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Heat-Shock Response in Heat-Tolerant and Nontolerant Variants of *Agrostis palustris* Huds.[1]

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The heat-shock response in heat-tolerant variants (SB) and nontolerant variants (NSB) of creeping bentgrass (*Agrostis palustris* Huds.) was investigated. Both variants were derived from callus initiated from a single seed of the cultivar Penncross. SB and NSB synthesized heat-shock proteins (HSPs) of 97, 83, 70, 40, 25, and 18 kD. There were no major differences between SB and NSB in the time or temperature required to induce the heat-shock response. When the HSPs synthesized by SB and NSB were analyzed by two-dimensional gel electrophoresis, it was apparent 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. Analysis of F_1 progeny of NSB × SB indicated that 7 of the 20 progeny did not synthesize the additional HSP25 polypeptides. These progeny were significantly less heat tolerant than progeny that did synthesize the additional HSP25 polypeptides. The χ^2 test of independence ($\chi^2 = 22.45$, $P < 0.001$) indicated that heat tolerance and the presence of the additional HSP25 polypeptides are linked traits.

Plants have evolved with the remarkable ability to adapt to many environmental stresses. Exposure to high temperatures is one of these stresses, and it limits plant productivity and survival (Boyer, 1982). Studies with a number of organisms, including higher plants, indicate that a brief exposure to moderately high temperatures improves the ability of the organism to survive subsequent exposures to potentially lethal temperatures (see reviews by Vierling, 1991; O'Connell, 1994). One postulated mechanism for the acquisition of thermotolerance is the synthesis of HSPs during exposure to high temperature. These proteins are believed to protect biomolecules and organelles in the plant cell during high-temperature stress. The synthesis of HSPs by cotton (Burke, 1985), soybean (Kimple and Key, 1985), and other legumes (Hernandez and Vierling, 1993) grown in the field suggests that their synthesis is a normal occurrence, which may protect plants from daily exposures to high temperatures.

HSPs are generally divided into two classes: the high-molecular-mass HSPs (60–110 kD) and low-molecular-mass HSPs (15–30 kD) (Vierling, 1991; O'Connell, 1994). The low-molecular-mass HSPs, which are encoded by a large gene family, are the most abundant HSP class found in plants (Vierling, 1991). The function of the low-molecular-mass HSPs is unknown, but it has been shown that their rate of synthesis and accumulation coincides with acquired thermotolerance in soybean (Lin et al., 1984; Vierling, 1991). Although there have been a number of studies in which heritable differences in thermotolerance in plants (Vierling, 1991; O'Connell, 1994) and qualitative differences in low-molecular-mass HSP synthesis in heat-tolerant and nontolerant cultivars of several species (Vierling, 1991; O'Connell, 1994; Jorgensen and Nguyen, 1995) were documented, there is no conclusive genetic evidence in plants linking HSP polymorphisms with thermotolerance (Fender and O'Connell, 1989, 1990; Marmiroli et al., 1989; Fova and Gorla, 1993; O'Connell, 1994). However, in *Neurospora crassa* it was demonstrated that disruption of HSP30 (an α-crystallin-related protein) expression reduces thermotolerance when the fungi are grown on a limiting Glc supply (Plesofsky-Vig and Brambl, 1995).

Creeping bentgrass (*Agrostis palustris* Huds., cv Penncross) is a commonly used, cool-season turfgrass grown in temperate regions of the United States. It cannot survive in subtropical regions without irrigation. Cell culture with heat as a selection pressure has been used to develop several somaclonal variants of creeping bentgrass with improved heat tolerance (Bayta-Blance, 1984). The original source of plant material was Penncross, a synthetic cultivar resulting from the open pollination of three selected vegetative clones of creeping bentgrass (Hanson, 1959). Because of the open pollination, each seed of Penncross will be heterozygous. To obtain the heat-tolerant variants, callus was initiated from a single seed (genotype) of Penncross, which was subjected to high-temperature stress (Fig. 1). One of the thermotolerant variants isolated

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Abbreviations: 1-D, one-dimensional; 2-D, two-dimensional; HSP, heat-shock protein; NSB, nonselected bentgrass; SB, selected bentgrass.

