

Dynamics of Endogenous Indole-3-acetic Acid and Cytokinins During Adventitious Shoot Formation in Ipecac

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Abstract For most plant species, auxins and cytokinins are added to the culture medium to induce adventitious shoot formation. In ipecac (*Carapichea ipecacuanha* (Brot.) L. Andersson), however, shoots form on internodal segments without phytohormone treatment. This creates an opportunity to analyze the dynamics of endogenous phytohormones during adventitious shoot formation. Ipecac is a medicinal plant whose root extract is used as an expectorant and emetic. We explored the relationships among adventitious shoot formation and levels of an auxin (indole-3-acetic acid) and four cytokinins in ipecac. Adventitious shoots that formed in the apical region of internodal segments were derived from epidermal cells. One of the shoots grew vigorously with a vascular bundle connected to that of the segment, and the others were suppressed in the outgrowth. When the biggest shoot was removed, another began to grow. During adventitious shoot formation, endogenous auxin accumulated in the basal region of segments, whereas cytokinins accumulated in the middle region. Thus, the distribution of auxin, not cytokinins, determined where adventitious shoots formed.

Keywords Adventitious shoots · *Carapichea ipecacuanha* · Internodal segment · Phytohormone-free medium · LC–MS/MS

Introduction

In 1902, Haberlandt proposed the concept of “totipotency,” by which plant cells of any differentiated cell type can divide and differentiate to ultimately regenerate the whole plant (Haberlandt 1902). In tobacco, a high ratio of auxins to cytokinins (CKs) in culture medium induces roots, a low ratio induces shoots, and high concentrations of both induce callus, indicating that plant cells have high plasticity for cell differentiation and organogenesis (Skoog and Miller 1957). The organ regeneration system has since been widely applied for the propagation of economically important crops and for the regeneration of transgenic plants (Ganeshan and others 2002). The effects of exogenously applied auxins and CKs in the formation of adventitious shoots and roots have been extensively studied, and many efforts have been made to determine the optimum concentrations of each in inducing shoots and roots (George and others 2008). Auxins and CKs antagonistically interact to regulate plant growth and development including embryogenesis, shoot and root development, and vascular tissue formation (Schaller and others 2015). However, the optimum concentrations and combinations of phytohormones differ by species and among tissues used as explants (such as leaf, stem, root, and embryo). In addition, little is known about the absorption of exogenous phytohormones in plant tissues and how they affect cell differentiation. To determine the effects of phytohormones in organogenesis, it is necessary to quantify the changes in endogenous phytohormone levels in explants (Djilianov and others 2013;

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Golovatskaya and Karnachuk 2007; Zhang and others 2012).

Adventitious shoots can be induced on phytohormone-free culture media in some plant species, such as *Dianthus caryophyllus* L. (Wataid and others 1996), *Aegle marmelos* (L.) Corrêa (Ajithkumar and Seeni 1998), *Bacopa monnieri* (L.) Pennell (Tiwari and others 2001), *Celastrus paniculatus* Willd. (Rao and Purohit 2006), *Kalanchoë blossfeldiana* Poelln. (Sanikhani and others 2006), and *Carapichea ipecacuanha* (Brot.) L. Andersson (Yoshimatsu and Shimomura 1991), and CK treatment can increase the number of shoots. Changes in endogenous phytohormones within plants can be analyzed during adventitious shoot formation.

Carapichea ipecacuanha (Brot.) L. Andersson (ipecac) is an important medicinal plant whose roots contain alkaloids such as emetine and cephaeline (Teshima and others 1988), which are used as an expectorant, emetic, and an amoebicide (Chatterjee and others 1982). In its native Brazil, ipecac grows as a shrub in tropical rainforests (Yoshimatsu and Shimomura 1993). In Japan, it is cultivated in greenhouses but rarely produces seeds, and the germination rate decreases during seed storage (Yoshimatsu and Shimomura 1993). Therefore, tissue culture methods have been developed to induce shoot formation from the shoot meristem (Ideda and others 1988), adventitious shoot formation on root segments (Yoshimatsu and Shimomura 1994) and internodal segments (Yoshimatsu and Shimomura 1991), and plant regeneration from callus (Rout and others 2000). Adventitious shoot formation on internodal segments induces the most shoots per explant (Yoshimatsu and Shimomura 1991). The method offers two significant clues to the pattern of formation of adventitious shoots: First, adventitious shoots form in the apical region of segments, whereas callus forms in the basal region, indicating polarity (Fig. 1a). Second, one shoot comes to dominate the other shoots that form, indicating an auxin effect (Fig. 1a). Shoots can form on the surface of internodal segments without callusing and can regenerate complete plants (Fig. 1b). In tissue culture, auxins and CKs are generally added to the culture medium to induce adventitious shoots (Skoog and Miller 1957). In ipecac, however, adventitious shoots can form even on phytohormone-free medium (Yoshimatsu and Shimomura 1991). Thus, ipecac culture allows us to analyze the dynamics and effects of endogenous phytohormones during adventitious shoot formation.

