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Cytokinin-induced mRNAs in cultured soybean cells

(*Glycine max*/phytohormones/plant development/ribosomal proteins/auxin)

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ABSTRACT **We have isolated 20 cDNA clones for which the corresponding mRNAs accumulate 2- to 20-fold within 4 hr of cytokinin addition to cytokinin-starved soybean suspension cultures. These changes in mRNA levels precede the greening and cell growth induced by cytokinin in this system. Treatment with cycloheximide before cytokinin addition enhances the accumulation of these messages. In addition, the abundance of these mRNAs is influenced by auxin. We demonstrate that the mRNAs corresponding to the cDNAs in this collection display a variety of patterns of accumulation in the tissues of an intact plant. We also show that different cytokinins induce similar increases in message levels. However, analogs of cytokinin that are much less active in bioassays induce lower levels of message accumulation. Sequence analyses indicate that two of the cDNAs correspond to ribosomal protein genes. The remaining cDNAs are likely to represent previously undiscovered genes that function early in the initiation of plant cell growth and/or chloroplast development.**

Cytokinins are plant growth substances that induce cell division (1). Most cytokinins are adenine derivatives and some, such as zeatin, induce cell division in tobacco and soybean callus cultures at concentrations as low as 0.1 nM (2). In whole plants, cytokinins affect many aspects of growth (3), including the development of lateral buds, chloroplasts, and roots, and the differentiation of vascular tissue.

The reported effects of cytokinin treatment include changes in cellular enzyme activities and mRNA levels. A 1-hr cytokinin treatment is sufficient to cause an increase in the activities of nitrate reductase (4) and cytokinin oxidase (5), and a 2-hr treatment causes a decrease in the level of phytochrome mRNA (6). Even shorter cytokinin treatments induce changes in oxidative metabolism (7, 8). Within 24 hr of treatment, cytokinin increases the levels of the mRNAs that encode the light-harvesting chlorophyll a/b binding protein and the small subunit of ribulose-1,5-bisphosphate carboxylase (9). Changes in mRNA and protein levels after several days of cytokinin treatment have also been reported (10–13).

This work was undertaken to identify mRNAs that accumulate rapidly after treatment of plant cells with cytokinin. In this paper, we report the isolation and characterization of cDNA clones corresponding to 20 mRNAs that begin to accumulate 1–4 hr after cytokinin treatment of cytokinin-starved soybean cells. Accumulation of these messages precedes greening and resumption of normal growth. Thus, further characterization of these messages may contribute to an understanding of the molecular events that lead to the induction of physiological responses by cytokinin.

MATERIALS AND METHODS

All phytohormones were from Sigma except thidiazuron, which was a gift from Nor-Am Chemical (Wilmington, DE). Unless otherwise noted, all nucleic acid manipulations were as described (14).

Tissue Culture. A suspension culture derived from soybean hypocotyl (*Glycine max* cv. Mandarin) was subcultured every 7 days into Murashige–Skoog liquid medium (15) containing 10 μM α-naphthaleneacetic acid (2 mg/liter), 5 μM zeatin, *myo*-inositol at 100 mg/liter, nicotinic acid at 1 mg/liter, pyridoxine·HCl at 1 mg/liter, and thiamine at 10 mg/liter and was maintained at 27°C in continuous fluorescent light. The cells were filtered through a 1.5-mm wire mesh before subculture, at which time 7 ml of settled cells were added to 30 ml of medium. Growth was monitored by measuring 10-min settled cell volumes and by microscopic examination of the cells (which indicated that no changes in average cell size occurred during the experiments).

Cell cultures were starved for either cytokinin or auxin 4 days after subculture. Cells were placed on Miracloth in a Buchner funnel, washed with ten 100-ml aliquots of the appropriate hormone-free medium, and then resuspended in the same hormone-free medium. Hormone inductions were done after 3 days of starvation by adding either 5 μM zeatin or 10 μM α-naphthaleneacetic acid. When indicated, cycloheximide (10 μg/ml) was added 30 min before hormone addition.