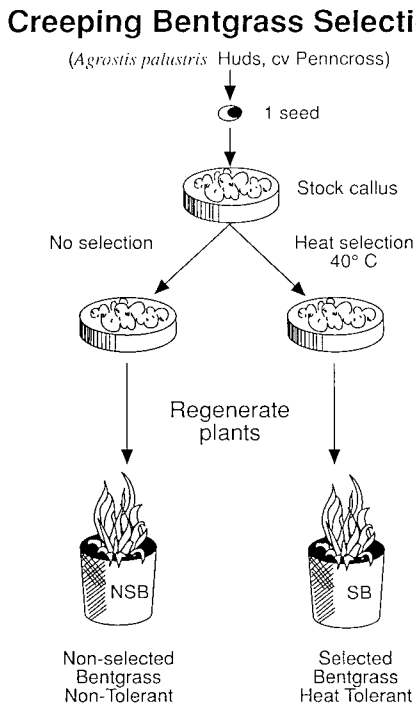

Figure 1. Development of heat-tolerant creeping bentgrass. Callus was generated from a single seed of Penncross and plants were regenerated from callus surviving heat selection at 40°C for 10 d. SB was one of several heat-tolerant variants recovered; NSB is the nontolerant variant.

was SB. NSB was regenerated from the same callus as SB, but this callus was not subjected to high-temperature selection and is not thermotolerant. Several other heat-tolerant variants have been isolated from the stock callus, suggesting that the increased heat tolerance was due to the selection and not to loss of tolerance in NSB. Clonally propagated SB has been grown in the field for approximately 10 years and has maintained thermotolerance. The Mississippi Agricultural and Forestry Experiment Station (Mississippi State, MS) has released SB germplasm for use in the development of heat-tolerant commercial cultivars.

We are using SB and NSB to investigate the molecular mechanisms of thermotolerance in higher plants. Since these two variants originated from the same seed, we expect fewer genetic differences than would be observed between different cultivars. Therefore, it is possible that some of the physiological, biochemical, and molecular differences between these variants may be related to heat tolerance. Karyotyping (U. Siregar and D.S. Luthe, unpublished data) indicated that both SB and NSB have the same number (14 pairs) of chromosomes, suggesting that phenotypic differences are probably not due to alterations in chromosome number that may have occurred during tissue culture.

The purpose of this study was to systematically investigate the heat-shock response in SB and NSB to ascertain whether there were differences in the time or temperature required to induce the heat-shock response or qualitative differences in the synthesis of HSPs. The results indicated that two to three additional HSP25 polypeptides were synthesized in SB but not NSB. Analyses of the progeny from these two variants indicated that the ability to synthesize the additional HSP25 polypeptides and thermotolerance were positively correlated.

MATERIALS AND METHODS

Development of SB and NSB

In vitro cell-selection techniques were used to isolate heat-tolerant variants of creeping bentgrass (*Agrostis palustris* Huds., cv Penncross) (Bayta-Blance, 1984). Stock callus was initiated from one seed of Penncross, portions of that callus were subjected to 40°C for 10 d, and high temperature-tolerant variants (SB) were recovered (Fig. 1). Plants (NSB) were also recovered from the same stock callus without selection at 40°C. Heat tolerance in the in vitro heat-selected variants was verified by growing plants hydroponically in a temperature-controlled water bath in a growth chamber. Water and air temperature was maintained at 40°C day and night for 2 weeks. The light intensity was 150 μmol m^{-1} s^{-2} and the photoperiod was 12 h. After high-temperature treatment, the air and water temperatures were reduced to 23°C for 2 weeks. The high-temperature treatment resulted in 88 to 93% plant mortality. Twenty-three variants survived heat treatment and were planted in field plots. Field plots of the SB and NSB were established using 10-cm-diameter plugs planted on 60-cm centers. All entries were replicated three times in a random complete block design. One of the 23 variants (SB) was selected for further study because of its superior field performance.

Generation of F_1 Progeny

Reciprocal crosses were made between SB and NSB at the Seed Testing Nursery (Hubbard, OR) in the summer of 1992. Seeds were germinated on Murashige and Skoog medium (Murashige and Skoog, 1962) in the presence of 0.5 mg/L GA_3. Nineteen plants from the NSB × SB cross and one plant from the SB × NSB cross were obtained. Seedlings were transferred to soil and placed in a growth chamber. F_1 progeny, NSB, and SB were maintained in a growth chamber with a 16-h photoperiod and 22°C day and 16°C night temperatures. The light intensity was 153 μE m^{-2} s^{-1}. NSB, SB, and F_1 progeny were vegetatively propagated by stolons as needed.

