HEAT SHOCK PROTEIN EXPRESSION IN THERMOTOLERANT AND THERMOSENSITIVE LINES OF COTTON

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ABSTRACT

A large proportion of the water absorbed by a plant is used to cool its leaves as the air temperature becomes too hot. If the molecular basis of heritable thermotolerance were understood, then improvements in water use by crop plants might be possible. The role of heat shock proteins (HSPs) in the expression of heritable thermotolerance in cotton was investigated. Comparisons were made between the expression of HSPs in genetically characterized heat-tolerant and heat-sensitive lines of cotton. These comparisons were based on electrophoretic analysis of in vivo labelled proteins. No differences were observed between the two lines with regard to (1) the temperature at which HSP synthesis was induced (37°C), or the temperature at which HSP synthesis was maximal (45°C), (2) the rates of recovery from HSP synthesis or in the duration of HSP synthesis, and (3) the major size classes of HSPs expressed in these two lines. Several unique HSPs were identified on two-dimensional gels: a 26 kDa HSP which was expressed in the tolerant cotton line and a 24 kDa and an 18 kDa HSP which were expressed in the sensitive cotton line. However, the HSP pattern displayed in a heat-tolerant BC_3 individual was that of the heat-sensitive parent. No alteration in HSP expression could be found which correlated with the heritable thermotolerance.

Key words: biotechnology, plant stress, water use efficiency

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INTRODUCTION

In semiarid and arid environments, where water stress is associated with temperature stress, leaf temperatures commonly increase above air temperature as a result of stomatal closure and reduced transpiration (Hatfield 1979). Plants can resist high temperature stress either by avoidance mechanisms or by tolerance mechanisms (Levitt 1980). Examples of heat avoidance mechanisms are insulation, decreased respiration, or transpirational cooling (Levitt 1980). A great proportion of the water absorbed through the roots of plants is lost through evaporation from the leaves. Plants use this evaporation to cool their leaves when the air temperature becomes too hot. Transpirational cooling allows the plant to maintain its normal physiological processes at air temperatures which would otherwise be lethal. Plants can control the rate of evapo-transpiration through structual devices, waxy coatings on leaves and by controlling the opening and closing of the stomata or pores on the leaves. Most of these mechanisms decrease photosynthetic efficiency and therefore decrease crop yields. Obviously, expression of some of these avoidance mechanisms requires the coordination of many cell and tissue types. Potential mechanisms of heat tolerance are the synthesis of protectants (Levitt 1980), increased thermostability of enzymes (Downton, Berry, and Seeman 1984), and increased saturation of fatty acids (Pearcy 1978).

The discovery of specific proteins which are expressed at high temperatures, the heat shock proteins (HSPs), has caused considerable speculation in their possible role in mechanisms controlling heat tolerance (Lindquist 1986). The major effect of heat shock is the rapid induction of a discrete set of messenger RNAs (mRNAs) coupled with the predominant synthesis of the corresponding proteins, called HSPs. The occurrence of the heat shock response over a wide range of species, in many different tissues,

and at several developmental stages suggests that HSPs have adaptive significance, and somehow protect organisms from heat damage or enable organisms to recover from heat damage (Altschuler and Mascarenhas 1982a; Nover et al. 1984; Atkinson and Walden 1985; Lindquist 1986). It has long been observed that when plants are pretreated with sublethal high temperature, they become tolerant to normally lethal temperatures in later exposures; this phenomenon is referred to as acquired thermotolerance or thermoprotection (Levitt 1980). HSPs in plants have been shown to correlate with thermotolerance acquired from heat pretreatments (Altschuler and Mascarenhas 1982a; Lin, Roberts, and Key 1984; Atkinson and Walden 1985; Kimpel and Key 1985a; Kimpel and Key 1985b). It is well established that in animal systems, expression of HSPs, specifically HSP70, is necessary for acute or acquired thermotolerance (Li and Laszlo 1985; Riabowal, Mizzen, and Welch 1988; Johnston and Kucey 1988).

Plants synthesize HSPs of 92, 84, 70, and 68 kDa, along with a complex group of low molecular weight HSPs between 15 and 27 kDa (Kimpel and Key 1985b; Vierling et al. 1986). HSPs are synthesized when plant temperature is raised either abruptly or gradually (1°C-3°C per hour) 8°C to 10°C higher than optimum. HSPs are induced immèdiately after a shift to high temperature and their synthesis continues to increase for approximately 3 to 4 hours after which time it begins to decline (Altschuler and Mascarenhas 1982b; Atkinson and Walden 1985; Vierling and Key 1985). When plants are returned to more optimum temperatures, synthesis of HSPs declines and eventually stops although some HSPs may be stable for as long as 20 to 21 hours (Atkinson and Walden 1985; Vierling and Key 1985; Kimpel and Key 1985b).

Expression of HSPs is not limited to occurrences in laboratory water

baths. Field grown soybeans have been shown to accumulate mRNAs for HSPs in the heat of the afternoon, and levels of HSP mRNA are higher in plants sampled from unirrigated plots than in irrigated plots (Kimpel and Key 1985a). Field grown cotton has been shown to accumulate the same HSPs under water deficit conditions as those induced during heat shock experiments under laboratory settings (Burke et al. 1985). These results suggest that heat and water stressed plants turn off normal protein synthesis every stressful day and synthesize instead the heat shock family of proteins. If these proteins are protectants and therefore valuable, it will be important to identify plant germplasm which optimally expresses these proteins.

Despite the crop losses suffered due to heat stress, breeding or selection for heat tolerant crop plants has not received much specific effort. This is in part due to the limited understanding of the genetic bases of heat tolerance in plants, and in part due to the lack of accurate laboratory tests to select for the components of heat tolerance (Marshall 1982). Heritable variation in resistance to heat stress has been found in several crop species: alfalfa (McDaniel 1982), cotton (Rodriguez-Garay 1985), sorghum, corn, soybean, and oats (Marshall 1982, review).

If expression of HSPs contributes to an individual's ability to perform during heat stress, then one would predict that, during heat shock, differences in HSP expression would be observed between selected lines which differ only in their heritable thermotolerance. There have been a few reports in the literature which suggest that this phenomenon may be occurring: differences in the amount of HSPs synthesized during heat shock have been correlated with differences in thermotolerances in lines of sorghum (Ougham and Stoddart 1986); unique HSPs have been associated with thermotolerant

lines of wheat (Krishnan, Nguyen, and Burke 1989); and expression of high molecular weight HSPs have been proposed to confer thermoadaptation in heat tolerant lines of cowpea cell cultures (Heuss-LaRosa, Mayer, and Cherry 1987).

