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RESEARCH PAPERS

Plastome Transcription Machinery and Peculiarities of the Expression of Its Genes during Cytokinin-Dependent Deetiolation of *Arabidopsis thaliana*

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Abstract—Molecular mechanisms of cytokinin effect as the key activators of chloroplast biogenesis have been thoroughly investigated in recent decades; however, the role of this class of phytohormones in the regulation of expression of the plastome transcription machinery genes is obscure. In order to look into the effect of the components of the cytokinin signal system on plastid transcription machinery during deetiolation, we analyzed light- and cytokinin-dependent expression dynamics of chloroplastic RNA-polymerases, PAP proteins, and transcription factors genes upon light exposure of 4-day-old seedlings of wild type *Arabidopsis thaliana* (L.) Heynh. (Columbia-0) and knockout mutants for perception and transduction of the cytokinin signal. Both agents exerted a selective influence on the expression of different genes of the plastome transcription machinery. The positive effect of light and cytokinin on deetiolation probably depended on the operation of receptors AHK3 and AHK4 and response regulator genes *ARR1, ARR10,* and *ARR12.*

Keywords: Arabidopsis thaliana, deetiolation, light, cytokinins, receptors, plastome, gene expression, etioplasts, chloroplasts

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INTRODUCTION

The key role of light in plant vital activities is well known. Light-mediated growth and development of plants (photomorphogenesis) depends on the quick formation of the photosynthetic apparatus accompanied by activation of transcription of numerous plastid and nuclear genes. This can be seen particularly clear when proplastids or etioplasts are differentiated to become chloroplasts [1]. Etioplasts are a special type of plastids, typical for seedlings whose growth and development occur in complete darkness as a rule, under the soil surface. The process occurring in the seedlings and manifesting in specific morphological features (elongation of hypocotyl, few true leaves, and formation of apical hook), as well as physiological and biochemical characteristics (lack of chlorophyll and heterotrophic nutrition), is called etiolation (from French *étioler*: to make pale) or skotomorphogenesis. The formation of etioplasts from proplastids occurs in the dark and depends on operation in the cell of the systems of endogenous control over development and, primarily, on the hormonal system [2].

On exposure to light, etiolation gives place to deetiolation: a brief initial stage of photomorphogenesis. It is chiefly regulated by photoreceptors that initiate transformation of colorless etioplasts into green chloroplasts by means of an intricate signal network. Outwardly, deetiolation is manifested in the greening of seedling cotyledons owing to accumulation of photosynthetic pigments, while inwardly it is in the start of numerous processes, such as reprogramming of cell genome and biosynthesis of proteins and their import into chloroplasts, which ultimately promotes initiation of photosynthesis and realization of the trophic function of the light [2, 3].

Plant cell plastids are the main target for light and hormones in the cell; however, biogenesis of chloroplasts is an extremely intricate process and its molecular mechanisms are not fully decoded. Since operation of the chloroplasts depends on the coordinated synthesis of photosynthetic proteins and chlorophyll, transcription regulation of the chloroplast protein genes is very important for biogenesis of chloroplasts. The role of light in this process is crucial since one third of the genes of nuclear genome are known to modify their activity under the effect of illumination [4, 5]. Most of these genes encode chloroplast proteins. Endogenous factors are also very important; among them, cytokinins (CK)

Abbreviations: CK—cytokinins; EPR—endoplasmic reticulum; NEP—phage-type nuclear-encoded RNA-polymerase; PEP—multi-subunit plastid-encoded RNA-polymerase; PSII—photosystem 2.

are of special significance since they may regulate deetiolation owing partly to direct binding of type B ARR *trans*-factors with *cis*-elements of promoters of the genes encoding chloroplast proteins [6]. The ability of CK to simulate the effect of light was clearly shown in the seedlings of wild type *Arabidopsis thaliana* grown in the dark on nutrient medium supplemented with cytokinin. Such seedlings contained lenticular etioplasts and, instead of prolamellar bodies, they formed thylakoid membranes as it occurs during deetiolation [7]. A similar effect of cytokinin on plastid ultrastructure was shown in the cotyledons of yellow lupine [8]. According to Riefler et al. [9], CK-dependent morphogenesis in the dark predominantly depended on AHK3 sensor histidine kinase: one of the three membrane receptors of cytokinin. We showed earlier an important role of this receptor in the regulation of plastid gene expression in etiolated plants [10].

In spite of considerable morphological and functional differences between etioplasts and chloroplasts, these organelles contain identical DNA (plastome) in the same plants. However, gene expression therein differs owing to formation of more complex transcription machinery in the light. According to current notions, plastome transcription is performed by polymerases of two types: single-subunit nuclear-encoded RNA-polymerase (NEP) of the phage type, which is represented by two genes in the genome of *A. thaliana—RPOTp* and *RPOTmp*—and multi-subunit plastid-encoded RNA-polymerase (PEP) of a bacterial type, whose core subunits α, β, β', and β'' are encoded in the plastome by the genes *rpoA*, *rpoB*, *rpoC1,* and *rpoC2*. Recognition of promoter regions of PEP plastid genes requires trans-factors of the sigma family (SIG1–SIG6), which perform selective binding and initiate transcription from PEP-specific promoters [11–13]. In etioplasts of plants grown in the dark, the transcription of the plastome equally depends on polymerases of NEP and PEP type. PEP is represented by a soluble form PEP-B composed of four main subunits (α, β, β', and β'') and one of six σ-factors (SIG1–SIG6). In the light, PEP-B binds with extra PEP-associated proteins (PAP) and forms a more complex enzyme structure PEP-A [12, 13]. PEP then becomes the main polymerase of green plastids responsible for transcription of most photosynthesis genes. Light- and cytokinin-dependent expression of key genes of plastome transcription machinery in this critical stage has been hardly explored.

The aim of this work was to study the role of the components of cytokinin signal perception and transduction in the regulation of expression of the genes of plastome transcription machinery during deetiolation.

MATERIALS AND METHODS

Plant material. We investigated 4-day-old etiolated seedlings of wild type *Arabidopsis thaliana* (L.) Heynh. (Columbia-0) and its insertion knockout mutants for receptor genes *ahk2*, *ahk3*, *ahk4*, *ahk2/ahk3*, *ahk2/ahk4*, and *ahk3/ahk4* and the genes responsible for cytokinin signal transduction *arr1/10/12*.

Growth conditions. The seeds were surface-sterilized in sodium hypochlorite solution and sown on Petri dishes containing half-strength Murashige-Skoog nutrient medium (Duchefa, the Netherlands) without sucrose and hormone (0 μM *trans*-zeatin; control material) or supplemented with cytokinin (1 μM *trans*-zeatin; experiment). In order to synchronize seed germination, the Petri dishes were kept for 2 days at 4°C, and then seed germination was stimulated by 3-h exposure to white light at intensity of 120 μmol/(m^2 s). The seedlings were then grown in complete darkness for 4 days at a temperature of 22°C.

