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ORIGINAL ARTICLE

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Cytokinin-induced hypocotyl elongation in light-grown *Arabidopsis* plants with inhibited ethylene action or indole-3-acetic acid transport

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Abstract Cytokinins inhibit hypocotyl elongation in darkness but have no obvious effect on hypocotyl length in the light. However, we found that cytokinins do promote hypocotyl elongation in the light when ethylene action is blocked. A 50% increase in *Arabidopsis thaliana* (L.) Heynh. hypocotyl length was observed in response to N^6-benzyladenine (BA) treatment in the presence of Ag^+. The level of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid was strongly increased, indicating that ethylene biosynthesis was up-regulated by treatment with cytokinin. Furthermore, the effects of cytokinins on hypocotyl elongation were also tested using a series of mutants in the cascade of the ethylene-signal pathway. In the ethylene-insensitive mutants *etr1-3* and *ein2-1*, cytokinin treatment resulted in hypocotyl lengths comparable to those of wild-type seedlings treated with both Ag^+ and BA. A similar phenotypical response to cytokinin was observed when auxin transport was blocked by α-naphthylphthalamic acid (NPA). Applied cytokinin largely restored cell elongation in the basal and middle parts of the hypocotyls of NPA-treated seedlings and at the same time abolished the NPA-induced decrease in indole-3-acetic acid levels. Our data support the hypothesis that, in the light, cytokinins interact with the ethylene-signalling pathway and conditionally up-regulate ethylene and auxin synthesis.

Keywords *Arabidopsis* · Auxin · Cytokinin · Ethylene · Hypocotyl · Light

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Abbreviations: ACC: 1-Aminocyclopropane-1-carboxylic acid · AVG: Aminoethoxyvinylglycine · BA: N^6-Benzyladenine · GUS: β-Glucuronidase · IAA: Indole-3-acetic acid · NPA: α-Naphthylphthalamic acid · LNM: Low-nutrition medium

Introduction

The responses of seedlings to specific environmental parameters or growth conditions can be used to elucidate signal transduction pathways in plants. The hypocotyls of *Arabidopsis thaliana* seedlings respond differently to exogenous plant hormones in light or darkness.

Ethylene inhibits hypocotyl elongation as part of the "triple response" in etiolated *Arabidopsis* seedlings, but not in light-grown seedlings (Bleecker et al. 1988). Most ethylene mutants have been identified using this triple response (Ecker 1995). Smalle et al. (1997), however, demonstrated that ethylene or its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) affects hypocotyl elongation in light-grown nutrient-starved seedlings. On a low-nutrition medium (LNM), ACC caused hypocotyl elongation up to twice the size observed in controls, a response that was abolished by treatment with Ag^+.

Similar to the effect of ethylene on hypocotyl development, a correlation between auxin levels and hypocotyl length has also been demonstrated. In the dark, transgenic auxin overproducers exhibit the same hypocotyl lengths as wild-type plants (Romano et al. 1995), but the *axr2-1* mutant, which demonstrates auxin-resistant root growth, was found to have shorter hypocotyls (Timpte et al. 1992). In light, however, the transgenic plants showed increased hypocotyl elongation and displayed up to 4-fold higher levels of free indole-3-acetic acid (IAA; Romano et al. 1995), whereas the *axr2* mutant once more demonstrated a decrease in hypocotyl length. Furthermore, the *sur1* mutant (Boerjan et al.

1995) displayed increased IAA levels together with an increased hypocotyl length in the light; and in nutrient-starved *Arabidopsis* wild-type seedlings, hypocotyl length was increased after IAA treatment in the light (Smalle et al. 1997). Friml et al. (2002) have demonstrated a link between auxin concentrations and cell elongation in the hypocotyl. These authors analysed the relationship between auxin distribution and growth responses in *Arabidopsis* hypocotyls using the synthetic auxin-responsive reporter *DR5::GUS* (Ulmasov et al. 1997). During phototropic and gravitropic curvature, *DR5::GUS* expression was enhanced on the convex side of the hypocotyl, indicating a relation between auxin levels and cell elongation.

Cytokinins inhibit hypocotyl elongation in dark-grown *Arabidopsis* seedlings (Su and Howell 1995), an effect that is due to cytokinin-induced ethylene production (Cary et al. 1995). Based on this knowledge, Vogel et al. (1998a, 1998b) isolated *Arabidopsis* mutants defective in cytokinin-induced ethylene production. In dark-grown mungbean hypocotyls, N^6-benzyladenine (BA) was shown to synergistically enhance ethylene production in the presence of IAA (Lau et al. 1977; Yoshii and Imaseki 1982). Kim et al. (2001) suggested that in dark-grown mungbean hypocotyls, IAA and BA inhibit ethylene action, thereby influencing the ethylene feedback mechanism that in turn causes an up-regulated ACC synthase expression. However, cytokinins had no effect on hypocotyl elongation in *Arabidopsis* seedlings grown in the light (Su and Howell 1995).

