Cytokinin Stimulates Chloroplast Transcription in Detached Barley Leaves[1][OA]

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Chloroplasts are among the main targets of cytokinin action in the plant cell. We report here on the activation of transcription by cytokinin as detected by run-on assays with chloroplasts isolated from apical parts of first leaves detached from 9-d-old barley (*Hordeum vulgare*) seedlings and incubated for 3 h on a 2.2×10^{-5} M solution of benzyladenine (BA). Northern-blot analysis also detected a BA-induced increase in the accumulation of chloroplast mRNAs. A prerequisite for BA activation of chloroplast transcription was preincubation of leaves for 24 h on water in the light, resulting in a decreased chloroplast transcription and a drastic accumulation of abscisic acid. Cytokinin enhanced the transcription of several chloroplast genes above the initial level measured before BA treatment, and in the case of *rrn16* and *petD* even before preincubation. Cytokinin effects on basal (youngest), middle, and apical (oldest) segments of primary leaves detached from plants of different ages revealed an age dependence of chloroplast gene response to BA. BA-induced stimulation of transcription of *rrn16*, *rrn23*, *rps4*, *rps16*, *rbcL*, *atpB*, and *ndhC* required light during the period of preincubation and was further enhanced by light during the incubation on BA, whereas activation of transcription of *trnEY*, *rps14*, *rpl16*, *matK*, *petD*, and *petLG* depended on light during both periods. Our data reveal positive and differential effects of cytokinin on the transcription of chloroplast genes that were dependent on light and on the age (developmental stage) of cells and leaves.

Cytokinins are hormones that play an important role in development and senescence of plants and in division and differentiation of their cells (Mok and Mok, 2001). In recent years, principal components of the systems for cytokinin perception, signal transduction, and biosynthesis and degradation have been elucidated (Ferreira and Kieber, 2005; Sakakibara, 2006; Müller and Sheen, 2007; Hirose et al., 2008). Cytokinins are involved in the control of chloroplast biogenesis and function. They affect chloroplast and etioplast ultrastructure, chloroplast enzyme activities, pigment accumulation, and the rate of photosynthesis (Lerbs et al., 1984; Chory et al., 1994; Kusnetsov et al., 1994, 1998; Yaronskaya et al., 2006; for older reports, see Parthier, 1979). Exogenously applied cytokinins delay senescence of detached leaves and keep chloroplasts photosynthetically active longer than in control leaves not treated with cytokinins (Romanko et al., 1969). Chloroplasts harbor enzymes for the biosynthesis of cytokinins and contain a set of natural cytokinins, including free bases, ribosides, ribotides, and *N*-glucosides (Benkova et al., 1999; Kasahara et al., 2004; Polanska et al., 2007). The developmental and/or metabolic state of plastids influences the response of leaves to exogenous cytokinins (Kulaeva et al., 2002). Plastids also play a role in the biosynthesis of abscisic acid (ABA), a plant hormone that, among other functions, acts as a cytokinin antagonist in the regulation of chloroplast biogenesis (Khokhlova et al., 1978; Koiwai et al., 2004).

Although it is obvious that chloroplasts are among the targets of cytokinin action, it is not understood how cytokinins exert their effects on plastids/chloroplasts. At least in part, chloroplast responses to cytokinin may result from hormone effects on the expression of nuclear genes encoding chloroplast proteins (Chory et al., 1994; Kusnetsov et al., 1994; Hutchison and Kieber, 2002; Rashotte et al., 2003; Brenner et al., 2005; Kiba et al., 2005). Since plastids possess genes and the machinery for their expression, cytokinins may also affect the activity of plastid/chloroplast genes. Plastid genes are transcribed by

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two types of RNA polymerases: a plastid-encoded plastid RNA polymerase (PEP) related to bacterial enzymes, and one or more nucleus-encoded plastid RNA polymerases (NEPs) related to phage enzymes. Plastid transcripts are regularly processed, which may include splicing and editing. Translation occurs on bacteria-type 70S ribosomes. All steps of plastid gene expression are targets of regulatory processes (Choquet and Wollman, 2002; Herrin and Nickelsen, 2004; Liere and Börner, 2007). Only little and contradictory information is available on cytokinin effects on the expression of plastid genes. A cytokinin-binding protein was isolated from barley (*Hordeum vulgare*) chloroplasts, which stimulated total chloroplast transcription in a hormone-dependent manner (Kulaeva et al., 2000). The functional activity of this protein was shown to depend on the age of plants (Lukevich et al., 2002). Several studies indicated that cytokinin might enhance or decrease the transcript levels of certain chloroplast genes (Lerbs et al., 1984; Stabel et al., 1991; Masuda et al., 1994; Hande et al., 1996; Kasten et al., 1997; Brenner et al., 2005). In contrast, cytokinin did not affect the levels of several chloroplast mRNAs in detached lupine (*Lupinus luteus*) cotyledons, although it could differentially regulate the accumulation of polypeptides encoded by the corresponding genes (Kusnetsov et al., 1994). These results suggest that cytokinins act under certain conditions on transcript accumulation, posttranscriptional processes, and translation in plastids.

Since previous studies used different plant species and different conditions of growth and treatment, it is difficult to compare the results obtained. Moreover, although effects on transcript accumulation in chloroplasts have been reported, the influence of cytokinins on plastid transcription per se has not been demonstrated yet. Therefore, we performed a series of experiments to investigate cytokinin effects on the transcription of plastid genes. Detached primary barley leaves were selected as the experimental system for studying the cytokinin effects on chloroplast gene expression because previous experiments with this material have demonstrated chloroplast responses to cytokinin treatment (Kulaeva et al., 2000; Lukevich et al., 2002). Moreover, leaves of barley and other grasses allow for effective analyses of age- and development-dependent effects of cytokinin, since they grow from a basal meristem and, therefore, display a developmental spatial gradient with the youngest cells positioned in the basal zone and the oldest cells at the tip. The meristematic cells at the leaf base contain small, nonphotosynthetic proplastids, whereas the mesophyll cells located in the leaf upper parts harbor photosynthetically active chloroplasts. The tips of fully expanded leaves may already show signs of senescence (Baumgartner et al., 1989). Here, we report on studies employing run-on transcription in chloroplasts isolated from primary barley leaves. Our data showed a cytokinin-induced stimulation of chloroplast gene transcription that depended on light and the age of leaves and cells.