Hydroponic Heat Stress

Plants were grown hydroponically in one-half-strength Hoagland solution (Hoagland and Arnon, 1950) in a growth chamber under the same conditions described in the previous section. After acclimation at 22 and 16°C, small plants (three to six leaf blades per plant) were transferred to a temperature-controlled water bath, in another growth chamber with a light intensity of 150 μE m^{-2} s^{-1} and a 12-h photoperiod. Plants were suspended in pressed foam and placed in a glass container containing one-half-strength Hoagland solution. The container was placed in

the water bath and the level of the Hoagland solution was monitored daily. The air and water temperatures were maintained at 40°C. Leaf damage (brown, necrotic regions) was visually assessed every 3 d for 9 d and scored. A damage score of 1 was given when less than one-half of the leaf blade contained necrotic regions. A damage score of 5 was given when more than one-half of the leaf blade contained necrotic regions, but green areas were still apparent. A damage score of 10 was given when the leaf blade was totally necrotic and desiccated. The scores for each leaf blade of the plant were added and divided by the number of leaf blades per plant. Plants with the highest score were the most damaged. This experiment was repeated six times with different sets of plants each time. Statistical analysis was conducted using the SAS-General Linear Model procedure (SAS Institute, Cary, NC).

Radiolabeling of Leaf Segments for SDS-PAGE

Several leaves from each variant were cut into 1-cm segments. Seven randomly selected segments of each variant were pooled and placed in 1 mL of sterile incubation buffer containing 1% (w/v) Suc, 1 mM potassium phosphate buffer, pH 6.0, and 0.02% (v/v) Tween 20 (Lin et al., 1984). No chloramphenicol was added to the incubation buffer. Samples were incubated in a water bath, and proteins were labeled by the addition of Tran^{35}S label (L-Met ^{35}S; L-Cys ^{35}S; >1000 Ci/mmol; ICN). Following labeling leaf segments were homogenized in 300 μL of sample buffer (Laemmli, 1970) containing 1 mM PMSF. Samples were boiled for 5 min and centrifuged at 15,000*g* for 10 min. The amount of Tran^{35}S label incorporated into protein was determined by spotting an aliquot of the supernatant on Whatman 3MM filter paper (2.3 cm diameter), washing once with cold 10% TCA for 15 min and twice for 10 min, and finally washing in acetone for 10 min. The amount of radioactivity was determined by scintillation spectroscopy in a Mark III scintillation counter (Tracor Analytic, Elk Grove, IL). Samples were analyzed by SDS-PAGE (Laemmli, 1970) on gels containing a 10 to 15% (w/v) gradient of acrylamide. Gels were processed for fluorography using Resolution (EM, Chesnut Hill, MA) and the directions of the manufacturer. In all experiments, equivalent numbers of TCA-precipitable counts per minute (5–8 $\times$ 10^4) were placed in each lane of the gel.

Determination of the Temperature and Time Required for HSP Induction

Six leaf segments were placed in 1 mL of incubation buffer that was equilibrated at 25, 30, 34, 36, and 40°C. The samples were incubated at the temperatures indicated for 1.5 h and then were labeled with Tran^{35}S label for 1.5 h at 25°C. Samples were prepared for SDS-PAGE as described above.

2-D Gel Electrophoresis

For 2-D gel electrophoresis twenty 1-cm leaf segments from each variant were pooled and incubated in 1.2 mL of incubation buffer containing 500 μCi of Tran^{35}S label for 1.5 h at control (28°C) or heat-shock (40°C) temperatures. Leaf segments were homogenized in protein isolation buffer containing 0.7 M Suc, 0.5 M Tris-HCl, pH 8.5, 0.05 M Na_2EDTA, 0.1 M KCl, and 2% (v/v) 2-mercaptoethanol and extracted with water-saturated phenol (Hurkman and Tanaka, 1986). Samples were dissolved in modified lysis buffer (O'Farrell, 1975) containing 1% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate in addition to the other components. Samples were then analyzed by 2-D gel electrophoresis (O'Farrell, 1975). Equal amounts of TCA-precipitable counts per minute were placed on each gel. Radioactive polypeptides were visualized by fluorography as described above.

