Journal of Visualized Experiments

Quantification of endogenous auxin and cytokinin during internode culture of ipecac

--Manuscript Draft--

Manuscript Number:	JoVE56902R1
Full Title:	Quantification of endogenous auxin and cytokinin during internode culture of ipecac
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	adventitious shoot; auxin; Carapichea ipecacuanha; cytokinin; LC-MS/MS; phytohormone-free medium; plant regeneration
Manuscript Classifications:	95.51.11: botany; 95.51.30: plants
Corresponding Author:	Mikihisa Umehara, Ph.D. Toyo University Itakura-machi, Ora-gun, Gunma JAPAN
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	umehara@toyo.jp
Corresponding Author's Institution:	Toyo University
Corresponding Author's Secondary Institution:	
First Author:	Imari Koike
First Author Secondary Information:	
Other Authors:	Imari Koike
	Koichiro Shimomura
Order of Authors Secondary Information:	
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 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 cytokinins in the segments by LC-MS/MS. This method allows us to analyze endogenous indole-3-acetic acid and cytokinins levels simply. It can be applied to investigate dynamics of the endogenous auxin and cytokinin during organogenesis in other plant species.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

September 18, 2017

Mikihisa Umehara
Graduate School of Life Sciences
1-1-1 Izumino, Itakura-machi,
Ora-gun, Gunma 374-0193, Japan
Tel. & Fax: +81-276-82-9144
E-mail: umehara@toyo.jp

Journal of Visualized Experiments
Alisha DSouza
Review Editor

Dear Dr. Alisha DSouza,

With this letter, we are submitting a revised version of our manuscript (Ms. No. JoVE56902) entitled "**Quantification of endogenous auxin and cytokinin during internode culture in ipecac**".

We thank the review editor and reviewers for their comments on our manuscript. We have revised it according to their comments, and describe our responses in detail in this letter. The revised parts of the manuscript are highlighted in green.

This manuscript contains 3 figures and 3 Tables.

We would appreciate it very much if you could consider the revised version of our manuscript for publication in *Journal of Visualized Experiments* again. We hope that our responses and the resulting changes are satisfactory, but we are happy to work with you and the reviewers to resolve any remaining issues. We look forward to your and reviewers' comments.

Sincerely,

Mikihisa Umehara (on behalf of all authors)

TITLE:

Quantification of Endogenous Auxin and Cytokinin During Internode Culture of Ipecac

AUTHORS & AFFILIATIONS:
Imari Koike, Koichiro Shimomura, Mikihisa Umehara

Graduate School of Life Sciences, Toyo University

EMAIL ADDRESSES:
Imari Koike (imr.413.gently@icloud.com)
Koichiro Shimomura (shimomur@toyo.jp)
Mikihisa Umehara (umehara@toyo.jp)

CORRESPONDING AUTHOR:
Mikihisa Umehara
Email: umehara@toyo.jp
Tel: +81-276-82-9144.

KEY WORDS:
Adventitious shoot, auxin, *Carapichea ipecacuanha*, cytokinin, LC-MS/MS, phytohormone-free medium, plant regeneration

SHORT ABSTRACT:
Adventitious shoots can be induced on internodal segments of ipecac without phytohormone treatment. To evaluate phytohormone dynamics during adventitious shoot formation, we measured endogenous auxin and cytokinin in internodal segments by LC-MS/MS.

LONG 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.

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

1.1. Prepare phytohormone-free B5 medium adjusted to pH 5.7[10], and add 0.2% gellan gum. Sterilize by autoclaving.

1.2. Pour 25 mL of the autoclaved medium into a sterile Petri dish (90 mm × 20 mm).

1.3. 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**).

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.

1.5. 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

2.1. Put a 5-mm zirconia bead in each of the four 2-mL sample tubes.

2.2. Cut the segments into regions I to IV (each 2 mm in length) using a surgical scalpel on a sterile acrylic plate.

2.3. Collect eight segments of each region in separate sample tubes (10–30 mg fresh weight).

2.4. Weigh, and then freeze the samples in liquid nitrogen.

2.5. Crush the frozen samples using a bead-based homogenizer.

2.6. 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.

