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The requirements for Ca^{2+}, protein phosphorylation and concurrent protein synthesis for zeatin signaling of acidic chitinase transcript accumulation in *Cucumis sativus* L.

Sonali P. Barwe, Muthukrishnan Sathiyabama, Chelliah Jayabaskaran*

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

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Summary

We have previously reported that both Ca^{2+} and staurosporine-sensitive protein kinase(s) are involved in the cytokinin zeatin induction of cucumber chitinase activity and its protein content (Barwe et al. 2001). To further characterize signal transduction events involved in this cytokinin induction of chitinase gene expression, Northern hybridizations of total RNAs prepared from excised, dark-grown cucumber cotyledons treated with cytokinins and/or various agonists and antagonists of signal transduction components, were carried out using a cucumber acidic chitinase (CACHT) cDNA probe (Metraux et al. 1989). CACHT mRNA increased by approximately 5- to 6-fold in response to exogenous zeatin (Z), zeatin riboside (ZR), and benzyladenine (BA) treatment, but failed to accumulate in response to kinetin (K). Among the cytokinins tested, Z was most effective. The Z-induced accumulation of CACHT mRNA was inhibited by a plasma membrane Ca^{2+} channel blocker verapamil. Treatment of cotyledons with exogenous $CaCl_2$ and calcium ionophore A23187 in the presence and absence of cytokinin enhanced CACHT mRNA accumulation. These two observations suggest the participation of extracellular calcium in signaling Z-induction. Furthermore, the presence of staurosporine (an inhibitor of protein kinase) in Z treatment reduced CACHT mRNA, suggesting the involvement of phosphorylation of one or more cellular proteins. In addition, we provide evidence that the Z-induction of CACHT mRNA is blocked by protein synthesis inhibitor cycloheximide treatment. Taken together, these results suggest that Ca^{2+} influx from extracellular space, protein phosphorylation, and concurrent protein synthesis events participate in cytokinin signaling during Z-induced CACHT transcript accumulation.

Key words: Ca^{2+} – chitinase-cytokinin signaling – *Cucumis sativus* L. – protein phosphorylation – transcript level – zeatin

Abbreviations: BA benzyladenine. – CACHT cucumber acidic chitinase. – CHX cycloheximide. – EGTA ethylene glycol. – bis(2-amino-ethylether) tetraacetic acid. – K kinetin. – NaF sodium fluoride. – ST staurosporine. – Z zeatin. – ZR zeatin riboside

* E-mail corresponding author: cjb@biochem.iisc.ernet.in

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Introduction

Cytokinins are of interest in view of their possible regulatory roles in several stages of plant development. They induce cell division, cell expansion in cotyledon, chloroplast and etioplast development, suppression of apical dominance and senescence, and differentiation of *in vitro* cultured cells (Letham and Palni 1983, Evans 1984). There is a considerable amount of evidence to show that treatment of intact plants or excised organs with exogenous cytokinins stimulates the activity of several enzymes and/or their respective mRNAs. These include ribulose-1,5-biosphophate carboxylase/oxygenase (Flores and Tobin 1986), PEP carboxylase (Suzuki et al. 1994), protein kinase (Sano and Youssefian 1994), nitrate reductase (Dilworth and Kende 1974, Lu et al. 1990), chitinase (Memelink et al. 1987), chalcone synthase, chalcone isomerase, dihydrofolate reductase, and phenylalanine ammonia lyase (Deikman and Hammer 1995). These studies demonstrate that although gene expression is enhanced by cytokinin, the molecular mechanisms controlling the expression of these genes appear to be different for different enzymes. For instance, the expression of cytokinin-inducible genes encoding PEP carboxylase, nitrate reductase, chalcone synthase, and dihydrofolate reductase is regulated at the level of transcription (Dilworth and Kende 1974, Lu et al. 1990, Suzuki et al. 1994, Deikman and Hammer 1995), whereas the expression of genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, chalcone isomerase and phenylalanine ammonia lyase is regulated at the post-transcriptional level (Flores and Tobin 1986, Deikman and Hammer 1995). Even with this present knowledge of the role of these hormones in the enhancement of expression of a variety of genes, very little is known about how cytokinin signals are perceived and the components required for transducing them.