Chloroplast Isolation

Chloroplasts were isolated from fully expanded leaf blades harvested during the light cycle. The chloroplast isolation method was modified from that of Fish and Jagendorf (1982). Leaf blades were cut in 1-cm sections and 0.2 g were labeled in incubation buffer containing 500 μCi of Tran^{35}S label. Labeling was conducted at 40°C for 1.5 h followed by incubation at 25°C for 0.5 h. Leaf segments were rinsed twice with distilled water and homogenized with a mortar and pestle in grinding buffer (Fish and Jagendorf, 1982). All centrifugations were done in the Sorvall HB-4 swinging bucket rotor. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 1,500*g* for 3 min. The pellet was resuspended in 0.5 mL of grinding buffer and was layered on a 10-mL gradient of 10 to 50% Percoll. The Percoll gradient contained the same ingredients as the grinding buffer and was formed over a 2-mL cushion of 3 M sorbitol. The gradient was centrifuged at 13,000*g* for 7 min. The lower green band (between approximately 20 and 30% Percoll) containing intact chloroplasts was collected. This fraction was diluted with 2 volumes of resuspension buffer (Fish and Jagendorf, 1982) and centrifuged at 4,000*g* for 3 min. The pellet was resuspended in 400 μL of resuspension buffer. One portion (200 μL) of the chloroplast suspension was treated with thermolysin (Smeekens et al., 1986) (dissolved in 10 mM $CaCl_2$) at a final concentration of 0.1 μg/mL at 4°C for 30 min. Following thermolysin treatment, the mixture was diluted with 500 μL of resuspension buffer and centrifuged for 5 min at 4,000*g*. The chloroplast pellet was gently resuspended in resuspension buffer and pelleted at 4,000*g* for 5 min. This washing procedure was repeated three times. The remaining sample was not treated with the proteinase. In a separate experiment, the chloroplast suspension was simultaneously treated with thermolysin and 1% (v/v) Nonidet P-40. Chloroplast samples were dissolved in protein isolation buffer and extracted with phenol for 2-D gel analysis as described above. Equivalent amounts of radioactivity (20,000 cpm) were applied to each 2-D gel.

RESULTS

Thermotolerance in SB and NSB

Previous field observations and regrowth experiments (Kemp, 1987) indicated that SB was more heat tolerant than

NSB. To confirm these observations, thermotolerance was measured hydroponically at 40°C over a 9-d period. Damage was assessed by scoring brown necrotic regions on the leaf blades. The results in Table I indicate that NSB incurred more damage, especially after 9 d of continuous stress at 40°C. Figure 2 shows SB and NSB after a growth chamber malfunction that resulted in a temperature increase to 37°C for 24 h. Since both NSB and SB were well watered on the evening prior to the malfunction, it is unlikely that the damage was due to water stress. Following this high-temperature stress, SB appeared relatively undamaged, whereas the leaf blades of NSB were severely withered. At control temperatures, SB cannot be distinguished from NSB.

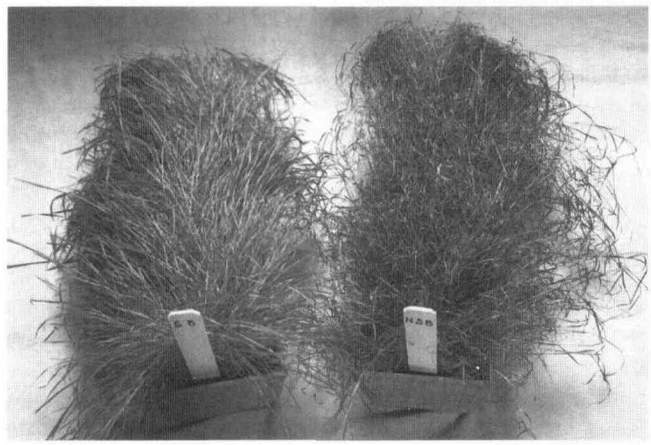

Figure 2. SB (heat tolerant, left) and NSB (nontolerant, right) after exposure to 37°C for 24 h following growth chamber malfunction.

HSPs in SB and NSB

Because it is possible that differences in HSP synthesis might be correlated with the observed presence or absence of heat tolerance, initial experiments were conducted to determine whether there were qualitative or quantitative differences in HSPs synthesized by SB and NSB. Leaf segments were labeled at heat-shock (40°C) or control (28°C) temperatures and the products were analyzed by SDS-PAGE. The major HSPs synthesized by NSB and SB were those with molecular masses of 97, 83, 70, 40, 27, and 18 kD (Fig. 3). Small amounts of HSP97, HSP83, and HSP70 were synthesized at the control temperatures, and their synthesis increased following heat shock at 40°C. The HSPs were not induced by crushing the leaves prior to labeling, but treatment with arsenate increased the synthesis of HSP83, HSP70, HSP27, and HSP18 (data not shown).