RESULTS

Responses of Chlorophyll Content to Cytokinin Depend on Cell Age

Cytokinin is known to delay senescence and the breakdown of chlorophylls in detached leaves (Gan and Amasino, 1997; Lim et al., 2007). Senescing leaves differ from young ones by a more pronounced response to cytokinin. Barley leaves (like leaves of other cereals) display a spatial longitudinal gradient of the cell age: the youngest cells are positioned in the basal leaf zone, and the oldest ones are found at the tip. Therefore, the responses to cytokinin were studied separately in basal, middle, and apical (oldest) parts of the leaves. The leaf age most sensitive to cytokinin treatment was previously determined in experiments showing that cytokinin enhanced total transcription most efficiently in the first mature leaves of 8- to 10-d-old barley plants (Selivankina et al., 1980; Kulaeva et al., 1996). Therefore, we chose the first leaf of 9-d-old barley plants for our experiments. Detached leaves were incubated in the light on filter paper moistened with water or benzyladenine (BA) solution (2.2×10^{-5} M) for 5 d. The chlorophyll content was determined separately in apical, middle, and basal parts of the leaves. The apical parts contained the highest amount of chlorophyll (about 2-fold higher than the basal part), demonstrating an advanced developmental stage of the chloroplasts at the beginning of the experiment (Fig. 1). The apical part was also characterized by the highest rate of chlorophyll loss. After 3 d of leaf incubation on water, the chlorophyll amount in the apical part was 23%; after 5 d, it was only 6% of the initial level. In contrast, the youngest basal leaf parts maintained about 50% of their initial chlorophyll content after 3 d and one-third of it after 5 d. The middle

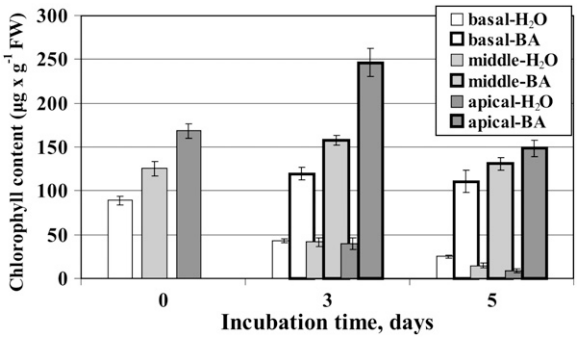

Figure 1. Cytokinin effect on chlorophyll content in detached barley leaves. First leaves were detached from 9-d-old barley seedlings and incubated in the light on filter paper moistened with water (thin outline) or BA (2.2×10^{-5} M; thick outline) solution during 5 d. Chlorophyll content in basal (white columns), middle (light gray columns), and apical (dark gray columns) parts of the leaves was analyzed spectrophotometrically after extraction with acetone from freshly detached leaves (incubation time 0) and after 3 and 5 d of incubation, respectively. The means from three independent experiments performed in three replicates ± SE are presented. FW, Fresh weight.

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part, however, showed an intermediate rate of chlorophyll loss.

The chlorophyll contents of the three leaf zones differed from each other also in their response to cytokinin (Fig. 1). Three days of incubation with BA increased the chlorophyll content above the initial level by 50%, 23%, and 40% in the apical, middle, and basal parts, respectively. After 5 d of incubation on BA, however, cytokinin could not prevent chlorophyll degradation anymore, although the chlorophyll content was still distinctly higher in all leaf zones than in control leaves (15 times in apical parts, 6.5 and 4.6 times, respectively, in middle and basal sections).

Responses of Chloroplast Transcription to Cytokinin Depend on the Age of Cells and Leaves

As in previous experiments the first leaves detached from 9-d-old barley plants were used.

In preliminary experiments, preincubation of the detached leaves for 24 h on water in the light before BA application was found to be a prerequisite for pronounced cytokinin effects on chloroplast transcription. Therefore, detached leaves were first incubated for 24 h in the light on water, then for 3 h on a BA solution (2.2×10^{-5} M) or water (control), also in the light. After incubation, chloroplasts were isolated from the basal (youngest), middle, and apical (oldest) sections of the leaves and used for run-on transcription assays in the presence of [α-^{32}P]UTP. Labeled transcripts were hybridized to DNA fragments of 36 chloroplast genes on a nylon membrane, similar to those shown in Figure 2. The selected chloroplast genes (*psaA*, *psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbH*, *psbK*, *atpA*, *atpB*, *atpF*, *atpH*, *atpI*, *petB*, *petD*, *petL/G*, *rbcL*, *ndhA*, *ndhC*, *ndhF*, *rpoB*, *rps2*, *rps4*, *3′rps12*, *rps14*, *rps16*, *rpl16*, *rpl23/rpl2*, *rrn16*, *rrn23*, *trnE/Y*, *trnV-intron*, *matK*, *clpP*, and *ycf4*) represented all functionally important groups of chloroplast proteins and RNAs, all chloroplast operons, as well as NEP- and PEP-transcribed genes. After a series of preliminary experiments, 10 genes that showed a reproducible response to BA were selected, together with two genes that did not respond to BA, for further studies, as shown in Figure 2. After hybridization, the signals obtained with transcripts of the run-on assays (Fig. 2, B–G) were quantified (Fig. 2H). Transcription in chloroplasts from the basal part of the leaves did not respond to cytokinin (Fig. 2, B and C). Similarly, transcription in chloroplasts isolated from the middle part of the leaf blade was not found to be stimulated by BA, with the exception of the *rrn16* gene, which showed a weak yet significant increase in transcription (Fig. 2, D, E, and H). In accordance with cytokinin effects on chlorophyll content in the three leaf sections, a strong positive effect of cytokinin on chloroplast transcription was observed in the apical parts (i.e. the oldest leaf cells; Fig. 2, F–H). An at least 2-fold higher rate of transcription as a result of cytokinin effect could be observed for *rrn16*, *trnEY*, *rps14*, *rps16*, *rpl16*, *matK*, *petD*, *petLG*, *rbcL*, and *atpB* in chloroplasts of the apical leaf segments (Fig. 2H). Although a weak stimulation of transcription of *psbA* and *psbD* might have occurred, we could not find a significant response of these genes to BA. Therefore, we used *psbA* as an internal standard in the quantification of hybridization signals (Fig. 2).

Subsequently, we studied the response of chloroplast transcription to cytokinin in different parts of the leaves detached from barley plants at the age of 4 d, when the first leaf was still growing, and 22 d, when the first leaf showed signs of senescence. In the case of 4-d-old leaves, all studied genes were transcribed in all investigated sections of the leaf, but we could not detect any significant effect of BA on transcription, regardless of the leaf segment tested (data not shown). In the apical part of leaves detached from 22-d-old plants, we observed a drastic decrease in transcriptional activity. We could not detect transcripts of *rps14*, *rps16*, *rpl16*, *petD*, and *petLG* in the water control (Fig. 3). Moreover, the chloroplasts responded only very weakly to cytokinin. Only transcription of *rrn16* was higher after incubation on BA than in the water control (Fig. 3). Most likely, progression of senescence in those leaves reduced transcriptional activity and susceptibility to cytokinin in the apical section. The strong response of chloroplast transcription to BA treatment was clearly shifted from the apical to the middle section of leaves from 22-d-old plants compared with 9-d-old plants (Figs. 2 and 3), suggesting that a certain stage of leaf development and a certain cell age are preconditions for BA effects on chloroplast transcription.

