

Cytokinin regulation of a soybean pollen allergen gene

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Abstract

Cytokinin treatment of suspension-cultured soybean cells stimulated the accumulation of an mRNA, called cim 1, by a factor of ca. 20 within 4 h. Induction of cim 1 mRNA accumulation occurred at benzyladenine concentrations as low as 10^{-8} M. Furthermore, cim 1 mRNA accumulation was stimulated in the absence of cytokinin by staurosporine (an inhibitor of protein kinases) and inhibited in the presence of cytokinin by okadaic acid (an inhibitor of protein phosphatases 1 and 2a), suggesting that cim 1 accumulation in response to cytokinin is dependent on cytokinin-induced dephosphorylation of one or more cellular proteins. The deduced amino acid sequence of the cim 1 protein product, derived from the complete nucleotide sequence of a cim 1 cDNA, was 40% identical to that of a perennial rye grass pollen allergen cDNA (Lol PI). This sequence also indicated that the cim 1 protein product contains a putative signal peptide followed by predominantly hydrophilic residues, consistent with the hypothesis that it is exported to the apoplast.

Introduction

Cytokinin, discovered in 1955, was shown to stimulate division and sustained growth of tobacco pith cells when provided in combination with auxin and was, thus, described as a 'cell division factor' [27]. The name 'cytokinin' was later adopted to describe this effect on cytokinesis [36]. However, since its discovery, cytokinin has been shown to exert other effects on plant growth and development as well. For example, cytokinin delays tissue senescence [29, 33] and promotes shoot organogenesis [35], lateral bud release [34], chloroplast development [30, 37],

leaf expansion [26], and tumor formation [1, 2, 17]. The dramatic and sustained nature of these effects suggests that changes in gene expression mediate the response of plant cells to cytokinin.

Cytokinin has been shown to induce the accumulation of various mRNAs and proteins in plant cells [reviewed in 6]. Among the mRNAs that have been shown to accumulate in response to cytokinin are those encoding the light-harvesting chlorophyll *a/b*-binding protein [14, 15, 23, 40], the small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) [13, 14, 21, 24], nitrate reductase [10, 11, 19, 22, 25], an early nodulin

protein from *Sesbania rostrata* [8], and a 'multiple stimulus response' protein from *Nicotiana plumbaginifolia* [12]. In some cases, cytokinin has been shown to increase the rate of transcription of the corresponding genes. For example, Lu *et al.* have obtained direct evidence in etiolated barley leaves for the role of transcription in cytokinin enhancement of light induction of nitrate reductase mRNA accumulation by measuring changes in the rate of transcription of the nitrate reductase gene [25]. Similarly, a 'multiple stimulus response' protein gene from *N. plumbaginifolia* has been shown to be under transcriptional control [12]. In contrast, Flores and Tobin have reported that the cytokinin-induced accumulation of the mRNAs for the light-harvesting chlorophyll *a/b*-binding protein and the small subunit of Rubisco occurs primarily by a post-transcriptional mechanism in *Lemna gibba* [14, 15].

Crowell *et al.* have reported the isolation of 20 cDNAs that correspond to cytokinin-inducible soybean mRNAs [7]. One of these mRNAs, called cim 1 (cytokinin-induced message 1), accumulates approximately 20-fold within 4 h of cytokinin addition to cytokinin-depleted soybean suspension cultures. This mRNA accumulates in response to various biologically active cytokinins, but is relatively unresponsive to auxin or to inactive cytokinin analogues such as adenine or 6-methyladenine. Thus, the accumulation of the cim 1 mRNA appears to be moderately cytokinin-specific. In this report, it is shown that cim 1 mRNA accumulation occurs at physiological concentrations of cytokinin (as low as 10^{-8} M) and that this accumulation is sensitive to inhibitors of protein phosphorylation and dephosphorylation. Furthermore, the complete nucleotide sequence of a cim 1 cDNA indicates that the cim 1 gene encodes a soybean homologue of the perennial rye grass pollen allergen Lol PI [31].

