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Recovery from Heat Shock in Heat-Tolerant and Nontolerant Variants of Creeping Bentgrass[1]

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Recovery from the heat-shock response was tested in heat-tolerant (selected bentgrass [SB]) and nontolerant (nonselected bentgrass [NSB]) variants of creeping bentgrass (*Agrostis palustris* Huds.) SB increased incorporation of radioactive amino acids into protein 2 h earlier than NSB when leaf blades were incubated at the recovery temperature following heat shock. Electrophoresis indicated that heat-shock protein (HSP) synthesis decreased and normal protein synthesis increased at 4 h in SB and at 6 to 8 h in NSB. Increased synthesis of normal proteins was not due to increased abundance of normal mRNAs, which were equivalent in SB and NSB at 4 h. But at 4 h, more of the normal mRNA population was associated with polysomes in SB than in NSB. Synthesis of HSP70 and HSP18 decreased earlier in SB than in NSB. The decreased synthesis of these HSPs appeared to be correlated with decreased mRNA abundance. But at 4 h, some of the HSP18 mRNA may have been associated with heat-shock granules in SB. Synthesis of HSP25 continued through the 8-h recovery in both variants. Although the abundance of HSP25 was equivalent in SB and NSB during heat shock and recovery, more HSP25 mRNA was associated with polysomes in SB than in NSB.

Plants must endure both chronic and acute exposures to high temperatures. To cope with high-temperature stress, plants have developed mechanisms that include both avoidance and tolerance (Levitt, 1980; Kappen, 1981). Avoidance may result from specific morphological characteristics such as altered leaf shape. Tolerance, on the other hand, results from altered physiological processes (Kappen, 1981). In many cases, heat stress is due to a brief exposure to sublethal temperatures, which results in reversible damage to cellular and subcellular structures and functions (Kappen, 1981). Heat-tolerant plants are capable of repairing this damage and resuming normal metabolic functions faster than nontolerant plants (Levitt, 1980; Kappen, 1981; Howarth, 1991). These plants have a competitive advantage because they can resume normal cellular functions, such as photosynthesis, sooner than nontolerant plants.

Because the synthesis of HSPs has been correlated with the acquisition of thermotolerance in a number of organisms (for review, see Vierling, 1991), the heat-shock response of two somaclonal variants of creeping bentgrass (*Agrostis palustris* Huds.) that differ in thermotolerance was investigated (Park et al., 1996). Both the heat-tolerant SB and nontolerant NSB variants were derived from a single seed of the cv Penncross using cell culture (Park et al., 1996). The major difference between the two variants was that SB synthesized two to three additional members of the HSP27 family, which were smaller (25 kD) and more basic than those synthesized by NSB (Park et al., 1996). Analysis of the F_1 progeny of NSB × SB indicated that 7 of 20 progeny did not synthesize the additional HSP25 polypeptides. These progeny were significantly less heat tolerant than those that synthesized the additional HSP25 polypeptides. Chi square analysis indicated that heat tolerance and the synthesis of the additional HSP25 polypeptides were linked traits (Park et al., 1996). Other aspects of the heat-shock response in SB and NSB were investigated, and there were no major differences between the two variants in the time or temperature required to induce HSP synthesis. However, when conditions for in vivo labeling were being optimized, we consistently noticed that SB increased the incorporation of radioactive amino acids into protein 2 h earlier than NSB, when leaf blades were incubated at the recovery temperature following heat shock. This study was conducted to determine what factors might account for the difference in recovery time between the two bentgrass variants.

MATERIALS AND METHODS

Two variants of creeping bentgrass (*Agrostis palustris* Huds.), the nontolerant variant NSB and a heat-tolerant variant SB, were grown as previously described (Park et al., 1996). Under these growing conditions SB and NSB were morphologically indistinguishable.

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Abbreviations: 2-D, two-dimensional; HSG(s), heat-shock granule(s); HSP(s), heat-shock protein(s); LSU, Rubisco large subunit; NSB, nonselected bentgrass; SB, selected bentgrass; SSU, Rubisco small subunit.

In Vivo Labeling of SB and NSB with ^{3}H-Leu during Recovery from Heat Shock

Intact leaf blades of NSB and SB, with one cut at the base to minimize wounding, were used. Duplicate samples at the same developmental stage of SB and NSB were prepared for each time point during the recovery period. Each sample contained 100 mg of leaf blades in 3 mL of sterile incubation buffer without chloramphenicol (Lin et al., 1984). All samples were heat-shocked at 40°C for 1.5 h in a water bath, then 100 μCi of ^{3}H-Leu (110 Ci/mmol; ICN) was added to each tube and the tubes were placed in a 25°C water bath. Samples (100 mg each) were removed at 1, 2, 4, and 6 h after recovery at 25°C. Following incubation, leaf blades were homogenized in 3 mL of sample buffer (Laemmli, 1970) containing 1 mM PMSF, boiled for 5 min, and centrifuged at 15,000*g* for 5 min. The amount of ^{3}H-Leu incorporated into protein was determined by spotting 50-μL aliquots of the supernatant onto filter paper, washing with TCA, and scintillation spectroscopy as previously described (Park et al., 1996).

Electrophoretic Analysis of Proteins Synthesized during Heat Shock and Recovery

For this experiment six samples were prepared for each variant. Each sample consisted of six randomly selected, 1-cm leaf blade segments from plants at the same developmental stage, which were pooled and placed in 1 mL of sterile incubation buffer. Samples were heat-shocked in a water bath at 40°C for 1.5 h and transferred to 25°C for 2, 4, 6, and 8 h. Proteins synthesized during heat shock and recovery were labeled by the addition of 250 μCi of Tran^{35}S label (L-Met ^{35}S; L-Cys ^{35}S; >1000Ci/mmol; ICN) for 1.5 h at 40 and 25°C, respectively. Recovery samples were labeled during the 1.5 h of the recovery period at 25°C. Samples were prepared for SDS-PAGE and 2-D gel electrophoresis using the phenol-extraction method (Hurkman and Tanaka, 1986) as previously described (Park et al., 1996). 2-D gel electrophoresis was conducted as described by O'Farrell (1975). Gels were processed for fluorography using Resolution (EM Corp., Chesnut Hill, MA) and the directions of the manufacturer.

Preparation of RNA from Leaf Blades

Leaf blades (5 g per treatment) from NSB and SB were incubated in 50 mL of incubation buffer (Lin et al., 1984). Control and heat-shock samples were incubated for 1.5 h at 25 and 40°C, respectively. Following heat shock at 40°C for 1.5 h, recovery samples were allowed to recover at 25°C for 4 and 8 h. Following these treatments, total RNA was extracted from leaf blades (Bowden and Lord, 1979). Electrophoresis of RNA in 1.5% agarose gels containing 0.5% methylmercuric hydroxide (Gruenwedel and Davidson, 1966) indicated that the RNA was not degraded.

Isolation of Polysomes

Polysomes were isolated from NSB and SB using the method of Jackson and Larkins (1976). Leaf samples (6 g per treatment) for polysome isolation were cut and incubated at the same time as those used for total RNA extraction. Polysome pellets were resuspended in 60 μL of resuspension buffer (40 mM Tris-HCl [pH 9.0], 40 mM KCl, 10 mM $MgAc_2$, and 10% glycerol) and stored at −80°C.