Experimental conditions. Etiolated seedlings were transferred to a MLR-352H-PE plant growth chamber (Sanyo, Japan) with photon flux density of 120 μmol/(m^2 s) and temperature of 22°C. In order to study the effect of light and cytokinin on expression of the genes of chloroplast proteins in the course of deetiolation, wild type seedlings were fixed in liquid nitrogen 1, 3, 6, 9, 12, and 16 h after the start of illumination. CK-signaling mutants were exposed to light for 6 h. All the manipulations with etiolated seedlings were performed in green light of low intensity. When determining the lengths of hypocotyls, for each type of treatment, at least 50 seedlings were measured.

Pigment assay. Total chlorophyll was determined according to Lichtenthaler [14].

Gene expression. Relative level of transcripts was evaluated by the method of real-time quantitative polymerase chain reaction (RT-PCR) after reverse transcription using a LigthCyclerR96 system (Roche, Switzerland) according to the procedure described earlier [15]. The content of transcripts of target genes was normalized to the level of transcripts of a polyubiquitin gene (*UBQ10*). Primers used for RT-PCR analysis are shown in Table 1.

Statistic analysis. Independent experiments were repeated 3–4 times. Bar graphs show the means and their standard errors. Significance of differences between the means was determined using Student's *t*-test.

RESULTS

Optimization of the Model System

At first, we conducted a number of preliminary experiments to estimate changes in the expression of two marker nuclear genes whose transcription is positively regulated by light and CK: light-regulated gene *LHCB2.4* and cytokinin-regulated gene *ARR4*. For this purpose, we transferred 4-day-old etiolated seedlings of wild type *A. thaliana* grown with and without cytokinin to the light and compared the expression of these genes in the dark and after 16-h exposure to light. At the same time, we determined the dynamics

Table 1. Nucleotide sequences of primers used for RT-PCR

Locus	Name	Function	Localization of encoded proteins	5' → 3' nucleotide sequence
At3g27690	*LHCB2.4*	protein of PSII light-harvesting complex	chloroplast	ccaacgatctcctccgcaaa; agacttgacggtacgacgca
At1g10470	*ARR4*	negative regulator of response to CK	nucleus	cggagaatgtattgaccagaatc; agaaatcttgagcaccttcctc
At1g27320	*AHK3*	cytokinin receptor, histidine kinase 3	EPR, plasma membrane	ttcttgccactgtttcacat; gcggtcctaacataatcctg
At2g01830	*AHK4/CRE1*	cytokinin receptor, histidine kinase 4	EPR, plasma membrane	ttcagattccacacactttcg; cgtaaccatccatcccactt
At2g24120	*RPOTp*	phage type RNA-polymerase	plastids	ccttcacttgtcgtctcctca; gactgtttatgcaagacccacc
At5g15700	*RPOTmp*	phage type RNA-polymerase	plastids and mitochondria	cacgaggtttgggaactgacg; tcgatcacttgattccattgc
AtCg00740	*rpoA*	α-subunit of PEP	plastids	cgccaagtaaagctcttcgc; aaggccaagccgacacaata
AtCg00190	*rpoB*	β-subunit of PEP	plastids	atagccgaacacgagggaa; gcttagagtatcaccattgccc
AtCg00180	*rpoC1*	β'-subunit of PEP	plastids	aggcaatccacagcgatgtaa; cggtgattgtcgttggacct
AtCg00170	*rpoC2*	β"-subunit of PEP	plastids	cgtctgctaagacacgacca; cggatttcggtgagtcctcg
At2g34640	*PAP5/pTAC12*	protein associated with PEP	chloroplasts	cgacgagaaagtgccgagga; agttctcagcatcatccacggc
At1g64860	*SIG1*	PEP *trans*-factor sigma 1	plastids	cattgcggatactcgtttgga; cccgttctccgagtgttgc
At1g08540	*SIG2*	PEP *trans*-factor sigma 2	plastids	ctggtgcggaagtctctctctg; tcggtttaggagagagtaga
At5g24120	*SIG5*	PEP *trans*-factor sigma 5	plastids	gactctctttcggcttcaatg; agatgttgatggtgttggagc
At2g36990	*SIG6*	PEP *trans*-factor sigma 6	plastids	tcgcctattgttggttcgc; gggctgataatgatgatgcg
At4g05320	*UBQ10*	ubiquitin 10	cytoplasm	gcgtcttcgtggtggtttctaa; gaaagagataacaggaacggaaaca

of chlorophyll accumulation. Table 2 shows that exogenous cytokinin reliably intensified production of chlorophyll in the light in our experimental model.

Transfer of *A. thaliana* etiolated seedlings to the light induced an the elevation of the content of gene *LHCB2.4* templates after 3-h illumination; it reached a peak level in 6 h (Fig. 1a). In the course of deetiolation of the seedlings, exogenous cytokinin induced a more pronounced accumulation of templates of photosynthetic gene *LHCB2.4* as compared with plant material exposed to light without the hormone. Similar results were obtained for *ARR4* gene. The content of its transcripts in the seedlings grown in the presence of hormone was much greater than in control material both in the dark and in the light (Fig. 1b). Changes in the expression of marker genes in etiolated seedlings in response to light and hormones, as well as a more pronounced accumulation of chlorophyll, indicate that our experimental conditions were appropriate. Therefore, this experimental design was employed for subsequent estimation of the expression of the genes for cytokinin signaling and plastome transcription machinery.

Expression of the Genes for Cytokinin Signaling

Changes in the expression of the genes of chloroplast proteins in the course of deetiolation may depend on the level of endogenous cytokinins, content of signaling proteins, or their activity. Therefore, in order to determine possible ways of plastid gene regulation by hormones during deetiolation, we analyzed the expression of the genes of CK receptors in the seed-

Table 2. Content of total chlorophyll ($a + b$) upon illumination of 4-day-old etiolated seedlings of wild type *A. thaliana,* Columbia-0

Time after transfer of plants to light, h	Chlorophyll ($a + b$), mg/g fr wt	
	control seedlings (0 μM *trans*-zeatin)	experimental seedlings (1 μM *trans*-zeatin)
3	0.027 ± 0.004	0.020 ± 0.008
6	0.037 ± 0.007[c*]	0.053 ± 0.006[be]
12	0.060 ± 0.009[d]	0.075 ± 0.002[af]

* Reliable differences between the seedlings grown without *trans*-zeatin and plants grown on the nutrient medium with cytokinin at $P < 0.05$ (a) and $P < 0.01$ (b); between control plants without cytokinin treatment (3 h) and plants grown without hormone after 6 and 12 h of illumination, at $P < 0.05$ (c) and $P < 0.01$ (d); between the plants grown on *trans*-zeatin (3 h) and seedlings grown on hormone after 6 and 12 h of illumination, at $P < 0.01$ (e) and $P < 0.01$ (f).