Now, the question arose of whether cytokinin truly stimulates chloroplast transcription to reach higher activities or just prevents a senescence-induced decline in transcription that might occur after detachment of leaves. To answer this question, transcription was measured immediately after detachment of leaves, then after the 24-h preincubation on water in the light (the observed prerequisite of cytokinin effect on chloroplast transcription; see below), and after a further 3 h of incubation on water or BA solution. As is evident from Figure 4, the 24-h preincubation on water indeed resulted in a sharp decline of the transcriptional activity of all genes tested, probably an indication of rapid leaf senescence under these conditions. During the further 3-h incubation of control leaves on water, the transcription of chloroplast genes was further reduced, whereas the 3-h leaf incubation on BA solution not only prevented this drop in transcriptional activity but also substantially (two to four times) activated transcription compared with the transcription rate after the 24-h preincubation on water (Fig. 4). Transcription of the *rrn16* and *petD* genes was even enhanced to higher activities than in leaves immediately after their detachment. Transcription of the *psbA* and *psbD* genes was not increased significantly by cytokinin and remained at the low level reached during incubation on water for 24 or 27 h. Thus, cytokinin actually differentially stimulates the transcription of chloroplast genes.

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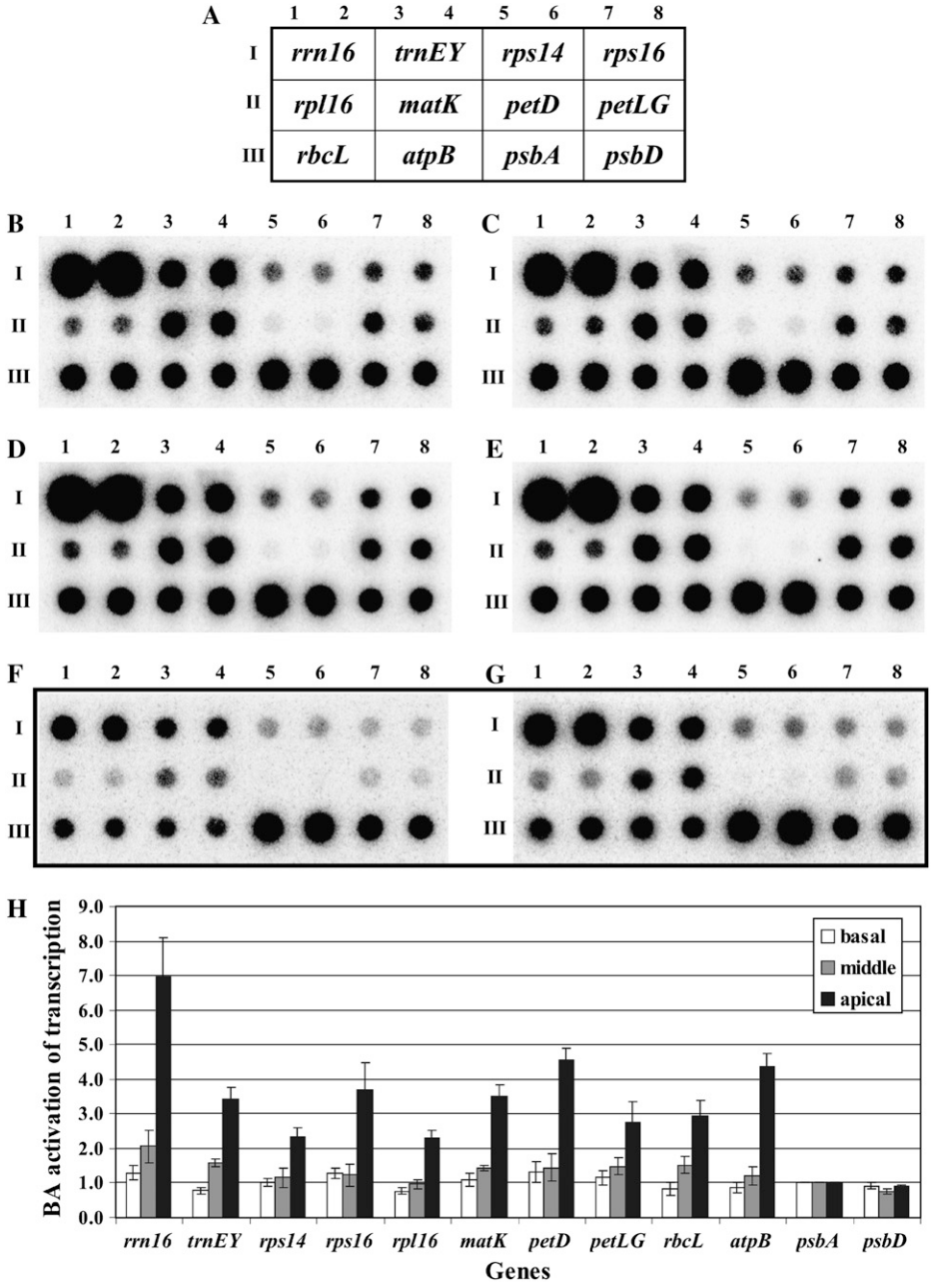

Figure 2. Effect of cytokinin on chloroplast transcription in detached barley leaves. First leaves were detached from 9-d-old plants, preincubated for 24 h on water in the light, and then incubated for 3 h in the light on water or BA. Chloroplasts were isolated from basal (B and C), middle (D and E), and apical (F and G) parts of leaves and used for run-on transcription assay. ^{32}P-labeled transcripts were isolated and hybridized to plastid gene probes (Table II) blotted on nylon membranes according to the scheme presented in A. Radioactive signals were detected and quantified as described in "Materials and Methods." B to G, Autoradiograms for selected genes probed with run-on transcripts isolated from chloroplasts of various parts of leaves, incubated on water (B, D, and F) or BA solution (C, E, and G). Autoradiograms demonstrating an effect of cytokinin on chloroplast transcription in the apical part of leaves are framed. Data shown for the representative dot blots may be slightly different from the averages of multiple experiments shown in H. H, Ratios of the transcription rates in chloroplasts from cytokinin-treated leaves to the rates in chloroplasts from control leaves (on water) are presented as means from three independent experiments ± SE and relative to transcription of *psbA* (=1).

