BIOLOGIA PLANTARUM 35 (1): 53-62, 1993

# Calcium mediated cytokinin action on chlorophyll synthesis in isolated embryo of Scots pine

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## Abstract

Chlorophyll (Chl) synthesis in isolated Scots pine embryos depended on exogenous application of cytokinin (CK) and $Ca^{2+}$. At a constant benzyladenine (BA) level ($4.4 \times 10^{-5}$ M) $10^{-4}$ to $10^{-2}$ M $Ca^{2+}$ concentrations in mineral medium were optimum for Chl biosynthesis under both light and dark. At a zero or very low ($10^{-6}$ M) concentration of external $Ca^{2+}$, Chl synthesis was relatively more $Ca^{2+}$-dependent in embryos cultured in darkness than in the light, which suggested that the light: (*a*) stimulated the transport of $Ca^{2+}$ from external sources to cytosol, and/or (*b*) interacted with $Ca^{2+}$ directly in the pathway of Chl biosynthesis. The need of external $Ca^{2+}$ was evidenced in experiments with modulators of $Ca^{2+}$-transport systems. The reduction of the inward current of $Ca^{2+}$ from readily accessible external sites by chelating agent (ethylene glycol-bis (beta-aminoethyl ether - N,N,N'N' - tetraacetic acid, EGTA) and $Ca^{2+}$-channel blockers canceled the formation of Chl. The effect of EGTA depended on the level of external $Ca^{2+}$. Inhibitory action of $Ca^{2+}$-channel blockers depended on their kind and concentration: at the $10^{-5}$ M concentration $La^{3+}$ > verapamil > nifedipine inhibited Chl formation. In the presence of $Ca^{2+}$, the $Ca^{2+}$-agonist A 23187 mimicked the BA effect and about 92 % of Chl was synthesized as compared with the BA variant. Low concentrations of calmodulin antagonists reduced the amounts of Chl. Calmodulin was included in a second messenger system for BA action in promoting Chl biosynthesis in isolated Scots pine embryos.

## Introduction

According to Bogorad (1950) cotyledons of gymnosperms are capable of Chl synthesis in darkness but the embryos must be connected with megagametophyte. However, isolated embryos of gymnosperms grown on mineral medium synthesize

*Received* 4 February 1992, *accepted* 2 April 1992.

*Abbreviations used*: BA - benzyladenine; CaM - calmodulin; Chl - chlorophyll; CK - cytokinin; DMSO - dimethylsulfoxide; EGTA - ethylene glycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid; ER - endoplasmatic reticulum; PChl - protochlorophyll; PChlide - protochlorophyllide, TFP- trifluoroperazine; W-7 - N-(6-aminohexyl)-5-chloro-1-naphtalene-sulfonamide.

Chl in the light (Engvild 1964). External supply of CK to the mineral medium with isolated embryos of *Pinus nigra* resulted in the synthesis of Chl in darkness (Jelić and Bogdanović 1988). In intact gymnosperm cotyledons CK may act as an endogenous factor in the regulation of greening (Jelić and Bogdanović 1990), in the dark CK may trigger Chl biosynthesis. This effect may include the activation of the enzymatic system involved in the synthesis of 5-amino-levulinic acid or it may influence membrane organization in such a way as to facilitate the enzyme reduction of PChlide (Jelić and Bogdanović 1988).

CK application causes a rise in intracellular $Ca^{2+}$ content, contributing to the formation of CaM, while CaM in turn causes a series of physiological changes (Hanson 1984, Hepler and Wayne 1985, Marmé 1986, Thomas 1986). In certain tissues CK induces an increased $Ca^{2+}$ absorption (Lejohn and Stevenson 1973, Lau and Yang 1975, Saunders and Hepler 1981, 1982, 1983, Kubowicz *at al.* 1982), probably by increasing the activity of $Ca^{2+}$-channels (Hepler and Wayne 1985, Saunders 1986). However, in excised cucumber cotyledons Zhao and Ross (1989) were not able to assess the connection of CK action with an increase in $Ca^{2+}$ content in cytosol and in CaM activation, and hence their effects on Chl synthesis. On the other hand, Kotzabasis *at al.* (1990) discovered that the levels of PChlide and PChl in the pigment mutant C-2A' of *Scenedesmus obliquus* grown in darkness depended upon $Ca^{2+}$ concentration in the growth medium.