There are many ways a plant can resist high temperature stress--through insulation, decreased absorption of radiant energy, transpirational cooling, etc. (Levitt 1980). Expression of some if not all of these tolerance mechanisms requires the coordination of many cell and tissue types. If HSP expression is to contribute to heritable thermotolerance, one would need to identify examples of thermotolerances which were manifest at the cell level. The two cotton lines tested in this study satisfied that requirement: they displayed thermotolerance and thermosensitivity at a number of developmental stages both sporophytic and gametophytic (Rodrgiuez-Garay and Barrow 1988) and also as a cell suspension (Rodriguez-Garay, personal communication, 9/86). Selections of cotton germplasm were made based on the ability of heat treated flowers to produce viable pollen and produce seed. The heat tolerant line also displayed drought and salt tolerances at the cell culture level when compared with the heat sensitive line (Rodriguez-Garay 1985).

The goal of this project was to characterize and compare the heat shock proteins produced in heat sensitive and heat resistant lines of cotton using gel electrophoretic analysis of in vivo labelled proteins from selected cotton lines. To test the hypothesis that differences in HSPs or in HSP expression are correlated with genotypic differences in plant heat tolerance, we investigated the following parameters of HSP expression in the two selected lines of cotton: the temperatures of HSP induction and maximal synthesis, quantitative and qualitative changes in HSP patterns, and the recovery rates and duration of HSP synthesis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *Gossypium hirsutum*, L. var Paymaster 404, and *G. barbadense*, L. line 7456, were generously supplied by Dr. Rodriguez-Garay, New Mexico State University. Seeds were planted in MetroMix growing medium #352, and germinated in growth chambers set at 25°C with a 16 hour photoperiod. Plants were watered with a Nitsch nutrient solution (O'Connell and Hanson 1985). All analyses were performed on plant tissue grown from seed under controlled conditions.

In Vivo Labelling

Labelling experiments were performed on either seedling tissue (minus roots and cotyledons), leaves from young plants, or immature floral buds as indicated. Samples were incubated in 1% (w/v) sucrose, 1 mM K phosphate, pH 6.0, 50 µg/ml chloramphenicol, at the indicated temperatures (25°C-48°C) for 20 minutes. [^{35}S]methionine (Trans ^{35}S-Label, ICN, 1100 Ci/mmole) 0.5 µCi/µl was added and the incubation continued at the indicated temperature for the indicated time period, usually 1 to 2 hours. Samples were rinsed in distilled water and ground in SDS sample buffer (2% (w/v) sodium dodecyl sulfate, 5% (v/v)ß-mercaptoethanol, 62 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 0.00125% (w/v) bromophenol blue). Samples were boiled for 2 min and insoluble debris removed by centrifugation in a microfuge. [^{35}S]methionine incorporation into protein was determined by trichloroacetic acid (TCA) precipitation (Mans and Novelli 1961).

One- and Two-Dimensional Gel Electrophoresis

Proteins were examined on 10-15% (w/v) polyacrylamide gradient gels with a discontinuous buffer system (Laemmli 1970). Gel lanes were loaded with equal amounts of precipitable counts, low molecular weight protein standards (Bio-Rad) were run as size markers. Gels were stained with Coomassie brilliant blue, destained and processed for fluorography using the liquid scintillant preparation, Enhance (New England Nuclear/DuPont). Alternatively, samples were processed for two-dimensional gel electrophoresis as described by O'Farrell (1975) with modifications (Adams 1987). Prior to loading on isoelectricfocusing gels, samples were precipitated by the addition of 9 volumes of ice cold ethanol, kept at -20°C for 30 min, spun, dried and resuspended in (9.5M urea, 4% (w/v) CHAPS (3-[(3-Cholamido- propyl)-dimethylammonio]-1-propanesulfonate), 5% (v/v) ß-mercaptoethanol, 1.6% (v/v) Biolyte 5/7 ampholyte mixture, 0.4% (v/v) Biolyte 3/10 ampholyte mixture). Isoelectricfocusing gels were loaded with equal amounts of TCA precipitable counts; with sample loaded at the basic end. The pH gradient after the run was determined to range from pH 6.6 to pH 4.2. The second dimension was run on 12% (w/v) polyacrylamide discontinuous gels (Laemmli 1970); for size reference, a side lane was loaded with the same sample prior to isoelectricfocusing.

Duration and Recovery

The persistence of HSP synthesis was characterized by incubating cotton tissue at 44°C for increasing times, 6 to 12 hours. [^{35}S]methionine was added to the sample for a 2 hour period immediately prior to sample preparation for SDS-gel analysis. Recovery from heat shock was characterized by incubating

cotton tissue at specific elevated temperatures (38°C to 44°C) for 2 h, transferring the sample to 25°C and incubating for increasing periods of time, 1, 2, 3, or 4 hours. [^{35}S]methionine was added for the last hour of incubation at 25°C. Samples were processed for SDS-gel analysis as described above.

RESULTS

The cotton germplasm utilized in these experiments had been extensively characterized for a variety of stress tolerances (Rodriguez-Garay 1985; Rodriguez-Garay and Barrow 1988). Cotton germplasm was tested for the ability of heat-treated flowers to produce viable pollen and produce seed. Two lines were extensively characterized which represented heat tolerant (Gossypium barbadense line 7456) and heat sensitive (G. hirsutum cv Paymaster 404) phenotypes; line 7456 also displayed thermotolerance at the cell culture level (Rodriuguez-Garay, personal communication, 9/86) and displayed drought and salt tolerances at the cell culture level when compared with Paymaster 404 (Rodriguez-Garay 1985). The inheritance of this phenotype was examined in the F_1 and BC_1 generations, and heritability estimates suggested that one or two factors with partial dominance were responsible for the heat tolerant phenotype (Rodriguez-Garay 1985). A series of experiments were designed to determine if specific HSPs or regulators controlling HSP expression were the heritable factors. All of the results described in the following sections were replicated at least once. All of the results described in the following sections were based on comparisons of in vivo labelled protein synthesis following a variety of treatments. The method of detection of the protein synthesis was visual comparison of autoradiograph images. We chose to normalize the samples compared in these images with equal numbers of TCA precipitable counts rather than equal amounts of total protein; in this way we were comparing protein synthetic capactity during the experimental treatment. A report of some of these results has been published (Fender and O'Connell, 1989a).