There have been few reports on the possible involvement of the two-component regulatory system, typically consisting of a sensor histidine kinase and a response regulator in the signal transduction of cytokinins (Kakimoto 1996, Schmulling et al. 1997, Kakimoto 1998, Sakakibara et al. 1999). Sakakibara et al. (1998) isolated a cytokinin-inducible gene, ZmCip1, encoding a homologue of a response-regulator of a bacterial two-component signaling system. The authors showed that the steady-state level of the ZmCip1 transcript was markedly enhanced by cytokinin treatment of the excised maize leaves. In *Arabidosis thaliana*, CKI1, a gene encoding homologues domains of His-protein kinase and response regulator, was isolated using activation tagging, and overexpression of this gene induced shoot development from callus in the absence of exogenous cytokinins, suggesting that the two-component system is involved in cytokinin signal transduction (Kakimoto 1996). Further, a role for protein phosphorylation/dephosphorylation has been suggested on the basis of studies using inhibitors of protein kinases and protein phosphatases in the expression of cytokinin inducible genes such as soybean β-expansin gene (Crowell 1994, Downes and Crowell 1998) and *Sesbania rostrata* early nodulin gene *SrEnod2* (Schmulling et al. 1997).

This study presents an effort to elucidate the mechanism of cytokinin-induced gene activation and includes the identification of the components of cytokinin signal transduction. We have earlier shown by TaqMan (Quantitative RT-PCR) analyses (Rajesh 2000) that cytokinin treatment of dark-grown, excised cucumber cotyledons induced the expression of the genes encoding a calcium-dependent protein kinase (CuCDPK3) and the FtsZ plastid division protein. We have also demonstrated the induction of chitinase activity by exogenous cytokinin in dark-grown, excised cucumber cotyledons and the involvement of Ca^{2+} and staurosporine-sensitive protein kinase in cytokinin-induced stimulation of chitinase activity and its protein accumulation (Barwe et al. 2001). In this paper we have investigated the effects of cytokinins on CACHT transcript accumulation, and possible second messengers and signaling components involved in the cytokinin-mediated induction of CACHT transcript accumulation. The results demonstrate that CACHT transcript accumulation is enhanced by cytokinin zeatin, and that this mechanism requires Ca^{2+} influx from extracellular space, protein phosphorylation, and ongoing protein synthesis.

Materials and Methods

Chemicals and materials

The various chemicals and other materials used in this study were obtained from commercial sources as follows: acrylamide, benzyladenine, calcium chloride, cycloheximide, Ca^{2+} ionophore A23187, EGTA, kinetin, staurosporine, Tris base, zeatin, and zeatin riboside from Sigma Chemical Co., USA; nylon membranes from Amersham Pharmacia Biotech, USA, cucumber (*Cucumis sativus* L. cv. Poinsette) seeds from National Seed Corporation, Bangalore, India. Cucumber acidic chitinase cDNA clone was a kind gift from Dr. Metraux, Ciba-Geigy, Agricultural Centre, Switzerland. [α^{32}-P]-dCTP was obtained from New England Nuclear, USA. All other chemicals used were of analytical grade.

