ORIGINAL ARTICLE

Endogenous auxin determines the pattern of adventitious shoot formation on internodal segments of ipecac

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Abstract

Main conclusion **Endogenous auxin determines the pattern of adventitious shoot formation. Auxin produced in the dominant shoot is transported to the internodal segment and suppresses growth of other shoots.**

Abstract Adventitious shoot formation is required for the propagation of economically important crops and for the regeneration of transgenic plants. In most plant species, phytohormones are added to culture medium to induce adventitious shoots. In ipecac (*Carapichea ipecacuanha* (Brot.) L. Andersson), however, adventitious shoots can be formed without phytohormone treatment. Thus, ipecac culture allows us to investigate the effects of endogenous phytohormones during adventitious shoot formation. In phytohormone-free culture, adventitious shoots were formed on the apical region of the internodal segments, and a high concentration of IAA was detected in the basal region. To explore the relationship between endogenous auxin and adventitious shoot formation, we evaluated the effects of auxin transport inhibitors, auxin antagonists, and auxin biosynthesis inhibitors on adventitious shoot formation in ipecac. Auxin antagonists and biosynthesis inhibitors strongly suppressed adventitious shoot formation, which was restored by exogenously applied auxin. Auxin biosynthesis and transport inhibitors significantly decreased the IAA level in the basal region and shifted the positions of adventitious shoot formation from the apical region to the middle region of the segments. These data indicate that auxin determines the positions of the shoots formed on internodal segments of ipecac. Only one of the shoots formed grew vigorously; this phenomenon is similar to apical dominance. When the largest shoot was cut off, other shoots started to grow. Naphthalene-1-acetic acid treatment of the cut surface suppressed shoot growth, indicating that auxin produced in the dominant shoot is transported to the internodal segment and suppresses growth of other shoots.

Keywords Adventitious shoots · Apical dominance · *Carapichea ipecacuanha* · Indole-3-acetic acid · Polar auxin transport

Abbreviations

4-Cl-PEO-IAA	4-Chloro-α-(phenyl ethyl-2-one)-indole-3-acetic acid
Kyn	L-Kynurenine
NAA	Naphthalene-1-acetic acid
NPA	*N*-(1-Naphthyl)phthalamic acid

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PIN	PIN-FORMED
PPBo	4-Phenoxybenzeneboronic acid
TIBA	2,3,5-Triiodobenzoic acid

Introduction

Haberlandt (1902) was the first to propose the concept of totipotency, which postulates that differentiated plant cells can de-differentiate and re-differentiate to regenerate the whole plant (Haberlandt 1902). In plant tissue culture, two phytohormones, auxin and cytokinin, influence the developmental fates of cells. Under certain conditions, plant somatic cells can regenerate the whole body (Steward et al. 1958). In tobacco, a high ratio of auxin to cytokinin in culture medium induces roots, a low ratio induces shoots, and high concentrations of both induce callus formation, indicating high plasticity of plant cells in differentiation and organogenesis (Skoog and Miller 1957). Callus can be induced by auxin, cytokinin, wounding, and acquisition of embryonic fate (Ikeuchi et al. 2013). Organ regeneration is widely used for the propagation of economically important crops and the regeneration of transgenic plants (Ganeshan et al. 2002). The effects of exogenously applied auxins and cytokinins on the formation of adventitious shoots and roots have been extensively studied, and many efforts have been made to determine the optimum concentrations of each phytohormone for inducing shoots and roots (Gahan and George 2008). On the other hand, little is known about the contribution of endogenous phytohormones produced in explants used in plant tissue culture.

In the tissue culture of many plant species, exogenous auxin and cytokinin are added to the culture medium to induce adventitious shoots (Ganeshan et al. 2002). In *Carapichea ipecacuanha* (Brot.) L. Andersson (ipecac), however, adventitious shoots can be induced even on phytohormone-free medium (Yoshimatsu and Shimomura 1991). This unique characteristic allows us to analyze the dynamics and effects of endogenous phytohormones during adventitious shoot formation. Ipecac is a medicinal plant whose roots contain alkaloids such as emetine and cephaeline (Teshima et al. 1988), which are used as an expectorant, emetic, and amoebicide (Chatterjee et al. 1982; Trease and Evans 1989). Ipecac grows as a shrub in tropical rainforests of Costa Rica, Nicaragua, Panama, and Brazil (Yoshimatsu and Shimomura 1993). Internodal segments of ipecac produce many adventitious shoots per explant (Yoshimatsu and Shimomura 1991). In our previous study, to analyze the relationships between adventitious shoot formation and the dynamics of endogenous phytohormones, we counted adventitious shoots formed on internodal segments of ipecac and measured endogenous IAA and cytokinin levels (Koike et al. 2017). Adventitious shoots were formed without callusing on the epidermis of the apical region of internodal segments, whereas IAA accumulated in the basal region and cytokinins accumulated in the middle region. These results suggest that the distribution of IAA, not cytokinins, determines the position of adventitious shoot formation in ipecac. We also found that only one of the shoots grew vigorously, with its vascular bundle connected to that of the segment, and the outgrowth of the others was suppressed. This phenomenon resembles shoot apical dominance, i.e. control of the outgrowth of axillary buds by the apical bud (Cline 1991). These data suggest an important role of endogenous IAA in determining the patterns of adventitious shoot formation on internodal segments of ipecac.