Analysis of Polysomes on Suc Gradients

For Suc gradient analysis (Luthe, 1983), polysome samples with A_{260} of 3.5 were loaded onto 4-mL linear Suc gradients (18–50% Suc in 40 mM Tris-HCl, pH 9.0, 40 mM KCl, and 10 mM $MgAc_2$). Gradients were centrifuged for 1 h at 150,000*g* with slow acceleration and deceleration. The polysomes were fractionated using a density gradient fractionator (model 185, ISCO, Lincoln, NE) at a flow rate 0.75 mL/min. The absorbance was measured at 254 nm using a UA-5 absorbance monitor (ISCO). Five fractions (0.75 mL/fraction) were collected and kept on ice for RNA extraction.

RNA Isolation from Polysomes

RNA was isolated from polysome fractions using the procedure of Apuya and Zimmerman (1992) with some modifications. An equal volume of hot (55°C) SDS buffer (1% SDS, 200 mM NaCl, 40 mM EDTA, and 20 mM Tris-HCl [pH 7.5]) was added to the polysome suspension. The mixture was incubated at 55°C for 5 min and then cooled to room temperature. SDS was added to a final concentration of 1% before extracting two times with phenol:$CHCl_3$:isoamyl alcohol (25:24:1, v/v) and one time with $CHCl_3$:isoamyl alcohol (24:1, v/v). The RNA was precipitated from the aqueous phase by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% EtOH. The mixture was stored overnight at −20°C. The RNA was pelleted by centrifugation at 10,000*g* for 25 min and resuspended in 20 μL of distilled H_2O.

Preparation of Probe DNA

Inserts used as DNA probes for RNA-blot analysis were isolated from the following plasmids: pMon9508, encoding a maize HSP70 (Rochester et al., 1986); pWHSP16.9, encoding a wheat HSP18 (McElwain and Spiker, 1989); Tahsp26.6, encoding a chloroplast-localized wheat HSP25 (Weng et al., 1991); and pTayss, encoding the wheat SSU, which was obtained from Agrigenetics Corp (Dr. M. Murray, Madison, WI). Plasmids were purified from bacterial cultures using the method of Birnboim and Doly (1979). Inserts were excised with the appropriate restriction endonucleases. The reactions were conducted by incubating 10 μg of DNA in 1× restriction buffer provided by the manufacturer with 10 to 20 units of restriction enzyme at 37°C for 3 h. After digestion, the DNA was separated by electrophoresis on a 0.7% agarose gel and the insert was purified using the method of Zhen and Swank (1993). Following the directions of the manufacturer, DNA probes were radiolabeled using the nick-translation system from Promega.

Preparation of the Total cDNA Probe from Control Tissue

A total cDNA probe, which corresponded to the mRNA population expressed under control conditions, was prepared by reverse transcription of total RNA isolated from leaf blades of NSB incubated at 25°C. Total RNA (15 μg) from NSB and 1 μg of oligo(dT)(12–18), in a final volume of 50 μL, were incubated at 75°C for 3 min and immediately cooled on ice. Then 5 μL of 10x buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$), 10 mM DTT, 350 μM of each dNTP except dCTP, 20 units of murine leukemia reverse transcriptase (Stratagene), and 8 μL of [a-^{32}P]dCTP (ICN) were added. The reaction was incubated at 42°C for 1 h. The size of the total cDNA probe, which was determined by electrophoresis on a 1% agarose gel followed by autoradiography, ranged from 3 to 0.5 kb. This probe did not hybridize to rRNA on a northern blot.

RNA-Blot Analysis

RNA-blot analysis was conducted by applying either 20 μg of total RNA, or equivalent volumes of polysomal RNA, to Gene Screen Plus membrane (DuPont) using a slot-blot template (VacuSlot System, American Bionetics, Hayward, CA). The membrane was prepared, prehybridized, and hybridized using the manufacturer's protocol. Following hybridization, membranes were washed in 2× SSC containing 0.1% SDS at room temperature for 10 min, and then for 8 min at 50 to 60°C. The final wash was 0.1%× SSC/0.1% SDS at 25°C for 10 min. Hybridization was detected by autoradiography on X-Omat AR x-ray film (Kodak) at room temperature with an intensifying screen. For all slot-blot hybridizations, band intensities were quantified using a densitometer (model FB910, Fisher Scientific) and an integrator (model 3396A, Hewlett-Packard). For the mRNA hybridization in Figures 4 and 5 the relative abundance of each mRNA was presented as a percentage of the NSB value. For the polysome data presented in Figures 4 and 5 the relative amount of hybridization in each gradient fraction was determined by densitometry. The amount of RNA hybridizing in the polysome region was determined by adding the areas of fractions 2 through 5 and was designated as the total polysome area. Data are presented as a percentage of the NSB value.

Dissociation of mRNA Attached to Polysomes

Polysomes were treated with EDTA and KCl as described by Berry et al. (1988) to release the mRNA from the ribosomes. Polysomes were isolated from control (25°C), heat-shock, and recovery (4 h) leaf blades of SB using the previously described method. Three A_{260} units of polysomes were pretreated with 40 mM EDTA and 250 mM KCl at 37°C for 15 min prior to loading on 18 to 52% Suc gradients. Following centrifugation at 150,000*g* for 1.5 h, the gradients were fractionated and RNA was isolated from each fraction as described previously. Equal volumes of RNA from each fraction were applied to the slot blot for RNA-hybridization analysis.

RESULTS

Protein Synthesis during Recovery from Heat Shock

Since previous work (Park et al., 1996) indicated that there were no differences between NSB and SB in the time or temperature required to induce the heat-shock response, experiments were conducted to determine if there were differences in the ability of NSB and SB to recover normal levels of protein synthesis following heat shock. Figure 1 shows ^{3}H-Leu incorporation into TCA-insoluble material during recovery at 25°C following heat shock at 40°C. Both variants had reduced protein synthesis during the first 2 h of recovery, which is probably due to a continuation of the heat-shock response. In SB, however, amino acid incorporation into TCA-insoluble material increased between 2 and 4 h. There was a longer lag period in the recovery time for NSB; amino acid incorporation did not reach the level of SB until 6 h of recovery. It appears that SB can resume normal levels of amino acid incorporation following heat shock about 2 h earlier than NSB. Since there were no differences in ^{3}H-Leu uptake between SB and NSB during heat shock and recovery (data not shown), the difference in the recovery times could be due to differences in mRNA abundance, translational efficiency, or protein turnover.

SDS-PAGE (Fig. 2) was conducted to visualize the pattern of proteins synthesized by NSB and SB during heat shock and recovery. During recovery in NSB there was the increased synthesis of an unidentified 32-kD polypeptide, which did not appear in SB. An additional polypeptide was also present in the 25-kD region of SB (Fig. 2). This protein is probably one of the additional HSP25 polypeptide(s) present in SB and not NSB (Park et al., 1996).