Temperature Required to Induce HSP Synthesis

Differences in heat tolerance between SB and NSB may be due to differences in the temperature required to induce HSP synthesis. In both variants the increased synthesis of HSPs 97, 83, and 70 occurred between 30 and 32°C (Fig. 4). These bands were most intense at 36°C for both SB and NSB. At 40°C there appeared to be decreased synthesis of the high-molecular-mass HSPs in SB. Synthesis of HSP40 was detected at 30°C in SB but was not apparent in NSB until 34°C. Synthesis of the HSP27 increased between 30 and 32°C, and synthesis of HSP18 increased between 32 and 34°C for SB and NSB. The maximum level of HSP synthesis occurred at 36 to 40°C for both variants. When leaves were incubated at 45°C almost all protein synthesis was inhibited (data not shown), suggesting that this temperature was lethal.

The temperature shift from 22°C, the temperature of the growth chamber, to 30 to 32°C appeared to be sufficient to induce the low-level synthesis of HSPs. Induction at this lower temperature is not surprising, since HSP induction begins at 10 to 15°C above the optimal growing temperature (Vierling, 1991). The temperature range for HSP induction was similar to that of a number of other plant species (Baszczynski and Walden, 1982; Ougham and Stoddard, 1986; Necchi et al., 1987; Hwang and Zimmerman, 1989; Fender and O'Connell, 1990).

The synthesis of proteins other than HSPs (e.g., 32, 37, and 55 kD) decreased in both variants as the temperature increased. The abundant 55-kD protein is the large subunit of Rubisco. Vierling and Key (1985) have demonstrated that the synthesis of this protein decreases when soybean cells are heat shocked. Synthesis of the 32-kD protein stopped at 36°C in NSB and 40°C in SB. The differences between the two variants in response to increasing temperature were the induction of HSP40 synthesis at lower temperatures in SB and the inhibition of synthesis of the 32-kD normal protein at 36°C in NSB.

There were no differences between SB and NSB in the length of time required to induce HSP synthesis (data not shown). Synthesis of HSP83 and HSP70 began after 5 min at 40°C and was greatest at 30 and 60 min. Synthesis of HSP27 and HSP18 began after 15 min of incubation at 40°C and was greatest at 30 and 60 min. Incubation of leaf blades at 25°C for the same times did not induce the synthesis of HSPs. The induction period of 15 min for HSP18 and HSP27 was similar to that found in corn (Baszczynski and Walden, 1982) and sugar cane cells (Moisyadis and Harrington, 1989).

Table I. *Leaf damage scores[a] of SB and NSB during hydroponic heat stress at 40°C*

Plant	d 3	d 6	d 9
NSB	1.17 ± 0.49	4.00 ± 0.69	7.35 ± 3.06
SB	0.84 ± 0.16	2.09 ± 0.67	1.55 ± 0.53

[a] A damage score of 1 was given when less than one-half of the leaf blade contained necrotic regions. A damage score of 5 was given when more than one-half of the leaf blade contained necrotic regions, but green areas were still apparent. A damage score of 10 was given when the leaf blade was totally necrotic and desiccated. The scores for each leaf blade of the plant were added and divided by the number of leaf blades. The results are the averages of triplicate experiments.

2-D Gel Analysis of Heat-Tolerant Variants

To determine whether there were size or charge variants of HSPs that were not apparent when proteins were ana-

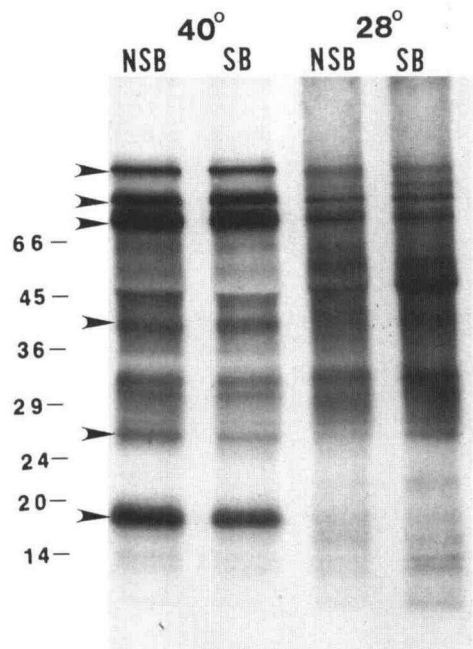

Figure 3. HSP synthesis in SB and NSB. Leaf segments were incubated at heat-shock (40°C) or control (28°C) temperatures for 1.5 h and were labeled in vivo with Tran^{35}S label for 1 h at the same temperature. Equivalent numbers of TCA-precipitable counts per minute were applied to each lane of the SDS-PAGE gel, and bands were visualized by fluorography. Numbers in the left margin are molecular mass markers in kD. Arrowheads mark the major HSPs listed in the text.

lyzed by SDS-PAGE, 2-D gel electrophoresis was conducted. Figure 5 shows the pattern of proteins synthesized at the control and heat-shock temperatures for both variants. Following heat shock there was increased synthesis of the high-molecular-mass HSPs. Several additional variants of HSP70, HSP83, and HSP97 were synthesized during heat shock at 40°C. Because of the large number of polypeptides differing in size and charge in this region of the gel, it was difficult to determine whether there were specific polymorphisms in the high-molecular-mass HSPs synthesized by NSB and SB.

