Video Article

Quantification of Endogenous Auxin and Cytokinin During Internode Culture of Ipecac

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Abstract

Adventitious shoot formation is an important technique for the propagation of economically important crops and for the regeneration of transgenic plants. Phytohormone treatment is required for the induction of adventitious shoots in most species. Whether adventitious shoots can be induced is determined by the balance between auxin and cytokinin (CK) levels. Much effort goes into determining optimum concentrations and combinations of phytohormones in each tissue used as explants and in each plant species. In ipecac, however, adventitious shoots can be induced on internodal segments in culture medium without phytohormone treatment. This allows the inherent plasticity of ipecac for cell differentiation to be evaluated. To induce adventitious shoots in ipecac, we cultured internodal segments at 24 °C under 15 µmol m^{-2} s^{-1} of light in a 14-h light/10-h dark cycle on phytohormone-free B5 medium solidified with 0.2% gellan gum for 5 weeks. To investigate phytohormone dynamics during adventitious shoot formation, we measured endogenous indole-3-acetic acid and CKs in the segments by liquid chromatography-tandem mass spectrometry LC-MS/MS. This method allows analysis of endogenous indole-3-acetic acid and CKs levels in a simple manner. It can be applied to investigate the dynamics of endogenous auxin and CK during organogenesis in other plant species.

Video Link

The video component of this article can be found at https://www.jove.com/video/56902/

Introduction

Gottlieb Haberlandt (1854-1945) proposed the concept of "totipotency", by which plant cells can divide, differentiate, and regenerate whole plants even after their prior differentiation into specific cell types in mature plants[1]. In tissue culture, whether plant regeneration can be induced or not is determined by the combination and concentration of exogenously applied phytohormones in the growth medium. Skoog and Miller found that adventitious shoots could be induced from tobacco callus on culture medium containing a high ratio of CKs to auxins, whereas adventitious roots could be induced on medium containing a low ratio[2]. Since that finding, tissue culture has been widely used for the propagation of economically important crops and for the regeneration of transgenic plants[3]. Adventitious shoots can be induced from tissues other than shoot apical meristem, such as leaves, roots, and internodes. Phytohormone treatment is required for the induction of adventitious shoots in most plant species. However, the optimum concentrations and combinations differ by species and among tissues used as explants. Thus, much effort goes into determining the optimum concentrations and combinations of phytohormones for experiments.

Carapichea ipecacuanha (Brot.) L. Andersson (ipecac) is a medicinal plant that contains alkaloids such as emetine and cephaeline, mainly in the roots[4]. Root extracts are used as an expectorant, an emetic, and an amoebicide[5]. Although ipecac grows naturally in the tropical rainforests of Brazil, it is reluctant to set seeds in culture, and the germination rate decreases during seed storage in Japan, with its colder climate[6]. Instead, it is propagated by tissue culture, in which adventitious shoot formation on internodes is the most efficient method[7,8]. Interestingly, adventitious shoots can be induced in this species without phytohormone treatment[8].

Adventitious shoots are formed on the epidermis in the apical region of internodal segments without callusing, but not in the basal region[9]. This difference indicates tissue polarity in internodal segments, which is probably under phytohormonal regulation. The ipecac culture system allows a unique opportunity to analyze changes in endogenous phytohormone levels during adventitious shoot formation. Here we introduce our method for the analysis of the endogenous levels of one auxin (indole-3-acetic acid (IAA)) and four CKs (isopentenyl adenine (iP), isopentenyl adenine riboside (iPR), *trans*-zeatin (tZ), and *trans*-zeatin riboside (tZR)) in internodal segments through the use of LC-MS/MS.

Protocol

Note: Ipecac (*C. ipecacuanha*) was used in this study because it facilitates the analysis of endogenous phytohormones.