The mechanism of CK action on Chl synthesis in embryo of pine is still not clear. The aim of the present work was to determine the effect of external $Ca^{2+}$ application on Chl synthesis in isolated Scots pine embryos grown in mineral medium in light and dark. We tested this effect by applying external CK and different drugs which modulated the opening of $Ca^{2+}$-channels (antagonist and an agonist), a $Ca^{2+}$ chelating agent, and CaM inhibitors.

## Materials and methods

**Embryos**: Seeds of *Pinus silvestris* L. were collected in a forest near Krzeszowice (Poland) and kept in refrigerator during three months. Seeds of black coloration and uniform mass of 6 - 9 mg with possibly largest embryos were selected for the trials (Wrześniewski 1982). They were paced in Petri dishes lined with one layer of *Whatman* No.*1* filter paper soaked with bidistilled water, and then transferred to a lightproof chamber at 20 °C. After 24 h imbibition the germs were isolated from seeds using a stereomicroscope; this moment was accepted as zero time of the experiment. The preparation of embryos was carried out in the very dim green safelight (about 0.5 μmol $m^{-2}$ $s^{-1}$). Embryos were divided into two groups for light and dark experiments. The isolated embryos were placed in plastic Petri dishes, 9 cm in diameter (30 embryos per dish); the dishes contained two layers of *Whatman* No.*1* filter paper and 10 $cm^3$ of the tested solution.

**Experimental procedure**: The embryos were cultivated in basal mineral medium (Reid and York 1958) or in tested medium. Depending on the kind of trial, different concentrations of $CaCl_2$, BA, modulators of $Ca^{2+}$ level in cells, and CaM

antagonists were added to the basal medium. The Petri dishes were pre-washed with $10^{-3}$ mol EGTA to remove $Ca^{2+}$ and then repeatedly washed with bi-distilled water. The tested solutions were changed every day during the experimental period. The embryos were cultured during 10 d in complete darkness or in continuous light. Each treatment was carried out eight times. The photosynthetically active irradiance was 500 μmol $m^{-2}$ $s^{-1}$ (mercury 400 W and fluorescence 40 W lamps, *LF-F, Polamp,* Poland). The photon flux density was measured with the *Li-Cor* model *LI-185 A* PFD meter (*Lambda Instruments*, Lincoln, Nebraska, U.S.A.). The temperature during the experiments was kept at 20 °C. All dark manipulations (*e.g.*, changes of the medium, Chl extraction) were carried out under a very dim green radiation.

**Chlorophyll determination**: In each experimental sample, Chl was extracted from 30 embryos in 80 % (v/v) acetone. The absorbance of acetone extracts were measured with the *Specord M 40* spectrophotometer (*Zeiss*, Jena, Germany). The amount of total Chl (*a+b*) was calculated according to Arnon (1949). In diagrams means obtained from about 240 embryos (eight series of 30 embryos each) are presented.

**Chemicals**: The chelating agent EGTA, CaM-antagonists W-7 and TFP, and $Ca^{2+}$-channel blockers $LaCl_3$ and verapamil were added to the experimental medium in aqueous solution. The nifedipine $Ca^{2+}$-channel blocker was dissolved in DMSO. The final concentration of DMSO never exceeded 0.1 % (v/v) of the tested medium. Control with 0.1 % DMSO showed that this concentration of organic solvent had no effect on Chl formation (about 99 % of the control without DMSO). The calcium ionophore *A 23187* was dissolved in a 1:1 mixture of absolute ethanol and DMSO to make a $10^{-3}$ M stock solution, and then diluted with mineral medium. The tested solutions were adjusted to pH 6.7 before using. All chemicals were purchased from *Sigma* (U.S.A.).

## Results

**Effect of $Ca^{2+}$ concentration**: Isolated embryos were cultured in a mineral medium containing $4.4 \times 10^{-5}$ M BA (synthetic cytokinin) (Jelić and Bogdanović 1988) and different concentrations of $CaCl_2$ (from zero to $10^{-1}$ M). During the 10-d cultivation period (Fig. 1) in a $CaCl_2$-free medium the embryos synthesized in the light and dark about 15 and 4.5 %, respectively, of Chl compared with embryos cultured in the medium containing $10^{-3}$ M $CaCl_2$, and at $10^{-6}$ M $Ca^{2+}$ about 69 and 12 %, respectively, of the amounts synthesized at $10^{-3}$ M $CaCl_2$.

