Induction of Specific mRNAs in Cultured Soybean Cells during Cytokinin or Auxin Starvation

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ABSTRACT

We report the isolation of five cDNA clones whose corresponding mRNAs accumulate in cultured soybean cells (Glycine max cv Mandarin) during cytokinin or auxin starvation. The levels of three of these mRNAs decrease rapidly after addition of 5 micromolar zeatin to cytokinin-starved cells or after addition of 10 micromolar α-naphthaleneacetic acid to auxin-starved cells. These mRNAs also exhibit various patterns of accumulation in the tissues of intact soybean plants. Partial nucleotide sequence analysis demonstrates that one of the cDNAs in the collection, called SAM46, is 46% identical at the amino acid level to the iron superoxide dismutase gene of Escherichia coli. Expression of this cDNA in Escherichia coli cells results in detectable iron superoxide dismutase activity, confirming the identity of the cDNA.

Cytokinins and auxins are plant growth substances that promote cell division in many cell culture systems at or below 10^{-5} M and are required in combination for the sustained growth of certain cell cultures (e.g. soybean). However, their effects on many aspects of the growth and development of whole plants are dramatically different. For example, the development of lateral buds is stimulated by cytokinin but inhibited by auxin (12).

Recently, much work has been done in various plant systems on the induction of gene expression by cytokinins and auxins. For example, cytokinin treatment of Lemna gibba has been shown to cause increases in the levels of the mRNAs that encode the light-harvesting Chl a/b binding protein and the small subunit of ribulose 1,5-bisphosphate carboxylase (9). Cytokinin-induced mRNAs have also been detected in pumpkin cotyledons (4) and soybean suspension cultures (6). Similarly, auxin-induced mRNAs have been reported in tobacco (20, 22), pea (21), and soybean (10, 13, 24).

The inhibition of expression of plant genes by cytokinins and auxins has also been recently examined. For example, phytochrome mRNA levels have been shown to decrease in response to cytokinin (5) and β-glucanase mRNA levels have been shown to decrease in response to cytokinin and auxin (15). However, much remains to be learned about the mechanism(s), direct or indirect, by which cytokinins and auxins decrease the levels of specific mRNAs. We report the isolation of five cDNAs from soybean (Glycine max cv Mandarin) whose corresponding mRNAs increase in abundance during cytokinin or auxin starvation. Three of these mRNAs decrease in abundance within 4 h of adding either cytokinin to cytokinin-starved cells or auxin to auxin-starved cells. We also report the tissue-specific accumulation of four of these mRNAs in whole soybean plants and show that one of the mRNAs encodes an FeSOD. Further study of the genes that correspond to these mRNAs may lead to an understanding of the mechanism(s) that mediate cytokinin-repressed or auxin-repressed gene expression.

MATERIALS AND METHODS

All phytohormones were obtained from Sigma (St. Louis, MO). Unless otherwise indicated, all nucleic acid manipulations were as described (18).

Tissue Culture

Cultured soybean cells (Glycine max cv Mandarin) were maintained by weekly subculture as described (6). Suspension cultures of these cells were starved 3 d for either cytokinin or auxin, beginning on the fourth day of subculture, after which 5 × 10^{-5} M zeatin was added to cytokinin-starved cells and 10^{-5} M NAA was added to auxin-starved cells. Cells were harvested 4 h after phytohormone treatment.

Isolation of Tissues from Soybean Seedlings

Except where noted, tissues were excised from seedlings of G. max cv Elgin that were grown for 8 d at 27°C in Jiffy Mix under continuous light. The tissues harvested were as follows: unexpanded primary leaves; shoot tip (the 2 mm region of the shoot above the point of attachment of the primary leaves); epicotyl (the 5 mm region of elongating stem between the primary leaves and the cotyledons); cotyledons; hypocotyl (1 cm of stem just below the cotyledons); root tip (the terminal 2–5 mm); whole roots; expanded primary leaves of 16 d old soybeans. These tissues were excised directly into liquid nitrogen and stored at −80°C before RNA isolation.

