

# CALCIUM VARIOUSLY MEDIATES THE EFFECT OF CYTOKININ ON CHLOROPHYLL AND LHCPII ACCUMULATION DURING GREENING IN BARLEY LEAVES AND CUCUMBER COTYLEDONS

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During greening, excised etiolated barley leaves and cucumber cotyledons that were depleted of exogenous  $\text{Ca}^{2+}$  by a chelating agent (ethylene glycol-bis (beta aminoethyl ether)-N,N,N',N'-tetraacetic acid, EGTA) showed ~50% reduced chlorophyll (Chl) accumulation and ~30% accumulation of apoprotein of the light-harvesting chlorophyll a/b-binding protein complex of photosystem II (LHCPII). The  $\text{Ca}^{2+}$  channel blocker lanthanum chloride ( $\text{LaCl}_3$ ) applied to cucumber cotyledons reduced LHCPII accumulation more than EGTA did. In both plant materials, cytokinins enhanced chlorophyll accumulation by 50–60% and this effect was completely canceled by EGTA application. Hormones significantly increased LHCPII accumulation but EGTA application reduced that effect in barley leaves by ~30% and in cucumber cotyledons by ~80%. A similar effect was observed in  $\text{LaCl}_3$ -treated cotyledons.  $\text{CaCl}_2$  application boosted chlorophyll accumulation in both plant materials.  $\text{CaCl}_2$  applied together with cytokinin reduced the hormonal effect on chlorophyll accumulation by ~38% in barley leaves and 23% in cucumber cotyledons, but almost totally inhibited cytokinin-stimulated LHCPII accumulation. Our results indicate that calcium variously mediates the effect of cytokinin on chlorophyll and LHCPII accumulation. Cytokinin-induced enhancement of chlorophyll accumulation seems totally dependent on the exogenous pool of  $\text{Ca}^{2+}$ , while  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent pathways are involved in the hormonal effect on LHCPII accumulation. The effect of cytokinin on the increase of light-induced LHCPII accumulation appears to be sensitive to exogenously applied  $\text{Ca}^{2+}$ , which almost totally blocked the hormonal effect. Our results give indirect evidence that the responses to cytokinin and light act on different events leading to Chl and LHCPII accumulation.

**Key words:** Barley leaves, calcium, chelating agent – EGTA, chlorophyll, cucumber cotyledons, cytokinin, greening, LHCPII, lanthanum chloride, light.

## INTRODUCTION

De-etiolation is a complex developmental process that occurs when dark-grown angiosperm seedlings or their organs are exposed to light. Light exposure induces the transformation of proplastids or etioplasts into photosynthetically competent chloroplasts. Phytochrome and cytokinin have been implicated in the greening process (Fletcher and McCullagh, 1971; Briggs et al., 1988) but the signal transduction pathways through which both are linked to the induction of Chl accumulation have not been identified. All the enzymes needed for conversion of 5-aminolevulinic acid (ALA) to protochlorophyllide must be present in dark-grown seedlings (Reinbothe and Reinbothe, 1996), and they are nuclear gene products (Grimm, 1990; Ilag et al., 1994). Cytokinins (CKs) are involved in controlling chloro-

plast biogenesis and function. They affect chloroplast and etioplast ultrastructure, chloroplast enzyme activities, pigment accumulation and the rate of photosynthesis (Parthier, 1979; Chory et al., 1994; Yaronkaya et al., 2006). Application of CK shortens or eliminates the lag phase of Chl accumulation (Fletcher and McCullagh, 1971; Lew and Tsuji, 1982; Yaronkaya et al., 2006). Several laboratories have shown that CKs increase the accumulation of proteins encoded by *cab* and *rbcS* genes (Ohya and Suzuki, 1991; Chory et al., 1994; Kusnetsov et al., 1994). Cytokinin-controlled expression of LHCII has been observed in tobacco cell suspension, cucumber cotyledons, and tissue culture of *Dianthus caryophyllus* (Teyssendier de la Serve et al., 1985; Ohya and Suzuki, 1991; Winiarska et al., 1994). In *Lemna* studies it was found that kinetin regulation of *cab* and *rbcS*

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mRNA was primarily post-transcriptional (Flores and Tobin, 1988).

