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RESEARCH PAPERS

Expression of Plastid Genome and Development of *Arabidopsis thaliana* with Disturbed Synthesis of Brassinosteroids

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Abstract—Comparative analysis of growth characteristics, content of auxins and cytokinins, and the level of photosynthetic pigments was conducted in *det2* mutant plants of *Arabidopsis thaliana* (L.) Heynh notable for a disturbed synthesis of brassinosteroids and therefore their low endogenous level. In this mutant line, we observed de-etiolation: the formation of large cotyledons and long hypocotyls in the dark already shown earlier. For the first time, we analyzed transcription of 12 chloroplast genes encoding functionally different proteins and RNAs in rosette leaves of wild type and *det2* plants. In rosette leaves of mutant plants, transcription activity of the investigated plastid genes was much higher than in the parental line. On these grounds, we assumed that the low level of brassinosteroids was correlated with the activation of transcription of some chloroplastic genes and realization of the de-etiolation program.

Keywords: Arabidopsis thaliana, brassinosteroids, photomorphogenesis, phytohormones, photosynthetic pigments, run-on transcription, transcription of plastid genes.

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INTRODUCTION

Presently it is generally accepted that phytohormones play an important role in the realization of light regulatory and photosynthetic functions. Some phytohormones are known to imitate the regulatory function of light because they similarly control the rate and nature of morphophysiological processes in plants [1]. This function of phytohormones was shown in the course of investigation of phenotypic features of plants differing in the endogenous level of hormones or sensitivity to them [2–6]. Brassinosteroids occupy a specific place among phytohormones; along with cytokinins, they can induce in the dark phenotypic changes and trigger the features characteristic of light-regulated development [7–9]. At the same time, experimental data concerning the contribution of brassinosteroids to regulation of photomorphogenesis in plants are highly contradictory. For instance, some researchers showed that in the dark brassinosteroids were capable of suppressing the development of embryonic stems (hypocotyls) and enhancing cotyledon growth [5, 10]. On the other hand, some pea mutants with disturbed biosynthesis of brassinosteroids, such as *lka* and *lkb*, preserved the etiolated phenotype in the dark [11, 12]. Moreover, there are indications that brassinosteroids were detrimental to photomorphogenesis when the mutants with a disturbed synthesis of brassinosteroids or modified sensitivity to them were used as an experimental model [2, 13].

In order to look into the role of brassinosteroids in the regulation of photomorphogenesis, *det2* mutant plants of *Arabidopsis thaliana* with a disturbed brassinosteroid biosynthesis and their low content were used. In the course of dark development, this mutant accumulates mRNAs of several nuclear and plastid genes encoding the proteins of the photosynthetic machinery [2, 14]. In this work, we have made an attempt to investigate a possible role of brassinosteroids in the regulation of photomorphogenesis by following growth characteristics and hormonal balance in the seedlings of *A. thaliana* of wild type and *det2* mutant line as well as the content of photosynthetic pigments and transcription activity of some plastid genes. In addition, it was important to elucidate the level of regulation of expression of these genes in the *det2* mutant notable for a pronounced shortage of endogenous brassinosteroids.

MATERIALS AND METHODS

The experiments were conducted with the plants of *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia, and its mutant form *det2* with a disturbed brassinosteroid synthesis [2]. The seeds of both lines were obtained

from Arabidopsis Biological Resource Center, Ohio State University, Columbus (United States).

Growth characteristics were determined in 7-day-old etiolated seedlings. Seeds of *A. thaliana* were decontaminated with 3% H_2O_2 in 80% ethanol and placed in Petri dishes with a half-strength modified Murashige-Skoog liquid medium. In order to stimulate and synchronize germination, seeds were incubated for three days at 4–6°C and illuminated with white light for 3 h (LD-40 luminescent lamps, 3700 lx); then seeds were germinated for seven days in the dark. The lengths of seedling hypocotyls and roots were determined with a BM-51-2 magnifier, the cotyledon area was measured under a Micros MC 100 microscope (Austria) using a Moticam 2000 digital camera (Spain) and a Motic Images Plus 2.0 computer program. Each type of treatment comprised three replicates, each comprising 30 seedlings.

