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Cytokinin and Ethylene Affect Auxin Transport-Dependent Rhizogenesis in Hypocotyls of Common Ice Plant (*Mesembryanthemum crystallinum* L.)

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Abstract Hypocotyl explants of *Mesembryanthemum crystallinum* regenerated roots when cultured vertically with either the apical end (AE) or basal end (BE) in media containing indole-3-acetic acid (IAA). IAA alone induced roots regularly from the basal end of the explants, either from the cut surface immersed in the medium or from the opposite side. The inhibitors of auxin efflux carriers, α-naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA), inhibited rhizogenesis only from AE-cultured explants, indicating the role of polar auxin transport in root regeneration in this system. Cytokinin (zeatin, kinetin, BAP) added to auxin-containing medium reduced rhizogenesis from the explants maintained with BE and AE and additionally changed the IAA-induced pattern of rooting in AE-cultured explants by favoring rooting from the apical end and middle part of the hypocotyl with its concomitant reduction from the basal end. The addition of kinetin did not influence the content of IAA in the explants maintained with AE, suggesting that the cytokinin effect on root patterning was not dependent on auxin biosynthesis. Kinetin, however, strongly enhanced ethylene production. The importance of ethylene in regulating PAT-dependent rhizogenesis was tested by using an ethylene antagonist $AgNO_3$, an inhibitor of ethylene synthesis aminoethoxyvinylglycine (AVG), and a precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC). $AgNO_3$ applied together with IAA or with IAA and kinetin strongly reduced the production of ethylene, inhibited rhizogenesis, and induced nonregenerative callus from BE, suggesting the need for ethylene signaling to elicit the rhizogenic action of auxin. A reduction of rhizogenesis and decrease of ethylene biosynthesis was also caused by AVG. In addition, AVG at 10 μM reversed the effect of cytokinin on root patterning, resulting in roots emerging only from BE on the medium with IAA and kinetin. Conversely, ACC at 200 μM markedly enhanced the production of ethylene and partly mimicked the effect of cytokinin when applied with IAA alone, thus confirming that in cultured hypocotyls of ice plant, cytokinin affects IAA-induced rhizogenesis through an ethylene-dependent pathway.

Keywords Adventitious roots · Auxin · Cytokinin · Ethylene · *Mesembryanthemum crystallinum* · Polar auxin transport

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Introduction

Auxin, indole-3-acetic acid (IAA), is a key signaling molecule involved in almost all aspects of root growth and differentiation. It was shown to influence the induction of rhizogenic competence (Dubrovsky and others 2008),

differentiation of the root apical meristem (Jiang and Feldman 2005), development of the root cap (Ponce and others 2005), formation of the root vascular system (Aloni 2004), and tropic responses (Swarup and others 2005). These physiologic auxin effects are directly related to unique auxin movement throughout the plant body, called polar auxin transport (PAT). PAT is a specialized delivery system whereby IAA is transported from cell to cell with a strict directionality: from the shoot apex toward the root apex in the stele and in the opposite direction within the root cortex and epidermis. The polarity of auxin flux is believed to be determined by the specific cellular distribution of auxin influx carriers (AUX1 proteins) and efflux carriers (PIN and MDR/PGP proteins) that mediate the flow of hormone into and out of the cells, respectively (for review see Vieten and others 2007).

Since the classic studies of Skoog and Miller (1957), it has been known that cytokinins interact with auxin and that the relative ratio of these two hormones determines the type of organs regenerated in vitro. Subsequent studies on whole plants and excised tissues revealed that cytokinins are negative regulators of both lateral and adventitious root development and that they can reverse the inductive effect of auxin (for review see Rashotte and others 2005). The inhibitory effect of cytokinin on root formation is ascribed to the downregulation of expression of root-specific cyclin-dependent kinases (John and others 1993). However, the more recent report of Laplaze and others (2007) proved that the inhibition of rhizogenesis by cytokinin could also be due to the disruption of PAT by this hormone, which prevents the formation of the proper auxin gradient and the patterning of root primordia. Further studies by Kuderová and others (2008) confirmed that cytokinins can interact with auxin via the regulation of its polar flow. Cytokinins were also shown to regulate the biosynthesis of auxin, which in turn is known to modify the activity of its own transport machinery (for review see Vieten and others 2007).

Numerous studies have demonstrated the cross-talk between cytokinin and ethylene (for review see Casson and Lindsey 2003; Rashotte and others 2005). Cytokinins are well-known ethylene-inducing factors and cytokinin-induced ethylene was shown to be involved in the inhibition of elongation of roots and hypocotyls of *Arabidopsis* (Cary and others 1995). In addition, cytokinins can interact synergistically with auxin to stimulate ethylene production (Woeste and others 1999) and, conversely, auxin production is regulated by ethylene (Stepanova and others 2007). Data in the literature concerning the role of ethylene in root differentiation are contradictory. In some species, the treatment that enhances ethylene production was stimulatory for root formation (for example, Mergemann and Sauter 2000; Yamamoto and others 1995). However, the negative effect of ethylene on both lateral and adventitious root initiation was also reported (Kępczyński and others 2006; Negi and others 2008). Recent studies link the role of ethylene in rhizogenesis to its effect on the capability of auxin transport (Negi and others 2008). It was reported that ethylene regulates the transcription of some auxin transport components, which may result in inhibition (Morgan and Gausman 1966; Prayitno and others 2006; Suttle 1988) or acceleration (Negi and others 2008; Růžička and others 2007) of auxin flow in different plant species and organs. Considering the interactions between auxin, cytokinin, and ethylene, Aloni and others (2006) formulated the "root initiation hypothesis," whereby the disruption of PAT in root or shoot by ethylene is the prime cue for auxin accumulation and subsequent formation of root initials just around the IAA-inhibition site. According to these authors, cytokinins that diffuse throughout the cells of root and stem can locally antagonize IAA action as the main determinant of longitudinal spacing of primordia.

Mesembryanthemum crystallinum L. (common ice plant) is an important model for studying the plant responses to different environmental stresses. Previously, we reported the importance of hydrogen peroxide and some antioxidant enzymes in regulating the rhizogenesis in hypocotyl-derived callus of common ice plant (Libik and others 2005). As part of the investigation on molecular mechanisms that underlie root formation in this species, we have focused now on the hormonal control of this process. In this study we examined the relationship among auxin, cytokinin, and ethylene during direct root regeneration and tested the hypothesis as to whether cytokinin affects root regeneration by modulating ethylene levels and polar auxin transport.