Sizes of HSPs in Cotton

The HSPs synthesized by leaf tissue of line 7456 and Paymaster 404 are shown in Figure 1. In general, the control temperature protein synthetic pattern is still present in the heat shocked samples, however the heat shock induced proteins are made in a greater abundance. The major HSPs in the two lines have similar molecular weights (97, 92, 86, 78, 67, 26, 24, 19 and 18 kDa) and are present in equivalent amounts in the two lines. There are no differences in the relative abundances of the different classes of HSPs nor are there differences in the sizes of the major classes of HSPs synthesized in the heat tolerant and sensitive lines. The spectrum of HSPs synthesized in these two cotton lines is similar but not identical to the HSP profile reported by Burke et al., (1985) for a different strain of cotton, G. hirsutum T185.

There is an appreciable amount of protein in the control lane of both line 7456 and Paymaster 404 which co-migrates with HSP 67. We presume this protein is analogous to the ubiquitous HSP70 of other organisms. There is no consistent difference in the control levels of this protein between the tolerant and sensitive cotton lines, but cotton as compared with other crop plants appears to have significant constitutive quantities of this protein.

Temperature Response of HSP Synthesis in Cotton

The effect of increasing temperature on in vivo protein synthesis in Paymaster 404 and line 7456 is shown in Figure 2. From this analysis, the temperature of induction and of maximal synthesis of HSP expression can be determined for the two cotton lines. The temperature at which HSPs could first be detected was the same for both lines, 37°C, and the temperature of maximal HSP synthesis was the same for both lines, 45°C. Both lines ceased protein

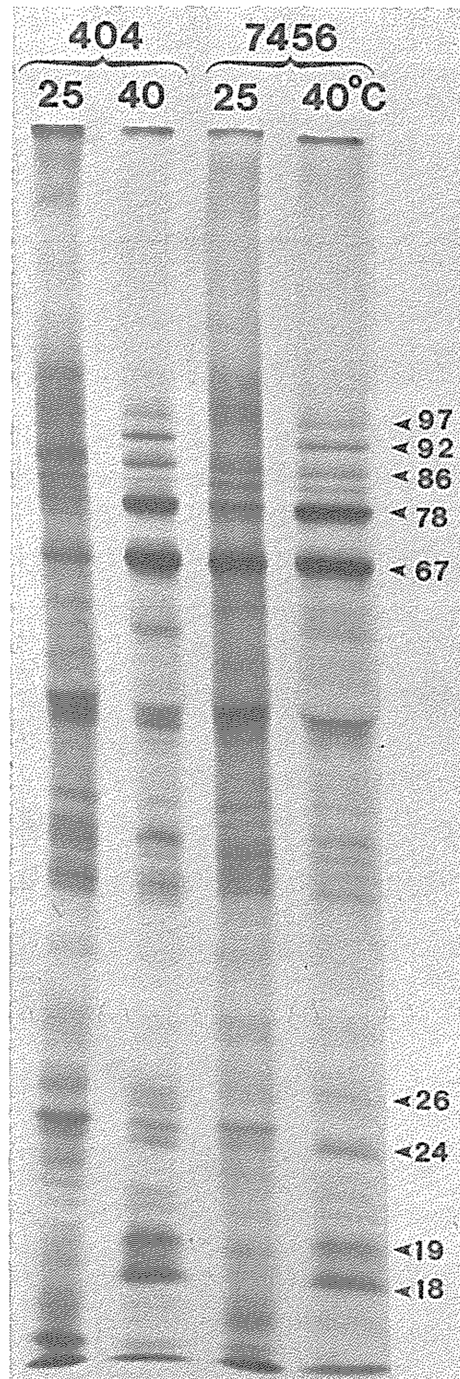

Figure 1. In vivo labelled proteins synthesized in cotton leaf tissue during heat shock. Leaf tissue from Paymaster 404, or line 7456 were incubated in the presence of [^{35}S]methionine at 25°C or 40°C for 2 hours. Samples were prepared for electrophoresis as described in Materials and Methods. Gel lanes were loaded with equal TCA precipitable counts. The molecular weights of the major HSPs are indicated.