lings of a wild type. The key receptors for the cytokinin signal in *A. thaliana* are the three sensor histidine kinases AHK2, AHK3, and AHK4. Binding of histidine kinases with CK results in phosphorylation of phosphotransmitter proteins (AHP) that move to the nucleus where they transfer the phosphate to ARR response regulators, type B (*trans*-factors) and type A (cytokinin response regulators) [16]. Comparison of the levels of gene matrices of three receptors in etiolated seedlings of a wild type showed a positive effect of light on mRNA of *AHK3* and *AHK4* with identical dynamics of their activation. The content of transcripts reached maximum after 3 and 6 h of illumination (Figs. 2a and 2b). The content of transcripts of *AHK2* gene did not significantly differ from the level of templates in control etiolated seedlings grown on the media with and without the hormone (data not shown). In contrast to *AHK2* and *AHK3* genes, the level of transcripts of *AHK4* receptor gene considerably increased in response to CK in the dark, and it remained steadily high in the light throughout the experiment after some decrease in the beginning of deetiolation (Fig. 2b). Thus, the genes of individual CK receptors are selectively regulated by light and exogenous cytokinin, which probably results in differentiated changes in the activity of the cytokinin signal system upon germination of *Arabidopsis* depending on the effect of exogenous and endogenous factors.

Expression of the Genes for Plastome Transcription Machinery

NEP and PEP are known to differ by their contribution to the process of plastid transcription at different stages of ontogenesis. The expression of the genes for sigma factors also depends on the stage of plant development and environmental conditions, which implies a differential regulation of their activity by exogenous and endogenous factors. In order to investigate light- and cytokinin-induced regulation of expression of the key genes of plastid transcription machinery in the course of deetiolation of the seedlings, we selected nuclear genes of phage type monosubunit RNA-polymerases (*RPOTp* and *RPOTmp*) and the genes for a complex structure of bacterial type multisubunit RNA-polymerase: *rpoA, rpoB, rpoC1, rpoC2,* as well as *SIG1-SIG6* and *PAP5*.

Accumulation of *RPOTp* and *RPOTmp* transcripts rose since the beginning of illumination and reached peak values by the sixth and ninth hour, respectively (Figs. 3a and 3b). The most pronounced (approximately fivefold) growth of the expression under the effect of light was observed for *RPOTp* gene encoding plastid RNA-polymerase directed exclusively into plastids (Fig. 3a). Activation of *RPOTmp* gene operating in the plastids and mitochondria was somewhat less pronounced (approximately three times). In the light, exogenous cytokinin stimulated almost equally accumulation of templates of the genes for both NEPs except 1 h exposure, when the level of transcripts of both genes sharply decreased approaching control values in the absence of the hormone. It is of interest that the greatest activating effect of cytokinin occurred after 16-h light exposure. The obtained results show (Fig. 3) that light is absolutely necessary for activation of the expression of the *RPOTp* and *RPOTmp* genes by cytokinin.

Dynamics of the expression of NEP-dependent chloroplast genes encoding core subunits of PEP was quite different. The level of mRNA of these genes decreased upon the transfer of seedlings to the light. Certain growth started only after 6-h light exposure. However, accumulation of templates of *rpoB*, *rpoC1*, and *rpoC2* genes belonging to the same operon exceeded mRNA level in etiolated seedlings by 1.5 to 4 times after 16 h (Figs. 4b–4d), and mRNA of *rpoA* gene transcribed from another NEP-dependent promoter exceeded it 7 times (Fig. 4a). At the same time, exogenous cytokinin did not essentially regulate any of the *rpo* genes in the dark or in the light (Fig. 4).

Deetiolation ambiguously affected the dynamics of template accumulation of six different members of the *SIG* family. Illumination stimulated accumulation of

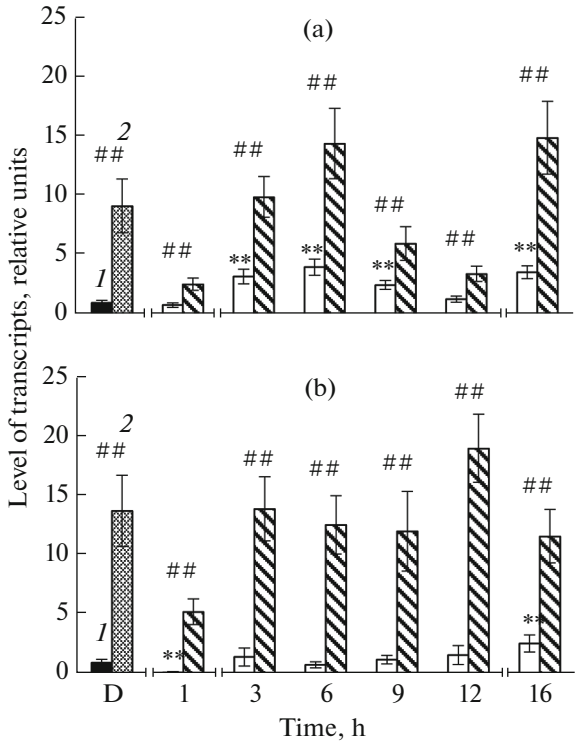

Fig. 1. Effect of cytokinin and light on the content of transcripts of marker genes of response to light—*LHCB2.4* (a) and cytokinin—*ARR4* (b) in 4-day-old etiolated seedlings of wild type *Arabidopsis*. *1*—0 μM *trans*-zeatin; *2*—1 μM *trans*-zeatin. D—dark. Changes in the levels of transcripts in all the types of treatment were normalized to the level of transcripts in wild type plants grown in the dark without hormones. * Reliable differences between average values of expression without cytokinin in the light and in the dark at $P \le 0.05$ and at ** $P \le 0.01$. # Reliable differences between average values of expression with and without hormones at $P \le 0.05$ and at ## $P \le 0.01$.

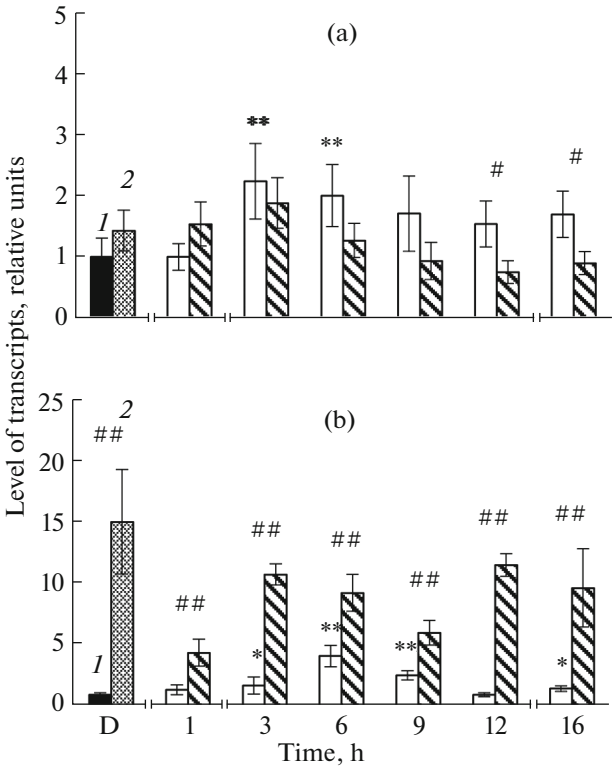

Fig. 2. Effect of cytokinin and light on the content of transcripts of the genes of cytokinin receptors *AHK3* (a) and *AHK4* (b) in 4-day-old etiolated seedlings of wild type *Arabidopsis*. *1*—0 μM *trans*-zeatin; *2*—1 μM *trans*-zeatin. D—dark. Changes in the levels of transcripts in all the types of treatment were normalized to the level of transcripts in wild type plants grown in the dark without hormones. * Reliable differences between average values of expression without cytokinin in the light and in the dark at $P \le 0.05$ and at ** $P \le 0.01$. # Reliable differences between average values of expression with and without hormones at $P \le 0.05$ and at ## $P \le 0.01$.