IAA is the most abundant endogenous auxin (Ljung et al. 2001) and is synthesized mainly in two steps (Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011). In the first step, aminotransferase encoded by *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* converts L-tryptophan to indole-3-pyruvic acid. In the second step, flavin monooxygenase encoded by *YUCCA* catalyzes the conversion of indole-3-pyruvic acid to IAA. IAA is then directionally transported into auxin-responsive cells by auxin influx and efflux carrier proteins (Adamowski and Friml 2015; Zhu et al. 2016). This auxin polar transport system generates an asymmetric auxin concentration gradient. Auxin influx is mediated by the AUXIN-RESISTANT 1 proton gradient–driven symporter (Marchant et al. 1999). PIN-FORMED (PIN) and ATP-BINDING CASSETTE subfamily B transporters mediate auxin efflux and regulate the direction and rate of intercellular auxin flow (Zazimalova et al. 2010). In the nuclei of auxin-responsive cells, IAA binds to the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1), which is an F-box protein that forms an SCF E3 ubiquitin ligase complex with Skp1 and Cullin (Dharmasiri et al. 2005; Kepinski and Leyser 2005). Auxin/IAA transcriptional repressors are ubiquitinated by SCF^{TIR1} complexes and degraded, and IAA promotes this process by enhancing the interaction between the TIR1 and Auxin/IAA proteins (Gray et al. 2001; Tan et al. 2007). Appropriate auxin distribution and responsiveness are important for organogenesis such as generation of shoots and roots (Petrasek and Friml 2009; Bohn-Courseau 2010).

In this study, to explore the roles of endogenous auxin in adventitious shoot formation in ipecac, we evaluated the effects of auxin polar transport inhibitors, auxin biosynthesis inhibitors, and auxin antagonists on adventitious shoot formation in the internodal segments. Disturbance of IAA levels shifted the positions of adventitious shoots from the apical region to the middle region of the segments. Our data show that endogenous auxin determines the positions of adventitious shoots formed on internodal segments of ipecac. In addition, only one shoot becomes dominant

during culture and auxin produced in this shoot suppresses the growth of other shoots.

Materials and methods

Plant material

The ipecac (*Carapichea ipecacuanha* (Brot.) L. Andersson) shoot culture system used in this study was established by Yoshimatsu and Shimomura (1991) at Tsukuba Medicinal Plant Research Station in Japan (now Tsukuba Division, Research Center for Medicinal Plant Resources, National Institutes of Biomedical Innovation, Japan). Ipecac plants were maintained at Toyo University. Sterile ipecac plants were propagated from shoot tips, nodes, and internodes. To induce adventitious shoots, internodal segments (5 mm) were placed horizontally on 25 ml phytohormone-free B5 medium (Gamborg et al. 1968) solidified with 0.2% Gelrite in a Petri dish (90 mm i.d. × 20 mm height) and cultured at 24 °C under a 14-h light / 10-h dark photoperiod (10–15 μmol photons m^{-2} s^{-1}) for 2 months. The total number of adventitious shoots longer than 0.3 mm was counted under a digital microscope (DHS1000; Leica Microsystems, Wetzlar, Germany). The biggest among adventitious shoots formed was defined as the dominant shoot. In some experiments, internodal segments were partitioned into four regions (apical to basal, I–IV) and adventitious shoots were counted in each region.

Chemicals

IAA and naphthalene-1-acetic acid (NAA) were purchased from Wako, Osaka, Japan. [2,4,5,6,7-^{2}H]IAA was purchased from OlChemim (Olomouc, Czech Republic). IAA and [2,4,5,6,7-^{2}H]IAA were dissolved in acetonitrile to prepare a 100 pg μl^{-1} stock and used to generate a standard curve in liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

Auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and *N*-(1-naphthyl)phthalamic acid (NPA) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Among auxin biosynthesis inhibitors, L-kynurenine (Kyn) was purchased from Tocris Bioscience (Bristol, UK) and 4-phenoxybenzeneboronic acid (PPBo) from Frontier Scientific (Logan, UT, USA). Auxin antagonists α-[2,4-dimethylphenyl ethyl-2-oxo]-IAA (auxinole) and 4-chloro-α-(phenyl ethyl-2-one)-IAA (4-Cl-PEO-IAA) were synthesized according to Hayashi et al. (2012). TIBA, NPA, and Kyn were dissolved in alkaline water to prepare 10 mM stocks and were stored at 4 °C. NAA was dissolved in alkaline water to prepare a 100 μM stock and was stored at 4 °C. PPBo, auxinole, and 4-Cl-PEO-IAA were

dissolved in acetone to prepare 100 mM stocks and were stored at − 30 °C. To estimate the effective concentrations of auxinole, 4-Cl-PEO-IAA, Kyn, PPBo, TIBA, and NPA, each chemical was tested at 0.1, 1, 10, or 100 μM (data not shown). The chemicals were added in culture medium. Eight internodal segments were used in each chemical treatment.

Preparation of frozen sections

Internodal segments were treated with auxin polar transport inhibitors (100 μM TIBA or NPA) for 4 weeks, fixed in a fixative solution (formalin: acetic acid: 50% ethanol = 5:5:90, by vol.) for 1 day at 4 °C, washed with Milli-Q water to remove the fixative solution, 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 attached to 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 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 sections were observed under an optical microscope (BX63; Olympus, Tokyo, Japan).

IAA extraction and purification

Internodal segments were cultured for 1 week and were cut into four regions (apical to basal, I–IV). Four internodal segments were used in each experiment. Solid-phase extraction and purification of IAA from each region were performed as described previously (Koike et al. 2018) with some modifications. Each region (20–50 mg) was collected in a 2.0-ml tube with a zirconia bead, frozen in liquid nitrogen, and crushed in a TissueLyser II (Qiagen, Hilden, Germany). The crushed sample was suspended in 1 ml of acetonitrile containing 500 pg of [2,4,5,6,7-^{2}H]IAA as an internal standard. The sample was incubated at 4 °C for 1 h and was centrifuged at 3500*g* for 5 min at room temperature. The pellet was washed with 80% (v/v) acetonitrile containing 1% (v/v) acetic acid and centrifuged as above. Both supernatants were combined and mixed with 600 μl of water containing 1% acetic acid, and acetonitrile was evaporated. Each sample was loaded onto a pre-equilibrated Oasis HLB cartridge column (Waters, Milford, MA, USA). The cartridge was washed with 1 ml of water containing 1% (v/v) acetic acid. IAA was eluted with 2 ml of 30% (v/v) acetonitrile containing 1% (v/v) acetic acid. Each fraction was evaporated and stored at − 30 °C until LC–MS/MS analysis.