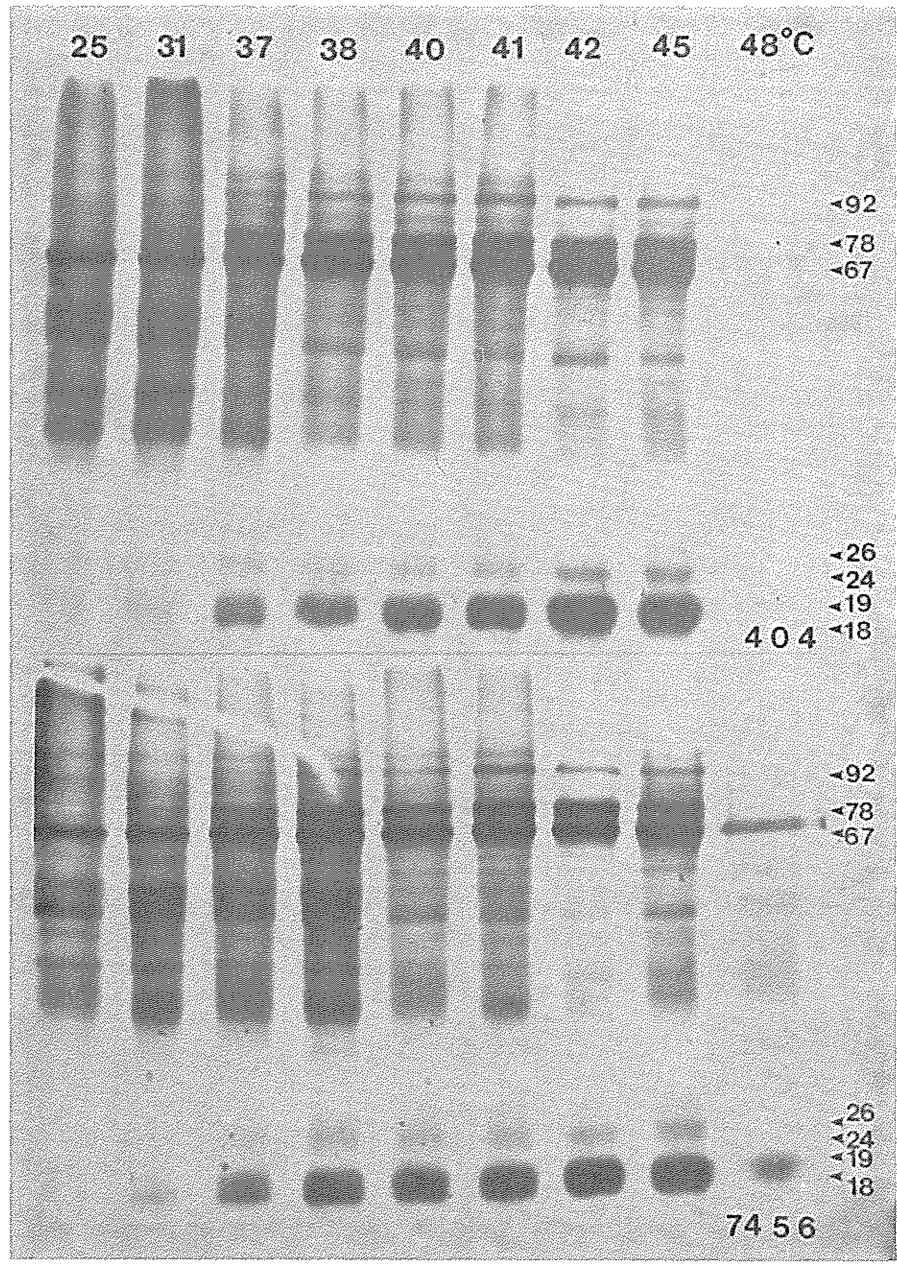

Figure 2. Effect of increasing temperature on HSP synthesis in cotton seedling tissue. Paymaster 404 seedling tissue, upper panel, or line 7456 seedling tissue, lower panel, were incubated in the presence of [^{35}S]methionine at the indicated temperatures (25°C to 48°C) for 2 hours. Samples were prepared for electrophoresis as described in Materials and Methods. Gel lanes were loaded with equal TCA precipitable counts. The molecular weights of the major HSPs are indicated.

synthesis at temperatures between 45°C and 48°C. These analyses were also performed on cotton leaf tissue and the same results were obtained (data not shown). There was no difference in either the temperature of induction of HSP synthesis nor in the temperature of maximal HSP synthesis between the heat sensitive and heat resistant lines of cotton.

Expression of HSPs in Cotton At Different Developmental Stages

HSP expression was characterized at three different developmental stages: seedling (Figure 2), leaf (Figure 1), and floral bud (data not shown). All three developmental stages displayed the same classes of HSPs and the same temperatures of induction and of maximal synthesis, using one-dimensional gel for analysis of in vivo labelled HSPs. There was no difference between the heat sensitive and heat resistant lines of cotton at any developmental stage tested in any of the HSP parameters tested.

Duration of HSP Synthesis in Cotton During Continuous Heat Shock

The duration of HSP synthesis over a 12 h period is shown for leaf tissue of line 7456 (Figure 3) and Paymaster 404 (Figure 4). HSP synthesis persists in both line 7456 and Paymaster 404 for the duration of the heat shock, 12h. In replicate experiments we have observed the continued synthesis of HSPs during a 24 h heat shock (data not shown). There was no difference between the two cotton lines in the duration of HSP synthesis during a constant heat shock.

The observed persistance of HSP synthesis in cotton during a persistent heat shock is in contrast with results obtained by other workers. HSP synthesis in corn (Baszczynski, Walden, and Atkinson 1985) and soybean (Key et al. 1985) is transient during a persistent heat shock. The lack of a