Nucleic Acid Isolation. RNA and mRNA [poly(A)-enriched RNA] were prepared as described (16).

Construction of the cDNA Plasmid Library. The mRNA used to make the cDNA library was isolated from cells that were cytokinin-induced in the presence of cycloheximide. First-strand cDNA synthesis was done with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) using an oligo(dT)-*Xba* I primer-adapter. Second-strand cDNA synthesis was then done in the presence of RNase H (BRL) and *Escherichia coli* DNA polymerase I (New England Biolabs), and blunt ends were generated with T4 DNA polymerase. Unphosphorylated *Eco*RI–*Not* I linkers with a 5′ AATT overhang were then ligated to the cDNA using T4 DNA ligase, and the 5′ ends were phosphorylated by polynucleotide kinase reaction. The cDNA was then digested with *Xba* I. These manipulations resulted in *Eco*RI-compatible 5′ ends and *Xba* I-compatible 3′ ends. The cDNA was size-fractionated by agarose gel electrophoresis, and cDNA longer than ≈300 base pairs was ligated into *Eco*RI–*Xba* I-digested, dephosphorylated vector DNA (Bluescript SK$^+$, Stratagene). Recombinants of *E. coli* strain DH1αF′ were selected on LB plates containing carbenicillin at 100 μg/ml and pooled.

Single-stranded library DNA was prepared as described (17) and subtracted by solution hybridization to a 10-fold excess of mRNA from cytokinin-starved cells. This mRNA was modified before hybridization by the covalent addition of biotin (18). Hybridization occurred at 40°C in 5 μl of 0.2% SDS/50 mM Hepes, pH 7.6/0.5 M NaCl/2 mM EDTA/50% (vol/vol) formamide for 16 hr. After hybridization, DNA–

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Abbreviation: cim, cytokinin-induced message.

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RNA hybrids and excess RNA were removed by streptavidin treatment and phenol/chloroform extraction as described (18). The single-stranded, subtracted cDNA was then used to transform *E. coli* strain DH5α to carbenicillin resistance.

Differential Screen of the cDNA Library. The subtracted cDNA library described above was differentially screened with subtracted cDNA probes made from the mRNAs of cytokinin-induced and cytokinin-starved cells. cDNA probes were synthesized as described above using Moloney murine leukemia virus reverse transcriptase in the presence of deoxyadenosine 5′-[α-^{32}P]triphosphate. These cDNA probes were subtracted with mRNA from cytokinin-starved cells as described (18). Filters were made by replica printing after growing the equivalent of one library on nylon filter discs (Gelman). These filters were hybridized to cDNA probes as described (19).

RNA Blot Analysis. RNA [5 μg of total RNA or 0.2 μg of poly(A)$^+$ RNA] was fractionated by formaldehyde-agarose gel electrophoresis, transferred to nylon membranes (16), and hybridized to antisense RNA probes, which were made by T3 RNA polymerase.

RESULTS

Properties of the Soybean Cell Line. We chose a suspension culture of soybean for these experiments because it exhibited three useful properties. (*i*) The cell line requires exogenous cytokinin, in addition to auxin, for growth. (*ii*) The cells retain the ability to respond to exogenous cytokinin for at least 5 days after removal of cytokinin from the medium. Addition of cytokinin during this time causes normal growth to resume. (*iii*) The cells in suspension culture are in direct contact (or nearly so) with the medium and respond rapidly to changes in exogenous hormone levels.

Cytokinin starvations were begun on the fourth day after subculture, at which time the cells were washed extensively with, and resuspended in, cytokinin-free medium. The rate of cell division and enlargement decreased, relative to the rate in cultures containing cytokinin, within 3 days of the start of starvation. In addition, yellowing of the cells could be seen after 2 days of starvation. After 5 days, starved cells that were subcultured again into fresh cytokinin-free medium ceased growth and continued to yellow, whereas starved cells that were subcultured into medium containing zeatin resumed normal growth and became greener. Typical growth curves for starved and unstarved cells are shown in Fig. 1.