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2 Abbreviations: FeSOD, iron superoxide dismutase; NAA, α-naphthaleneacetic acid; SOD, superoxide dismutase; MnSOD, manganese superoxide dismutase; SAM, starvation-associated message.
Construction and Differential Screening of the cDNA Library

A cDNA library was constructed from the mRNA of cultured soybean cells that were starved 3 d for zeatin. This construction was done as described (6) except that an oligo dT-NotI primer-adapter was used to prime first-strand cDNA synthesis, an EcoRI linker-adapter was ligated to the 5' ends of the cDNAs, and Bluescript SKII+ was used as a vector (Stratagene, LaJolla, CA). This library was differentially screened as described (6, 16) with cDNA probes made from the mRNAs of cytokinin-starved and unstarved cells.

Cross-Hybridization Analysis

Probes corresponding to cDNA inserts were prepared using random primers as described (8) and hybridized to dot blots of candidate plasmids (16).

RNA Isolations and RNA Blot Analyses

Total and poly(A)+ RNA (mRNA) isolations were as described (6, 11). All RNA blot analyses were performed on poly(A)+ RNA. Briefly, mRNA samples (0.2 μg per lane) were 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 made by T3 RNA polymerase reaction (6, 11).

DNA Sequencing

DNA sequencing reactions were performed on supercoiled DNA by the dideoxynucleotide chain-termination method (19) using T7 DNA polymerase (Pharmacia), the KS 17mer sequencing primer (Stratagene) and [α-35S]deoxyadenosine triphosphate (Amersham, Arlington Heights, IL). Sequencing products were resolved by 8 M urea/8% (w/v) polyacrylamide gel electrophoresis as described (18).

Expression of FeSOD in Escherichia coli

The cDNA insert corresponding to SAM46 was excised by EcoRI-NotI digestion, purified by agarose gel electrophoresis, and ligated into EcoRI-NotI digested vector DNA (Bluescript KSII+, Stratagene, La Jolla, CA). Recombinants of E. coli strain JM83 (23) were then selected on LB plates containing 100 μg/mL carbenicillin. Cultures of E. coli strain JM83 containing either the recombinant plasmid SAM46/KSII+ or the vector KSII+ were grown in LB liquid medium containing 100 μg/mL carbenicillin at 37°C for 16 h. Crude cell extracts of these stationary phase cultures were then prepared in 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA by passage through a French pressure cell at 14,000 psi. Soluble supernatants of these extracts were prepared by centrifugation at 20,000g for 15 min and stored at −80°C. One hundred μg of protein, as determined by the method of Bradford (2), was fractionated per lane on a nondenaturing 10% (w/v) polyacrylamide slab gel. Superoxide dismutase assays were performed on gel slices as described (1) after a 45-min incubation at 25°C in either 50 mM potassium phosphate (pH 7.8), 50 mM potassium phosphate (pH 7.8) containing 2 mM KCN, or 50 mM potassium phosphate (pH 7.8) containing 2 mM H2O2.

RESULTS AND DISCUSSION

We were interested in determining whether cytokinin or auxin depletion of cultured soybean cells was accompanied by increases in the levels of specific mRNAs. For this study, we used a suspension-cultured soybean cell line that requires exogenous cytokinin, as well as auxin, for sustained growth in vitro. This cell line ceases growth upon starvation for either hormone and resumes normal growth upon addition of exogenous hormone for at least 5 d after the beginning of starvation. Growth curves for normal and cytokinin-starved soybean cell cultures have previously been reported (6).

We constructed a plasmid cDNA library containing 105 clones from the mRNA of soybean cells that were starved 3 d for cytokinin. Half of this library was differentially screened with cDNA probes made from the mRNAs of cytokinin-starved and unstarved cells. (The control cDNA probe from unstarved cells was made from the mRNA of log phase cultures growing in the presence of cytokinin and auxin, 2 d postsubculture.) Fifty colonies that hybridized more strongly to the cDNA probe from cytokinin-starved cells than to the cDNA probe from unstarved cells were selected. However, cross-hybridization analysis indicated that all 50 of these candidates belonged to one of five cross-hybridizing groups. The longest cDNA insert in each group was chosen for further study.

We confirmed by RNA blot analysis (Fig. 1) that the five cDNAs in this collection correspond to starvation associated messages (SAMS). This confirmation was obtained in three separate experiments. We have previously shown data on specific control mRNAs whose levels decrease or do not change significantly during identical cytokinin and auxin starvations (6).

