formation on day 6 after culture, IAA rapidly increased from 1.49 to 1.72 ng g^{-1} fresh weight (Fw), ABA rapidly decreased from 52.61 to 13.47 ng g^{-1} Fw, and zeatin increased from 1.68 to 5.98 ng g^{-1} Fw. During primordia formation on day 12 after culture, IAA

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Wanmei Jin jwm0809@163.com rapidly increased to 1.88 ng g^{-1} Fw, ABA rapidly decreased to 2.69 ng g^{-1} Fw, GA₃ peaked at 73.91 ng g^{-1} Fw, and zeatin peaked at 7.69 ng g^{-1} Fw. Our results suggested that the histological variations were consistent with the plant hormonal changes during shoot regeneration, and that changes in hormone concentration could be used as a reference to characterize the mode of shoot regeneration.

Keywords 'Honeoye' · Leaf disk regeneration · Histology · Plant growth regulators · HPLC–MS/MS

Abbreviations

Abscisic acid
Gibberellic acid
Mercuric chloride
6-benzyladenine
Indole-3-butyric acid
Thidiazuron
Iindole-3-acetic acid
3-Benzo[b]selenienyl acetic acid
N ⁶ (2-isopentenyl)adenosine
N ⁶ (2-isopentenyl)adenine

Introduction

Strawberry (*Fragaria* \times *ananassa*), belonging to the Rosaceae family, is a very popular fruit with high market demand because of its unique aroma, sweet taste, bright color and nutritional value. It is an ideal material for genetic research because it has a short life cycle and an abundant seed set on self-pollination and a high regeneration rate, which overcomes the limitations associated with the traditional breeding

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methods. *F. ananassa* has been used in *Agrobacterium tumefaciens*-mediated transformation over the past 25 years (Barceló et al. 1998; Gruchała et al. 2004; Nehra et al. 1990a). Strawberry cultivar 'Honeoye', with excellent traits, is a particularly suitable material for genetic transformation because of its high regeneration rate in vitro conditions.

Adventitious shoots regeneration by strawberry is affected by internal signals and external conditions. In vitro techniques for its regeneration have been reported using different genotypes, explant types, basal medium components, plant growth regulators, ethylene inhibitor (silver thiosulfate), and the duration of dark culture (Cosic et al. 2015; Haddadi et al. 2013; Jones et al. 1988; Nehra et al. 1990b; Passey et al. 2003; San et al. 2015; Sorvari et al. 1993). Considerable evidence suggests an important role for plant growth regulators in regulating shoot regeneration. Each class of hormones has characteristic biological effects (Pan et al. 2010). Recent results suggested that cytokinins are an important regulatory factor of plant meristem activity and morphogenesis, with opposing roles in shoots and roots (Werner et al. 2003). Both auxins and cytokinins are required for shoot regeneration (Cosic et al. 2015; Jones et al. 1988; Landi and Mezzetti 2006; Passey et al. 2003). Exogenously applied abscisic acid (ABA) at low concentrations has a positive role on callus growth and organogenesis (Paulraj et al. 2014). Preculturing stock plants on regeneration medium with hormones, including gibberellic acid (GA₃) promoted shoot regeneration in the Finnish 'Hiku' strawberry and the Norwegian 'Jonsok' strawberry (Sorvari et al. 1993). Despite the recognized importance of plant hormone levels on shoot organization in vitro, few studies have been published on endogenous hormone through shoot regeneration.

Histological studies have been widely used during in vitro culture, including shoot regeneration (Cosic et al. 2015; Mandal and Gupta 2001; Pereira et al. 2000; Takagi et al. 2011; Trivedi 2014; Varshney et al. 2011; Vatankhah et al. 2014; Yin et al. 2013). On the one hand, histology reveals the regeneration pathway, thereby providing a strong basis for establishing the proper regeneration system. On the other hand, the number of cells involved in the shoot regeneration process is almost always a very small fraction of the total number of cells of explants (De Klerk et al. 1997). In molecular and biochemical studies, typically all of the cell material is analyzed. Thus, to establish fully investigate regeneration, molecular and biochemical studies should be accompanied by appropriate histological analyses. However, few precise histological analyses have been carried out to determine the in vitro shoot regeneration in F. ananassa. Furthermore, hormonal analysis and histological studies have not been analyzed together during the developmental stages of shoots regeneration in vitro.