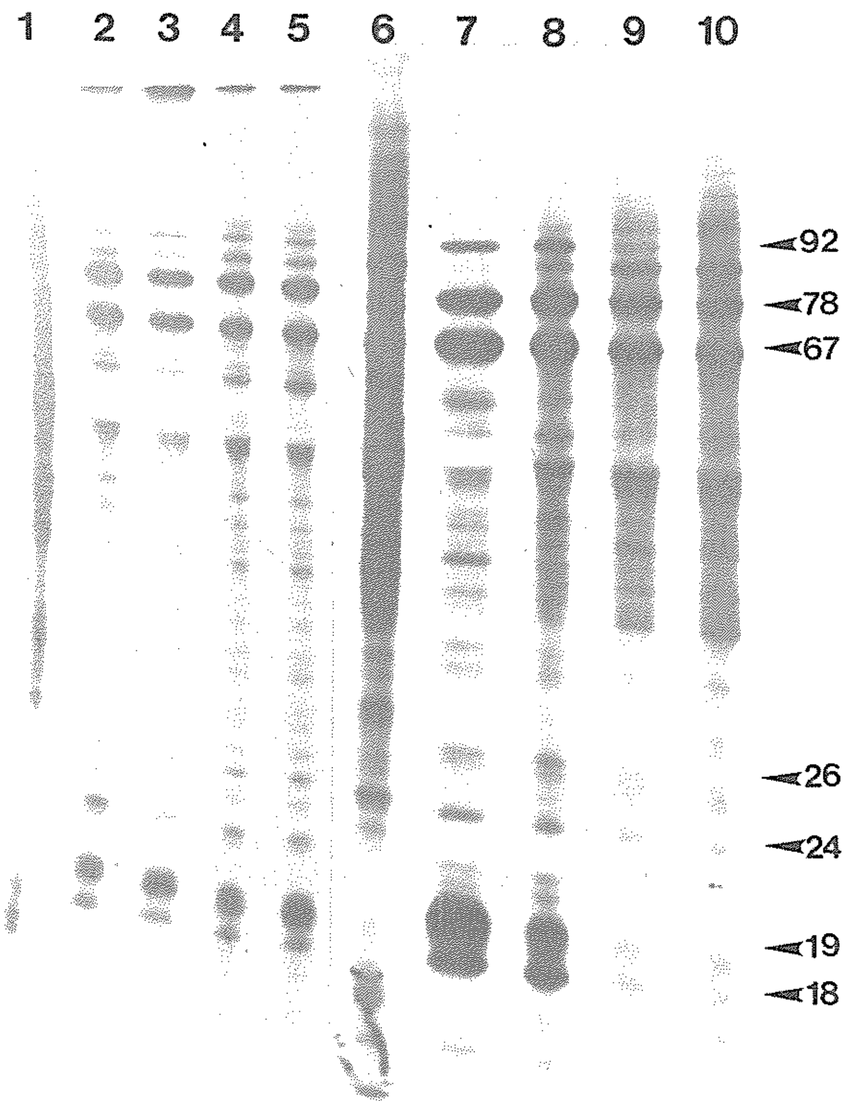

Figure 3. Duration and recovery of HSP synthesis in line 7456 leaf tissue. Leaf tissue from line 7456 was incubated at 44°C for 6 h (lane 2), 8 h (lane 3), 10 h (lane 4), or 12 h (lane 5). [^{35}S]methionine was added to the incubation buffer for the last 2 h of the incubation period at 44°C. Lane 1 contains leaf tissue incubated at 25°C for 12 h and labelled for the last 2 h of incubation. Leaf tissue from line 7456 was incubated at 44°C for 2 h and then transferred to 25°C and incubated for 1 h (lane 7), 2 h (lane 8), 3 h (lane 9) or 4 h (lane 10). [^{35}S]methionine was added to the incubation buffer for the last 1 h at 25°C. Lane 7 contains leaf tissue incubated at 25°C for 4 h and labelled for the last 1 h of incubation. Samples were prepared for electrophoresis as described in Materials and Methods. Gel lanes were loaded with equal TCA precipitable counts. The molecular weights of the major HSPs are indicated.

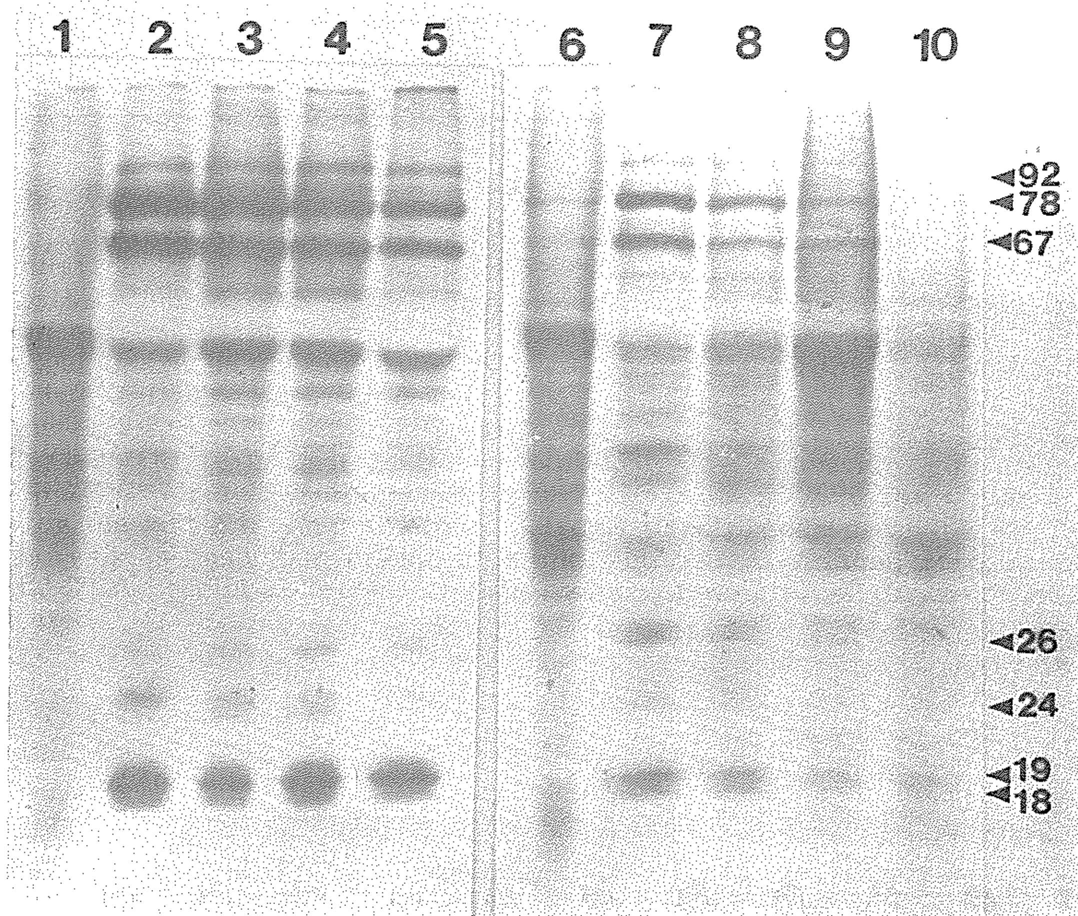

Figure 4. Duration and recovery of HSP synthesis in Paymaster 404 leaf tissue. Leaf tissue from Paymaster 404 was incubated at 44°C for 6 h (lane 2), 8 h (lane 3), 10 h (lane 4), or 12 h (lane 5). [^{35}S]methionine was added to the incubation buffer for the last 2 h of the incubation period at 44°C. Lane 1 contains leaf tissue incubated at 25°C for 12 h and labelled for the last 2 h of incubation. Leaf tissue from Paymaster 404 was incubated at 44°C for 2 h and then transferred to 25°C and incubated for 1 h (lane 7), 2 h (lane 8), 3 h (lane 9) or 4 h (lane 10). [^{35}S]methionine was added to the incubation buffer for the last 1 h at 25°C. Lane 7 contains leaf tissue incubated at 25°C for 4 h and labelled for the last 1 h of incubation. Samples were prepared for electrophoresis as described in Materials and Methods. Gel lanes were loaded with equal TCA precipitable counts. The molecular weights of the major HSPs are indicated.

transient expression pattern of HSP synthesis in cotton may be the result of a species difference or due to the temperature at which the assay was conducted.The cotton tissue was heat shocked at the temperature of maximal HSP synthesis. If assayed at a lower temperature, the cotton tissue might have displayed a transient accumulation of HSPs during a persistent heat shock. We have also characterized the heat shock response of two tomato species, and have observed a persistance of HSP synthesis during a persistent heat shock in these species (Fender and O'Connell, 1989b). Since these results with tomato were observed at the temperature of induction and at the temperature of maximal synthesis, the transient expression pattern observed in corn and soybean tissue during a persistent heat shock may be the exception rather than the rule.