Plant material and excised cotyledon treatments

Cucumber (*Cucumis sativus* L. var. Poinsette) seeds were germinated, and grown in the dark at 28 ˚C, as previously described (Barwe et al. 2001). After 7 days, cotyledons were excised under dim green light, transferred to small Petri dishes, and pre-incubated in distilled water for 24 h in the dark to deplete endogenous cytokinins. Treatments of the cotyledons with cytokinin (BA, Z, ZR, or K) and/or with one of the other chemicals, such as staurosporine, sodium fluoride, $CaCl_2$, calcium ionophore A23187, calcium antagonists (EGTA, verapamil), $MgCl_2$, or cycloheximide were carried out as described previously (Barwe et al. 2001). The treated as well as water-treated (control) cotyledons were collected, rinsed with distilled water, and surface blotted carefully with filter paper. Cotyledon samples were frozen in liquid nitrogen and stored at –80 ˚C until extraction of total RNA.

Total RNA isolation and Northern blot analysis

Total RNA was isolated from frozen cucumber cotyledons according to the method described by Verwoerd et al. (1989). The RNA was subjected to electrophoresis on agarose gels that contained formaldehyde, and blotted onto nylon membranes (Sambrook et al. 1989). The blots were probed with a cucumber acidic chitinase cDNA (Metraux et al. 1989) that had been labeled by random-primed labelling with [α^{32}-P]-dCTP (Feinberg and Vogelstein 1984). The blots were placed in hybridization bottles, subjected to pre-hybridization in hurch buffer containing 0.5 mol/L sodium phosphate buffer, pH 7.2, 10 mmol/L EDTA, and 7% (w/v) SDS for 3 h at 65 °C in a rotating hybridization oven, and subsequently hybridized to the probe in the same condition. After hybridization, the membranes were washed once with 2×SSC and 0.5% SDS at room temperature for 15 min, and then twice with 0.1×SSC and 0.5% SDS at 65 °C for 30 min. They were then exposed to X-ray film with an intensifying screen at –70 °C. Relative levels of RNA were determined by densitometry scanning of the autoradiogram using the TotalLab Analysis software from Phoretix.

Results

The effects of cytokinins on cucumber acidic chitinase (CACHT) transcript accumulation

In order to determine if cytokinin treatment influences CACHT transcript level by increasing the rate of transcriptional initiation and/or by altering some post transcriptional event, total RNA was isolated from cotyledons treated with various cytokinins and Northern blot analysis was performed. The Northern blot hybridization and CACHT transcript quantitation data are shown in Figures 1A and C. Hybridization with the ^{32}P-labeled CACHT cDNA probe revealed a major transcript of 1.0 kb corresponding to the size of the CACHT mRNA (Metraux et al. 1989). The level of CACHT transcript increased by approximately 5- to 6-fold following BA, ZR, and Z treatment. BA and ZR were equally effective while Z appeared to be a slightly stronger effector among other cytokinins. K was totally ineffective and did not affect the level of the transcript. We also examined the same blot by hybridization with an 18S rDNA probe. As anticipated the level of 18S rRNA detected with its probe did not change upon treatment with cytokinins (Fig. 1B). This indicates that the observed effects of cytokinins in the accumulation of CACHT transcripts were specific.