HSP83 and HSP70 were synthesized at low levels at the control temperature, but their synthesis increased in NSB and SB during heat shock (Fig. 2). Synthesis of HSP104,

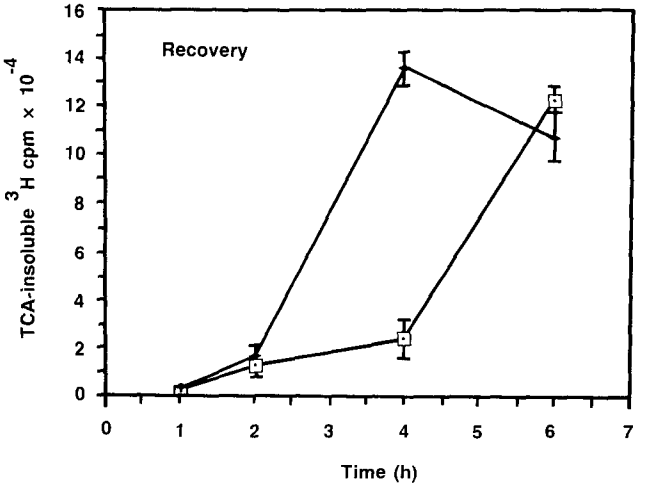

Figure 1. Incorporation of ^{3}H-Leu into leaf protein in NSB (□–□) and SB (◆–◆) during recovery at 25°C following 1.5 h of heat shock at 40°C. Leaf blades were incubated at 40°C for 1.5 h and were then continuously labeled with ^{3}H-Leu at 25°C. Samples were removed at 2, 4, and 6 h and the amount of TCA-insoluble cpm was determined. Duplicate samples were averaged for each experiment. Data reported are the average of three independent experiments.

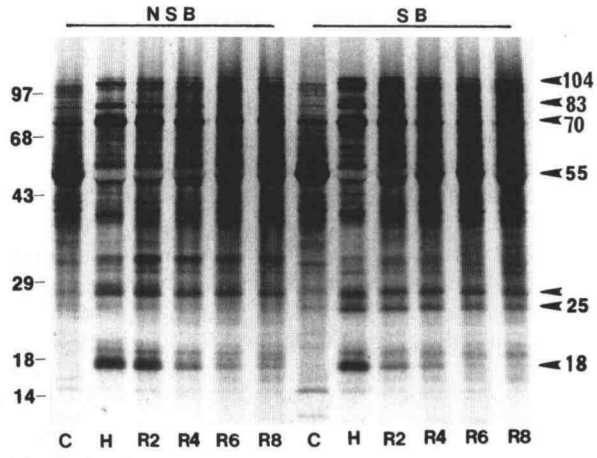

Figure 2. Autoradiogram of SDS-PAGE analysis of leaf proteins from NSB and SB labeled during heat shock and recovery. Following heat shock at 40°C for 1.5 h leaf segments were incubated at 25°C for 2, 4, 6, and 8 h. Recovery samples were labeled in vivo with Tran^{35}S-label during the last 1.5 h of the recovery. Equal amounts of TCA-precipitable cpm (6×10^4) were loaded in each lane. Lanes C, Control sample incubated and labeled at 25°C for 1.5 h; lanes H, heat-shocked and labeled at 40°C for 1.5 h; lanes R2 through R8, incubated at the recovery temperature (25°C) for 2, 4, 6, and 8 h. Numbers in the left margin are the molecular weight markers in kD. Arrowheads in the right margin indicate the major HSPs. The 55-kD protein is LSU.

HSP25, and HSP18 was not detected at the control temperature, but they were synthesized during heat shock in both variants (Fig. 2). During recovery from heat shock there was a gradual decrease in the synthesis of HSP104, HSP83, HSP70, and HSP18 in both variants (Fig. 2). The synthesis of HSP104, HSP83, and HSP70 appeared to decrease faster in SB than in NSB. The synthesis of the HSP25 polypetides increased in both NSB and SB during heat shock, and there was no appreciable decrease in their synthesis during the recovery period in either variant.

The major difference in HSP synthesis was the synthesis of HSP18, which decreased more rapidly in SB than in NSB during recovery from heat shock (Fig. 2). HSP18 comprised the same proportion of total HSP synthesis during heat shock in SB and NSB. After 2 h of recovery the proportion of HSP18 synthesized by SB was approximately 50% lower than by NSB. In NSB HSP18 was still synthesized at the heat-shock level at 2 h, and only after 4 h had its synthesis decreased. There was no significant difference between SB and NSB in the amount of total protein per g fresh weight during heat shock and recovery. Therefore, the amount of HSP18 synthesized by SB at 2 and 4 h of recovery was probably less than that synthesized by NSB.

There was a gradual increase in the synthesis of some normal proteins during recovery from heat shock (Fig. 2). After 8 h of recovery the pattern was similar, but not identical to the control in both variants. The transition from HSP synthesis to normal protein synthesis began earlier in SB than in NSB. At 4 h of recovery there were fewer HSPs and more normal proteins synthesized in SB than in NSB. The pattern of proteins synthesized by SB at 4 h was very similar to the 8-h pattern, whereas in NSB the 4-h pattern was more typical of the heat-shock pattern. In both variants the transition from the heat-shock pattern to the control pattern corresponded to the time when increased amino acid incorporation occurred (Fig. 1).

One of the normal proteins present on the gel was the 55-kD LSU. Its synthesis decreased in both variants during heat shock. Although synthesis of LSU did not recover to the control levels during the 8-h recovery period, its synthesis increased earlier (4 h) in SB than in NSB (6–8 h).

2-D Gel Electrophoresis of Proteins Synthesized during Heat Shock and Recovery

Proteins synthesized in vivo were analyzed by 2-D electrophoresis to determine if there was a selective decrease in any of the members of the HSP18 family during recovery from heat shock (Fig. 3). Following heat shock there were approximately 17 HSP18 polypeptides synthesized by both variants. The absence of two HSP18 polypeptides in SB (see boxes in Fig. 3) has been previously observed (Park et al., 1996). At 2 h the same set of HSP18 polypeptides was present in both variants, but the intensity of the spots appeared to be less in SB than in NSB. By 4 h both the number and intensity of the HSP18 polypeptides diminished in SB compared with NSB. Two HSP18 polypeptides in the basic region of the gel were absent from SB, but not NSB, at this time. There were no major differences between SB and NSB at 6 h, but at 8 h there were approximately eight HSP18 polypeptides remaining in NSB and only four in SB. Thus, there appears to be a gradual decrease in number and intensity of HSP18 polypeptides during recovery. This decrease occurs faster in SB than in NSB. In contrast, the synthesis of two high-molecular-weight HSP70 polypeptides (see dark arrowheads in Fig. 3) did not appreciably decrease during the recovery period in either variant. The synthesis of the HSP25 family also continued during the recovery period in both SB and NSB.

The synthesis of several normal proteins was monitored during heat shock and recovery. These included LSU and a polypeptide marked ss, which has a molecular mass (14 kD) and pI similar to the SSU of soybean (Vierling and Key, 1985). In both variants the synthesis of LSU decreased during heat shock and remained low after 2 h. LSU synthesis increased at 4 h in SB, but not NSB. At 6 and 8 h LSU synthesis decreased in SB. This may be due to the prolonged incubation of the leaf blades. In NSB LSU synthesis did not increase beyond the heat-shock level during the entire 8-h recovery period. In both variants the synthesis of ss (SSU) did not decrease during heat shock. Its synthesis decreased in both variants at 2 and 4 h, and at 6 h ss (SSU) could not be detected.

In addition to the two polypeptides mentioned above, the synthesis of several other non-HSPs (a, b, c, d, and e) was monitored. These proteins were selected because they were clearly separated from the other polypeptides on the gel and could be easily visualized (Fig. 3). During heat shock synthesis of polypeptides a, c, d, and e decreased in both variants, whereas synthesis of polypeptide b remained relatively constant. Renewed synthesis of polypetides a and e occurred at 2 h of recovery in SB, but not

until 4 h in NSB. Polypeptides c and d reappeared at between 2 and 4 h, but there was no difference between SB and NSB in the time of their appearance. There were several sets of normal polypeptides present in SB and NSB. Synthesis of some polypeptides (e.g. b and ss) was not inhibited by heat shock in either variant. Synthesis of another set of polypeptides (e.g. c and d) decreased during heat shock and the first 2 h of recovery, but increased at 4 h in both variants. Finally, synthesis of some polypeptides (e.g. a and e) increased about 2 h earlier in SB than in NSB.