Rate of Recovery from HSP Synthesis

The persistence of HSP synthesis following a shift from heat shock temperatures to control temperatures was compared between the two cottonlines. Leaf tissue of line 7456 (Figure 3) or Paymaster 404 (Figure 4) was incubated at 44°C for 2 h, transferred to 25°C and incubated for increasing times, 1 to 4 hours. During the final hour of incubation at 25°C, [^{35}S]methionine was added to the incubation buffer. The proteins synthesized during the indicated times were characterized on SDS gels. From these analyses, the time at which HSP expression ceased and control protein synthesis patterns resumed could be determined for the two cotton lines. In general, the recovery times were dependent on the temperature of heat shock and the duration of the heat shock; i.e., recovery times are less than 1 h following a 2 h, 38°C heat shock, whereas, return to control protein synthesis patterns had started after 3 h

following a 2 h 44°C heat shock (Figure 3 and 4). For a given heat shock condition, both lines displayed the same recovery times.

Analysis of Cotton HSPs Using Two-Dimensional Gel Electrophoresis

Genetic analysis of the thermotolerance trait had suggested that one or two genes were involved in controlling the phenotype. While no differences in molecular weights of the major HSP classes were observed at the level of one-dimensional gel analysis between the two cotton lines (Figure 1), a more sensitive assay might be needed to identify single gene products. To determine if these gene products were HSPs, it was necessary to analyze the HSPs synthesized in the two lines with two-dimensional gel electrophoresis (Figure 5). This technique will allow the characterization of individual proteins since the proteins are resolved as functions of both their size and their isoelectric point.

The two-dimensional pattern of the proteins synthesized at 25°C by the two cotton lines were superimposable (Figure 5). The pattern of proteins synthesized by the two cotton lines at the control temperature is complex, with many proteins ranging in size from over 100 kDa to under 15 kDa and ranging in charge from pIs of 6.6 to 4.2.` It was anticipated that the pattern of proteins syntheisized by the two cotton lines would be similar since the one-dimensional patterns were identical, however it is remarkable that the two-dimensional patterns were identical since the two cotton lines are different species.

Under heat shock conditions, cotton synthesizes only HSPs. A comparison of the protein patterns at the control and heat shock temperatur using two-dimensional gels indicates that most of the developmentally controlled

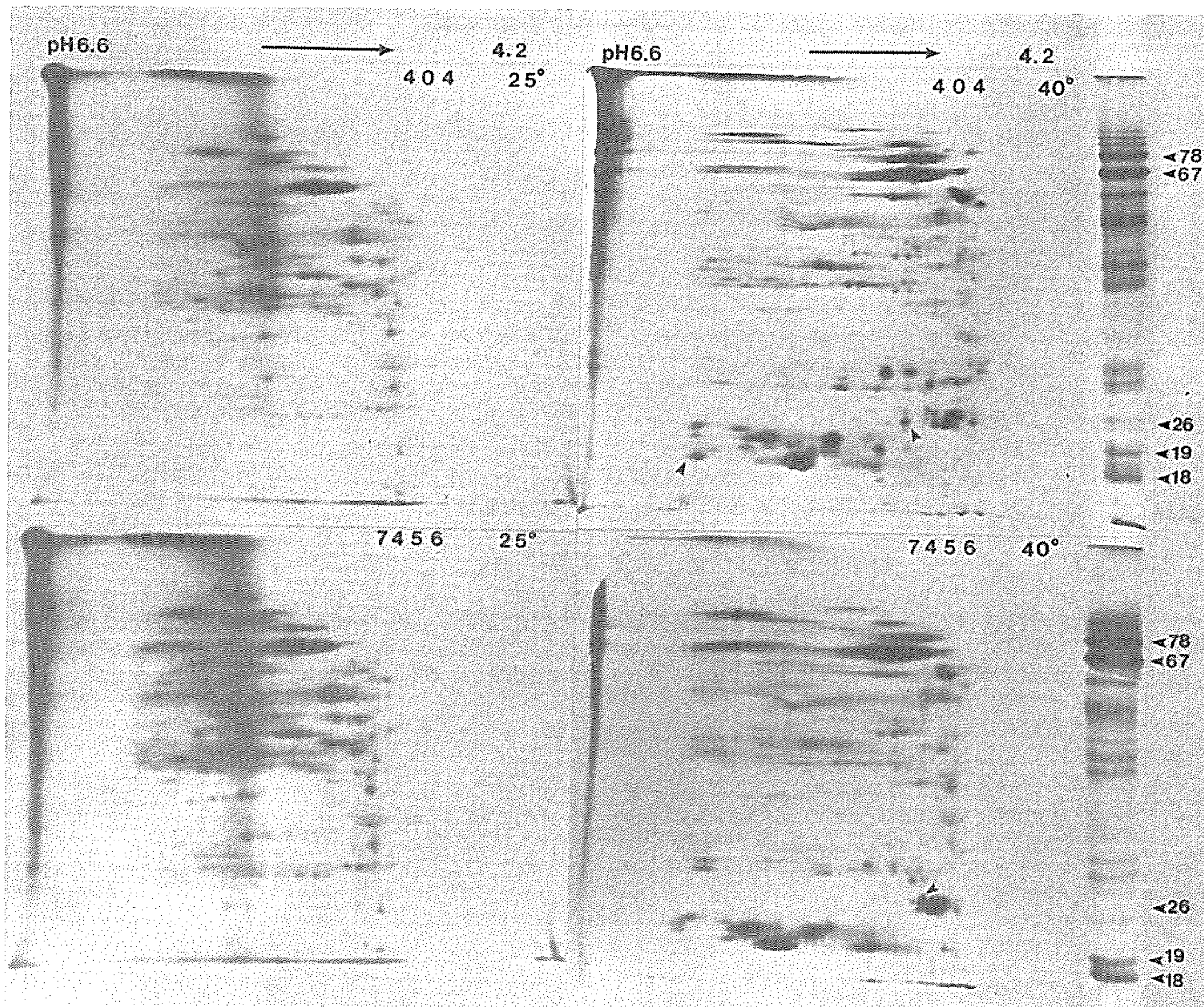

Figure 5. Two dimensional gel analysis of HSP synthesis following heat shock of cotton leaf tissue. Paymaster 404 or line 7456 leaf tissue was incubated in the presence of [^{35}S]methionine for 2 h at 25°C or 40°C. Samples were processed for two-dimensional gel analysis as described in Materials and Methods. The isoelectricfocusing gels were loaded with equal TCA precipitable counts. The molecular weights of the major HSPs are indicated.

or constitutive protein synthesis ceases during heat shock in cotton (Figure 5). As expected, the two-dimensional pattern of HSPs synthesized by the two cotton lines are similar. There are only a few differences between the two linesbased on visual inspection of the fluorograms, and all of the differences involve the low molecular weight HSPs. Line 7456 has a 26 kDa protein not present in the profile of Paymaster 404. Paymaster 404 has two proteins not found in the profile of line 7456, 18 kDa and 24 kDa proteins. These unique proteins were reproducibly observed.

