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Induction and Regulation of Heat-Shock Gene Expression by an Amino Acid Analog in Soybean Seedlings[1]

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The effect of the proline analog azetidine-2-carboxylic acid (Aze) on the induction and the regulation of heat-shock (HS) mRNA accumulation and heat-shock protein (HSP) synthesis in soybean (*Glycine max*) seedlings was studied. Treatment with Aze elicited an HS-like response at the normal growth temperature, 28°C, with seven of nine HS cDNA clones tested. Two cDNA clones, GmHsp22.5 and pFS2033, share 78% identity; however, transcripts hybridizing to GmHsp22.5 but not pFS2033 accumulated with Aze treatment at 28°C. Substantial incorporation of radioactive amino acid into high molecular weight HSPs but not low molecular weight HSPs was observed in vivo during Aze treatment at 28°C. Low molecular weight HSPs were detected using antibodies raised against an abundant member of low molecular weight class I HSPs, indicating that low molecular weight HSPs were synthesized at normal growth temperatures during Aze treatment despite a lack of substantial in vivo radioactive amino acid incorporation. In summary, Aze treatment induced accumulation of most but not all HS mRNAs and HSPs in soybean seedlings; the observations presented here suggest differential regulation among various HS genes at the transcriptional and posttranscriptional levels.

Increases in environmental temperatures of about 5 to 10°C above the normal growth temperatures leads to altered gene expression in bacteria, lower eukaryotes, animals, and plants, a response commonly referred to as the HS response (for reviews, see Nagao et al., 1990; Nover, 1991; Vierling, 1991; Parsell and Lindquist, 1993; Morimoto et al., 1994). At the onset of the HS response, transcriptional activation of HS genes occurs, and the transcription of most previously active genes is decreased. Relative to most mRNAs present at control temperature, the HS mRNAs are preferentially translated into HSPs at heat-stress temperatures.

Even though HSPs were first characterized because their expression increased in response to elevated temperatures, many HSPs are also synthesized in response to other stresses, including, but not limited to, amino acid analogs, arsenite, and cadmium (Edelman et al., 1988; Nover, 1991). The signal transduction pathway of HS gene expression remains unclear. Since the stresses that induce HSP synthesis share the common property of either damaging proteins directly or causing cells to synthesize aberrant proteins, it has been hypothesized that accumulation of abnormal proteins in cells causes the onset of HS gene expression (Ananthan et al., 1986; Edington et al., 1989).

Among the agents that elicit an HS-like response at normal growth temperatures, amino acid analogs have attracted research interest because they often disrupt the function of proteins into which they have been incorporated. In *Drosophila*, the inclusion of the amino acid analog canavanine in the incubation medium resulted in the synthesis of nonfunctional HSPs based on the inability of the analog-containing HSPs to undergo selective localization during heat treatment (DiDomenico et al., 1982). Studies using amino acid analogs in *Drosophila* and animals have shown that the synthesis of functional HSPs is important to the normal regulation of HS gene expression (DiDomenico et al., 1982; Thomas and Mathews, 1984; White and Hightower, 1984).

To help understand the regulation of HS gene expression in higher plants, the effect of treatment with a Pro analog, Aze, on the expression of soybean (*Glycine max*) HS genes was initiated. A collection of soybean HS cDNA clones representing different molecular weight classes or genes localized to different cellular sites were tested to evaluate mRNA induction and accumulation following Aze treatment. Novel observations of differential HSP gene expression and HSP synthesis following incubation with Aze are presented in this paper.

MATERIALS AND METHODS

Materials

Soybean (*Glycine max* cv Williams 82) seeds were surface sterilized with 10% Clorox for 10 min and allowed to germinate in rolls of moist Kimpak (Nationwide Papers, Atlanta, GA) at 28°C in a dark growth chamber (Lin et al., 1984). Two-day-old seedlings with cotyledons removed were incubated in buffer containing 1 mM potassium phosphate (pH 6.0), 1% Suc (w/v), and 50 μg/mL chloramphenicol. Aze and cycloheximide (Sigma) were added in the

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Abbreviations: Aze, azetidine-2-carboxylic acid; HS, heat shock; HSP, HS protein; TBS, Tris-buffered saline (20 mM Tris, pH 7.5 and 500 mM NaCl).

incubation buffer as indicated. The treatments were done in shaking water baths equilibrated at the desired temperatures.

