Plant Physiology, September 1999, Vol. 121, pp. 245–251, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Cytokinins in Tobacco and Wheat Chloroplasts. Occurrence and Changes Due to Light/Dark Treatment[1]

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Although cytokinins (CKs) affect a number of processes connected with chloroplasts, it has never been rigorously proven that chloroplasts contain CKs. We isolated intact chloroplasts from tobacco (*Nicotiana tabacum* L. cv SR1) and wheat (*Triticum aestivum* L. cv Ritmo) leaves and determined their CKs by liquid chromatography/tandem mass spectroscopy. Chloroplasts from both species contained a whole spectrum of CKs, including free bases (zeatin and isopentenyladenine), ribosides (zeatin riboside, and isopentenyladenosine), ribotides (isopentenyladenosine-5′-monophosphate, zeatin riboside-5′-monophosphate, and dihydrozeatin riboside-5′-monophosphate), and *N*-glucosides (zeatin-N_9-glucoside, dihydrozeatin-N_9-glucoside, zeatin-N_7-glucoside, and isopentenyladenine-*N*-glucosides). In chloroplasts there was a moderately higher relative amount of bases, ribosides, and ribotides than in leaves, and a significantly increased level of N_9-glucosides of zeatin and dihydrozeatin. Tobacco and wheat chloroplasts were prepared from leaves at the end of either a dark or light period. After a dark period, chloroplasts accumulated more CKs than after a light period. The differences were moderate for free bases and ribosides, but highly significant for glucosides. Tobacco chloroplasts from dark-treated leaves contained zeatin riboside-*O*-glucoside and dihydrozeatin riboside-*O*-glucoside, as well as a relatively high CK oxidase activity. These data show that chloroplasts contain a whole spectrum of CKs and the enzymatic activity necessary for their metabolism.

Cytokinins (CKs) regulate a number of growth and developmental processes in plants, including activation of cell division, stimulation of growth of axillary buds (suppression of apical dominance), inhibition of root growth, and suppression of senescence (for reviews, see Binns, 1994; Mok and Mok, 1994). CK-suppressed senescence was first reported by Richmond and Lang (1957) in detached *Xanthium* leaves kept in the dark. CKs were shown to maintain protein synthesis and to prevent chlorophyll degradation. Numerous subsequent studies initiated by the pioneering work of Mothes (1960) have shown that CKs also affect many other processes connected with chloroplasts. Exogenous CKs stimulate de-etiolation, i.e. the transition of etioplasts into chloroplasts in detached leaves and cell cultures (for reviews, see Parthier, 1979; Reski, 1994).

CKs affect the abundance of transcripts and proteins encoded both by nuclear and plastid genomes, the most notable being genes coding for the small subunit of Rubisco and chlorophyll *a/b* binding protein (for reviews, see Link, 1988; Parthier, 1989). The mechanism by which this occurs has not been completely resolved, but there is evidence for translational and posttranslational control (Link, 1988). This control may be mediated by polyribosome formation (Ohya and Suzuki, 1998), modification of the secondary structure of poly(A^+) RNA (Jackowski et al., 1987), phosphorylation of the ribosomal proteins (Yakovleva et al., 1992), or regulation of the activity of tRNAs (Romanov, 1990). CKs (specifically, benzylaminopurine [BAP]) have also been reported to increase the activity of aminoacyl-tRNA synthetases in senescing bean leaves (Jayabaskaran et al., 1990). A correlation between two CK-regulated processes, cell division and chloroplast biogenesis, has been reported (Reski and Abel, 1992; Suzuki et al., 1992). The coordinated action of light and CKs in many processes in chloroplasts has also been described in a number of reports (e.g. Teyssandier de la Serve et al., 1985; Parthier, 1989).