Materials and Methods

Plant Material

Seeds of *Mesembryanthemum crystallinum* (L.) were obtained from plants grown in phytotron chambers at 25/20°C (light/dark), light regime of 16 h light (250-300 μmol m^{-2} s^{-1}, cool-fluorescent tubes) and 8 h dark at humidity of 65%. The seeds were surface sterilized with 70% (v/v) ethanol for 60 s, commercial bleach solution diluted in water (1:2 v/v) for 15 min, and next rinsed three times with sterile distilled water. Then they were germinated on 9-cm petri dishes containing 20 ml of MS medium (Murashige and Skoog 1962) with 30 g L^{-1} sucrose and 5 g L^{-1} agar (Difco Bacto, USA), pH 5.7. Dishes with seeds were kept in a growth chamber under 25/20°C (light/dark), 16-h/8-h (light/dark) photoperiod (light intensity = 100–120 μmol m^{-2} s^{-1}).

After 14 days of germination, hypocotyls (5-7 mm in length) were excised from the seedlings and mounted

vertically with the apical end (AE) down on the culture medium. Where indicated, cultures of hypocotyls embedded with the basal end (BE) down on the culture medium were also established.

Culture Media and Culture Conditions

The basal medium used in all experiments consisted of MS salts and vitamins (Murashige and Skoog 1962) supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar (Difco Bacto, USA). For induction of rhizogenesis, the basal medium was supplemented with 2 μM of auxin (IAA) and/or 1 μM of cytokinin (kinetin, zeatin, or BAP). The effect of hormones and orientation of explants on culture media were compared with respect to the efficiency of root production and pattern of root formation.

Inhibitors of auxin efflux carriers, such as 2,3,5-triiodobenzoic acid (TIBA) and α-naphthylphthalamic acid (NPA), at concentrations of 1, 10, 100, and 200 μM were used to determine the relationship between the pattern of root formation and PAT from AE-cultured explants on medium containing 2 μM IAA.

To evaluate the effect of ethylene on rhizogenesis (efficiency of root regeneration, pattern of root initiation), the media containing 2 μM IAA alone and containing 2 μM IAA + 1 μM kinetin were additionally amended with 50, 100, or 200 μM of the ethylene biosynthesis precursor 1-aminocyclopropane-1-carboxylic acid (ACC), 1, 5, or 10 μM of inhibitor of ethylene signaling, $AgNO_3$, or 1 and 10 μM of inhibitor of ethylene biosynthesis aminoethoxyvinylglycine (AVG).

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Germany). Growth regulators and $AgNO_3$ were added to the medium before sterilization; ACC, AVG, NPA, and TIBA were filter-sterilized and aseptically introduced into previously autoclaved and cooled medium. Stock solutions of 100 mM ACC and 10 mM AVG were prepared in water, whereas NPA and TIBA were dissolved in dimethyl sulfoxide (DMSO) as 20 mM stocks. The pH of all media was adjusted to 5.7 with 0.1 M NaOH and/or 0.1 M HCl before autoclaving.

In all in vitro experiments the hypocotyls were maintained under the same conditions as described for seed germination. Each treatment involved five replicates with ten hypocotyls per petri dish (9 cm in diameter and containing 20 ml of medium). After 14 days of culture, the percentage of rhizogenesis (root-producing explants ÷ total number of explants in each treatment × 100) and total number of roots induced from different hypocotyl regions (apical end, middle part, and basal end) were calculated. The terms "apical end" and "basal end" used in this study define the region of the hypocotyl within a distance of 2 mm from cut surfaces.

IAA Analysis

First we estimated IAA concentrations in freshly cut hypocotyls, just before explantation; these represent control values of endogenous IAA levels in the hypocotyls (day 0 in Fig. 4). To determine the effect of exogenous IAA and kinetin on endogenous auxin content, the explants were cultured for 3 days with AE on basic medium without hormones and on media supplemented with 2 μM IAA or 2 μM IAA with 1 μM kinetin. Weighted samples of plant material (~100 mg) were preserved in 1 ml methanol and kept at −70°C until analyzed. When a batch of samples had accumulated, methanol from each one was drawn out and kept aside for further processing, while the sample was homogenized in 500 μl methanol with 1% of acetic acid and extracted for 1 h with frequent vortexing. Then the samples were centrifuged, the supernatants collected, and the extraction repeated. The supernatants and methanol, in which the sample was stored, were combined, 10 ng of $^{13}C_6$-IAA was added as internal standard, and the solution was evaporated to dryness under vacuum in a centrifuge evaporator (Concentrator 5301, Eppendorf). The dry residues were dissolved in 150 μl methanol, 1 ml ether solution of diazomethane was added, and after 10 min the sample was evaporated to dryness under nitrogen. Methylated samples were dissolved in 30 μl ethyl acetate and 1-μl aliquots were analyzed on a gas chromatograph (Agilent 6890 N) equipped with a DB5MS capillary column (J&W) and mass spectrometric detector (Agilent 5975B inert XL MSD). The mass spectrometer was set to monitor ions at *m/z* 130, 136, 189, 195 (mass and molecular ions of methyl ester of IAA and its ^{13}C-labeled standard, respectively). The content of endogenous compounds in the analyzed samples was calculated as follows:

$$\text{ng IAA} = 10\frac{A130}{A136}$$

where A_X is the relative abundance of a given ion.

Ethylene Analysis

To determine the effect of exogenous auxin and cytokinin on ethylene production, the explants were cultured for 3 days with AE on media supplemented with 2 μM IAA and 2 μM IAA + 1 μM kinetin. Culture on hormone-free medium was used as a control. In the second set of experiments, the effect of 10 μM $AgNO_3$, 10 μM AVG, and 200 μM ACC on ethylene production by hypocotyls maintained on media with IAA alone and IAA together with kinetin was determined. The hypocotyls (30 per vial) were incubated in 8-ml gas chromatography vials containing 3 ml medium at the conditions described for seed germination. The vials were capped with screw caps with a

hole for 24 h before the ethylene measurements were done. After 24 h, vials were opened for 5 min and capped again, followed by incubation under the same conditions for 2 h. Then a 1-ml gas sample was withdrawn and injected into a gas chromatograph (Hewlett-Packard 5890 II) equipped with a flame ionization detector and stainless-steel column packed with 80/100 Poropack-Q. The oven temperature was 60°C. Ethylene production was expressed in nM/30 hypocotyls/2 h.