It has been shown that the use of nutrient-starved seedlings is of benefit to understanding the roles of both IAA and ethylene (Smalle et al. 1997; Vandenbussche et al. 2003). We therefore analysed the effect of cytokinins on *Arabidopsis* hypocotyls grown in long-day conditions on normal Murashige and Skoog medium and on LNM. To define possible mutual interactions between cytokinins, ethylene and IAA, we used the ethylene-action inhibitor Ag^+, ethylene-signalling mutants and the IAA-transport inhibitor α-naphthylphthalamic acid (NPA). Our findings show that cytokinins promote hypocotyl elongation in the light when ethylene action or IAA transport is blocked. This elongation is characterised by cell elongation at the hypocotyl base and middle portion, and might involve ethylene or IAA biosynthesis and/or increased cytokinin sensitivity.

Materials and methods

Plant material and growth conditions

All *Arabidopsis thaliana* (L.) Heynh. plants used were the Columbia-0 ecotype. The wild type was purchased from Lehle Seeds (Tucson, AZ, USA) while the ethylene-response mutants and the *DR5::GUS* line were gifts from Dominique Van Der Straeten and Tom Beeckman (University of Ghent, Ghent, Belgium), respectively. The mutants used in this study were *etr1-3* (Bleecker et al. 1988), *ein2-1* (Guzman and Ecker 1990) and *ctr1-1* (Kieber et al. 1993). Seeds were surface-sterilised for 15 min in 5% sodium hypochlorite, rinsed at least 5 times with sterile water and transferred to the medium with sterile, fine tweezers. Two growth media were used. A half-strength Murashige and Skoog (MS/2) medium including vitamins (MS; Duchefa, The Netherlands) supplemented with 1% sucrose (pH 5.8) and the low-nutrient medium LNM. This LNM consisted of 0.8% agar in SPA Reine water (Spa Monopole, Spa, Belgium) containing 3 mg l^{-1} Na^+, 0.5 mg l^{-1} K^+, 4.5 mg l^{-1} Ca^{2+}, 1.3 mg l^{-1} Mg^{2+}, 5 mg l^{-1} Cl^-, 4 mg l^{-1} SO_4^{2-}, 1.9 mg l^{-1} NO_3^-, 15 mg l^{-1} HCO_3^-, and 7 mg l^{-1} SiO_4, as determined by the producer, and was brought to pH 5.8 with 0.01 N HCl before autoclaving. BA, IAA, aminoethoxyvinylglycine (AVG) and NPA were obtained from Sigma. $AgNO_3$ was from Merck. After sowing, plates were stored overnight at 4°C and then put for 10 days in a vertical position in a growth chamber at 22 ± 2°C with white light from fluorescent lamps (40 μmol photons m^{-2} s^{-1}) under long-day conditions (16 h light/8 h dark).

Determination of hypocotyl and cell lengths and the number of cells

In order to measure hypocotyl lengths, cell numbers and cell lengths, images of the hypocotyl were collected with a Zeiss Axioskop microscope equipped with a Nikon DXM1200 digital camera. Pictures were subsequently analysed by means of Scion Image (http://www.scion-corp.com) software.

The number of cells per hypocotyls and the cell length were calculated from 10-day-old plants. Because a clear gradient in cell length was observed across the hypocotyls, measurements of cell length were performed on the base, middle and top parts of the hypocotyl. Cell lengths were scored using a graticule as reference.

Mean values are presented with standard error of the mean (SE). The statistical significance of the data on hypocotyl length, cell number and cell length was analysed using a univariate analysis of variance (ANOVA; SPSS for Windows, v10.0). Kolmogorov–Smirnov tests of normality were positive ($P > 0.05$) for measurements on cell number and hypocotyl length. Data on cell length were log-transformed to meet the conditions for ANOVA testing (H_0: $\mu = \mu_0$) and evaluated by repeated measures ANOVA taking individual plant variation into account.

Assay of β-glucuronidase

β-Glucuronidase (GUS) assays were based on Jefferson (1987). Four-day-old *DR5::GUS* seedlings were fixed for 30 min in 90% acetone at 4°C, washed with 1 M phosphate buffer and transferred to incubation buffer (100 mM sodium phosphate, pH 7.0, 10 mM EDTA,

0.5 mM $K_4Fe[CN]_6$, 0.5 mM $K_3Fe[CN]_6$, 0.1% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide), vacuum-infiltrated for 1 min and then incubated for 18 h at 37°C. Chlorophyll was removed from green tissues by 70% ethanol. GUS staining patterns were recorded using a Nikon DXM1200 digital camera mounted on a Zeiss Axioskop microscope.

Hormone analyses

Wild-type *Arabidopsis* plants were grown as described on LNM supplemented with 10 μM BA, 100 μM $AgNO_3$, 10 μM BA plus 100 μM $AgNO_3$, 5 μM NPA, or 10 μM BA plus 5 μM NPA. The non-supplemented LNM medium was used as control. After 10 days, hypocotyls plus cotyledons of about 80 seedlings were pooled per sample. The samples were weighed, immediately frozen in liquid nitrogen and stored at −70°C. Three replicates were used for each treatment.