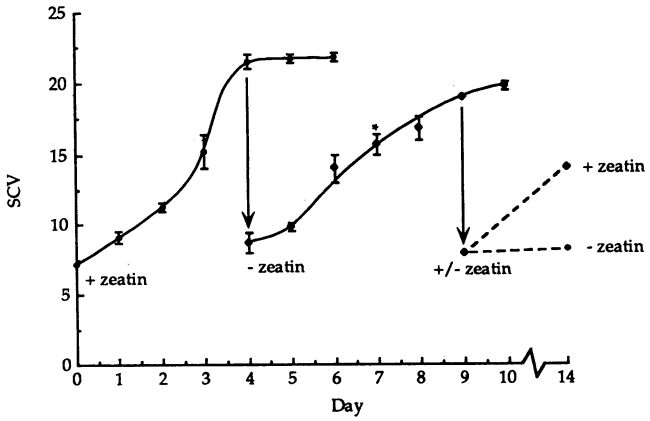

FIG. 1. Soybean suspension culture growth curves. Arrows indicate the times at which cells were subcultured into fresh medium, and the asterisk indicates the day on which inductions were done. The points connected by dotted lines are single values that were confirmed in a parallel experiment. All other points are the averages of three values. Error bars indicate the range of values seen. SCV, settled cell volume.

Isolation of cDNA Clones from Cytokinin-Induced Cells. mRNA was isolated from induced cells (i.e., cells that were cytokinin-starved for 3 days and then treated with 5 μM zeatin for 4 hr). Cycloheximide at 10 μg/ml was added to the cells 30 min before zeatin addition to increase the probability of isolating transcripts that accumulate early in the sequence of cytokinin-induced events. The cDNA library constructed from this mRNA contained 8 × 10^4 clones.

Single-stranded DNA from this library was subtracted with control mRNA (prepared from cells that were cytokinin-starved and then treated only with cycloheximide for 4.5 hr). This removed 70% of the cDNAs, reducing the library to 2.3 × 10^4 clones. One library equivalent was then differentially screened with ^{32}P-labeled cDNA probes made from the mRNAs of induced and control cells (both probes were subtracted with control mRNA). Colonies corresponding to cDNAs that hybridized more strongly to the induced probe than to the control probe were picked from the master plates and rescreened. We confirmed that these cDNAs correspond to cytokinin-induced messages (cims) by hybridizing blots of control and induced RNAs (both total RNA and mRNA preparations) to probes corresponding to the cDNAs. We found that 82 of the probes hybridized to mRNAs that accumulate within 4 hr of cytokinin treatment (Fig. 2*a*). The accumulation of cim2, -11, and -12 (Fig. 2*a*) and cim10, -13, and -16–19 (data not shown) is more pronounced in mRNA preparations than in total RNA. Accumulation of cim12 and cim17 is detectable only in mRNA preparations. Some of the probes used to characterize these messages cross-hybridized to other RNAs that were not induced by cytokinin (data not shown).

We determined 200–400 nucleotides of DNA sequence at the 3′ end of each cDNA in the collection (data not shown). Comparison of these sequences allowed us to eliminate redundancy in the collection and identify 20 cDNAs that correspond to different cytokinin-induced mRNAs. The sizes of these mRNAs were estimated by gel electrophoresis and are shown in Table 1.

Message Accumulation in the Absence of Cycloheximide. All 20 cDNAs correspond to mRNAs that accumulate within 4 hr of cytokinin treatment in the presence of cycloheximide. To show that these mRNAs accumulate in response to cytokinin alone, we examined message induction in the absence of protein synthesis inhibitors. We found that the magnitude of induction is generally higher in cells that are exposed to cycloheximide, due to two effects of the cycloheximide treatment (compare inductions in Fig. 2*a* to those in Fig. 2*b*, lanes 3 and 8). The first is a general decrease in residual message levels, which lowers the background in control mRNA and increases the apparent magnitude of induction. The second effect is an enhancement of the cytokinin-induced message accumulation. An extreme example of this enhancement is exhibited by cim4, which was strongly induced in the presence of cycloheximide but which accumulated only 2-fold over the control level in the absence of cycloheximide.