2.7. Hold at 4 °C for 1 h, then centrifuge at 3,500 × *g* for 5 min at 4 °C.

2.8. Wash the pellet in 80% (v/v) acetonitrile containing 1% (v/v) acetic acid. Centrifuge again at 3,500 × *g* for 5 min at 4 °C. Combine the supernatants (from steps 2.7 and 2.8) in a disposable glass tube.

2.9. Add 600 µL water containing 1% (v/v) acetic acid to each combined supernatant, and evaporate the acetonitrile using a vacuum concentrator.

2.10. Equilibrate hydrophilic-lipophilic-balanced (HLB) column cartridges by applying 1 mL each of acetonitrile, methanol, and water containing 1% (v/v) acetic acid.

2.11. Apply one sample solution per equilibrated HLB cartridge.

2.12. Wash the cartridges with 1 mL water containing 1% (v/v) acetic acid.

2.13. Elute all hormones with 2 mL 80% (v/v) acetonitrile containing 1% (v/v) acetic acid in a glass

tube.

2.14. 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.

2.15. 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.

2.16. Apply one sample solution per equilibrated MCX cartridge.

2.17. Wash the cartridges with 1 mL water containing 1% (v/v) acetic acid.

2.18. Elute IAA with 2 mL 30% (v/v) acetonitrile containing 1% (v/v) acetic acid in a glass tube.

2.19. Wash the cartridges with 2 mL 80% (v/v) acetonitrile containing 1% (v/v) acetic acid.

2.20. Wash the cartridges with 2 mL water and 1 mL water containing 5% aqueous ammonia.

2.21. Elute the CKs with 2 mL 60% (v/v) acetonitrile containing 5% aqueous ammonia in a glass tube.

2.22. 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

3.1. 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.

3.2. 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.

3.3. 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**.

3.3.1. 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.

3.3.2. 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.

3.3.4. Set the electrospray ionization (ESI)-MS parameters of ion source as listed in **Table 2**.

3.4. Use the multiple reaction monitoring (MRM) transition for quantification of each analyte listed in **Table 3**.

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 AND TABLE LEGENDS:

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.

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]

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]

Table 1: HPLC condition in IAA and CK analysis.

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

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.

ACKNOWLEDGMENTS:

We are grateful to Mr. Akira Murakami of the Department of Applied Biosciences, Toyo University, and Mr. Koudai Taniguchi of the Gunma Agricultural Technology Center for their technical assistance. We are also grateful to Professor Shosaku Kashiwada and Dr. Uma Maheswari Rajagopalan, Toyo University for their suggestions. This study was supported in part by the Research Center for Life and Environmental Sciences, Toyo University.

DISCLOSURES:

The authors declare that they have no conflicts of interest.

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, doi:10.1007/bf00272542, (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, doi:10.1007/bf00272545, (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, doi:10.1007/bf00232333, (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, doi:10.1007/s00344-017-9684-8, (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,

doi:http://dx.doi.org/10.1016/0304-4238(96)00874-6, (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, doi:10.1007/s002990050418, (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, doi:10.1023/a:1010652006417, (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, doi:10.1007/s10535-006-0079-0, (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, doi:10.1007/s11240-005-9050-6, (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, doi:10.1105/tpc.109.068635, (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, doi:10.1105/tpc.114.133595, (2015).