Statistical Evaluation

For each experiment the means from five (in vitro culture, ethylene assay) or three (IAA measurements) replicates were calculated. Statistical differences between mean values ($p \leq 0.05$) were determined with the analysis of variance (two-way ANOVA) followed by Duncan's multiple-range test using Statistica for Windows v8.0 (StatSoft, Inc., Tulsa, Oklahoma, USA).

Results

Effect of Auxins and Cytokinins

Cultures on hormone-free basic medium and in the presence of cytokinin only did not form roots (Fig. 1a, b). The onset of regeneration became evident after 5 days of culture when maintained on medium containing IAA alone. The hypocotyls produced about three times more roots and the efficiency (%) of regeneration was about twice as high when they were mounted AE compared to BE (Table 1). Notably, IAA alone promoted rhizogenesis only from the basal end of the hypocotyl, that is, either from the region in direct contact with the medium or from the opposite one when cultured BE or AE down, respectively (Table 1, Fig. 1c, d).

The addition of cytokinin (BAP, zeatin, or kinetin) to IAA-containing medium resulted in a significant decrease in the final percentage of rhizogenesis and number of roots formed (Table 1). For all treatments rhizogenesis was direct and occurred from significantly swollen explants after 6 days of culture (Fig. 1e–h). Comparing the roots obtained from cultures on media containing auxin alone, those induced in the presence of cytokinins were thicker and more densely coated with long root hairs. All cytokinins strongly influenced the pattern of root formation from the explants mounted AE, leading to the formation of roots along the whole length of the hypocotyl, with a higher number of roots induced from AE and/or the middle part of the explants than from BE (Fig. 1e–g). Notably, the addition of cytokinins did not affect the site of root production from BE-cultured explants (Table 1, Fig. 1h).

Effect of Auxin Transport Inhibitors

Increasing the concentration of TIBA and NPA caused a gradual decrease in the induction of rhizogenesis, leading finally to complete inhibition of root formation from the explants mounted AE (Fig. 2a). On the contrary to

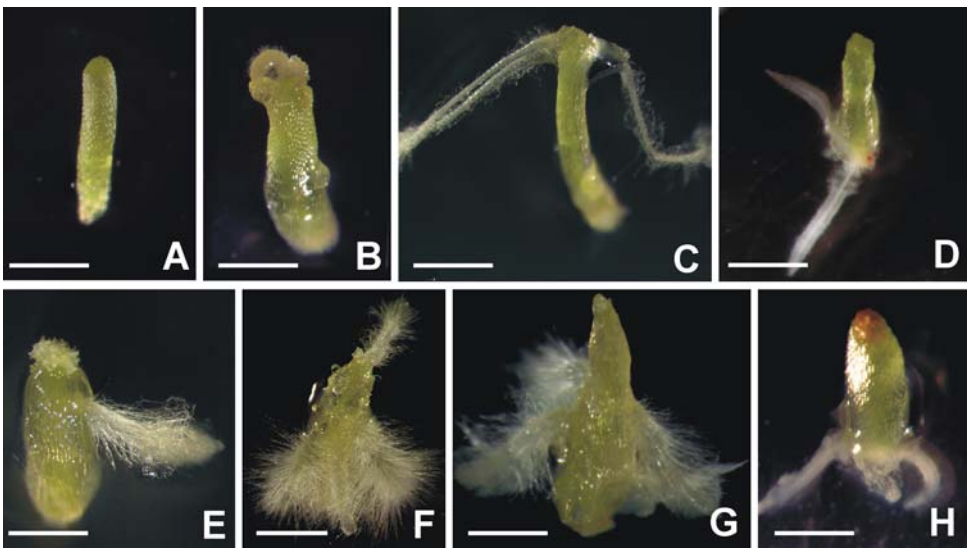

Fig. 1 Effect of auxin (2 μM), cytokinin (1 μM), and mode of placing of hypocotyls onto culture medium on root formation after 14 days of culture. **a** AE-cultured hypocotyl on hormone-free medium. **b** AE-cultured hypocotyl on medium with kinetin alone. **c**, **d** Root formation from hypocotyls placed AE down (**c**) and BE down (**d**) on medium containing IAA alone. **e–g** Effect of addition of BAP (**e**), zeatin (**f**), or kinetin (**g**) to IAA-containing medium on root formation from AE-cultured explants. **h** Rhizogenesis from BE-placed hypocotyl on medium containing IAA with kinetin. Scale bar = 2.5 mm

Table 1 Effect of auxins (2 μM), cytokinins (1 μM), and orientation of the explant on induction of rhizogenesis (%) and number of roots produced from different parts of hypocotyl

Growth regulators	AE immersed in the medium				BE immersed in the medium			
	(%)	No. of roots			(%)	No. of roots		
		AE	Middle part	BE		AE	Middle part	BE
—	0	0	0	0	0	0	0	0
IAA	100a	0	0	255	52a	0	0	82
BAP	0	0	0	0	0	0	0	0
Kinetin	0	0	0	0	0	0	0	0
Zeatin	0	0	0	0	0	0	0	0
IAA + BAP	22d	10	10	2	14c	0	0	10
IAA + kinetin	62b	36	40	14	20c	0	0	26
IAA + zeatin	58bc	4	31	11	26bc	0	0	21