Thus the dependence of Chl synthesis on external $Ca^{2+}$ was relatively more expressed in the dark than in light. This may suggest that light: (*a*) stimulates the transport of $Ca^{2+}$ to cytosol from external sources (cell wall, apoplastic or external medium) or internal reservoirs (vacuole, ER, mitochondria) enhancing its concentration, and/or (*b*) reacts with $Ca^{2+}$ directly in the pathway of Chl biosynthesis.

The optimum of external $Ca^{2+}$ concentration occurred in range from $10^{-4}$ - $10^{-2}$ M for embryos cultivated both in light and darkness. External $Ca^{2+}$ concentration of $10^{-1}$ M was toxic for Chl synthesis.

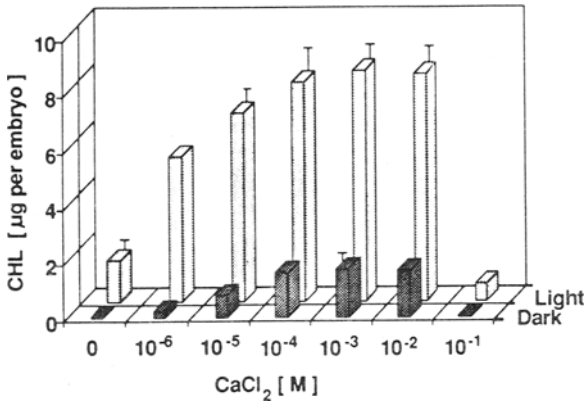


Fig.1. Concentrations of chlorophyll (Chl) in isolated embryos of *Pinus silvestris* L. after a 10-d cultivation in mineral solution containing benzyladenine (BA) $4.4 \times 10^{-5}$ M and different $CaCl_2$ concentrations. Bars represented the SE.

**Effect of BA and *A 23187*:** $4.4 \times 10^{-5}$ M BA was given to mineral solution with or without $10^{-3}$ M $CaCl_2$. BA applied together with $CaCl_2$ during a 10-d period induced maximum Chl synthesis in isolated Scots pine embryos (Fig. 2). In comparison with this combination taken as reference (100 %), Chl content in BA and $Ca^{2+}$-free mineral solutions was decreased to about 12 and 5 % in the light and dark, respectively. Similar Chl contents were found in embryos cultivated in $Ca^{2+}$-free mineral solution containing BA. Thus without a simultaneous accessibility of $Ca^{2+}$ the application of BA did not affect Chl biosynthesis.

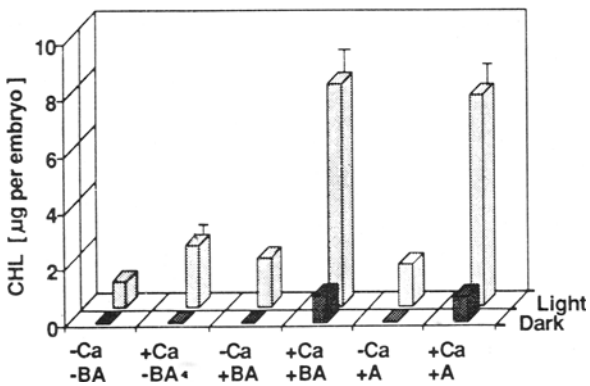


Fig.2. Chlorophyll (Chl) concentrations in isolated embryos of *Pinus silvestris* L. after a 10-d culture in mineral solution with or without $4.4\ 10^{-5}$ M benzyladenine (BA), $10^{-3}$ M $CaCl_2$ and $10^{-6}$ M *A 23187* (*A*).

The application of $Ca^{2+}$ to BA-free mineral solution caused an increase in Chl biosynthesis to 28 and 10 % in light and dark, respectively. A larger increase in Chl formation in light than in darkness confirmed the existence of processes, which occur solely or at a higher rate under irradiance (*cf.* Fig. 1). The application of $10^{-6}$ M calcium ionophore *A 23187* additionally confirmed the role of $Ca^{2+}$, in Chl formation (Fig. 2). *A 23187*, a known agonist of $Ca^{2+}$ thus, mimicked the action of BA.