Fig. 1

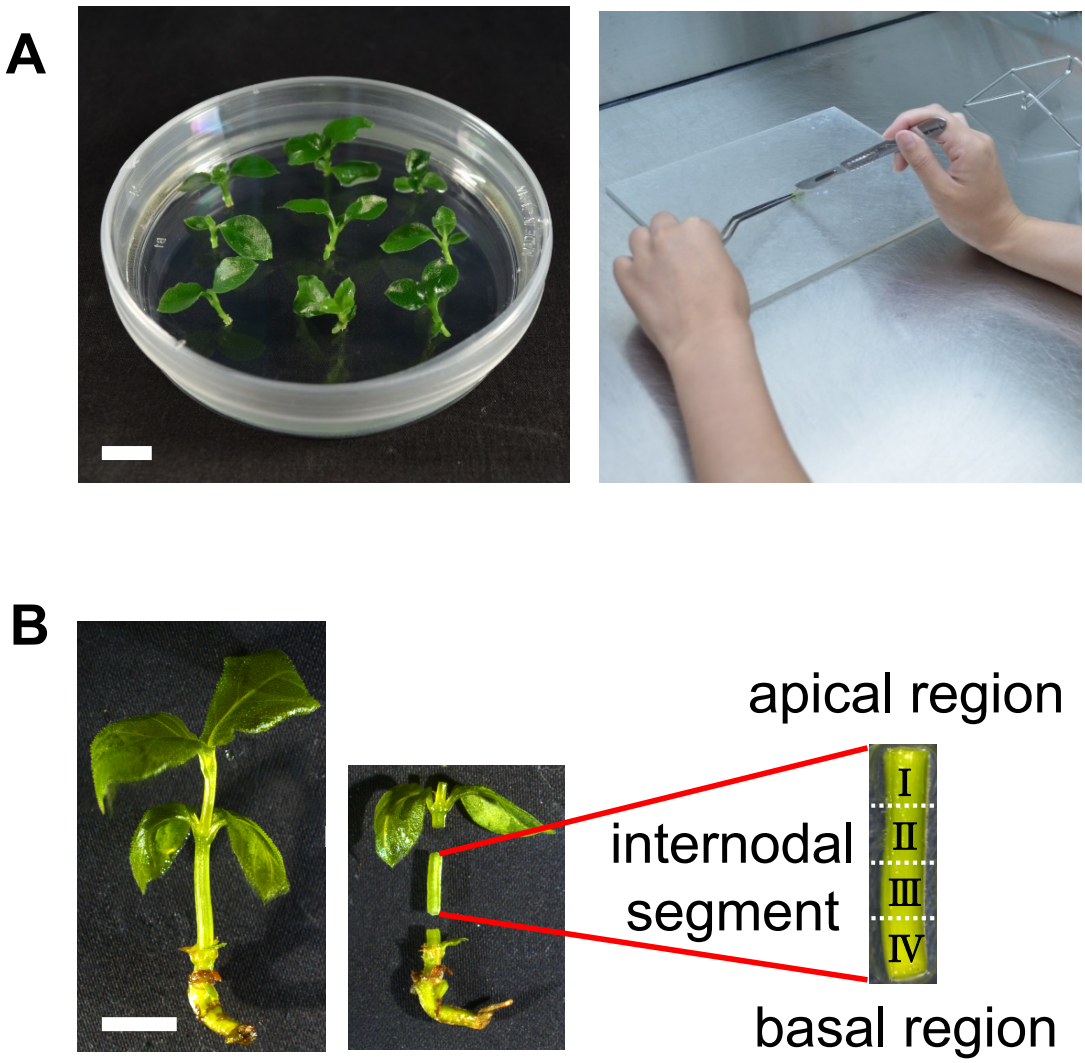