In this study, we aimed to develop an efficient protocol for in vitro shoot regeneration of 'Honeoye' and to carry out detailed histological observations and hormonal analysis during shoot regeneration from cultured leaf explants. The links between histological changes and hormonal variations were also determined for the first time.

Materials and methods

Plant materials preparation

Plant materials of 'Honeoye' were obtained from the Beijing Academy of Agriculture and Forestry Sciences, Beijing, China. The protocols for sterilization and sample preparation were optimized according to the protocol described by Jin et al. (2014). Buds were collected from runners of 'Honeove' in the fall, and then washed in tap water for 3 h. The buds were surface disinfected with 0.1 % (wt/vol) mercuric chloride (HgCl₂) for 6 min and washed three times with sterile distilled water. The disinfected buds were then placed on basic MS medium (Murashige and Skoog 1962) containing 0.2 mg L^{-1} 6-benzyladenine (BA) and 0.1 mg L^{-1} indole-3-butyric acid (IBA). After 30 days, the shoots that developed from the buds were subcultured onto MS medium containing 0.5 mg L^{-1} BA and 0.1 mg L^{-1} IBA. The basic MS medium contained 30 g L^{-1} sucrose and 6.0 g L^{-1} agar (Shishi Co., Fujian, China). The pH was adjusted to 5.9 and media were autoclaved for 20 min at 121 °C. Cultures were maintained in a growth room at 25 ± 2 °C under a 16-h light photoperiod at approximately 25 μ mol m⁻² s⁻¹ supplied by cool-white fluorescent lamps.

Adventitious shoot regeneration

The protocol for in vitro shoot regeneration were optimized according to that described by Jin et al. (2014). The first, second, and third leaves from the apex were obtained from in vitro cultures after 30-35 days. These leaves were cut on the vein into $3-5 \text{ mm}^{-2}$ discs and cultured on MS medium containing 2.0 mg L^{-1} thidiazuron (TDZ) for use as explants (Yin et al. 2003; Fig. 2a). 25 explants were randomly placed into each 100 mL Erlenmeyer flask (90 mm in diameter, adding 40 mL of medium). To study the effects of an initial dark period on adventitious shoot formation, the explants were kept in the dark for day 0, 7, 14, 21, and 28 days under the growth conditions described in "Plant materials preparation". Adventitious shoot regeneration rate (%) and number of shoots per explant were recorded after 42 days of culture (0:42 days at 16-h light photoperiod; 7:7 days at dark and 35 at 16-h light photoperiod; 14:14 days at dark and 28 at 16-h light photoperiod: 21:21 days at dark and 21 at 16-h light photoperiod; 28:28 days at dark and 14 at 16-h light photoperiod). There were five replications for each treatment and the experiment was repeated three times. The best regeneration conditions (initial 0, or 7, or 14, or 21, or 28 days of dark culture) will be applied for preparing leaf explants at 0, 3, 6, 9, 12, 15, 18, and 21 days of culture for histological observations and plant growth regulators analysis. In the present study, the best regeneration condition was initial 14 days of dark culture, then 0, 3, 6, 9, 12, 15, 18, and 21 days of culture would be as follows: 0:0 days at dark; 3:3 days at dark; 6:6 days at dark; 9:9 days at dark; 12:12 days at dark; 15:14 days at dark and 1 day at 16-h light photoperiod; 18:14 days at dark and 4 days at 16-h light photoperiod; 21:14 days at dark and 7 days at 16-h light photoperiod.

The adventitious shoot regeneration rate was calculated as the total number of regenerating explants divided by total number of explant 100 %. The number of regenerated shoots per explant was calculated as the total number of adventitious shoots divided by total number of regenerating explants.