We examined the kinetics of accumulation in the absence of cycloheximide for each of the mRNAs. These data allowed us to identify aspects of message accumulation that vary among our clones. First, four of the messages [cim1 and -4 (shown in Fig. 2*b*) and cim3 and -6 (data not shown)] begin to accumulate 1 hr after cytokinin addition. Most of the other messages [cim2, -5, -7, -11, and -15 (Fig. 2*b*) and cim8, -10, -13, and -16–19 (data not shown)] begin to accumulate 2 hr after cytokinin addition, but four [cim9 (Fig. 2*b*) and cim12, -14, and -20 (data not shown)] do not accumulate until 4 hr of exposure to cytokinin. A second interesting feature is an initial decrease in the abundance of eight of the mRNAs [cim1, -2, -5, -9, -11, and -15 (Fig. 2*b*) and cim17 and -18 (data not shown)] after 30 min of cytokinin treatment. These

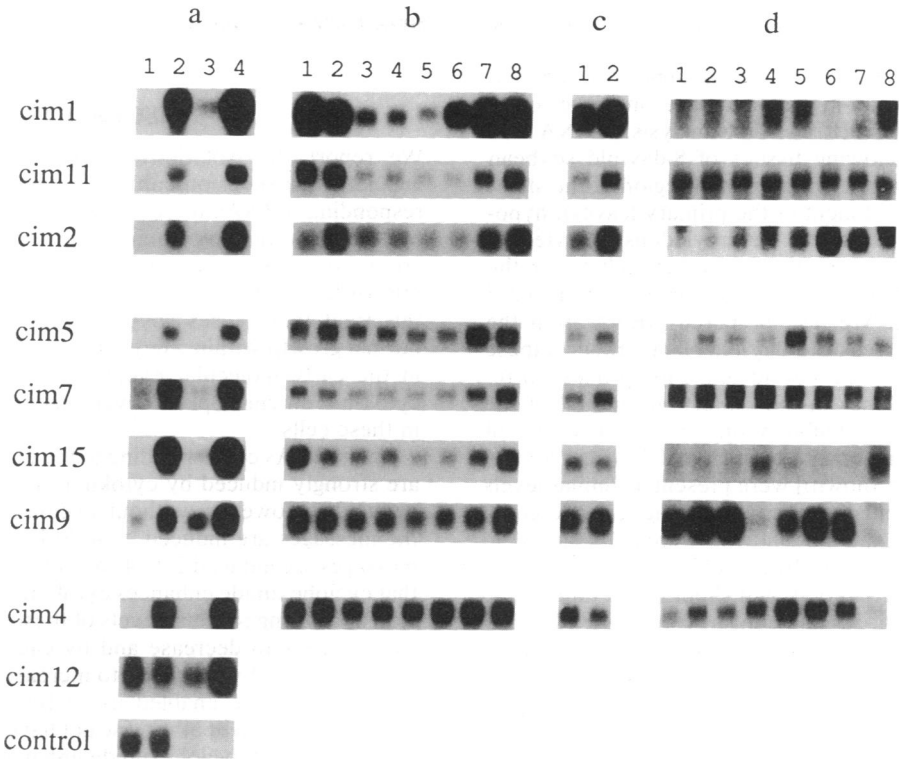

FIG. 2. Northern blots of soybean RNA showing representative cytokinin-induced messages. (*a*) Total RNA and mRNA from cytokinin-starved cells treated with or without 5 μM zeatin (Z) for 4 hr (in the presence of cycloheximide at 10 μg/ml). Lanes: 1, total RNA − Z; 2, total RNA + Z; 3, mRNA − Z; 4, mRNA + Z. (*b*) mRNA from cells during normal growth, zeatin starvation, and induction (without cycloheximide). Lanes: 1, day 2 of subculture; 2, day 4 of subculture (day of starvation); 3, day 3 of starvation. Lanes 4–8 show mRNA levels at various times after zeatin addition: 4, 15 min; 5, 30 min; 6, 1 hr; 7, 2 hr; 8, 4 hr. (*c*) mRNA from auxin-starved and induced cells. Lanes: 1, day 3 of auxin starvation; 2, 4 hr after addition of 10 μM α-naphthaleneacetic acid. (*d*) mRNA from soybean tissues (8-day-old plant, unless otherwise noted). Lanes: 1, primary leaf; 2, shoot tip; 3, hypocotyl; 4, cotyledon; 5, elongation region of the stem; 6, root tip; 7, whole root; 8, 16-day-old primary leaf.