Abundance of mRNAs in SB and NSB during Heat Shock and Recovery

To ascertain if the changes in HSP synthesis during recovery from heat shock were due to changes in mRNA availability, the relative abundance of several mRNA families was measured. Total RNA isolated from both variants was hybridized to probes encoding HSP70, HSP25, HSP18, and SSU mRNA. All of these proteins belong to multigene families. Since these are not gene-specific probes, we assumed that they could hybridize to some or all members of their respective families.

There were low levels of HSP70 mRNA in leaves of both variants incubated at the control temperature (Fig. 4). This mRNA was most likely used for the translation of HSP70 cognates, which were synthesized at the control temperature (Figs. 2 and 3). Following heat shock the abundance of HSP70 mRNAs increased approximately 4-fold in SB and 2-fold in NSB, relative to the amount present at the control temperature. After 4 h there was a large decrease of HSP70 mRNA abundance in SB, whereas in NSB the abundance was nearly equivalent to the heat-shock level. By 8 h there was no difference between the variants. A comparison of HSP70 synthesis (Fig. 2) with the mRNA abundance indicated that HSP70 synthesis continues until 8 h of recovery despite the decrease in the abundance of HSP70 mRNA.

Unlike HSP70 mRNA, the abundance of HSP18 mRNA was low at the control temperature (Fig. 4). In both variants, HSP18 mRNA increased during heat shock, but its abundance was approximately 2.5-fold greater in SB than in NSB. At 4 h the abundance of HSP18 mRNA in SB decreased about 3-fold from the heat-shock level, whereas in NSB the level of HSP18 mRNA increased approximately 2-fold. The amount of HSP18 mRNA decreased again at 8 h, but there was no major difference between SB and NSB at this time. In another experiment we observed a 3.5-fold decrease in HSP18 mRNA in SB, and only a 2-fold decrease in NSB at 2 h of recovery (data not shown). Since the abundance of HSP18 mRNA and synthesis of HSP18 both decrease rapidly and coordinately in SB during recovery from heat shock, this suggests that the expression of the HSP18 family is regulated by the availability of HSP18 mRNA.

Low levels of mRNA encoding the HSP25 the family were present at the control temperature (Fig. 4). Upon heat shock, the abundance of HSP25 mRNA increased in both variants with slightly more mRNA being present in SB than in NSB. The amount of HSP25 mRNA decreased during 4 and 8 h of recovery, but there were no differences between SB and NSB in the relative mRNA abundance.

Because the synthesis of normal proteins during recovery from heat shock occurs earlier in SB than in NSB, we wanted to determine if there was a greater abundance of mRNAs encoding normal proteins in SB. As an example of a specific control protein/mRNA, SSU was selected. At the control temperature, SSU mRNA was approximately twice as abundant in SB than in NSB. In SB the abundance of SSU mRNA decreased during heat shock and recovery. In NSB SSU mRNA abundance decreased during heat shock and increased at 4 and 8 h. During recovery (4 and 8 h), there was no major difference in SSU mRNA abundance between SB and NSB.

The fate of the total normal mRNA population was determined by preparing the following cDNA probe. Total RNA, isolated from NSB leaf blades incubated at the control temperature (25°C), was used as a template for cDNA synthesis. The resulting cDNA should represent all normal mRNAs expressed at the control temperature. This cDNA was used to probe the slot blot of total RNA isolated from SB and NSB during recovery from heat shock (Fig. 5). The amount of normal mRNA was nearly equal in both variants at all time points. This result indicated that the abundance of the normal mRNA population did not change significantly during the heat-shock and recovery periods, and that there were no major differences between SB and NSB.

Polysome Analysis during Heat Shock and Recovery

In vivo labeling and one-dimensional and 2-D gel electrophoresis indicated that the synthesis of certain sets of normal proteins occurred earlier in SB than in NSB during recovery from heat shock. Since there were no differences between SB and NSB in normal mRNA abundance (Fig. 5), this suggests that increased levels of normal protein synthesis may be regulated at the translational or posttranslational level.

Polysomes were isolated from SB and NSB incubated at the control temperature (25°C), at heat shock (40°C), and at 4 and 8 h of recovery and were analyzed on Suc gradients (Fig. 6). At the control temperature there was a large proportion of polysomes in both variants, and the ratio of the area of polysome/monosome was 8.1 and 6.1 for SB and NSB. Following heat shock polysomes decreased and monosomes increased in both variants, reflecting the decrease in protein synthesis during heat shock. During heat shock the ratio of polysome to monosome was 4:1 in SB and 3.2:1 in NSB. At 4 h the polysomal area increased in both variants, but it was greater in SB than in NSB. The polysome to monosome ratio was 13:1 for SB and 8.5:1 for NSB. This corresponds to the time when there were the greatest differences in protein synthesis between SB and NSB. At 8 h the proportion of polysomes in both variants was higher than the control, which may be indicative of the need to increase protein synthesis to compensate for the damage caused by heat shock. At this time, the polysome to monosome ratio was nearly equivalent in SB (13.2:1) and NSB (12.8:1).