Cytokinin Effects on Steady-State Levels of *rbcL* and *atpB* Transcripts

To elucidate whether the observed effects of cytokinin on transcription were associated with the altered accumulation of RNAs in chloroplasts, we studied BA effects on steady-state levels of *rbcL* and *atpB* mRNAs by northern hybridization. RNA was isolated from apical segments of the first leaves of 9-d-old barley plants. Detached leaves were preincubated on water for 24 h in the light and then on BA solution (2.2×10^{-5} M) or water under identical conditions of illumination for an additional 24 h. After 3 h of incubation with cytokinin, we observed an enhanced accumulation of the *rbcL* and *atpB* transcripts (Fig. 5) relative to the accumulation of rRNA, which served as a loading control. Cytoplasmic and chloroplast rRNA levels were found to remain stable during the first 48 h of incubation with cytokinin in previous experiments with detached barley leaves (Kulaeva et al., 1967; Y.O. Zubo, unpublished data). We also did not observe a change in the ratio of chloroplast to cytoplasmic rRNAs in this study (Fig. 5), although BA had a noticeable effect on the transcription of plastid rRNA genes (Figs. 2–4). Due to the large amounts of rRNAs per cell, enhanced transcription may require more time to become detectable at the level of RNA accumulation compared with mRNAs, and posttranscriptional processes might be active to stabilize rRNA

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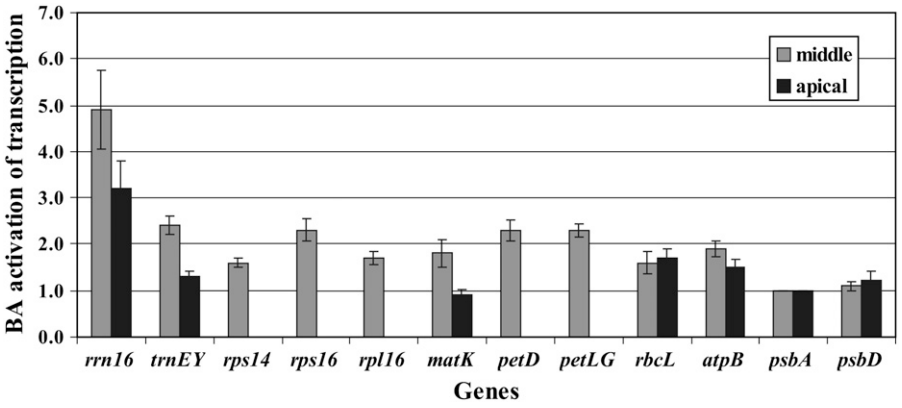

Figure 3. Effect of cytokinin on transcription of chloroplast genes in first leaves detached from 22-d-old barley plants. Preincubation, incubation, and run-on assays with chloroplasts isolated from different parts of the leaves were performed as in Figure 2. The ratios of the transcription rates in chloroplasts from cytokinin-treated leaves to transcription rates in chloroplasts from control leaves incubated on water are presented (BA activation) relative to *psbA* transcription. Chloroplasts were isolated from middle parts of the leaves (gray columns) and from apical parts of the leaves (black columns). Activity of several genes was not detectable in apical sections. The means from three independent experiments ± SE are shown.

levels. After 6 h of BA treatment, the *rbcL* and *atpB* transcripts reached the point of maximal accumulation. During further incubation with BA, the amounts of *rbcL* and *atpB* mRNAs decreased again, probably because of an increased degradation in the detached leaves.

Interaction between Cytokinin and Light in the Control of Chloroplast Transcription

To examine the role of light in the response of plastid transcription to BA, run-on assays were performed with chloroplasts isolated from apical segments of the first leaves detached from 9-d-old plants after a series of different treatments: (1) 24 h of preincubation on water in darkness followed by incubation for 3 h on BA solution in darkness or in the light; and (2) 24 h of preincubation on water in the light followed by incubation for 3 h on BA solution in darkness or in the light. The results confirmed that illumination during the period of preincubation (24 h) on water is a prerequisite for the effects on chloroplast transcription during subsequent incubation with cytokinin (Fig. 6). After preincubation in darkness, 3 h of incubation on BA solution could not activate transcription even if this incubation was performed in the light. Light was required during both preincubation and incubation with BA to stimulate the transcription of *trnEY*, *rps14*, *rpl16*, *matK*, *petD*, and *petLG* (Fig. 6A). In the case of *rrn16*, *rrn23*, *rps4*, *rps16*, *rbcL*, *atpB*, and *ndhC*, however, leaf preincubation in the light followed by incubation in darkness was sufficient for a stimulatory effect of BA. Incubation under illumination further enhanced the positive effect of cytokinin on the transcription of *rrn16* and *atpB*, indicating that cytokinin and light synergistically interact to enhance the rate of chloro-

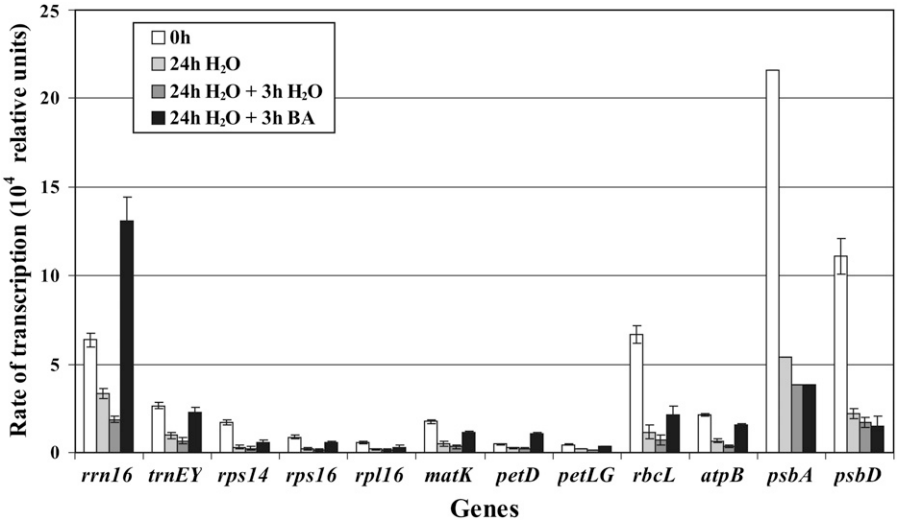

Figure 4. Effect of preincubation of leaves on water in the light (24 h) followed by a 3-h incubation on water or BA solution on the transcription of chloroplast genes. Transcription of chloroplast genes was determined in apical parts of the first leaves detached from 9-d-old barley plants immediately after detachment (0 h), after a 24-h preincubation on water (24 h H_2O), or after a 24-h preincubation and subsequent incubation for 3 h on water (24 h H_2O + 3 h H_2O) or BA solution (24 h H_2O + 3 h BA). Preincubation and incubation of the leaves were carried out in the light. The means of relative units of transcription activity (related to *psbA* transcription) from three independent experiments ± SE are presented.