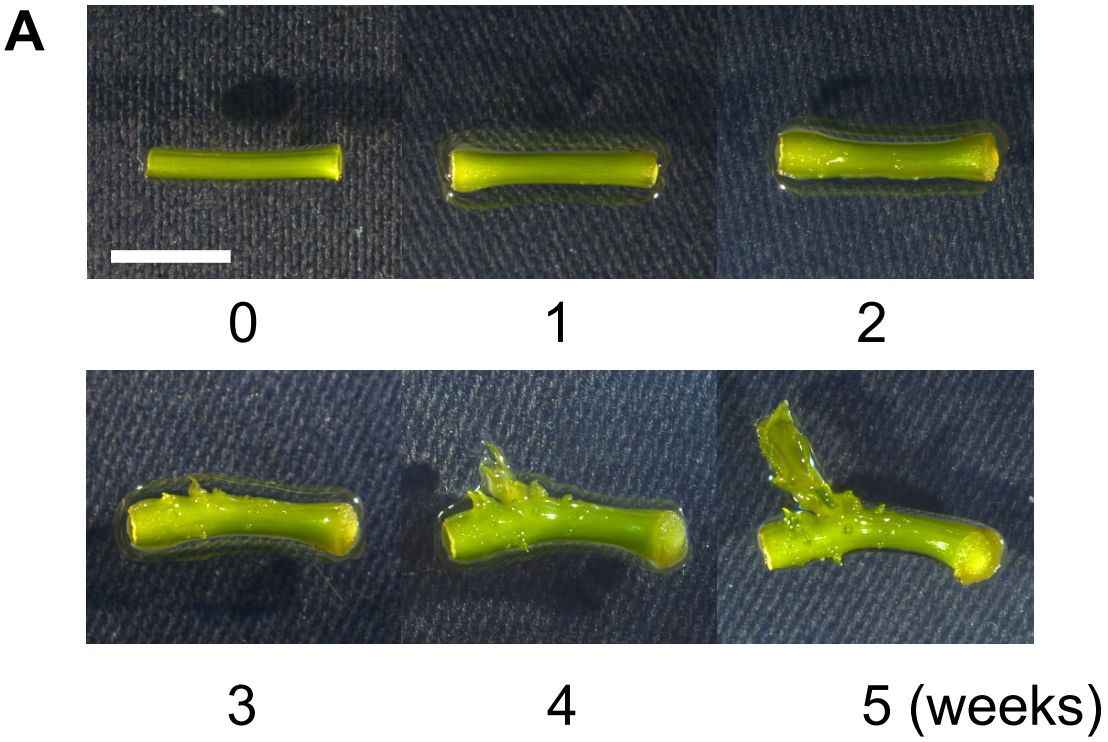