Phytohormones in 7-day-old etiolated seedlings were determined by a classic solid-phase immunoenzyme method. Plant material (1.0–1.5 g fr. wt.) was fixed in liquid nitrogen with subsequent ethanol extraction as described earlier [5]. Phytohormones were separated on Sorbfil TLC plates (Sorbiopolimer, Russia) using standard markers of IAA (Serva, Germany), zeatin, and zeatin riboside (Sigma, United States). Phytohormones were assayed with 3 replicates and 6 repetitions.

The rate of transcription of chloroplastic genes and the content of photosynthetic pigments were investigated in rosette leaves of 3.5-week-old plants grown in a controlled-climate chamber under white light (flux density of incident quanta was 220 μmol/(m^2 s), photoperiod of 16 h, and air temperature of 20–22°C.

In order to estimate the level of photosynthetic pigments, leaves were ground in 96% ethanol, and the slurry was centrifuged for 15 min at 14 000 rpm in a MiniSpin microfuge (Eppendorf, Germany). Optical density was measured using a Genesys 10UV spectrophotometer (Thermo Fisher Scientific, United States). The concentration of pigments in the ethanolic extract was calculated according to Lichtenthaler [15].

Isolation of plastids and run-on transcription in plastid lysates were conducted as described earlier [16]. Chloroplasts were isolated from rosette leaves using a stepwise gradient of Percoll (40 and 70%) at 4°C. In vitro transcription was performed for 15 min at 25°C in the lysate containing 5×10^7 chloroplasts in 100 μl of buffer of the following composition: 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 0.2 mM CTP, GTP, and ATP, 0.01 mM UTP, 50 μCi [α-^{32}P]-UTP (Amersham, United Kingdom), 20 units of activity of RNAase inhibitor (Fermentas, Lithuania), and 10 mM β-mercaptoethanol. Transcription was terminated by the addition of equal volume of stop buffer (50 mM Tris–HCl, pH 8.0, 25 mM EDTA, and 5% sarcosyl). ^{32}P-labeled RNA produced as a result of transcription was isolated from the reaction medium and used for hybridization. We analyzed transcription of 12 chloroplast genes highly homologous to the barley genes, which belong to functionally different groups of plastome genes. Barley gene-specific fragments used as hybridization probes are described elsewhere [17–19]. About 2 μg of each fragment of the genes under study was applied in duplicate on a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, United Kingdom). Following hybridization with ^{32}P-labeled RNA produced as a result of in vitro transcription, radioactive signals were scanned and digitized using a Phosphorimager Typhoon Trio (a Typhoon TRIO+ Variable Mode Imager scanner equipped with a Typhoon Scanner Control package) and an ImagerQuant TL Control Centre (GE Healthcare, United States). The changes in the rate of transcription were considered reliable when the signal exceeded no less than twice that in the control,

Table 1. Growth characteristics of 7-day-old etiolated seedlings of *Arabidopsis thaliana*

Line	Dimensions of hypocotyl		Area of cotyledons, 1000 μm^2	Root length, mm
	length, mm	width, mm		
Col-0	14.30 ± 0.22	0.190 ± 0.002	141.9 ± 4.1	3.12 ± 0.10
det2	6.60 ± 0.12	0.200 ± 0.002	189.0 ± 6.5	1.43 ± 0.06

The figures show the results of the experiments as the means and their standard errors. Independent samples obeying the law of normal distribution were compared using the parametric Student's test. The values of *t*-test were significant at 95% level ($P < 0.05$).

RESULTS

Growth Characteristics and Content of Photosynthetic Pigments in A. thaliana

The results of morphometric examination of 7-day-old etiolated seedlings of *A. thaliana* of wild type Columbia (Col-0) and its mutant *det2* indicate that *det2* seedlings grown in the dark showed a de-etiolated phenotype. De-etiolation was manifested in a considerable (by 2.2 times) shortening of axial organs (hypocotyls and roots) and expansion of cotyledons (by 1.3 times) (Table 1). Suppression of cell elongation induced by mutation in *DET2* gene [2] also affected the development of true leaves. For instance, the dimensions and shape of rosette leaves in 3.5-week-old *det2* plants changed due to underdeveloped petioles (Fig. 1).