Histological observations

Histological studies were conducted to understand the pattern of adventitious shoot initiation and formation from the leaf explants of 'Honeoye'. 25 leaf explants cultured on MS medium containing 2.0 mg L^{-1} TDZ were sampled from the medium every 3 days after culture initiation. The explants were fixed in formaldehyde-acetic acid fixative solution (70 % ethanol-formalin-acetic acid = 18:1:1) for 48 h, dehydrated through an increasing ethanol series (70, 85, 95 and 100 %) according to Yin et al. (2013). The tissue was transparent through an increasing dimethylbenzene series (50 and 100 %). After that, the tissue was infiltrated by an increasing liquid paraffin series (50, 70 and 100 %). The wax infiltrated tissue was transferred to a brown paper rectangular dish filled with melted wax at 62 °C. The block containing the tissue sample was solidified at room temperature. Samples were sectioned in 7-10 µm slices with a microtome (Leica RM2245, Leica Microsystems, Heidelberg, Germany), stained with safranin (1 % prepared in ultrapure water) followed by fast green (1 % prepared in 95 % ethanol), and then dried at 35 °C for 4-6 h. The stained sections were permanently mounted on slides and observed using a light microscope (Motic MC 2000, Motic Corp., Germany).

Quantitative analysis of major plant growth regulators

Leaf explants of 'Honeoye' cultured on MS medium containing 2.0 mg L^{-1} TDZ were also sampled every 3 days after culture initiation for quantitative analysis of major plant growth regulators, including indole-3-acetic acid (IAA), ABA, GA₃ and zeatin, according to the method proposed by Pan et al. (2010). The protocol provides quantification of most major plant hormones in a single run from 50 mg of fresh plant tissue using reverse-phase liquid chromatography-tandem mass spectrometry with multiple reaction monitoring (Pan et al. 2010). 125 leaf explants of each developmental stage were frozen in liquid nitrogen and ground into powder. Around 50 mg powder was then transferred to 2 mL screw-cap tubes. 50 µl of the resulting supernatant was injected into a Agilent 1260 Infinity series HPLC system (Agilent Technologies, Santa Clara, CA USA) for chromatographic separation, before detection by tandem mass spectrometry (MS/MS) using an AB SCIEX QTRAP 5500 LC/MS/MS System (AB SCIEX Deutschland GmbH, Darmstadt, Germany).

Statistical analysis

Statistical analyses were performed in SPSS 11.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was applied for parameters (adventitious shoot regeneration rate, number of shoots per explant) to evaluate the effect of dark treatment over time. The significance of differences was determined according to Duncan's multiple range test. Differences at p values <0.05 were considered significant. Variation partitioning was conducted following Anderson and Cribble (1998). Variation partitioning would partition the variance of adventitious shoots regeneration into three fractions namely, the individual effects of exogenous plant growth regulators (IAA, ABA, GA3, and zeatin), joint effects of TDZ and endogenous plant growth regulators changes.

Results and discussion

Overall process of shoot regeneration

The process of adventitious shoot regeneration of 'Honeoye' is shown in Fig. 1. Leaf explants cultured on medium with TDZ at 2.0 mg L^{-1} were sampled for histological evidence of morphogenesis and plant hormone measurement. On day 0, the leaf explants were dark green. On day 6, the leaf explants appeared lighter green, and showed elongation and enlargement. The leaf explants then continued to enlarge, and bridge-like structures appeared on day 12 of culture. On day 15, indirect shoot organogenesis from very small callus was formed from the cuts of explants. A large callus mass formed on day 18 of culture. Relatively small shoots were observed that were sporadically dispersed within the callus on day 21 of culture and

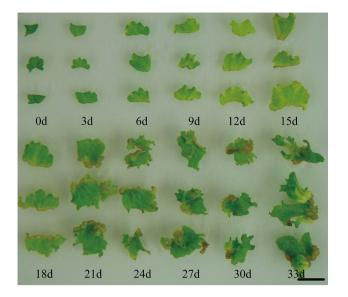


Fig. 1 Adventitious shoot regeneration process of leaf explants of 'Honeoye' with initial 14 days of dark culture on MS medium containing 2.0 mg L^{-1} TDZ. *Bar* 1 cm

clustered shoots were observed on day 27. The number of adventitious shoots and their growth increased over time, with most shoots being longer on day 33 of culture. Interestingly, a polarity phenomenon was observed. Adventitious shoots mainly occurred from the main vein of the leaf. More adventitious shoots occurred from the cuts near the petiole base, and fewer adventitious shoots occurred from the cuts near the leaf tips.