1. Growth Conditions to Induce Adventitious Shoots of Ipecac

1. Prepare phytohormone-free B5 medium adjusted to pH 5.7[10], and add 0.2% gellan gum. Sterilize by autoclaving.
2. Pour 25 mL of the autoclaved medium into a sterile Petri dish (90 mm × 20 mm).
3. Cut 8-mm internodal segments of ipecac plantlets using a surgical scalpel with a blade No. 22 on a sterile acrylic plate, and place on the medium (**Figure 1**).
4. Culture on phytohormone-free B5 medium at 24 °C under 15 µmol m^{-2} s^{-1} of light in a 14-h light/10-h dark cycle for 5 weeks.
5. Identify regions I (apical) to IV (basal) in each segment (**Figure 1B**). Count the number of adventitious shoots > 0.3 mm length in each region under a microscope once a week.

2. Extraction and Purification of Phytohormones

1. Put a 5-mm zirconia bead in each of the four 2-mL sample tubes.
2. Cut the segments into regions I to IV (each 2 mm in length) using a surgical scalpel on a sterile acrylic plate.
3. Collect eight segments of each region in separate sample tubes (10-30 mg fresh weight).
4. Weigh, and then freeze the samples in liquid nitrogen.
5. Crush the frozen samples using a bead-based homogenizer.
6. Suspend the crushed samples in 1 mL acetonitrile containing 500 pg of each internal standard of auxin and CKs (d_5-IAA, d_5-tZ, d_5-tZR, d_6-iP, d_6-iPR) using a vortex mixer.
7. Hold at 4 °C for 1 h, then centrifuge at 3,500 × *g* for 5 min at room temperature.
8. Wash the pellet in 80% (v/v) acetonitrile containing 1% (v/v) acetic acid. Centrifuge again at 3,500 × *g* for 5 min at room temperature. Combine the supernatants (from steps 2.7 and 2.8) in a disposable glass tube.
9. Add 600 µL water containing 1% (v/v) acetic acid to each combined supernatant, and evaporate the acetonitrile using a vacuum concentrator.
10. Equilibrate hydrophilic-lipophilic-balanced (HLB) column cartridges by applying 1 mL each of acetonitrile, methanol, and water containing 1% (v/v) acetic acid.
11. Apply one sample solution per equilibrated HLB cartridge.
12. Wash the cartridges with 1 mL water containing 1% (v/v) acetic acid.
13. Elute all hormones with 2 mL 80% (v/v) acetonitrile containing 1% (v/v) acetic acid in a glass tube.
14. Evaporate the acetonitrile in the eluate to obtain extract in water containing 1% (v/v) acetic acid using a vacuum concentrator.
    NOTE: Do not dry this completely.
15. Equilibrate mixed-mode, strong cation-exchange (MCX) column cartridges by applying 1 mL acetonitrile, 1 mL methanol, 0.5 mL 0.1 M HCl, and 1 mL water containing 1% (v/v) acetic acid.
16. Apply one sample solution per equilibrated MCX cartridge.
17. Wash the cartridges with 1 mL water containing 1% (v/v) acetic acid.
18. Elute IAA with 2 mL 30% (v/v) acetonitrile containing 1% (v/v) acetic acid in a glass tube.
19. Wash the cartridges with 2 mL 80% (v/v) acetonitrile containing 1% (v/v) acetic acid.
20. Wash the cartridges with 2 mL water and 1 mL water containing 5% aqueous ammonia.
21. Elute the CKs with 2 mL 60% (v/v) acetonitrile containing 5% aqueous ammonia in a glass tube.
22. Evaporate the solvent of each hormone fraction using a vacuum concentrator and store at −30 °C until the LC-MS/MS analysis.