Despite this considerable knowledge of CK effects on chloroplast development and function, it has not yet been proven that chloroplasts contain CKs. Davey and Van Staden (1981) showed the presence of zeatin-type CKs in isolated spinach chloroplasts using paper chromatography and a soybean callus bioassay, but did not demonstrate that the chloroplast preparation was intact and uncontaminated by other cell fractions. There is substantial evidence for the occurrence of CKs in chloroplast tRNAs. Swaminathan and Bock (1977) described the occurrence of isopentenyladenine, 2-methylthioisopentenyladenine, and 2-methylthioisopentenyladenosine in tRNA of *Euglena gracilis* chloroplasts. The presence of these and other CKs was later shown in tRNAs of chloroplasts of various plant species (for reviews, see Taller, 1994; Prinsen et al., 1997). The predominant CK-containing tRNA species are tRNA-Phe and tRNA-Leu. It is not yet clear whether the modifications of tRNA molecules occur in the chloroplast. Although chloroplasts seem to be the site of methylthiola-

[1] This research was supported by the Grant Agency of the Czech Republic (grant no. 206/96/K188 to I.M.) and by the Kontakt by the Ministry of Education of the Czech Republic (grant no. ES040 to B.B.).

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tion, the genes for tRNA-modifying enzymes are probably encoded by nuclear genes (see Taller, 1994, and refs. therein).

The occurrence of CK-binding sites in chloroplasts has been demonstrated previously. Romanko et al. (1986) isolated two CK-binding proteins from oat leaf chloroplasts, which in an in vitro assay promoted chloroplast-specific transcription in the presence of CKs. Another CK-binding protein was isolated from wheat chloroplast membranes (Zhang et al., 1991). This binding site shows a high affinity for BAP and was partially inhibited by kinetin and zeatin, but not by adenine (Zhang et al., 1996). The prevailing opinion that CKs contained in tRNA molecules do not significantly contribute to the pool of free CKs (Kamínek, 1974; Chen, 1997; Prinsen et al., 1997) and reports on CK-binding proteins in chloroplasts raise the question of whether CKs are present and metabolized in chloroplasts. This paper aims to address this question.

MATERIALS AND METHODS

Plant Material

Tobacco (*Nicotiana tabacum* L. cv SR1) plants were grown in soil in a greenhouse for about 12 weeks under a day/night temperature regime of 25°C/20°C. Two types of leaves were taken for chloroplast preparation and CK analyses: younger ones (not smaller than 100 mm in length) and mature ones (non-senescing). Leaves for chloroplast preparation were immediately processed as described below. Leaves for CK analyses were stored at −70°C.

Wheat (*Triticum aestivum* L. cv Ritmo) plants were grown in soil in a growth cabinet at a day/night temperature regime of 25°C/20°C and 14 h of light daily provided by sodium lamps (PPFD, 216 μmol m^{-2} s^{-1} at plant level) for 10 d. The aboveground parts were used for chloroplast preparation and CK analyses.

Chloroplast Preparation

Tobacco leaves were deribbed, cut into thin strips, and kept on ice. About 40 g was mixed with 200 mL of homogenization medium (0.33 M sorbitol, 50 mM Tris, pH 7.5, 0.4 mM KCl, 0.04 mM Na_2EDTA, 0.1% [w/v] BSA, 1% [w/v] PVP, and 5 mM isoascorbic acid), which was used in a semifrozen state. Homogenization was performed with a polytron for 10 to 20 s. The brei was squeezed through two layers of muslin to remove coarse debris, and filtered through cotton wool between eight layers of muslin. The chloroplast fraction was recovered from the homogenization medium by centrifugation at 1,000*g* for 1 min at 4°C, washed twice with 100 mL of resuspension medium (0.33 M sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 mM HEPES, pH 7.6), and resuspended in 4 mL of resuspension medium. This suspension was underlayed with 20 mL of 40% (w/v) and 10 mL of 80% (w/v) Percoll solution in the same medium and centrifuged at 1,000*g* for tobacco and 2,500 g for wheat for 15 min at 4°C. Intact chloroplasts moved to the interface between the different density Percoll solutions. Chloroplasts were washed with 10 mL of resuspension medium, and after centrifugation were resuspended in 1 to 1.5 mL of the medium (Jensen and Bassham, 1966) and kept frozen until analysis.