Samples were ground in liquid nitrogen, transferred into 80% methanol and extracted overnight at −20°C. Phenyl-$^{13}C_6$-IAA (10 pmol; Cambridge Isotope Laboratories Inc., Andover, MA, USA) and $^2H_4$-ACC (10 pmol; Sigma) were added for isotope-dilution purposes. After centrifugation (24,000 *g*, 15 min, 4°C), IAA and ACC were further purified by a combined solid-phase extraction procedure (Prinsen et al. 2000; Persson and Näsholm 2001). While IAA was methylated prior to analyses (Prinsen et al. 2000), ACC was derivatised by pentafluorobenzyl bromide (Sigma; Netting and Duffield 1985). Quantification was done by microLC–(ES^+)MS/MS in MRM mode for IAA (Prinsen et al. 1998) and GC–(CI^-)MS in SRM mode for ACC (Smets et al. 2003). The chromatograms obtained were processed by means of Masslynx software (Micromass). Concentrations were expressed in picomoles per gram fresh weight.

Results

Cytokinins induce hypocotyl elongation when ethylene action is blocked

Adding 1 μM or 10 μM BA had no effect on the growth of hypocotyls of wild-type seedlings grown on LNM (Table 1). Supplementing 100 μM Ag^+, a concentration used for blocking ethylene perception (Vandenbussche et al. 2003), however, caused a small (14%) yet significant decrease in the hypocotyl length. This effect was completely abolished by adding 1 μM BA to the Ag^+-treated seedlings, and in the presence of 10 μM BA the hypocotyls of the Ag^+-treated seedlings were even significantly longer (23%) than the hypocotyls of the untreated wild type. Figure 1 illustrates the phenotypes of the 10-day-old *Arabidopsis* seedlings grown on LNM (Fig. 1a) and on LNM supplemented with BA (Fig. 1b), Ag^+ (Fig. 1c) or BA plus Ag^+ (Fig. 1d).

Table 1 Dose response of *Arabidopsis thaliana* hypocotyl length to BA. Results are expressed in mm ± SE and as percentages. Seeds were germinated under long-day conditions on LNM containing different concentrations of BA with or without Ag^+ or AVG. Each value represents the measurements of at least 15 seedlings. The data obtained were ranked in separate classes according to their hypocotyl length, with (a) and (h) having the smallest and largest hypocotyl lengths, respectively. Classes were significantly different according to a 2-factor ANOVA test ($P < 0.05$)

	BA (μM)		
	0	1	10
Wild type	1.93 ± 0.05(d) 100%	1.91 ± 0.03(d) 99%	1.87 ± 0.08(d) 97%
Wild type + AVG	1.04 ± 0.03(a) 54%	1.41 ± 0.05(b) 73%	1.44 ± 0.02(b) 75%
Wild type + Ag^+	1.66 ± 0.05(c) 86%	1.90 ± 0.05(d) 98%	2.37 ± 0.04(f) 123%
etr1-3	1.65 ± 0.03(c) 85%	2.56 ± 0.08(h) 132%	1.72 ± 0.03(d) 89%
ctr1-1	2.27 ± 0.04(f) 118%	2.24 ± 0.05(f) 116%	2.05 ± 0.04(e) 106%
ctr1-1 + Ag^+	1.58 ± 0.07(c) 82%	2.62 ± 0.07(h) 136%	2.39 ± 0.08(g) 124%
ein2-1	1.57 ± 0.05(c) 81%	2.48 ± 0.06(h) 128%	2.19 ± 0.08(f) 113%
ein2-1 + Ag^+	1.45 ± 0.03(b) 75%	1.74 ± 0.04(d) 90%	2.49 ± 0.06(h) 129%

Applying Ag^+ to *Arabidopsis* seedlings grown on a rich nutrient medium (MS/2) also caused a small but not significant decrease in the hypocotyl length (Table 2). Compared to LNM, a comparable effect of BA on hypocotyl length was obtained at lower concentrations of BA on MS/2. For instance, a 30% increase in hypocotyl length compared to the untreated control occurred when Ag^+-treated seedlings were supplemented with 0.01 μM BA. Because of the greater uniformity in plant size obtained on LNM and the unique responses (Saibo et al. 2003), which are different with MS/2, it was decided to use LNM for further experimental work.