3. LC-MS/MS Analysis of IAA and CKs

1. Dissolve each hormone extract in a tube with 600 µL of methanol and transfer the solution to a screw neck total recovery vial. Evaporate the solvent using a vacuum concentrator.
2. Dissolve the IAA fraction in 20 µL 30% (v/v) acetonitrile, and the CK fractions in 20 µL water containing 1% (v/v) acetic acid in screw neck total recovery vials.
3. Analyze the samples in positive ion mode on a triple-quadrupole MS system equipped with an HPLC system.
   NOTE: We set the HPLC conditions as listed in **Table 1**.
   1. For the IAA elution, use a binary gradient of 5%-50% solvent B over 7 min, then increase by 98% solvent B and hold for 1 min, and then equilibrate for 2 min at 5% solvent B by nest injection.
   2. For the CK elution, use a binary gradient of 2%-40% solvent B over 5 min, 40%-70% solvent B in 7 min, then increase by 95% solvent B and hold for 1 min, and then equilibrate for 2 min at 2% solvent B by nest injection.
   3. Set the electrospray ionization (ESI)-MS parameters of ion source as listed in **Table 2**.
4. Use the multiple reaction monitoring (MRM) transition for quantification of each analyte listed in **Table 3**.
5. Quantify endogenous IAA and CK levels against a standard curve of the ratio of unlabeled to deuterium-labeled standards.

Representative Results

At the 1st week, no adventitious shoots had formed. At the 2nd week, small shoots appeared. At the 3rd and 4th weeks, the number of shoots increased mostly in the apical regions (I and II) (**Figure 2A**). At the 5th week, the number of shoots was approximately 7 in region I and 5 in region II (**Figure 2B**). In contrast, only a few shoots were formed in regions III and IV.

Before culture, the IAA level was slightly higher in region I (4.1 pg/mg, fresh weight (FW)) than in regions II-IV (~ 2.5 pg/mg FW; **Figure 3**). At the 1st week, the IAA level increased greatly in region IV (11.4 pg/mg FW) and decreased slightly in regions I-III (1.5-2.2 pg/mg FW). At the 2nd week, the IAA level in region IV decreased to ~ 4.4 pg/mg FW, indicating that IAA accumulation in the basal region was transient. By 5 weeks of culture, an IAA concentration gradient emerged, with levels increasing from region I to region IV.

Before culture, there were only trace levels of most CKs (**Figure 3**). At the 1st week, the tZR level increased to 13.8 pg/mg FW in region II and to 18.1 pg/mg FW in region III. The levels then decreased gradually over 5 weeks of culture. On the other hand, the levels of tZ, iP, and iPR changed only slightly during culture.

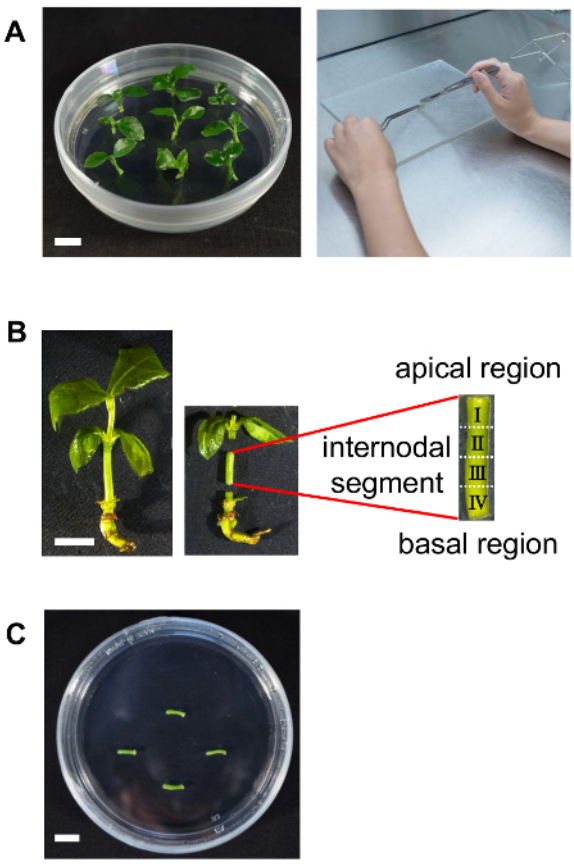

Figure 1: Preparation of ipecac for adventitious shoot formation. (**A**) An internodal segment (8 mm long) is cut from regenerated ipecac on a clean bench. (**B**) The first internode was used for adventitious shoot formation. (**C**) Segments are cultured on 25 mL phytohormone-free B5 medium in Petri dishes to induce adventitious shoots. Scale bar = 1 cm. Please click here to view a larger version of this figure.