Chlorophyll Determination

Chlorophyll was extracted into 80% (w/v) acetone and then centrifuged at 500*g* for 5 min. The absorption was measured at 652 nm and the chlorophyll concentration (in micrograms per milliliter) was calculated as $A_{652} \times 1{,}000/34.5$ (Arnon, 1949).

Glyceraldehyde-3-P Dehydrogenase Assay

The glyceraldehyde-3-P dehydrogenase assay was chosen for checking the intactness of chloroplasts (Latzko and Gibbs, 1968). The assay solution contained the chloroplast fraction (20 μL), 0.033 M Tris/HCl buffer, pH 8.5, 17 mM sodium arsenate, 4 mM Cys, 20 mM sodium fluoride, and 4×10^{-5} M $NADP^+$. The reaction was initiated with 50 μL of 0.1 M glyceraldehyde-3-P. Reduction of $NADP^+$ was followed at 340 nm. The same assay was run with chloroplasts disrupted with 0.01 M $MgCl_2$.

Cyt *c* Oxidase Assay

To determine possible contamination of the chloroplast preparation by mitochondria, Cyt *c* oxidase, a mitochondrial marker enzyme, was assayed according to the method of Tolbert (1974). The chloroplast sample (20 μL) was mixed with 10 μL of 3% (w/v) digitonin and incubated 60 s before the addition of 920 μL of 40 mM potassium phosphate buffer, pH 7.4. The reaction was initiated with 50 μL of reduced Cyt *c* (4 mg/mL). Cyt *c* was reduced with 1 M DTT until the A_{550}/A_{565} ratio was 9 to 10, and excess reducing agent was removed by bubbling air through the solution. Oxidation of Cyt *c* was followed at 550 nm.

Cyt *c* Reductase Assay

To determine possible contamination with membranes of the ER, Cyt *c* reductase, an ER marker, was assayed using oxidized Cyt *c* (Tolbert, 1974). The reaction mixture contained chloroplasts (20 μL) and 50 mM Tris/MES buffer, pH 7.5, 1 mM KCN, 0.748 mM NADH, 0.0747 mM Cyt *c*, and 14.8 μg/mL antimycin A. Reduction of Cyt *c* was followed at 550 nm.

Glc-6-P Dehydrogenase Assay

To determine the presence of trace cytoplasm in the chloroplast preparation, Glc-6-P dehydrogenase, a cytosolic marker enzyme, was assayed. The reaction mixture contained 10 mM $MgCl_2$, 0.1% (w/v) Triton X-100, 0.17 mM $NaDP^+$, 0.33 mM Glc-6-P, and 20 mM TES NaOH buffer, pH 7.5, in a final volume of 1 mL. The reduction of $NADP^+$ was measured by monitoring A_{340} (Simcox et al., 1977).

Catalase Assay

H_2O_2 was dissolved in 50 mM sodium phosphate buffer, pH 7.4, to an A_{240} of 0.6 to 0.8. The reaction was initiated with a 20-μL chloroplast fraction, and the reduction of H_2O_2 was followed at 240 nm (Gregory and Fridovich, 1974).