Calcium has been implicated as a second messenger in several signal transduction pathways in plants, including those involving phytochrome and CK (Shacklock et al., 1992; White and Broadley, 2003). Specifically, transduction signals are thought to cause a transient influx of exogenous  $\text{Ca}^{2+}$  from the apoplast, a major reservoir of cations (especially calcium), or release of internally compartmental  $\text{Ca}^{2+}$ , thereby raising the cytoplasmic  $\text{Ca}^{2+}$  concentration (Demarty et al., 1984; Tretny et al., 1991). A stimulus that causes elevation of specific  $\text{Ca}^{2+}$  signals may cause  $\text{Ca}^{2+}$  to either activate or repress the activity of  $\text{Ca}^{2+}$ -responding proteins (e.g., protein kinase or phosphatase) or, through the interaction of  $\text{Ca}^{2+}$  with  $\text{Ca}^{2+}$  sensors such as calmodulin (CaM), it may inhibit or enhance binding to a target protein, such as the transcription factor (TF), and thus cause it to activate or repress the transcription of target genes, depending on whether the TF itself is a transcriptional activator or repressor (Galon et al., 2010; Batistic and Kudla, 2012).

Phytochrome phototransduction, which stimulates synthesis of the chlorophyll precursor ALA, and induced gene encoding of the major light-harvesting chlorophyll *a/b*-proteins of photosystem II, are involved in calcium/calmodulin-dependent and -independent signal transduction pathways (Shacklock et al., 1992; Neuhaus et al., 1993; Bowler et al., 1994; Grover et al., 1998). Some phytochrome-activated transcription factors are  $\text{Ca}^{2+}$ -dependent (Galon et al., 2010). Kushwaha et al. (2008) showed that one of the four *Arabidopsis thaliana*  $\text{Ca}^{2+}$  sensors, CaM isoform CaM7, is transcriptional regulator that interacts with the promoters of light-inducible genes *cab1* and *rbcS* and promotes photomorphogenesis. In certain tissues CK increased the cytosolic concentration of  $\text{Ca}^{2+}$  through activation of  $\text{Ca}^{2+}$  channels in the plasma membrane (Elliot, 1986; Silverman et al., 1998). Černý et al. (2010) analyzed the involvement of calcium signaling in early phosphoproteome regulation by CK in *Arabidopsis* seedlings in the presence or absence of calcium channel blocker D 600 and competitive inhibitor of calcium uptake  $\text{LaCl}_3$ . They identified five phosphoproteins in which regulation by T-zeatin was lost in the presence of calcium-signaling inhibitors. There are few publications on the involvement of  $\text{Ca}^{2+}$  in the cytokinin signaling pathway in higher plants.

A good protocol for studying the role of  $\text{Ca}^{2+}$  in the studied processes is to change the level of  $\text{Ca}^{2+}$  either by treating the plant material with a hyperphysiological concentration of  $\text{Ca}^{2+}$  or by removing free available  $\text{Ca}^{2+}$  in the extracellular solution with EGTA, a selective calcium chelator which is not likely to enter the cell and reduce calcium ions in the

apoplast. Here we studied the effect of changes in the exogenous concentration of calcium on quantitative changes in Chl and LHCPII accumulation during greening enhanced by CKs in excised etiolated barley leaves and cucumber cotyledons. We examined the effect of EGTA as well as  $\text{CaCl}_2$  on the accumulation of these two compounds in detached etiolated barley leaves and cucumber cotyledons incubated under light in the presence of benzyladenine (BA) and kinetin (KIN) respectively. We also used  $\text{LaCl}_3$  in experiments on cucumber cotyledons.