Northern Hybridization Analyses

Total RNAs were isolated from soybean seedlings as previously described (Schöffl and Key, 1982), separated by electrophoresis in 2% agarose gels containing 6% formaldehyde and 10 mM Mops (pH 7.0), and blotted onto nitrocellulose filters (Sambrook et al., 1989). Probes for northern blots were prepared by random primer labeling of cDNA inserts using [α-^{32}P]dATP (5000 Ci/mmol, New England Nuclear). All blots were prehybridized for 4 h in hybridization buffer containing 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 7.0), 5 × Denhardt's solution, 100 μg/mL salmon sperm DNA, 100 μg/mL yeast RNA, and 0.1% SDS. ^{32}P-labeled probe was added to fresh hybridization buffer, and blots were hybridized for 24 h at 42°C and washed as described by Kimpel and Key (1985).

Nine cDNA clones that hybridize to soybean HS-induced poly(A)$^+$ RNAs (see "Results") were used in the experiments. According to the deduced molecular weights of the encoded peptides, near-full-length cDNA clones have been named GmHsp23.9, GmHsp22.3, GmHsp20.7, and GmHsp22.5 (LaFayette et al., 1995). The partial cDNA clones pCE53, pCE75, and pFS2033 represent the soybean genes GmHsp17.5E, GmHsp17.9D, and GmHsp22, respectively.

In Vivo Labeling of Seedlings

Soybean seedlings (2 g) were incubated at the desired temperatures as described in "Materials." During the last 2 h of incubation, Tran^{35}S-Label (ICN) equivalent to 200 μCi of [^{35}S]Met was added. At the end of the incubation, total proteins were extracted as described previously (Lin et al., 1984), except that 60 mM DTT was used instead of 2% β-mercaptoethanol in the extraction buffer.

In Vitro Translation

In vitro translation of soybean seedling total RNAs (5 μg per reaction) was carried out in a cell-free wheat germ S-30 system supplemented with [^{35}S]Met (Key et al., 1981).

Electrophoresis

SDS-PAGE was performed using 12.5% (w/v) polyacrylamide gels according to the method of Lin et al. (1984). Proteins were resolved by two-dimensional gel electrophoresis using IEF in the first dimension and 12.5% (w/v) polyacrylamide SDS-PAGE in the second dimension (Mansfield and Key, 1987). Proteins were loaded onto the acidic end of IEF gels from pH 4 to 7. Radioactive proteins were visualized by fluorography (Lin et al., 1984).

Immunoblots

Proteins were separated by two-dimensional PAGE and blotted onto a polyvinylidene fluoride microporous membrane (Millipore) according to the manufacturer's instructions, except that electroblotting was done with 60 V for 3 h followed by 100 V for 1 h. Nonspecific protein-binding sites were blocked by incubation in TBS with 3% gelatin for 1 h. Antibodies were raised in rabbits against an abundant member of the class I low molecular weight HSPs (Hsieh et al., 1992). Blots were incubated with whole sera at a 1:1000 dilution in TBS with 1% gelatin overnight followed by the incubation in goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical) diluted 1:2000 in TBS with 1% gelatin for 1 h. Antigenic polypeptides were visualized as described by Knecht and Dimond (1984).

RESULTS

HS mRNA Accumulation Induced by Aze Treatment

Nine cDNA clones, which are described in Table I, were used to assess whether Aze induces HS mRNA accumulation in soybean seedlings. Emphasis was placed on low molecular weight HSP genes because of their unique abundance and complexity in plants, and selected cDNA clones were chosen because they represent different soybean HS gene classes. The cDNA clone pSB70 encodes a major cytoplasmic heat-inducible HSP70 (Roberts and Key, 1991) and was included as a high molecular weight comparison. The cDNA clone pCE54 encodes a general stress-induced protein with similarity to glutathione *S*-transferase (Czarnecka et al., 1984; Droog et al., 1993). Other cDNA clones used in this study encode HSPs in the molecular mass range of 15 to 24 kD, and these peptides are localized in the cytoplasm (pCE53 and pCE75), chloroplasts (GmHsp20.7), endomembranes (GmHsp22.5 and pFS2033), and mitochondria (GmHsp23.9) (Key et al., 1985; Vierling et al., 1988; LaFayette et al., 1995).

