

Effects of benzylaminopurine and irradiance on cytokinin contents, α -tubulin gene expression and cucumber cotyledon expansion

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Abstract

The fluctuation of endogenous cytokinins was determined in the excised cucumber (*Cucumis sativa* L.) cotyledons incubated with benzylaminopurine (BA) under irradiance or in darkness. The data indicated that light stimulated the cotyledon expansion compared with dark and BA further enhanced the expansion of cotyledons. However, only BA treatment markedly increased the contents of endogenous cytokinins and induced α -tubulin gene expression. Actinomycin D, a well-known inhibitor of gene transcription, strongly inhibited both light- and BA-induced cotyledon expansions and increase of endogenous cytokinin contents and α -tubulin gene expression. Colchicine, an antimicrotubular reagent, partially inhibited the cotyledon expansion without affecting the cytokinin contents.

Additional key words: actinomycin D, colchicine, *Cucumis sativa*.

Introduction

Cytokinins constitute a major class of plant growth regulators that have been shown to be involved in a wide range of physiological processes (Davies 1995). The actions of cytokinins are involved in the cross-talk with many other signals. A particular example is the cytokinin interaction with light in photomorphogenesis (Moller and Chua 1999). Light effects on plant growth and development can be observed at almost every stage of the life cycle (Neff *et al.* 2000). Cytokinins have been shown to induce, in darkness, a number of processes normally controlled by light. Examples include amaranthin synthesis, chloroplast development, and differentiation of leaves and cotyledons (Chory 1993).

Both cytokinins and light cause profound changes in the morphology of the developing young dicotyledonous seedling. It was first reported by Ikuma and Thimann (1963) that the action of cytokinins in causing expansion of lettuce cotyledon during germination. The similar effects have been found on excised cotyledons of other plants, including mustard (Lovell and Moor 1970), watermelon (Longo *et al.* 1981) and cucumber (Narain and Laloraya 1974). The role of cytokinin-induced cotyledon expansion has been studied by a number of groups. It has been demonstrated that cytokinin-induced cotyledon expansion was dependent on nucleic acid

synthesis (Teramoto *et al.* 1993). Metabolism of the excised cucumber cotyledons was extensively studied during cytokinin-induced expansion process (Tsui *et al.* 1983). However, the actions of exogenous cytokinins on endogenous cytokinins during cotyledon expansion are still unclear and whether the gene expression is involved in this fast response and which specific genes play roles during this process are still under investigation. Furthermore, relationship between cytokinins and light in inducing cotyledon expansion is not fully understood.

The microtubules in elongating cells can re-orient in response to endogenous and exogenous signals such as light, gravity, plant hormones, abiotic and biotic stresses (Nick 1998), accompanied by corresponding changes in the proportionality of cell expansion. Therefore, it is interesting to know if α -tubulin is involved in cotyledon expansion process and its interaction with cytokinins. In this report, the effects of benzylaminopurine (BA) on endogenous cytokinin contents during cotyledon expansion were investigated. The relationship among BA, light and endogenous cytokinins were analyzed. The mechanisms of light and cytokinins in inducing cotyledon expansion are evaluated in connection with gene expression and particularly with α -tubulin actions.

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Abbreviations: Act D - actinomycin D; BA - benzylaminopurine; Col - colchicine; PCR - polymerase chain reaction.

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Materials and methods

Plants and incubation of excised cotyledons: Seeds of cucumber (*Cucumis sativa* L. cv. Jing-Yan No. 4) were washed with running water for 12 h, then soaked in 70 % ethanol for 10 min and rinsed five times with sterile water. The seeds were germinated on *Whatman* paper saturated with sterile water in Petri dishes at 25 °C in the dark. The cotyledons from 5-d-old seedlings were excised under a green safe light and incubated on *Whatman* paper saturated with sterile water in Petri dishes at 25 °C in the dark for 6 h. After this pretreatment, irradiance of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at plant level was provided by white fluorescent lamp. The cotyledons were harvested at intervals up to 24 h. Benzyladenine (BA), actinomycin D (Act-D) and colchicine (Col) were used for the different treatments.