mRNA of *SIG1, SIG2,* and *SIG6* genes (up to twofold; Figs. 5a, 5b, 5d), whereas the content of templates of *SIG3* and *SIG4* essentially did not change as compared with control dark level (data not shown). Accumulation of *SIG5* gene transcripts in etiolated seedlings sharply rose (almost seven times) during the first hour of light exposure and remained high throughout the following 12 h (Fig. 5c). At the same time, cytokinin negatively regulated the level of templates of *SIG5* gene in the light; however, the content of transcripts of this gene in the light considerably exceeded the level of mRNA in etiolated seedlings grown with hormones in the dark. Cytokinin activated the expression of *SIG2* gene in the light and maintained an elevated content of transcripts of *SIG2* and *SIG6* genes in the dark (Figs. 5c and 5d). Out of twelve PAP proteins, the most important upon deetiolation is HEMERA/pTAC12/PAP5. This protein of dual location (in the nucleus and plastids) is responsible for a quick adaptation of seedlings to light, which requires a strict coordination between the expression of plastid and nuclear genomes [5].

According to our data, etiolated seedlings transferred to light accumulated the greatest level of *PAP5* gene transcripts after 6-h light exposure. The content of *PAP5* templates also reliably increased in response to CK in the light and in the dark and it exceeded the figures of control seedlings cultured without hormones during the whole investigation (Fig. 6).

Role of Cytokinin Signaling Components in the Expression Regulation of the Plastid Transcription Machinery Genes

Specificity of morphogenetic responses to cytokinin primarily depends on the systems of signal perception and transduction; therefore, we subsequently looked into participation of CK signaling genes in hormone-dependent regulation of the genes of plastome transcription machinery upon deetiolation. The functions of individual cytokinin receptors and response regulators were estimated using *Arabidopsis* knockout

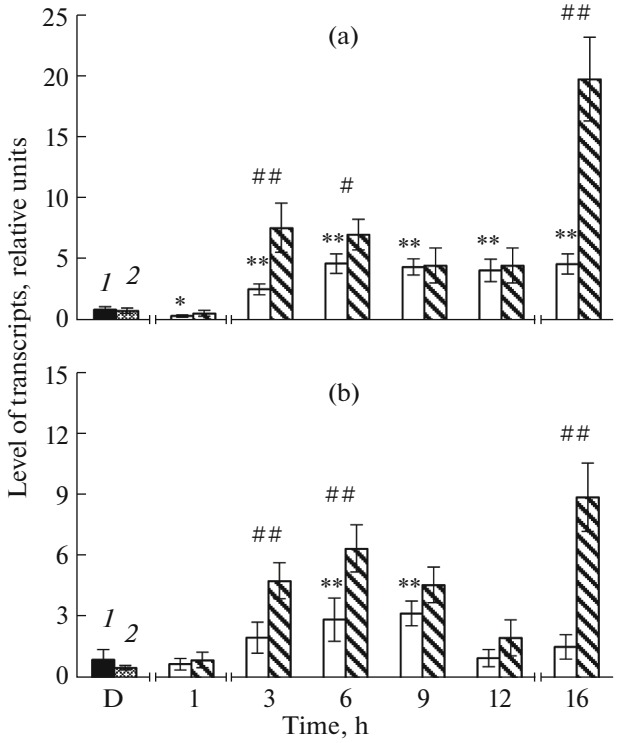

Fig. 3. Effect of cytokinin and light on the content of transcripts of nuclear genes of monosubunit RNA-polymerases *RPOTp* (a) and *RPOTmp* (b) in 4-day-old etiolated seedlings of wild type *Arabidopsis*. *1*—0 μM *trans*-zeatin; *2*—1 μM *trans*-zeatin. D—dark. Changes in the levels of transcripts in all the types of treatment were normalized to the level of transcripts in wild type plants grown in the dark without hormones. * Reliable differences between average values of expression without cytokinin in the light and in the dark at $P \le 0.05$ and at ** $P \le 0.01$. # Reliable differences between average values of expression with and without hormones at $P \le 0.05$ and at ## $P \le 0.01$.

mutants for the genes of hormonal signal perception and transduction.

One of the standard tests for sensitivity to CK is a shortening of hypocotyls in the seedlings cultured in complete darkness [7]. Comparison of the length of hypocotyls of 4-day-old seedlings grown on a nutrient medium with 1 μM *trans*-zeatin and without it showed that the shortening of hypocotyls was least pronounced in the mutants *ahk3*, *ahk4, ahk2/3,* and *ahk3/4* as well as in a triple mutant *arr1/10/12* (Figs. 7a and 7b). These results suggest that all the investigated components of cytokinin signaling could contribute to CK-dependent response in our experimental design.

In order to investigate the role of cytokinin signaling components in regulation of cytokinin-dependent deetiolation, we chose 6-h exposure to light. This is accounted for by the fact that the majority of studied genes showed a steady response to light and hormone in 6 h. We dealt with the genes of transcription machinery, which showed a pronounced response in wild type plants. *AHK3* gene turned out to be a key participant in the regulation of the transcript level of *ARR4* marker gene encoding response to cytokinin: its inactivation brought about a considerable decrease in the content of transcripts of both the marker gene *ARR4* and marker gene *LHCB2.4* associated with photomorphogenesis (Figs. 8a and 8b). Analysis of knockout mutants showed the following patterns. Light-dependent level of transcripts of *RPOTp* and *RPOTmp* genes was lower in all mutants, except *ahk2*, than the wild type level (Figs. 8c and 8d). At the same time, the greatest decrease in the content of transcripts as compared with wild type material was observed in lines *ahk3* (*RPOTmp*) and *ahk4* (*RPOTp*). Cytokinin-dependent activation of the NEP genes was also reliably reduced in the mutants, especially in single ones defective for *AHK3* and *AHK4* genes and in a double mutant *ahk3/4* (Figs. 8c and 8d). Lack of CK receptors practically did not reflect upon light-dependent level of the templates of *SIG2* gene, except the line with knocked out *AHK4* gene. At the same time, cytokinin-dependent response of *SIG2* gene was determined by the genotype of seedlings and, first of all, by the presence of operating *AHK3* receptor gene and the genes of response regulators. Expression of *SIG5* gene hardly differed in the wild type seedlings and the CK receptor mutant lines (Fig. 8f). The only exception was mutant *arr1/10/12*, where a decrease in light-induced activation and a twofold rise in the accumulation of *SIG5* transcripts were observed upon CK treatment as compared with the level of transcripts in wild type plants.