Values in each column sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test

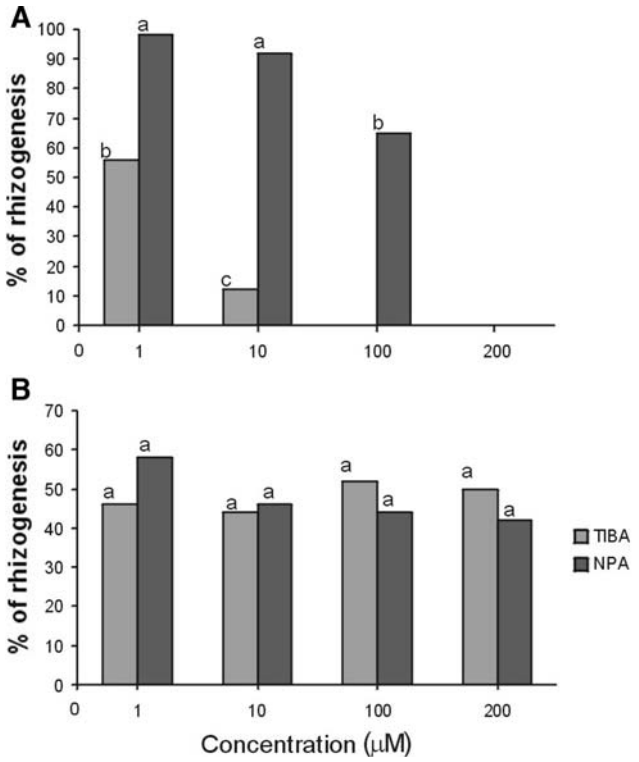

Fig. 2 The influence of different concentrations of TIBA and NPA on root formation from hypocotyls maintained AE down (**a**) and BE down (**b**) on medium containing 2 μM IAA. Treatments sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test

AE-placed hypocotyls, no influence of TIBA and NPA on root formation from BE-cultured explants was observed (Fig. 2b).

Manipulation of Ethylene Level and Ethylene Signaling

The results of the experiments with ACC, AVG, and $AgNO_3$ are summarized in Table 2. In combination with 2 μM IAA, 50 and 100 μM ACC had no effect on the percentage of rooting and caused only a little decrease in the number of roots when compared with cultures on IAA alone. At these two concentrations, ACC delayed the first appearance of roots until day 8 and promoted root initiation from both the apical and basal ends of only slightly swollen hypocotyls (Fig. 3a). The highest concentration of ACC (200 μM) strongly reduced the rhizogenic response, leading to the formation of single roots from AE after 10–12 days of culture (Fig. 3b). Unlike cultures with IAA as the sole hormone, the application of ACC to the medium with IAA + kinetin resulted in a failure of root production (Table 2). Instead, the explants became markedly swollen and developed smooth, nonregenerative callus at the place of contact with the culture medium (Fig. 3c).

AVG at concentrations of 1 and 5 μM exerted similar, slightly inhibitory effects on root formation in the presence of auxin and cytokinin, but reduced around twofold the percentage of induction (1 μM) and completely inhibited

Table 2 Effect of ethylene precursor (ACC), inhibitor of ethylene synthesis (AVG), and inhibitor of ethylene signaling ($AgNO_3$) on induction of rhizogenesis (%) and number of roots produced from different parts of hypocotyls cultured with AE on media containing 2 μM IAA or 2 μM IAA + 1 μM kinetin

Treatment (μM)	IAA				IAA + kinetin			
	(%)	No. of roots			(%)	No. of roots		
		AE	Middle part	BE		AE	Middle part	BE
–	100a	0	0	255	62a	36	40	14
ACC 50	94a	46	0	192	0	0	0	0
ACC 100	80a	42	0	167	0	0	0	0
ACC 200	10c	12	0	0	0	0	0	0
AVG 1	28c	0	0	14	42b	14	26	12
AVG 5	0	0	0	0	36b	21	0	34
AVG 10	0	0	0	0	18c	0	0	30
$AgNO_3$ 1	54b	0	0	60	36b	6	9	4
Ag NO_3 10	0	0	0	0	0	0	0	0

Values in each column sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test

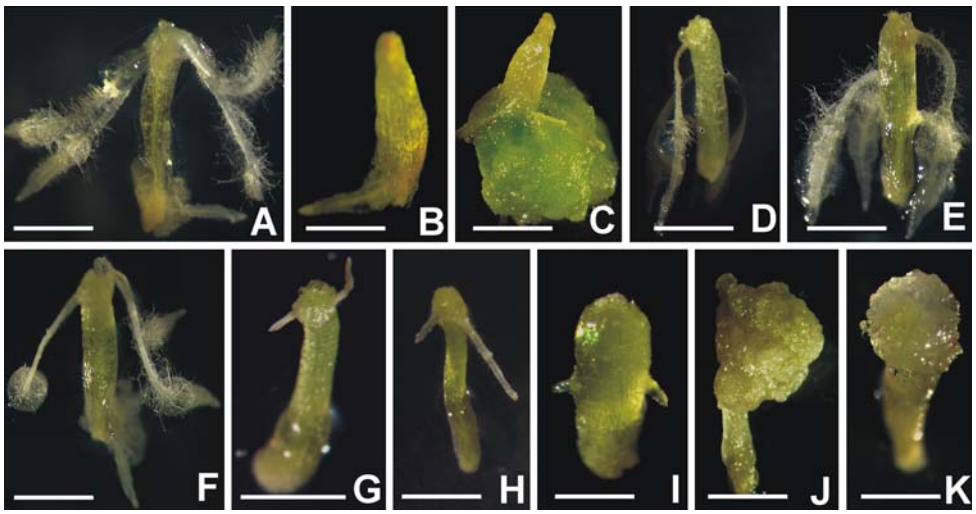

Fig. 3 Effect of ethylene precursor (ACC), inhibitor of ethylene synthesis (AVG), and ethylene antagonist ($AgNO_3$) on root formation from hypocotyls cultured AE down on media containing 2 μM IAA and 2 μM IAA together with 1 μM kinetin. **a**, **b** Effect of ACC at 100 μM (**a**) and 200 μM (**b**) on rhizogenesis induced on medium with IAA alone. **c** Callus formation on medium supplemented with IAA, kinetin, and 100 μM ACC. **d** Rhizogenesis from hypocotyl maintained on medium with IAA and 1 μM AVG. **e**–**g** Effect of AVG at 1 μM (**e**), 5 μM (**f**), and 10 μM (**g**) on rhizogenesis from explants maintained on medium with IAA together with kinetin. **h**, **i** Effect of 1 μM $AgNO_3$ on root formation from hypocotyls cultured on medium with IAA alone (**h**) and IAA together with kinetin (**i**). **j**, **k** Polar callus formation from the explants maintained on medium with IAA (**j**) and IAA with kinetin (**k**) after addition of 10 μm $AgNO_3$. Scale bar = 2.5 mm

rhizogenesis (5 μM) when added to the medium containing IAA alone (Table 2). Similar to that with ACC, the addition of AVG delayed the rhizogenic response until day 8. AVG at 1 μM had no effect on the pattern of root formation: The roots were induced from BE of the explant or along the hypocotyl when AVG was added to IAA- and IAA + kinetin-containing media, respectively (Fig. 3d, e). However, when applied at 5 μM to cytokinin-enriched medium, AVG partly abolished the effect of kinetin and restored the polar manner of rhizogenesis by induction of roots from BE and AE explants (Table 2, Fig. 3f). The application of higher doses of AVG (10 μM) was detrimental and led to degeneration of hypocotyls on IAA medium. On the other hand, when AVG was added to kinetin-containing medium, the explants remained green throughout the culture and formed thin roots regularly from the basal end (Fig. 3g). These roots were almost devoid of root hairs and died soon.