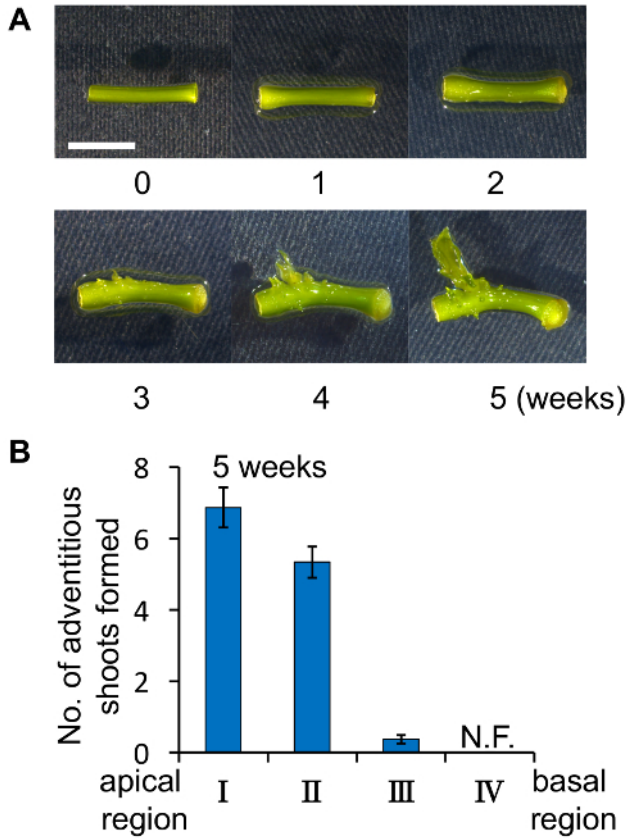

Figure 2: Distribution of adventitious shoots formed on an internodal segment. (**A**) Adventitious shoots formed after 0 to 5 weeks of culture. Scale bar = 5 mm. (**B**) Segments were partitioned into four regions (I-IV), and the number of adventitious shoots in each region was counted at week 5. Data are means ± SEM (*n* = 3). Ten segments were used in each experiment. N.F. = not found. This figure has been modified from Koike *et al.*[9] Please click here to view a larger version of this figure.

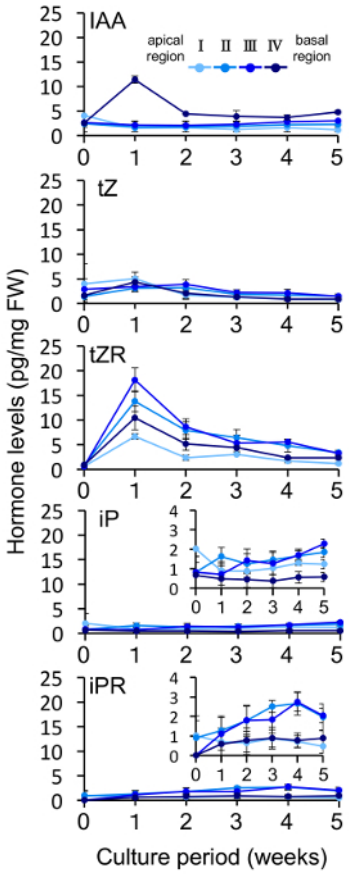

Figure 3: Time-course analysis of phytohormone levels in internodal segments. Segments were separated into four regions (I-IV). Endogenous IAA and CKs (tZ, tZR, iP, and iPR) in each region were quantified by LC-MS/MS. Because iP and iPR levels were very low, a zoomed graph was inserted inside the same graph. Data are means ± SEM (*n* = 3). Eight segments were used in each experiment. This figure has been modified from Koike *et al.*[9] Please click here to view a larger version of this figure.