LC–MS/MS analysis

Each fraction was dissolved in 20 µl of 30% (v/v) acetonitrile containing 1% (v/v) acetic acid. The samples were analyzed on an LC–MS/MS system consisting of a triple quadrupole mass-spectrometer (3200 QTRAP; Sciex, Framingham, MA, USA) and a high-performance liquid chromatograph (Prominence; Shimadzu, Kyoto, Japan) equipped with an Acuity BEH C18 column (ø 2.1 mm × 100 mm; Waters). HPLC conditions and parameters of the ion source were as described previously (Koike et al. 2018). LC–MS/MS analysis was controlled by Analyst v. 1.5.1 software, and IAA was quantified on a standard curve of the ratio of unlabeled IAA to [2,4,5,6,7-^{2}H] IAA in MultiQuant v. 2.0.2 software (Sciex).

Statistical analysis

All experiments were arranged in a completely randomized design. Statistical analyses were carried out in SPSS 23.0 software (IBM SPSS Inc., Armonk, NY, USA). The data were assumed to follow normal distribution. Pairwise comparisons were performed by t-test after evaluation of variance by F-test. Multiple comparisons were performed by Tukey's honestly significant difference test after ANOVA.

Results

Effects of exogenous auxin and auxin antagonists on adventitious shoot formation

To evaluate the effect of exogenously applied auxin, we added NAA to culture medium. NAA strongly suppressed adventitious shoot formation at very low concentrations (Fig. 1). Adventitious shoots were formed mainly in regions I and II of internodal segments in the control, and their formation was strongly suppressed in all regions by NAA treatment (Fig. S1). Auxinole (100 µM) decreased the total number of shoots to approx. 75% of that in the control (Fig. 2a). The number of shoots in region I decreased drastically, whereas that in region III and IV increased. When we applied 10 µM 4-Cl-PEO-IAA, the total number of shoots decreased to approx. 55% of that in the control (Fig. 2b). The number of shoots decreased strongly in regions I and II but increased in region III. There was a dominant shoot in the control but not in auxin antagonist treatment (Fig. 2). Supplementation with 0.05 µM NAA partially restored the number of shoots and the positions of shoot formation altered by the antagonists (Fig. 2).
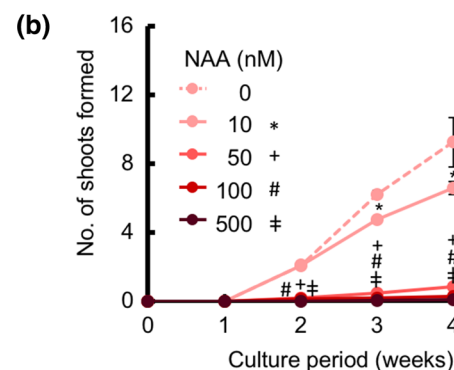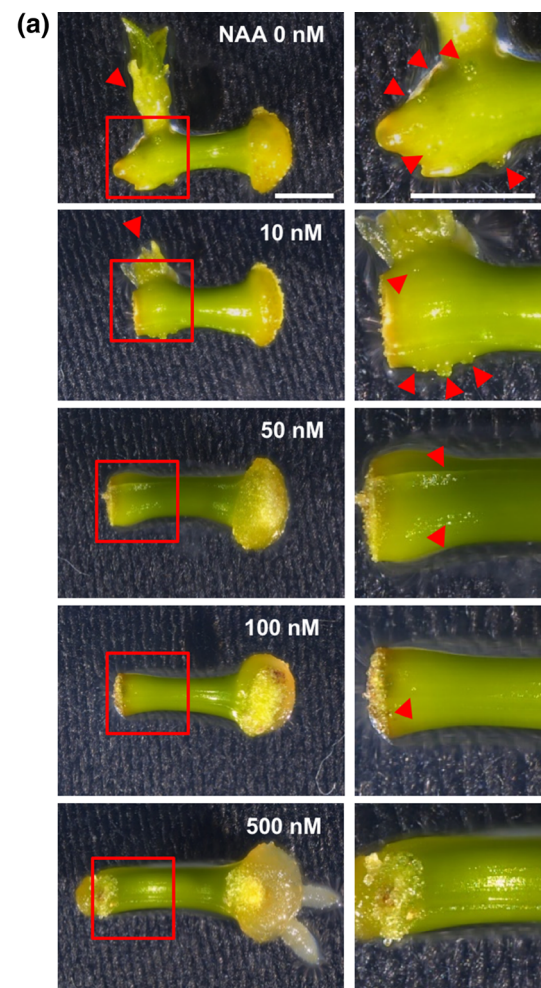
Fig. 1 Effect of NAA on adventitious shoot formation. **a** Adventitious shoots after 5 weeks of culture. Red arrowheads indicate adventitious shoots formed. Right panels are magnified photographs of the areas shown by red squares in left panels. Bars 2 mm. **b** Number of adventitious shoots formed on internodal segments treated with NAA. Data are means ± SE (n = 3). "Asterisk, plus, ash, alveolar" indicates significant difference compared with control (t test, $P < 0.05$)

(a)

Auxinole   0 μM   100 μM   100 μM
NAA   0 μM   0 μM   0.05 μM

(b)

