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.

Received 23 September 2005, accepted 17 May 2006.

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

Acknowledgments: This work was supported by the National Natural Science Foundation of China (No. 30671043, 30400222, 30570133), the Natural Science Foundation of Beijing (No. 5063047) and the Innovation Project of Chinese Academy of Sciences.

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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 µmol m⁻² 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 cm³ 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 cm³ 80 % methanol. The cytokinins were then separated by HPLC using a reverse-phase Zorbax ODS (Act-D) and colchicine (Col) were used for the different treatments.

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 µmol m⁻² s⁻¹]. Data presented here are the mean ± SD of three replicates with 10 cotyledons per replicate.

<table>
<thead>
<tr>
<th>BA [µM]</th>
<th>0</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
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<tbody>
<tr>
<td>Dark</td>
<td>15.2±1.4</td>
<td>20.1±1.4</td>
<td>24.7±2.1</td>
<td>27.8±2.1</td>
<td>28.9±2.5</td>
</tr>
<tr>
<td>Light</td>
<td>20.2±2.1</td>
<td>24.1±1.9</td>
<td>28.2±1.7</td>
<td>32.7±3.1</td>
<td>34.3±4.1</td>
</tr>
</tbody>
</table>

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, 20 µM of BA was used in the following experiments.

| Dark | 24.7±2.1 | 27.8±2.1 | 28.9±2.5 |
| Light | 32.7±3.1 | 34.3±4.1 | 34.3±4.1 |

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'-GACCTGAGTCGACATCGA(T)17-3' and 3'-primer as 5'-CTCAGACCAAACCTCCTTC-3'. These primers corresponded to pea α-tubulin cDNA (GenBank no U12589) 3’ terminus (Briere 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× SSC, 5× Denhardt, 0.5 % SDS, 100 µg cm⁻³ salmon sperm DNA with 50 % formamide and washed with 0.1× 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.

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 µmol m⁻² s⁻¹]. Benzyladenine (BA) of 20 µM was used for cytokinin treatment plus different concentration of actinomycin D. Data presented here are the mean ± SD of three replicates with 10 cotyledons per replicate.

<table>
<thead>
<tr>
<th>Act-D [µM]</th>
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<th>45</th>
<th>60</th>
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<td>15.1±1.4</td>
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<td>17.2±0.7</td>
<td>15.9±1.1</td>
<td>16.2±1.2</td>
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<tr>
<td>Dark+BA</td>
<td>28.9±2.5</td>
<td>24.1±2.3</td>
<td>18.7±1.1</td>
<td>16.2±1.5</td>
<td>15.8±1.2</td>
</tr>
<tr>
<td>Light+BA</td>
<td>34.3±4.1</td>
<td>28.3±3.2</td>
<td>20.5±1.5</td>
<td>17.0±1.6</td>
<td>16.1±1.4</td>
</tr>
</tbody>
</table>

*Fig. 1. The time course of the cucumber cotyledon expansion under darkness or white light [30 µmol m⁻² 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 µmol m⁻² 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).

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. Previous data also showed 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

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 µmol m⁻² s⁻¹]. Benzyladenine (BA) of 20 µM was used for cytokinin treatment plus different concentration of colchicine. Data presented here are the mean ± SD of three replicates with 10 cotyledons per replicate.

<table>
<thead>
<tr>
<th>Col [µM]</th>
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<th>1</th>
<th>2</th>
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</tr>
<tr>
<td>Light</td>
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<td>19.9±1.9</td>
<td>19.2±0.7</td>
<td>19.0±1.1</td>
<td>18.7±1.7</td>
</tr>
<tr>
<td>Dark+BA</td>
<td>28.9±2.5</td>
<td>28.1±2.1</td>
<td>25.3±2.2</td>
<td>23.8±2.5</td>
<td>23.3±2.6</td>
</tr>
<tr>
<td>Light+BA</td>
<td>34.3±4.1</td>
<td>33.7±3.2</td>
<td>30.5±2.5</td>
<td>27.0±1.9</td>
<td>25.5±2.9</td>
</tr>
</tbody>
</table>
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 inhibited the cotyledon expansion when its concentration reached 45 µM. 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 fisZ 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 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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