It is likely that endogenous phytohormone levels change dramatically during adventitious shoot formation, triggering the expression of genes required for shoot formation. To explore the relationships between shoot formation and phytohormone levels in ipecac, we counted the number of adventitious shoots formed and measured the endogenous levels of one auxin and four CKs in internodal segments by liquid chromatography–tandem mass spectrometry

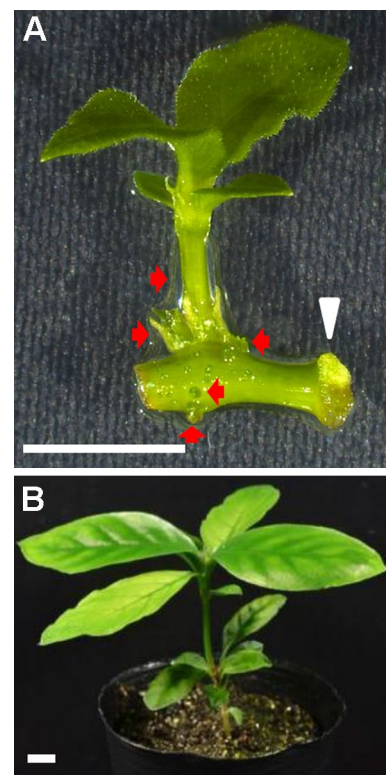


Fig. 1 Adventitious shoot formation in ipecac. **a** Adventitious shoots formed on an internodal segment. *Arrows* adventitious shoots; *Arrow-head* callus; *bar* 5 mm. **b** A regenerated plant; *bar* 1 cm. (Color figure online)

(LC–MS/MS). We analyzed indole-3-acetic acid (IAA), the most abundant type of auxin (Ljung and others 2001), and four major active types of CKs: isopentenyl adenine (iP), isopentenyl adenine riboside (iPR), *trans*-zeatin (tZ), and *trans*-zeatin riboside (tZR), whose biosynthesis is regulated by auxins (Nordström and others 2004; Stolz and others 2011). Here we showed that the level of IAA was low in the apical region of internodal segments, where adventitious shoots formed, but high in the basal region, where shoots did not form. CKs were distributed throughout the segments; in particular, the production of tZR was high before shoot formation.

Materials and Methods

Induction of Adventitious Shoots

We induced adventitious shoot formation on internodal segments according to a previous method (Yoshimatsu and Shimomura 1991). Axenic ipecac shoots (Fig. S1) were subcultured in test tubes (30 mm i.d.×120 mm) containing 20 mL of phytohormone-free B5 medium (Gamborg and others 1968) solidified with 0.2% Gellan Gum (Wako,

Osaka, Japan) at 24 °C under a 14-h light/10-h dark photoperiod (photosynthetic photon flux density, 10–15 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for every 3 months. To induce adventitious shoots, we put 7- to 8-mm-long internodal segments on 25 mL of the same medium in Petri dishes (90 mm diam. \times 20 mm) and cultured them under the same conditions. We used the first (lower) internodes for shoot induction because the number of shoots formed did not differ between the first and second internodes (Fig. S1). Eight to ten segments were used in each experiment, and biologically independent experiments were repeated three times. We counted all shoots longer than 0.3 mm under a digital microscope (DHS1000; Leica Microsystems, Wetzlar, Germany) every week for 10 weeks. To investigate where the shoots formed, we partitioned the segments into four regions (apical to basal region, I–IV) and counted the shoots every week for the first 5 weeks, while their number increased.