Two sets of low-molecular-mass HSPs, the HSP27 and HSP18 families, were induced by heat shock. In the HSP18 family there were at least 17 polypeptides distributed in the pH range 5.7 to 7.5. Two of the polypeptides with pIs of approximately 6.5 and 6.8 were present in NSB but not in SB.

The major HSP27 proteins were in the acidic region of the gel. These proteins were distributed in the pH range of approximately 5.6 to 5.4. There are two to three major HSP27 polypeptides synthesized by both SB and NSB; however, in SB there were two to three additional polypeptides in this group. The polypeptides in SB were smaller (approximately 25 kD) and slightly more basic than the HSP27 polypeptides. These HSPs were designated HSP25, in contrast to the larger HSP27 group. Since the HSP25 polypeptides were found in the heat-tolerant variant SB, it is possible that they may be correlated with thermotolerance. Several other heat-tolerant variants, derived from Penncross in a different series of selections, were tested to determine whether the additional HSP25 polypeptides were present. These variants were judged to be thermotolerant by increased growth in the field during thermal stress (J.V. Krans, unpublished data). All of the heat-tolerant variants tested synthesized the additional HSP25 polypeptides (data not shown).

Localization of HSP25

It has been shown that some members of the low-molecular-mass HSP25 family are localized in the chloroplast (see review by Vierling, 1991; O'Connell, 1994). To determine whether this was the case for bentgrass, leaves were labeled in vivo under heat-shock conditions, chloroplasts were isolated, and proteins were analyzed on 2-D gels. Figure 6 shows that one to two members of the HSP27 family were localized in the chloroplast in both NSB and SB. In SB it appears that at least one of the additional HSP25 polypeptides was present in the chloroplast. The same pattern of HSP27 and HSP25 was apparent following thermolysin treatment, indicating that the proteins were localized within the chloroplast, not attached to the chloroplast outer membrane (Smeekens et al., 1986). When chloroplasts were simultaneously treated with detergent and thermolysin, no proteins were visible on the gel (data not shown).

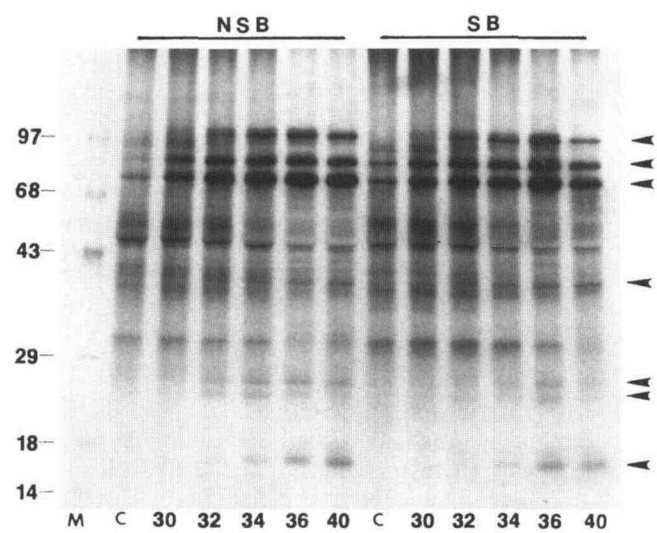

Figure 4. Temperature required for the induction of HSP synthesis in NSB and SB. Leaf segments were incubated at the temperatures indicated at the bottom margin for 1.5 h and then were labeled with Tran^{35}S label for 1.5 h at 25°C. Lane C represents samples incubated at control temperature (25°C) for 1.5 h and then labeled with Trans^{35}S label at 25°C for 1.5 h. Proteins were extracted, separated by SDS-PAGE, and visualized by fluorography. Equivalent numbers of TCA-precipitable counts per minute were placed in each lane. Numbers in the left margin are molecular mass markers in kD; arrowheads in the right margin indicate the molecular masses and positions of the major HSPs. Lane M contains the molecular mass markers.