Materials and methods

Tissue culture

All experiments were performed on suspension cultures of soybean (*Glycine max* cv. Mandarin),

which were grown in Murashige-Skoog liquid medium [28] containing $10\text{ }\mu\text{M}$ α -naphthaleneacetic acid (NAA) (2 mg/l) and $4\text{ }\mu\text{M}$ benzyladenine (BA) (1 mg/l) at $26 \pm 1\text{ }^{\circ}\text{C}$ in continuous fluorescent light on a rotary shaker set to 110 rpm. These cultures were subcultured every 14 days by transferring 10 ml of stationary phase cells into 30 ml of fresh medium. Cytokinin depletions were performed by washing stationary-phase cells with 1 liter of cytokinin-free Murashige-Skoog liquid medium containing $10\text{ }\mu\text{M}$ α -NAA (5 washes of 200 ml each) on a sterile Buchner funnel and then culturing the cells in 40 ml of fresh cytokinin-free medium for 6 days. Cytokinin-depleted cells were either harvested directly or were harvested after treatment with (1) various concentrations of BA for 4 h, (2) $1\text{ }\mu\text{M}$ staurosporine in dimethyl sulfoxide (DMSO) for various times (the final DMSO concentration was 0.1%), (3) 0.1% DMSO for various times, (4) $1\text{ }\mu\text{M}$ BA for 4 h in the presence of $0.5\text{ }\mu\text{M}$ okadaic acid (added in 0.15% ethanol 45 min prior to the addition of benzyladenine), or (5) $1\text{ }\mu\text{M}$ BA for 4 h in the presence of 0.15% ethanol (added 45 min prior to the addition of benzyladenine). Cells were harvested by suction filtration, frozen in liquid nitrogen, and ground to a fine powder with a liquid nitrogen-cooled mortar and pestle. Total RNA was then prepared from these samples as described [18].

RNA blot analyses

RNA ($5\text{ }\mu\text{g}$ of total RNA per lane) from soybean suspension cultures was size-fractionated by 7.2% (v/v) formaldehyde 1% (w/v) agarose gel electrophoresis, transferred to nylon membranes by capillary blotting, and hybridized to antisense RNA probes, which were prepared *in vitro* by T3 RNA polymerase-mediated transcription of a linearized cim 1 cDNA. Hybridizations were as described except that the hybridization buffer contained 0.5 mg/ml yeast RNA [32]. Quantitation of changes in cim 1 mRNA levels was done by excising the radioactive bands corresponding to cim 1 from the blots and measuring the radioactivity in the bands by liquid scintillation.

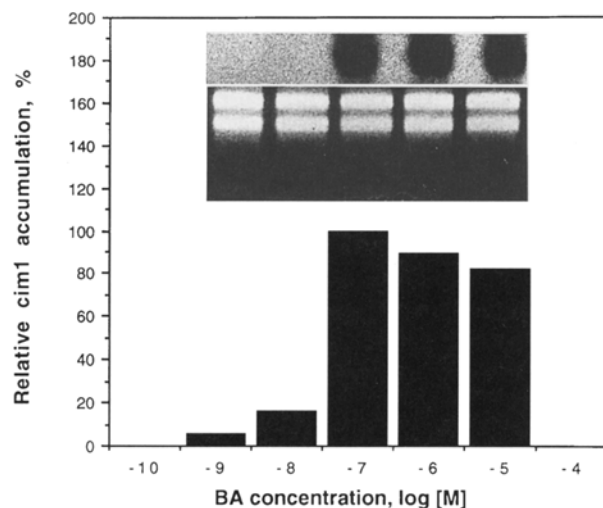


Fig. 1. Cim 1 mRNA accumulation at various doses of cytokinin. Cytokinin-depleted soybean cells were treated for 4 h with benzyladenine at various concentrations. Total RNA was then analyzed for cim 1 abundance. The RNA blot from which these data were obtained is shown. An RNA gel stained with ethidium bromide is also shown to control for sample load. BA, N⁶-benzyladenine.