As shown in Figure 1, SAM22 is present at relatively low levels in log phase cells that are growing in the presence of cytokinin and auxin. However, this mRNA accumulates approximately 10-fold (as determined by laser scanning densitometry) by the fourth day of subculture, when cells enter stationary phase. No further increase in the level of SAM22 occurs during 3 d of cytokinin starvation, perhaps because a sufficiently low level of hormone is present by the fourth day of subculture to fully induce SAM22 accumulation. Consistent with this hypothesis, SAM22 decreases in abundance within 4 h of adding cytokinin to cytokinin-starved cells. Furthermore, auxin starvation and readdition causes similar, if not more dramatic, changes in SAM22 accumulation.

SAM26 and SAM46 also increase in abundance between the second and the fourth days of subculture (Fig. 1), but accumulate to even higher levels during 3 d of cytokinin starvation (SAM26 and SAM46 levels increase approximately 10- and 5-fold, respectively, over the levels found in log phase cells by the end of the cytokinin starvation). Furthermore, as with SAM22, the levels of SAM26 and SAM46 decrease rapidly upon cytokinin treatment of cytokinin-starved cells. Auxin starvation also induces the accumulation of SAM26 and SAM46, which then decrease dramatically in abundance within 4 h of adding auxin to auxin-starved cells.
Unlike the mRNAs discussed above, SAM36 and SAM45 decrease in abundance between d 2 and d 4 of subculture (Fig. 1), but increase in abundance 5- to 10-fold during cytokinin or auxin starvation. Since these two messages are present at higher levels in cells starved 3 d for cytokinin or auxin than in log phase cells growing in the presence of both hormones, we classify them as starvation-associated messages. However, the decrease in SAM36 and SAM45 levels between d 2 and d 4 of subculture is inconsistent with these messages being induced solely by cytokinin or auxin depletion. Furthermore, neither of these mRNAs decreases significantly in abundance within 4 h of adding cytokinin to cytokinin-starved cells or auxin to auxin-starved cells.

**Figure 1.** Accumulation of SAMs during hormone starvation. Lanes (from left to right) are as follows: mRNA from log phase soybean cells grown in the presence of cytokinin and auxin (d 2 of subculture); mRNA from early stationary phase cells grown in the presence of cytokinin and auxin (d 4 of subculture); mRNA from cells starved 3 d for zeatin, beginning on the fourth day of subculture; mRNA from cells starved 3 d for zeatin and then treated for 4 h with \( 5 \times 10^{-6} \) M zeatin; mRNA from cells starved 3 d for auxin, beginning on the fourth day of subculture; mRNA from cells starved 3 d for auxin and then treated for 4 h with 10^{-5} M NAA. Probes are indicated on the left.

**Figure 2.** Tissue specificity of SAM accumulation in soybean plants. All mRNAs are from 8 d old soybean plants unless otherwise indicated. Lanes (from left to right) are as follows: mRNA from unexpanded primary leaves; mRNA from shoot tip; mRNA from epicotyl; mRNA from cotyledons; mRNA from hypocotyl; mRNA from root tip; mRNA from whole roots; mRNA from expanded primary leaves of 16 d old soybeans; mRNA from cultured cells starved 3 d for zeatin (for reference). Probes are indicated on the left.
Because phytohormone levels can vary in different plant tissues, we analyzed the tissue specificity of SAM accumulation in whole soybean plants. As shown in Figure 2, SAM accumulation appears to be strongly root-specific in soybean seedlings. SAM26, on the other hand, is detectable in all tissues at various levels. SAM36 is present at low levels in root tip and whole root, while SAM45 is most abundant in epicotyl and cotyledon. SAM45 and SAM46 appear to be developmentally regulated in leaves, for neither are detectable in the unexpanded primary leaves of 8 d old plants but are present in the expanded primary leaves of 16 d old plants.

To help identify the cDNAs in our collection, all of which are approximately the same length as their corresponding mRNAs (data not shown), we determined 200 to 400 nucleotides of DNA sequence at the 5' end of each cDNA (data not shown), translated the sequences, and searched for sequence similarities in a complete translation of the GenEMBL version 6.0 nucleic acid sequence data base (7). This search indicated (Fig. 3) that the 5' end of SAM46 is 46% identical to the amino acid level to the 5' end of the Escherichia coli FeSOD gene (3).