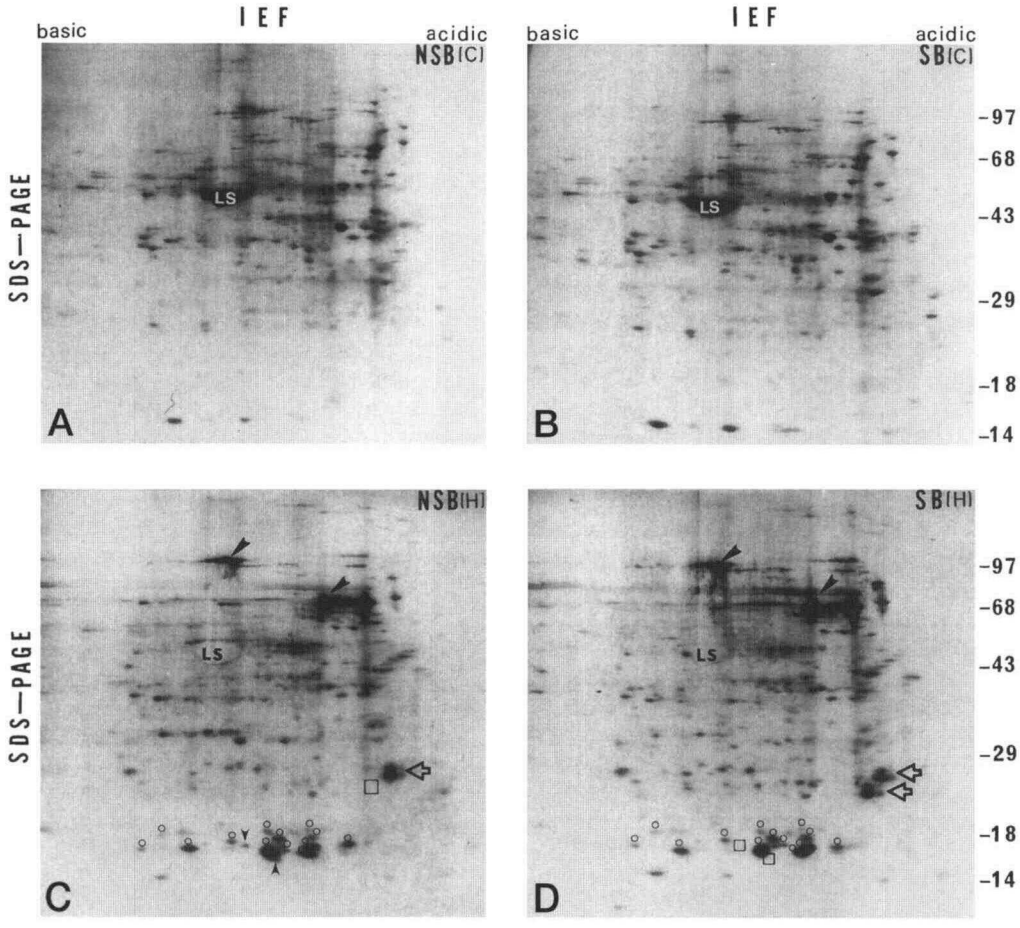

Figure 5. 2-D gel electrophoresis of HSPs and normal proteins synthesized by SB and NSB. Leaf segments were incubated at heat-shock (40°C) or control (25°C) temperatures for 1.5 h and were labeled with Tran^{35}S label for 1.5 h at the same temperature. Equivalent numbers of TCA-precipitable counts per min were applied to each gel. Proteins were visualized by fluorography. A, NSB, 25°C; B, SB, 25°C; C, NSB, 40°C; D, SB, 40°C. Numbers in the right margin are the molecular mass markers in kD. LS refers to the large subunit of Rubisco. C and D, Large, solid arrowheads mark the high-molecular-mass HSPs, and open arrows indicate the HSP27 group. The large open boxes indicate the missing HSP25 polypeptides in NSB. The open circles mark the HSP18 family; the small arrowheads in this region mark the polypeptides that are present in NSB but that are absent in SB; the small open squares indicate the positions of the missing peptides in SB. C and H in the upper right-hand corners refer to control and heat shock, respectively.