Column	IAA: ACQUITY BEH C18 φ2.1×100 1.7 μm
	CKs: Poroshell EC-C18 φ2.1×50 2.7 μm
Temperature	40ºC
Mobile phase	Solvent A: distilled water + 0.05% acetic acid
	Solvent B: acetonitrile + 0.05% acetic acid
Flow rate	0.35 ml / min
Injection volume	18 μl

Table 1: HPLC condition in IAA and CK analysis.

Curtain gas (a.u.)*	10 / 40**
Collision gas (a.u.)*	5 / 3**
Ion spray voltage (V)	5500
Temperature (ºC)	600
Ion source gas 1 (a.u.)*	30
Ion source gas 2 (a.u.)*	40 / 80**
Resolution	Unit

Table 2: Parameters of ion source. *arbitrary units. **IAA analysis/CK analysis. This table has been modified from Koike *et al.*[9]

	Q1 (m/z)	Q3 (m/z)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)
IAA	176	130	26	6.5	21
d_5-IAA	181	134	36	4.0	23
tZ	220	136	36	5.0	23
d_5-tZ	225	137	31	5.0	21
tZR	352	220	41	5.5	23
d_5-tZR	357	225	51	5.0	21
iP	204	136	31	9.0	19
d_6-iP	210	137	31	5.5	21
iPR	336	204	31	5.0	21
d_6-iPR	342	210	36	5.5	21

Table 3: MRM transitions of IAA and CKs in LC-MS/MS analysis. This table has been modified from Koike *et al.*[9]

Discussion

To identify the distribution of phytohormones involved in organogenesis, it is important to use plant materials in which organogenesis can be observed on phytohormone-free medium, because when phytohormones are exogenously applied to explants for inducing shoots or roots, they affect the whole explant, making it difficult to evaluate the inherent plasticity of plants in cell differentiation and organogenesis. Adventitious shoots can be induced on phytohormone-free culture media in other plant species such as *Dianthus caryophyllus* L.[11], *Aegle marmelos* (L.) Corrêa[12], *Bacopa monnieri* (L.) Pennell[13], *Celastrus paniculatus* Willd.[14], and *Kalanchoë blossfeldiana* Poelln.[15]. It would be possible to apply the protocol in these plant species.

We extracted IAA, tZ, tZR, iP, and iPR in acetonitrile and purified them by solid-phase extraction. The original method uses three types of cartridge columns (HLB, MCX, and weak anion exchange (WAX)) because all phytohormones are purified (including gibberellins, abscisic acid, jasmonic acid, and salicylic acid)[16]. The HLB column uses a polymeric reverse-phase sorbent, the MCX column uses the same with cation-exchange groups, and the WAX column uses the same with weak anion-exchange groups. The original method elutes CKs (basic) with 60% acetonitrile containing aqueous ammonia on an MCX column in the second step, and then IAA (acidic) with 80% acetonitrile containing 1% acetic acid on a WAX column in the last step. As our focus is auxin and CKs, which interact antagonistically to regulate plant growth[17], the simplified protocol uses only the HLB and MCX columns; IAA is eluted with 30% acetonitrile containing 1% acetic acid on the HLB column in the first step. It takes two days from sample preparation to LC-MS/MS analysis.

The acetonitrile solvent should not be allowed to dry out during phytohormone purification in the cartridge columns. If it does, resuspend the sample in acetonitrile to prevent the phytohormones from becoming stuck to the glass tube and lost from the sample. In this protocol, the detection limit with the ion-trap MS system is ~ 10 pg for IAA and CKs from 10-30 mg fresh tissues. To analyze smaller amounts, it would be necessary to collect much more sample or to use MS with higher sensitivity.

Phytohormone analysis is an important technique for the evaluation of plant growth and development. Using this method, we might be able to determine the timing of auxin and CK treatment for adventitious shoot formation in plant species where the optimum culture condition is still unknown. As phytohormone quantification becomes increasingly important, the LC-MS/MS protocol described here will enable the analysis of small samples with high sensitivity and resolution. Our simplification of a previous method will facilitate purification and analysis, and bring high versatility and reproducibility. In the future, this method can be applied to investigate the dynamics of the endogenous auxin and CK during organogenesis in other plant species.