observations have been verified in four separate time-course experiments.

The magnitude of message accumulation also varies among these clones. We evaluated the extent of mRNA induction in the absence of cycloheximide by laser scanning densitometry (data not shown). cim1 levels increase 21-fold within 4 hr of cytokinin treatment, cim11 levels increase 6-fold, and cim2 levels increase 5-fold. The other messages accumulate to 2–4 times the level in starved cells.

Evaluation of the Cytokinin Specificity of the Inductions. We performed two experiments to examine the cytokinin specificity of the accumulation of these messages. First, we compared message levels in cells growing at different rates to determine whether message levels correlate with growth rate. mRNA preparations from log-phase cells (day 2 after subculture), early stationary-phase cells (day 4 after subculture), and cytokinin-starved cells (day 3 after starvation) were probed with RNA probes corresponding to each cDNA (Fig. 2*b* lanes, 1–3). The levels of five of the messages [cim1, -7, -9, and -15 (Fig. 2*b*) and cim20 (data not shown)] decreased between the second and fourth days after subculture, but after 3 days of starvation the levels of these messages were less than or equal to the levels in stationary-phase cells. Because the cytokinin-starved cells were growing, these mRNAs do not accumulate in a growth-inducible manner. Seven of the messages [cim2, -4, -5, and -11 (Fig. 2*b*), and cim10, -16, and -17 (data not shown)] were present at higher levels in stationary-phase cells than in log-phase cells. The levels of these messages decreased during the 3-day starvation. The remaining messages were present at the same level in log-phase and stationary-phase cells, and the levels of these messages decreased or remained the same during the starvation. Thus, the levels of these mRNAs do not correlate with the growth rate of the cells.

Table 1. Approximate mRNA sizes in nucleotides of cim1–20

cim	Size	cim	Size
1	1200	11	1800
2	450	12	800
3	2800	13	1400
4	2500	14	200
5	2500	15	400
6	2300	16	250
7	2900	17	500
8	1200	18	1000
9	700	19	600
10	900	20	600

In the second experiment, we compared message levels in cells that had been treated with 10 μM naphthaleneacetic acid for 4 hr, after a 3-day auxin starvation, to the levels in uninduced, auxin-starved cells (Fig. 2*c*). All of the messages were induced by this auxin treatment, except cim3, -4, -15, and -18. We also compared the residual levels of these mRNAs after 3 days of auxin starvation to the levels after 3 days of cytokinin starvation. We found that the levels of 18 of the mRNAs after auxin starvation were comparable to the levels detected after cytokinin starvation. The remaining mRNAs (cim1 and cim8) were more abundant after auxin starvation than after cytokinin starvation. This observation suggests that the levels of these two messages are more responsive to changes in exogenous cytokinin levels than to changes in auxin levels. Nonetheless, auxin appears to in-

fluence the accumulation of all of these cytokinin-induced messages.