Chemicals

IAA and tZR were purchased from Wako Pure Chemical Industry (Osaka, Japan), tZ and iP from Sigma (St. Louis, MO, USA), iPR from Kanto Chemical (Tokyo, Japan), and stable-isotope-labeled compounds used as internal standards (D_5 -IAA, D_5 -tZ, D_5 -tZR, D_6 -iP, and D_6 -iPR) from OlchemIm (Olomouc, Czech Republic).

Preparation of Cryo-Sections

Internodal segments were fixed in FAA solution (formalin:acetic acid:50% ethanol=5:5:90 v/v/v) for 1 day at 4 °C, washed with Milli-Q water to remove the FAA, and placed in 20% sucrose solution for 1 day. They were then embedded in super cryo-embedding medium (Section-Lab, Hiroshima, Japan) and frozen at -100°C in a UT-2000F freezer (Eyela, Tokyo, Japan). The frozen segments were stuck on Cryofilm type 2C(9) adhesive film (Section-Lab) and sliced at 5 μm on a CM3050 S Research Cryostat (Leica Microsystems) at -20°C in a cryo-chamber (Kawamoto 2003). The frozen sections were stained with 0.05% toluidine blue O, mounted on a glass slide, and covered with super cryo-mounting medium type R3 (Section-Lab), which was solidified with a UV Quick Cryosection Mounter (Leica Microsystems). The frozen sections were observed under an optical microscope (BX63; Olympus, Tokyo, Japan).

Phytohormone Extraction and Purification

To analyze the dynamics of endogenous phytohormones during shoot formation, we measured phytohormone levels in regions I–IV every week for the first 5 weeks, while the number of shoots increased. IAA, tZ, tZR, iP, and iPR

in each region were extracted and purified by solid-phase extraction, as described previously (Yoshimoto and others 2009). We placed 20–50 mg of each internodal segment in a 2.0-mL tube with zirconia beads and froze it in liquid nitrogen. The frozen sample was crushed in a TissueLyser II (Qiagen, Hilden, Germany) and suspended in 1 mL of acetonitrile containing 500 pg of each internal standard. The samples were centrifuged at $11,000\times g$ for 5 min at 4 °C, and the pellet was washed with 80% (v/v) acetonitrile containing 1% (v/v) acetic acid. We added 600 μL of water containing 1% acetic acid to the bulked supernatant, evaporated the acetonitrile, and applied the sample to pre-equilibrated Oasis HLB column cartridges (Waters, Milford, MA, USA). After washing with 1 mL of water containing 1% acetic acid, all hormones were simultaneously eluted with 1 mL of 80% acetonitrile containing 1% acetic acid. The eluate was evaporated to leave the extract in the aqueous fraction and applied to pre-equilibrated Oasis MCX column cartridges (Waters). After the cartridges were washed with 1 mL of water containing 1% acetic acid, IAA was eluted with 2 mL of 30% acetonitrile containing 1% acetic acid. The cartridges were then washed with 2 mL of 80% acetonitrile containing 1% acetic acid, then 1 mL of water, and finally 1 mL of water containing 5% aqueous ammonia. CKs were eluted with 2 mL of 60% methanol containing 5% aqueous ammonia. Each fraction was evaporated and stored at -30°C until analysis. All phytohormones were measured by LC–MS/MS.

Quantitative Analysis of IAA and CKs

The IAA fraction was resolved in 20 μL of 30% (v/v) acetonitrile, and CK fractions in 20 μL of water containing 1% (v/v) acetic acid. The samples were analyzed by LC–MS/MS using a triple quadrupole MS system (3200 QTRAP; Sciex, Framingham, MA, USA; parameters in Supp. Tables S1, S2) and an HPLC system (Prominence; Shimadzu, Kyoto, Japan) equipped with an Acuity BEH C18 column (ϕ 2.1 mm \times 100 mm; Waters) for IAA analysis and with a Poroshell EC-C18 column (ϕ 2.1 mm \times 50 mm; Agilent Technologies, Santa Clara, CA, USA) for CK analysis, under the control of the Analyst v. 1.5.1 spectrometer software. In IAA analysis, the mobile phase was changed from 5% acetonitrile containing 0.1% acetic acid to 50% over 7 min after injection, at a flow rate of 0.34 mL min^{-1} . In CK analysis, the mobile phase was changed from 2% acetonitrile containing 0.1% acetic acid to 40% over 5 min after injection, at a flow rate of 0.40 mL min^{-1} . The temperature of the column oven was set at 40 °C. IAA and CKs were quantified on a standard curve of the ratio of unlabeled to deuterium-labeled standards using MultiQuant v. 2.0.2 software (Sciex).