## MATERIAL AND METHODS

### PLANT MATERIAL

Barley (*Hordeum vulgare* L. cv. Nagrat) and cucumber (*Cucumis sativus* L. cv. Racibór) seeds were surface-sterilized and soaked for 2–3 h in distilled water and placed on several layers of tissue paper moistened with distilled water at 25°C in the dark. Excised etiolated five-day-old barley leaves were incubated in water (control), 50  $\mu\text{M}$  BA, 20 mM EGTA, BA and EGTA, 5 mM  $\text{CaCl}_2$ , BA and  $\text{CaCl}_2$  for 14 h under continuous light (146  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ). Six-day-old detached etiolated cucumber cotyledons were pre-incubated in the dark for 14 h in water (control), 100  $\mu\text{M}$  KIN, 15 mM EGTA, KIN and EGTA, 1 mM  $\text{CaCl}_2$ , KIN and  $\text{CaCl}_2$ , 10 mM  $\text{LaCl}_3$  or KIN and  $\text{LaCl}_3$  and then exposed to light for 6 h. The experimental conditions (CKs, concentration of hormones, time of incubation) were selected in preliminary studies. The Chl level was the marker in these experiments. The concentration of EGTA and  $\text{LaCl}_3$  applied in the experiments inhibited Chl accumulation by ~50%.

### ISOLATION OF THYLAKOID-ENRICHED FRACTION

Thylakoid membranes from plant material were isolated according to Hilditch (1986), with modifications. Plant material was homogenized in a mortar in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.4 mM sucrose, 0.01 M KCl, 0.01 M  $\text{MgCl}_2$  and 6 mM  $\beta$ -mercaptoethanol. The homogenate was filtered and centrifuged for 10 min at 1500  $\times$  g. The chloroplast pellet was suspended in isolation buffer, layered on 3 ml of it containing 65% sucrose, and centrifuged as above. The chloroplasts were collected from the top of the sucrose layer and centrifuged as previously. Then the chloroplasts were osmotically shocked in isolation buffer without sucrose and chloroplast membranes were collected by centrifugation at 12,000  $\times$  g for 15 min. The membrane pellet was washed twice in buffer without sucrose and centrifuged as above. The thylakoid pellet was suspended in the buffer used for osmotic shock and

proteins were precipitated in 80% acetone and kept overnight at  $-20^{\circ}\text{C}$ . The protein pellet was resolved in buffer containing 125 mM Tris-HCl, pH 6.8, 10% mercaptoethanol, 3% SDS and 20% glycerol, and used for SDS-PAGE. Protein concentration was measured by Bradford's (1979) method.

#### PROTEIN BLOTTING

SDS-PAGE was performed according to Laemmli (1970). Proteins (10  $\mu\text{g}$ ) from isolated thylakoids were separated using 12% polyacrylamide slab gel electrophoresis and transferred onto PVDF membranes using a semi-dry blotting system. Immunoblotting was performed using the ExtrAvidin Peroxidase Staining Kit (Sigma, St. Louis, MO, USA). Anti-LHCPII antibodies raised by injection of rabbit with LHCPII isolated from carnation leaves (Legocka et al., 1990) were used to recognize LHCPII. These antibodies recognized two polypeptides: a major ( $27.5 \pm 0.7$  kDa) and a minor one ( $26.5 \pm 0.8$  kDa). Immunoblotting showed only the presence of the major polypeptide in the thylakoids isolated from barley leaves and cucumber cotyledons. The optimal dilution of antibodies as estimated by dot-blot was 1:5000. For quantification of the immunostained band of LHCPII we scanned the PVDF sheets using UMAX Alpha Vista II, and measured and calculated the staining intensity of the bands using Multi Gauge (Fuji Photo Film Co., Tokyo, Japan). Because the measurement system was not calibrated, the values are given in arbitrary units.

#### CHL DETERMINATION

Chlorophyll concentration was assayed according to Hiscox and Israelstam (1979).

#### STATISTICAL ANALYSIS

Each value is the mean of three or four biological replicates from three or four independent experiments (see figure captions). Statistical analyses employed Standard Methods STATISTICA (StatSoft, Tulsa, OK, USA).