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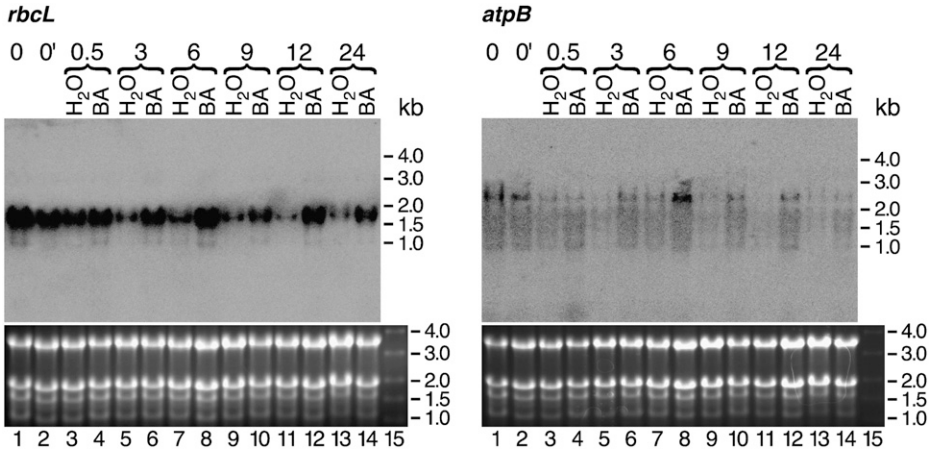

Figure 5. Effect of cytokinin on the steady-state levels of *rbcL* and *atpB* transcripts in the first barley leaves. Leaves were detached from 9-d-old barley plants and preincubated for 24 h on water in the light followed by incubation on water or BA for 0.5, 3, 6, 9, 12, and 24 h in the same light conditions. Equal amounts of total RNA isolated from apical parts of leaves were electrophoretically separated on agarose-formaldehyde gels and hybridized with DNA probes specific for the chloroplast *rbcL* and *atpB* genes. Total RNA served as a loading control (bottom panels). The hybridization data are representative for three replicates with independently isolated RNAs.

plast transcription under our experimental conditions (Fig. 6B).

Endogenous Cytokinin and ABA Content in Apical, Middle, and Basal Parts of Detached Leaves

Because the effect of cytokinin treatment may depend on both the content of endogenous cytokinins and the ratio between cytokinins and ABA, its antagonist in the regulation of chloroplast biogenesis in leaf tissues, we determined the content of cytokinins altogether (trans-zeatin and zeatin riboside) and ABA in the basal, middle, and apical segments of barley leaves immediately after detachment from 9-d-old plants and after preincubation of detached leaves on water for 24 h under illumination or in darkness (Table I). In freshly detached leaves, the content of cytokinin in basal segments was half the content in the middle and upper parts, while ABA showed similar levels in all parts of the leaf. During preincubation, the content of endogenous cytokinins doubled in all parts of the leaf in the light. The increase was less pronounced after preincubation in darkness. The content of ABA changed dramatically during the 24-h preincubation of the leaf in the light. In the apical and middle leaf parts, the ABA levels increased approximately 7-fold, and in the basal part they increased up to 12-fold. As a result, the ABA to cytokinin ratio, which was 1.5 in freshly detached leaves, increased 5-fold in the apical zone, 8-fold in the middle zone, and 23-fold in the basal zone after the 24-h leaf preincubation on water in the light. The ABA level also rose during preincubation in darkness, but this increase was much less pronounced than in the light. Thus, preincubation of the leaf on water in the light enhanced the content of cytokinins and ABA and sharply increased the ratio of ABA to cytokinins in detached leaves.

DISCUSSION

In spite of the important role of cytokinins in the control of chloroplast biogenesis, nothing was known about their potential role in the control of transcription of individual chloroplast genes. This study demonstrated that the cytokinin BA differentially activated the transcription of chloroplast genes and counteracted the reduction of transcriptional activity in chloroplasts of barley leaves that occurred when leaves were incubated on water after their detachment. The positive effect of BA on the transcription of certain chloroplast genes was dependent on light and the age of cells and leaves.

The response to cytokinin was different in the three sections of 9-d-old primary leaves that were investigated in this study: the basal, middle, and apical leaf zones (Figs. 1 and 2). A principal difference between apical, middle, and basal parts of barley leaves is the different age and developmental stage of their cells. The cells of cereal leaves display striking variations in plastid gene expression related to their age gradient. Baumgartner et al. (1989) demonstrated that the youngest cells, positioned in the basal part of the barley leaf, contain small plastids with low transcriptional activity. The highest transcriptional activity of chloroplasts was observed in the middle part of the leaf. In the oldest, apical zone, chloroplast transcription activity declined because of the beginning of senescence (Baumgartner et al., 1989). We made similar observations; however, a direct comparison of our results with

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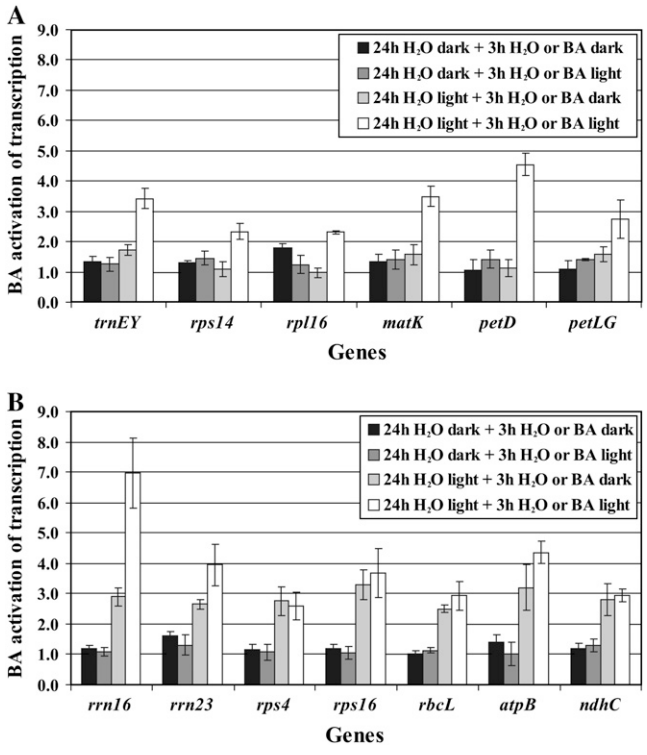

Figure 6. Effect of light during leaf preincubation on water and following incubation on water or BA solution on chloroplast transcription. Detached first leaves of 9-d-old barley plants were preincubated for 24 h on water in the dark or in the light and incubated for an additional 3 h on BA solution or water in the dark or in the light. Thereafter, chloroplasts were isolated from the apical parts of leaves and used in run-on transcription assays. A, Genes that responded to cytokinin only after illumination during both preincubation on water and incubation on BA solution. B, Genes that responded to cytokinin after preincubation in the light and incubation in darkness. Transcriptional activities of *psbA* served as an internal standard. The means of the ratios of water control to BA-treated samples from three independent experiments ± SE are presented.

those of Baumgartner et al. (1989) is not possible, since we did not study transcription in freshly harvested leaves but only at 27 h after their detachment (Fig. 2). We observed the most pronounced cytokinin effect on chloroplast transcription in the apical segments of first leaves of 9-d-old plants. The basal leaf zone never responded to cytokinin treatment. In the middle zone of the leaf, cytokinin weakly enhanced transcription of only the *rrn16* gene (Fig. 2). Thus, the ability to respond to cytokinin treatment depends on the age of the cells. The analysis of cytokinin effects on chlorophyll content in these three sectors revealed that the apical part of the leaf has the highest sensitivity to cytokinin, which also showed the highest chlorophyll content in freshly harvested leaves and the most drastic loss of chlorophyll (i.e. fastest senescence during incubation; Fig. 1).