Statistical Analysis

To confirm the differences in the number of adventitious shoots between internodal segments with and without a dominant shoot, a pairwise *t* test was performed. To compare phytohormone levels among different regions (I–IV) in the segments, a multiple comparison was performed by Tukey's honestly significant difference (HSD) test. These analyses were carried out using SPSS 23.0 (IBM SPSS Inc., Armonk, NY, USA).

Results

Time Course of Adventitious Shoot Formation in Ipecac

To investigate when adventitious shoots are formed during internode culture, we observed their formation for 10 weeks. Small shoots formed on internodal segments by 2 weeks of culture (Fig. 2a). By 6 weeks, the number increased to approximately 15 and plateaued (Fig. 2b). By 7 weeks, one shoot became dominant, whereas the others seemed almost to stop growing, indicating that the dominant shoot might suppress the others via apical dominance (Fig. 2a). To confirm the effect of the dominant shoot, we removed it and found that another shoot started to enlarge, which resembles a release from apical dominance (Fig. 3). The number of shoots did not increase further in intact segments, but when the biggest shoot was removed, the number increased to approximately 20 after another 3 weeks, and adventitious roots elongated from callus that formed from the basal region of the segments (Fig. 3).

Internal Structure of Adventitious Shoots and Internodal Segments

To find which tissues formed adventitious shoots in internodal segments, we prepared frozen sections of these segments and examined their internal structure. Very small adventitious shoots were visible by 2 weeks of culture (Fig. 4b). In segments in which adventitious shoots were not observed on the surface, epidermal cells started to divide (Fig. 4b). Shoots were clear on the surface of segments by 3 weeks (Fig. 4a). Cell division activity increased in the cortex and endodermis; a vascular bundle started to differentiate in shoots by 4 weeks and connected to that of the segment by 5 weeks (Fig. 4b).

Distribution of adventitious shoots and endogenous phytohormone levels in internodal segments

To confirm which region of an internodal segment formed adventitious shoots, we partitioned the segments into four

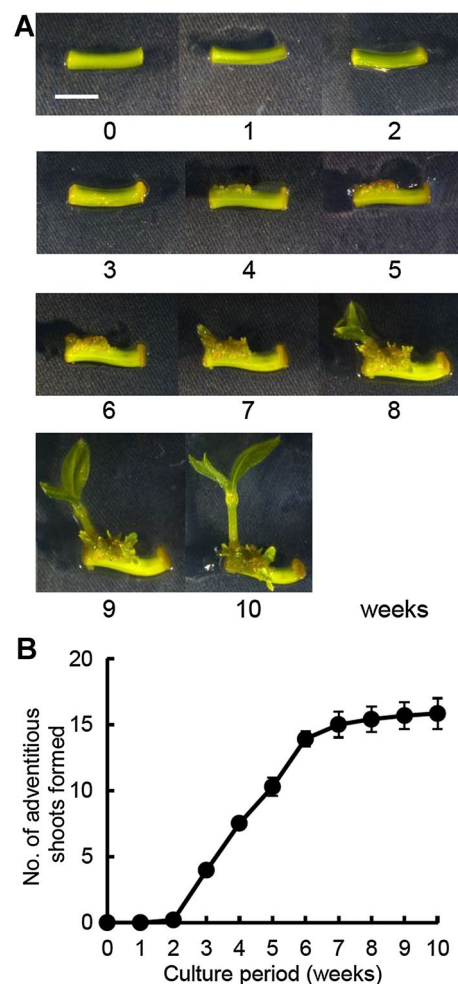


Fig. 2 Time course of adventitious shoot formation in ipecac. **a** Adventitious shoots were observed every week. Photographs show morphological changes in a representative segment; bar 5 mm. **b** Number of adventitious shoots (>0.3 mm) formed. Values are means \pm SEM ($n=3$). Ten segments were used in each experiment

regions (apical to basal regions, I–IV) and counted the shoots formed in each of them every week for the first 5 weeks. Adventitious shoots were mainly formed in the apical regions (I and II; Fig. 5). By 5 weeks of culture, there were approximately seven shoots in region I, 5.3 in region II, 0.4 in region III, and none in the basal region (IV) (Fig. 5).