Contents of endogenous cytokinins were determined by high-performance liquid chromatography (HPLC) combined with ELISA method. Cotyledons were ground to a fine powder with a pestle and mortar in liquid nitrogen and extracted in 10 cm^3 80 % methanol with 40 mg dm^{-3} butyl hydroxy-toluene at 4 °C overnight. The extraction was repeated twice. The extracts were purified through *Millipore* (Shanghai, China) filters (0.22 μm) and *SepPak C18* columns (*Millipore*). After filtration, eluates were reduced to dryness in a vacuum with a rotary evaporator and the residues redissolved in 0.5 cm^3 80 % methanol. The cytokinins were then separated by HPLC using a reverse-phase *Zorbax ODS* 5 \times 250 mm column (*Shimadzu*, Kyoto, Japan), at a flow rate of 1 $\text{cm}^3 \text{min}^{-1}$ with 10 % methanol in acid water (water plus 1 % acetic acid). Fractions were collected at 2-min intervals and the corresponding fractions were combined together and reduced to dryness in nitrogen gas flow. Cytokinin contents were quantified using an ELISA Kit (China Agricultural University, China) with anti-[9R]Z (for zeatin and zeatin riboside) and anti-[9R]iP (for iP and iP riboside). The hormone contents in each sample were

measured three times and the standard error was calculated.

Isolation of pea α -tubulin cDNA sequence for use as a probe: Total RNA was isolated from pea tissues by TRI reagent (*Molecular Research Center*, Cincinnati, USA) according to the manufacturer's instruction. Poly(A)⁺RNA was isolated using PolyAT tractR mRNA Isolation Kit (*Promega*, Madison, USA). cDNA synthesis was based on the rapid amplification of cDNA ends method (Frohman *et al.* 1988) using oligonucleotide primer 5'-GACTCGAGTCGACATCGA(T)17-3'. PCR was conducted using 5'-primer as 5'-CGTGGTGATGTTGTGCCT-3' and 3'-primer as 5'-CTCAGCACCAACCTCTTC-3'. These primers corresponded to pea α -tubulin cDNA (GenBank no U12589) 3' terminus (Brierley *et al.* 1995). The PCR products were resolved on a 1.0 % agarose gel and purified by using a *GlassMAX*[®] DNA Isolation Kit (*Gibco*, Grand Island, USA). The purified fragments were cloned into *pGEM-T Easy* vector (*Promega*). After sequencing, a clone of 357 bp in length showed identical to pea α -tubulin cDNA was used as a probe for RNA gel blot hybridization.

RNA gel blot analysis: Total RNA (10 μg) from cucumber was electrophoresed on 1.4 % (m/v) formaldehyde agarose gels. RNA was blotted onto *Hybond-N⁺* membrane (*Amersham*, Beijing, China) using established protocols (Sambrook *et al.* 1989). The blots were hybridized at 42 °C in 6 \times SSC, 5 \times Denhardt, 0.5 % SDS, 100 $\mu\text{g cm}^{-3}$ salmon sperm DNA with 50 % formamide and washed with 0.1 \times SSC plus 0.1 % SDS at 65 °C. Probes were ³²P-labelled using a *Ready-to-Go DNA Labeling Kit* (*Amersham*). RNA blots were quantified using Phosphor Image and mRNA levels were normalized by comparison to a soybean 18S rRNA.

Results

Both BA and light induced cotyledon expansion: The effects of BA on the induction of expansion reached maximum between 10 to 20 μM (Table 1). Therefore,

Table 1. Fresh mass [mg cotyledon⁻¹] of the excised cucumber cotyledon as affected by different concentrations of benzyladenine (BA) for 24 h under darkness or white light [30 $\mu\text{mol m}^{-2} \text{s}^{-1}$]. Data presented here are the mean \pm SD of three replicates with 10 cotyledons per replicate.

BA [μM]	0	2.5	5	10	20
Dark	15.2 \pm 1.4	20.1 \pm 1.4	24.7 \pm 2.1	27.8 \pm 2.1	28.9 \pm 2.5
Light	20.2 \pm 2.1	24.1 \pm 1.9	28.2 \pm 1.7	32.7 \pm 3.1	34.3 \pm 4.1

20 μM of BA was used in the following experiments.