### RESULTS

In excised etiolated barley leaves incubated for 14 h under light in the presence of 0.1 and 1 mM  $\text{CaCl}_2$  we found no differences in the Chl level; in the 5 mM  $\text{CaCl}_2$  treatment, Chl accumulation was 25% higher than in the control (Fig. 1). The higher concentration of  $\text{CaCl}_2$  (10 mM; 50 mM) inhibited Chl accumulation: in EGTA-treated leaves it was  $\sim 48\%$  lower than in the control (Fig. 2a, Tab. 1). In leaves incubated with BA the amount of Chl was  $\sim 48\%$  higher than in

the control (Fig. 2a, Tab. 1). Chl accumulation in leaves incubated in solution containing both BA and EGTA was  $\sim 56\%$  lower than in leaves treated with BA alone (Fig. 2a, Tab. 1). The hormonal effect on Chl accumulation was completely canceled by EGTA. Leaves treated with BA combined with 5 mM  $\text{CaCl}_2$  showed  $\sim 38\%$  lower Chl accumulation than leaves treated with BA alone (Fig. 2a, Tab. 1).

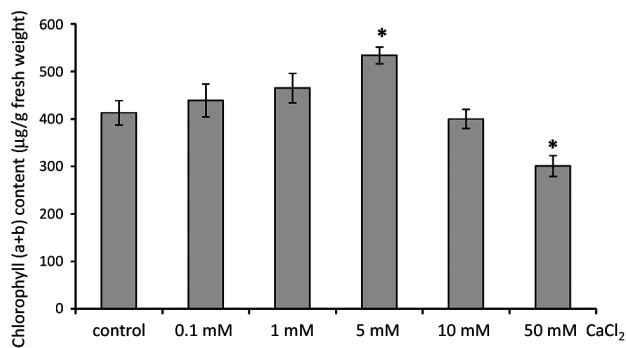
In leaves incubated under light in the presence of EGTA, LHCPII accumulation was  $\sim 28\%$  lower than in the control (Fig. 2b, Tab. 1). LHCPII accumulation significantly increased in BA-treated leaves, by  $\sim 150\%$  versus the control (Fig. 2b, Table 1). EGTA externally supplied to medium with BA decreased LHCPII accumulation by 30% versus treatment with BA only (Fig. 2b, Tab. 1). Dosing barley leaves with  $\text{CaCl}_2$  caused a 25% increase of LHCPII accumulation versus the control (Fig. 2b, Tab. 1).  $\text{CaCl}_2$  added to the BA medium almost totally canceled the cytokinin's effect on LHCPII accumulation (Fig. 2b, Tab. 1).

Cucumber cotyledons incubated with 1 mM  $\text{CaCl}_2$  showed  $\sim 21\%$  higher Chl accumulation than in the control; leaves incubated with 10 mM and 50 mM  $\text{CaCl}_2$  showed lower Chl levels (Fig. 3). EGTA suppressed Chl accumulation by  $\sim 55\%$  and KIN increased it by  $\sim 60\%$  versus the control (Fig. 4a, Tab. 1). As in the barley leaves, in cucumber cotyledons EGTA completely blocked the stimulatory effect of cytokinin on Chl accumulation (Fig. 4a, Tab. 1).  $\text{LaCl}_3$  lowered Chl accumulation in cotyledons by 53% versus the control (Fig. 4a, Tab. 1). This channel blocker inhibited the effect of KIN on Chl accumulation by only 20% (Fig. 4a, Tab. 1). In cotyledons treated with KIN and calcium the hormonal effect on Chl accumulation was reduced by  $\sim 23\%$  (Fig. 4a, Tab. 1).