Tissue Specificity of Message Accumulation. We examined the levels of the cytokinin-induced messages in tissues of an intact plant by Northern (RNA) blot analysis. mRNA was prepared from the following tissues of 8-day-old soybean plants: primary leaf, shoot tip (the 2-mm region of the shoot above the point of attachment of the primary leaves), hypocotyl (1 cm of stem just below the cotyledons), cotyledon, elongating region of the stem (the 5-mm section between the cotyledons and the primary leaves), root tip (the terminal 2–5 mm) and whole root. We also prepared mRNA from the expanded, primary leaves of 16-day-old plants. Blots of these mRNA preparations were hybridized with probes corresponding to the 20 clones in our collection, and all of the messages except cim1 exhibited one of five patterns of expression. Four of the mRNAs [cim7 and -11 (Fig. 2*d*) and cim6 and -14 (data not shown)] were present at similar levels in all of the tissues tested. Others [cim9 (Fig. 2*d*) and cim8, -10, -12, -19, and -20 (data not shown)] were abundant in all tissues except cotyledon and 16-day-old leaf and were most apparent in root tip, hypocotyl, and shoot tip. A third group of mRNAs [cim15 (Fig. 2*d*) and cim13, -16, and -17 (data not shown)] accumulate primarily in cotyledon and 16-day-old leaf. cim5 and, to a lesser degree, cim4 (and cim3, data not shown) accumulate mainly in the elongating region of the stem. Finally, cim2 appears to be root-specific. Many of the probes also hybridized to additional messages, some of which showed different patterns of accumulation.

Analysis of Induction by Different Cytokinins. We examined the levels of induction of cim1, cim2, and cim11 in total RNA prepared from starved cells that were treated for 4 hr with 5 μM of each of the following cytokinins: zeatin riboside, isopentenyladenine, isopentenyladenine riboside, benzyladenine, benzyladenine riboside, kinetin, and thidiazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-urea). In addition, we treated starved cells with 5 μM adenine or 6-methyladenine, which are structurally related compounds that are much less active in bioassays (20, 21). RNAs from cells treated with each of these compounds were blotted and hybridized to probes corresponding to cim1, cim2, and cim11. We found that the seven active cytokinins were equally effective at inducing accumulation of these messages within 4 hr. This accumulation equaled that observed after zeatin treatment (data not shown). Adenine and 6-methyladenine also induced some message accumulation but much less than the other compounds tested (see Fig. 3 for cim1 data).

Identification of Ribosomal Protein mRNAs. We determined 200–400 nucleotides of DNA sequence at the 5' end of each of our cDNA clones (data not shown). This allowed us to predict partial amino acid sequences and to search for sequence similarities in a complete translation of the GenEMBL version 6.0 nucleic acid sequence data base (22). This search indicated that cim9 is related to the ribosomal protein L30 gene from rat and the ribosomal protein L32 gene from yeast, and that cim20 is related to the ribosomal protein L44 genes from yeast and human (data not shown). All other cDNAs in the collection appear to be distinctive, although some were short and did not provide us with enough coding sequence to perform a reliable search.

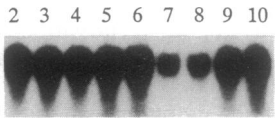

FIG. 3. Northern blot of cim1 induction in total RNA preparations by various cytokinins (5 μM). Lanes: 1, no cytokinin; 2, zeatin riboside; 3, benzyladenine; 4, benzyladenine riboside; 5, isopentenyladenine; 6, isopentenyladenine riboside; 7, adenine; 8, 6-methyladenine; 9, kinetin; 10, thidiazuron.

DISCUSSION

We report the isolation of 20 cDNAs from cytokinin-dependent, suspension-cultured soybean cells for which corresponding mRNAs increase in abundance within 1–4 hr of cytokinin addition to cytokinin-starved cells. These increases are more rapid than the cytokinin-induced increases in mRNA levels observed in other systems (9, 11–13). Because this treatment causes soybean cells to green and resume normal growth within 5 days, these mRNAs may participate in the early molecular events that mediate the effects of cytokinin on chloroplast development and growth initiation in these cells.

The mRNAs corresponding to the cDNAs in our collection are strongly induced by cytokinin in the presence of cycloheximide. However, without cycloheximide, only three of the messages are induced >5-fold by cytokinin. (The other messages are induced 2- to 4-fold.) Further analyses revealed that cycloheximide enhances cytokinin-induced gene expression by causing residual levels of these mRNAs in cytokinin-starved cells to decrease and by causing the magnitude of gene induction by cytokinin to increase. Hence, the addition of cycloheximide enabled us to find cytokinin-responsive messages that might otherwise not have been detectable. This phenomenon of cycloheximide-enhanced induction of genes by growth factors is commonly observed in animal systems (23, 24).