Effect of initial dark culture on adventitious shoot regeneration from the leaves of 'Honeoye'

To determine the conditions for high efficiency shoot regeneration from the leaves of 'Honeoye', various periods of initial dark culture (from 0 to 28 days) were applied. Control explants (0 day, no initial dark culture treatment) produced few adventitious shoots (Fig. 2b). When explants were kept in initial dark culture, adventitious shoot regeneration occurred (Fig. 2c-f). The adventitious shoot regeneration rate and the number of shoots per explant after the initial dark culture were all significantly higher than in the control (a; Table 1), but were not different from each other (b; Table 1). The highest adventitious shoot regeneration rate (94.67) was obtained after 14 days of dark culture. The highest number of shoots per explant was 4.14, which was achieved after 21 days of dark culture. A lower shoot regeneration rate was observed when the period of darkness was extended to 28 days. This result showed that the duration of dark culture was a key factor for adventitious shoot regeneration in 'Honeoye'.

Three types of response to dark treatment have been reported, including a promotion, no effect, and inhibition. For Malus domestica, dark treatment (1-3 weeks) was necessary to obtain a high frequency of shoot regeneration (Dobránszki and Teixeira da Silva 2010; Jin et al. 2014). For Prunus domestica, dark treatment had no effect (Petri and Scorza 2010). For Prunus armeniaca, dark treatment inhibited shoot regeneration (Wang et al. 2011). Little information is available on the effect of dark culture on strawberries. Barceló et al. (1998) reported that the optimum incubation conditions for strawberries included 2 weeks in the dark, with subsequent transfer to light. Similarly, in our study, the highest adventitious shoot regeneration was also achieved with 14 days of initial dark culture, which indicated that dark culture is a key factor in the regeneration of 'Honeoye'.

Histology during shoot regeneration

The adventitious shoots were derived from subepidermal cells (Fig. 3; Fig. S1) and the epidermal cells of the vein near the cut of the leaf explants of 'Honeoye' (Fig. 4; Fig. S2). On day 0 of culture, anatomical observations of transverse sections of the leaf explant showed a typical dicotyledonous leaf-like structure, with an intact upper and lower epidermal surface, normally spaced veins, palisade tissue and spongy tissue. The leaf had no apparent meristematic tissues (Fig. 3a). After 3 days of culture, the cultured explants exhibited enlarged epidermal cells and mesophyll cell expansion (Fig. 3b). During the early stages of callus formation, subepidermal cells resumed their meristematic activity and produced primary callus. Dense contents of the subepidermal cells also became more obvious. From day 6 to 9 of culture, meristemoids (MS) formed with densely stained cytoplasmic cells (Fig. 3c, d). A vascular (VS) connection between the MS and the vein was also observed. The shoot primordia (SP) formed and enlarged from day 12 to 18 of culture (Fig. 3e-g). Fully developed adventitious shoots (ADS) with vascular structures appeared on the surface of these callus within 21 days (Fig. 3h, i). The regeneration pattern from subepidermal cells was similar to those reported in Carthamus tinctorius (Mandal and Gupta 2001), Lilium Oriental hybrid 'Siberia' (Yin et al. 2013), and Brassica oleracea var. gongylodes (Cosic et al. 2015). When the adventitious shoots were derived from the epidermal cells, the MS and SP also formed on day 6 and day 12 of culture (Fig. 4). We did not observe any vascular connection between the MS and the leaf vein (Fig. 4c, d). The regeneration pattern from epidermal cells was similar to that reported in T. oldhamii (Takagi et al. 2011). The process of organogenesis varied from explant to explant, including shoot-like structures or