Application of 1 μM $AgNO_3$ caused a reduction by half of the percentage of responding explants, and the number of roots was reduced about fourfold in comparison to media containing IAA and IAA + kinetin, but without silver nitrate (Table 2). An evident effect of $AgNO_3$ on the morphology of induced roots was that it made them relatively thin and almost devoid of root hairs (Fig. 3h). Occasionally, little green outgrowths, which never developed further, formed on the surface of hypocotyls (Fig. 3i). Contrary to ACC and AVG, the addition of $AgNO_3$ had no effect on the pattern of root formation. Roots were induced only from BE of the explant on the medium containing $AgNO_3$ + IAA, or along the whole length of hypocotyls on the medium with $AgNO_3$ + IAA + kinetin (Table 2). The explants cultured in the presence of 10 μM $AgNO_3$ failed to produce roots with concurrent formation of nonregenerative callus at their BE (Fig. 3j, k).

IAA Content and Ethylene Production

The addition of kinetin to IAA-containing medium did not influence the endogenous level of auxin. During the first 3 days of culture, the endogenous content of IAA in explants was similar on both media tested and comparable to that found in freshly cut hypocotyls. In contrast, when cultured on hormone-free basal medium, the level of endogenous auxin decreased significantly after 1 day and remained constant the following next 2 days (Fig. 4).

During the first 3 days of culture, the production of ethylene by hypocotyls maintained on hormone-free medium was constant (~70 nM/30 hypocotyls/2 h), but it was about fivefold and eightfold less than that found for the explants cultured on media with IAA alone or IAA with kinetin, respectively (Fig. 5). AVG at 10 μM and $AgNO_3$ at 10 μM exerted a similar, strong inhibitory effect on ethylene synthesis, both on media containing IAA alone or IAA with kinetin (Fig. 6a, b). On the other hand, the addition of 200 μM ACC to the medium supplemented

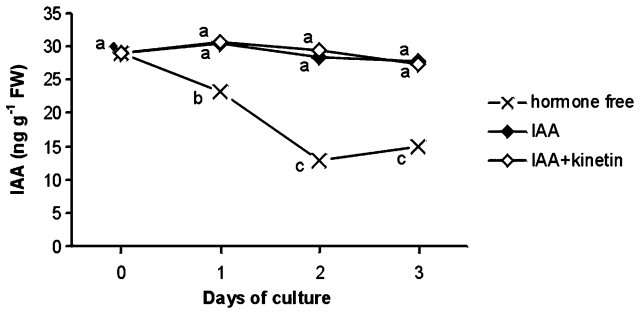

Fig. 4 The endogenous content of IAA in the hypocotyls maintained AE down on media containing 2 μM IAA or 2 μM IAA + 1 μM kinetin. Treatments sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test

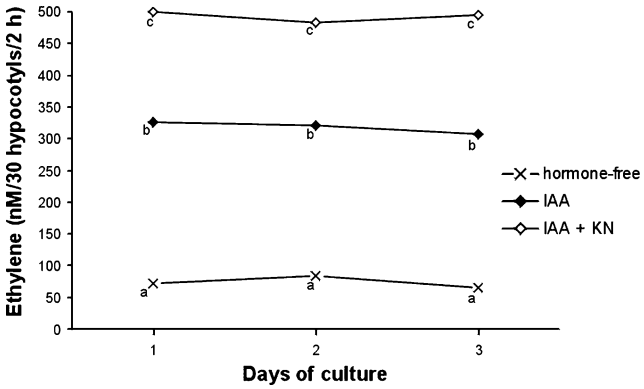

Fig. 5 The production of ethylene by hypocotyls maintained AE down on medium devoid of hormones and on media containing 2 μM IAA or 2 μM IAA + 1 μM kinetin. Treatments sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test

with 2 μM IAA or 2 μM IAA + 1 μM kinetin did not influence the ethylene production at day 1, followed by its significant increase at days 2 and 3 (Fig. 6a, b).

Discussion

The results of this study revealed the interactions among auxin, cytokinin, and ethylene during rhizogenesis from hypocotyls of common ice plant. In our study, the presence of IAA in the medium was necessary for the induction of root regeneration. When auxin was applied alone, the hypocotyls produced roots regularly from the basal end whether they were mounted BE or AE (Table 1, Fig. 1c, d). IAA is transported basipetally through the hypocotyl according to the antipolar alignment of auxin influx and efflux carriers in the cells (Rashotte and others 2003). In agreement with this, both TIBA and NPA reduced in a dose-dependent manner rhizogenesis from the explants maintained AE (Fig. 2a), confirming the involvement of