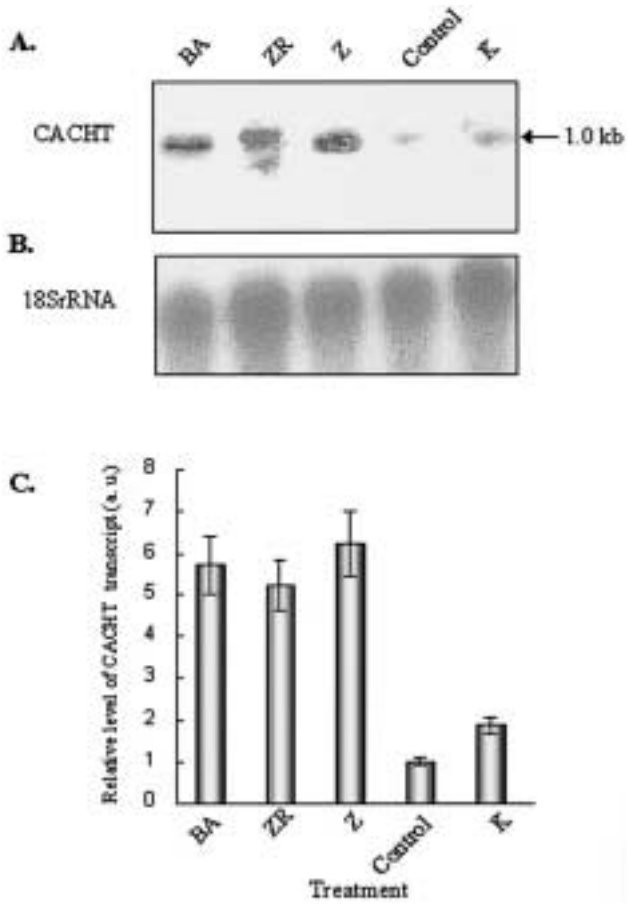

Figure 1. Effects of various cytokinins on the accumulation of CACHT transcripts (A) and 18S rRNA (B). Total RNA was isolated from excised, dark-grown cucumber cotyledons 48 h after treatment with water (control), benzyladenine (BA), zeatin riboside (ZR), zeatin (Z), and kinetin (K). All tested cytokinins were used at a concentration of 100 µmol/L. Ten micrograms of total RNA from each sample were separated electrophoretically in each lane and analysed by Northern hybridization with ^{32}P-labeled CACHT cDNA and 18S rRNA gene probes (see Materials and Methods). The Northern blot analysis was repeated twice, with similar results. C. Quantifications of CACHT transcripts. The relative levels of CACHT transcript were measured by densitometric scanning of autoradiograms. The level of CACHT transcript present in the water-treated (control) cotyledons was set as 1. Values are the means and SD from two separate experiments for each treatment.

Role of Ca^{2+} in Z-induced CACHT transcript accumulation

We investigated the role of Ca^{2+} in the accumulation of CACHT transcript in general, and zeatin-induced accumulation of CACHT transcript in particular, using the CACHT cDNA probe. Northern blot was carried out using total RNA isolated from cotyledons treated with agents that influence intracellular Ca^{2+} concentrations. The results of the Northern analysis are presented in Figures 2A and B. Interestingly, the CACHT transcripts increased upon either $CaCl_2$ or zeatin treatment; however, no further increase was observed in the $CaCl_2$ plus Z-treated cotyledons. This observation clearly indicates that an increase in the intracellular Ca^{2+} level might be involved in the observed zeatin stimulation of CACHT transcript accumulation. To confirm the involvement of Ca^{2+} in CACHT transcript accumulation, the cotyledons were treated with a specific calcium chelator, EGTA, and the Ca^{2+}-induced CACHT transcript accumulation was examined. Chelation of extracellular Ca^{2+} with EGTA inhibited the Ca^{2+}-induced expression of CACHT mRNA to a level similar to that of the water-treated