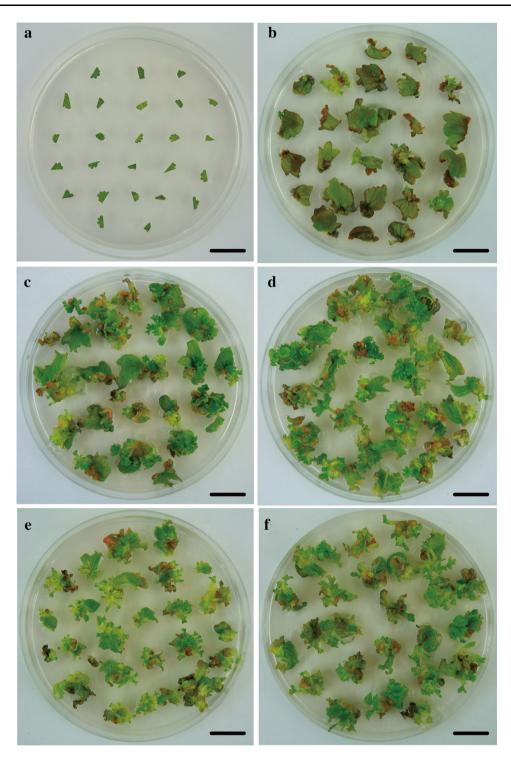


Fig. 2 Adventitious shoot regeneration from cultured leaves of 'Honeoye'. **a** Leaf discs at the time of culture initiation (day 0). **b** Adventitious shoot regeneration from leaves when explants were kept in the light after 42 days on MS medium containing 2.0 mg L^{-1}

TDZ. **c-f** Adventitious shoot regeneration from leaves when explants were kept in the dark for 7, 14, 21 or 28 days after 42 days on MS medium containing 2.0 mg L^{-1} TDZ. *Bars* 1 cm

well developed shoots (Figs. 3, 4h, i). The differences in morphogenic responses could reflect the pre-disposed genetic condition of the explant (Varshney et al. 2011).

Moreover, Pereira et al. (2000) reported that shoot formation on *Pothomorphe umbellata* originated from epidermal and subepidermal cells of the midrib abaxial surface. Table 1Mean values on shootregeneration rate and shootnumber/explant of *F.ananassa*cv. Honeoye leaf explants fordifferent dark culture durationtreatments in TDZconcentration of 2.0 mg L^{-1}

Dark treatment (day)	Adventitious shoot regeneration rate (%)	No. shoots per explant
Control (0)	$40.0\pm4.0^{\rm a}$	$2.3 \pm 0.2^{\mathrm{a}}$
7	86. 7 \pm 6.7 ^b	3.3 ± 0.4^{b}
14	$94.7 \pm 5.3^{\rm b}$	3.4 ± 0.3^{b}
21	$90.7 \pm 7.4^{\rm b}$	4.1 ± 0.3^{b}
28	$85.3 \pm 8.7^{\rm b}$	3.5 ± 0.1^{b}

MS basal medium contained 2.0 mg L⁻¹ TDZ. Values were calculated after 42 day. Values represent mean \pm SE. a, b letters indicate statistically significant differences within treatments (Duncan's honestly significant difference test, p < 0.05)

0:42 days at 16-h light photoperiod

7:7 days at dark and 35 days at 16-h light photoperiod

14:14 days at dark and 28 days at 16-h light photoperiod

21:21 days at dark and 21 days at 16-h light photoperiod

28:28 days at dark and 14 days at 16-h light photoperiod

The present study reported a detailed histological description of the adventitious shoot regeneration from the subepidermal cells and epidermal cells, which eventually developed into adventitious shoots, with callus formation. El Mansouri et al. (1996) made similar observations in *Fragaria vesca*, which revealed active proliferation of subepidermal cells at 2 weeks, after which meristem-like structures could be observed, and shoot regeneration along the periphery of the explants was observed within 4–6 weeks: most explants showed well developed shoots (5 mm) within 8 weeks.