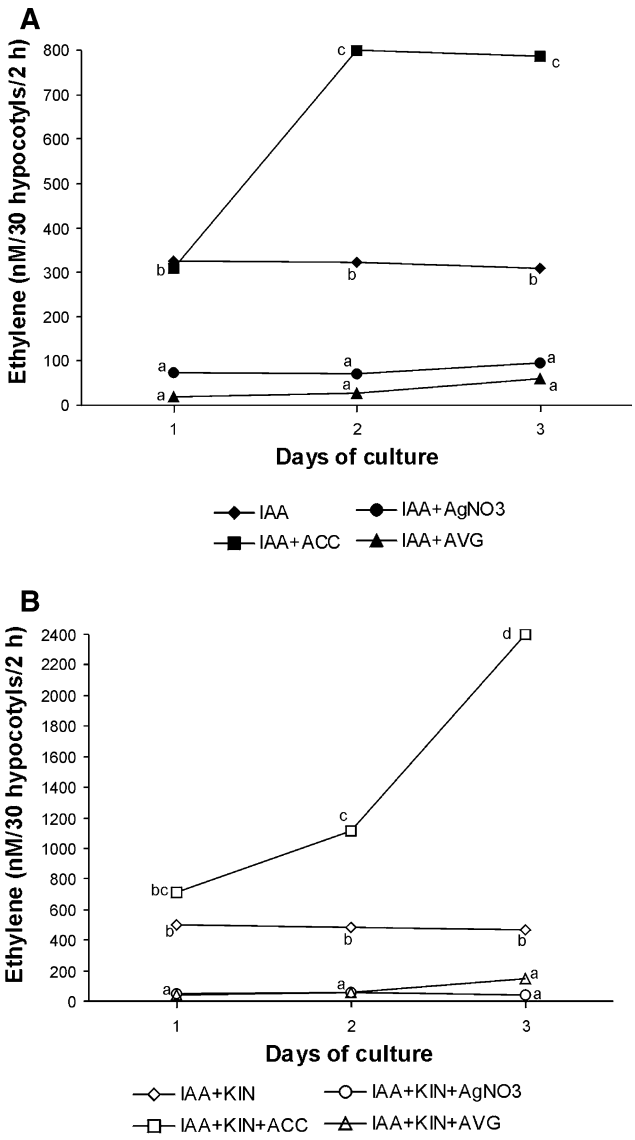

Fig. 6 Effect of 200 μM of ethylene precursor (ACC), 10 μM of inhibitor of ethylene synthesis (AVG), and 10 μM of ethylene antagonist ($AgNO_3$) on the ethylene production by hypocotyls maintained with AE down on media containing either 2 μM IAA (**a**) or 2 μM IAA + 1 μM kinetin (**b**). Treatments sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test

PAT in root formation in this system. It is worth mentioning that the concentration of TIBA sufficient for inhibition of rhizogenesis from AE of explants was twofold lower than NPA. The reason for this difference may be related to the different mode of action of TIBA and NPA. Aside from the effect on auxin transport, TIBA can also act as a weak auxin antagonist, being able to suppress the activity of IAA (Katekar and Geissler 1980). The role of PAT in the rooting of hypocotyl cuttings has already been reported in other species (for example, Liu and Reid 1992; López Nicólas and others 2004). However, hypocotyls of

the common ice plant could also regenerate roots in a PAT-independent way if they were placed BE, as revealed in our experiments. In this position, the roots were produced only from the region in direct contact with the medium and the process was not affected by PAT inhibitors (Fig. 2b), suggesting a passive uptake of exogenous auxin as the possible mechanism underlying regeneration.

The addition of cytokinin (kinetin, zeatin, or BAP) to auxin-containing medium strongly reduced the rhizogenic response of the explanted hypocotyls (Table 1), confirming the antagonistic role of these hormones in controlling rhizogenesis (for example, Li and others 2006; Lloret and Casero 2002; Zhang and Hasenstein 1999). In addition, each of the three cytokinins applied here could affect the IAA-induced pattern of rooting by favoring rhizogenesis from the middle and the apical end of the explant, with concomitant reduction of root production from the basal end (Fig. 1e–g). The position of root initiation is thought to be determined by local auxin accumulation in the tissue, which in turn depends on the activity of the auxin transport machinery (Benková and others 2003; Petrášek and others 2006). Recently, Laplaze and others (2007) reported that cytokinins can affect rhizogenesis via the downregulation of expression of some *PIN* genes. Notably, in our culture the effect of cytokinins on root patterning was visible only in the hypocotyls maintained AE down, where the activity of auxin efflux carriers was shown to be necessary to elicit the rhizogenic response (Fig. 2a). Thus, we hypothesized that the new pattern of rooting observed after addition of cytokinin to IAA-containing medium could be the result of modification of PAT in the hypocotyls maintained AE down.

Cytokinins can modulate the endogenous content of auxin, which in turn is known to regulate its own transport (Rashotte and others 2005; Vieten and others 2007). The elevated level of cytokinin achieved either via exogenous supply or transformation with bacterial *IPT* genes was shown to increase (Bertell and Eliasson 1992; Bourquin and Pilet 1990) or decrease the auxin content (Eklöf and others 1997; Nordström and others 2004) in different plant species. In our study, no differences in IAA content between ice plant explants maintained on media with IAA alone versus IAA together with kinetin were observed. Nordström and others (2004) concluded from studies on *Arabidopsis* that cytokinin affects auxin production via developmental changes rather than by a direct effect. In our study, the hypocotyls maintained on medium containing IAA alone and IAA with kinetin started to form roots at about the same time, that is, after 5 and 6 days of culture, respectively. Thus, it may not be ruled out that the observed similarity in the respective results could be due to the similar stages of rhizogenesis in the cultured material. When studying the interaction between auxin and cytokinin on the whole plant/organ level, one should also take into account the possible local changes in hormone homeostasis, which could be restricted to the single cells of the organ or respective tissue. To investigate this in further detail, studies on auxin distribution with the use of auxin-responsive reporter constructs and/or antiauxin antibodies are needed. Unfortunately, *M. crystallinum* is a plant species that has remained recalcitrant with respect to gene transformation techniques.

It is worth noting that the concentration of IAA in the explants displaying rhizogenesis was comparable to that found in freshly cut hypocotyls but significantly higher than that measured in explants grown on medium devoid of hormones (Fig. 4). This observation may indicate that at the time of explantation the hypocotyls of the common ice plant could already be competent to rhizogenesis and that the exogenous application of IAA was necessary only to keep the steady-state level of auxin to allow root formation. However, the results presented here showed that auxin itself was not a sufficient signal for root formation; it required ethylene. The ethylene antagonist ($AgNO_3$) at 1 μM reduced rhizogenesis by half and at 10 μM even completely inhibited it, on media with IAA alone and with IAA and kinetin (Table 2). The results of ethylene measurements after application of 10 μM of $AgNO_3$ revealed a conspicuous decrease in ethylene production when compared with media without silver ions (Fig. 6a, b). Because $AgNO_3$ inhibits ethylene action through the Ag^{2+} ions, reducing the receptor capacity to bind ethylene, the observed inhibition of ethylene production could be associated with negative feedback regulation of ethylene biosynthesis due to an excess of unbound gas (Kende 1993). Notably, in hypocotyls of the common ice plant the similar strong reduction of ethylene biosynthesis was also obtained after addition of 10 μM AVG, but in the presence of $AgNO_3$ the inhibition of rhizogenesis was accompanied by the induction of nonrhizogenic callus regularly from the basal end of the explants (Figs. 3j, k, and 6a, b). Thus, it may be suggested that the blocking of ethylene signaling abolished the rhizogenic action of IAA but did not influence its mitotic activity, which was manifested in the form of callus production. Moreover, the presence of callus only at the basal end of hypocotyls may indirectly indicate that inhibition of the ethylene response did not disturb the main auxin transport pathway in cultured explants. Nevertheless, when comparing the effect of AVG and $AgNO_3$, it is important to keep in mind the possible nonspecific, stress-related effect of silver ions.