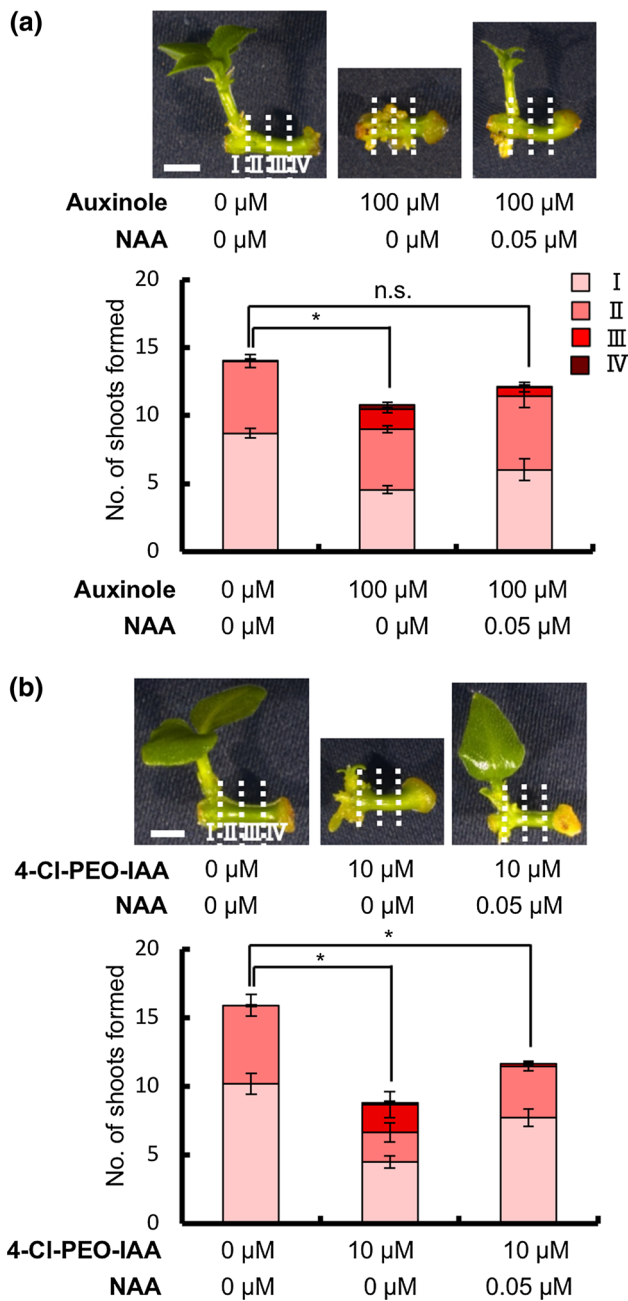4-Cl-PEO-IAA   0 μM   10 μM   10 μM
NAA   0 μM   0 μM   0.05 μM

Fig. 2 Effects of auxin antagonists on adventitious shoot formation. Numbers of adventitious shoots formed on internodal segments treated with **a** auxinole or **b** 4-Cl-PEO-IAA in the presence or absence of NAA were counted after 8 weeks of culture. Bars 2 mm. Data are means ± SE (*n* = 3). "Asterisk" indicates significant difference from control in the total number of shoots (*t* test, *P* < 0.05); *n.s.* not significant

Effects of auxin biosynthesis inhibitors on adventitious shoot formation

Endogenous IAA levels increase in the basal region of internodal segments after 1 week of culture (Koike et al.
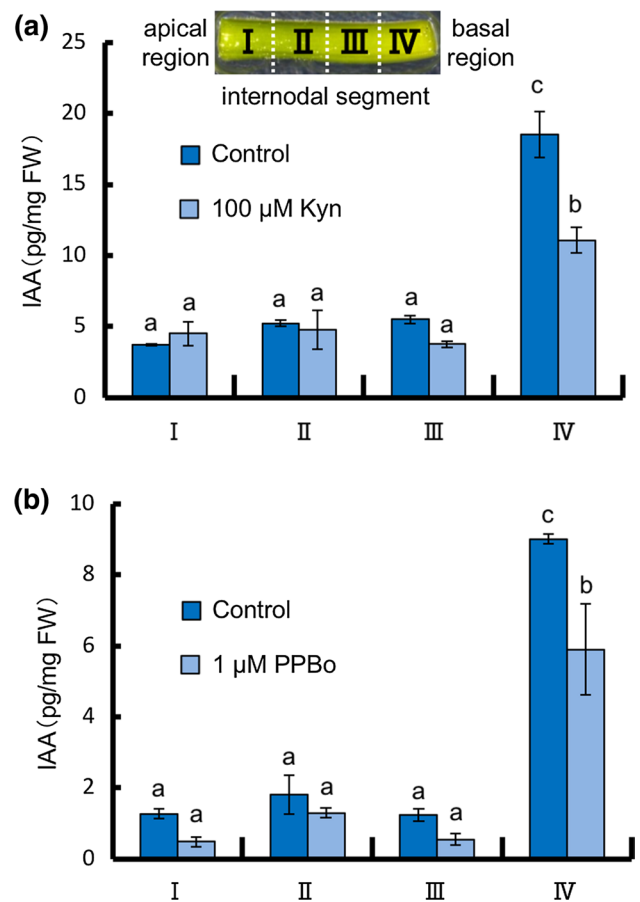

Fig. 3 IAA levels in internodal segments treated or not with auxin biosynthesis inhibitors. Effects of **a** Kyn and **b** PPBo were determined after 1 week of culture. Data are means ± SE (*n* = 3). Different letters above bars indicate significant difference (Tukey's HSD, *P* < 0.05)