Fig. 2



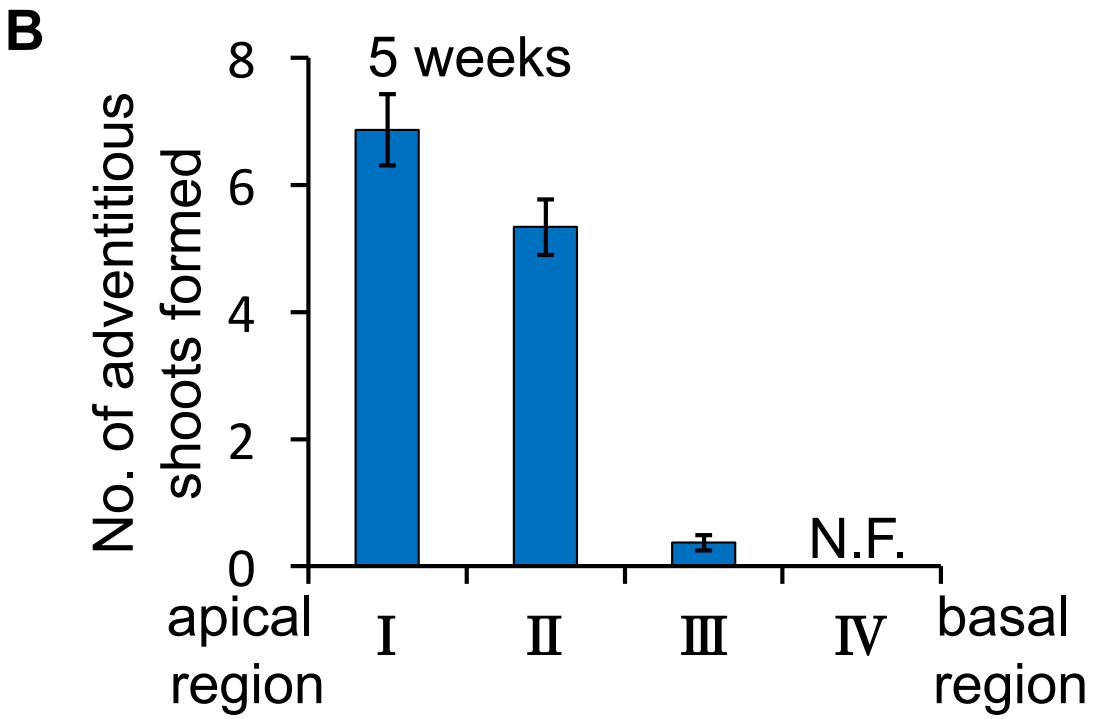


Fig. 3

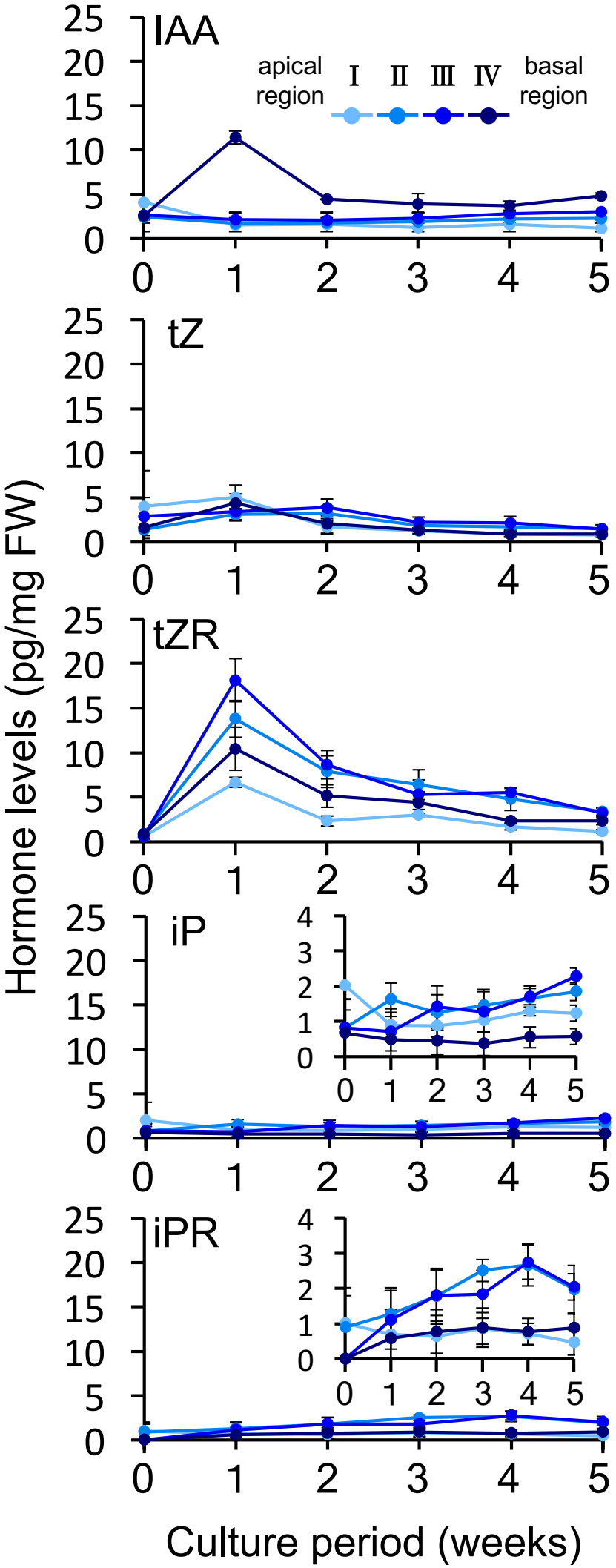


Table 1. HPLC condition in IAA and CK analysis

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 2. Parameters of ion source

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

* arbitrary units

**IAA analysis / CK analysis

This table has been modified from [Koike et al. 2017][9].