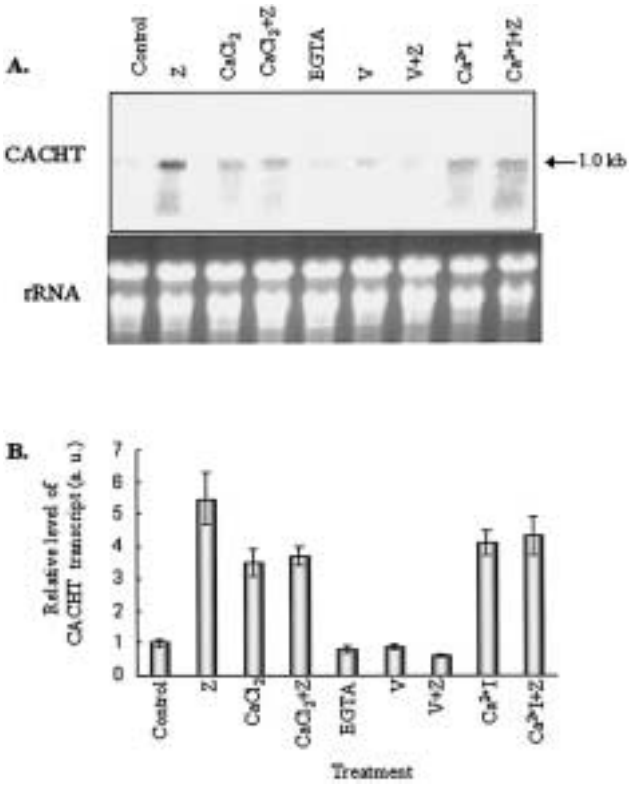

Figure 2. Effects of Ca^{2+}, EGTA, Ca^{2+} ionophore (Ca^{2+}I), and Ca^{2+} channel blocker verapamil (V) on uninduced and Z-induced accumulation of CACHT transcripts. Total RNA was isolated from excised, dark-grown cucumber cotyledons 48 h after treatment with water (control), 100 µmol/L zeatin (Z), 5 mmol/L calcium chloride ($CaCl_2$), 5 mmol/L calcium chloride plus 100 µmol/L zeatin ($CaCl_2$+Z), 5 mmol/L EGTA (EGTA), 10 µmol/L verapamil (V), 10 µmol/L verapamil plus 100 µmol/L zeatin (V+Z), 100 µmol/L calcium ionophore (Ca^{2+}I), and 100 µmol/L calcium ionophore plus 100 µmol/L zeatin (Ca^{2+}I+Z). Northern blot analysis was carried out as described in the legend to Figure 1. The rRNA detected with ethidium bromide was indicated as the loading control. B. Densitometric quantitation of CACHT transcript levels. Other explanations are as described in the legend to Figure 1C.

(control) cotyledons. To further test the role of extracellular Ca^{2+} in the Z-induced CACHT transcript accumulation, the plasma membrane Ca^{2+} channel blocker, verapamil, and Ca^{2+} ionophore A23187 were used. Treatment of cotyledons with the Ca^{2+} ionophore reproduced the regulatory effect of Ca^{2+} on Z-induced accumulation of CACHT transcript. Verapamil treatment inhibited the Z-induced CACHT transcript accumulation. These results indicate that Z-induced CACHT transcript accumulation is due to the elevations in cellular Ca^{2+} concentrations as a result of Ca^{2+} uptake from the extracellular space through plasma-membrane channels.

Role of protein phosphorylation in Z-induced CACHT transcript accumulation

To determine whether protein phosphorylation/dephosphorylation is involved in Z-induced CACHT transcript accumulation, the cotyledons were treated with zeatin in the presence or absence of inhibitors of protein kinase and protein phosphatase, and the Z-induced CACHT transcript accumulation was examined by Northern analysis. As shown in Figures 3 A and B, staurosporine strongly inhibited Z-induced CACHT transcript accumulation, whereas NaF treatment showed no change. Water-treated (control) cotyledons indicated the basal level of CACHT transcript accumulation, which was not affected by treatment with dimethylsulfoxide (DMSO), the solvent used for dissolving staurosporine. The former result indicates that protein kinase activity is involved in cytokinin regulation of CACHT transcript level.