Both auxins and cytokinins stimulate ethylene biosynthesis, and the effect is more pronounced when they are applied together (Abel and others 1995; Cary and others 1995; Chae and others 2003). Consistent with this conclusion is that the production of ethylene was significantly

higher when explants were grown on medium containing auxin with kinetin than on medium with IAA alone or medium devoid of hormones (Fig. 5). Manipulation of the endogenous ethylene level by the application of a precursor (ACC) or inhibitor (AVG) of its biosynthesis strongly affected the frequency of root regeneration and the pattern of rooting, confirming the regulatory role of this hormone in root regeneration in the material studied. Notably, the inhibitory effect of ACC and AVG differed according to the presence of kinetin in the medium. Thus, the hypocotyls cultured in the presence of IAA alone were more sensitive to AVG than those upon ACC treatments, whereas hypocotyls overproducing ethylene on medium with IAA + kinetin responded to inhibitor/precursor treatments in an opposite way (Table 2). These observations may indicate that there is an exact range of ethylene concentrations in the hypocotyls of the common ice plant that is needed to either stimulate or to inhibit root initiation. Aloni and others (2006) suggested that the disruption of PAT by an elevated level of ethylene in roots and shoots is the prime cue for local auxin accumulation and subsequent formation of root initials just around the IAA inhibition site. In our experiment, the addition of ACC at 50 and 100 μM to IAA-containing medium induced root production from both AE and BE (Table 2, Fig. 3a), but when it was added at 200 μM, rhizogenesis from BE was completely inhibited and the hypocotyls regenerated roots only from AE immersed in the medium (Fig. 3b). A similar pattern of root formation occurred in BE-cultured explants, where the activity of the auxin transport machinery was not required for root initiation (Fig. 1d). Interestingly, when ACC was added to the medium containing auxin and kinetin, the production of callus from the cultured explants was also restricted to the region in direct contact with the medium (Fig. 3c). Ethylene was shown to inhibit PAT in shoot tissues (Morgan and Gausman 1966; Suttle 1988). As shown in Fig. 6, the addition of 200 μM of ACC caused an approximately 2.5-fold or 5-fold increase in ethylene production by the explants maintained on media with IAA alone or supplemented with IAA and kinetin, respectively. Taking this fact into account, it may be suggested that the elevated level of ethylene achieved by ACC treatments could hamper the transport of exogenous IAA throughout the hypocotyls, leading to its accumulation in the basally located cells of the explants and resulting in subsequent root or callus formation.

Contrary to ACC, the addition of AVG did not influence the root patterning of explants cultured on medium with IAA (Fig. 3d). However, when it was applied together with auxin and kinetin, the cytokinin-induced pattern of rooting was changed and the explants produced smooth and thin roots regularly from the BE (Fig. 3g). Thus, AVG apparently reversed the effect of kinetin on root patterning, indicating the involvement of ethylene in the cytokinin-induced response. Indeed, in explants maintained for 3 days in the presence of IAA, kinetin, and 10 μM AVG, the production of ethylene was significantly lower (38–150 nM/30 hypocotyls/2 h) compared to the medium without inhibitor (500 nM/30 hypocotyls/2 h) (Fig. 6b). The involvement of ethylene in the cytokinin-induced response of hypocotyls of the common ice plant can also be confirmed by macroscopic observations. Thus, application of AVG abolished the kinetin-induced effect on root morphology, making roots relatively thin and almost devoid of root hairs (Figs. 1e–g and 3g).

In summary, our results reveal the regulatory role of cytokinin and ethylene in IAA-induced rhizogenesis. Cytokinin strongly affects root induction in terms of both efficiency and root patterning, and its final effect could be partly accounted for by the production of ethylene. The obtained data also indirectly suggest the possible involvement of cytokinin-induced ethylene in the regulation of PAT. However, to confirm it unambiguously, further studies on root formation with the use of IAA transport assays are needed.

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References

Abel S, Nguyen MD, Chow W, Theologis A (1995) ACS4, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1caroxylate synthase in *Arabidopsis thaliana*. J Biol Chem 270:19093–19099

Aloni R (2004) The induction of vascular tissue by auxin. In: Davis PJ (ed) Plant hormones: biosynthesis, signal transduction, action. Kluwer, Dordrecht, pp 471–492

Aloni R, Aloni E, Langhans M, Ullrich CI (2006) Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Ann Bot 97:883–893

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jürgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115:591–602

Bertell G, Eliasson L (1992) Cytokinin effects on root growth and possible interactions with ethylene and indole-3-acetic acid. Physiol Plant 84:255–261

Bourquin M, Pilet PE (1990) Effect of zeatin on the growth and indolyl-3-acetic acid and abscisic levels in maize roots. Physiol Plant 80:342–349

Cary AJ, Liu W, Howell SH (1995) Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyls elongation in *Arabidopsis thaliana* seedlings. Plant Physiol 107:1075–1082

Casson SA, Lindsey K (2003) Genes and signaling in root development. New Phytol 158:11–38

Chae HS, Faure F, Kieber JJ (2003) The *eto1*, *eto2* and *eto3* mutations and cytokinin treatment elevate ethylene biosynthesis in *Arabidopsis* by increasing the stability of the ACS5 protein. Plant Cell 15:545–559

Dubrovsky JG, Sauer M, Mapsucialy-Mendivil S, Ivanchenko MG, Friml J, Shishkova S, Celenza J, Benkova E (2008) Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. Proc Natl Acad Sci USA 105:8790–8794