Extraction and Purification of CKs

Extraction and purification was performed according to the method of Redig et al. (1996). Samples were extracted with Bieleski solvent (Bieleski, 1964) containing a mixture of deuterated standards (Z, ZR, DZ, DZR, ZMP, DZMP, ZN7G, ZN9G, ZOG, ZROG, DZOG, DZROG, 2iP, 2iPA, 2iPMP, and 2iPN9G, Apex International, Honiton, UK) and 80% methanol. Extracts were then purified using DEAE-Sephadex (to separate nucleotides), C_{18} cartridges, and immunoaffinity columns. The combination of these methods separates CKs into three fractions: 1, free bases, ribosides, and N_9-glucosides; 2, ribotides, determined as ribosides after cleavage by alkaline phosphatase; and 3, N_7- and *O*-glucosides. At the time of analyses, the standard of 2iPN7G was not available and, thus, the separation of N_7 and N_9 glucosides of 2iP was not tested. Therefore, the only 2iP glucoside that was quantified was that which occurred in the fraction 1 together with free bases and ribosides. On the basis of analogy with ZN7G and ZN9G, we may assume that it was iPN9G. N_3-glucosides were not determined because standards were not available.

Quantitative Analyses of CKs

Analyses were performed according to methods described by Prinsen et al. (1995) and Witters et al. (1999) using HPLC linked to a mass spectrometer (Quatro II, Fisons, Beverly, MA) equipped with an electro-spray interface ([+] electro-spray LC-tandem MS). Samples were injected onto a C8 column (5 μm, 125 $\times$ 4 mm; LiChrosphere 60 RP Select B, Merck, Rahway, NJ) and eluted with methanol:0.01 M ammonium acetate (70:30, v/v) at 800 μL min^{-1}. The effluent was introduced into the electro-spray source (80°C source, +3.5 V capillary, 20 V cone) using a post-column split 1/20. Quantitation was performed by multiple ion monitoring (Prinsen et al., 1995). The recovery of deuterated standards was about 25% to 50% for free bases and ribosides, respectively, and about 10% to 20% for glucosides. All results were calculated using the recovery value of each of the standards in the given sample.

Test for CK Absorption

A mixture of ^{3}H-labeled zeatin, zeatin riboside, and isopentenyladenosine (each, 50 kBq mL^{-1}) was prepared by Dr. J. Hanuš of the Isotope Laboratory, Institute of Experimental Botany (Prague) by alkylation of [^{3}H]adenosine with 4-tert-butoxy-3-methyl-*trans*-but-2-enyl bromide, followed by Dimroth rearrangement and hydrolysis of the tert-butyl group. This mixture was incubated with 1 mL of the chloroplast preparation at 20°C for 1 h. Chloroplasts were then sedimented at 1,000*g* and washed three times with resuspension solution. The radioacivity of the chloroplast fraction was measured by scintillation counting.

CK Oxidase Activity

The method described by Motyka and Kamínek (1994) was used to measure CK oxidase activity. The chloroplast suspension was mixed with 0.1 M Tris-HCl, pH 7.5, in the presence of insoluble PVP. After filtration, centrifugation, and removal of nucleic acids by Polymin P (Serva Feinbiochemico, Heidelberg), proteins were precipitated with 80% (w/v) saturated ammonium sulfate. Protein content was determined according to the method of Bradford (1976) using

Table I. *Levels of cytokinins in tobacco leaves (pmol mg^{-1} chlorophyll), in their chloroplasts (fmol mg^{-1} chlorophyll), and in the second-to-last washing liquid (pmol mL^{-1}) in two independent experiments*