**Effect of EGTA**: EGTA chelates $Ca^{2+}$ from readily accessible external sites and modifies the inward current of $Ca^{2+}$ to cytosol. EGTA may thus affect the response induced by BA. The reduction of $Ca^{2+}$ effect controlled by EGTA suggests that external $Ca^{2+}$ may be involved in the action mechanism of Chl synthesis in isolated Scots pine embryos. At the constant BA level ($4.4 \times 10^{-5}$ M) the effect of EGTA on Chl synthesis depended on its and $CaCl_2$ concentrations in the medium (Fig. 3).

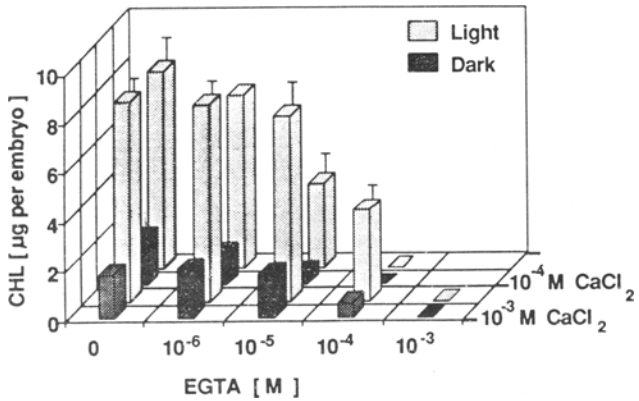


Fig.3. Effect of different ethylene glycol-bis(beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and $CaCl_2$ concentrations in mineral solution on chlorophyll (Chl) accumulation in isolated embryos of *Pinus silvestris* L. after 10-d cultivation in mineral solution containing benzyladenine, (BA) $4.4 \times 10^{-5}$ M.

EGTA externally supplied to the medium with $CaCl_2$ reduced the extent of Chl synthesis, especially at a lower ($10^{-4}$ M) $CaCl_2$ concentration in the medium. At $10^{-3}$ M $CaCl_2$ and $10^{-4}$ M EGTA in the medium, Chl formation fell to about 42 and 30 % in embryos in the light and darkness, respectively, as compared with the respective control (0 M EGTA). A decrease in $CaCl_2$ concentration to $10^{-4}$ M reduced Chl synthesis to about 5 %, both in the light and dark. At $10^{-3}$ M EGTA in the medium, Chl synthesis was completely inhibited. This result indicated that the limited accessibility of external $Ca^{2+}$ inhibited the effect of BA on Chl formation.

**Effect of $Ca^{2+}$-channel blockers:** The necessity of $Ca^{2+}$ transport from external sources (*e.g.* wall space, medium) was ascertained by experiments showing that both the inorganic ($La^{3+}$) and organic (verapamil-class: phenylakylamine, and nifedipine-class: dihydropyridine) $Ca^{2+}$-channel blockers inhibited Chl biosynthesis.

Different concentrations of these blockers were applied to mineral solutions containing $10^{-3}$ M $CaCl_2$ and $4.4 \times 10^{-5}$ M BA (Fig. 4).

At all tested concentrations, $La^{3+}$ was the most effective in inhibiting Chl formation in both light and dark. The organic blockers were less effective: in light verapamil was more effective than nifedipine, but in the dark both blockers were equally effective. $La^{3+}$ is known as irreversibly binding and blocking $Ca^{2+}$-channels (Saunders and Hepler 1983, Tsutsui *et al.* 1987) with a low specifity. $La^{3+}$ does not enter plant cells (Thomson *et al.*1973) and may inhibit the action of $Ca^{2+}$ on the external site of plasmalemma. As the used growth medium contained $10^{-3}$ M $CaCl_2$, the competition between $Ca^{2+}$ and the applied organic $Ca^{2+}$-channel blockers might cause the high concentrations of blockers needed for complete Chl inhibition in our long-term experiments.