To follow up on the results from the wild-type seedlings, we analysed the hypocotyl lengths of a set of ethylene signal-transduction mutants (*etr1-3*, *ctr1-1* and *ein2-1*) grown on LNM in the presence or absence of BA (Table 1). On LNM, the *etr1-3* and *ein2-1* mutants both had shorter hypocotyls than the wild type. When the medium was supplemented with 1 μM BA, the hypocotyls of both mutants were significantly longer (about 30%) than those of the wild type. At 10 μM BA, *etr1-3* hypocotyls were similar to those of the wild type, whereas hypocotyls of the *ein2-1* plants were 13% longer than those of the wild type. On non-supplemented LNM, seedlings of the constitutive ethylene-responsive *ctr1-1* seedlings had significantly longer (18%) hypocotyls than wild-type seedlings. The hypocotyl growth of this mutant was not affected by 1 μM BA, and only marginally (6% longer than the wild type) by 10 μM BA. Adding Ag^+ caused a significant decrease in hypocotyl length of *ctr1-1* seedlings (30%) and *ein2-1* (6%) seedlings. This inhibitory effect of Ag^+ was completely reversed by BA, even causing hypocotyls of the

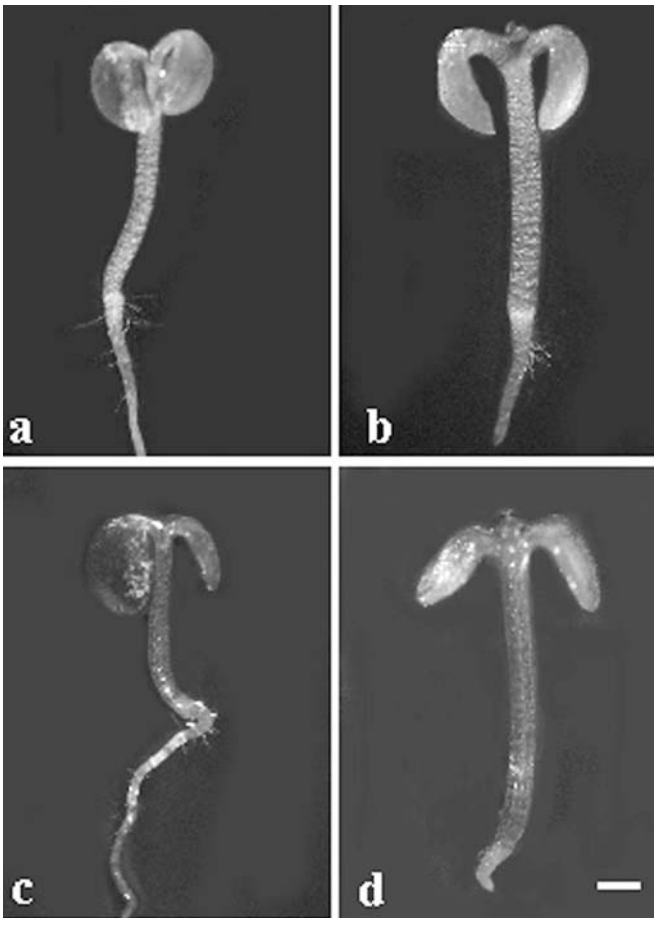

Fig. 1a–d Hypocotyl development of *Arabidopsis thaliana* wild-type seedlings grown on LNM (**a**) or on LNM supplemented with 10 μM BA (**b**), 100 μM Ag^+ (**c**) or 10 μM BA plus 100 μM Ag^+ (**d**). Seeds were incubated overnight at 4°C in the dark and then grown in sterile conditions for 10 days in a vertical position at 22°C with white fluorescent light and long day conditions. Bar = 0.5 mm

ctr1-1 and *ein2-1* mutants to become significantly longer than those of the wild type.

When ethylene biosynthesis was blocked with 10 μM AVG in wild-type seedlings (Table 1), hypocotyl development was strongly inhibited (46%). This inhibition was partially reversed (75%) by 1 or 10 μM BA.

Cytokinins restore hypocotyl elongation when IAA transport is inhibited

Seedlings grown on LNM supplemented with NPA, demonstrated a strongly (48% of wild type) reduced hypocotyl length (Table 3). Addition of 10 μM BA to the medium partially rescued (84% of wild type) this NPA induced inhibition.

The cytokinin effect on hypocotyl length is caused by cell elongation

To find out whether the elongation responses observed are due to an increase in cell elongation and/or cell division, we determined the size and number of epidermal cells after 10 days of treatment.

No significant differences in total cell number per cell file (not shown) were found between seedlings grown on LNM, on LNM with Ag^+, or on LNM with Ag^+ and BA (ANOVA analysis; Tukey–Kramer multiple comparisons test; $P > 0.05$). Similar results were obtained when NPA instead of Ag^+ was added to the medium (data not shown). The observed differences in hypocotyl length must therefore thus result from differences in cell elongation.