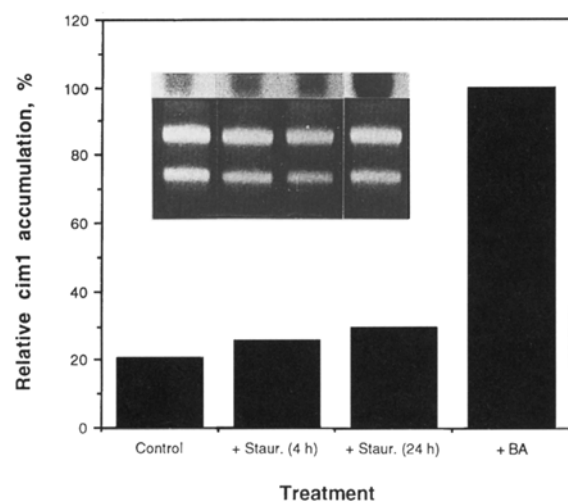


Fig. 2. Induction of cim 1 mRNA accumulation by staurosporine. Cytokinin-depleted soybean cells were treated with 1 μ M staurosporine for 4 or 24 h or with 1 μ M benzyladenine for 4 h. Total RNA was then analyzed for cim 1 abundance. The RNA blot from which these data were obtained is shown. An RNA gel stained with ethidium bromide is also shown to control for sample load. BA, N⁶-benzyladenine; staur., staurosporine.

DNA sequence analysis

Sequence analysis of the cim 1 cDNA was done as described [3, 38]. DNA sequence manipulations and database searches were performed with the sequence software of the University of Wisconsin Genetics Computer Group [9].

Results

Cytokinin induction of cim 1 mRNA accumulation

The addition of N⁶-benzyladenine to cytokinin-depleted soybean suspension cultures at concentrations between 10^{-7} and 10^{-8} M induced the accumulation of cim 1 in 4 h (see Fig. 1). Thus, cim 1 accumulation occurred at physiological concentrations [29] of cytokinin. Maximal accumulation

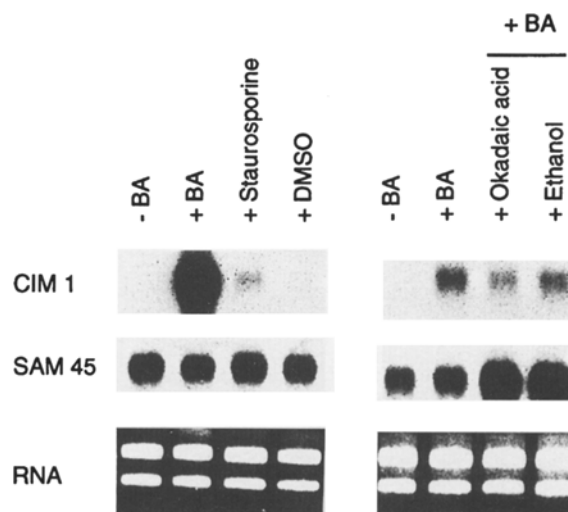


Fig. 3. Effects of staurosporine and okadaic acid on cim 1 mRNA accumulation. Cytokinin-depleted soybean cells were either harvested directly or were harvested following treatment with (1) 1 μ M benzyladenine for 4 h, (2) 1 μ M staurosporine (in 0.1% DMSO) for 24 h, (3) 0.1% DMSO for 24 h, (4) 1 μ M benzyladenine for 4 h in the presence of 0.5 μ M okadaic acid (added in 0.15% ethanol 45 min prior to the addition of benzyladenine), or (5) 1 μ M benzyladenine for 4 h in the presence of 0.15% ethanol (added 45 min prior to the addition of benzyladenine). Total RNA was then analyzed for cim 1 or sam45 abundance. The autoradiogram shown in the upper right corner is under-exposed relative to the autoradiogram shown in the upper left corner. RNA gels stained with ethidium bromide are also shown to control for sample load. BA, N⁶-benzyladenine; DMSO, dimethyl sulfoxide.