To confirm the identity of SAM46, the corresponding cDNA insert was expressed in E. coli cells, resulting in detectable FeSOD activity. This was accomplished by inserting the cDNA into EcoRI-NotI-digested KSII+ vector DNA, which generated a recombinant plasmid, called SAM46/KSII+, containing a translational fusion between the lacZ sequences on the vector and the SAM46 sequences on the insert. SAM46/KSII+ and KSII+ were then introduced into E. coli strain JM83 and crude supernatants were prepared from stationary phase cultures of the resulting transformants. These supernatants were fractionated on a nondenaturing polyacrylamide gel and slices of the gel were either stained with Coomassie brilliant blue R-250 (Fig. 4, panel A) or assayed for SOD activity (Fig. 4, panels B, C, and D). As shown in Figure 4, the supernatant from cells bearing the control vector KSII+ contains three bands of SOD activity. The lowest band is resistant to 2 mM KCN but sensitive to 2 mM H2O2, indicating that it corresponds to endogenous E. coli FeSOD (17). The upper two bands are resistant to both KCN and H2O2 treatments and, therefore, correspond to endogenous E. coli MnSOD (17). The supernatant from cells bearing SAM46/KSII+ contains the same three bands of E. coli SOD activity and, in addition, contains SAM46-dependent SOD activity distributed at the top of the gel above and below the E. coli MnSOD bands. This additional activity is resistant to 2 mM KCN but sensitive to 2 mM H2O2, confirming the FeSOD identity of SAM46 (the distribution of SAM46-dependent FeSOD activity in the gel probably reflects the aggregation or degradation state of the expressed protein).

The results shown in Figures 1 and 2, along with the demonstration that SAM46 encodes an FeSOD, suggest that the soybean FeSOD gene is regulated by cytokinin and auxin in cultured cells and by developmental signals in the leaves of whole plants (whether this mRNA accumulates in other tissues of 16 d old plants is currently under investigation). The induction of FeSOD mRNA accumulation in cultured cells by cytokinin or auxin starvation may reflect the need to turn-over superoxide formed by oxidative enzymes that cat-

Figure 3. Identity between the deduced amino acid sequence of SAM46 and the N-terminus of E. coli iron superoxide dismutase (3). Vertical lines indicate sequence identity and dots indicate conservative substitutions. Asterisks indicate the start of translation for the E. coli FeSOD and the presumed start of translation for the soybean FeSOD. A putative leader sequence for import of the soybean FeSOD into chloroplasts is indicated.

Figure 4. Detection of SAM46-dependent FeSOD activity in E. coli cells. Lanes (from left to right) are as follows: Coomassie blue-stained protein from JM83 cells containing the plasmid SAM46/KSII+; Coomassie blue-stained protein from JM83 cells containing the plasmid KSII+; total SOD activity from JM83 cells containing the plasmid SAM46/KSII+; total SOD activity from JM83 cells containing the plasmid KSII+; KCN-resistant SOD activity from JM83 cells containing the plasmid SAM46/KSII+; H2O2-resistant SOD activity from JM83 cells containing the plasmid SAM46/KSII+. The positions of the endogenous E. coli SOD activities are indicated by arrows on the right.
alyze the breakdown of macromolecules during hormone deprivation. In the leaves of whole plants, FeSOD mRNA accumulates during the process of expansion, at which point photosynthetic activity increases. This is particularly interesting because FeSOD is present in chloroplasts (17), which generate superoxide upon illumination (14, 17).

The phytohormone responsiveness of the starvation-associated messages described in this paper will require further study. It is currently unclear whether this responsiveness is specific to phytohormones or simply a general phenomenon associated with the stress caused by phytohormone deprivation. Accordingly, experiments are planned to test the possibility that other stresses will induce the accumulation of these mRNAs.

The work in this paper demonstrates that five unique mRNAs accumulate during cytokinin or auxin starvation of cultured soybean cells, that exogenous cytokinin or auxin causes a rapid decrease in the levels of three of these mRNAs and that fluctuations in the levels of these mRNAs occur during different phases of the growth of normal soybean cultures. Whether these changes in mRNA levels reflect altered rates of transcription of the corresponding genes, changes in RNA processing, or changes in mRNA turnover is currently being studied. This work also demonstrates developmental regulation of the accumulation of these mRNAs in soybean seedlings. Further studies on the mechanism(s) by which cytokinin and auxin regulate the expression of the corresponding genes in cultured cells and on the mechanism(s) that mediate the developmental regulation of these genes in whole plants may lead to a better understanding of the role of cytokinin and auxin in the developmental program of plants.

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