2017). To explore activation of IAA biosynthesis in the basal region, we next investigated the effects of auxin biosynthesis inhibitors on endogenous IAA levels in regions I–IV after 1 week of culture (Fig. 3). IAA levels in regions I–III were not significantly affected by 100 μM Kyn or 1 μM PPBo, but decreased to 60%–65% of the control level in region IV in the presence of either inhibitor. In the presence of 100 μM Kyn or 1 μM PPBo, the number of shoots in region I decreased drastically, whereas those in regions III and IV increased; the total number of shoots decreased to half of that in the control (Fig. 4). One shoot became dominant in the control, but no dominant shoot was observed in the presence of Kyn or PPBo. Supplementation with 0.05 μM NAA partially restored the number of shoots decreased and the positions of shoot formation by treatment with the biosynthesis inhibitors.
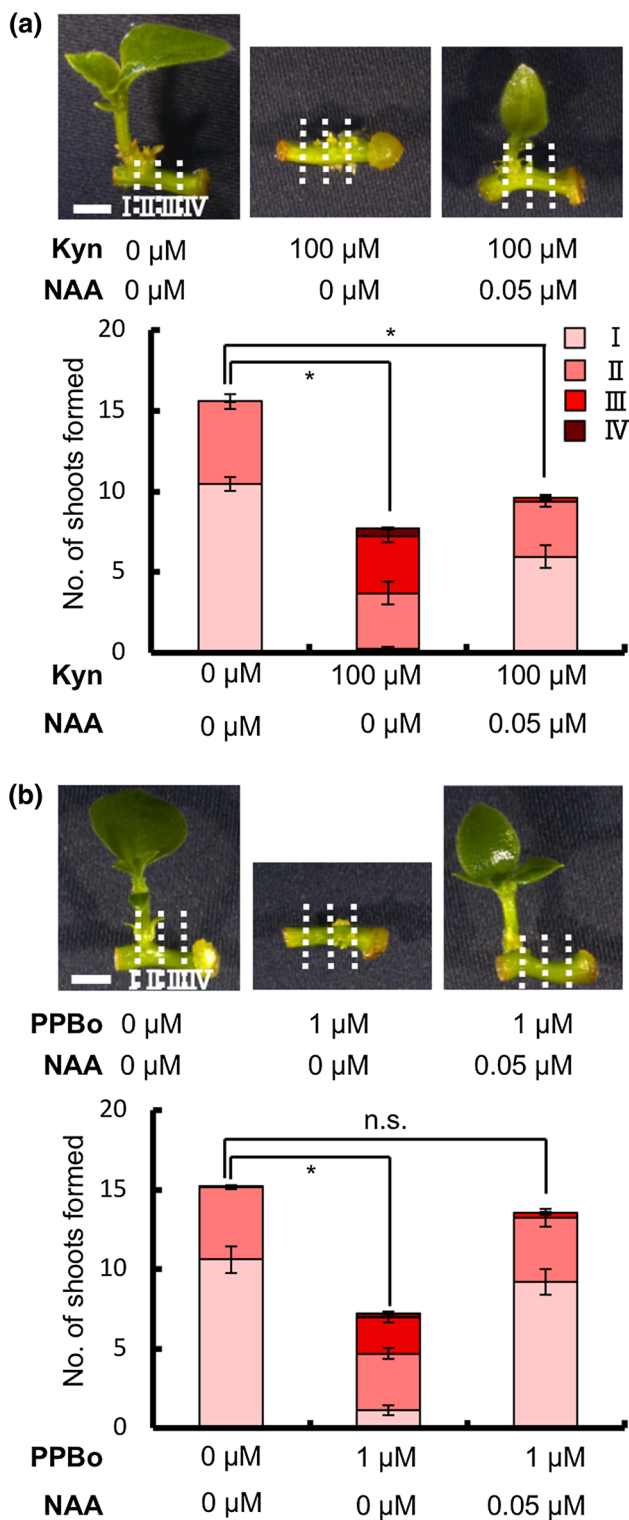
(a)

Kyn 0 µM   100 µM   100 µM
NAA 0 µM   0 µM   0.05 µM

| Kyn | 0 µM | 100 µM | 100 µM |
| NAA | 0 µM | 0 µM | 0.05 µM |

(b)

PPBo 0 µM   1 µM   1 µM
NAA 0 µM   0 µM   0.05 µM

| PPBo | 0 µM | 1 µM | 1 µM |
| NAA | 0 µM | 0 µM | 0.05 µM |

Fig. 4 Effects of auxin biosynthesis inhibitors on adventitious shoot formation. Numbers of adventitious shoots formed on internodal segments treated with **a** Kyn or **b** PPBo in the presence or absence of NAA were counted after 8 weeks of culture. Bars, 2 mm. Data are means ± SE ($n = 3$). "Asterisk" indicates significant difference from control in the total number of shoots (*t* test, $P < 0.05$); n.s., not significant
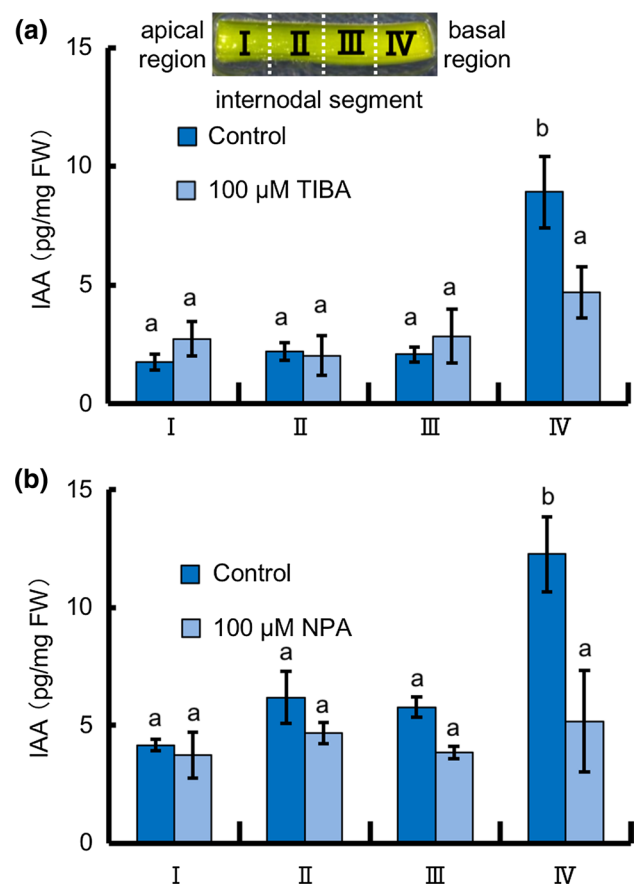

Fig. 5 IAA levels in internodal segments treated or not with auxin transport inhibitors. Effects of **a** TIBA and **b** NPA were determined after 1 week of culture. Data are means ± SE ($n = 3$). Different letters above bars indicate significant difference (Tukey's HSD, $P < 0.05$)

Effects of auxin transport inhibitors on adventitious shoot formation

A strong increase in IAA in the basal region of internodal segments (Koike et al. 2017) might also suggest that IAA is transported from the apical region to the basal region and accumulates in the basal region.