The data indicated that light stimulated the cotyledon expansion, the fresh mass increased by 44.3 % in the light as compared to 8.6 % increase in the dark after 24 h treatment (Fig. 1A). BA strongly enhanced the expansion of cotyledons, the fresh mass of the cotyledon increased by 106.4 % after 24 h treatment with BA in the dark. BA and light combined together had an additive effect on cucumber cotyledon expansion. The fresh mass increase in that treatment was 145 %.

Effects of BA and light on endogenous cytokinins: The cytokinin contents remained almost unchanged in the excised cotyledons incubated in the light or dark without BA treatment, it was only slightly decreased after 24-h

incubation. BA treatment markedly increased the cytokinin contents in the excised cotyledons (by 89 % after 24 h). BA treatment plus light did not further promote the increase of cytokinin content, contrasting with the fresh mass increase in the same treatment (Fig. 2A).

Effects of actinomycin D on cucumber cotyledon expansion: The actinomycin D, a well-known inhibitor of gene transcription, strongly inhibited both light- and BA-induced cotyledon expansions. The inhibiting effects were already noticed at 15 μM actinomycin D, but the maximum inhibition was observed at concentration reached 45 μM (Table 2).

The time-course of actinomycin D inhibition on cotyledon expansion was checked at 30 μM concentration. Actinomycin D slightly inhibited BA and light-induced cotyledon expansion after 4 h and this inhibition reached maximum after 12 h (Fig. 1B). In the presence of actinomycin D, endogenous cytokinin contents were markedly reduced compared to the control without

actinomycin D (Fig. 2B). After 4 h of treatment with actinomycin D, cytokinins contents showed about 29.7 % increase in BA treatment. However, they decreased to their initial levels after 24-h treatment with actinomycin D.

Table 2. Fresh mass [mg cotyledon⁻¹] of the excised cucumber cotyledon as affected by different concentrations of actinomycin D (Act-D) for 24 h under darkness or white light [30 $\mu\text{mol m}^{-2} \text{s}^{-1}$]. Benzyladenine (BA) of 20 μM was used for cytokinin treatment plus different concentration of actinomycin D. Data presented here are the mean \pm SD of three replicates with 10 cotyledons per replicate.

Act-D [μM]	0	15	30	45	60
Dark	15.2 \pm 1.4	15.1 \pm 1.4	15.6 \pm 2.1	15.4 \pm 1.6	15.5 \pm 1.3
Light	20.2 \pm 2.1	18.9 \pm 0.9	17.2 \pm 0.7	15.9 \pm 1.1	16.2 \pm 1.2
Dark+BA	28.9 \pm 2.5	24.1 \pm 2.3	18.7 \pm 1.1	16.2 \pm 1.5	15.8 \pm 1.2
Light+BA	34.3 \pm 4.1	28.3 \pm 3.2	20.5 \pm 1.5	17.0 \pm 1.6	16.1 \pm 1.4