That chloroplast transcription in the oldest cells does not necessarily respond most strongly to BA, but rather a certain developmental stage of the cells (and chloroplasts) is a precondition for susceptibility of the transcriptional apparatus to cytokinin, was demonstrated by another set of experiments. A drastic decrease in chloroplast transcription was observed for the apical part of leaves detached from 22-d-old plants, which is likely a sign of their advanced senescence. Under these conditions, chloroplast transcription was much less efficiently activated by cytokinin than in the apical part of 9-d-old plants (Fig. 3). In the 22-d-old plants, the zone of the highest sensitivity of chloroplast transcription to BA shifted from the apical (as observed for 9-d-old leaves; Fig. 2) to the middle part of the leaf (Fig. 3). Cytokinin did not affect chloroplast transcription in any parts of young growing leaves detached from 4-d-old barley seedlings (data not shown). This is in agreement with previous data showing that cytokinin treatment has no effect on total transcription (Selivankina et al., 1979) and on transcriptional activation in chloroplasts by a cytokinin-binding protein in young barley leaves (Lukevich et al., 2002).

Thus, the response to cytokinin operated during a clear developmental window. We can define three developmental stages with respect to the BA effects on chloroplast transcription: stage 1 (all parts of 4-d-old-leaves, basal and mid sections of 9-d-old leaves) showed constitutive chloroplast transcription, not affected by exogenous BA; in stage 2 (apical part of 9-d-old leaves and mid section of 22-d-old leaves), all studied genes were actively transcribed, and certain chloroplast genes responded to cytokinin by enhanced transcriptional activity; stage 3 (apical part of 22-d-old leaves) exhibited an overall low transcriptional activity of chloroplasts and nearly no response to BA. Similarly, a developmental window of the response to light has been reported for the nuclear *cab1* gene (encoding a plastid protein involved in photosynthetic light harvesting) during the first days of development of Arabidopsis (*Arabidopsis thaliana*) seedlings (Brusslan and Tobin, 1992). A dependence of the transcriptional activity of chloroplasts on the age of barley leaves was also reported by Klein and Mullet (1990). Illumination of etiolated 4.5-d-old barley seedlings did not activate the transcription of plastid genes. However, transcriptional activation of plastid gene expression was observed after illumination of 8-d-old etiolated seedlings (Klein and Mullet, 1990). It is well documented that the degree of leaf senescence is one of the crucial factors for the response to cytokinin (Gan and Amasino, 1997; Jordi et al., 2000; Yang et al., 2002), and our data suggest that transcription in chloroplasts responds positively to cytokinin in rather earlier (stage 2) than advanced (stage 3) stages of senescence.

Detachment of leaves induces senescence and may quickly lead to a decrease of chloroplast transcription. Therefore, the question arose whether the observed effect of cytokinin on chloroplast transcription (Fig. 2) was an indication of keeping the transcriptional activity of certain genes at levels reached at the beginning

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Table I. *Content of cytokinin and ABA in basal, middle, and apical segments (2 cm) of detached barley leaves as affected by their incubation for 24 h in the light or in darkness*

Cytokinin (trans-zeatin and zeatin riboside) and ABA were determined by ELISA using polyclonal antibodies against cytokinins and ABA.

Treatment	Phytohormone	Content of Phytohormones in Leaf Segment		
		Apical	Middle	Basal
		ng g^{-1} fresh weight		
Leaf immediately after detachment	Cytokinin	37 ± 2	30 ± 2	15 ± 1
	ABA	55 ± 2	75 ± 2	64 ± 2
24 h of incubation in darkness	Cytokinin	50 ± 3	48 ± 3	26 ± 2
	ABA	94 ± 3	194 ± 6	121 ± 5
24 h of incubation in the light	Cytokinin	76 ± 5	62 ± 4	32 ± 2
	ABA	375 ± 11	498 ± 15	762 ± 29

of incubation while their activity declined in control leaves during incubation on water, or, alternatively, whether cytokinin induced a genuine increase of transcriptional activity above the original levels. In fact, 24 h of preincubation of the leaf on water in the light resulted in a decline in transcription (1.8-fold for *petD* and 5.7-fold for *rbcL*; Fig. 4), an indication of ongoing senescence. The following incubation for 3 h on water further suppressed chloroplast transcription. Interestingly, 3 h of incubation of the leaf on the cytokinin solution not only prevented this further suppression but enhanced transcriptional activity of the investigated genes (with the exception of *psbA* and *psbD*) clearly above the levels reached before incubation with cytokinin, and in the case of *rrn16* and *petD*, even above the levels measured in freshly harvested leaves (Fig. 4). We conclude, therefore, that cytokinin not only stabilizes transcriptional activity by retarding senescence (see also BA effect on chlorophyll content; Fig. 1) but also activates the transcription of several genes above control levels.

Our results demonstrate that light was needed together with cytokinin to activate chloroplast transcription. Neither light nor BA alone stimulated chloroplast transcription under our experimental conditions (Fig. 6). Light was required during the 24 h of preincubation on water to observe enhanced transcription of all genes activated by BA compared with the water control, but the studied genes differed in their requirement for light during subsequent leaf exposure to cytokinin. A group of genes comprising *trnEY*, *rps14*, *rpl16*, *matK*, *petD*, and *petLG* required light for stimulation of their transcription not only during leaf preincubation on water (24 h) but also during incubation for 3 h with cytokinin (Fig. 6A). Another group (*rrn16*, *rrn23*, *rps4*, *rps16*, *rbcL*, *atpB*, and *ndhC*) needed light only during preincubation, although ongoing illumination during cytokinin treatment further increased the effect (Fig. 6B). These data demonstrate the synergistic interaction of light and cytokinin in our system. A cooperative role of light and cytokinin in the synthesis of fatty acids in plastids of greening cucumber (*Cucumis sativus*) cotyledons was demonstrated by Yamaryo et al. (2003). A requirement of long-term illumination in the period preceding cytokinin application implies that light might trigger the synthesis of one or more unknown factors required for the response of the plastid transcription system to cytokinin. The role of such regulatory factors could be fulfilled, for example, by cytokinin-binding proteins in the chloroplast (Kulaeva et al., 2000) or by σ-factors that are needed by PEP for promoter recognition. The genes for σ-factors are expressed differentially in different plant tissues, under different light regimes, and during different stages of chloroplast development (Lysenko, 2007). Evidently, factors and/or signals produced during 24 h of illumination and involved in the response to cytokinin of the second group of genes were maintained in the period of the short-term exposure to cytokinin in darkness. For both gene groups, light-triggered processes occurred during leaf preincubation on water, because preincubation in darkness prevented responses to cytokinin independently of the illumination regime during cytokinin treatment. The interaction between regulatory factors was demonstrated for light and cytokinin effects (Ferreira and Kieber, 2005). Light- and cytokinin-dependent signaling pathways may merge on identical cis-elements of nuclear promoters (Kusnetsov et al., 1999). It remains to be shown if this is also the case in chloroplasts.