In the *det2* seedlings, there were changes in the balance between photosynthetic pigments. Analysis of pigment composition in rosette leaves showed a significant increase in the level of chlorophylls *a* and *b* and carotenoids in the mutant as compared with the wild-type plants (Fig. 2), with the chlorophyll *a* to chlorophyll *b* ratio 2.3 and 2.2 for the wild type and *det2* plants, respectively.

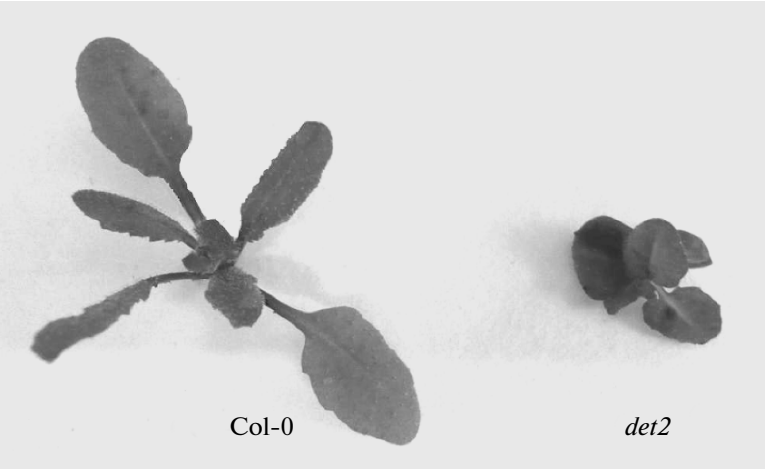

Fig. 1. 3.5-week-old plants of *Arabidopsis thaliana* grown in the light.
Col-0—wild-type plant, *det2*—mutant plant.

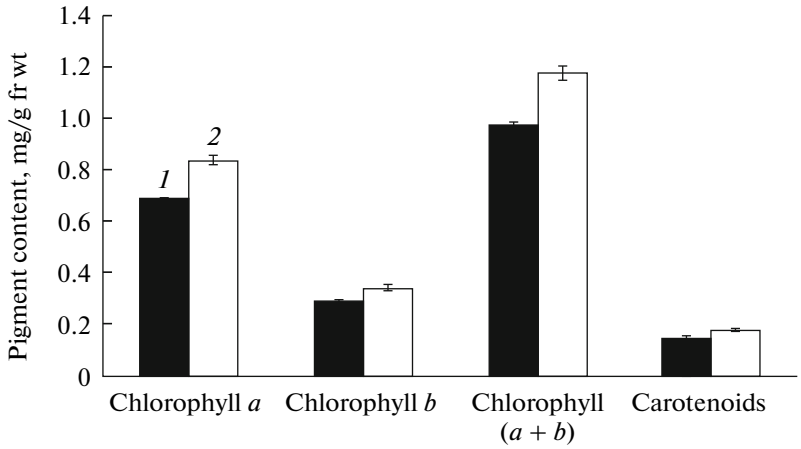

Fig. 2. Content of chlorophylls *a* and *b* and carotenoids in rosette leaves of 3.5-week-old plants of *A. thaliana* grown in the light.
(*1*) Col-0; (*2*) *det2*.

Phytohormone Content in the Etiolated Seedlings of Arabidopsis

Mutation in the gene *DET2* disturbed the initial stages of the synthesis of brassinosteroids and induced a ten-fold decrease in their endogenous content in the *det2* seedlings as compared with the wild type [14]. One could expect that such an upset of the balance between steroid hormones will be accompanied by changes in the content of other phytohormones. Analysis of IAA content (free and bound forms) and cytokinins (zeatin and zeatin riboside) showed that, in the mutant *det2* (as compared with wild type), the level of free auxin was twice lower and the content of bound form 1.4 times higher while the total content of endogenous auxins was the same. The content of zeatin and zeatin riboside in the *det2* seedlings as compared with the parental form also rose by 3.8 and 3.2 times, respectively. On this basis, one can assume that among the causes for the development of larger cotyledons in the mutant (Table 1) is their ability to accumulate more cytokinins (Table 2) which are known to promote cell division and elongation [20].