(ca. 20-fold) was observed at 10^{-7} M benzyladenine (previous data have shown that naturally occurring cytokinins are as effective as benzyladenine at inducing cim 1 accumulation when used at 5×10^{-6} M [7]).

The accumulation of cim 1 mRNA was influenced by inhibitors of protein phosphorylation and dephosphorylation. As shown in Figs. 2 and 3, the addition of 1 μ M staurosporine, a protein kinase inhibitor [39], to cytokinin-depleted soybean suspension cultures caused a gradual

increase in the abundance of the cim 1 mRNA over 24 h. Staurosporine also enhanced cytokinin induction of cim 1 mRNA accumulation (data not shown). Furthermore, as shown in Fig. 3, a control SAM45 mRNA [5] showed no induction by staurosporine. Since staurosporine is a broad spectrum protein kinase inhibitor [39], these data suggest that cytokinin-depleted cells contain predominantly the phosphorylated form of one or more protein(s) that promote the accumulation of cim 1 when converted to the dephosphorylated



Fig. 4. Nucleotide and deduced amino acid sequences of the soybean cim 1 cDNA.

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Cim1      MALTQRAISELLTLIAILSIFLVIPSFNFKLYNASYYSPSSSDWSP
Lol pI    MASSSSVLLVVALFAVELGSAH--GIATVPPGPNITAEGDKALD

Cim1      AVATWYGFANGDGS--EGGACGYGNVAGQPFSSLSAGSPLIITDYGKGC
Lol pI    AKSTWYGKPTGAGPKDNGGACGY-KNMDKAPFNGMTGCCNTPTFKDGRGC

Cim1      GSCYEVKCTGNSACSGNFKVWVITDECAGCGSDAQYHFDLSGNAFGAMAI
Lol pI    GSCFFTKCTIKPESCSGEAMITVITLDNE--EPTAFYHFDLSGHAFGSMAR

Cim1      SGQDENLRNAGKINIOHRRTEONYFGRS-IAFHVDSGSNDQYFFAILVEYE
Lol pI    KGEENVRVSAGELELOFRVVKQRYPDPTKPTFHVKEASNPNYLAILVKKV

Cim1      DGDGDLAKVELKEALDSGSMDSMOQSWGAVWKFQKGSPLRAPFSIKLITL
Lol pI    DGDGDIVVANDIKKK-GKDKMTLKEKSWGAVWRITPTDKLTGPTVRYITL-

Cim1      ESGQTIVANNVIFAGWTPGQTYRSIVNFAT
Lol pI    EGCTKSEFEDVLEGGWKADTSMSAK

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Fig. 5. Identity between the deduced amino acid sequences of the soybean cim 1 and perennial rye Lol pl cDNAs.

form. Furthermore, since this protein kinase inhibitor caused only a gradual accumulation of cim 1, the rate of dephosphorylation of the protein(s) involved in this process may be low in cytokinin-depleted soybean cells. Thus, the rapid induction of cim 1 mRNA accumulation by cytokinin may involve the activation of a protein phosphatase rather than the inactivation of a protein kinase. To test this hypothesis, the effect of okadaic acid, an inhibitor of type 1 and 2a serine/threonine protein phosphatases [4, 16], on the cytokinin induction of cim 1 mRNA accumulation was examined. As shown in Fig. 3, cim 1 mRNA accumulation in the presence of 1 μ M benzyladenine was moderately inhibited by 0.5 μ M okadaic acid. Complete inhibition was observed at 1 μ M okadaic acid, but the additional ethanol contributed to the inhibition (data not

shown). Thus, inhibition of protein phosphatases 1 and 2a inhibited the cytokinin induction of cim 1 mRNA accumulation, consistent with the hypothesis that a protein phosphatase mediates the effects of cytokinin on cim 1 abundance. The accumulation of a control SAM45 mRNA [5] was unaffected by okadaic acid (Fig. 3). These results are in contrast to those reported by Dominov *et al.* [12], who showed that staurosporine inhibited cytokinin- or auxin-induced accumulation of a 'multiple stimulus response' mRNA in *N. plumbaginifolia* cells, whereas okadaic acid stimulated accumulation, especially in cells pre-treated with auxin (these cells were not starved for cytokinin and do not require exogenous cytokinin for growth). Hence, it is likely that different signal transduction pathways regulate the expression of different cytokinin-modulated plant genes.