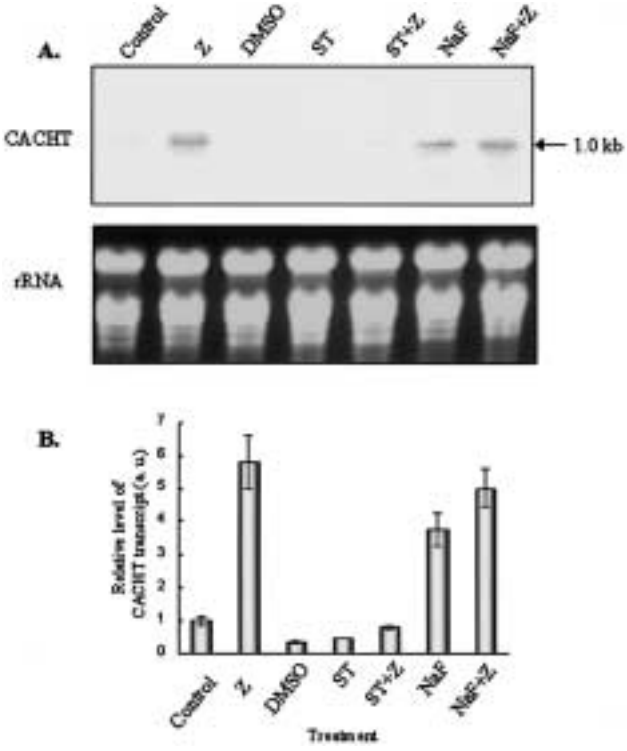

Figure 3. Effects of inhibitors of protein kinase (staurosporine, ST) and protein phosphatase (sodium fluoride, NaF) on uninduced and Z-induced accumulation of CACHT transcripts. Total RNA was isolated from excised, dark-grown cucumber cotyledons 48 h after treatment with water (control), 100 µmol/L zeatin (Z), 0.1 % DMSO, 10 nmol/L staurosporine (ST), 10 nmol/L staurosporine plus 100 µmol/L zeatin (ST+Z), 1 mmol/L sodium fluoride (NaF), 1 mmol/L sodium fluoride plus 100 µmol/L zeatin (NaF+Z). Northern blot analysis was carried out as described in the legend to Figure 1. The rRNA detected with ethidium bromide was indicated as the loading control. B. Densitometric quantification of CACHT transcript levels. Other explanations are as described in the legend to Figure 1C.

Requirement of protein synthesis in the Z-induced CACHT transcript accumulation

To investigate whether the enhancement of CACHT transcript accumulation by zeatin was dependent on concurrent protein synthesis, the effect of cycloheximide, a protein synthesis inhibitor, on the basal as well as Z-induced CACHT transcript level was studied. Excised, dark-grown cucumber cotyledons were treated with 100 µmol/L cycloheximide in the presence or absence of 100 µmol/L Z, after which RNA was isolated, and CACHT transcript levels were examined by Northern blot hybridization. As shown in Figures 4 A and B, cycloheximide treatment completely inhibited the Z-induced accumulation of CACHT transcript. The decrease in the basal level of CACHT transcript after treatment with cycloheximide was not as pronounced as the decrease in the level of Z-induced CACHT transcript. This data suggests that ongoing protein synthesis is required for the cytokinin-stimulated increased level of CACHT transcript accumulation.