To explore the relationships between the number of shoots formed and phytohormone dynamics in the internode during adventitious shoot formation, we measured the endogenous levels of IAA and four CKs in each region using LC–MS/MS. Before culture, the IAA level was slightly higher in the apical region (I, 4.1 pg/mg FW) than in the other regions (II–IV, ~2.5 pg/mg FW) (Fig. 6a). After 1 week of culture, it had increased to 11.4 pg/mg FW in region IV, but had decreased to 1.5–2.2 pg/mg FW in

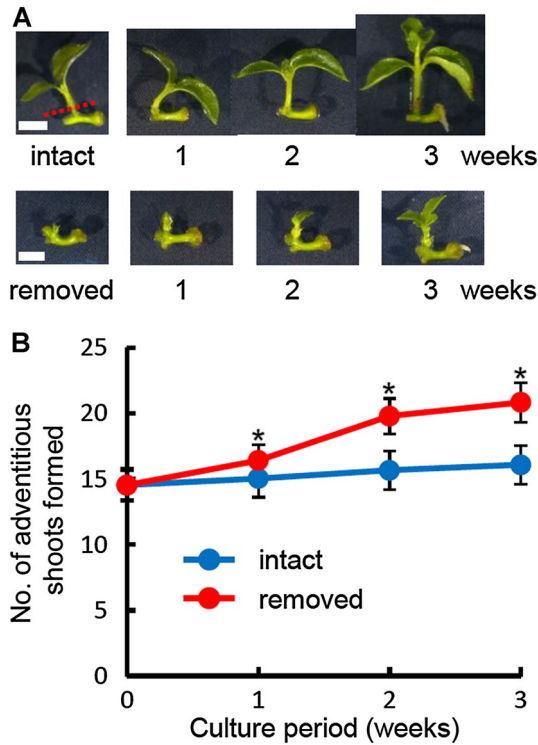


Fig. 3 Removal of the dominant adventitious shoot (*dotted line*). **a** The dominant shoot was removed after 7 weeks of culture and the remained segment cultured for further 3 weeks. Photographs show morphological changes in a representative segment; *bar* 5 mm. **b** Total number of adventitious shoots including newly developed shoots. Values are means \pm SEM. Eight segments were used in each experiment (* $P < 0.05$ by *t* test)

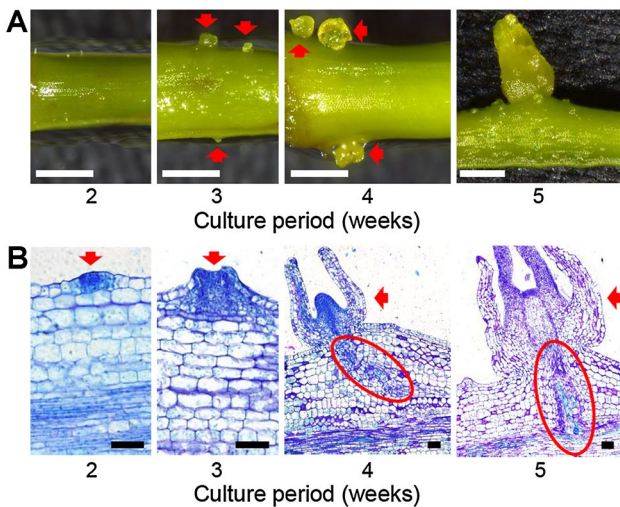


Fig. 4 **a** Surface of an internodal segment. *Arrows* adventitious shoots; *bar* 1 mm. **b** Cross-sections of internodal segments stained with *toluidine blue* O. *Ellipses* vascular bundles; *Arrows* adventitious shoots. *Bars* 100 μ m