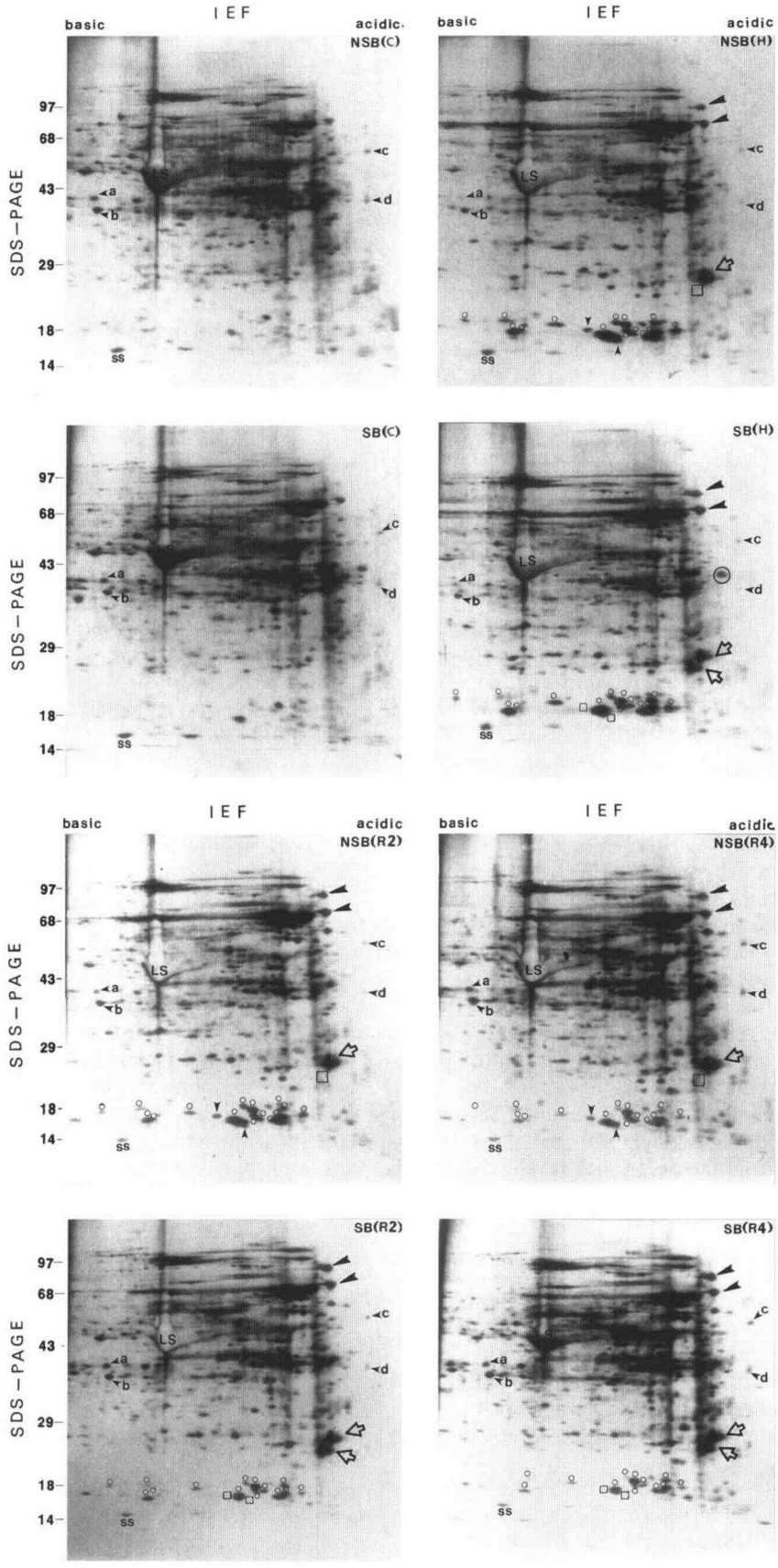

Figure 3. (Figure continued on next page.)

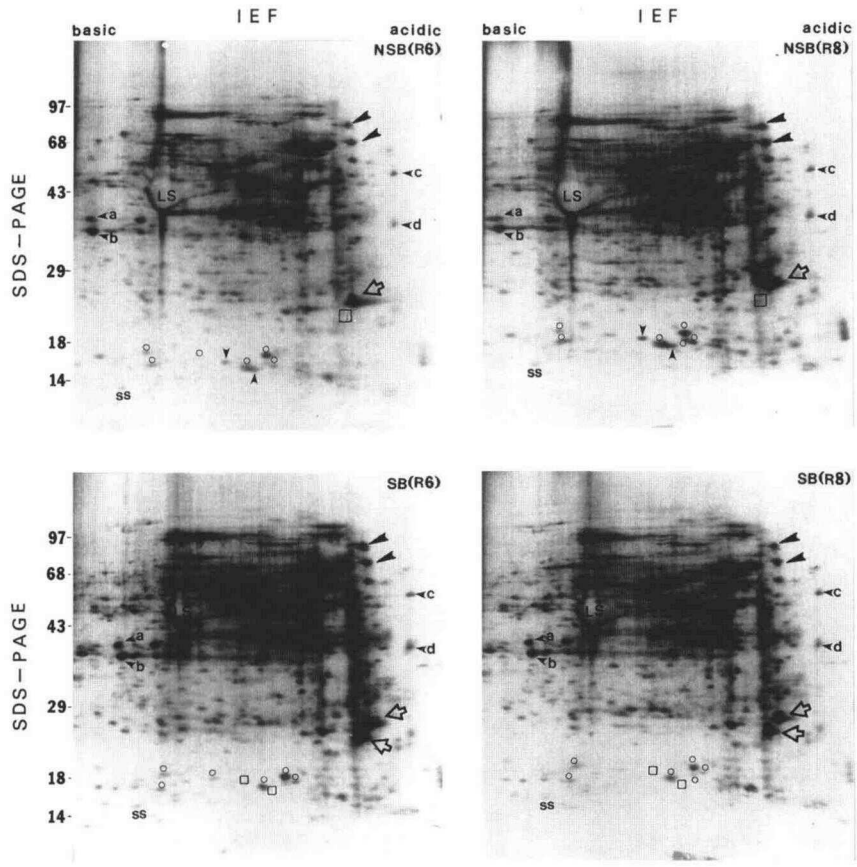

Figure 3. (Figure continued from previous page.) 2-D gel electrophoresis of leaf segments from NSB and SB, labeled in vivo at the control temperature, during heat shock and recovery. Leaf segments were labeled and proteins were extracted as described in Figure 2. Equal amounts of TCA-precipitable cpm (1.5×10^5) were loaded on each gel and polypeptides were visualized by fluorography. C, Control sample incubated and labeled at 25°C of 1.5 h; H, heat-shocked and labeled at 40°C for 1.5 h; R2 through R8, incubated at the recovery temperature (25°C) for 2, 4, 6, and 8 h. Numbers in the left margin are the molecular mass markers in kD. The letters a, b, c, d, e, and ss indicate normal proteins. LS marks LSU. The large, dark arrowheads indicate two high-molecular-weight HSPs. The open arrows mark the HSP25 group; the large, open box indicates the absence of two HSP 25 polypeptides in NSB. The small, open circles indicate HSP18 polypeptides; the small, dark arrowheads indicate two polypeptides that are present in NSB, but absent in SB. The small, open squares indicate the absence of these polypeptides in SB. The large, open circle in SB (H) indicates a 40-kD protein that occasionally appears as a HSP in that variant.

The sedimentation positions of the polysome peaks indicated that the number of ribosomes per mRNA (about 5–6) was the same in both variants in all treatments, suggesting that the efficiency of ribosome binding to mRNA was equivalent in both variants. However, at 4 h more mRNA was associated with polysomes in SB than in NSB.

Polysomal RNA-Blot Analysis

The polysome profiles (Fig. 6) indicated that there was an earlier recruitment of normal mRNAs onto polysomes in SB. The purpose of this experiment was to determine if there were differences in mRNA loading onto polysomes between SB and NSB during recovery from heat shock. Polysome gradients were fractionated, RNA was extracted from each fraction, and equal volumes of RNA were applied to a slot blot, which was probed with the same clones that were used for total RNA hybridization.

There were low levels of HSP70 mRNA on polysomes in both variants at the control temperature (Fig. 4). The abundance of polysomal HSP70 mRNA increased dramatically during heat shock, and was 2-fold higher in SB than in NSB. During recovery the amount associated with polysomes decreased in both variants, and there was no significant difference between NSB and SB. The parallel decrease in the abundance of total HSP70 mRNA and HSP70 polysomal mRNA suggests that HSP70 synthesis may be regulated at the transcriptional or posttranscriptional level.