Northern blot analyses revealed that mRNAs hybridizing to cDNA clones pSB70, pCE53, pCE75, GmHsp23.9, GmHsp22.3, GmHsp22.5, and pCE54 accumulated at 28°C, the normal growing temperature, in the presence of 5 mM Aze (Fig. 1). On the other hand, HS mRNAs represented by cDNA clones GmHsp20.7 and pFS2033 were not significantly induced by treatment with 5 mM Aze at 28°C (Fig. 1). Data presented in Figure 1 are for 2 h of Aze treatment, and autoradiograms for pFS2033 and GmHsp20.7 were exposed 5 times longer than GmHsp22.5 in an attempt to enhance visualization of hybridization signal. Longer Aze treatment (6 and 12 h) resulted in increased message accumulation for those genes induced by Aze but did not result in accumulation of mRNA hybridizing to pFS2033 or GmHsp20.7 (data not shown). Aze treatment (5 mM) at 40°C resulted in accumulation of HS mRNAs corresponding to all of the cDNA clones noted above, including GmHsp20.7 and pFS2033 (Fig. 1).

As one test of the hypothesis that the accumulation of abnormal proteins due to the incorporation of amino acid analog into proteins may cause the onset of HS gene expression, the protein translation inhibitor cycloheximide was added in the experiments. Cycloheximide (2 μg/mL) in the incubation medium for 30 min prior to a 40°C heat treatment inhibited amino acid incorporation into protein 98% but did not inhibit the induction of HS mRNA accumulation corresponding to all cDNA clones tested (data not shown).

Table I. *Summary of HS-induced cDNA clones used in northern hybridization analyses*

Probe	Hybrid-Select/Translation Proteins[a]		References
	Number detected	Size(s)	
		kD	
pSB70	1	70	Roberts and Key (1991)
pCE54	4	26–27	Czarnecka et al. (1984)
LMW class I[b](cytoplasm-localized) pCE53	13	15–19	Key et al. (1985)
LMW class II (cytoplasm-localized) pCE75	5–8	15–16	Key et al. (1985)
LMW class III (chloroplast-localized) GmHsp20.7	2	28[c]	Vierling et al. (1988)
LMW class IV (endomembrane-localized) GmHsp22.5	3–4	21–24	Nagao et al. (1990) LaFayette et al. (1995)
pFS2033	3	21–24	Schöffl and Key (1982) LaFayette et al. (1995)
LMW class V GmHsp22.3	4	21–24	Nagao et al. (1990) LaFayette et al. (1995)
LMW class VI GmHsp23.9	4	21–24	Nagao et al. (1990) LaFayette et al. (1995)

[a] The number and size(s) of the proteins detected by two-dimensional gel electrophoresis following hybrid-select and in vitro translation of HS poly(A)$^+$ RNAs using each of the probes. [b] Low molecular weight (LMW) HSPs are classified as suggested by Vierling (1991) and by LaFayette et al. (1995). [c] Predicted processed protein is 20.7 kD.

The cycloheximide treatment alone did not induce accumulation of the pSB70 and pCE53 HS mRNAs at 28°C (Fig. 2). The HS mRNAs corresponding to pSB70 and pCE53 were induced by Aze at 28°C in the presence of cycloheximide; however, their accumulation was delayed because significant levels of mRNA were not detected after 2 h of cycloheximide plus Aze treatment but were detected after 6 h (as pSB70 and pCE53 in Fig. 2). Similar results were observed with pCE75, GmHsp23.9, GmHsp22.3, and GmHsp22.5 (data not shown). Cycloheximide alone, however, did induce the accumulation of pCE54 mRNAs at 28°C, and the accumulation was much greater with the combined treatment of cycloheximide and Aze at 28°C (Fig. 2). Cycloheximide treatment alone or in combination with Aze treatment did not induce the accumulation of the GmHsp20.7 and pFS2033 mRNAs (as pFS2033 shown in Fig. 2).

During a prolonged 40°C heat treatment, the steady-state levels of HS mRNAs were maximal after 1 to 2 h and were much reduced after 6 h at 40°C as previously reported (Kimpel et al., 1990; Fig. 2). Except for that hybridizing with the pCE54 clone, the levels of Aze-induced HS mRNAs that accumulated at 28°C for 2 h were lower than the corresponding levels after a 2-h treatment at 40°C; however, in contrast to 40°C heat treatments, the levels of HS mRNAs continued to increase between 2 and 6 h of Aze treatment (Fig. 2). The decline of HS mRNA steady-state levels was not observed in the Aze-treated seedlings, as occurs after 1 to 2 h of continuous 40°C treatment. The level of pCE54 mRNA increased to well above the levels typically induced by a 2-h 40°C treatment (Fig. 2).