Transcription of Chloroplast Genes

Using the run-on method, we investigated the transcription of certain chloroplast genes in the wild-type and its mutant *det2* plants. The transcription system was based on lysed chloroplasts isolated from the rosette leaves of 3.5-week-old Arabidopsis plants grown under white light. In the course of transcription, newly produced molecules of RNA became labeled with α-^{32}P-UMP, which made it possible to subsequently analyze only newly produced transcripts. Typical autoradiograms produced in the course of the run-on experiment with the chloroplasts of Arabidop-

Table 2. Phytohormone content in 7-day-old etiolated seedlings of *Arabidopsis thaliana*

Line	IAA content, ng/g fr wt		Cytokinin content, ng/g fr wt	
	free	bound	zeatin	zeatin riboside
Col-0	98.08 ± 10.10	117.30 ± 21.00	0.86 ± 0.20	1.15 ± 0.10
det2	53.26 ± 6.70	166.50 ± 15.00	3.30 ± 1.00	37.24 ± 5.40

sis rosette leaves and the rates of transcription expressed as arbitrary units are shown in Fig. 3.

We compared the transcription rates of 12 chloroplast genes belonging to functionally different groups of plastome genes (Fig. 3). First of all, they are the genes encoding the products that play an important role in the process of photosynthesis: the photosystem I genes *psaA* and *psaB*, the photosystem II genes *psbA*, *psbD*, and the *psbK*, gene of the large subunit of Rubisco (*rbcL*), the ATP-synthase complex genes *atpB*, and the subunit F of NADPH-plastoquinone oxidoreductase *ndhF*. Among the household genes, we investigated transcription of the gene encoding β-subunit of RNA-polymerase of bacterial type (*rpoB*), the genes of 16S and 23S ribosomal RNA (*rrn16* and *rrn23*), and the genes of tRNA-Glu and tRNA-Tyr (*trnE-trnY*).

The obtained results showed that among the investigated plastid genes in the rosette leaves of the parental and mutant lines, the highest transcription rate was characteristic of the genes encoding 16S and 23S rRNA (*rrn16* and *rrn23*), several proteins of PSI and PSII (*psaA*, *psbA*, and *psbD*), large subunit of Rubisco, and Glu/Tyr transport RNAs. The lowest transcription activity in the investigated lines was manifested by the genes *psaB*, *psbK, ndhF*, and *rpoB*. Some of the genes under study were differently transcribed in the two compared lines. For instance, if the genes *psaB* and *atpB* showed moderate activity in *det2* plants, the rate of their transcription in the wild type was insignificant. On the whole, the obtained results showed that the transcription rate of all the plastid genes under study in the *det2* rosette leaves considerably exceeded the transcription activity in the seedlings of the initial line.

Twofold (and greater) differences in the gene transcription rate were considered significant. For nine chloroplast genes of *det2* plants, the transcription rate was 2–4 times higher than in the parental line. The greatest differences (8–12-fold) were observed for weakly transcribed genes, such as *psbK*, *ndhF*, and *atpB*. Thus, in the *det2* mutant, differential regulation of the rate of transcription of some chloroplastic genes was shown.

DISCUSSION

One of the major regulatory functions performed by brassinosteroids is promoting plant growth through the activation of cell division and elongation [21]. This hormonal effect is attained owing to the regulation by brassinosteroids of expression of the genes encoding expansin proteins and the enzymes of cell wall (xyloglucan endotransglycosylase/hydrolase, pectin-like lyase, and glucanase), activity of aquaporines and vacuolar H^+-ATPase. Expression of some of the above-listed genes solely depended on the level of endogenous brassinosteroids because other phytohormones such as auxins, ethylene, and gibberellins considerably affecting cell elongation were not affected [21].

Specific regulation with brassinosteroids was exemplified by expression of the Korrigan gene encoding endo-β-1,4-glucanase essential for correct assembly of cell wall components [22]. It was insufficient synthesis of brassinosteroids that most probably led to the formation of dwarf axial organs (hypocotyl and root) in the seedlings of Arabidopsis in the dark (Table 1). At the stage of rosette leaves in *det2* in the light, such a trend also manifested as the shortening of embryonic stem (hypocotyl) and the petioles of rosette leaves (Fig. 1).