On the whole, the results obtained correspond to the notion of a positive impact of cytokinin signaling components on the accumulation of mRNA of the genes for plastid transcription machinery upon deetiolation. However, the expression of *PAP5* gene in mutants conflicts with the pattern. Inactivation of AHK2 and AHK3 receptors promoted a rise in light- and hormone-dependent accumulation of *PAP5* gene transcripts, which implies a negative regulation of its expression by CK but does not agree with the conclusion about the positive effect of exogenous hormone on expression of *PAP5* gene in wild type plants. Therefore, peculiarities of regulation and division of labor between individual members of the AHK family depend not only on their functional specificity but also on the genotype.

DISCUSSION

Conversion of etioplasts into chloroplasts in the seedlings under the influence of various exogenous and endogenous agents requires a coordinated expression of the genes in the nucleus and plastids. Therefore, investigation of molecular mechanisms ensuring interaction between the nucleus and plastids in the plant cell during chloroplast differentiation is very important. A large-scale activation of transcription in the course of deetiolation probably depends on inte-

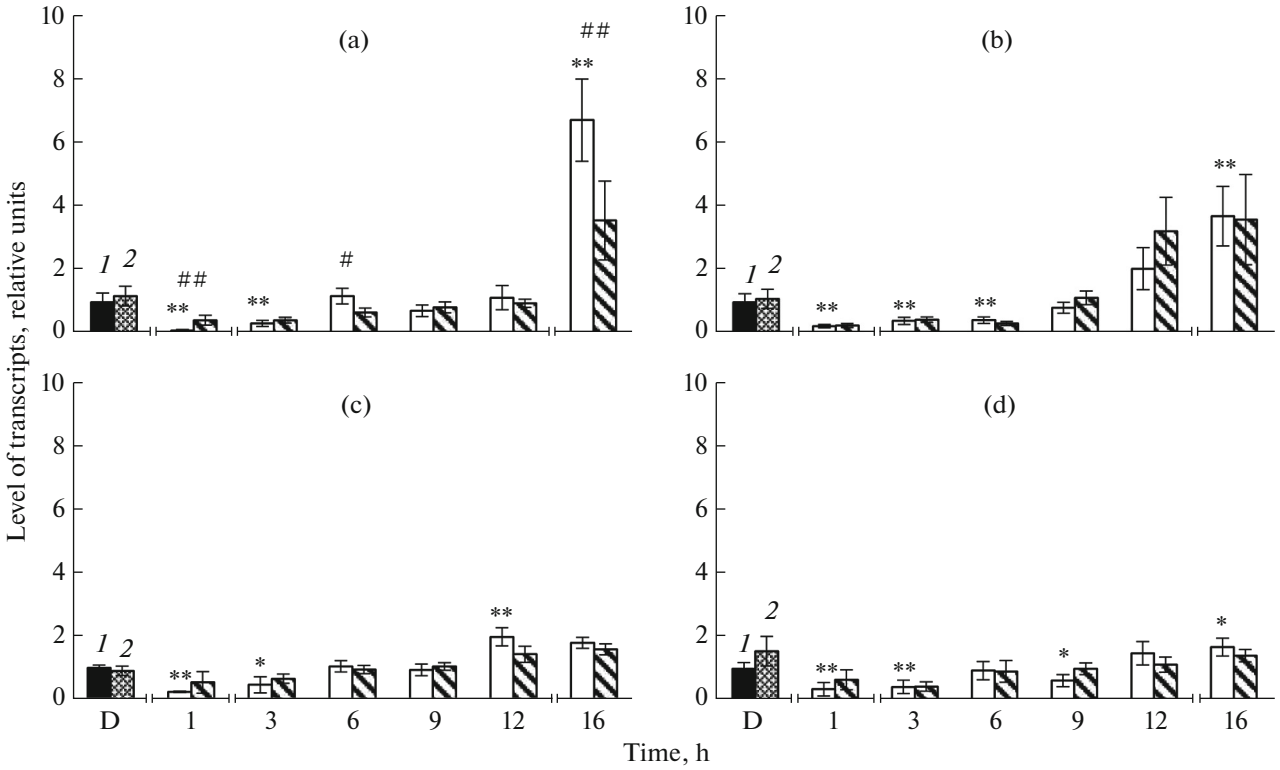

Fig. 4. Effect of cytokinin and light on the content of transcripts of chloroplast genes of PEP RNA-polymerase core subunits *rpoA* (a), *rpoB* (b), *rpoC1* (c), and *rpoC2* (d) in 4-day-old etiolated seedlings of wild type *Arabidopsis*. *1*—0 μM *trans*-zeatin; *2*—1 μM *trans*-zeatin. D—dark. Changes in the levels of transcripts in all the types of treatment were normalized to the level of transcripts in wild type plants grown in the dark without hormones. * Reliable differences between average values of expression without cytokinin in the light and in the dark at $P \le 0.05$ and at ** $P \le 0.01$. # Reliable differences between average values of expression with and without hormones at $P \le 0.05$ and at ## $P \le 0.01$.

gration between light signaling systems and phytohormones on a molecular level. A special role in regulation of these processes belongs to cytokinins.

In this work, we examined CK- and light-regulated expression of the genes for plastome transcription machinery upon deetiolation as a possible mechanism of realization of the CK signal in plastids at the stage of their differentiation into chloroplasts. Our investigation has shown that the expression of *NEP* genes (*RPOTp* and *RPOTmp*) and NEP-dependent genes of *PEP* core subunits (*rpoA, rpoB, rpoC1,* and *rpoC2*) was activated with a considerable time gap. Accumulation of transcripts for PEP core subunits occurred with a 13-h lag against NEP genes. The profile of transcription activation of the genes for both types of polymerases reflected the role of these gene products in realization of plastid genetic information during chloroplast differentiation. Activation of NEP genes after 3-h light exposure of etiolated seedlings is accounted for by a predominantly NEP-dependent transcription at the initial stage when expression of household genes prevails [17]. As the chloroplasts become more mature, PEP activity grows and transcription of the genes of photosynthesis is triggered [12]. It is interesting that exogenous cytokinin activated accumulation of NEP transcripts solely upon illumination throughout the whole investigated period starting from the third hour after the beginning of deetiolation, whereas accumulation of transcripts of the main PEP subunits returned to the initial dark level only in 6 h and more.