The marked decrease in transcriptional activity during 24 h of preincubation on water may provide one explanation for our finding that this period was a precondition for detectable cytokinin effects on chloroplast transcription. Although darkness, like detachment of leaves, is a factor that induces senescence that can be retarded by cytokinin (Weaver et al., 1998; To et al., 2004), preincubation of leaves for 24 h in darkness did not permit subsequent transcription activation by cytokinin (Fig. 6). In this context, it could be relevant that leaf senescence in darkness has both common and different features with natural senescence. In particular, the regulatory systems of cytokinin-induced retardation of senescence differ in these two cases (Kim et al., 2006). Interestingly, leaf preincubation in darkness and in the light differently affected the hormonal status of the investigated leaf sectors (Table

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I). The content of cytokinins increased in both cases, although it was stronger in the light, implying synthesis of cytokinins under our experimental conditions. Cytokinin synthesis in detached leaves was demonstrated previously (Nordström et al., 2004). Much more remarkable changes occurred with regard to the ABA content during preincubation on water, particularly in illuminated leaves. Leaves preincubated in the light differed from those preincubated in darkness by a substantial increase in the ABA to cytokinin ratio, and apical, middle, and basal sectors of the leaf accumulated different amounts of ABA (Table I). ABA antagonizes cytokinin effects in various processes, including chloroplast biogenesis and leaf senescence. The structural and metabolic differentiation of chloroplasts is activated by cytokinin but suppressed by ABA (Khokhlova et al., 1978; Kusnetsov et al., 1998; Kulaeva et al., 2002). A subunit of Mg-chelatase, a plastid enzyme that is a key component in both chlorophyll biosynthesis and plastid-to-nucleus signaling, was found to be also an ABA receptor (Shen et al., 2006). Increased ABA accumulation during the period of leaf preincubation on water in the light for 24 h could be related to stress induced by both leaf detachment and continuous illumination. High light accelerates leaf senescence (Humbeck and Krupinska, 2003) and induces ABA accumulation (Weatherwax et al., 1996; Symons and Reid, 2003). ABA is one of the hormones known to accelerate plant senescence, antagonistically to cytokinins (Nooden, 1988; Yang et al., 2002). Antagonism in the impacts of ABA and cytokinin depends on the ratio between the concentrations of these hormones. Thus, for the observed stimulatory effects of BA on chloroplast gene transcription, the ratio between ABA and cytokinin concentrations might be important. We have observed a reduced transcription of certain chloroplast genes in detached barley leaves after treatment with externally added ABA (M.V. Yamburenko and Y.O. Zubo, unpublished data). Although this finding is in agreement with the hypothesis that internal ABA to cytokinin ratios could be involved in the limitation of cytokinin effects to certain developmental stages and to a certain pretreatment with light, it should be pointed out that the effects of endogenous ABA (and cytokinin) could be different from the effects of externally added hormones. Therefore, it would be interesting to analyze chloroplast transcription in mutants with altered internal levels of ABA and cytokinins.

Although the transcriptional machinery of chloroplasts in higher plants with at least two types of RNA polymerase, several σ-factors, various other transcription factors, and multiple promoters is highly complex, examples for a differential regulation of the transcription of chloroplast genes remained rare (Liere and Börner, 2007). This study revealed a differential effect of cytokinin on the transcription of chloroplast genes in barley leaves. While numerous genes did not respond to cytokinin under our experimental conditions (Table II; Fig. 2), the *rrn16*, *atpB*, and *petD* genes were found to be most responsive to cytokinin treatment. The rate of their transcription increased 4- to 7-fold in comparison with control leaves. Transcription rates of *rbcL*, *rps16*, *rps14*, *petLG*, *matK*, and some other genes were less affected by cytokinin treatment and showed only a 2.5- to 3-fold increase. Cytokinin primarily enhanced transcription of genes for the components of the gene expression machinery and electron transport (*rrn16*, *matK*, *trnEY*, *rps14*, *rps16*, *rbcL*, *petD*, and *atpB*). Most of these genes have multiple promoters and are expected to be transcribed by PEP and NEP (Liere and Börner, 2007). We found similar patterns of changes caused by cytokinin with respect to polypeptide complex accumulation in the

Table II. *Chloroplast genes studied, and length and position of DNA fragments used in run-on assays*

Gene	Product	Size of DNA Fragment	Location of DNA Fragment in the Barley Chloroplast Genome
		no. of bases	*bp*
rrn16	16S ribosomal RNA	1,401	92,761–94,161 123,973–125,373
rrn23	23S ribosomal RNA	624	97,930–98,553 119,581–120,204
trnE-trnY	tRNAGlu (UCC) and tRNATyr (GUA)	824	15,401–16,224
matK	Putative maturase K	918	2,606–3,523
ndhC	NADH dehydrogenase ND3	315	50,117–50,431
rbcL	Rubisco large subunit	1,297	55,031–56,328
atpB	ATPase β-subunit CF1	986	53,023–54,008
rps4	Ribosomal protein S4	192	45,782–45,973
rps14	Ribosomal protein S14	291	36,949–37,239
rps16	Ribosomal protein S16	775	5,196–5,970
rpl16	Ribosomal protein L16	1,138	78,503–79,640
petD	Cytochrome *b*/*f* subunit IV	276	74,259–74,534
petL-petG	Cytochrome b_6/*f* complex subunits V and VI	338	64,106–64,443
psbA	PSII 32-kD protein	770	803–1,572
psbD	PSII D2 protein	989	9,176–10,165

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thylakoid membranes of chloroplasts in lupine cotyledons (Kusnetsov et al., 1994). In agreement with published data (Lerbs et al., 1984; Kasten et al., 1997; Brenner et al., 2005), we observed a positive effect of BA also on the steady-state levels of chloroplast RNAs. Northern analysis detected an increase in the accumulation of *rbcL* and *atpB* transcripts after a 3-h treatment of the leaf with cytokinin (Fig. 5). The highest levels were observed after 6 h of treatment with the phytohormone followed by a decrease in the transcript amounts. However, a positive effect of cytokinin was still evident after 24 h of incubation. It remains to be shown if the enhanced transcript levels are only a consequence of increased rates of transcription or also of an impact of cytokinin on transcript stability.