The 20 mRNAs we have characterized differ with respect to several aspects of induction. (*i*) The earliest time at which mRNA accumulation is detectable varies from 1 to 4 hr after cytokinin treatment. (*ii*) Cytokinin causes some of the mRNAs to first decrease in abundance and then accumulate to levels that exceed the levels in starved cells. (*iii*) The cytokinin-inducibility of some of the messages is much more apparent in preparations of mRNA than it is in total RNA, suggesting that control at the level of polyadenylylation might play a role in cytokinin-mediated effects.

To determine whether the increases in mRNA levels we observed were specific to cytokinin treatment or more generally associated with growth rate, we compared the amount of mRNA present 2 days after subculture, when cells are growing rapidly, to the amount present 4 days after subculture, when cells cease growth. We found that some of these mRNAs were more abundant 4 days after subculture than 2 days after subculture. Furthermore, most of the mRNAs were less abundant 3 days after cytokinin starvation than 4 days after subculture, even though the cytokinin-starved cells were growing more rapidly. These observations indicate that the levels of these mRNAs do not correlate with the growth rate of the cells.

Because the soybean cells require both auxin and cytokinin for growth, we were able to further define the cytokinin specificity of mRNA accumulation by examining the effect of auxin on mRNA levels. Accordingly, we assessed the auxin responsiveness of the messages in two ways: (*i*) by comparing mRNA levels in auxin-starved cells to the levels in auxin-induced cells, and (*ii*) by comparing the levels in auxin-starved cells to the levels in cytokinin-starved cells. For most of our clones, corresponding mRNA levels increase within 4 hr after auxin treatment of auxin-starved cells, and in most cases mRNA levels after 3 days of auxin starvation are comparable to levels detected 3 days after cytokinin starvation. Hence, although it remains clear that the cDNA clones in our collection correspond to messages that are cytokinin-inducible, other factors such as auxin appear to influence the accumulation of these messages.

From the patterns of accumulation of the cytokinin-induced messages in whole soybean plants, we were able to put most of the messages into five classes: (*i*) those that are present uniformly throughout all tissues, (*ii*) those that preferentially accumulate in all tissues except expanded leaves and cotyledons, (*iii*) those most abundant in expanded leaves and cotyledons, (*iv*) those that accumulate primarily in the elongating region of the stem, and (*v*) those that are most abundant in roots and root tips. These results suggest that the mRNAs present mainly in growing tissues (i.e., classes 2, 4, and 5) may correspond to genes that are involved in growth initiation. Conversely, those present mainly in expanded leaves and cotyledons may correspond to genes that are involved in greening (i.e., chloroplast development).

To determine whether biologically active cytokinins induce greater message accumulation than less active analogs, we tested various compounds for their ability to induce the accumulation of cim1, cim2, and cim11. In this experiment, zeatin, isopentenyladenine, benzyladenine, and their corresponding ribonucleosides, as well as kinetin and thidiazuron, induced similar levels of mRNA accumulation within 4 hr. However, adenine and 6-methyladenine were much less active. These results agree with physiological studies that show adenine and 6-methyladenine to be weak inducers of growth in bioassays compared with the other compounds tested (20, 21).

Sequence analyses indicated that two of the cDNAs in the collection correspond to ribosomal protein mRNAs. Because growth of plant cells requires protein synthesis, it is not surprising that cytokinin induces the accumulation of these mRNAs. Other ribosomal protein mRNAs have been shown to be induced by auxin (25).

The results shown herein demonstrate that induction of gene expression is one of the early molecular events following cytokinin treatment of cytokinin-starved soybean cells. Whether this induction is due to increased transcription or to decreased degradation of specific mRNAs is currently under investigation. Further studies of the genes in this collection, as well as the promoters for these genes, will provide information on the mechanism of cytokinin-induced responses in soybean.

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