Table 3. MRM transitions of IAA and CKs in LC-MS/MS analysis

	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

This table has been modified from [Koike et al. 2017][9].

Table 2. Parameters of ion source

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

* arbitrary units

**IAA analysis / CK analysis

This table has been modified from [Koike et al. 2017][9].

Table 3. MRM transitions of IAA and CKs in LC-MS/MS analysis

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

This table has been modified from [Koike et al. 2017][9].

Collision energy (V)
21
23
23
21
23
21
19
21
21
21

Materials Table

Click here to download Materials Table JoVE Materials-3.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
[2H5]indole-3-acetic acid	Olchemlm Ltd	031 1531	Internal standard for LC-MS/MS
[2H5]*trans*-zeatin	Olchemlm Ltd	030 0301	Internal standard for LC-MS/MS
[2H5]*trans*-zeatin riboside	Olchemlm Ltd	030 0311	Internal standard for LC-MS/MS
[2H6]N6-isopentenyl adenine	Olchemlm Ltd	030 0161	Internal standard for LC-MS/MS
[2H6]N6-isopentenyl adenosine	Olchemlm Ltd	030 0171	Internal standard for LC-MS/MS
indole-3-acetic acid	Wako	098 00181	standard for LC-MS/MS
trans-zeatin	SIGMA-ALDRICH	Z0876 5MG	standard for LC-MS/MS
trans-zeatin riboside	Wako	262 01081	standard for LC-MS/MS
N6-isopentenyl adenine	SIGMA-ALDRICH	D7674 1G	standard for LC-MS/MS
N6-isopentenyl adenosine	ACROS ORGANICS	22648 1000	standard for LC-MS/MS
acetonitrile hypergrade for LC-MS LiChrosolv	MERCK	1.00029.1000	solvent for LC-MS/MS
Water for chromatography LiChrosolv	MERCK	1.15333.1000	solvent for LC-MS/MS
HPLC	SHIMADZU	Prominence	
MS	Sciex	3200QTRAP	
Oasis HLB 30 mg/1 cc	Waters	WAT094225	cartridge column
Oasis MCX 30 mg/1 cc	Waters	186000252	cartridge column
screw neck total recovery vial	Waters	186002805	
blue, 12 x 32mm screw neck cap and PTFE/silicone septum	Waters	186000274	
Acquity UPLC BEH C18, 2.1x100 mm	Waters	186002350	UPLC column
Proshell 120 EC-C18, 2.1x50 mm	Agilent	699775-902	UPLC column
Digital microscope	Leica	DHS1000	

TissueLyser II	QIAGEN	85300	
Surgical blade	Feather	No. 22	
Scalpel handle	Feather	No. 4	
Savant SpeedVac/Refregerated vapor trap	Thermo Fisher Scientific	SPD111/RVT4104	vacuum concentrartor
Disposable glass tobe (13x100 mm)	IWAKI	9832-1310	
Sterile petri dish	INA OPTICA	I-90-20	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Quantification of endogenous auxin and cytokinin during internode culture in Ipecac

Author(s): Imari Koike, Koichiro Shimomura, Mikihisa Umehara

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Mikihisa Umehara

Department: Graduate school of life sciences

Institution: Toyo University

Article Title: Quantification of endogenous auxin and cytokinin during internode culture in ipecac

Signature: Mikihisa Umehara Date: 18/9/2017

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Response to comments from Review editor

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have corrected some small errors that are not described in this letter, and have also highlighted these changes in green.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

1) E.g. Line 66 should be a note.

L. 68, We have added 'Note: ' before ipecac (*Carapichea ipecacuanha*).

In addition, L. 82-83, We have revised the second sentence to imperative tense.

L. 131, We have deleted 'Further'.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to the following protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

1) Line 72: cute using scissors?

L. 76, 82, We have added 'using a surgical scalpel with a blade No. 22 on a sterile acryl plate'.

2) Line 74: What is the culture medium/substrate?