In conclusion, we demonstrated that exogenously applied cytokinin stimulated transcription of chloroplast genes in detached barley leaves and that the cytokinin effect depended on light and was only found in segments of leaves at an early stage of senescence. Cytokinin enhanced substantially and differentially the transcription of certain, but not all, chloroplast genes compared with the gene activities before cytokinin treatment. For example, the two enzymes with highest transcriptional activity in our studies, *rrn16* and *psbA*, differed extremely in their response to BA. The transcription of *rrn16* was particularly sensitive against cytokinin, while *psbA* was among those genes that did not exhibit a reproducible and strong response to BA. The observed differential response of chloroplast genes to cytokinin cannot be explained by an overall transcriptional activation of the whole chloroplast genome by cytokinin (e.g. due to changes in DNA topology, plastome copy numbers, or general activation of nucleus- and plastid-encoded plastid RNA polymerases). It is not clear yet from this study whether BA acted in a direct or indirect way on chloroplast transcription. It seems likely that cytokinin regulates the transcription of certain plastid genes via cytokinin-dependent trans-factors (Kulaeva et al., 2000). Further experiments have to identify the cytokinin receptors, components of signal transduction pathways, and cis- and trans-acting factors involved in cytokinin effects on chloroplast gene expression and to elucidate whether cytokinin acts on such factors within the organelle and/or indirectly via its effects on known and unknown nuclear and cytosolic components.

MATERIALS AND METHODS

Plant Material and Treatment with Cytokinin

Barley seedlings (*Hordeum vulgare* 'Luch') were grown in soil at 22°C under illumination of 270 μmol m^{-2} s^{-1} from luminescent tubes (Lamp Master HPI-T Plus 400W E40; Philips) with a 16-h photoperiod. The first leaves were detached from 4-, 9-, and 22-d-old plants. If not otherwise stated, the leaves were incubated on filter paper moistened with water under continuous illumination of 270 μmol m^{-2} s^{-1} for 24 h. Subsequently, the leaves were transferred to water or a solution of the synthetic cytokinin BA (2.2×10^{-5} M) and kept for 3 h under the same light conditions. For chloroplast isolation, we used the apical, middle, and basal segments (each 2 cm in length) of the first leaves.

Chloroplast Isolation

Leaf segments (10 g) were homogenized in 80 mL of buffer A (0.33 M sorbitol, 50 mM Tricine, pH 8.0, 2 mM EDTA, and 5 mM β-mercaptoethanol). The homogenate was squeezed through Miracloth (Calbiochem-Behring) and centrifuged at 2,700*g* for 6 min. The pellet was resuspended in 1.5 mL of buffer A and fractionated in a 40%/70% discontinuous Percoll gradient by centrifugation at 4,000*g* for 30 min. Intact chloroplasts were collected at the interface between 40% and 70% Percoll, washed in buffer A, and resuspended in 0.5 to 1 mL of buffer B (50 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 10 mM KCl, and 4 mM β-mercaptoethanol). All procedures were performed at 4°C. The number of chloroplasts in the samples was determined by counting the organelles with a light microscope using a Fuchs-Rosenthal hemocytometer (Brown and Rickless, 1949), and chloroplast were used for run-on transcription.

Run-On Transcription Assay

Run-on transcription assay with 5×10^7 lysed plastids was carried out in a 100-μL volume by the method of Mullet and Klein (1987) modified as follows. Transcription was performed for 15 min at 25°C in buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.2 mM CTP, GTP, and ATP, 0.01 mM UTP, 50 μCi of [α-^{32}P]UTP (Amersham), 20 units of RNasin (Fermentas), and 10 mM β-mercaptoethanol. The reaction was stopped by the addition of an equal volume of stop buffer (50 mM Tris-HCl, pH 8.0, 25 mM EDTA, and 5% sarcosyl). ^{32}P-labeled transcripts were isolated from chloroplasts as described by Zubo and Kusnetsov (2008) and hybridized to plastid genes blotted on a nylon membrane in a buffer containing 250 mM Na_2HPO_4, 7% SDS, and 2.5 mM EDTA. Radioactive signals were detected and quantified by scanning using Molecular Imager FX and Quantity One software (Bio-Rad). Cytokinin effects on transcription were considered significant if the signals differed at least 2-fold from the water control. Every experiment was repeated at least three times. Transcriptional activities of *psbA* were used as an internal standard.

Blotting of Chloroplast Genes

Plastid DNA was isolated from intact plastids by the phenol-chloroform method (Kusnetsov et al., 1994), treated with DNase-free RNase (Fermentas), and precipitated with ethanol. Fragments of 26 plastid genes were amplified by PCR and cloned into pUC57 A/T vector (Fermentas). Fragments of 10 other genes were kindly provided by Prof. R.G. Herrmann (Botanical Institute, University of Munich) and subcloned into the pUC57 vector. The gene-specific fragments used in this study are listed in Table II. The gene fragments were dotted onto nylon Hybond-N^+ membrane (Amersham Pharmacia Biotech). One microgram of DNA of each gene fragment treated as described by Zubo and Kusnetsov (2008) was loaded onto the membrane in two replicates using a Bio-Dot apparatus (Bio-Rad).

Northern Hybridization

Total RNA was extracted from leaf segments using TRizol reagent (Gibco/BRL) according to the manufacturer's protocol. RNA was electrophoretically separated in 1.2% agarose-formaldehyde gels and blotted onto Hybond-N^+ membrane (Amersham Pharmacia Biotech) by capillary transfer (Sambrook et al., 1989). Radioactive probes for hybridization were produced by PCR in the presence of [α-^{32}P]dCTP (chloroplast genes *rbcL* and *atpB* were cloned into pUC57; radioactive probes for *rbcL* and *atpB* were generated using standard M13 primers). PCR-generated and purified fragments of corresponding nuclear and chloroplast genes served as templates. RNA gel-blot hybridization with ^{32}P-labeled probes and subsequent membrane washing was carried out as described (Sambrook et al., 1989). Radioactive signals were detected and quantified using a Fujifilm BAS1500 Phosphoimager (Fuji) or by autoradiography.

Chlorophyll Determination

Chlorophyll was extracted from plant material with 80% acetone. Samples were analyzed in a Beckmann DU 65 spectrophotometer, and total chlorophyll was estimated as described by Oelmüller et al. (1986).

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Determination of Endogenous Cytokinins and ABA

Free cytokinins and ABA were estimated in the leaf samples by competitive ELISAs using polyclonal antibodies specific for a particular hormone and anti-rabbit antibodies conjugated with peroxidase. The samples (0.5–0.8 g) of apical, middle, or basal leaf segments were ground in liquid nitrogen, and cytokinins were extracted with 80% ethanol for 16 h at 4°C. The homogenate was centrifuged at 18,000*g* for 10 min, and the supernatant was concentrated to an aqueous residue in vacuo. An aliquot of the aqueous residue was subsequently used to determine the content of cytokinins by cross-reaction with rabbit antibodies raised against zeatin riboside (Shakirova et al., 2004). ABA was extracted from the aqueous residue with ethyl ester and methylated with diazomethanol, and, after evaporation, the dry residue was dissolved in 80% ethanol; aliquots were used for determination of ABA by ELISA with polyclonal antibody against ABA as described previously (Shakirova et al., 2004).

All molecular-biological procedures, such as isolation of plasmid DNA, digestion by restriction endonucleases, ligation, bacterial transformation, and others were performed as described by Sambrook et al. (1989).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: barley chloroplast chromosome, EF115541; *rbcL*, X00630; *atpB*, EF115541.

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