Identical phenotypic changes were earlier pronounced in *det2* mutant plants of Arabidopsis [2], where endogenous level of physiologically active brassinosteroids did not exceed 10–15% of their level in the wild-type plants, such a drop resulted from disturbed operation of the key gene of hormone biosynthesis (*DET2*) [14]. This mutation was organ-specific: while the length of axial organs decreased, the cotyledons became larger. This phenomenon may depend on considerable changes in the endogenous level of some other hormones. In particular, in etiolated *det2* seedlings, the content of free auxins decreased and the level of cytokinins considerably rose (Table 2). Elevation of the endogenous cytokinin content would promote cell division and elongation, which could probably cause the expansion of cotyledons in the *det2* seedlings in the course of dark development (Table 1). The formation of such a de-etiolated phenotype (short hypocotyl and large cotyledons) in the seedlings grown in the dark

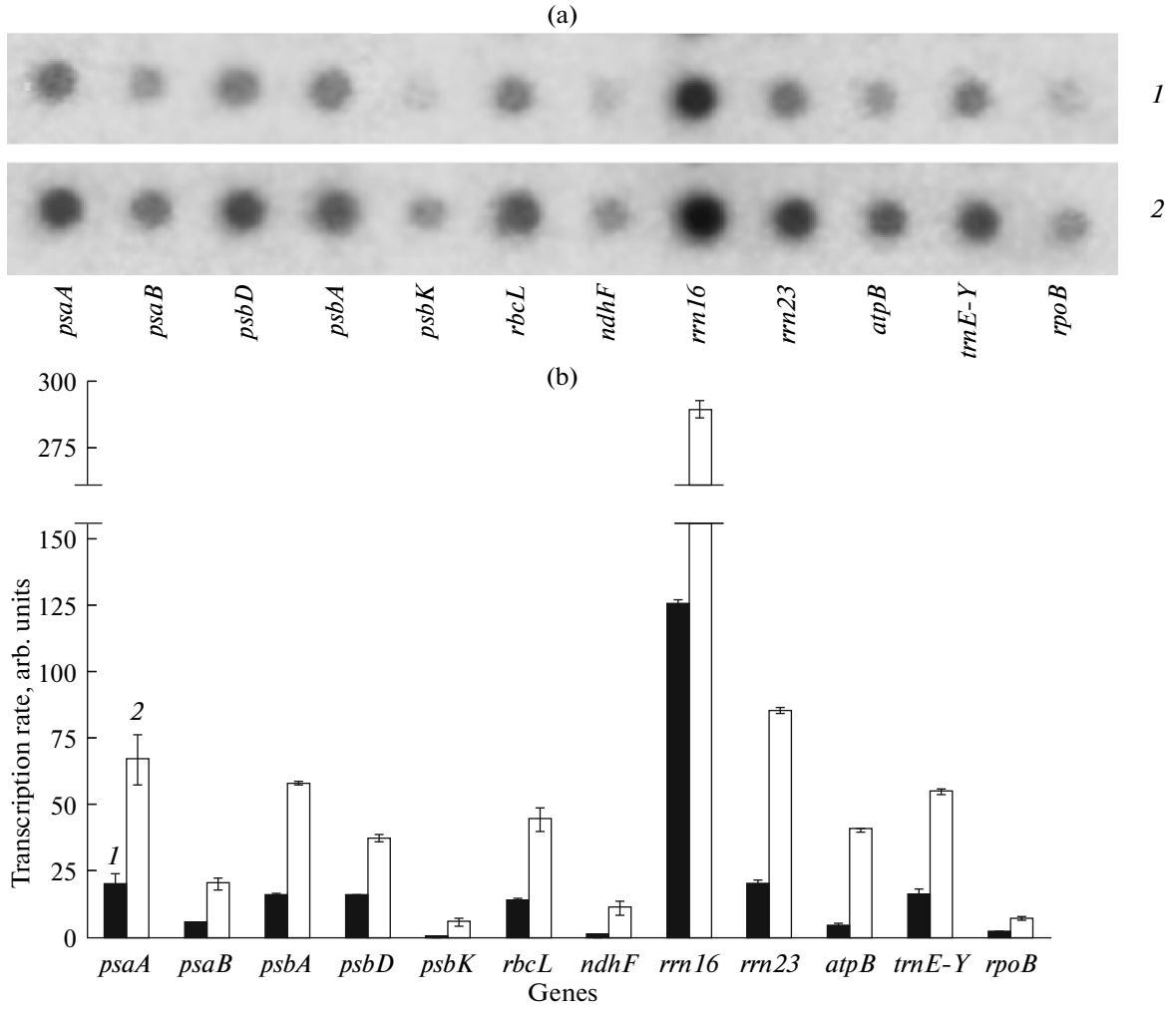

Fig. 3. Transcription rate of plastid genes in rosette leaves of 3.5-week-old plants of *A. thaliana* grown in the light.
(a) Autoradiogram showing the results of run-on transcription with the chloroplasts from *Arabidopsis thaliana* of wild type (Col-0) and mutant *det2*; (b) the results of autoradiogram scanning: (*1*) Col-0, (*2*) *det2*.

points to the manifestation of the features of light-regulated development.

Regulation of light development of plants by phytohormones occurs not only at the level of morphological changes but also on the gene level. Our studies made it possible to show that phytohormones participated in the regulation of expression of the genes located in nuclei and chloroplasts, whose products are essential for realization of the photosynthetic function. In this context, some researchers emphasized an important role of hormones antagonistic to cytokinins and ABA that along with the light were involved in the regulation of the formation of photosynthetic machinery in dicots and monocots [23–25]. Probably, such an effect of phytohormones on realization of the photosynthetic role of light depends on the fact that plastids of the plants accommodate not only the enzymes of biosynthesis of some hormones but also certain stages of their biosynthesis [26].

Experimental data obtained by the run-on transcription indicate that the greatest rate of transcription of chloroplast genes irrespective of the endogenous level of brassinosteroids was observed in two genes encoding proteins D1 and D2 of PSII, *psbA* and *psbD*. The products of these genes form a reaction center that, as a key component of PSII, ensures the primary charge separation at chlorophyll P680 and transfer of electron from the water splitting complex to the pool of plastoquinones. High transcriptional activity was also shown for the gene *psbA* encoding subunit A of PSI reaction center.