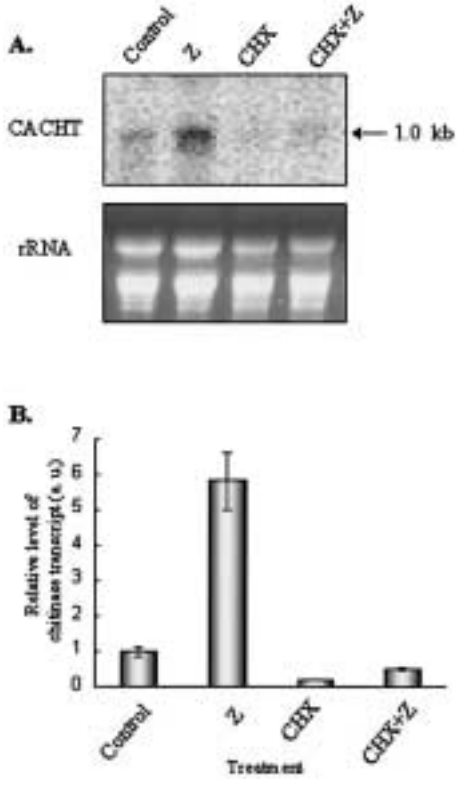

Figure 4. Effect of cycloheximide on uninduced and Z-induced accumulation of CACHT transcripts. Total RNA was isolated from excised, dark-grown cucumber cotyledons 48 h after treatment with water (control), 100 µmol/L zeatin (Z), 100 µmol/L cycloheximide (CHX) and 100 µmol/L cycloheximide plus 100 µmol/L zeatin (CHX + Z). Northern blot analysis was carried out as described in legend to Figure 1. The rRNA detected with ethidium bromide was indicated as the loading control. B. Densitometric quantification of CACHT transcript levels. Other explanations are as described in the legend to Figure 1C.

Discussion

Chitinase has been shown to be expressed constitutively in cucumber (Majean et al. 1990, Barwe et al. 2001), and was induced by elicitors, wounding, pathogens, salicylic acid, and phytohormones, such as abscisic acid, ethylene, and cytokinins (Mauch et al. 1988, Siegrist and Kauss 1990, Zhang and Punja 1994, Yu et al. 1998, Petruzzelli et al. 1999, Barwe et al. 2001). A cDNA encoding an acidic chitinase has been isolated and characterized from cucumber: its gene has been shown to be present as a single copy gene in the cucumber genome, and its protein was found to have no significant homology with either the basic chitinase isolated from bean or tobacco or the chitinase isolated from *Serratia marcescens* (Metraux et al. 1989). We were able to use this cDNA as probe to determine effects of cytokinins and/or various agonists and antagonists of signal transduction as part of a study to characterize cytokinin regulation of CACHT gene expression. In this paper we present evidence that there is an approximately 5- to 6-fold induction in CACHT mRNA in response to three of the four cytokinins tested (zeatin, zeatin-riboside, and benzyladenine). The cytokinin-induced enhancement of the CACHT transcript appears to be dependent of influx of extracellular Ca^{2+}, protein phosphorylation and concurrent protein synthesis.

The primary action of cytokinins has been associated with enhanced calcium ion influx and the action of calmodulin (Hepler and Wayne 1985, Poovaiah and Reddy 1987). A rise in intracellular Ca^{2+} was shown to mediate cytokinin-induced bud formation in *Funaria* by the use of calcium antagonists such as EGTA, La^{3+}, and TMB and Ca^{2+} channel blockers, such as verapamil (Saunders and Hepler 1983). We used a Ca^{2+} chelator EGTA, Ca^{2+} channel blocker verapamil and calcium ionophore, to investigate the role of Ca^{2+} in our system. Both EGTA and verapamil blocked the basal and/or the cytokinin-induced accumulation of CACHT transcript. Because EGTA and verapamil are unlikely to enter the cells and verapamil blocks the Ca^{2+} channels localized in the plasma membrane (Graziana et al. 1988, Knight et al. 1992), our data indicates that the influx of Ca^{2+} from the extracellular space via the Ca^{2+} channels localized in the plasma membrane is required for the transduction of the cytokinin signal, and that the cytokinin may influence the activity of the Ca^{2+} channels. This conclusion is supported by the fact that increasing intracellular Ca^{2+} levels by enhancing uptake from the extracellular space using a Ca^{2+} ionophore, reproduced the effects of exogenous cytokinin or $CaCl_2$ treatment on accumulation of CACHT transcripts. Nevertheless, our study does not rule out the possibility of mobilization of calcium from intracellular compartments. Ca^{2+} signaling often coordinates parallel and/or sequential use of different sources of Ca^{2+} and different Ca^{2+} channels in different subcellular locations. It was demonstrated in tobacco cells that hypo-osmotic shock stimulates Ca^{2+} fluxes in a sequential manner, deriving first from external

and then internal Ca^{2+} stores, and that these fluxes are mediated by Ca^{2+} channels (Cessna et al. 1998).