Further investigation revealed that significant differences in cell elongation (Fig. 2) were related to both cell location and plant treatment ($P < 0.001$ for the two parameters). Untreated cells in the base and middle of the hypocotyl were found to be longer than those of the upper portion. At the base and in the middle part of hypocotyls, Ag^+ caused a decrease of around 35% in cell length (Fig. 2a), whereas with NPA treatment, (Fig. 2b) a decrease in cell length of 50–60% was observed. In the upper portions of the hypocotyl the effect of NPA was limited to a 20% decrease in cell length (Fig. 2b), whereas Ag^+ treatment had no effect (Fig. 2a). Compared to untreated cells, cells located at

Table 2 Dose response of *Arabidopsis* hypocotyls length to BA. Seeds were germinated under long-day conditions on MS/2 containing different concentrations of BA with or without Ag^+. Results are expressed in mm ± SE and as percentages. Each value represents the measurements of at least 15 seedlings. The data obtained were ranked in separate classes according to their hypocotyl length with (a) and (b) having the smallest and largest hypocotyl lengths, respectively. Classes were significantly different according to a 2-factor ANOVA test ($P < 0.05$)

	BA (μM)		
	0	0.01	0.1
Wild type	3.62 ± 0.16(a) 100%	3.21 ± 0.09(a) 89%	3.18 ± 0.07(a) 88%
Wild type + $AgNO_3$	3.08 ± 0.12(a) 85%	4.71 ± 0.21(b) 130%	4.60 ± 0.25(b) 127%

Table 3 The effect of NPA and BA on elongation of *Arabidopsis* hypocotyls. Results are expressed in mm ± SE and as percentages. Seedlings were grown on LNM with or without BA and NPA. Each value represents the measurements of at least 20 seedlings. The data obtained were ranked in separate classes according to their hypocotyl length with (a) and (c) having the smallest and largest hypocotyl lengths, respectively. Classes were significantly different according to a 2-factor ANOVA test ($P < 0.05$)

	BA (μM)	
	0	10
Wild type	2.14 ± 0.06(c) 100%	2.20 ± 0.05(c) 103%
Wild type + NPA	1.11 ± 0.03(a) 52%	1.80 ± 0.05(b) 84%

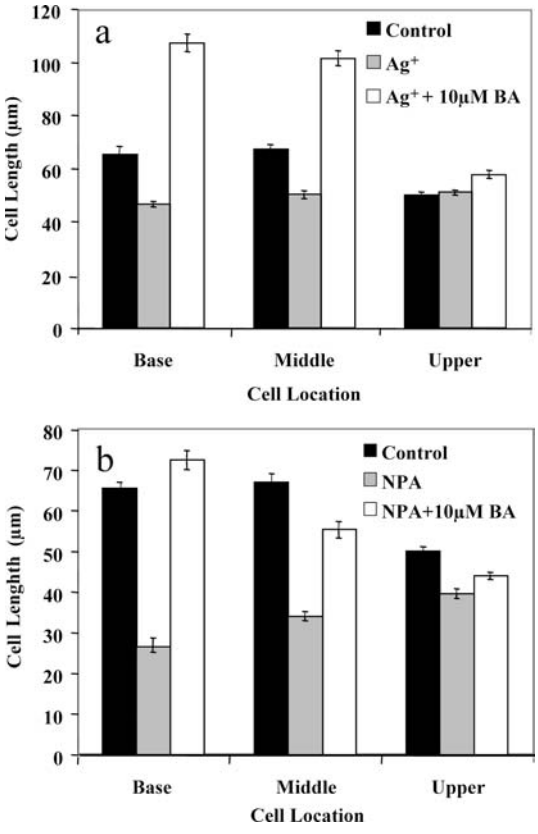

Fig. 2a, b Effects of Ag^+ (**a**) or NPA (**b**), in the presence and absence of BA, on the cell length of *Arabidopsis* wild-type hypocotyls. Seedlings were grown and treated as described in Fig. 1. Cell lengths were determined for three randomly selected cells from each region of the hypocotyl (base, middle and top). Each point represents the mean ± SE for measurements of at least 20 hypocotyls

the hypocotyl base and in the middle part of plantlets grown on Ag^+ plus BA were about 50% longer (Fig. 2a); cells of the upper portion of the hypocotyls showed only a minor increase in cell length (18%) under these conditions. In NPA-treated plantlets (Fig. 2b), BA restored cell length at the hypocotyl base to the level of non-treated plants. In the middle and upper portions of the hypocotyls of NPA treated seedlings, BA partially rescued cell elongation, but the cells never reached the final length observed in untreated controls (Fig. 2b).

Cytokinins indirectly affect IAA and ACC levels

The above results demonstrate that cytokinins induce hypocotyl elongation in light-grown *Arabidopsis* seedlings through interactions with auxin and ethylene. To further investigate these interactions, we first used IAA-responsive GUS lines to monitor endogenous auxin levels in response to the different treatments.

The major differences in hypocotyl growth rates between IAA-treated and control seedlings take place 4 days after germination. It is interesting that this growth phase coincides with an increase in GUS activity in the hypocotyls of IAA-treated *DR5::GUS* plants (Vandenbussche et al. 2003; D. Van Der Straeten, personal communication). Therefore, we tested whether or not treatment with BA induced an enhancement of *DR5::GUS* expression in the hypocotyls of 4-day-old seedlings when ethylene action was blocked. As shown in Fig. 3, GUS was only expressed in hypocotyls of plants treated with 1 μM IAA. None of the other treatments or the untreated control showed any visible GUS activity in the hypocotyl.