Analysis of Unique HSPs in BC_3 Generation

A BC_3 generation was obtained following recurrent selection for heat tolerant pollen production on Paymaster 404 background. This generation has heat tolerant vegetative growth similar to the line 7456 parent (data not shown). The HSP expression was examined in one-dimensional SDS gels and no differences were observed between line 7456, BC-3 and Paymaster 404 (data not shown). Two-dimensional gels were prepared of heat shocked and control leaf tissue from a BC_3 individual (Figure 6). The HSP profile of the BC_3 was compared with the profiles of the two parental lines, (compare Figure 6, with Figure 5). The line 7456 unique HSP, 26 kDa, is not observed in the BC_3 sample and both of the Paymaster 404 unique HSPs, 18 and 24 kDa are present. While the HSP expression of only one BC_3 individual is presented in Figure 6, one individual is sufficient since the HSP profile is unambiguous. If a combination of both parental specific HSPs had been observed in the BC_3 individual, or one of the Paymater 404 unique HSPs and the 7456 unique HSP, then additional individuals would be needed to establish the segregation.

The differences observed in unique HSPs between line 7456 and

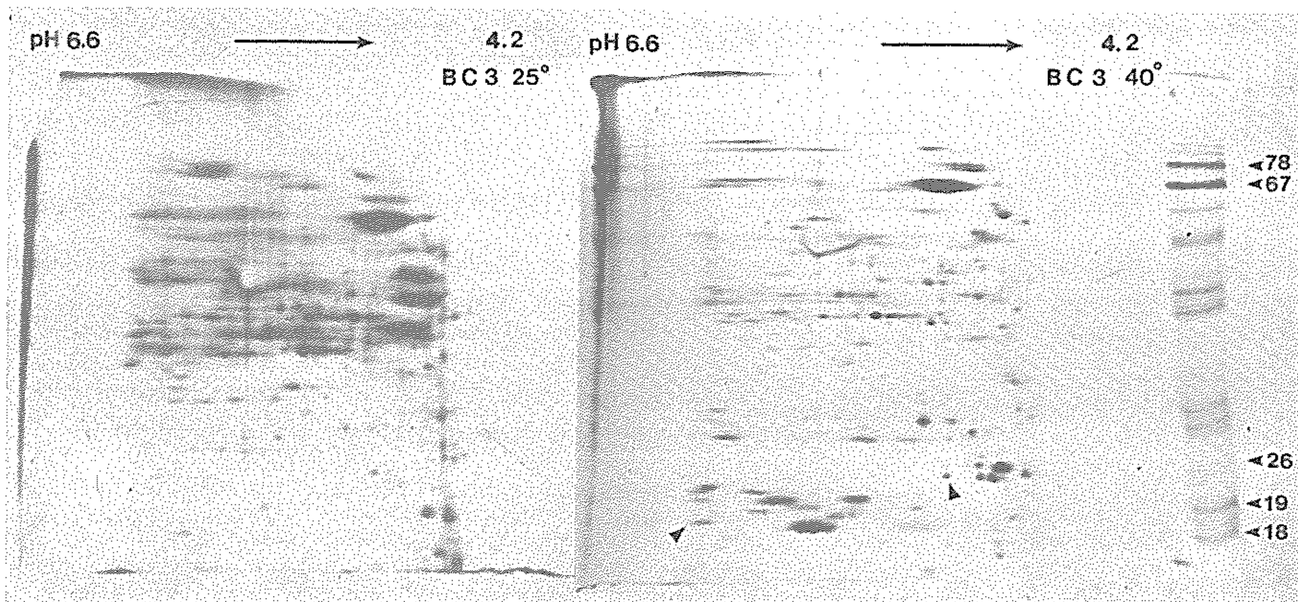

Figure 6. Two-dimensional gel analysis of HSP synthesis following heat shock of cotton leaf tissue from a BC-3 individual. Leaf tissue from a BC-3 individual was incubated at 25°C or 40°C for 2 h in the presence of [^{35}S]methionine. Samples were processed for two dimensional gel analysis as described in Materials and Methods. The isoelectricfocusing gels were loaded with equal TCA precipitable counts. The molecular weights of the major HSPs are indicated.

Paymaster 404 did not co-segregate with the thermotolerance trait in the BC_3 generation. These differences are, therefore, unlikely to contribute to the thermotolerance phenotype.

DISCUSSION

It has been assumed that one of the HSPs' functions is to provide thermoprotection. There is much evidence to suggest this positive correlation between HSP expression and thermotolerance (Altschuler and Mascarenhas 1982a; Kimpel and Key 1985a; Kimpel and Key 1985b; Lin, Roberts, and Key 1984; Lindquist 1986). However, there are also lines of evidence which do not support this function for HSP expression (Ramsay 1988; Lindquist 1986). If the hypothesis is correct that HSP expression is causal in thermotolerance, then HSP expression should be different in genotypes which differ in thermotolerance. We tested this hypothesis by examining the expression of HSPs under a variety of conditions in two lines of cotton selected for their heritable differences in thermotolerance.