Radioactive Amino Acid Incorporation into HSPs during Aze Treatment

The effects of Aze treatment on HSP synthesis were also evaluated by incubating soybean seedlings with radioac-

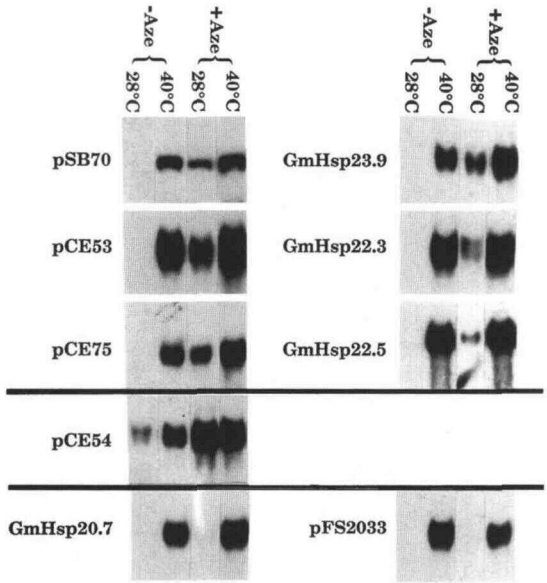

Figure 1. Northern blot analyses of the HS mRNA accumulation during the treatments of heat and Aze for 2 h. Fifteen micrograms of total RNA were applied to each lane. The ^{32}P-labeled cDNA probes used for each blot are shown on the left. +Aze, 5 mM Aze; −Aze, no added Aze.

tive amino acid. Incorporation of exogenously supplied radioactive amino acid into HSPs was prominent during the first 2 to 4 h of continuous 40°C treatment. Incorporation into HSPs was much reduced after about 6 h at 40°C, and the protein synthesis pattern gradually returned to the 28°C pattern (Kimpel et al., 1990; Fig. 3A). This marked decline in HSP synthesis during a continuous 40°C treatment was not due to a general decrease in the uptake or incorporation of radioactive amino acid by the seedlings (Kimpel et al., 1990).

There was substantial incorporation of radioactive amino acid into high molecular mass HSPs in Aze-treated seedlings (e.g. 70, 83, and 100 kD), and the synthesis of these HSPs appeared to be almost linear for up to 12 h (Fig. 3B). Conversely, the incorporation into proteins in the molecular mass range of 15 to 18 kD (e.g. 15- to 18-kD HSPs are normally the most abundant; Mansfield and Key, 1987) was hardly detectable with either [^{35}S]Met (Fig. 3B) or [^{3}H]Leu (data not shown). Further analysis by two-dimensional gel electrophoresis of in vivo labeled proteins with Aze treatment at 28°C, in which the radioactive incorporation into low molecular weight HSPs was too low to be detected, confirmed the differential incorporation of radioactive amino acids into the high molecular weight HSPs relative to the low molecular weight HSPs (data not shown). This phenomenon will be discussed below.

Aze treatment at 40°C resulted in a normal pattern of HSP synthesis, including both high molecular weight and low molecular weight HSPs, similar to a 40°C treatment alone. The synthesis of HSPs also appeared to be linear for up to 12 h (Fig. 3C). The marked decline of HSP synthesis at 6 to 8 h normally observed during a continuous 40°C treatment (Fig. 3A) was not observed in Aze-treated seedlings, in which HSP synthesis continued for up to 12 h both at 28 and at 40°C.

Immunoblot Analyses of HSP Synthesis during Aze Treatment

During in vivo labeling in the presence of Aze at 28°C, radioactive amino acid incorporation into high molecular mass HSPs (e.g. 70, 83, and 100 kD) was detected but was hardly detected in low molecular mass HSPs (e.g. 15–18 kD) as noted above. The analysis by two-dimensional gel electrophoresis of in vitro translation products from mRNAs isolated from soybean seedlings treated with Aze at 28°C gave very similar protein patterns to those from 40°C-induced mRNAs, especially in the 15- to 18-kD range (Fig. 4). This suggests that the Aze-induced mRNAs translated into the same products as 40°C-induced mRNAs, including both high and low molecular weight HSPs.