Plant Part[a]	ZR	DZ	DZR	2iP	2iPA	ZMP	DZMP	2iPMP	ZN7G	DZN7G	ZN9G	DZN9G	2iPNG
Experiment 1													
IYL	0.29	Tr[b]	Tr	0.27	0.05	0.24	0.23	0.47	0.94	0.14	0.02	0.02	0.27
IML	0.08	0.02	Tr	0.07	Tr	0.07	0.07	0.11	0.36	0.05	0.02	0.03	0.10
CYL	0.27	0.06	0.02	0.27	0.02	0.17	0.20	0:55	0.77	0.17	0.04	0.03	0.33
CML	0.08	0.03	Tr	0.06	Tr	0.04	0.05	0.12	0.30	0.05	0.03	0.03	0.13
YLC	22.32	Tr	0.14	15.42	8.15	16.33	14.46	18.21	5.80	Tr	11.63	7.63	6.92
MLC	12.62	0.23	0.11	8.42	5.05	5.90	6.54	9.66	3.76	Tr	10.32	4.48	3.45
WL	14.05	9.03	3.01	10.07	7.07	78.08	7.06	18.00	Tr	Tr	2.03	2.08	3.04
Experiment 2													
IYL	0.37	0.11	0.10	0.39	0.08	0.32	0.28	0.57	1.22	0.18	0.04	0.05	0.34
IML	0.11	0.08	Tr	0.12	Tr	0.12	0.10	0.18	0.49	0.08	0.04	0.07	0.16
CYL	0.35	0.10	0.04	0.37	0.04	0.24	0.23	0.64	1.03	0.20	0.07	0.06	0.41
CML	0.10	0.06	Tr	0.12	Tr	0.09	0.09	0.17	0.42	0.07	0.05	0.06	0.19
YLC	28.62	0.83	0.25	18.76	11.31	20.54	19.17	23.00	7.28	0.73	16.22	9.86	8.47
MLC	13.18	1.16	0.33	10.84	6.93	7.36	9.12	12.45	4.59	0.55	14.76	5.62	4.37
WL	16.22	8.73	4.14	12.23	8.21	65.63	10.48	18.55	Tr	Tr	2.82	2.37	4.16

[a] IYL, Intact young leaves; IML, intact mature leaves; CYL, cut young leaves; CML, cut mature leaves; YLC, young leaf chloroplasts; MLC, mature leaf chloroplasts; WL, washing liquid. [b] Tr, Trace.

Table II. *Abundance of cytokinin bases + ribosides, ribotides, and glucosides in tobacco leaves and chloroplasts in two independent experiments*

Plant Part[a]	Free Bases + Ribosides	Ribotides	Glucosides
Experiment 1		*% of total CKs*	
IYL	19.8	32.1	48.1
IML	17.2	25.6	57.1
CYL	22.0	31.8	46.2
CML	19.5	22.6	57.9
YLC	36.2	38.6	25.2
MLC	37.2	31.6	31.2
Experiment 2			
IYL	25.9	28.9	45.2
IML	20.0	25.8	54.2
CYL	23.8	29.4	46.8
CML	19.7	24.6	49.3
YLC	36.2	38.1	25.7
MLC	35.1	32.0	32.9

[a] IYL, Intact young leaves; IML, intact mature leaves; CYL, cut young leaves; CML, cut mature leaves; YLC, young leaf chloroplasts; MLC, mature leaf chloroplasts.

BSA as a standard. The assay of CK oxidase activity was based on the conversion of [2,8-^{3}H]isopentenyladenine to adenine. Tritiated isopentenyladenine was prepared by Dr. J. Hanuš as described above. Separation of substrate from the product was achieved by TLC on microcrystalline cellulose plates developed with the upper phase of the 4:1:2 (v/v) mixture of ethylacetate:*n*-propanol:water (Motyka and Kamínek, 1994).

Presentation of the Results

Each experiment was repeated two to three times. Results of one or two representative studies of each type are given. The other experiments showed similar tendencies, including changes in individual CKs between leaves and chloroplasts or between light and dark, but the absolute values resulting from each study were different, so no statistical analyses of the data are given.

RESULTS AND DISCUSSION

Chloroplast Preparation

All of the chloroplast preparations used for CK analysis were determined to be intact using the Glc-6-P-dehydrogenase test, with only approximately 10% of the chloroplasts disrupted. The measurements of the other marker enzymes showed the following impurities in the chloroplast fraction: less than 5% ER, less than 4% mitochondria, and less than 2% cytosol and microbodies. These results show that highly purified intact chloroplasts were obtained.