The two cotton lines were indistinguishable in their general heat shock response. The classes of HSPs induced in the two lines were of the same molecular weight, and equivalent quantities of the major classes of HSPs were induced at the same temperatures (Figure 1). If HSPs were functioning to facilitate survival of the heat tolerant line, then one could expect to see more HSPs in the tolerant than the sensitive line. This was not observed.
The temperatures of induction of HSP synthesis and of maximal HSP synthesis in the two lines were the same (Figure 2). If HSPs were protectants, one could expect to see differences in the temperature necessary for induction of HSP expression. No such differences were observed. The rates of recovery and the duration of HSP synthesis were the same between the two lines (Figure 3 and 4). If HSPs were functioning as protectants, one could expect to see a more rapid return to normal protein synthesis in the tolerant line. No

differences were observed. In general, the overall regulation of the heat shock response in the thermotolerant and thermosensitive lines of cotton was the same.

An alternative relationship between HSP expression and thermotolerance is that the tolerant line expresses HSPs constitutively, i.e., sufficient quantities of the protecting proteins are present in the tolerant line prior to the heat shock. Examination of the proteins synthesized at control temperature by two-dimensional gel analysis (Figure 5) did not show any reproducible differences between the tolerant and sensitive lines which could be due to the presence of HSPs. The abundance of HSP67 (HSP70) in Figure 1 in the control temperature incubation of the two cotton lines appears different. The 7456 sample appears to have more of this protein under control conditions. This difference was not consistently observed and could easily be artifactual based on the way the samples are loaded on the gels. Equal amounts of radioactively labelled proteins are loaded. An apparent abundance of a protein or group of proteins could be the result of lower amounts of other proteins, rather than an actual increase in expression of the specific protein. A more accurate method to determine if there was an increase in the synthesis of a specific protein would be to compare the concentration of mRNA which encodes the protein in question under control and heat shock conditions. We have observed that the amount of HSP 67 synthesized under control conditions (Figures 1 and 5) in cotton is greater than the amount of analogous protein made in other crop plants, i.e., tomato (Fender and O'Connell, 1989b) under control conditions.

It is possible that the quantities of HSPs made under control temperatures would not be sufficient to detect on the background of developmentally controlled protein synthesis, but that the quantities would be sufficient to

provide thermotolerance. We can not rule out this possible positive relationship between HSP expression and heritable thermotolerance. A more direct measurement of the amount of HSP67/70 synthesized under control and heat shock conditions in the two lines of cotton would answer this question. Either analysis of the abundance of HSP 70 using antibodies, or analysis of the abundance of mRNA for HSP70 using quantitative Northerns, i.e., dot blots, should resolve this question.

A number of researchers have suggested that the control of thermotolerance is regulated by expression of HSP 70 (Heuss-LaRosa, Mayer, and Cherry 1987; Li and Laszlo 1985; Lindquist 1986). We saw no differences in the amount of this class of HSP synthesized by the two different cotton lines either at the level of one- or two-dimensional gels. We did observe differences in the amounts of specific low molecular weight HSPs which were unique to the tolerant and to the sensitive lines (Figure 5). If these proteins had been associated with the heritable thermotolerance in cotton, then one would expect that the line 7456 pattern should be expressed in the BC_3 generation. However, the Paymaster 404 pattern was expressed in the BC_3 generation (Figure 6) suggesting that these proteins are unrelated to thermotolerance but may be due to the diverse genetic background of the two cotton lines. While the HSP expression of only one BC_3 individual is presented in Figure 6, one individual is sufficient since the HSP profile is unambiguous. If a combination of both parental specific HSPs had been observed in the BC_3 individual, or one of the Paymater 404 unique HSPs and the 7456 unique HSP, then additional individuals would be needed to establish the segregation.

Pollen is unique in that it does not synthesize heat shock proteins (Altschler and Mascarenhas 1982b; Cooper and Ho 1984; Xiao and Mascarenhas

1985). One might argue that thermotolerance mechanisms which operate during pollen germination are unlikely to involve alterations in HSP expression. The cotton germplasm studied in this report was selected for thermotolerance initially at pollen germination, however, the thermotolerance was observed at other developmental stages including floral growth, seed production, vegetative growth, and cell suspension growth. If a thermotolerance mechanism unique to pollen was utilized in line 7456, the line also expressed a thermotolerance mechanism to support plant growth at other developmental stages. An HSP mediated mechanism could be expected to function during the sporophytic stages, but no indication of such a mechanism was found.

In conclusion, these results argue strongly that the differences in heritable thermotolerance observed in these lines of cotton are not the result of differences in regulation of HSP expression. Since these cotton lines display cell level thermotolerance, and thermotolerance at different developmental stages, it is reasonable to assume that the mechanism of thermotolerance in line 7456 is mediated at the cellular level. This rules out any tactic which requires organ or whole plant organization for expression, i.e., transpirational cooling, leaf angle, etc. Alternative possibilities need to fulfill two criteria: (1) the mechanism needs to operate at the molecular or cellular level and (2) the mechanism needs to be controlled by one or two genes. This second criterion rules out global differences in the thermostability of the enzymes of metabolism. It is still possible that there is one or two key enzymes which are thermosensitive and are important in the regulation of cell growth, likely enzyme or gene product candidates would be activities associated with DNA replication, RNA transcription, or translation.

The species specific differences in the unique HSPs involved proteins in

the same size range as those associated with water stress induced proteins in tobacco (Ericson and Alfinito 1984; Singh et al. 1985) and in soybean (Czarnecka et al. 1984). The cotton lines tested which showed these differences also differ in their sensitivity to water stress. It would be interesting in future studies to investigate the alterations in gene expression in these cotton lines under water deficit stress. It is quite possible that there are unique genes responsible for the different abiotic stress tolerances displayed by cotton line 7456. If that is true, then the unique HSP associated with this line may segregate with the drought tolerance phenotype. Selections of segregating populations based on drought tolerance would be necessary to test this hypothesis.

SUMMARY

Comparisons were made between the expression of HSPs in genetically characterized heat tolerant and heat sensitive lines of cotton. These comparisons were based on electrophoretic analysis of in vivo labelled proteins. No differences were observed between the two lines with regard to the temperature at which HSP synthesis was induced (37°C), or the temperature at which HSP synthesis was maximal (45°C). No major differences were observed in the rates of recovery from HSP synthesis between these two lines nor were differences observed in the duration of HSP synthesis between the two lines. In addition, no differences were observed in the major size classes of HSPs expressed in these two lines. However, several HSPs were identified on two-dimensional gels which were expressed uniquely in either the tolerant (26 kDa) or sensitive (18 and 24 kDa). However, the HSP pattern displayed in a heat tolerant BC_3 individual was that of the heat sensitive parent.

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