To test this possibility, we investigated the effects of auxin transport inhibitors on endogenous IAA levels in the four internodal segment regions. After 1 week of culture in the presence of 100 µM TIBA or NPA, IAA levels in regions I–III did not differ significantly from those in control segments, but those in region IV decreased to approx. 40%–50% of the control level (Fig. 5). Next, we counted shoots formed in each of the four regions every week for 8 weeks. In the presence of TIBA, the number of shoots in regions III and IV increased, resulting in a concentration-dependent increase in the total number of shoots (Fig. 6a, Fig. S2). In the presence of NPA, the number of shoots in regions III and IV also increased, but that in regions I and II decreased, resulting in
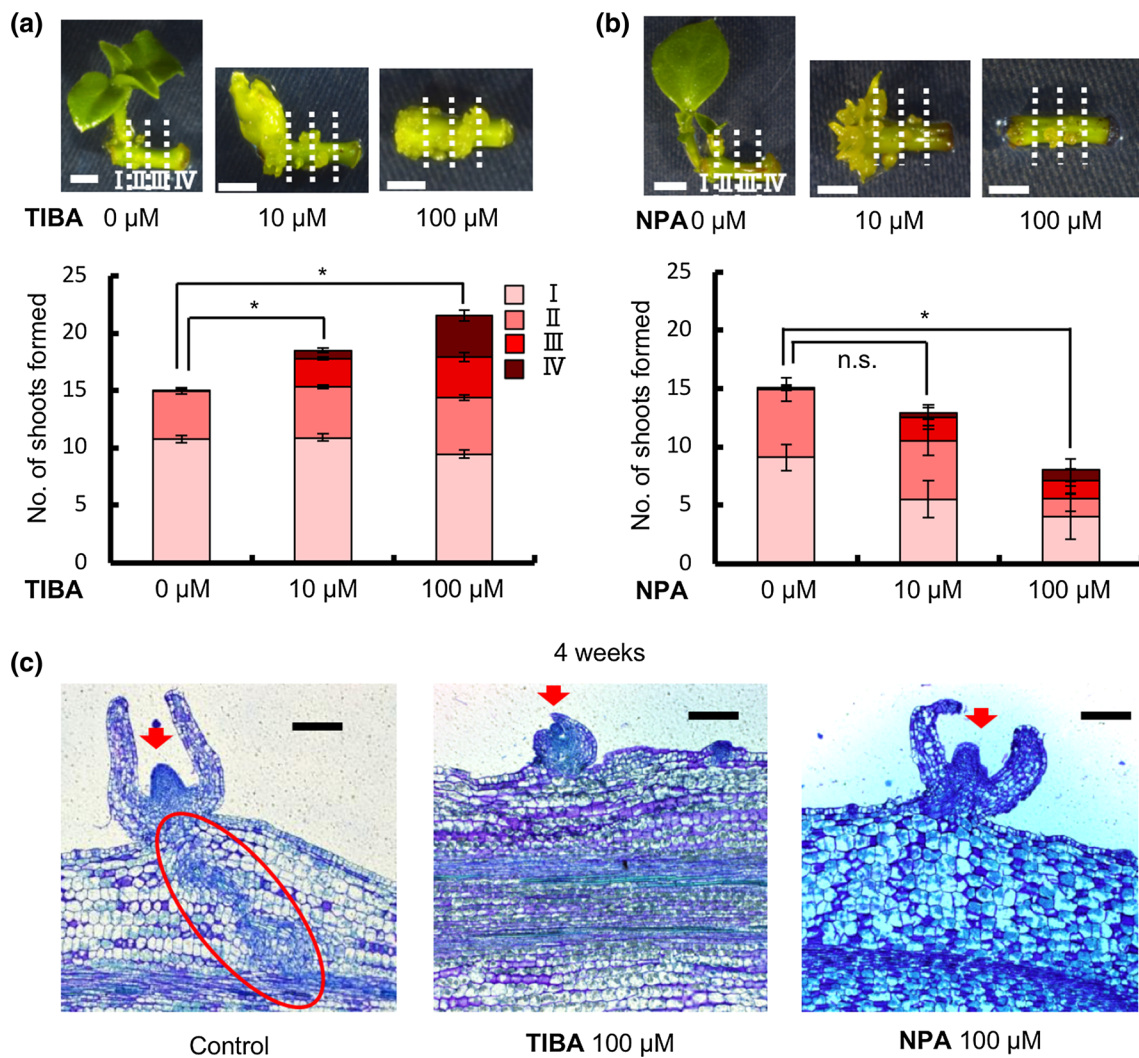
Fig. 6 Effects of auxin transport inhibitors on adventitious shoot formation. Numbers of adventitious shoots formed on internodal segments treated with **a** TIBA or **b** NPA were counted after 8 weeks of culture. Bars 2 mm. Data are means ± SE ($n = 3$). "Asterisk" indicates significant difference from control in the total number of shoots (t test, $P < 0.05$); *n.s.* not significant. **c** Internal structure of internodal segments after 4 weeks of culture in the presence or absence of TIBA or NPA. Cross-sections were stained with 0.05% toluidine blue O. Red ellipse, vascular bundles; arrows, adventitious shoots. Bars 500 μm

a concentration-dependent decrease in the total number of shoots (Fig. 6b, Fig. S3). In the absence of auxin transport inhibitors, one dominant shoot was observed after 8 weeks of culture (Fig. 6a, b). Its growth was suppressed by auxin transport inhibitors. Examination of frozen sections revealed that the vascular bundle of the untreated dominant shoot was connected with that of the internodal segment, but we could not find any development of vascular tissues in shoots treated with auxin transport inhibitors (Fig. 6c).

After 8 weeks of culture, adventitious shoots formed in the apical regions (I and II) of internodal segments placed horizontally on phytohormone-free medium (Figs. 1, 2, 4, 6). Shoots formed normally even if internodal segments were placed on the medium vertically, regardless of whether the apical region faced upward or downward (Fig. S4). These results indicate that internodal segments have tissue polarity that determines the position of adventitious shoot formation.