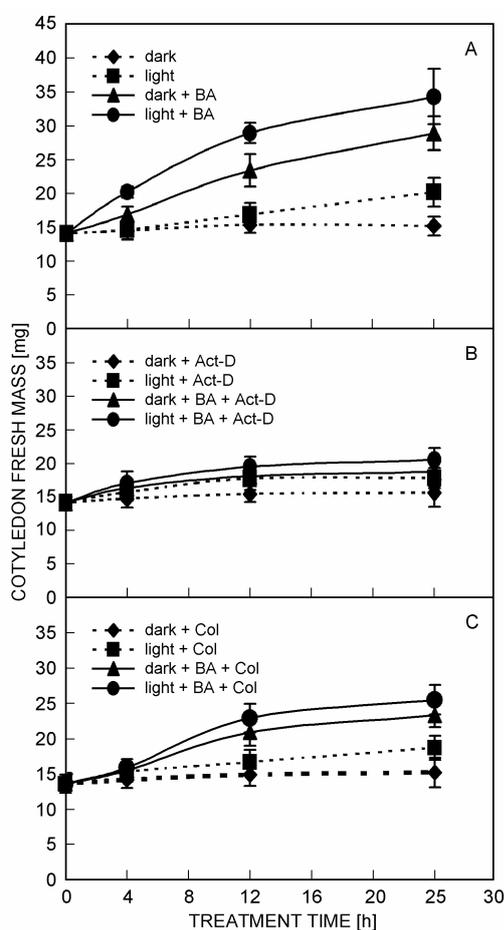


Fig. 1. The time course of the cucumber cotyledon expansion under darkness or white light [30 $\mu\text{mol m}^{-2} \text{s}^{-1}$]. Each value is the mean of three independent replicates with 10 cotyledons per replicate and vertical bars represent SD. A - treatment with 20 μM benzyladenine (BA); B - treatment with 30 μM actinomycin D (Act-D) plus 20 μM BA; C - treatment with 10 mM colchicine (Col) plus 20 μM BA.

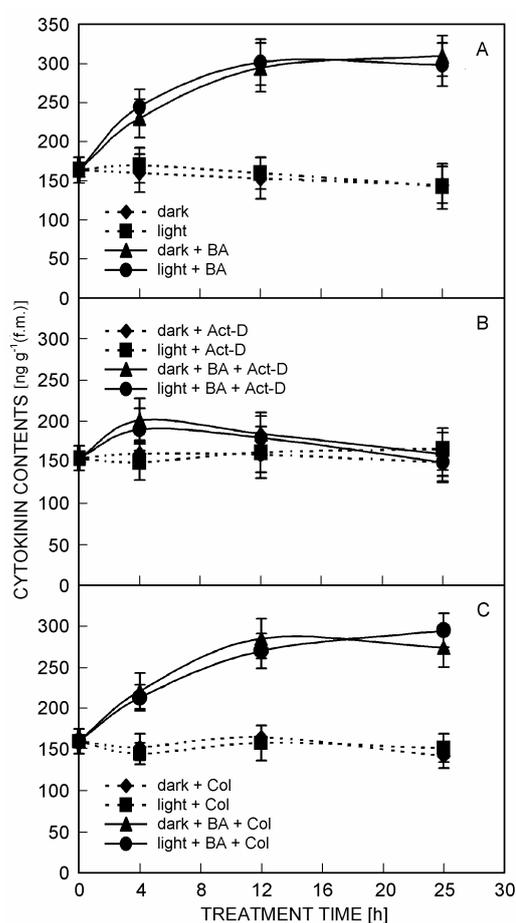


Fig. 2. The cytokinin contents in the excised cotyledons of cucumber [ng g⁻¹(f.m.)] under darkness or white light [30 $\mu\text{mol m}^{-2} \text{s}^{-1}$]. Each value is the mean of three independent replicates and vertical bars represent SD. A - treatment with 20 μM benzyladenine (BA); B - treatment with 30 μM actinomycin D (Act-D) plus 20 μM BA; C - treatment with 10 mM colchicine (Col) plus 20 μM BA.

The α -tubulin gene during cucumber cotyledon expansion: α -tubulin mRNA levels exhibited little variation in cotyledon under dark treatment (Fig. 3A). The accumulation of α -tubulin transcripts was induced after treatment with BA. This induction was observable after 4 h of treatment and reached its maximum after 12 h of treatment (Fig. 3B). However, addition of actinomycin D markedly decreased the BA induction on the α -tubulin gene expression (Fig. 3C). These results were confirmed after the signals were normalized relative to the 18S rRNA signal (data not shown).

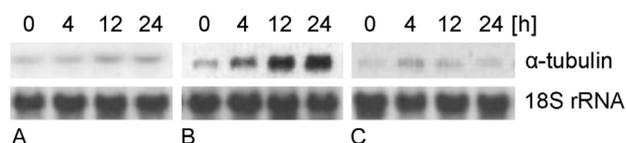


Fig. 3. RNA gel blot analysis of α -tubulin gene expression in cucumber cotyledons after treatment with water (A) 20 μ M BA (B) or 20 μ M BA plus 30 μ M actinomycin D (C) for different times as indicated. The treated cotyledons were collected, total RNA was isolated by TRI reagent and hybridized with pea α -tubulin gene probe. Hybridization with a soybean 18S rDNA probe has been included to confirm that the RNA preparations are undegraded and to serve as an internal control for variations in gel loading and blotting.