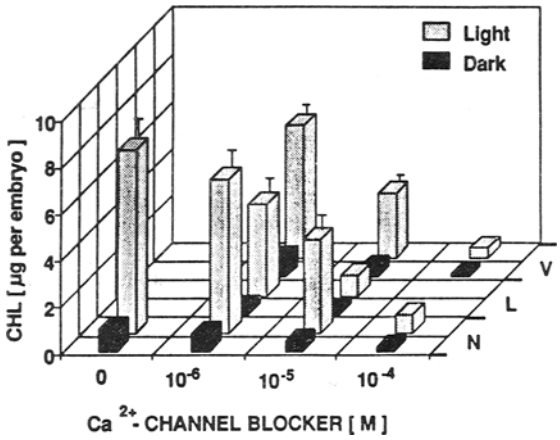


Fig.4. Effect of different concentrations of $Ca^{2+}$-channel blockers (L - $La^{3+}$ , N -nifedipine, V - verapamil) on chlorophyll (Chl) formation in isolated embryos of *Pinus silvestris* L. after a 10-d cultivation in mineral solution containing $4.4 \times 10^{-5}$ M benzyladenine and $10^{-3}$ M $CaCl_2$.

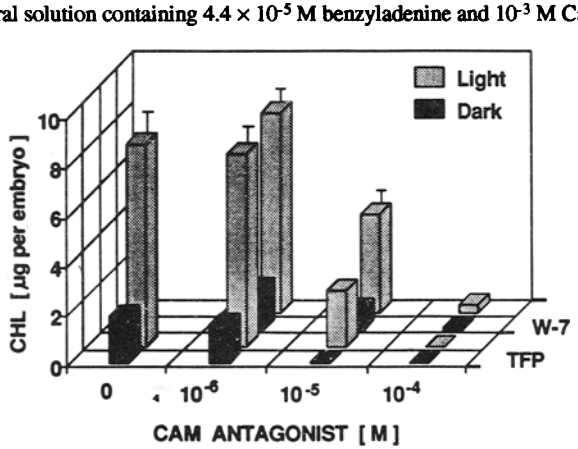


Fig.5. Chlorophyll (Chl) accumulation in isolated embryos of *Pinus silvestris* L. after a 10-d cultivation in mineral solution containing 4.4 × Ń $10^{-5}$ M benzyladenine and different concentrations of calmodulin (CaM) antagonists.

**Effect of CaM antagonists**: CaM is activated by $Ca^{2+}$ after its concentration in cytosol is enhanced to a micromolar level (Poovaiah and Reddy 1987).
CaM antagonists (phenothiazine class: TFP, naphtalenesulphonamide class: W-7) in $10^{-6}$ M concentration did not affect Chl formation (Fig. 5). At $10^{-5}$ M the action of TFP was over 40 % stronger than that of W-7. At higher concentrations the two drugs completely inhibited Chl synthesis, *i.e.* they suppressed the BA action. Our results also confirmed that the effects of CK required the formation of $Ca^{2+}$-CaM.

## Discussion

Phytohormone action is mediated by an increase in $Ca^{2+}$ level in cytosol and activation of CaM (Poovaiah and Reddy 1987). $Ca^{2+}$ concentration in cytosol is less than $10^{-6}$ M and the action of CaM depends on precisely regulated increases in $Ca^{2+}$ concentration only (Marmé 1986, Thomas 1986). Increases in $Ca^{2+}$ concentration in cytosol might result from the enhanced import from extracellular media or from a subcellular organelle (wall, ER, and vacuole). The effect of CK on an increase in internal $Ca^{2+}$ level in cell was determined in some plant organs (Lau and Yang 1975, Saunders and Hepler 1981, 1982, 1983, Kubowicz *et al.* 1982), being probably connected with the action of $Ca^{2+}$-channel in plasma membranes (Saunders 1986). CK causes a rise in intracellular $Ca^{2+}$; $Ca^{2+}$ -CaM is then formed, and CaM in turn causes a series of physiological changes which lead to the response. The level of (endo- and exogenous) CK is responsible for Chl accumulation, as shown by Jelić and Bogdanović (1988, 1990) in isolated embryos of *Pinus nigra* grown on mineral nutrients. In the dark the accumulation of Chl in isolated embryos is possible only after a CK treatment. Moreover, within 10 d of BA treatment in the light cultivated embryos the Chl level increased about four times as compared with the control (Jelić and Bogdanović 1988).