Rabbit polyclonal antibodies that recognize the soybean class I (15–18 kD) HSPs (Hsieh et al., 1992) were used to evaluate the accumulation of these HSPs during Aze treatment. The class I HSPs represent the translation products of mRNA hybrid selected by the cDNA clone, pCE53 (Table I). The pCE53 mRNAs were induced to accumulate by Aze treatment at 28°C as noted above (Figs. 1 and 2). Whereas radioactive amino acid incorporation into these low molecular weight HSPs during Aze treatment at 28°C was minimal (Fig. 3B), immunoblot analysis demonstrated that these HSPs accumulated to detectable levels with Aze treatment,

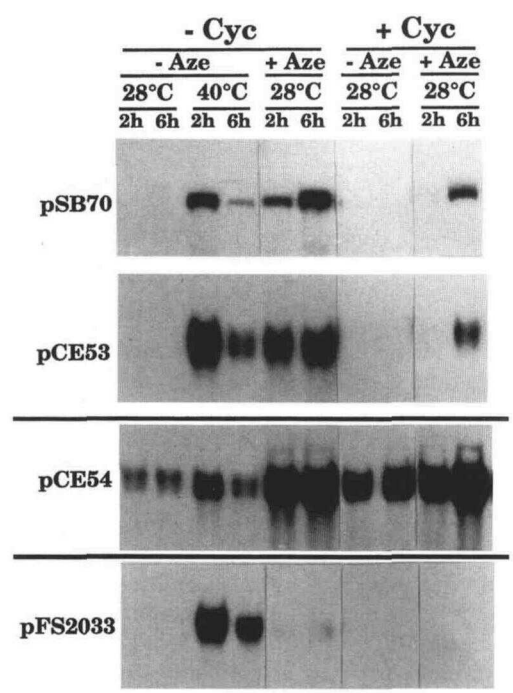

Figure 2. Northern blot analyses of the HS mRNA accumulation during the treatments of heat, Aze, and cycloheximide. Fifteen micrograms of total RNA were applied to each lane. The ^{32}P-labeled cDNA probes used for each blot are shown on the left. +Aze, 5 mM Aze; −Aze, no added Aze; +Cyc, 2 μg/mL cycloheximide; −Cyc, no added cycloheximide.

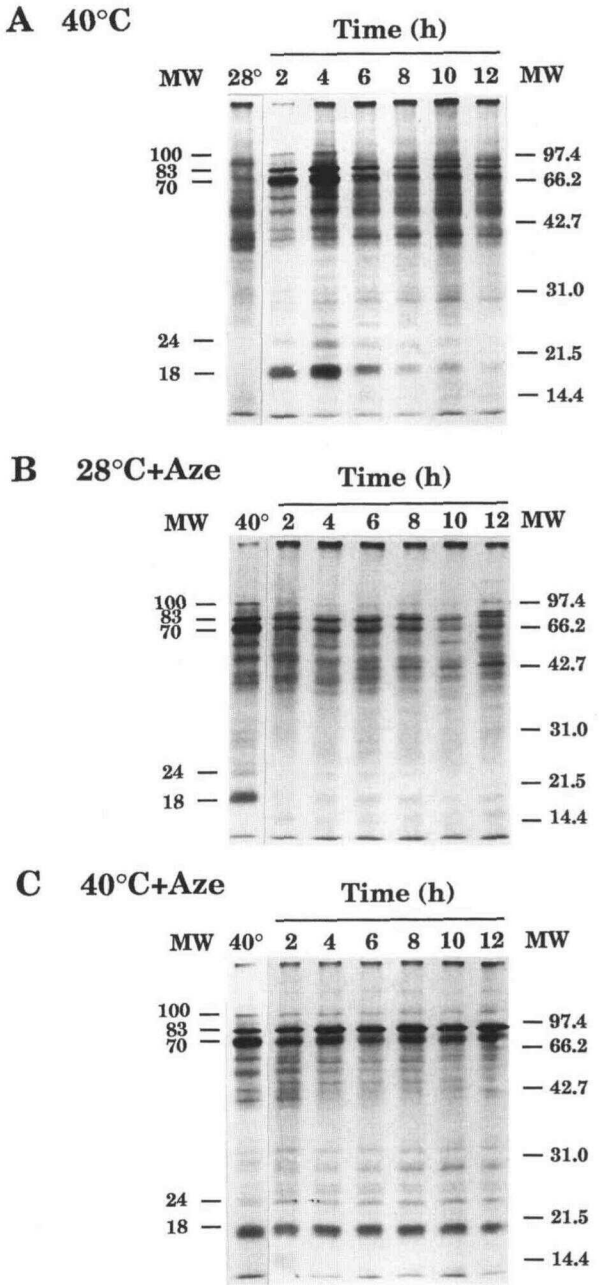

Figure 3. Incorporation of Tran^{35}S-Label during prolonged 40°C and Aze (5 mM) treatments. Fifteen seedlings were pulse labeled with Tran^{35}S-Label at consecutive 2-h intervals. Equal counts per minute of each protein extract were analyzed by electrophoresis on 12.5% SDS-PAGE. A, 40°C treatment; B, 28°C and Aze treatment; C, 40°C and Aze treatment. Lanes 28° and 40° show the labeled protein pattern after 2 h of treatment at the indicated temperatures. Major HSPs are indicated on the left and molecular weight markers are shown on the right of each gel (MW, in thousands).

although the levels were lower than in 40°C-treated seedlings (Fig. 5A). The Aze-induced class I HSPs were barely detected in the first 2 h at 28°C, but the level of accumulation increased during the 12 h of treatment (Fig. 5A).