Effects of auxin in an outgrowing adventitious shoot

Removal of the dominant shoot at 7 weeks of culture resulted in the growth of another dominant shoot and an increase in the number of newly formed shoots after three additional weeks of culture, resembling a release from apical dominance (Fig. 7). When 1 μM NAA was applied on the cut surface after dominant shoot removal, shoot outgrowth was strongly suppressed (Fig. 7). Therefore, we analyzed endogenous IAA levels in the leaves and stem of the dominant
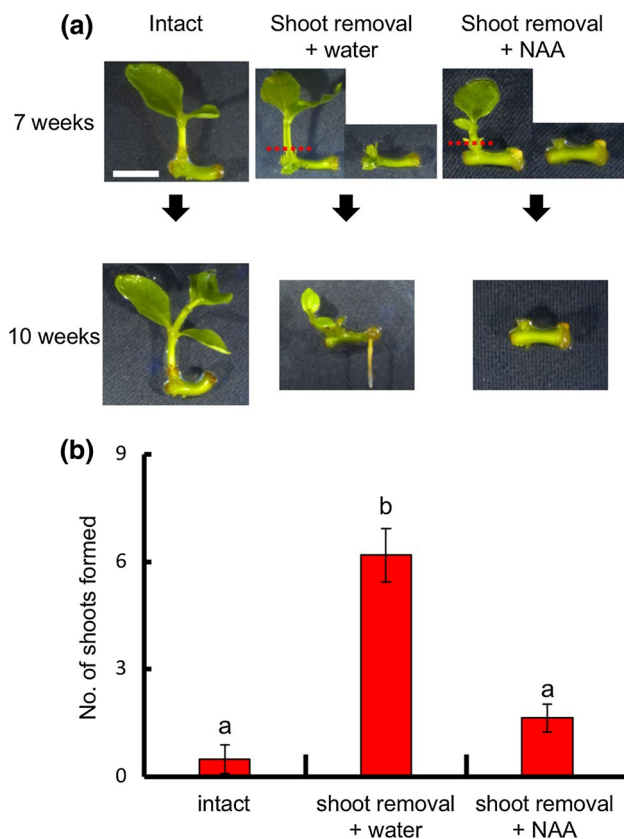
Fig. 7 Effect of NAA applied after removal of a dominant adventitious shoot on formation of new adventitious shoots. **a** Internodal segments either intact or with a shoot removed with or without 1 μM NAA treatment. The dominant shoot was removed after 7 weeks of culture, and the internodal segment was cultured for a further 3 weeks. Bar 5 mm. **b** Newly formed adventitious shoots were counted 3 weeks after shoot removal. Data are means ± SE (*n* = 3). "Asterisk" indicates Tukey's HSD, *P* < 0.05

shoot and found them to be higher than those in the apical region of the internodal segment (Fig. S5).

Discussion

Our work addresses the mechanism of the role of endogenous auxin in adventitious shoot formation. Using inhibitors of auxin signaling, biosynthesis, and transport, we showed that the inhibition of auxin signaling, biosynthesis, or transport shifted the position of adventitious shoot formation from the apical to the middle region of internodal segments, indicating that endogenous auxin determines the position of shoots formed on internodal segments of ipecac (Figs. 2, 4, 6). This is the first demonstration of the effects of endogenous auxin on plant regeneration without callusing in tissue culture.

Excess auxin strongly suppressed adventitious shoot formation (Fig. 1). However, auxin antagonists and auxin biosynthesis inhibitors also suppressed adventitious shoot formation (Figs. 2, 4), suggesting that an optimum auxin level is required for adventitious shoot formation on internodal segments (Fig. S6). Auxinole and 4-Cl-PEO-IAA, recently developed auxin antagonists, bind the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Hayashi et al. 2012) and are used for the analysis of auxin action (Smekalova et al. 2014; Dindas et al. 2018). Treatment with either antagonist shifted the positions of adventitious shoot formation from the apical region to the middle region (Fig. 2). Kyn is a competitive inhibitor of *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* in auxin biosynthesis (He et al. 2011), and PPBo inhibits YUCCA (Kakei et al. 2015). The application of these auxin biosynthesis inhibitors reduced endogenous auxin production, reduced the total amount of auxin transport and decreased IAA levels in region IV but did not affect IAA levels in regions I–III (Fig. 3). Free IAA might be released from IAA–amino acid conjugates in internodal segments, because the inhibitor treatment was short (1 week).

NPA and TIBA are well characterized auxin transport inhibitors (Thomson et al. 1973; Katekar and Geissler 1980). Both shifted the position of adventitious shoot formation from the apical region to the middle region. Interestingly, the total number of adventitious shoots was increased by TIBA treatment but decreased by NPA treatment (Fig. 6). The mechanisms of action of NPA and TIBA are different: NPA targets both ATP-BINDING CASSETTE subfamily B transporters and PIN proteins (Geldner et al. 2001; Noh et al. 2001), whereas TIBA disrupts the membrane localization of PIN proteins by blocking actin-dependent vesicle trafficking, but has no effect on ATP-BINDING CASSETTE subfamily B transporters (Dhonukshe et al. 2008). This difference probably explains why NPA is a stronger inhibitor of auxin efflux than TIBA. Nevertheless, TIBA and NPA each suppressed the development of vascular bundles (Fig. 6c). Auxin signaling is required for vasculature patterning in leaves and stems (Sachs 1981; Berleth and Sachs 2001; Mattsson et al. 2003).

One adventitious shoot became dominant after 7 weeks of culture (Fig. 7). This phenomenon resembles shoot apical dominance (Cline 1991). Removal of the dominant shoot induced the growth of another dominant shoot and formation of new shoots, which were suppressed by exogenous NAA applied on the cut surface (Fig. 7). When auxin is applied to the top of a decapitated plant, it mimics the effect of the removed shoot apex, preventing axillary bud outgrowth (Thimann and Skoog 1934). A dominant adventitious shoot and other shoots have characteristics similar to those of the shoot apex and axillary buds, respectively (Koike et al. 2017).