Plant growth regulators variation and its connection with histological changes during shoot regeneration

To determine the links between histological and plant growth regulators changes during shoot regeneration of strawberry, the plant growth regulators content (IAA, ABA, GA_3 , and zeatin) in samples of 0, 3, 6, 9, 12, 15, and 18 days of culture, were measured using HPLC-MS/MS. Qualified HPLC-MS/MS spectra allowed us to identify plant growth regulators, including IAA, ABA, GA₃, and zeatin, derived from the explants during shoot regeneration. Representative HPLC-MS/MS spectra of ABA during shoot regeneration in 'Honeoye' leaves are shown in Fig. S3. The retention times for samples of 0, 3, 6, 9, 12, 15, and 18 days of culture were 4.21 min for both the external standard (D6-ABA) and ABA analytes, with a total run time of 8.0 min. We observed a stable intensity of D6-ABA and a decrease in intensity of ABA during adventitious shoot regeneration of 'Honeoye' leaves.

During the course of adventitious shoots regeneration in 'Honeoye' leaves, the IAA concentration gradually increased from 1.49 ng g^{-1} fresh weight (Fw) to 1.98 ng g^{-1} Fw (Fig. 5a). During the time when MS developed (day 3–day 6) and shoot primordia formed (day

12-day 15), the IAA concentration increased most rapidly. During the course of adventitious shoots regeneration, the ABA concentration gradually decreased from 52.61 to 2.69 ng g^{-1} Fw (Fig. 5b). When the MS and shoot primordia appeared, the ABA concentration decreased most rapidly. During the time course, the GA₃ concentrations varied from 25.08 to 73.91 ng g^{-1} Fw (Fig. 5c). After 12 days, the GA₃ concentration peaked. During the time course, zeatin concentrations varied from 1.08 to 7.69 ng g^{-1} Fw (Fig. 5d). The zeatin concentrations peaked at 5.98 ng g^{-1} Fw during MS formation and reached 7.69 ng g^{-1} Fw during the formation of shoot primordia; little change in zeatin concentration occurred in the remaining period. Many reports have clarified that TDZ alone, or in combination with IBA, 3-benzo[b]selenienyl acetic acid (BSAA) or 2,4-D were effective for shoot regeneration from strawberry leaves (Haddadi et al. 2013; Landi and Mezzetti 2006; Passey et al. 2003). In the present study, adventitious shoots regeneration in 'Honeoye' leaves by interaction between the exogenous plant growth regulator (TDZ) and endogenous plant growth regulators (IAA, ABA, GA3, and zeatin) was observed. However, for the lack of a concentration gradient of TDZ, we failed to obtain the effects of interaction between TDZ and endogenous plant growth regulators changes using statistical methods.

Plant hormone changes in *Arabidopsis thaliana* leaves have been studied extensively. Pan et al. (2010) determined plant growth regulators in *A. thaliana* leaves over 4–5 weeks, revealing the following maxima: Zeatin 9.38 ng g⁻¹ Fw; IAA 43.75 \pm 12.5 ng g⁻¹ Fw; ABA 1.77 \pm 0.61 ng g⁻¹ Fw; and GA₃ 2.29 \pm 0.09 ng g⁻¹ Fw. Farrow and Emery (2012) estimated the concentrations of zeatin (1.02 ng g⁻¹ Fw), IAA (140.35 ng g⁻¹ Fw), and ABA (26.85 ng g⁻¹ Fw) in *A. thaliana* leaves. López-Carbonell and Jáuregui (2005) found