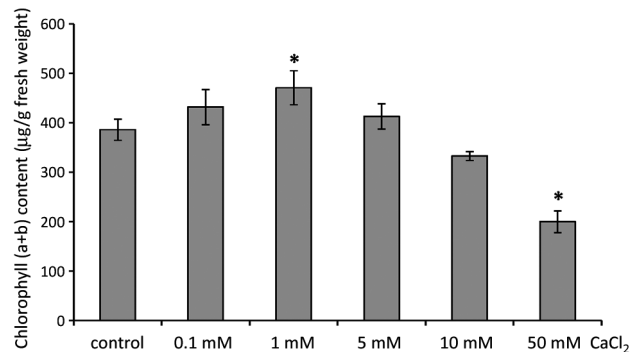
As in barley leaves, cucumber cotyledons incubated with KIN showed significantly higher LHCPII (160%) than the control (Fig. 4b, Tab. 1). EGTA decreased LHCPII accumulation by 32% versus the control and reduced the effect of KIN by  $\sim 77\%$  (Fig. 4b, Tab. 1). Exogenous  $\text{CaCl}_2$  reduced LHCPII accumulation by 29% versus the control. The effect of KIN on LHCPII accumulation was significantly affected by  $\text{CaCl}_2$ . Adding  $\text{CaCl}_2$  to KIN solution suppressed the hormone's effect on LHCPII accumulation almost totally (Fig. 4b, Tab. 1). The calcium channel blocker  $\text{LaCl}_3$  decreased LHCPII accumulation by  $\sim 76\%$  versus the control, and reduced the KIN effect by  $\sim 80\%$  (Fig. 4b, Tab. 1).

### DISCUSSION

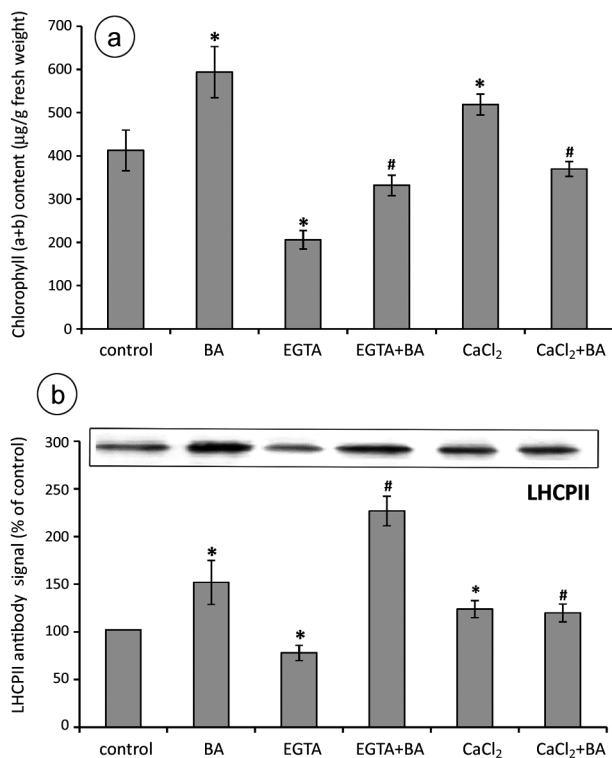
There is little published information on the potential role of  $\text{Ca}^{2+}$  during hormone-stimulated chloroplast development. Our results indicate that a source of



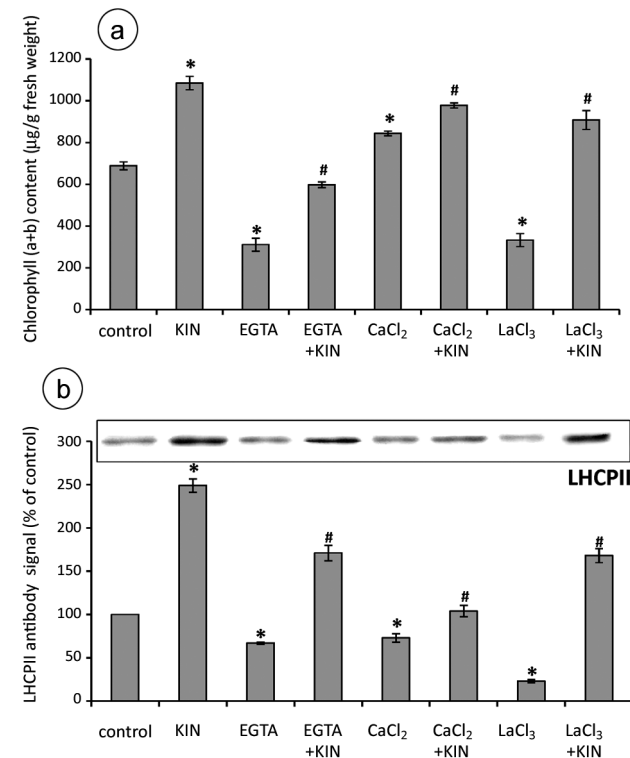
**Fig. 1.** Chlorophyll levels in excised etiolated barley leaves incubated under different CaCl<sub>2</sub> concentrations for 14 h under light. Values are means  $\pm$  SD of four independent experiments. Significant differences from the control are asterisked ( $p < 0.05$ ).