Effects of colchicine on cucumber cotyledon expansion: Incubation with increasing concentrations of

colchicine progressively inhibited cotyledon expansion (Table 3). However, differences in the effects of colchicine between 5 and 10 mM on the inhibition of expansion were negligible, suggesting the 10 mM concentration as suitable to test the physiological actions of colchicine.

The increase of fresh mass in cotyledons was partly inhibited by colchicine. The fresh mass of cotyledons increased by 46.5, 87.5 and 145 % when incubated with actinomycin D plus BA, colchicine plus BA and BA alone, respectively (Fig. 1C). However, the cytokinin contents were not affected by colchicine treatment (Fig. 2C).

Table 3. Fresh mass [mg cotyledon⁻¹] of the excised cucumber cotyledon as affected by different concentrations of colchicine (Col) for 24 h under darkness or white light [30 μ mol m⁻² s⁻¹]. Benzyladenine (BA) of 20 μ M was used for cytokinin treatment plus different concentration of colchicine. Data presented here are the mean \pm SD of three replicates with 10 cotyledons per replicate.

Col [μ M]	0	1	2	5	10
Dark	15.2 \pm 1.4	15.4 \pm 1.4	15.3 \pm 2.1	15.4 \pm 1.6	15.2 \pm 2.1
Light	20.2 \pm 2.1	19.9 \pm 1.9	19.2 \pm 0.7	19.0 \pm 1.1	18.7 \pm 1.7
Dark+BA	28.9 \pm 2.5	28.1 \pm 2.1	25.3 \pm 2.1	23.8 \pm 2.5	23.3 \pm 2.6
Light+BA	34.3 \pm 4.1	33.7 \pm 3.2	30.5 \pm 2.5	27.0 \pm 1.9	25.5 \pm 2.9

Discussion

Light has a profound influence on virtually all aspects of plant growth and development, including seed germination and seedling development (Kim *et al.* 2002). While the perception of light through photoreceptors is well understood, the mechanisms by which light mediates phenotypic change are not quite clear (Fankhauser 2002). The transition from a dark-grown (etiolated) to a light-grown (de-etiolated) morphology is marked by a number of dramatic phenotypic changes such as opening of the apical hook, expansion of cotyledons and the development of mature chloroplasts. Many of the light-induced changes during de-etiolation are also known to be regulated by plant hormones, particularly cytokinins (Symons and Reid 2003).

Excised cotyledons have been widely used as experimental system to study cytokinin-induced growth and related metabolism (Feng *et al.* 2003). In the present study, we showed that both BA and light could stimulate the cotyledon expansion in cucumber. The effects of BA were more pronounced than that of light on inducing cucumber cotyledon expansion. Although previous reports have demonstrated the cytokinin effects on cotyledon expansion, this was only based on exogenous application of BA. Whether this action is related to endogenous cytokinins is still unclear. In this report, we showed that the action of BA on cotyledon expansion was related to zeatin-type and iP-type cytokinins

(Fig. 2A). The antibodies we used are specific to zeatin-type and iP-type, the cross-reaction with BA is less than 1 % (data not shown). This ensures to distinguish between exogenous BA with endogenous zeatin-type and iP-type cytokinins. BA strongly induced the cucumber cotyledon expansion in the dark. In the same time, BA markedly increased endogenous cytokinin contents. Further analysis showed that the increase of endogenous cytokinins is earlier than that cotyledon expansion, it is reasonable to suggest that BA acts on cotyledon expansion by regulating endogenous cytokinins. A previous data also showed that the required exposure time to BA to be as short as half an hour (Tsui *et al.* 1983). These data suggest that the actions of BA are likely through trigger endogenous cytokinin signals. Compared with BA actions, light, however, did not induce the increase of endogenous cytokinin contents (Fig. 2A). Therefore, light is very likely to act on cotyledon expansion by other signal system, such as phytochrome. The relationship between cytokinins and light is complex and is likely to have different interactions in the different physiological processes (Kraepiel and Miginiac 1997). The data present in Fig. 2A indicated that light had no direct effects on endogenous cytokinin contents during the cucumber cotyledons expansion process, suggesting that light and cytokinins may act independently or sequentially through signal transduction to control

cotyledon expansion. This is in agreement with results from mustard cotyledons treated with cytokinins and light (Tong *et al.* 1983), indicating the effects of cytokinins and light on the cotyledon expansion is common in different plants.