When seedlings were treated with Aze at 40°C for 2 h, the level of class I HSP accumulation was lower than the level that accumulated at 40°C without Aze; however, the level of accumulation with the Aze treatment at 40°C was higher after both 6 and 12 h, compared to the corresponding 40°C treatment without Aze (Fig. 5A).

The antibodies used in these experiments detected at least 20 heat-inducible proteins ranging in molecular mass from 15 to 18 kD (Fig. 5B). The immunoblot patterns of HSPs produced by Aze treatment at 28 and 40°C were similar to the pattern of HSPs induced at 40°C (Fig. 5B). It is interesting that immunoblot analyses showed that one or two additional proteins that did not appear to be induced by 40°C treatment were possibly induced by Aze treatment. Additional regulatory control is suggested for proteins to be induced specifically or enhanced by Aze treatment.

DISCUSSION

Northern blot analyses revealed that the soybean HS genes that were tested, except for those represented by cDNA clones GmHsp20.7 and pFS2033, were induced by the Pro analog Aze at the normal growing temperature, 28°C (Fig. 1). The induction of HS genes by seemingly diverse conditions suggests a shared or common mechanism to initiate transcription of the HS genes. A common feature of heat and amino acid analog treatment is the tendency to generate abnormal polypeptides, which is thought to be a feature of HSP gene induction. Data consistent with this idea are presented in Figure 2, where protein synthesis was inhibited by cycloheximide and the accumulation of HS mRNAs was barely detected in Aze-treated seedlings growing at 28°C for 2 h; accumulation was also greatly reduced at 6 h.

Amino acid analogs were also shown to induce HSP synthesis in chicken embryo (Kelley and Schlesinger, 1978), cultured animal cells (Hightower, 1980), *Escherichia coli* (Goff and Goldberg, 1985), Chinese hamster fibroblasts (Li and Laszlo, 1985), and *Tetrahymena* (Jones and Findly, 1986), but differential HS gene expression was not observed. In soybean, however, expression of genes encoding low molecular weight HSPs represented by two cDNA clones, GmHsp20.7 and pFS2033, was not observed in Aze-treated seedlings at 28°C. This suggests a major difference in the regulation of expression of some HS genes, either transcriptionally or posttranscriptionally. One of the cDNA clones, GmHsp20.7, is representative of HS genes in class III, which encode cytoplasm-synthesized, chloroplast-localized HSPs (Vierling et al., 1986, 1988; Table I). The other cDNA clone, pFS2033, represents a small family of HS genes that encode proteins targeted to the endomembranes (Helm et al., 1993; LaFayette et al., 1995). The inability of Aze treatment to induce the accumulation of mRNAs corresponding to GmHsp20.7 and pFS2033 at 28°C might be the consequence of a different mechanism involved in the initiation of transcription of these HS genes; alternatively, these transcripts may be synthesized but somehow be less stable than other HS mRNAs in Aze-treated cells. Since these transcripts appear to be induced normally with Aze treatment at 40°C, a different mechanism must be involved