The observation that the inhibitory effect of protein kinase inhibitor staurosporine on cytokinin-induced CACHT transcript accumulation (Fig. 3) showed that a staurosporine-sensitive protein kinase(s) plays an important role in the transduction of the cytokinin signal, resulting in accumulation of the CACHT transcript. Thus, it is likely that the staurosporine-sensitive protein kinase(s) may participate in cytokinin-induced protein phosphorylation and, in turn, that the cytokinin-induced protein phosphorylation may ultimately cause the accumulation of CACHT transcript. Our results suggest the involvement of either Ca^{2+}-dependent protein kinase(s) or Ca-CaM (calmodulin)-dependent protein kinase(s) in the cytokinin response. Thus, it is possible that the influx of Ca^{2+} from the extracellular space by cytokinin regulates a complex network of a protein kinase cascade in regend to CACHT gene expression. Crowell (1994) has shown that in soybean, the Cim1 mRNA accumulation is stimulated by staurosporine in the absence of cytokinin, by okadaic acid, an inhibitor of protein phosphatases, and is inhibited in the presence of cytokinin. The authors suggest that dephosphorylation of one or more cellular proteins is involved in cytokinin regulation of Cim1 abundance. Moreover, it has been recently reported that cytokinin-induced Cim1 stability is blocked by the protein phosphatase inhibitor okadaic acid, and the involvement of protein phosphatase activity has been suggested for cytokinin-induced mRNA stability, and subsequent accumulation of Cim1 in soybean cells (Downes and Crowell 1998).

In another study, okadaic acid treatment of cultured tobacco cells has been shown to slightly stimulate the accumulation of msr1 mRNA in the absence of auxin or cytokinin, whereas staurosporine treatment blocked cytokinin- and auxin-induced msr1 mRNA accumulation (Dominov et al. 1992). Furthermore, in the tobacco cell cultures desensitized with auxin, okadaic acid treatment was shown to result in a strong stimulation of msrI mRNA accumulation. The authors thereby postulate the involvement of protein phosphorylation in msrI gene expression, and argue that the protein dephosphorylation might be involved in feedback inhibition of the hormone response. In contrast, Silver et al. (1996) have shown that the cytokinin induction of *Sesbania rostrata* early nodulin SrEnod2 mRNA accumulation was blocked by both okadaic acid and staurosporine. Based on these observations, the authors have suggested both phosphorylation and dephosphorylation processes. The evidence presented in this study indicates that a staurosporine-sensitive protein kinase is involved in the cytokinin-signaling pathway for the enhancement of CACHT transcript accumulation. An earlier report showed that cytokinin specifically increases wheat protein kinase *wpk4* mRNA accumulation, suggesting that cytokinins are involved in protein phosphorylation cascades (Sano and Youssefian 1994).

An additional factor to be considered was whether increases in CACHT transcript accumulation stimulated by cytokinin could involve concurrent synthesis of a transcriptional activator protein factor or some rapidly turned over protein involved in the control of mRNA stability. The data shown here indicates that concurrent protein synthesis is necessary for the cytokinin-enhanced accumulation of CACHT transcript. Treatment of cotyledons with cytokinin in the presence of protein synthesis inhibitor cycloheximide inhibited cytokinin-enhanced CACHT transcript accumulation (Fig. 4). Silver et al. (1996) reported the inhibition of cytokinin-induced SrEnod2 mRNA accumulation by cycloheximide, and proposed that concurrent protein synthesis is required for cytokinin enhancement of SrEnodZ mRNA accumulation. Since the cytokinin effect is dependent upon concurrent protein synthesis, it is possible that cytokinin induces synthesis and phosphorylation of some rapidly turned over protein, which, in turn, may ultimately cause the accumulation of CACHT transcript. It remains to be determined whether the cytokinin-induced accumulation of the chitinase transcript is solely caused by enhanced transcription, or is also partly due to post-transcriptional processes and/or stabilization of mRNA.

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