In another experiment, we determined the ability of cytokinins to alter ethylene or auxin production through the measurement of ACC and IAA concentrations (Table 4). Treatment with BA did not increase the ACC concentration. Ag^+ and NPA both caused a considerable increase in the endogenous ACC concentration. The effect of combining Ag^+ and BA was the most spectacular, resulting in a 10-fold increase in ACC concentration. The combination of NPA and BA increased endogenous ACC concentrations 3-fold.

Neither, BA, Ag^+, nor or a combination of both altered IAA concentrations. Addition of NPA, however, strongly reduced the endogenous IAA level. This effect was completely abolished by addition of BA.

Fig. 3a–e DR5::GUS activity in *Arabidopsis* seedlings grown on LNM (**a**) or on LNM supplemented with 1 μM IAA (**b**), 10 μM BA (**c**), 100 μM Ag^+ (**d**) or 10 μM BA plus 100 μM Ag^+ (**e**). Surface-sterilised seeds were stored overnight at 4°C in the dark and then put for 4 days in a vertical position in a growth chamber at 22°C with white fluorescent light and long day conditions. After vacuum infiltration, seedlings were incubated for 18 h at 37°C in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). Bar = 0.5 mm

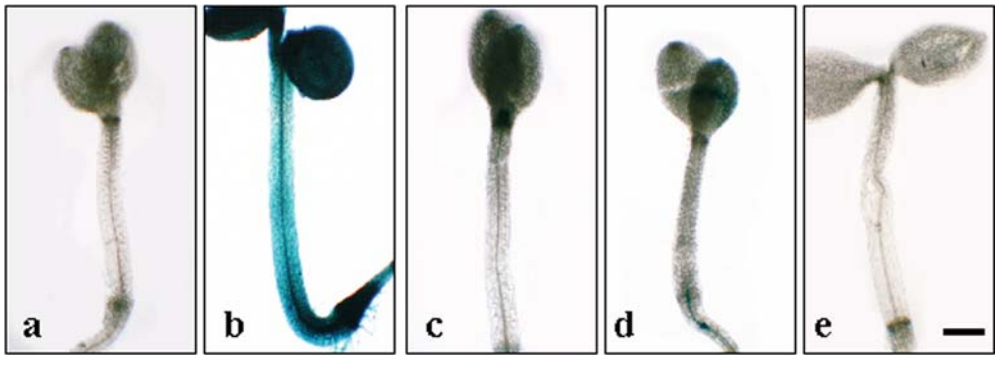

Table 4 ACC and IAA levels in *Arabidopsis* plants treated with Ag^+, NPA and/or BA. Seedlings were grown for 10 days in long-day conditions on LNM, after which the upper parts were collected. Hormone levels are expressed as pmol g^{-1} fresh weight. Each value represents the mean ± SD of measurements of 3 samples

	ACC	IAA
Control	1,510 ± 547	18.0 ± 5.7
BA	1,665 ± 642	20.1 ± 2.2
Ag^+	5,307 ± 1327	16.8 ± 1.6
Ag^+/BA	22,663 ± 6461	21.5 ± 3.4
NPA	4,143 ± 1733	11.7 ± 0.7
NPA/BA	6,117 ± 1426	21.6 ± 4.2

Discussion

In dark growth conditions, ethylene has an inhibitory effect on hypocotyl length (Kieber et al. 1993). Cytokinins also reduce hypocotyl length in the dark, and Cary et al. (1995) found that cytokinins can stimulate ethylene production. Under light growth conditions, ethylene and auxins are also involved in hypocotyl elongation. By growing seedlings on LNM, Smalle et al. (1997) demonstrated that exogenous ACC or IAA caused an increase in the hypocotyl length of *Arabidopsis*.

In the work reported here, no exogenous ethylene, ACC or IAA was applied. Instead, the signalling pathways of these endogenous hormones were inhibited during hypocotyl elongation in the light. The presence of AVG or Ag^+ in the medium led to reductions in hypocotyl length, even in *ctr1-1* seedlings, which exhibit a constitutive ethylene response and thus elongated hypocotyls. The final lengths of wild-type and *ctr1-1* seedling hypocotyls in Ag^+ treatments were similar to the hypocotyl lengths of untreated ethylene-insensitive *etr1-3* and *ein2-1* mutants (Smalle et al. 1997). Supply of Ag^+ to the ethylene-insensitive *ein2-1* mutant also caused a reduction in hypocotyl length, however. These latter results point towards an additional effect of Ag^+ on hypocotyl development that is not related to ethylene. Seedlings grown on LNM supplied with the auxin-transport inhibitor NPA (Morgan 1964) showed a severely reduced hypocotyl length. This corroborates the data of Jensen et al. (1998), who showed that NPA inhibited hypocotyl elongation in *Arabidopsis thaliana* seedlings grown in the light on normal medium. These results clearly confirm the involvement of ethylene and auxin in hypocotyl growth processes of *Arabidopsis* seedlings in the light.