Content of CKs in Chloroplasts from Tobacco Upper and Lower Leaves

Chloroplasts isolated from tobacco leaves contain a whole spectrum of CKs, including free bases (2iP), ribosides (2iPA and ZR), ribotides (2iPMP, ZMP, and DZMP), and *N*-glucosides (ZN9G, DZN9G, ZN7G, and 2iPNG). No *O*-glucosides were detected (Table I). The bases and ribosides represent 36.2% of total CKs in chloroplasts from both young and mature leaves, ribotides 38.4% and 31.8%, respectively, and *N*-glucosides 25.5% and 32.12%, respectively (Table II). Younger leaves contained 70% more CKs than mature leaves, while chloroplasts isolated from younger leaves contained 81% more CKs than those from mature leaves. These results show that although the age of the leaves affected the total level of CKs in chloroplasts, the proportions of individual CKs were not affected by leaf age.

To control the process of extraction, we analyzed CKs in the original leaves, in cut leaves (small pieces kept on ice), and in the penultimate and final washings. Comparisons between intact and cut leaves did not show many differences (Tables I and II). The predominant CKs were ZR, 2iP, 2iPNG, ZN7G, and all three ribotides. However, when the abundance of individual CK types were compared in leaves and chloroplasts, there were some striking differences. First, there was a lower relative amount of bases and ribosides in leaves than in chloroplasts; in cut leaves they represented on average 21% of the total CK amount compared with 37% in chloroplasts (Table II). Also, the relative level of ZN9G and DZN9G was significantly increased in chloroplasts compared with leaves. Additionally, the proportion of 2iPNG and ZN7G and DZN7G, and therefore also that of total glucosides, was lower in chloroplasts than in leaves. The relative level of ribotides was somewhat higher in chloroplasts than in leaves (Tables I and II).

The analysis of the last two washings showed that there were some CKs found in the second-to-last rinsate, especially ZMP, ZR, 2iP, 2iPMP, and DZ (Table I). In the last rinsate, no CKs were detected, showing that the intact chloroplasts were not leaky and that CKs did not diffuse out of isolated chloroplasts. To verify that CKs from cytoplasm were not absorbed on the chloroplast surface, we incubated isolated chloroplasts with ^{3}H-labeled CKs. Only

Table III. *Cytokinin contents of tobacco leaves (pmol mg^{-1} chlorophyll) and chloroplasts (fmol mg^{-1} chlorophyll) at the end of 12 h of light and 12 h of darkness*

Plant Part[a]	Z	ZR	DZ	DZR	2iP	2iPA	ZMP	DZMP	2iPMP	ZN7G	DZN7G	ZN9G	DZN9G	2iPNG	ZROG	DZROG
L-L	Tr[b]	0.26	Tr	0.07	0.10	Tr	0.10	0.04	0.10	Tr	Tr	0.16	0.18	0.11	ND[c]	ND
L-D	Tr	0.60	0.09	0.29	0.09	0.06	0.35	0.13	0.21	Tr	Tr	0.69	0.40	0.24	ND	ND
C-L	1.12	4.42	1.85	0.83	2.14	1.81	10.86	0.95	2.33	Tr	Tr	4.93	6.37	1.32	ND	ND
C-D	Tr	28.71	Tr	9.17	16.60	1.87	39.42	5.48	31.48	1.82	4.14	125.7	164.9	13.25	67.33	19.41

[a] L-L, Leaves in light; L-D, leaves in darkness; C-L, chloroplasts in light; C-D, chloroplasts in darkness. [b] Tr, Trace. [c] ND, Not detected.