L. 79, We have added 'on phytohormone-free B5 medium'.

In addition, we have added some details in the text. See green highlight.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

We have changed the numbering of protocol section as you proposed. In addition, we have inserted a one-line space between each step.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the

most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.
We have revised the protocol accordingly, but the total length was within 3 pages. Thus, we have not highlighted in yellow in the text.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
1), 3), 5) We compared comprehensive analysis of all phytohormones and described on critical steps in our protocol (L. 207-218).
2) We discussed the detection limit of phytohormone analysis (L. 219-224).
4) We added one sentence to the end of Discussion as the application of our method, 'In the future, this method would be applied to investigate dynamics of the endogenous auxin and cytokinin during organogenesis in other plant species.' (L. 231-233).

• **Figure/Table Legends:**:
1) Fig 3: Define the insets
We have defined the insets in Fig.3.
2) Tables 1-3: Add legends.
L. 198-203, Table legends have been added after figure legends.

• **References:**
1) Please abbreviate all journal titles.
We abbreviated the journal titles in references.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Leica Microsystems, TissueLyser II (Qiagen, Hilden, DHS1000, Oasis HLB, Oasis MCX, 3200 QTRAP; Sciex, HPLC, Prominence, MultiQuant v. 2.0.2 software (Sciex), etc.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have replaced commercial names with generic names in the text. And we have added the commercial names in a material file.

• Please define all abbreviations at first use.
We defined all abbreviations at first use.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.
We used abbreviations for SI units and non-SI units with a single space between the numerical value and unit.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
We have obtained the permission to re-use the figures and tables from the Springer. Text for the reprint permission was submitted as a supplemental file. In figure legends of Figs. 2 and 3, we added 'This figure has been modified from [Koike et al. 2017][9].' In Tables 2 and 3, we added 'This table has been modified from [Koike et al. 2017][9].'

Response to comments from Reviewer 1

1. I would like to know the hormone levels in other plant species that adventitious shoots can be induced on phytohormone free culture media.

Thank you for your comments. The JoVE is a scientific method journal. We hope that someone who read our paper would try to analyze the hormone levels in other plant species using our protocol.

2. I would like to know why ipecac does not need exogenously applied phytohormones for adventitious shoot formation.

This is an important question. However, nobody probably knows the mechanism at this moment. In the future, we will try to answer the question.

3. Authors described in abstract, 'the optimum concentrations and combinations differ by species' and `The results will allow us to estimate the most efficient timing of auxin

and cytokinin treatment to induce efficiently adventitious shoots in other plant species. I felt these two sentences are conflict a little bit.

We have revised the words 'the optimum concentrations and combinations differ by species' to be more clearly. And we have deleted a sentence, 'The results will allow us to estimate the most efficient timing of auxin and cytokinin treatment to induce efficiently adventitious shoots in other plant species'. We have added the advantages, limitations, and applications in our methods in stead of the sentence.

Response to comments from Reviewer 2

1. JoVE is a methods journal, I am not sure the manuscript is also a methods article. If it is, then I have a question. What are the advantages of the method you used? To clarify this, some comparison should be included in the results.

We have added the advantages of our method in Abstract (L. 29-31). Our method is simple compared with all hormone analysis established previously because we are focusing on two hormones, auxin and cytokinin. We have discussed it in Discussion (L. 216-227).

2. Why did you choose these two hormones (auxin and CKs) in your experiments, but not others such as cytokinins and gibberellins?

Because auxin and cytokinin mainly regulate adventitious shoot formation, we focused on quantification of these two phytohormones. To clarify the point, we have changed the title to ‘Quantification of endogenous auxin and cytokinin during internode culture of ipecac’.

3. Long abstract: The section should be re-written. In the "Long Abstract", the authors focused on the background, material and method, but not the results.

The instruction for authors requires that Abstract should be written on the method rather than the results of a specific method, and that we should state about the purpose of the method, advantages, limitations, and applications. Thus, we have revised the long abstract and we have added the advantages, limitations, and applications in our methods.