There was no detectable HSP18 mRNA on polysomes at the control temperature (Fig. 4). This was consistent with data showing that there was no HSP18 synthesis in the absence of heat shock (Fig. 2). The association of HSP18 mRNA with polysomes was greatest at the heat-shock temperature, and was about 2-fold higher in SB than in NSB. By 4 h the amount of HSP18 mRNA associated with polysomes decreased in SB, but there was no change in the

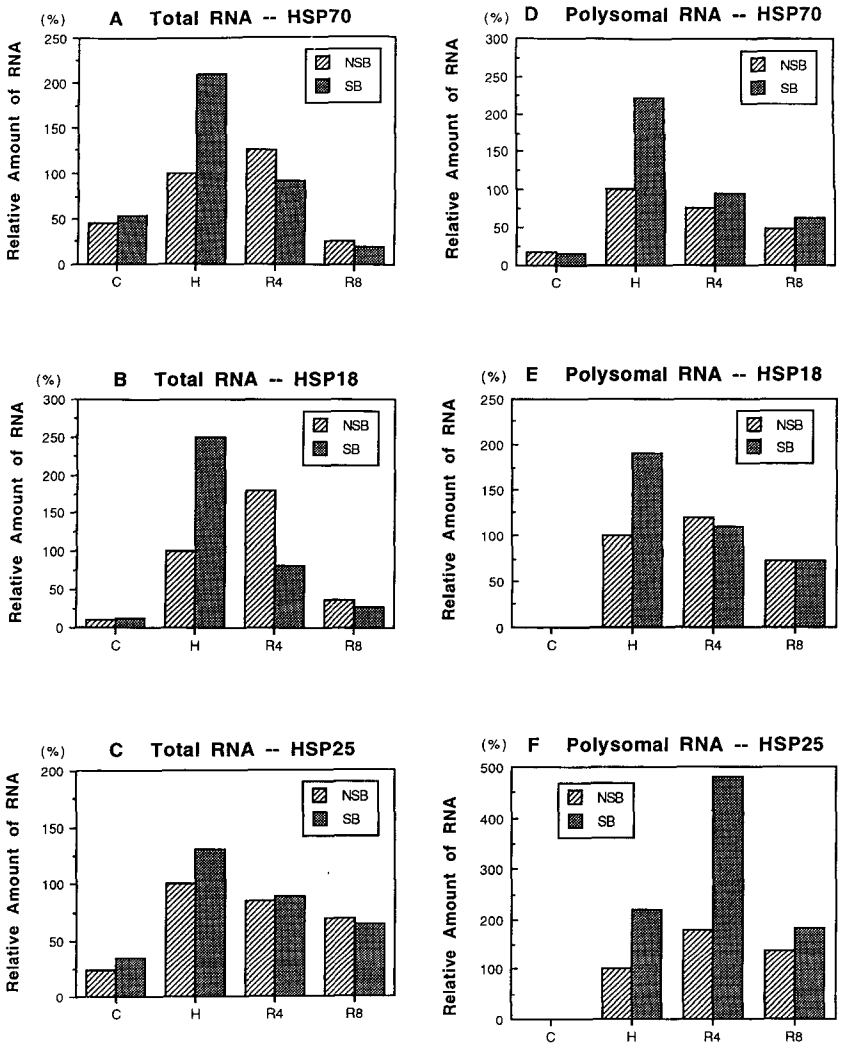

Figure 4. Relative abundance of HSP mRNAs and their distribution on polysomes at the control temperature and during heat shock and recovery. Total RNA (A, B, and C) and polysomes (D, E, and F) were isolated from leaf blades of SB and NSB and incubated at 25°C (C, control) or 40°C (H, heat shock) for 1.5 h, and from recovery samples incubated at 40°C for 1.5 h and then at 25°C for 4 (R4) and 8 (R8) h. RNA slot blots were probed with clones encoding HSP70 (A and D), HSP18 (B and E), and HSP25 (C and F). The relative abundance of the mRNAs and their association with polysomes is presented as a percentage of the NSB heat shock (40°C) value.

amount of polysome-associated HSP18 mRNA in NSB. The amount of HSP18 mRNA-associated polysomes decreased at 8 h of recovery in both SB and in NSB. Since both the amount of total HSP18 mRNA and the synthesis of HSP18 decreased faster in SB than in NSB, we did not expect to find equivalent amounts of HSP18 mRNA associated with polysomes in SB and NSB at 4 h of recovery.

There was no HSP25 mRNA associated with polysomes at the control temperature. In both variants the polysomal HSP25 mRNA increased rapidly upon heat shock and appeared to be about 2-fold greater in SB than in NSB at heat shock (Fig. 4). At 4 h the amount of polysomal HSP25 mRNA increased dramatically in SB and was about 3-fold higher than in NSB. According to in vivo-labeling data (Fig. 2) the synthesis of HSP25 was greater in SB than in NSB during heat shock and recovery. This was probably due to the synthesis of the additional HSP25 family members in SB. Although slot-blot analysis indicated that the amount of total HSP25 mRNA in the two variants was nearly equivalent (Fig. 4), there was a clear difference in the amount of HSP25 mRNA associated with polysomes in SB and NSB during heat shock and 4 h of recovery. This observation suggests that the synthesis of HSP25 may be translationally regulated. The translational regulation of small HSPs has been shown in carrot cell cultures (Apuyna and Zimmerman, 1992).

The association SSU mRNA and the normal mRNA population with polysomes was also determined (Fig. 5). At the control temperature there was no difference between SB and NSB in the amount of SSU mRNA associated with polysomes. During heat shock the amount of SSU mRNA associated with polysomes decreased in NSB and increased in SB. At 4 h the amount of SSU mRNA associated with polysomes in NSB increased over the heat-shock level, but it decreased in SB. The continued association of SSU with polysomes may be indicative of continued SSU synthesis during heat shock and early stages of recovery. At 8 h the amount of SSU mRNA associated with polysomes decreased in both variants.

When the cDNA probe, representing normal mRNAs expressed at the control temperature, was hybridized to the polysome fractions, there was no difference between the two variants in polysomal mRNA at the control temperature (Fig. 3). During heat shock and 4 h of recovery the

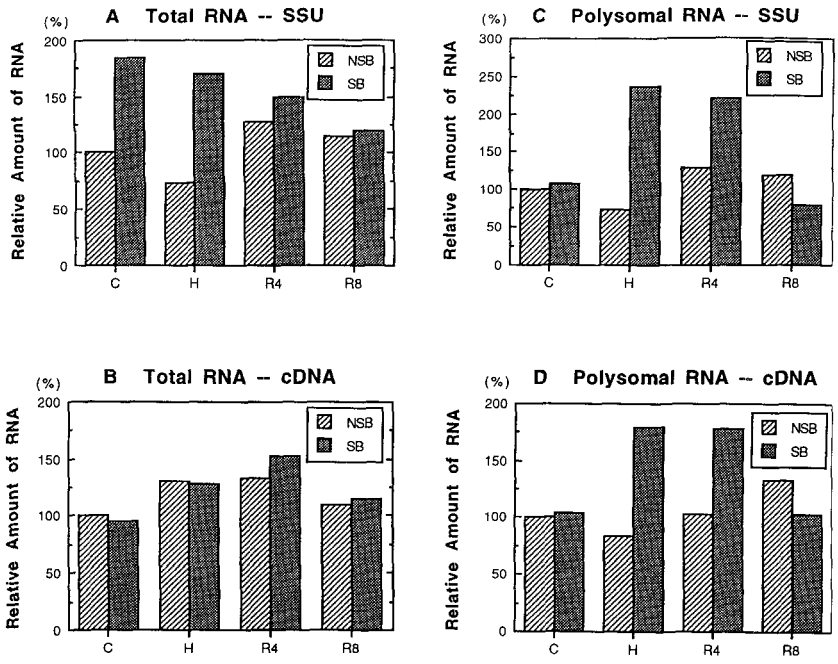

Figure 5. Relative abundance of normal mRNAs and their distribution on polysomes at the control temperature and during heat shock and recovery. Total RNA (A and B) and polysomes (C and D) were isolated from leaf blades of SB and NSB and incubated at 25°C (C, control) or 40°C (H, heat shock) for 1.5 h, and from recovery samples incubated at 40°C for 1.5 h and then at 25°C for 4 (R4) and 8 (R8) h. RNA slot blots were probed with a clone encoding SSU (A and C) or the total normal cDNA probe (B and D). The relative abundance of the mRNAs and their association with polysomes is presented as a percentage of the NSB control (25°C) value.

amount of polysomal mRNA remained relatively constant in NSB. In SB there was an approximately 2-fold increase in the amount of polysomal mRNA during the same treatments. After 8 h the amount of polysomal mRNA increased in NSB and decreased in SB. More of the normal mRNA population appeared to be associated with polysomes in SB than NSB during heat shock and 4 h of recovery.