The cytokinin BA did not affect hypocotyl elongation under light growth conditions, which is consistent with the observations of Su and Howell (1995) on seedlings grown on normal medium. However, BA reversed the inhibitory effect of Ag^+, AVG and NPA on hypocotyl length. This cytokinin-mediated hypocotyl elongation in the light did not involve cell division and was due only to a restoration of cell elongation.

As cytokinins have been shown to induce ethylene production in dark-grown seedlings (Cary et al. 1995), one might expect the same in BA-treated light-grown seedlings. However, BA did not increase the ACC content, which correlates well with the absence of a BA response on hypocotyl elongation. Ag^+, however, induced ethylene production, increasing ACC levels more than 2-fold. This observation is indicative of a putative feedback inhibition of ethylene perception on ACC production or metabolism, as has been previously observed (Kim et al. 1997; Yoon et al. 1997). The striking effect of BA on ACC levels in the presence of Ag^+ might possibly be explained through a feedback regulation mechanism on ethylene biosynthesis. Kim et al. (2001) demonstrated that BA inhibited both ethylene-induced expression of mungbean *VR-ACO1* and ethylene-suppressed expression of *VR-ACS1*, leading to moderately higher ethylene levels. However, since Ag^+ is known to be a strong inhibitor of ethylene action (Beyer 1976) it is very doubtful that the high endogenous ACC concentration observed in plants treated with Ag^+ and BA results in ethylene levels strong enough to overcome the Ag^+ inhibition (Guzman and Ecker 1990). Whereas ethylene inhibits, IAA is known to stimulate ACC synthase (Abel et al. 1995; Woeste et al. 1999). In our experiments however, we observed that NPA decreased the IAA concentration and yet slightly increased the ACC concentration. This increase in ACC could be important, as NPA is assumed not to block the ethylene pathway (Fujita and Syono 1997).

DR5::GUS-reporter seedlings, which are shown to be IAA (Ulmasov et al. 1997) and brassinosteroid (Nakamura et al. 2003) responsive, showed no staining in the hypocotyl in response to Ag^+ and BA at 4 days after germination. Moreover, IAA levels in 10-day-old seedlings grown on LNM supplemented with BA, Ag^+ or a combination of both, were unchanged. These results indicate that BA stimulation of hypocotyl elongation, in the presence of Ag^+, is probably not mediated through IAA. However, repartitioning of IAA in the BA- and Ag^+-treated samples beneath the GUS detection limit cannot be excluded. Treatment with NPA caused a decrease in IAA levels, which can be explained by a decrease in IAA biosynthesis. Ljung et al. (2001) described an NPA-mediated feedback inhibition of IAA biosynthesis in expanding leaves and cotyledons. An increased IAA conjugation or breakdown cannot, however, be excluded. BA seems to counteract this NPA effect, as IAA levels are restored to the level of control plants following BA treatment. BA treatment, however, does not fully restore the hypocotyl length. This indicates that another, BA-insensitive, factor may be needed to completely restore hypocotyl length.

As mentioned previously, ethylene-signalling mutants were also tested for BA-induced hypocotyl elongation. When treated with 1 μM BA, hypocotyl elongation was increased by 50% in *etr1-3* and *ein2-1*. Cross-talk between the signal transduction pathways of both ethylene and BA is thus to be expected downstream of *etr1-3* and *ein2-1*. Probably due to an emerging toxic response, higher BA concentrations caused a diminished increase

in hypocotyl length in the *etr1-3* and *ein2-1* mutants. In *ein2-1*, Ag^+ caused a shift in the maximal hypocotyl elongation response from 1 to 10 μM BA but had no influence on the maximal hypocotyl length. Therefore, these data may be indicative of a putative influence of Ag^+ on BA sensitivity.

Hall et al. (1999) observed that in *etr* leaves protein phosphorylation by cytokinin is much more pronounced than in wild-type plants, and concluded that ethylene acts as a cytokinin antagonist. Furthermore, both the ethylene receptor ETR1 and the cytokinin receptor CRE1 display histidine kinase activity and downstream phosphorelay components, allowing for possible cross-talk between them (reviewed by Deruère and Kieber 2002). Hamant et al. (2002) found that the KNAT2 (KNOTTED-like *Arabidopsis*) homeodomain protein acts synergistically with cytokinins and antagonistically with ethylene in light-grown *Arabidopsis* plants. To examine the interaction between ethylene and the *KNAT2* expression, a *KNAT2::GUS* line was crossed to the ethylene-resistant mutant *etr1-1* (Chang et al. 1993). The observed GUS signal was strongly expressed in the hypocotyl of the homozygous *etr1-1*/*KNAT2::GUS* line as compared to the wild-type *KNAT2::GUS* line, thus showing an increased cytokinin action in the hypocotyl when ethylene signaling is blocked.