Table IV. *The abundance of cytokinin free bases plus ribosides, ribotides, and glucosides in tobacco and wheat leaves and chloroplasts at the end of 12 h of light and 12 h of dark*

Plant Part[a]	Free Bases + Ribosides	Ribotides	Glucosides
		% of total CKs	
TL-L	38.4	21.4	40.2
TL-D	36.1	22.8	41.1
TC-L	31.2	36.4	32.4
TC-D	10.7	14.4	74.9
WL-L	38.0	26.8	35.2
WL-D	31.5	48.1	20.4
WC-L	28.4	48.4	23.2
WC-D	13.2	18.9	67.9

[a] TL-L, Tobacco leaves in light; TL-D, tobacco leaves in darkness; WL-L, wheat leaves in light; WL-D, wheat leaves in darkness; TC-L, tobacco chloroplasts in light; TC-D, tobacco chloroplasts in darkness; WC-L, wheat chloroplasts in light; WC-D, wheat chloroplasts in darkness.

1% to 2% of the added radioactivity was found associated with the chloroplast fraction.

The reason for the differences in the abundance of individual CKs in chloroplasts and leaf extracts is not clear. The most obvious changes occur in the levels of *N*-glucosides, which are considered to be very stable metabolites (Jameson, 1994). It is easy to envisage the formation of ZN9G and DZN9G from Z or DZ (zeatin was not detected), but the decrease in relative abundance of N_7-glucosides and 2iPNG is difficult to understand. This leads to the principal question of where CKs are synthesized. Are they synthesized in the cytoplasm and then transported into chloroplasts, where they are converted to other CK derivatives (or vice versa)? Or are CKs synthesized in both cytoplasm and chloroplasts? Our results do not allow us to differentiate between these possibilities, so it will be the goal of further research to answer these questions.

We do know from our recent work on the role of CKs in the flowering of *Chenopodium rubrum* (Macháčková et al., 1993) that the levels of free CKs (bases and ribosides) in leaves decrease during the dark period, probably due to changes in the conversion to ribotides. As the first step in unraveling this complex system, we analyzed the CK levels in wheat leaves and in the leaves and chloroplasts of tobacco taken for chloroplast preparation at the end of a 12-h period of light or dark. As a second step, we analyzed the CK oxidase activity in tobacco chloroplasts to determine whether chloroplasts were able to degrade CKs.

Content of CKs in Chloroplasts from Tobacco and Wheat Leaves in Relation to Light/Dark Treatment

In tobacco leaves the level of all CKs determined was about three times higher at the end of the dark period than at the end of the light period, which shows that the changes observed in *C. rubrum* (Macháčková et al., 1993) are not general and may be correlated with the photoperiodic type of the plant. The relative abundance of individual CKs remained almost unchanged (Tables III and IV). However, in chloroplasts isolated from leaves at the end of either a light or dark period the differences were more pronounced: chloroplasts from dark-treated leaves accumulated more CKs than those from light-treated leaves. The differences were moderate for free bases and ribosides but dramatic for glucosides, with a 25.6- and 25.3-fold increase in the content of ZN9G and DZN9G in the dark (Table IV). Some ZROG and DZROG were also detected in these chloroplasts. This was the only case where *O*-glucosides were found. The recovery for glucoside determinations was relatively low, so some lower levels might have escaped detection. This is a significant result because CK *O*-glucosides have been previously reported to occur in vacuoles (Fusseder and Ziegler, 1988).

The results for wheat were similar. In leaves, the levels of free CKs did not change significantly in response to dark or light. Darkness caused a slight increase in the level of ribotides and a slight decrease in the abundance of glucosides (Tables V and VI). Although the actual amounts of free bases and ribotides were about twice as high in chloroplasts from darkness than those from light, their relative contribution to the total amount of CKs was lower. This was again due to a substantial increase of glucosides in chloroplasts from darkness. In this case, the increase with respect to light was 18.6- and 18.1-fold for ZN9G and DZN9G, respectively (Tables V and VI). Therefore, the

Table V. *Cytokinin content in wheat leaves (pmol mg^{-1} chlorophyll) and chloroplasts (fmol mg^{-1} chlorophyll) at the end of 12-h light and 12-h darkness in two independent experiments*