**Fig. 3.** Level of chlorophyll in excised etiolated cucumber cotyledons incubated in the dark in different concentrations of CaCl<sub>2</sub> for 14 h and then exposed to light for 6 h. Values are means  $\pm$  SD of four independent experiments. Significant differences from the control are asterisked ( $p < 0.05$ ).



**Fig. 2.** Effect of calcium depletion or elevation on chlorophyll and LHCPII accumulation during light-dependent chloroplast development stimulated by BA in barley leaves. Detached etiolated barley leaves were incubated in water (control), 50  $\mu$ M BA, 20 mM EGTA, BA + EGTA, 5 mM CaCl<sub>2</sub> or BA + CaCl<sub>2</sub> for 14 h under light. (a) chlorophyll level after incubating leaves in variants described above, (b) LHCPII accumulation after incubating leaves in variants described above. Values are means  $\pm$  SD of three independent experiments. Significant differences from the control are asterisked ( $p < 0.05$ ). Significant differences from the cytokinin treatment are hashed ( $p < 0.05$ ).



**Fig. 4.** Effect of calcium depletion or elevation on chlorophyll and LHCPII accumulation during light-dependent chloroplast development stimulated by KIN in cucumber cotyledons. Detached etiolated cucumber cotyledons were incubated in the dark for 14 h in water (control), 100  $\mu$ M KIN, 15 mM EGTA, KIN+EGTA, 5 mM CaCl<sub>2</sub>, KIN+CaCl<sub>2</sub>, 10 mM LaCl<sub>3</sub> or KIN+LaCl<sub>3</sub> and then exposed to light for 6 h. (a) Chlorophyll levels after incubating cotyledons in variants described above, (b) LHCPII accumulation after incubating cotyledons in variants described above. Values are means  $\pm$  SD of three independent experiments. Significant differences from the control are asterisked ( $p < 0.05$ ). Significant differences from the cytokinin treatment are hashed ( $p < 0.05$ ).



TABLE 1. Percentage of increase or decrease of chlorophyll and LHCPII accumulation in detached etiolated barley leaves and cucumber cotyledons exposed to light after treatment specified in the table versus their levels in the control (100%) and cytokinin (CK, 100%, grey field)

Treatment	Percentage changes of chlorophyll and LHCPII accumulation versus the control or the CK (grey field); (+) higher than control/CK, (-) lower than control/CK			
	barley leaves		cucumber cotyledons	
	chlorophyll	LHCPII	chlorophyll	LHCPII
Control	100	100	100	100
CK	+ 48	+ 150	+ 60	+ 160
EGTA	- 48	- 28	- 55	- 32
CK + EGTA	- 56	- 30	- 73	- 77
CaCl <sub>2</sub>	+ 25	+ 25	+ 21	- 29
CK + CaCl <sub>2</sub>	- 38	- 125	- 23	- 147
LaCl <sub>3</sub>	-	-	- 53	- 76
CK + LaCl <sub>3</sub>	-	-	- 20	- 80