To explore whether BA- and light-induced the cotyledon expansion is dependent on the active gene expression, the cotyledons were treated with actinomycin D in combination with BA and light. The data (Table 2 and Fig. 1B) indicated that actinomycin D completely inhibit the cotyledon expansion when its concentration reached 45 μ M. This suggests that the synthesis of new mRNAs is essential in the process of BA- and light-induced the cotyledon expansion. Teramoto *et al.* (1993) showed that abundance of some translatable mRNAs in excised cucumber cotyledons changed within 1 to 2 h after application of BA. This is consistent with our results. Furthermore, actinomycin D also strongly inhibited increase endogenous cytokinin contents that was induced by BA (Fig. 2B), suggesting that the specific gene expressions are also involved in cytokinin signaling which will lead to cotyledon expansion.

A previous study indicated that BA induced cotyledon expansion in cucumber involved both cell expansion and cell division, in which cell expansion contribute more to cotyledon expansion (Tsui *et al.* 1983). Therefore, it is interesting to know whether the cytoskeletons, especially of microtubules, are involved in this process. Microtubules are capable of performing various tasks during the life cycle of eukaryotic cells, which may related to cell expansion and cell division (Meyer *et al.* 1998, Walczak 2000). RNA gel blot analysis indicated that BA strongly induced α -tubulin gene expression corresponding to cotyledon expansion (Fig. 3B). This induction is very fast as it becomes remarkable within 4 h of BA application. Actinomycin D completely inhibited α -tubulin gene expression in accompany with its inhibition of cotyledon expansion (Fig. 3C). Furthermore, inhibition of tubulin polymerization by colchicine, an antimicrotubular reagent, also showed to decrease the induction of BA on cotyledon expansion. This inhibition

is partial; consistent with the results that BA induction on cotyledon expansion was also related with water uptake (Tong *et al.* 1980). However, there is no detectable regulation on cytokinin contents in cotyledons exposed to colchicine treatment (Fig. 2C), suggesting that α -tubulin is the downstream target for cytokinins. Similar results have been reported that the α -tubulin genes were differentially expressed during leaf cell development in barley (Hellmann and Wernicke 1998, Schroder *et al.* 2001).

The extensive studies have been conducted to indicate the co-action or similarity of light and cytokinins in photomorphogenesis. However, these physiological evidences have prompted arguments and need to be further evaluated. Recently, it has reported that *ftsZ* gene expression was induced by light and cytokinins in excised cucumber cotyledons, which is responsible for plastid division. These results indicate that the same downstream gene is involved in light- and cytokinin- induced plastid division (Ullanat and Jayabaskaran 2002a). Molecular evidences further demonstrated that light and cytokinins play a co-operative role in monogalactosyldiacylglycerol (MGDG) synthesis in greening cucumber cotyledons, which is thought to determine the physicochemical properties of the thylakoid membrane that might be essential in supporting photosynthetic competence (Yamaryo *et al.* 2003). Exposure to light or treatment with BA was found to have a similar up-regulatory effect on *CsCDPK3* (a calcium dependent protein kinases) transcript levels in excised cotyledons in cucumber (Ullanat and Jayabaskaran 2002b). The results in this report indicate that the action of light on the cotyledon expansion is not related to cytokinins. However, both light and BA-induced cotyledon expansion requires the active gene expression, particularly α -tubulin gene expression. This suggests that the actions of cytokinins and light are independent at the initial signal transduction stage in the process of cotyledon expansion, although both signals may act on the same target proteins or genes in downstream.

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