Such an order of activation of gene expression of two different RNA-polymerases in the course of light-dependent transformation of etioplasts into chloroplasts well corresponds with the earlier-proposed model of RNA-polymerases division of labor: in the beginning, NEP is active and PEP becomes predominant as the chloroplasts become mature [18]. Switch over of plastid polymerase activities may involve PEP-dependent $tRNA^{GLU}$ which suppresses NEP activity [12]. Elevation of the content of *rpo* templates in a late period of illumination results in light-dependent formation of a minimum PEP complex and subsequently promotes activation of PEP-dependent plastome transcription. At the same time, active transcription of PEP-dependent photosynthesis genes requires nuclear-encoded PAP proteins and sigma factors ensuring specificity of PEP operation and recognition of gene promoter regions.

The obtained data concerning the effect of light and cytokinin on deetiolation of *Arabidopsis* seedlings point to a differential regulation of *trans*-factors of the *SIG* family by these two agents. It was found that, in

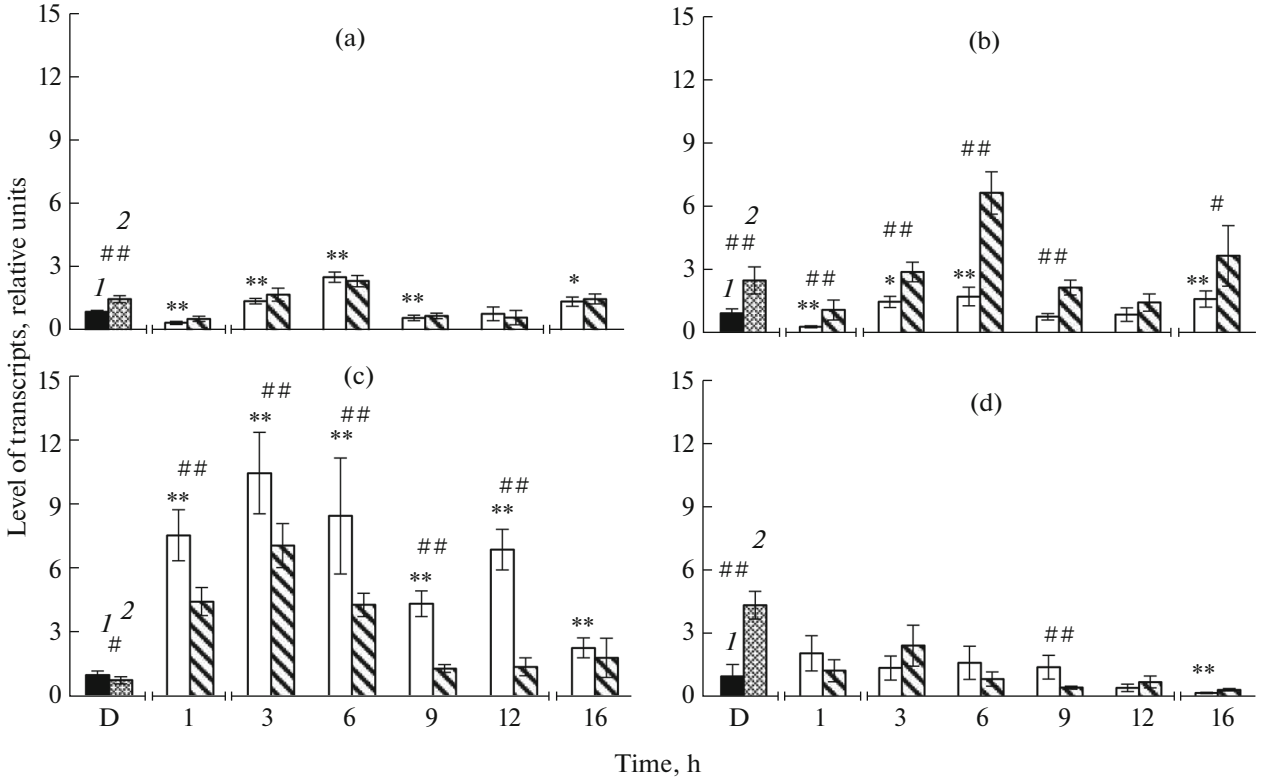

Fig. 5. Effect of cytokinin and light on the content of transcripts of nuclear genes of *trans*-factors of plastid multisubunit RNA-polymerase *SIG1* (a), *SIG2* (b), *SIG5* (c), and *SIG6* (d) in 4-day-old etiolated seedlings of wild type *Arabidopsis*. *1*—0 μM *trans*-zeatin; *2*—1 μM *trans*-zeatin. D—dark. Changes in the levels of transcripts in all the types of treatment were normalized to the level of transcripts in wild type plants grown in the dark without hormones. * Reliable differences between average values of expression without cytokinin in the light and in the dark at $P \le 0.05$ and at ** $P \le 0.01$. # Reliable differences between average values of expression with and without hormones at $P \le 0.05$ and at ## $P \le 0.01$.

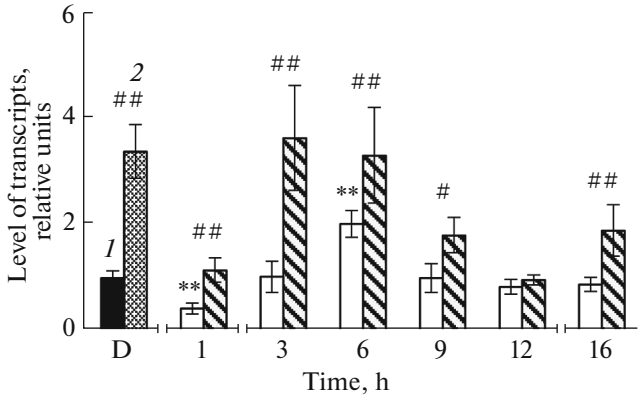

Fig. 6. Effect of cytokinin and light on the level of transcripts of gene *PAP5* in 4-day-old etiolated seedlings of wild type *Arabidopsis*. *1*—0 μM *trans*-zeatin; *2*—1 μM *trans*-zeatin. D—dark. Changes in the levels of transcripts in all the types of treatment were normalized to the level of transcripts in wild type plants grown in the dark without hormones. * Reliable differences between average values of expression without cytokinin in the light and in the dark at $P \le 0.05$ and at ** $P \le 0.01$. # Reliable differences between average values of expression with and without hormones at $P \le 0.05$ and at ## $P \le 0.01$.

respect to light regulation, six *SIG* genes could be subdivided into two groups. The first group comprises *SIG1*, *SIG2*, and *SIG5* genes whose transcription was activated by light. Light-dependent expression of these genes was earlier reported by Liere et al. [1]. The second group includes *SIG3*, *SIG4*, and *SIG6* genes whose expression did not change in response to light. According to the regulation by cytokinin, sigma genes were subdivided into those activated by cytokinin in the light (*SIG2*) and inhibited in response to hormone (*SIG5*) as well as the genes whose expression essentially did not change (*SIG1, SIG3, SIG4,* and *SIG6*). Such a complex nature of regulation apparently promotes differential expression of the plastome upon conversion of etioplasts into chloroplasts. It is known that in spite of overlapping functions in the control over PEP-depended transcription of plastome, individual sigma factors have a variable regulatory N-terminus ensuring a division of labor between individual members of the family [19]. For instance, promoters of *psbA* gene (protein D1 of PSII reaction center) and *rbcL* gene (large subunit of RBPC) are recognized by SIG2 during the early development of the seedling, and stress-induced SIG5 is necessary for activation of transcription of