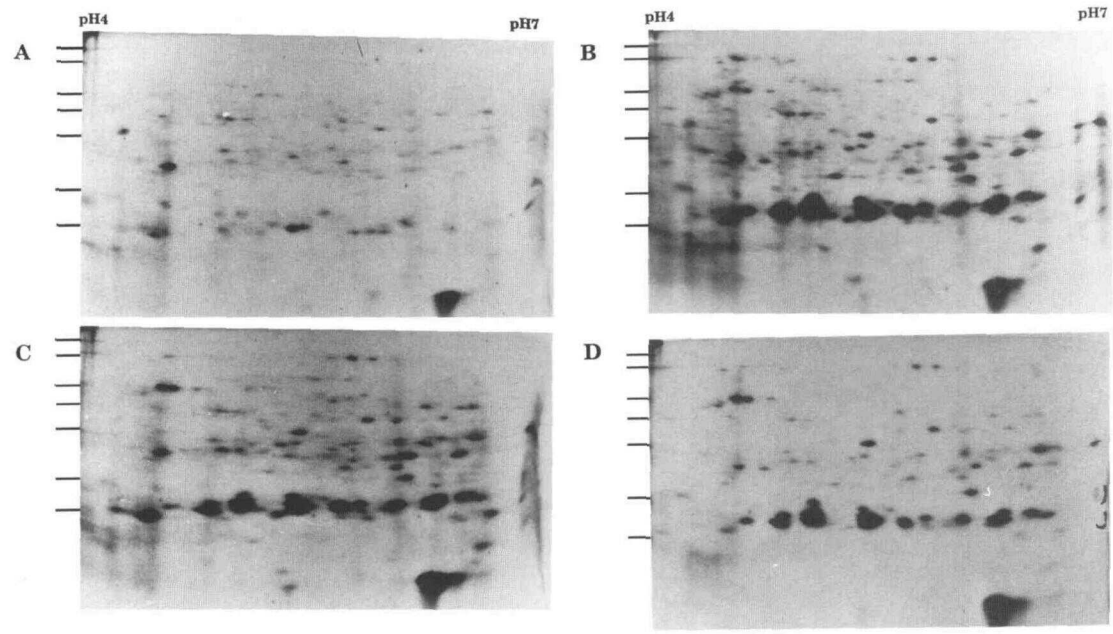

Figure 4. Two-dimensional gel electrophoresis of in vitro translation products labeled with [^{35}S]Met RNA samples were isolated after treatment for 2 h at 28°C (A), 40°C (B), 28°C in the presence of 5 mM Aze (C), and 40°C in the presence of 5 mM Aze (D). Molecular mass markers (200, 97.4, 68, 43, 29, 18.4, and 14.3 kD, from top to bottom, respectively) are shown on the left of each gel.

in the regulation of expression of these genes during Aze treatment.

The two cDNA clones pFS2033 and GmHsp22.5 encode strikingly similar proteins based on 78% identity and 90% conservation at the amino acid sequence level (LaFayette et al., 1995). The mRNAs for pFS2033 and GmHsp22.5 were preferentially associated with ER-bound polysomes in vivo, and both deduced polypeptides possess the carboxyl-terminal tetrapeptide KQEL, which is very similar to the consensus ER retention motif KDEL (LaFayette et al., 1995). Because of these similarities, it was concluded that these genes belong to the same class of low molecular weight HSPs; yet mRNAs corresponding to GmHsp22.5, but not to pFS2033, accumulated with Aze treatment at 28°C. The data presented in Figure 1 were for a 2-h Aze treatment, but longer treatment times (6 or 12 h) led to increasing accumulation of GmHsp22.5 mRNA without significant accumulation of mRNA hybridizing to pFS2033 (Fig. 2; data not shown). This example demonstrates that individual members of an HS gene class may be differently regulated. The selective difference between the regulation of expression by Aze and heat of the two highly homologous genes pFS2033 and GmHsp22.5 merits particular interest in future work.

The maximal level of HS mRNAs that accumulated in Aze-treated seedlings at either 28 or 40°C generally was similar to or lower than the heat-induced level. A marked exception to this pattern of mRNA accumulation was exhibited by the pCE54 class; these mRNAs accumulated to levels well above that induced by heat treatment. Genes of the pCE54 class are expressed at control temperatures, and synthesis is enhanced severalfold by heat as well as by numerous other stress agents, including Aze, cycloheximide, high salt concentration, anaerobiosis, water stress, 2,4-D, ABA, and heavy metals. Thus, this family is considered a general stress-responsive gene class rather than strictly an HS class (Czarnecka et al., 1984).

As noted in the literature, the expression of HS genes is transient, ranging from minutes in *E. coli* to a few hours in eukaryotes. During continuous heat treatment, this transient expression has been referred to as self-regulation (DiDomenico et al., 1982; Stone and Craig, 1990; Gurley and Key, 1991). In soybean seedlings, the accumulation of HS mRNAs was detected on northern blots within 3 to 5 min of exposure to 40°C. During continuous heat treatment, the HS mRNAs reached maximal steady-state levels at 1 to 2 h and were much reduced after 6 h but were still detectable after 12 h of exposure to heat (Kimpel et al., 1990). Transient HSP synthesis and accumulation was also observed during continuous heat treatment in which the incorporation of radioactive amino acid into HSPs reached maximal levels at 4 h and declined thereafter (Fig. 3A). The data presented here indicate that the synthesis of HSPs in the presence of Aze appeared to be linear up to 12 h at either 28 or 40°C (Fig. 3, B and C). Quantitative immunoblot analysis for pCE53 class I low molecular weight HSPs (Table I) showed that, after a 24-h Aze treatment, HSP accumulation was 80% of the maximum 3- to 6-h HS level (T.-L. Jinn and C.-Y. Lin, unpublished data). It is concluded that Aze treatment interfered with self-regulation of the HS response in soybean seedlings. It has been suggested that self-regulation is controlled by HSP70 in *E. coli* (Straus et al., 1990), in *Drosophila* (DiDomenico et al., 1982), and in yeast (Stone and Craig, 1990). There is currently no exper-