Eklöf S, Åstot C, Blackwell J, Mortiz T, Olsson O, Sandberg G (1997) Auxin–cytokinin interactions in wild-type and transgenic tobacco. Plant Cell Physiol 38:225–235

Jiang K, Feldman LJ (2005) Regulation of root apical meristem development. Ann Rev Cell Dev Biol 21:485–509

John PCL, Zhang K, Dong C, Diederich L, Wightman F (1993) $p34^{cdc2}$ related proteins in control of cell cycle progression, the switch between division and differentiation in tissue development and stimulation of division by auxin and cytokinin. Aust J Plant Physiol 20:503–526

Katekar GF, Geissler AE (1980) Auxin transport inhibitors. IV. Evidence of common mode of action for a proposed class of auxin transport inhibitors: phytotropins. Plant Physiol 66:1190–1195

Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 44:283–307

Kępczyński J, Nemoykina A, Kępczyńska E (2006) Ethylene and in vitro rooting of rose shoots. Plant Growth Regul 50:23–28

Kuderová A, Urbánková I, Válková M, Malbeck J, Brzobohatý B, Ménethova D, Hejátko J (2008) Effect of conditional IPT-dependent cytokinin overproduction on root architecture of *Arabidopsis* seedlings. Plant Cell Physiol 49:570–582

Laplaze L, Benkova E, Casimiro I, Maes L, Vanneste S, Swarup R, Weijers D, Calvo V, Parizot B, Herrera-Rodriguez MB, Offringa R, Graham N, Doumas P, Friml J, Bogusz D, Beeckman T, Bennett M (2007) Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell 19:3889–3900

Li X, Mo X, Shou H, Wu P (2006) Cytokinin-mediated cell cycling arrest of pericycle founder cells in lateral root initiation of *Arabidopsis*. Plant Cell Physiol 47:1112–1123

Libik M, Konieczny R, Pater B, Ślesak I, Miszalski Z (2005) Differences in the activities of some antioxidant enzymes and in H_2O_2 content during rhizogenesis and somatic embryogenesis in callus cultures of the ice plant. Plant Cell Rep 23:834–841

Liu JH, Reid DM (1992) Adventitious rooting in hypocotyls of sunflower (*Helianthus annuus* L.) seedlings. IV. The role of changes in endogenous and free and conjugated indole-3-acetic acid. Physiol Plant 86:285–292

Lloret PG, Casero PJ (2002) Lateral root initiation. In: Waisel Y, Eshel A, Kafkafi U (eds) Plant roots—the hidden half. Marcel Dekker, New York, pp 127–155

López Nicólas JL, Acosta M, Sánchez-Bravo J (2004) Role of basipetal auxin transport and lateral auxin movement in rooting and growth of etiolated lupin hypocotyls. Physiol Plant 121:294–304

Mergemann H, Sauter M (2000) Ethylene induces epidermal cell death at the site of adventitious root emergence in rice. Plant Physiol 124:609–614

Morgan P, Gausman H (1966) Effects of ethylene on auxin transport. Physiol Plant 41:45–52

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Negi S, Ivanchenko MG, Muday GK (2008) Ethylene regulates lateral root formation and auxin transport in *Arabidopsis thaliana*. Plant J 55:175–187

Nordström A, Tarkowski P, Tarkowska D, Norbaek R, Åstot C, Dolezal K, Sandberg G (2004) Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: a factor of potential importance for auxin–cytokinin-regulated development. Proc Natl Acad Sci USA 101:8039–8044

Petrášek J, Mravec J, Bouchard R, Blakeslee JJ, Abas M, Seifertová D, Wisniewska J, Tadele Z, Kubes M, Covanová M, Dhonukshe P, Skupa P, Benková E, Perry L, Krecek P, Lee OR, Fink GR, Geisler M, Murphy AS, Luschnig C, Zazímalová E, Friml J (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 312:914–918

Ponce G, Barlow PW, Feldmann LJ, Cassab GJ (2005) Auxin and ethylene interactions control mitotic activity of the quiescent centre, root cap size, and pattern of cap cell differentiation in maize. Plant Cell Environ 28:719–732

Prayitno J, Rolfe BG, Mathesius U (2006) The ethylene-insensitive sickle mutant of *Medicago truncatula* shows altered auxin transport regulation during nodulation. Plant Physiol 142:168–180

Rashotte AM, Poupart J, Waddell CS, Muday GK (2003) Transport of the two natural auxins, indole-3-butyric acid and indole-3-acetic acid, in *Arabidopsis*. Plant Physiol 133:761–772

Rashotte AM, Chae HS, Maxwell BB, Kieber JJ (2005) The interaction of cytokinin with other signals. Physiol Plant 123:184–194

Růžička K, Ljung K, Vanneste S, Podhorska R, Beeckman T, Friml J, Benková E (2007) Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell 19:2197–2212

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissue cultured in vitro. Symp Soc Exp Biol 11:118–131

Stepanova AN, Yun J, Likhacheva AV, Alonso JM (2007) Multilevel interactions between ethylene and auxin in *Arabidopsis* roots. Plant Cell 19:2169–2185

Suttle JC (1988) Effect of ethylene treatment on polar IAA transport, net IAA uptake and specific binding of N-1-naphthylphthalamic acid in tissues and microsomes isolated from etiolated pea epicotyls. Plant Physiol 88:795–799

Swarup R, Kramer EM, Perry P, Knox K, Leyser HM, Haseloff J, Beemster GT, Bhalerao R, Bennett MJ (2005) Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. Nat Cell Biol 7:1057–1065

Vieten A, Sauer S, Brewer PB, Friml J (2007) Molecular and cellular aspects of auxin-transport-mediated development. Trends Plant Sci 12:160–168

Woeste K, Vogel J, Kieber J (1999) Factors regulating ethylene biosynthesis in etiolated *Arabidopsis thaliana* seedlings. Physiol Plant 105:478–484

Yamamoto F, Sakata T, Terazawa K (1995) Physiological, morphological and anatomical responses of *Fraximus mandshurica* seedlings to flooding. Tree Physiol 15:713–719

Zhang NG, Hasenstein KH (1999) Initiation and elongation of lateral roots in *Lactuca sativa*. J Plant Sci 160:511–519