In light, insufficient synthesis of brassinosteroids produced a super de-etiolated phenotype in *det2* plants. This effect became apparent not only on the level of morphological changes but also regarding the content of chlorophylls *a* and *b* and carotenoids (Fig. 2). In addition, the transcription of numerous functionally different chloroplast genes was activated.

For instance, the transcription rate of the gene encoding F-subunit of NADH-plastoquinone oxidoreductase participating in plant respiratory metabolism was 12 times greater in the mutant *det2* than in the wild-type plants. Another pair of genes greatly differed (by 8–10 times) in the transcription rate in two lines under study was genes *psbK* encoding K-subunit (3.9 kD) of PSII and *atpB* responsible for β-subunit of ATP-synthase. The transcription activity of other nine genes in the rosette leaves of mutant plants was on the average 2–4 times greater than in the parental line.

The obtained results make it possible to assess the control of plastid gene expression in *det2* mutant plants. It was shown earlier [2] that in *det2* seedlings grown in the dark, there occurred a 10–30-fold increase in the level of mRNA for some plastid genes (*psaA*, *psaB*, *psbA*, *rbcL,* and *rrn16*). However, it was not clear whether an active constitutive expression of these genes was controlled at the level of transcription or post-transcription. The method of run-on transcription we employed for the evaluation of plastid gene expression unambiguously showed that, in the *det2* seedlings under illumination (as compared to the wild type plants), the transcription of chloroplast genes was more active (Fig. 3). This means that a ten-fold decrease [14] in the level of endogenous brassinosteroids in the mutant seedlings was accompanied by a derepression of some plastid genes at the transcription level. However, these results do not rule out the presence in the *det2* mutant of other levels of regulating the expression of these genes. In addition, these data suggest that the active expression of plastid genes in *det2* seedlings observed earlier in the dark [2] also occurs at the transcriptional level, although the regulation of the mRNA content by its stability is not ruled out.

Thus, the comparison of expression of some genes of the plastid genome and the development of *A. thaliana* of wild type and *det2* mutant with a disturbed synthesis and pronounced deficiency of brassinosteroids showed that the low level of brassinosteroids initiates some manifestation of the light-regulated development in the dark and more active transcription of plastid genes under illumination.

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