Alonso et al. (1999) proposed EIN2 to be located at the crossroads of multiple hormone signalling pathways. However, apart from *ein2-1*, the ethylene-insensitive *etr1-3* mutant also displayed enhanced cytokinin sensitivity. These results seem to indicate that the pathway defined by ETR1, CTR and EIN2 inhibits cytokinin signal transduction in hypocotyl elongation. A plausible interpretation of the data of the ethylene-signalling mutants is that the enhanced hypocotyl length in BA-treated *etr1-3* and *ein2-1* seedlings results from impaired ethylene signalling.

Arabidopsis ctr1-1 mutants, showing a constitutive ethylene response, have longer hypocotyls than the wild type (Smalle et al. 1997). Surprisingly, a substantial reduction in hypocotyl length was observed when these plants were treated with Ag^+, a response comparable to that of the wild type. Since Ag^+ acts upstream of the mutation, it is very surprising that it had an influence on the *ctr1-1* mutant plants. As in wild-type plants, BA was able to stimulate hypocotyl elongation only after Ag^+ treatment in the *ctr1-1* mutant. Moreover, hypocotyl elongation of the ethylene-insensitive *ein2-1* mutant, already displaying a reduced hypocotyl length, was inhibited after Ag^+ treatment. These results suggest a toxic side-effect of Ag^+ on hypocotyl elongation that can be reversed by BA. Also, long-term treatment of NPA may cause damage to plants (A. Murphy and J. Blakeslee, personal communication). If so, BA-stimulated hypocotyl elongation might then occur under general stress conditions and not only after inhibition of IAA transport or ethylene.

The cross-talk between the signalling pathways of the three hormones is presented in Fig. 4, which shows a model of cytokinin-stimulated hypocotyl elongation. In general, there is a 50% increase in hypocotyl length in response to cytokinins under light growth conditions, but only if ethylene action or IAA transport is blocked. Under these conditions, cytokinins stimulate ACC (and in NPA-treated seedlings also IAA) accumulation, possibly through a feedback regulation mechanism.

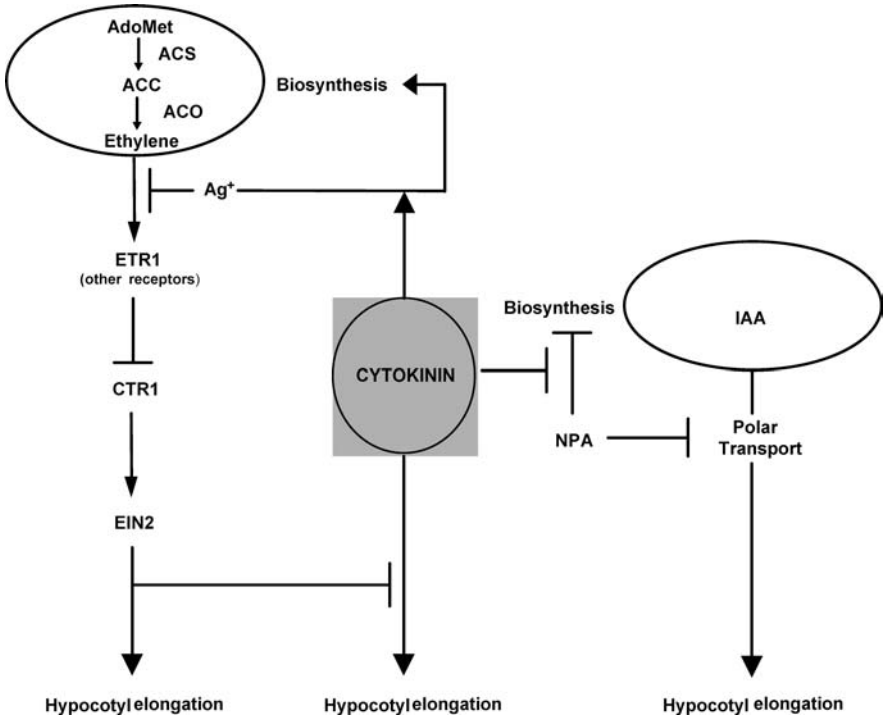

Fig. 4 Hypothetical model for cytokinin action on hypocotyl elongation. Cytokinins stimulate Ag^+-induced ethylene biosynthesis but antagonise the NPA-inhibited IAA production. Furthermore, higher cytokinin sensitivity was observed in an ethylene-insensitive background, indicating ethylene inhibition of cytokinin action. Ethylene (and in NPA-treated plantlets also IAA) production or an increased sensitivity to cytokinin, or a combination of both factors, might induce hypocotyl elongation

Furthermore, analysis of ethylene-insensitive mutants indicates that endogenous ethylene negatively regulates cytokinin-induced hypocotyl elongation through a downstream cross-talk mechanism.

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