Here we propose a hypothetical model of adventitious shoot formation of ipecac. Low endogenous auxin levels

Fig. 8 A hypothetical model of polar auxin transport in adventitious shoot formation. **a** Early stage of adventitious shoot formation. Auxin is transferred from the apical to the basal region of the internodal segment by PIN auxin efflux carriers, which maintain low endogenous auxin levels on the apical side, facilitating differentiation of adventitious shoots. Auxin accumulates on the basal side of the internodal segment, where it suppresses adventitious shoot formation and induces callus formation. **b** Late stage of adventitious shoot formation. One of the shoots formed becomes dominant. Auxin from its apical meristem is transported into the internodal segment, and the auxin transport stream suppresses growth of other shoots. Auxin is further transported from the apical side to the basal side of the internodal segment. Auxin flow is hypothesized to determine the pattern of adventitious shoot formation

(a) Early stage

(b) Late stage
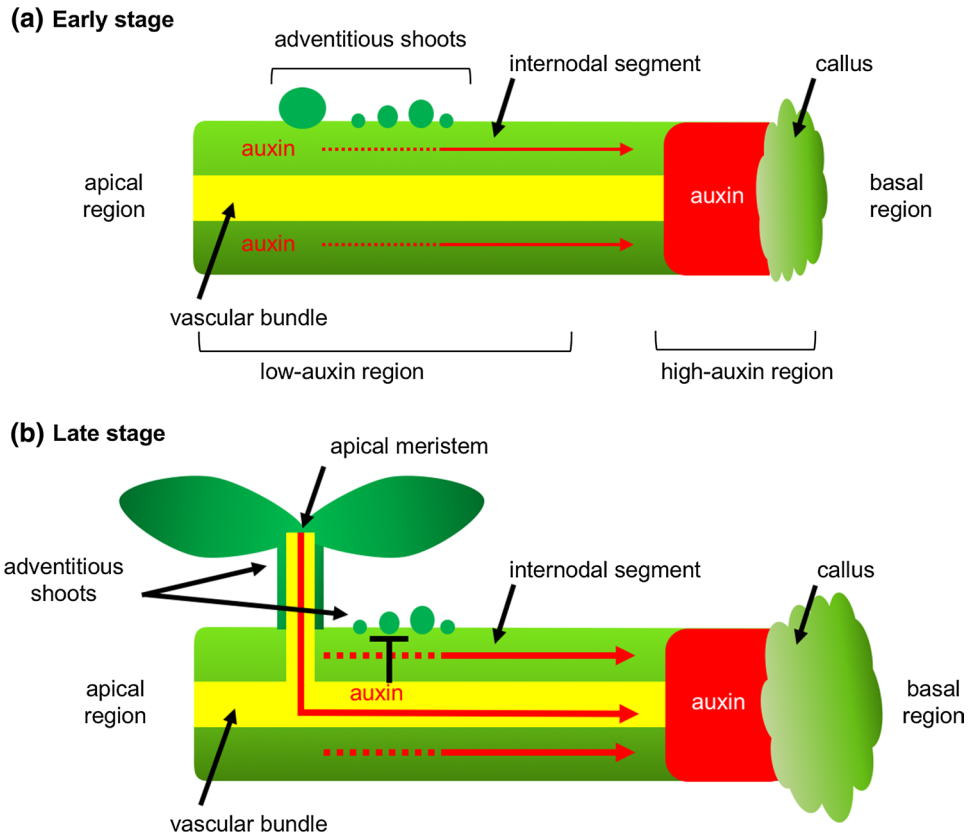
are critical for axillary meristem initiation (Wang et al. 2014a, b) and are caused by the activity of PIN auxin efflux carriers (Qi et al. 2014). In the early stage of adventitious shoot formation, auxin polar transport from the apical region to the basal region of internodal segments results in low endogenous auxin levels on the apical side, which facilitates differentiation of adventitious shoots from epidermal cells (Fig. 8a). In contrast, auxin accumulation in the basal region suppresses adventitious shoot formation and induces callus formation (Fig. 8a). Polar auxin transport forms tissue polarity of the internodal segment. In the late stage of adventitious shoot formation, auxin from an adventitious shoot is transported into the internodal segment, stimulating vascular bundle formation and promoting connection of the vasculature between the shoot and the segment, which results in the onset of dominant shoot growth. The dominant shoot produces high levels of IAA (Fig. S5); it acts as an auxin source, and the IAA transport stream derived from it suppresses the growth of other shoots, as in shoot apical dominance (Fig. 8b).

Endogenous cytokinin levels increase together with changes in endogenous auxin during adventitious shoot formation (Koike et al. 2017). In *Arabidopsis*, exogenously applied cytokinin modulates organogenesis via regulation of the expression of the *PIN* gene (Pernisova et al. 2009). AUXIN RESPONSE FACTOR 3, a component of auxin signaling, negatively regulates cytokinin biosynthesis (Cheng et al. 2013). Cytokinin signaling also promotes *WUSCHEL* expression to establish a new stem-shoot cell niche in *Arabidopsis* (Meng et al. 2017; Zhang et al. 2017). Strigolactone, another plant hormone, inhibits shoot branching in shoot apical dominance (Gomez-Roldan et al. 2008; Umehara et al. 2008). Strigolactone interacts with auxin and cytokinin in shoot branching regulation (Ferguson and Beveridge 2009; Hayward et al. 2009; Dun et al. 2012). To better understand the mechanism of adventitious shoot formation in ipecac, it is important to uncover interactions among auxin, cytokinin, and strigolactone. In ipecac, adventitious shoots are formed from epidermal cells of internodal segments (Koike et al. 2017). Which epidermal cells can be induced by endogenous hormones to differentiate into shoots is unknown, and it is also important to determine how epidermal cells become competent for adventitious shoot formation.

Author contribution statement HK, KH, KS, and MU conceived and designed research. KS maintained the ipecac plants. KH synthesized and provided auxin antagonists. IK, SW, and KO conducted experiments. IK, SW, KO, and MU analyzed the data. IK, KO, and MU wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

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

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