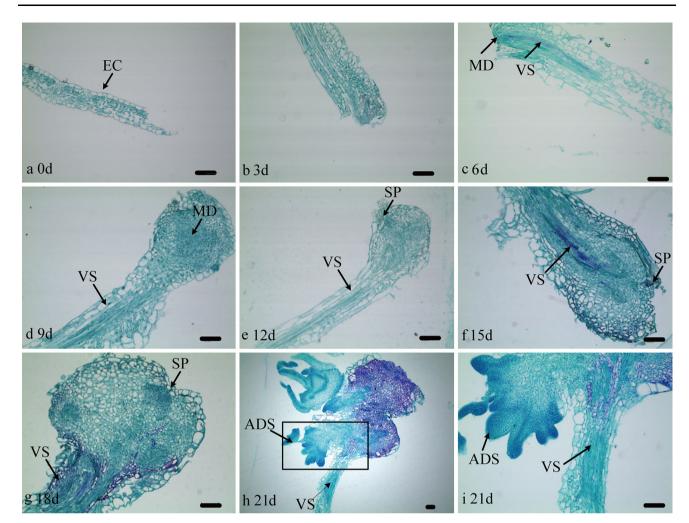


Fig. 3 Adventitious shoot regeneration derived from subepidermal cells of the midrib near the cut in transverse sections of leaf explants of 'Honeoye' by histological observation. **a** A leaf explant at the time of culture initiation (day 0). **b** A leaf explant on day 3 of culture. Some subepidermal cells on the adaxial side had dedifferentiated; these cells contained dense cytoplasm and a conspicuous nucleus. **c** The meristemoids (MD) and vascular structures (VS) formed on day

that control plants had almost constant, low levels of ABA $(2-3 \text{ ng g}^{-1} \text{ Fw})$, while stressed plants had increased ABA contents between the first and second week in *A. thaliana* (from 10 to 21 ng g⁻¹ Fw). The above-mentioned references indicated that IAA concentrations ranged from 40.35 to 143.35 ng g⁻¹ Fw, ABA concentrations ranged from 1.77 to 26.85 ng g⁻¹ Fw, GA₃ concentration was 2.29 ng g⁻¹ Fw, and zeatin concentrations ranged from 0.13 to 9.37 ng g⁻¹ Fw in *A. thaliana* leaves. During the adventitious shoots regeneration in 'Honeoye' leaves, IAA, ABA, GA₃, and zeatin concentrations ranged from 1.49 to 1.98 ng g⁻¹ Fw, 2.69 to 52.61 ng g⁻¹ Fw. The content ranges of plant growth regulators in the present study were comparable with these above mentioned results.

6 of culture. **d** A leaf explant on day 9 of culture. **e** The shoot primordium (SP) appeared on day 12 of culture. **f** A leaf explant on day 15 of culture. **g** A leaf explant on day 18 of culture. **h**, **i** Leaf explants on day 21 of culture, adventitious shoots (ADS) and VS included in the squares are enlarged in **i**. EC epidermal cells. *Bars* 100 μ m

Many studies have shown the effects of exogenous hormones, especially cytokinins, on shoot regeneration in strawberry (Haddadi et al. 2013; Landi and Mezzetti 2006; Passey et al. 2003). However, no endogenous hormone studies during shoot regeneration of strawberry have been reported. In this study, the IAA concentration gradually increased from day 0 to day 18, most rapidly from day 3 to day 6 and from day 12 to day 15. The zeatin concentrations significantly increased at day 6 and day 12 in 'Honeoye'. Consistent with our results, in *Saussurea involucrata*, zeatin and IAA concentrations increased from day 0 to day 15, with IAA increasing rapidly from day 5 to day 10 in the control explants, and the combination of increased zeatin and decreased IAA concentrations was observed in cold-treated explants (Guo et al. 2013). For ABA, Charrière et al. (1999)

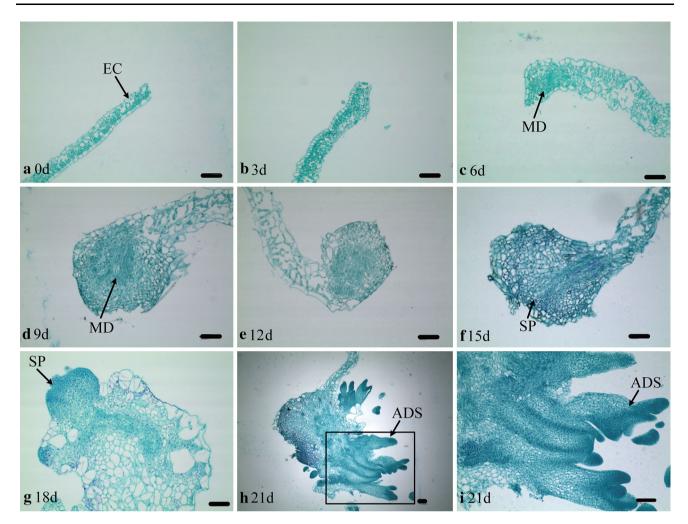
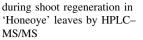