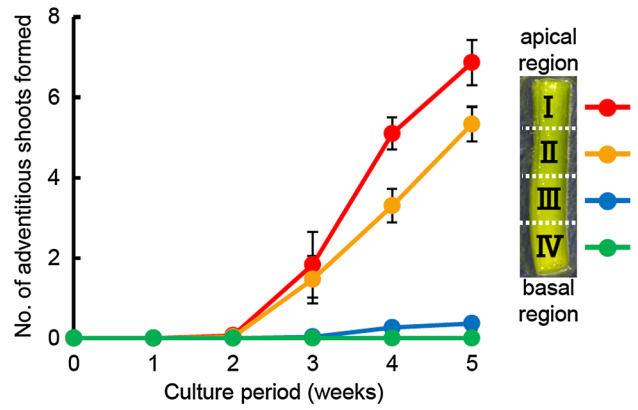


Fig. 5 Distribution of adventitious shoots formed on each of four regions of internodal segments. Values are means \pm SEM ($n = 3$). Ten segments were used in each experiment

regions I–III. After 2 weeks of culture, it had decreased to approximately 4.4 pg/mg FW in region IV, indicating that IAA accumulation was transient. Between 3 and 5 weeks, an IAA concentration gradient between regions I and IV emerged.

Analysis of CK levels in the same samples showed only trace levels of CKs before culture (Fig. 6b). During the culture, they were detected largely in regions II and III. Changes in levels differed between CK types. The tZ-type CKs increased by 1 week and then gradually decreased. In particular, tZR increased to 13.8 pg/mg FW in region II and to 18.1 pg/mg FW in region III. On the other hand, the iP-type CKs gradually increased during the culture from below the limit of quantification; iP increased to 1.8–2.2 pg/mg FW by 5 weeks in region II and iPR increased to around 2.7 pg/mg FW by 4 weeks in region III.

Discussion

Subepidermal cells of internodal segments of ipecac reportedly initiate cell division before adventitious shoot formation (Yoshimatsu and Shimomura 1991). However, our time-course observations revealed that epidermal cells differentiated adventitious shoots (Fig. 4b), as in *Antirrhinum majus* and *Neolamarckia cadamba* (Busse and others 2005; Huang and others 2014). However, it is still unclear why cells differentiate in some parts of the epidermal layer but not others; however, this may result from small-scale variations in phytohormone concentrations due to structural or physiological heterogeneity within a segment.

One of the adventitious shoots on each segment came to dominate (Fig. 3). When that shoot was removed, another shoot started to grow. This is similar to the breaking of apical dominance, a phenomenon in which the shoot