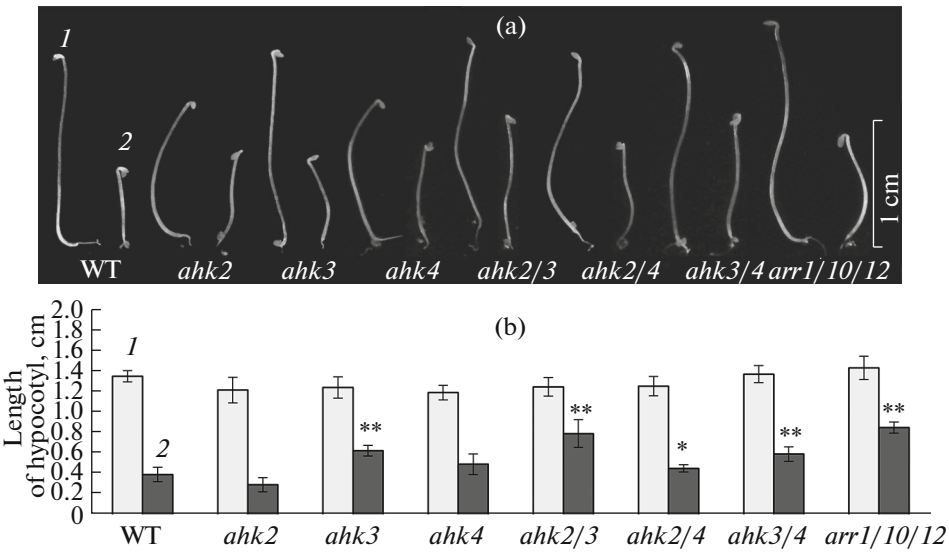

Fig. 7. (a) Exterior and (b) length of hypocotyls of 4-day-old etiolated seedlings of wild type *Arabidopsis* and knockout mutants by cytokinin signaling. *1*—0 μM *trans*-zeatin; *2*—1 μM *trans*-zeatin. WT—wild type. * Reliable differences between average values of hypocotyl length in the seedlings of mutants and wild type plants grown with hormones at $P \le 0.05$ and at ** $P \le 0.01$.

psbD operon (protein D2 of PSII reaction center) from blue light responsive promoter (BLRP) [20, 21].

Activation of *SIG2* gene and suppression of *SIG5* gene upon CK-dependent deetiolation is in agreement with the results of analysis of these gene expression in plants grown in the light [22]. It is of interest that apparent growth of the transcript level of stress-induced *SIG5* gene as compared with the dark type of treatment was much inferior to the level of light-dependent induction and pointed to an inhibitory effect of exogenous CK. Such an effect of CK on *SIG5* gene is accounted for by participation of SIG5 in protective reactions to photooxidative stress accompanying deetiolation. It is possible that exogenous cytokinin diminishes oxidative injury caused by free photoactive pigments upon deetiolation and partly lessens the stress [23], while decreasing *SIG5* expression at the same time. Photooxidative stress is apparently responsible for a reduced expression of a number of genes of transcription machinery during the first hours of deetiolation. Such a drop may depend on an adjustment of energy homeostasis and reorganization of the transcription/translation apparatus under stress conditions [24, 25].

Expression regulation of nuclear and chloroplast genes upon deetiolation is ensured by a direct participation of the components of CK signal transduction chain. Our results showed a decrease in the level of light- and hormone-dependent activation of *RPOTp* and *RPOTmp* gene expression in the mutants for CK receptors and a triple mutant for type B *trans*-factors. At the same time, expression of NEP genes by light and CK was chiefly activated owing to the operation of AHK3 and AHK4 receptors. This assumption is corroborated by the data on the activation of expression of these genes in wild type seedlings and the suppression of response to light and cytokinin in the mutants with inactivated *AHK3* and *AHK4* genes. Light-dependent regulation of *SIG2* gene expression was predominantly associated with receptor AHK4, and CK-dependent regulation was related to AHK3. Expression of *SIG5* gene hardly depended on the genotype of mutants for CK receptors in the light but changed in the mutants for the genes regulating response to this hormone, whereas CK-dependent expression of *SIG5* gene resulted from individual contributions of all the three cytokinin receptors.

Thus, analysis of the influence of exogenous cytokinin on mutant lines has shown that the effect of this phytohormone on the genes of transcription machinery depended on differential activity of individual receptors and transcription factors. This conclusion was made on the basis of a reduced response of mutants to the hormone as compared with wild type plants in the experiments with cytokinin-dependent deetiolation of seedlings. By their ability to control transcriptional changes in expression of the chloroplast proteins genes encoded in the plastids and nucleus, CK receptors may be arranged in the following order: AHK3 > AHK4 > AHK2. Response regulators also turned out to be very important for hormone-dependent expression of the genes of *Arabidopsis* plastome transcription machinery, which underlies morphogenetic changes in etioplasts occurring in the course of their conversion to chloroplasts.

Regulatory mechanisms ensuring participation of cytokinin membrane receptors in the control over chloroplast biogenesis may differ. Along with the changes in the rate of transcription of chloroplast genes, the components of CK signaling could stabilize