apoplast reservoir Ca<sup>2+</sup> is required for light-induced Chl and LHCPII accumulation in excised etiolated barley leaves and cucumber cotyledons, and that Ca<sup>2+</sup> is also involved in the cytokinin signal transduction pathways that enhance Chl and LHCPII accumulation. This is in agreement with reports showing that Ca<sup>2+</sup> and calmodulin are components of the signal transduction pathway linking phytochrome phototransformation (Neuhaus et al., 1993; Bowler et al., 1994; Reiss and Bale, 1995). In both of our plant materials we found that after chelation of exogenous Ca<sup>2+</sup> by EGTA the accumulation of Chl was reduced by about one half, and LHCPII accumulation by about one third, suggesting that light-induced accumulation of both compounds is only partially dependent on exogenous Ca<sup>2+</sup>. The changes in chlorophyll content caused by treatment with EGTA were much stronger than the changes in LHCPII content. The need for Ca<sup>2+</sup> transport from external sources was confirmed in the experiments with LaCl<sub>3</sub> on cucumber cotyledons, in which the reduction of Chl accumulation was similar to that in the EGTA treatment and LHCPII accumulation was double that in the EGTA treatment. La<sup>3+</sup> does not enter the plant cell; it inhibits the action of Ca<sup>2+</sup> on the outside of the cell membrane, preventing elevation of cytosolic Ca<sup>2+</sup> (Tester, 1990; Belyavskaya, 1996). Ca<sup>2+</sup> and CaM are involved in the signal transduction pathway in induction of the early chlorophyll biosynthetic enzyme glutamate 1-semialdehyde aminotransferase, and Chl *b* levels control the level of light-harvesting complexes at least partially (Im et al., 1996; Horn et al., 2007).

The sites of Ca<sup>2+</sup> in the pathways of Chl and LHCPII biosynthesis are not known. Ca<sup>2+</sup> may participate in Chl and LHCPII accumulation at different levels, for example in regulating the expression of the genes for enzymes involved in chlorophyll syn-

thesis, which are of nuclear origin (Grimm, 1990; Im et al., 1996), or expression of *cab* genes (Neuhaus et al., 1993; Kushwaka et al., 2008), and/or during transport of these proteins to chloroplasts, or in events occurring inside chloroplasts (Rocha and Vothknecht, 2012). Importation of nuclear-encoded proteins into chloroplasts via translocation at the outer/inner envelope of the chloroplast (TOC/TIC) complex has been shown to be affected by calcium (Chigri et al., 2005). Calcium regulation at the TIC complex on the inner surface of the envelope membrane is mediated by CaM binding to TIC3 (Chirgi et al., 2006). Some HSP proteins related to the hsp-90 family, which play a role in protein trafficking, are known to be autophosphorylated in the presence of Ca<sup>2+</sup> (Csermely and Kahn, 1991).

The data suggest that CKs affect greening via increased ALA synthesis (Lew and Tsuji, 1982; Lechowski and Białczyk, 1993; Reiss and Beale, 1995). The levels of the two ALA synthesis enzymes glutamyl t-RNA reductase and glutamate 1-semialdehyde aminotransferase were elevated in dark-grown kinetin-treated barley seedlings (Yaronskaya et al., 2006). All the steps of Chl synthesis occur in the chloroplast but the enzymes elevated by CK are of nuclear origin. The question of whether CK acts on the expression of these enzymes in the nucleus, cytoplasm or chloroplast is still open. In our study, EGTA application almost completely eliminated the cytokinin's effect on the increase of Chl accumulation in both plants. These results suggest that cytokinin-stimulated Chl accumulation is an external Ca<sup>2+</sup>-dependent process in etiolated barley leaves and cucumber cotyledons incubated under light.

The effect of EGTA depends on the level of external Ca<sup>2+</sup>. In our experiments we used a rather

high concentration of EGTA: 20 mM EGTA in the study with barley leaves and 15 mM in experiments with cucumber cotyledons. We wanted to obtain a marked effect of the chelator on Chl accumulation. These concentrations reduced Chl accumulation by ~50% but did not appear to affect the viability or general metabolism of the plants: the profiles of SDS-PAGE-separated thylakoid proteins were similar to those of untreated plant material (data not shown).