The main assumption of our work with $CaCl_2$, $Ca^{2+}$-channel blockers, $Ca^{2+}$ chelating agent EGTA, and calcium ionophore *A 23187*, which affected $Ca^{2+}$ level in cytosol and brought about Chl synthesis, was that these processes depended on $Ca^{2+}$ as a second messenger for BA action. Each compound modified the level of $Ca^{2+}$ in cytosol and thus affected the response to BA. The results of our experiments indicated a stronger influence of light than of darkness on Chl formation in isolated Scots pine embryos cultured on mineral nutrients containing BA and $CaCl_2$ (Figs. 1 and 2). In several kinds of plant cells the $Ca^{2+}$ transport was under light control (Hale and Roux 1980, Das and Sopory 1985). Enhanced $Ca^{2+}$ uptake after treatment in light was recently confirmed for characean cells (MacRobbie and Banfield 1988). Calcium moves through membranes using discrete channels regulated by ligand-receptor interactions; their properties can be elucidated through electrophysiological studies (Deitmer 1987). $Ca^{2+}$-channel blockers affected Chl synthesis to a different degree in isolated embryos (Fig. 4). Nevertheless, each of the applied blockers modified the investigated process by decreasing the activity of $Ca^{2+}$-channels and thus inhibiting Chl biosynthesis in the sequence $La^{3+} > V > N$. $La^{3+}$ binds and blocks $Ca^{2+}$-channels irreversibly (Saunders and Hepler 1983, Tsutsui *et al.* 1987), but it is not very specific (Segal 1986). Nifedipine binds a special kind of receptor molecule of the $Ca^{2+}$-channels and blocks them; verapamil also binds this molecule

but its specifity and the mode of binding are different (Murphy *et al.* 1983). The literature presents various, sometimes antagonistic activities of different $Ca^{2+}$-channels blockers, as depending on plant species and the kind of tissue or process (Shina and Tazawa 1987, Graziana *et al.* 1988, Takagi and Nagai 1988, Roberts and Haigler 1990).

The application of calcium ionophore *A 23187* which increased the concentration of $Ca^{2+}$ in cytosol (Artalejo and Garcio-Sancho 1988) induced Chl synthesis in embryos cultured in dark conditions, thus mimicking the BA effect (Fig. 5). Hence $Ca^{2+}$ ions are indispensable in Chl synthesis. The comparison of Figs. 1 and 2 suggested that the action of CK was mediated by $Ca^{2+}$. The results obtained with EGTA, an $Ca^{2+}$ chelating agent, confirmed this assumption (Fig. 3), showing that the $Ca^{2+}$ influx from extracellular compartments and the medium plays a major role in the formation and maintenance of Chl level.

Our results on the effect of $Ca^{2+}$ level of Chl synthesis agree with those of Peschek *et al.* (1989) and Kotzabasis *et al.* (1990). The site of $Ca^{2+}$ action on the pathway of Chl biosynthesis is not known. As shown by Kotzabasis *et al.* (1990), in the absence of external calcium no light-dependent PChlide formation and/or accumulation of PChl take place in the pigment mutant C-20 A' of *Scenedesmus obliquus*. The above authors suggest that this effect may be connected with: (*a*) inactivation of PChlide oxidoreductase and canceled possibility of binding PChlide to it, or (*b*) reduced chlorophyllide is photooxidized in the absence of $Ca^{2+}$. Peschek *et al.* (1989) reported that a light-independent PChlide oxidoreductase in plasma membrane isolated from cyanobacterium *Anacystis nidulans*, was active in the presence of $CaCl_2$, and that this activity was abolished by EGTA.

Both our data and those of Peschek *et al.* (1989) and Kotzabasis *et al.* (1990) on the role of $Ca^{2+}$ in Chl accumulation contradict those obtained by Zhao and Ross (1989) in experiments with cucumber cotyledons. According to Zhao and Ross (1989) there is no special requirement of $Ca^{2+}$ or CaM for the action of CK on Chl formation in these organs even if a high concentration of $Ca^{2+}$-channel blockers, CaM antagonists and *A 23187* were applied. These results might be associated with the kind of plant material or differences in the level of $Ca^{2+}$ stored in intracellular organelles. The results of our experiments (Fig. 5) showed that at low concentrations the CaM antagonists inhibited Chl formation. We conclude that CaM is included in second messenger system for CK promotion of Chl biosynthesis in isolated Scots pine embryos.

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