Is mRNA Associated with Polysomes or HSGs?

Instead of being associated with polysomes in SB at 4 h of recovery (Fig. 4), the HSP18 mRNA could have been complexed with HSGs, which cosediment with polysomes (Mansfield and Key, 1988). In this case, the mRNA would appear to be associated with polysomes, but would not be translated. To determine if heat shock and normal mRNAs were associated with polysomes or HSGs during heat shock and recovery, polysomes were prepared from SB and treated with 40 mM EDTA and 250 mM KCl prior to fractionation on Suc gradients. This treatment dissociates the ribosomes into subunits and releases the translated mRNAs (Pemberton et al., 1975), but it should not disrupt the HSGs (Mansfield and Key, 1988). If mRNAs are released from polysomes, they should migrate into the uppermost region of the Suc gradients.

The gradient profiles of the EDTA/KCl-treated polysomes from SB indicated that the polysomes were dissociated and the subunits sedimented as a large prominent peak on the top of the gradient (Fig. 7). RNA was isolated from each fraction of the gradient, and the distribution of mRNA across the gradient was determined. At the control temperature there was no HSP18 mRNA associated with polysomes. During heat shock most of the HSP18 mRNA sedimented in the monosome region of the gradient (Fig. 8, fractions 1 and 2), indicating that mRNA was released from the polysomes by EDTA/KCl treatment and was originally associated with polysomes. At 4 h the major hybridizing band was in the middle of the gradient (fraction 3). This indicated that the HSP18 mRNA could not be released with EDTA/KCl and suggested that it was associated with HSGs and not with polysomes. This confirmed our speculation that the HSP18 mRNA was not associated with polysomes in SB at 4 h of recovery (Fig. 4). If HSP18 mRNA was associated with HSGs in SB, it would be unavailable for translation and would account for the decreased synthesis of HSP18 (Neumann et al., 1989; Nover et al., 1989). Polysomes from SB and NSB not treated with KCl and EDTA were also fractionated and probed for the presence of HSP18 mRNA (data not shown). Although the total amount of HSP18 hybridizing in the polysome region of the two variants was equivalent, there was more HSP18 mRNA in fraction 3 of SB than NSB.

When the same blot was probed for HSP25 mRNA, most of the mRNA sedimented in the top region of the gradient during heat shock and recovery (Fig. 8). Therefore, the HSP25 mRNA appeared to be specifically associated with polysomes, and not with the putative HSGs. The slot blots were also probed for HSP70 mRNA, SSU RNA, and normal mRNA (cDNA probe). All of these mRNAs shifted to the top of the gradient during heat shock and recovery, indicating that they were also associated with polysomes (data not shown).

DISCUSSION

In a previous study (Park et al., 1996) we determined that SB synthesized two to three additional members of the HSP25 family that were not synthesized by NSB. Genetic analysis indicated that thermotolerance and the presence of these additional HSP25 polypeptides were linked traits (Park et al., 1996). These low-molecular-weight HSPs are generally more prevalent in plants than the high-

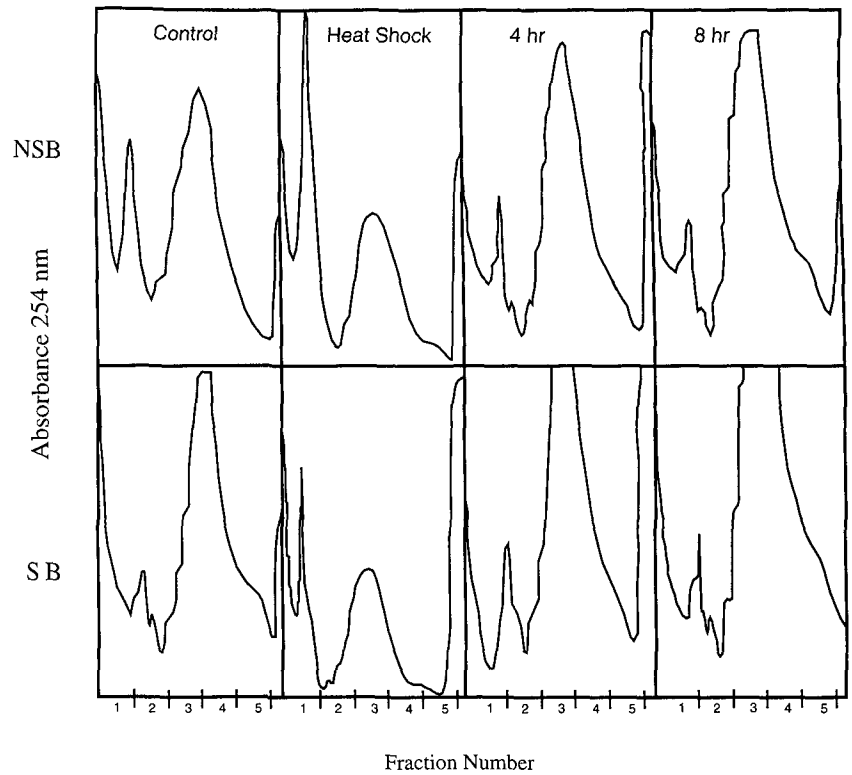

Figure 6. Suc-density gradient profiles of polysomes isolated from NSB and SB. Polysomes were isolated from leaf blades incubated at 25°C (Control) or 40°C (Heat Shock) for 1.5 h. Recovery samples were incubated at 40°C for 1.5 h and at 25°C (recovery) for 4 and 8 h. Equal amounts of polysomes (A_{260} of 3.5) were layered on each gradient. The top of the gradient is on the left. Polysomes sedimented in fractions 2 though 5.

molecular-weight HSPs. Their accumulation and persistence during heat shock and recovery have been correlated with increased thermotolerance (Waters et al., 1996). The low-molecular-weight HSPs probably function as molecular chaperones to prevent protein aggregation and misfolding during heat stress (Waters et al., 1996). One of the additional HSP25 polypeptides found in SB was localized in the chloroplast, where it may help to prevent inactivation of chloroplast proteins during heat stress (Park et al., 1996).

Although the additional HSP25 polypeptides were synthesized by SB, it seems unlikely that the presence of two to three additional HSPs from a multigene family could be responsible for the observed increase in thermal tolerance. Our hypothesis is that thermotolerance in SB may be the result of a regulatory mutation that has altered parts of the heat-shock response pathway, one being the expression of the additional HSP25 polypeptides, another being the ability to recover normal protein synthesis earlier following heat shock.