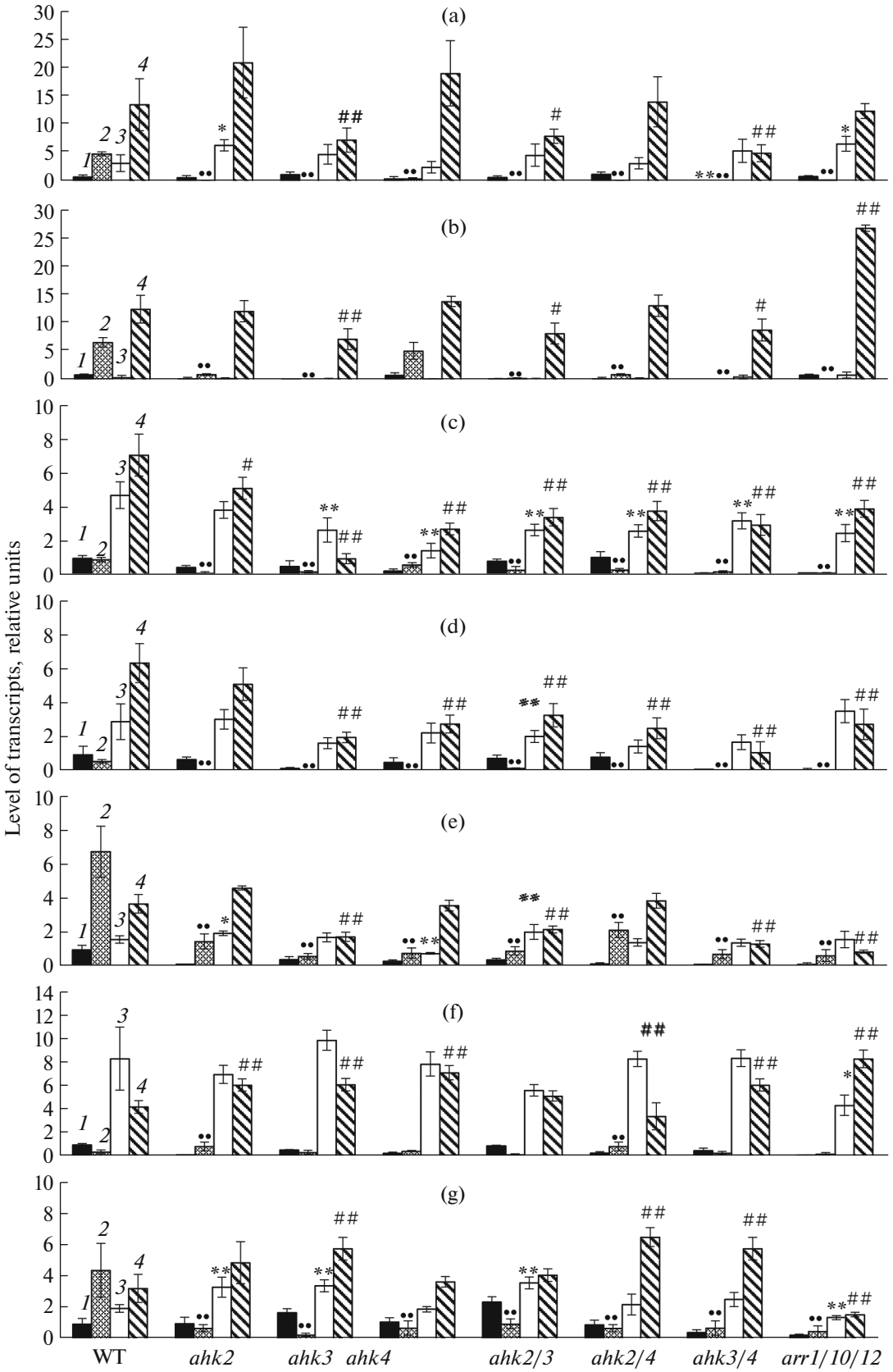
(a)
(b)
(c)
(d)
(e)
(f)
(g)
Level of transcripts, relative units
WT
ahk2
ahk3
ahk4
ahk2/3
ahk2/4
ahk3/4
arr1/10/12

Fig. 8. Effect of cytokinin and light on the content of genes transcripts *LHCB2.4* (a), *ARR4* (b), *RPOTp* (c), *RPOTmp* (d), *SIG2* (e), *SIG5* (f), and *PAP5* (g) in 4-day-old etiolated seedlings of wild type *Arabidopsis* and knockout mutants by the genes of cytokinin signaling after 6 h of illumination. *1*—darkness without hormones; *2*—darkness + 1 μM *trans*-zeatin; *3*—illumination without hormones; *4*—illumination + 1 μM *trans*-zeatin. Changes in the levels of transcripts in all the types of treatment were normalized to the level of transcripts in wild type plants grown in the dark without hormones. ● Reliable differences between average values of expression in the mutants and wild type plants in the darkness with cytokinin at $P \le 0.05$ and at ●● $P \le 0.01$. * Reliable differences between average values of expression in the mutants and wild type plants in the light at $P \le 0.05$ and at ** $P \le 0.01$. # Reliable differences between average values of expression in the mutants and wild type plants in the light with cytokinin at $P \le 0.05$ and at ## $P \le 0.01$.

(via RNA-polymerases) the levels of individual transcripts. Changes in the content of transcripts of chloroplast genes in the mutants with modified CK signaling upon deetiolation could also be accounted for by changes in the content of endogenous cytokinins in the seedlings of mutant plants or in the ratio between cytokinins and other phytohormones and trophic metabolites and/or in sensitivity to them.

Cytokinins may act as intermediaries in light-regulated processes. A number of investigations have shown integration between the systems of light and cytokinin signaling on a molecular level. For instance, CK-dependent *trans*-factor ARR4 stabilized the active form of phytochrome in the nucleus [26]. The complexity of the interaction of light and cytokinin is also indicated by the synergistic effect in the action of these factors on the level of transcripts of most examined genes in wild type plants (Fig. 8, all the genes except *SIG2*). The synergistic effect implies common stages in the action of cytokinin and light. This is corroborated by our results clearly showing that (at least in the regulation of expression of nuclear-encoded chloroplast *ATPC* gene) light and CK act via the same *cis*-element and probably involve the same *trans*-factors [27].

A key role in the transduction of the light signal belongs to COP1-dependent proteolysis of light-regulated transcription factors. In the dark, protein COP1 participates in proteolysis of light-regulated *trans*-factors and inhibits the expression of the genes for positive regulators of photomorphogenesis, such as HY5, LAF1, HFR1, and LZF1 [28]. Cytokinin may reduce the content of protein COP1 indirectly causing stabilization of protein HY5: one of the positive regulators of photomorphogenesis [29]. Along with COP1-dependent pathway of degradation of light-regulated transcription factors, there exists a COP1-independent pathway that involves protein HEMERA/pTAC12/PAP5. This protein of a dual location performs proteolysis of transcription factors PIF1 and PIF3 belonging to phytochrome nuclear bodies, which is an important stage in the transition from skotomorphogenesis to photomorphogenesis, and simultaneously participates in formation of a PEP complex in the chloroplasts activating transcription of photosynthesis genes [13, 28]. Expression of *PAP5* gene increased under the effect of light and cytokinin in wild type seedlings and was suppressed in the mutant with knocked out genes for cytokinin response regulators (Figs. 6 and 8), which suggests a positive regulation of *PAP5* by cytokinin. However, the lines with inactivated receptors AHK2 and AHK3 (Fig. 8) were notable for higher levels of transcripts of this gene as compared with wild type seedlings, i.e., regulation of *PAP5* gene depended on the genotype. This may be partly related to an elevated content of endogenous cytokinins in the receptor mutants [9] capable of compensating for a deterioration of their perception apparatus. Weakening of cytokinin signaling could also be compensated by signaling of other sensor histidine kinases (CKI1 or ETR) or by improvement of sensitivity to hormones [30]. Thus, the disturbance of cytokinin homeostasis brought about a modification of feedback regulating CK-dependent expression of *PAP5* gene; however, specific molecular mechanisms of this process are so far unknown.

In general, our investigations have shown an important role of cytokinin signal pathway in the control of the chloroplast development during deetiolation which is performed by means of a direct regulation of expression of the genes for plastome transcription machinery. A positive effect of cytokinin on deetiolation is chiefly realized via operation of AHK3 and AHK4 receptors and response regulator genes *ARR1, ARR10,* and *ARR12.*

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