Plant Part[a]	Z	ZR	DZ	DZR	2iP	2iPA	ZMP	DZMP	2iPMP	ZN9G	DZN9G	2iPNG
Experiment 1												
L-L	0.22	0.74	0.26	0.83	0.32	0.55	0.83	0.64	0.51	0.68	0.63	1.32
L-D	0.13	0.88	0.25	0.97	0.64	0.82	1.86	2.34	1.12	0.57	0.28	1.45
C-L	Tr[b]	1.42	3.28	1.76	2.52	1.64	9.23	3.74	4.83	1.82	2.57	3.61
C-D	Tr	2.15	Tr	6.84	6.35	4.80	13.57	6.51	8.75	38.98	54.82	9.55
Experiment 2												
L-L	0.24	1.12	0.22	1.06	0.40	0.75	1.21	0.97	0.63	0.92	0.81	1.56
L-D	0.21	1.21	0.23	1.18	0.57	0.90	2.33	2.47	1.36	0.75	0.38	1.60
C-L	Tr	2.50	2.63	1.94	3.56	2.08	1.92	5.29	4.85	2.40	3.55	4.21
C-D	Tr	3.43	1.12	5.64	8.19	5.72	11.36	9.83	8.05	36.81	49.74	12.11

[a] L-L, Leaves in light; L-D, leaves in darkness; C-L, chloroplasts in light; C-D, chloroplasts in darkness. [b] Tr, Trace.

Table VI. *The abundance of cytokinin free bases + ribosides, ribotides, and glucosides in extracts of wheat leaves and chloroplasts at the end of 12 h of light and 12 h of darkness*

Plant Part[a]	Free Bases + Ribosides	Ribotides	Glucosides
	% of total CKs		
Experiment 1			
L-L	38.0	26.8	35.2
L-D	31.5	48.1	20.4
C-L	28.4	48.4	23.2
C-D	13.2	23.2	67.9
Experiment 2			
L-L	38.3	28.4	33.3
L-D	32.6	46.7	20.7
C-L	29.4	46.8	23.8
C-D	15.8	19.2	65.0

[a] L-L, Leaves in light; L-D, leaves in darkness; C-L, chloroplasts in light; C-D, chloroplasts in darkness.

accumulation of glucosides in chloroplasts mentioned above was substantially potentiated by dark treatment of the leaves.

These results show a specific reaction of chloroplasts compared with the rest of the cell in their CK accumulation and/or metabolism in response to darkness. The effect of light (or darkness) on the content of individual CKs should be taken into consideration during the analysis of any plant material for CK content.

CK Oxidase Activity in Tobacco Chloroplasts

As the first aspect of CK metabolism in chloroplasts, CK oxidase activity was measured. The specific activity of CK oxidase was measured in TAPS-NaOH buffer, pH 8.5, and in Cu^{2+}-imidazole buffer, pH 6.0, in which the enzyme activity is stimulated (Chattfield and Armstrong, 1987; Motyka and Kamínek, 1994). When measured in TAPS-NaOH buffer (pH 8.5) and Cu^{2+}-imidazole buffer (pH 6.0), the activity expressed on a protein level was about three times higher in chloroplasts (0.039 and 0.363, respectively) than in leaves (0.011 and 0.158, respectively).

CONCLUSION

Chloroplasts contain a wide spectrum of CKs and a relatively high activity of CK oxidase, and they react to darkness with specific changes in the level of various CK metabolites. Our results suggest that the metabolism of CKs in chloroplasts may be of importance for their function in these organelles.

ACKNOWLEDGMENTS

The authors thank Prof. Dennis Baker (Wye College, London) and Dr. Laura Zonia (Institute of Experimental Botany, Prague) for language correction of the manuscript and Prof. Miroslav Kamínek (Institute of Experimental Botany, Prague) for critical reading of the manuscript.

Received May 27, 1999; accepted June 7, 1999.

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