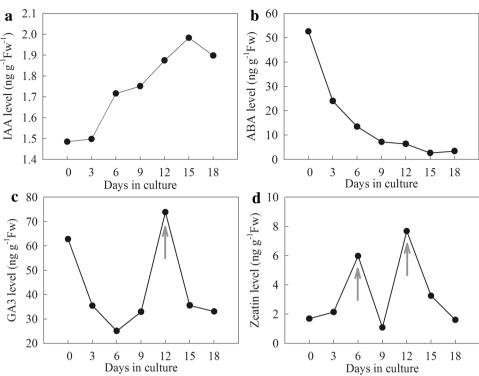
Fig. 4 Adventitious shoot regeneration derived from epiderma cells of the midrib near the cut in transverse sections of leaf explants of 'Honeoye' by histological observation. **a** A leaf explant at the time of culture initiation (day 0). **b** A leaf explant on day 3 of culture. **c** The meristemoids (MD) formed on day 6 of culture. **d** A leaf explant on

day 9 of culture. **e** A leaf explant on day 12 of culture. **f** The shoot primordium (SP) appeared on day 15 of culture. **g** A leaf explant on day 18 of culture. **h**, **i** Leaf explants observed on day 21 of culture, adventitious shoots (ADS) included in the square are enlarged in **i**. EC epidermal cells. *Bars* 100 μ m

showed that in sunflower (Helianthus annuus L.), the level of ABA was elevated at the time of excision, but decreased rapidly during the first 6 h of culture to a very low level. Similarly, the ABA concentration gradually decreased during the course of shoots regeneration. No previous studies on changes of GA₃ concentrations during shoot regeneration have been published. In our study, the GA₃ concentrations peaked during shoot primordial formation. Interestingly, Charrière et al. (1999) also reported that the BA content reached its maximum after 6 h of culture, and then decreased to a constant level after 24 h. N⁶(2-isopentenyl)adenosine (IPA) followed an accumulation pattern slower than BA, with a peak at 15 h of culture. N⁶(2-isopentenyl)adenine (2IP) accumulated later, but progressively, during the culture. These results indicated that the different types of cytokinins must be distinguished to study their effects on shoot regeneration. The mechanisms underlying the changes in endogenous hormones are unclear: molecular studies should be conducted in future research.

Changes in the contents of plant growth regulators during shoot regeneration might identify characteristic compounds specific for shoot regeneration. Nevertheless, few studies have been conducted into the links between histological and hormonal variation during shoot regeneration. In our study, during MS and primordia formation, IAA rapidly increased, ABA rapidly decreased, and GA₃ and zeatin peaked in 'Honeoye'. Similar to our results, Vatankhah et al. (2014) reported the histological and biochemical parameters (content of malondialdehyde, proline, phenolics and saccharides) of *Crocus sativus* during in vitro root and shoot organogenesis. Trivedi (2014) reported changes in antioxidative enzymes and performed histological studies of the in vitro regeneration of groundnut. These studies indicated that it is necessary to combine biochemical studies with





histological studies to fully understand shoot regeneration, and also suggested that changes in hormone concentrations or biochemical parameters could be used as markers to characterize the mode of shoot regeneration.

Conclusions

In the present work in vitro histological observations and plant hormonal measurements were carried out through adventitious shoot regeneration from leaves of strawberry 'Honeoye'. Our results showed that the histological variations were consistent with the plant hormonal changes during its shoot regeneration.

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