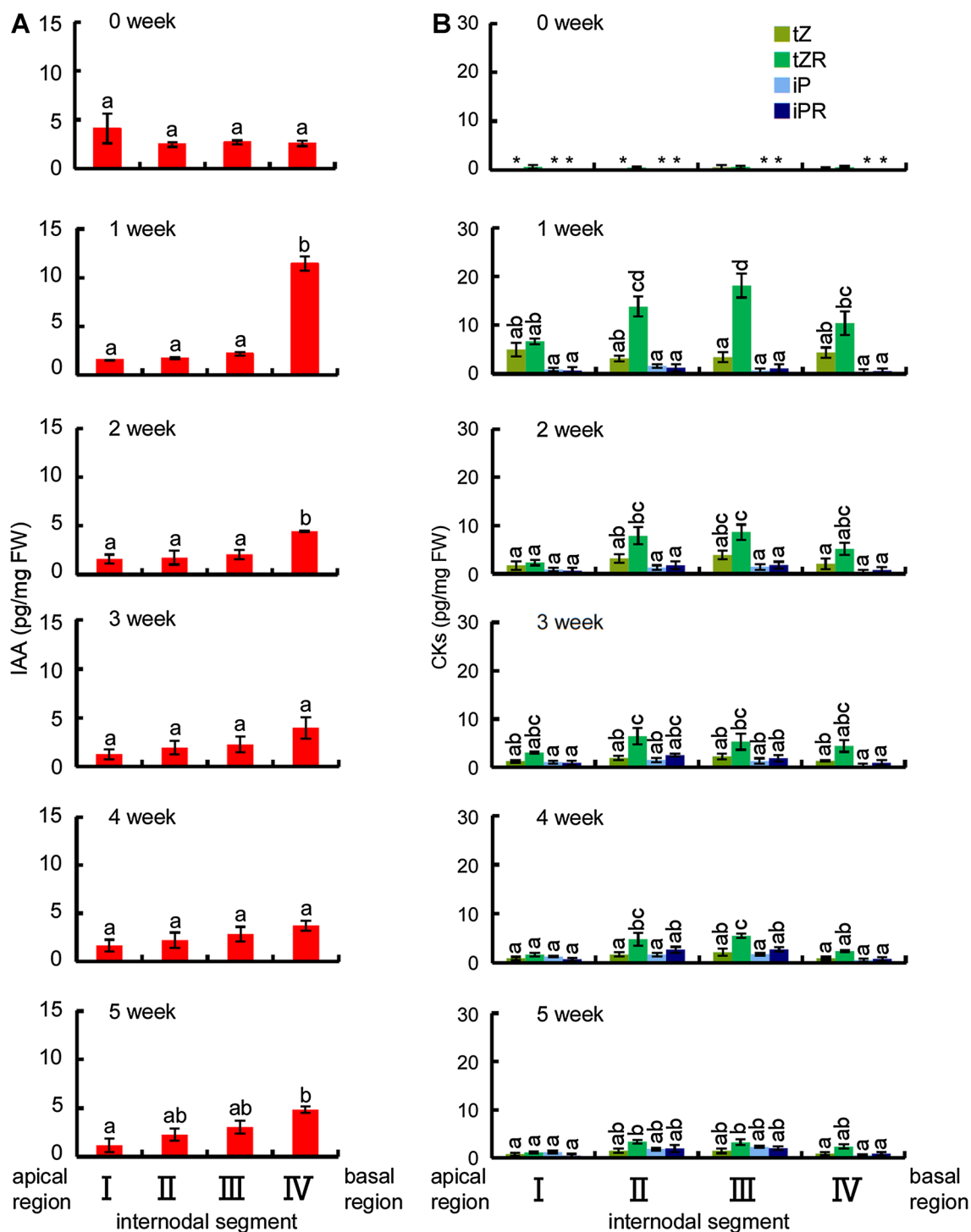


Fig. 6 Time-course analysis of **a** IAA and **b** CK levels in each of the four regions of internodal segments. Values are means \pm SEM ($n=3$). Eight segments were used in each experiment. *Trace levels

apex inhibits axillary bud outgrowth through the production of auxins reviewed by Cline (1991) and which can be mimicked by the application of auxins to the top of a decapitated plant (Thimann and Skoog 1933). The auxins produced in shoot tips are transported basipetally in the

high-conductance polar auxin transport stream (PATS), suppressing the biosynthesis of CKs that promote axillary bud outgrowth (Blakeslee and others 2005; Prusinkiewicz and others 2009; Shimizu-Sato and others 2009). Auxins suppress local CK biosynthesis by decreasing the

expression level of the isopentenyl transferase gene, which encodes a key enzyme in CK biosynthesis (Müller and others 2015; Tanaka and others 2006). We speculate that, in our experiments, the apex of the biggest shoot produced auxins, which were transported along the internodal segments. A vascular bundle started to differentiate in the shoot by 3 weeks of culture and connected to that of the segment by 5 weeks, becoming able to supply nutrients directly to the shoot, strengthening its growth (Fig. 4b). Auxin transport leads to the differentiation of new vascular tissues (Sachs 1981, 1991). In ipecac, auxin is released from the dominant adventitious shoot, which acts as an auxin sink, inducing the connection between its vascular tissues and those of the internodal segment. A recent model suggests that, in addition to PATS, widespread low-conductance auxin transport acts locally to exchange information between PATS and the surrounding tissues in shoot branching regulation (Bennett and others 2016). The widespread complex auxin flow might suppress the outgrowth of shoots surrounding the dominant shoot and stimulate vascular bundle formation in ipecac.