The question for our study was whether CK-stimulated expression of LHCP II is  $\text{Ca}^{2+}$ -dependent. The CK effect on enhancement of LHCP II in the presence of EGTA was reduced somewhat in barley leaves but more strongly in cucumber cotyledons, suggesting that calcium is only partially involved in the effect of CK on the increase of LHCP II accumulation. Earlier studies demonstrated that CK may increase the steady-state mRNA level of certain chloroplast genes that depend on mRNA synthesis, processing and stability (Lerbs et al., 1984; Kasten et al., 1997). Flores and Tobin (1988) sought to verify whether CK treatment influences LHCP mRNA by increasing the rate of transcription or by decreasing the rate of mRNA turnover. They analyzed the transcript produced by nuclei isolated from cytokinin-treated and control *Lemna*. Cytokinin caused only 1.5-fold enhancement of transcription but LHCP mRNA in leaves was about 5 times more abundant in cytokinin-treated plants, suggesting that cytokinin increases LHCP mRNA abundance at a posttranscriptional level, possibly by making the mRNA more stable. Currently there are no literature data indicating whether calcium is involved in mRNA stabilization in plants. The effect of 15 min treatment of 7-day-old *Arabidopsis thaliana* seedlings with CK indicates the involvement of calcium ions in CK signaling (Romanov et al., 2002). Most of the differentially regulated proteins and phosphoproteins are located in chloroplasts, suggesting an as-yet uncharacterized direct signaling chain responsible for the action of CK in chloroplasts (Černý et al., 2010). Interlinking of CK and calcium signaling is supported by the loss of CK regulation of several phosphoproteins in chloroplasts following inhibition of calcium signaling (Černý et al., 2010).

Unexpectedly, in leaves and cotyledons greening in the presence of CK together with  $\text{CaCl}_2$  we found that added  $\text{CaCl}_2$  depressed the hormonal effect on accumulation of Chl, and in the case of LHCP II the effect of CK was almost totally canceled. It is difficult to explain this phenomenon. We might hypothesize that the involvement of different  $\text{Ca}^{2+}$  channels in CK signal transduction pathways results in an influx of  $\text{Ca}^{2+}$  from an external area to the cytoplasm, and that it has a negative effect on the cytokinin signaling pathway involved in LHCP II accumulation. The effect of CK on the increase of  $\text{Ca}^{2+}$  levels inside the

cell has been determined in some plant organs as probably being connected with the action of  $\text{Ca}^{2+}$  channels in cell membranes (Conrad and Hepler, 1988). CKs activate plasma-membrane ion channels in *Funaria* (Hahm and Saunders, 1991) and voltage-dependent dihydropyridine-sensitive  $\text{Ca}^{2+}$  channels in the plasma membrane in moss protoplasts (Schumaker and Gizinski, 1993) and in *Physcomitrella* (Schumaker and Dietrich, 1998).

These results support both a role for  $\text{Ca}^{2+}$  in CK-enhanced LHCP II accumulation and the independence of the  $\text{Ca}^{2+}$  effect from that involving phytochrome phototransformation. Light enhancement of LHCP II accumulation in both plants required endogenous 1–5 mM  $\text{CaCl}_2$ , and the same concentrations of calcium in the presence of CK decreased LHCP II accumulation almost to the control level.

Our results confirm that an external source of  $\text{Ca}^{2+}$  is required for light-induced and CK-stimulated Chl and LHCP II accumulation during the greening process in barley leaves and cucumber cotyledons. We showed, for the first time in experimental conditions, that CK enhancement of Chl accumulation induced by light apparently is dependent on the external pool of  $\text{Ca}^{2+}$ , whereas CK stimulation of LHCP II accumulation induced by light is only partially dependent on that pool of  $\text{Ca}^{2+}$ . We also showed for the first time that combining exogenous  $\text{CaCl}_2$  with CK strongly disturbs the effect of CK on stimulation of LHCP II accumulation. Our results indirectly suggest that the responses to cytokinin and light act on different events leading to Chl and LHCP II accumulation.

#### AUTHORS' CONTRIBUTION

JL designed and coordinated the study and drafted the manuscript; ES-N grew and prepared the plant material and did the chlorophyll analysis, thylakoid fraction isolation, immunoblotting of thylakoid proteins and densitometry. The authors declare that they have no conflicts of interest.

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