Disclosures

The authors declare that they have no conflicts of interest.

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References

1. Haberlandt, G. Kulturversuche mit isolierten Pflanzenzellen. *Sitzungsber. Math.-Naturwiss. Kl. Akad. Wiss. Wien.* **111** 69-92 (1902).
2. Skoog, F., & Miller, C. O. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp Soc Exp Biol.* **11** 118-130 (1957).

3. Ganeshan, S., Caswell, K. L., Kartha, K. K., & Chibbar, R. N. in *Transgenic plants and crops.* eds G.G. Khachatourians, A. McHughen, R. Scorza, & W.K. Nip) Ch. 4, 69-84 CRC Press, (2002).
4. Teshima, D., Ikeda, K., Satake, M., Aoyama, T., & Shimomura, K. Production of emetic alkaloid by in vitro culture of *Cephaelis ipecacuanha.* A. Richard. *Plant Cell Rep.* **7** (4), 278-280 (1988).
5. Chatterjee, S. K., Nandi, R. P., & Ghosh, N. C. in *Cultivation and utilixzation of medicinal plants.* eds C.K. Atal & B.M. Kapur) 295-301 Regional Research Laboratory, Council of Scientific and Industrial Research, Jammu-Tawi, India, (1982).
6. Yoshimatsu, K., & Shimomura, K. in *Biotechnology in Agriculture and Forestry 21, Medicinal and Aromatic Plants IV.* (ed Y.P.S. Bajaj) 87-103 Springer-Verlag Berlin Heidelberg, (1993).
7. Ideda, K., Teshima, D., Aoyama, T., Satake, M., & Shimomura, K. Clonal propagation of *Cephaelis ipecacuanha. Plant Cell Rep.* **7** (4), 288-291 (1988).
8. Yoshimatsu, K., & Shimomura, K. Efficient shoot formation on internodal segments and alkaloid formation in the regenerates of *Cephaelis ipecacuanha.* A. Richard. *Plant Cell Rep.* **9** (10), 567-570 (1991).
9. Koike, I., Taniguchi, K., Shimomura, K., & Umehara, M. Dynamics of endogenous indole-3-acetic acid and cytokinins during adventitious shoot formation in ipecac. *J. Plant Growth Regul.* in press (2017).
10. Gamborg, O. L., Miller, R. A., & Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **50** (1), 151-158 (1968).
11. Watad, A. A. *et al.* Adventitious shoot formation from carnation stem segments: a comparison of different culture procedures. *Scientia Hortic.* **65** (4), 313-320 (1996).
12. Ajithkumar, D., & Seeni, S. Rapid clonal multiplication through in vitro axillary shoot proliferation of *Aegle marmelos.* (L.) Corr., a medicinal tree. *Plant Cell Rep.* **17** (5), 422-426 (1998).
13. Tiwari, V., Tiwari, K. N., & Singh, B. D. Comparative studies of cytokinins on *in vitro.* propagation of *Bacopa monniera. Plant Cell Tiss. Org. Cult.* **66** (1), 9-16 (2001).
14. Rao, M. S., & Purohit, S. D. *In vitro.* shoot bud differentiation and plantlet regeneration in *Celastrus paniculatus.* Willd. *Biol. Plant.* **50** (4), 501-506 (2006).
15. Sanikhani, M., Frello, S., & Serek, M. TDZ induces shoot regeneration in various *Kalanchoë blossfeldiana.* Poelln. cultivars in the absence of auxin. *Plant Cell Tiss. Org. Cult.* **85** (1), 75-82 (2006).
16. Yoshimoto, K. *et al.* Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in Arabidopsis. *Plant Cell.* **21** (9), 2914-2927 (2009).
17. Schaller, G. E., Bishopp, A., & Kieber, J. J. The yin-yang of hormones: cytokinin and auxin interactions in plant development. *Plant Cell.* **27** (1), 44-63 (2015).