Adventitious shoots were formed at the apical end of the internodal segments (regions I and II), indicating polarity in the segments (Fig. 5). IAA accumulated in the basal region (IV) by 1 week of culture, owing to either active IAA biosynthesis there or polar transport from the apical region, and induced callus formation in the basal region (Fig. 6a). Observing adventitious shoot formation in the apical region of internodal segments of *B. monniera* and *K. blossfeldiana*, Sanikhani and others proposed that an auxin gradient from the basal region to the apical region determines the position of shoot regeneration (Sanikhani and others 2006; Tiwari and others 1998). Our results strongly support this proposal.

tZ-type CKs (notably tZR) increased in the middle region of internodal segments after 1 week of culture, but the total amount of CKs in that region decreased by 2 weeks (Fig. 6b). Although the iP-type CKs increased slowly during culture, their levels were much lower (Fig. 6b). Therefore, we presume that tZR-type CKs are mainly involved in the induction of adventitious shoots in ipecac.

Auxins and CKs are normally required for cell dedifferentiation and redifferentiation. Their ratio is important for organogenesis. In ipecac, cutting of the internode triggers adventitious shoot formation. In *Arabidopsis*, *WOUND INDUCED DEDIFFERENTIATION 1* (*WIND1*), which encodes an AP2/ERF transcription factor, is involved in the control of cell dedifferentiation (Iwase and others 2011). *WIND1* is rapidly induced at wound sites, where it stimulates cell dedifferentiation and subsequent cell proliferation to form callus, and cells around wound sites greatly increased responsiveness to CKs. In ipecac, expression of *WIND1*-like genes might induce callus formation in

the high-auxin basal region of internodal segments. On the other hand, CK levels were lower in the apical region than in the middle region, suggesting that sensitivity to CK promoted by a *WIND1*-like gene, not increased CK levels, induces adventitious shoot formation in the apical region. Furthermore, a *wooden leg-3* (*wol-3*) mutant an allele of the *ARABIDOPSIS HISTIDINE KINASE 4* (*AHK4*) gene that encodes a CK receptor (Kuroha and others 2006). Analysis of *wol-3* suggests that CK receptors have an important role in the formation of auxin transporting vascular tissues in the hypocotyl.

In conclusion, we found that adventitious shoots were formed in the apical region of internodal segments. IAA levels gradually increased from the apical region to the basal region, but CKs accumulated in the middle region (Fig. 7). CKs probably induce adventitious shoot formation in ipecac, but the distribution of the shoots is determined by an auxin gradient. It is hypothesized that the root meristem of vascular plants has evolved from the shoot meristem as an adaptation to land habitats (Gensel and Edwards 2001). Although the shoot and root meristems are initiated at the opposite poles of the embryo, similar regulons act in both meristems (Stahl and Simon 2010). Adventitious shoot formation on the stem might be similar to primitive root generation. In the fern *Azolla*, which has the most basal euphyllophyte root, auxins restrict root meristem development, whereas CK promotes it (de Vries and others 2016). This is opposite to what is observed in *Arabidopsis*. Our results support the idea that adventitious shoot formation in ipecac has intermediate characteristics in shoot and lateral root development and uses an evolutionarily old system of meristem differentiation.

Large changes in endogenous phytohormone levels likely trigger the expression of genes required for adventitious shoot formation or inactivate genes that block

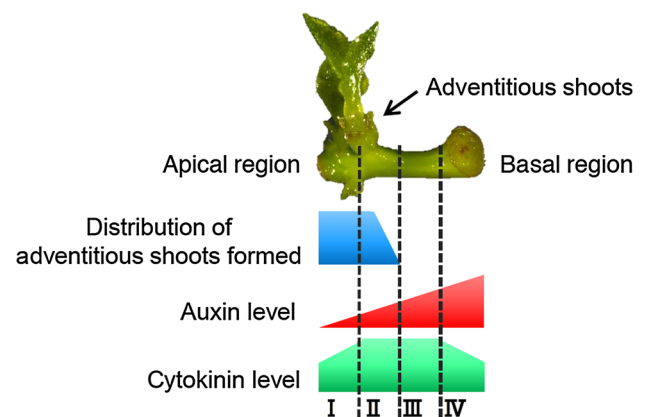


Fig. 7 Distribution of adventitious shoots and levels of auxin and CKs

dedifferentiation in epidermal cells by 1 week of culture. Identification of these genes and finding ways to control their expression will enable clonal propagation of plant species that are difficult to propagate by tissue culture.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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