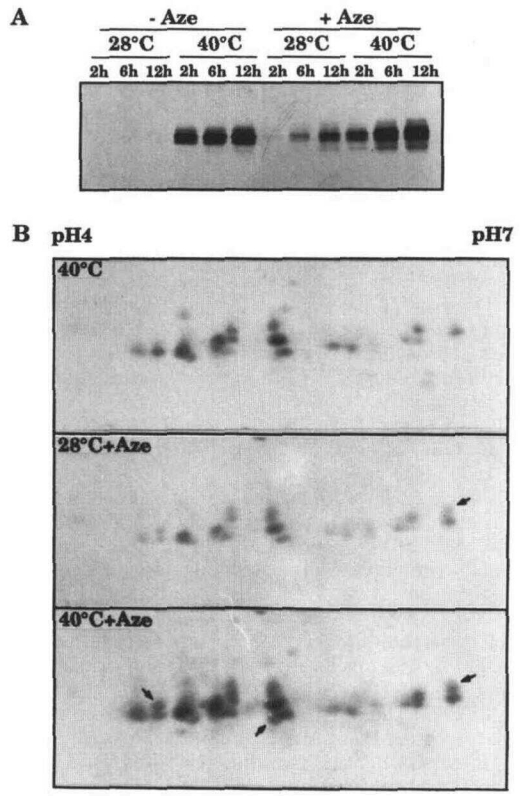

Figure 5. Immunoblot analysis of class I low molecular weight HSP accumulation during prolonged 40°C and +Aze (5 mM) treatments. A, One-dimensional SDS-PAGE of proteins extracted after indicated treatment; −Aze, no added Aze. B, Two-dimensional gel electrophoresis of proteins extracted after indicated treatment for 12 h. The left and right arrows indicate polypeptides possibly accumulating only in the presence of Aze; the center arrow indicates a polypeptide enhanced by Aze plus heat treatment.

imental basis to attach special significance to HPS70 in the self-regulation of the HS response in plants, although HSP70 is not excluded from such a role. In addition to HSPs, synthesis of other short-lived proteins that may be a part of the regulatory system could also be affected by Aze treatment. There is presently no evidence to eliminate the possibility that some short-lived non-HSP is involved in self-regulation of the HS response.

It was also interesting to observe that, although HS mRNAs were induced by Aze treatment at 28°C, radioactive amino acid incorporation into HSPs was not proportionally detected relative to mRNA abundance. Incorporation was readily detected in high molecular weight HSPs (in which mRNA abundance is lower), whereas very little incorporation into the abundant class I and class II low molecular weight HSPs was observed. Nonetheless, immunoblot analysis with antibodies to the class I low molecular weight HSPs detected substantial, but reduced, accumulation of this class of low molecular weight HSP with Aze treatment. The HSP70 and class I group of HSPs are both synthesized on free cytoplasmic polyribosomes (LaFayette et al., 1995); therefore, the disparity between radioactive amino acid incorporation into high and low molecular weight HSPs is unlikely to be explained by differential localization of translation (e.g. cytoplasmic ribosomes versus ER-bound polyribosomes) or different amino acid pools. Although an explanation of this observation is not yet obvious, results do suggest that conclusions about protein synthesis based on lack of radioactive amino acid incorporation into proteins should be interpreted with caution.

In summary, two novel observations, which have not been reported in other organisms to date, were made in these studies. First, unlike most other soybean HS genes, genes represented by the cDNA clones GmHsp20.7 and pFS2033 were not induced by Aze treatment at 28°C, suggesting a regulatory difference among soybean HS genes in response to Aze treatment. Second, although both high molecular weight and class I low molecular weight HSPs and their corresponding mRNAs accumulated with Aze treatment at 28°C, differential incorporation of radioactive amino acid into high molecular weight versus class I low molecular weight HSPs was observed. The regulatory mechanisms that account for these observations remain unknown. The selective regulation involved in differential HS gene expression described here represents an added sophistication of the HS response to other environmental stresses and warrants further investigation to specifically identify the mechanism(s) involved.

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