Following heat shock SB resumed normal protein synthesis 2 h earlier than NSB. Electrophoretic analysis of proteins synthesized during recovery indicated that the synthesis of several HSPs decreased and normal protein synthesis increased at 4 h of recovery in SB and at 6 to 8 h in NSB. Both quantitative and qualitative changes in protein synthesis occur sooner in SB than in NSB. Hybridization analysis using a total cDNA probe indicated that the abundance of normal mRNAs in SB and NSB was equivalent at 4 h of recovery. However, there were more polysomes and normal mRNAs associated with polysomes in SB than in NSB at this time. Gallie et al. (1995) determined that heat shock increased the stability of a reporter mRNA (lucifierase), but decreased its translational efficiency. The interaction between translational efficiency and mRNA stability, and its subsequent effect on protein synthesis, was dependent on the severity of the heat shock. Since there were no differences in the steady-state levels of normal mRNAs between SB and NSB, it appears that SB can more

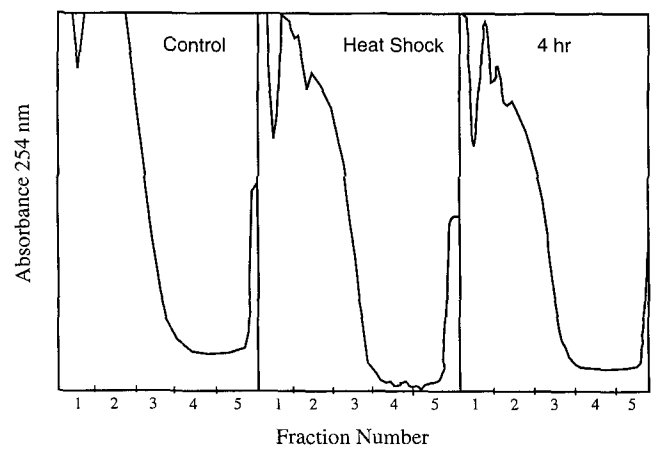

Figure 7. Suc-density gradient profiles of polysomes isolated from SB following treatment with EDTA and KCl. Polysomes were isolated from SB leaf blades incubated at 25°C (Control) or 40°C (Heat Shock) for 1.5 h. Recovery samples were incubated at 40°C for 1.5 h and at 25°C (recovery) for 4 h. Prior to centrifugation on 18 to 50% Suc gradients, polysomes were treated with 40 mM EDTA and 250 mM KCl. Equal amounts of polysomes (A_{260} of 3.0) were layered on each gradient. The top of the gradient is on the left.

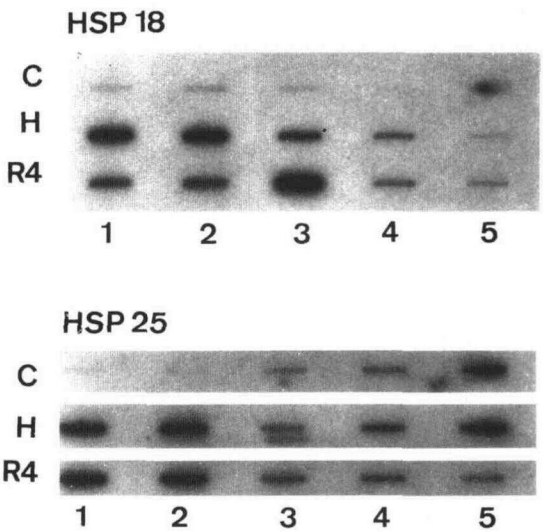

Figure 8. Distribution of mRNAs in EDTA/KCl-treated polysomes isolated from SB. The polysome gradients from Figure 7 were fractionated into fractions 1 though 5. RNA was extracted from each fraction and equal volumes of polysomal RNA were applied to a slot blot that was probed with clones encoding HSP18 and HSP25.

efficiently recruit normal mRNAs onto polysomes for translation during heat shock and recovery. The mechanism of this response is unknown. The abundance of a 40-kD acidic protein, which is associated with plant ribosomes, has been positively correlated with periods of active tissue growth and high polysome content (Garcia-Hernandez et al., 1996). The presence of this protein, an altered form of the SP6 ribosomal protein (Scharf and Nover, 1982), or some other translation factor may affect polysome formation in SB.

We observed that the synthesis of SSU (ss in Fig. 3), did not decrease in SB or NSB, and that SSU mRNA was associated with polysomes during heat shock. These results differ from those of Vierling and Key (1985), who observed a decrease in SSU synthesis at 40°C in soybean. However, in rice both the specific activity and amount of the Rubisco holoenzyme increased after 1 h of heat shock at 40°C (Bose and Gosh, 1995). The increased SSU synthesis was more pronounced in the heat-tolerant than nontolerant rice cultivar.

Not only does SB recover normal protein synthesis during recovery from heat shock faster than NSB, the synthesis of HSP83, HSP70, and HSP18 families decreases earlier. In contrast, synthesis of the HSP25 family continues throughout the 8-h recovery period in both NSB and SB. Three different mechanisms may be regulating the expression of the HSP70, HSP25, and HSP18 families during recovery from heat shock. First, synthesis of the HSP70 family appears to be correlated with the abundance of its mRNA (Fig. 4). Second, the synthesis of the HSP25 family, which continued throughout the recovery period in both bentgrass variants, appears to be regulated at the posttranscriptional/translational level. Both SB and NSB had similar amounts of HSP25 mRNA during heat shock and recovery, but more of the HSP25 mRNA was associated with polysomes in SB than in NSB. These results are similar to those of Apuya and Zimmerman (1992), who determined that heat-shocked globular embryos could more efficiently translate (recruit onto polysomes) HSP17.7 mRNA than callus cells. Third, synthesis of the HSP18 family decreased earlier in SB than in NSB and appeared to be correlated with mRNA abundance, which also decreased earlier in SB. However, at 4 h of recovery, there were equivalent amounts of HSP18 mRNA associated with polysomes in both SB and NSB. Since there was less HSP18 synthesis at 4 h in SB than in NSB, this seemed unlikely. When the polysome preparation was dissociated into ribosomal subunits, which should release all translated mRNA, the HSP18 mRNA migrated in the middle region of the Suc gradient suggesting that it was associated with a larger aggregate, such as a HSGs. In carrot callus cells the abundant HSP17.7 mRNA that was not associated with polysomes cofractionated with cytoplasmic particles (Apuya and Zimmerman, 1992). This sequestration of HSP mRNA probably prevented their translation.

Is there a relationship between the ability of SB to resume normal protein synthesis following heat shock and its physiological phenotype of increased thermotolerance? According to Howarth (1991), the ability of sorghum to withstand repeated diurnal heat stress in the field was dependent on its ability to conduct de novo HSP synthesis. Plants that did not completely recover from the first stress were unable to conduct de novo HSP synthesis, and could not withstand subsequent stresses. Failure to resume normal protein synthesis following heat shock has a deleterious effect on seedling growth and survival (Howarth and Skøt, 1994). The ability to resume normal protein synthesis following heat stress appears to be a characteristic of thermotolerant plants (Howarth and Skøt, 1994). In thermosensitive plants HSP synthesis will continue longer and recovery of normal protein synthesis will be delayed (Howarth and Skøt, 1994).

In the field HSP synthesis occurs when the temperature increases at midday. As the temperature decreases, plants that are capable of shutting down HSP synthesis and resuming normal protein synthesis earlier than others should be able to use the remaining sunlight more efficiently. Field studies (Kemp, 1987) demonstrated that when SB and NSB were grown at high temperatures in the field and then transferred to a growth chamber at the control temperature, SB accumulated dry matter faster than NSB. The ability of SB to recover from high-temperature stress at both the molecular and physiological levels may be linked to its increased thermotolerance. The linkage between the earlier resumption of normal protein synthesis and the presence of the additional HSP25 polypeptides has not been tested.

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