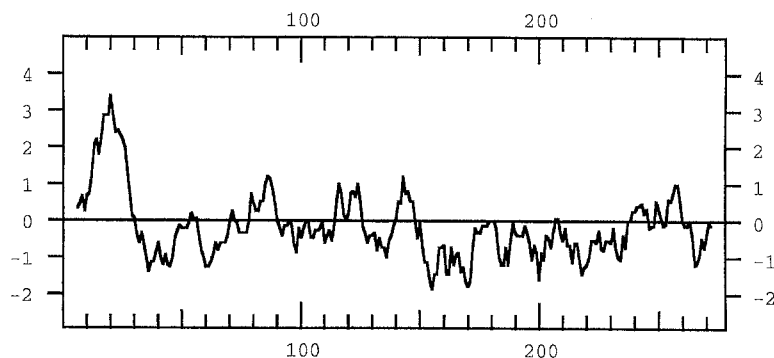


Fig. 6. Hydropathy plot for the soybean cim 1 protein product. The hydropathic character of the cim 1 protein product was determined as described by Kyte and Doolittle [20] and was based on the deduced cim 1 amino acid sequence shown in Figs. 4 and 5. Positive values represent hydrophobic regions.

Nucleotide sequence of a *cim 1* cDNA

The complete nucleotide sequence of the *cim 1* cDNA (Fig. 4) indicated that it contains a putative initiation codon and is approximately identical in length to the *cim 1* mRNA [7]. A considerable amount of secondary structure was found in the 3' untranslated region of the *cim 1* gene at a site rich in both A and U residues. The deduced *cim 1* amino acid sequence (Fig. 5) was 40% identical to that of a *Lol pl* perennial rye grass pollen allergen cDNA [31], suggesting that the *cim 1* protein product is targeted to the surface of pollen grains and perhaps other tissues as well. Indeed, a close inspection of the deduced *cim 1* amino acid sequence revealed a putative signal peptide. Furthermore, as shown in Fig. 6, a hydropathy plot of this sequence indicated that, with the exception of the putative signal peptide at the amino terminus, the *cim1* protein product is largely hydrophilic in nature [20], suggesting that it may be exported to the apoplast.

Discussion

The data shown in this paper demonstrate that *cim 1* mRNA accumulation occurs at benzyladenine concentrations as low as 10^{-8} M, is stimulated in the absence of cytokinin by an inhibitor of protein kinases, and is inhibited in the presence of cytokinin by an inhibitor of type 1 and 2a serine/threonine protein phosphatases. The observation that these inhibitors produce opposite effects on *cim 1* mRNA accumulation supports the hypothesis that protein dephosphorylation is involved in cytokinin induction of *cim 1* mRNA accumulation. Direct biochemical evidence for a cytokinin-induced serine/threonine protein phosphatase activity in cultured soybean cells would greatly strengthen this hypothesis.

The data shown herein indicate that the *cim 1* protein product corresponds to a soybean homologue of the perennial rye grass pollen allergen *Lol pl* [31]. This protein is expressed on the surface of pollen grains and perhaps other plant tissues as well. Consistent with this extracellular location, the *cim 1* polypeptide contains a putative

signal peptide followed by predominantly hydrophilic amino acid residues. The functional significance of cytokinin induction of a gene encoding an extracellular protein remains obscure, but perhaps the *cim 1* protein is involved in the deposition of cell wall material and the formation of the cell division plate during cytokinesis. Experiments to determine whether the *cim 1* protein is present in the cell division plate of dividing cells and in the cell wall of dividing and non-dividing cells are underway. These studies, along with those described above, will contribute to a better understanding of the mechanism by which cytokinin regulates the expression of plant genes and the role of these genes in plant growth and development.

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