Genetic Analysis

To ascertain whether the ability to synthesize the HSP25 polypeptides was associated with thermotolerance, reciprocal crosses were made between SB and NSB. Because of self-incompatibility, the yield and viability of the seed was low. However, 19 F_1 progeny were obtained from NSB × SB, and a single plant was obtained from SB × NSB. Figure 7 shows that 7 (nos. 7, 10, 11, 16, 17, 18, and 19) of the F_1 progeny of NSB × SB did not synthesize the HSP25 polypeptides. The single plant from the SB × NSB (no. 20) synthesized the HSP25 polypeptides, which indicated that the trait was not maternally inherited. The levels of synthesis of the HSP25 polypeptides varied among the progeny, but this could have been due to variation in labeling efficiency. We do not know why the F_1 progeny 10, which did not synthesize the extra HSP25, exhibited relatively high thermotolerance.

If the ability to synthesize the additional HSP25 polypeptides is a homozygous dominant trait, these proteins should be present in all F_1 progeny. However, 13 of the 20 F_1 progeny of NSB × SB and SB × NSB synthesized the HSP25 polypeptides and 7 did not. Because Penncross is a synthetic variety comprising three open-pollinated genotypes (Hanson, 1959), it is highly unlikely that the seed resulting in NSB or SB was a homozygous inbred. Therefore, we have predicted that the synthesis of the HSP25 polypeptides is a heterozygous dominant trait in SB and that NSB is homozygous recessive for this trait. χ^2 analysis ($\chi^2 = 1.8$, $P = 0.18$) supports this hypothesis.

Thermotolerance of F_1 progeny was assessed by hydroponic heat stress at 40°C for 9 d (Table II), which was when the first plant died. Six of the seven F_1 progeny (nos. 7, 16, 17, 11, 18, and 19) that did not synthesize the HSP25 polypeptides were the most severely damaged (i.e. had the

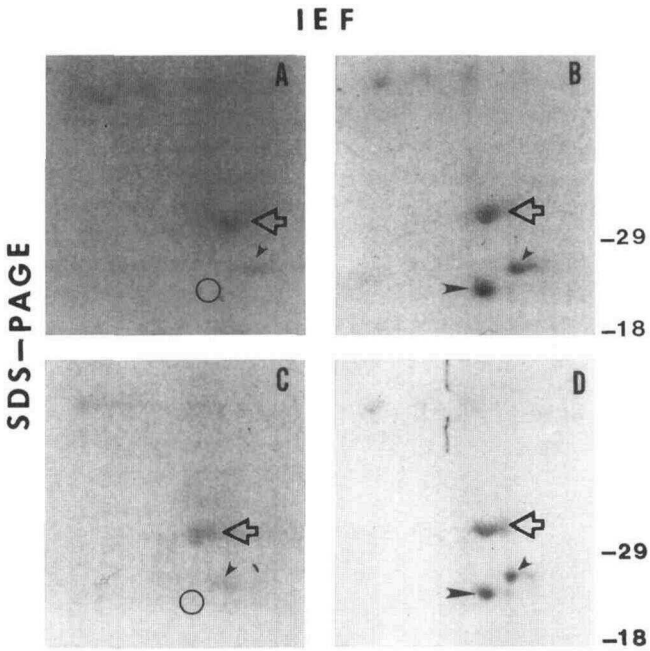

Figure 6. 2-D gel electrophoresis of chloroplast-localized HSP27 and HSP25 synthesized in vivo by SB and NSB. The region of the 2-D gel containing the HSP25 polypeptides has been enlarged. Equivalent numbers of TCA-precipitable counts per min were placed on each gel. Radioactive proteins were visualized by fluorography. Small arrowheads indicate the HSP27 polypeptides found in both NSB and SB. Large arrowheads indicate the HSP25 polypeptides found in SB. Circles indicate absence of the additional HSP25 polypeptides in NSB. Numbers in the right margin indicate the molecular mass of protein standards in kD. A, Chloroplasts isolated from NSB; B, chloroplast isolated from SB; C, chloroplasts isolated from NSB and treated with thermolysin prior to protein isolation; D, chloroplasts isolated from SB and treated with thermolysin prior to protein isolation. The open arrow indicates the position of a 32-kD chloroplast protein that is present at both heat-shock and control temperatures.

highest damage ranking). Analysis of variance (data not shown) indicated that there was a highly significant ($P < 0.0001$) difference in thermotolerance between progeny that synthesized the additional HSP25 polypeptides and those that did not. The *t* test (Table III) also indicated that the difference in thermotolerance between the two classes of F_1 progeny was significant. There was a positive correlation ($r = 0.743$, $P < 0.0002$) between the ability to synthesize the HSP25 polypeptides and thermotolerance. The χ^2 test of independence was used to determine whether there was a relationship between the presence of the HSP25 polypeptides and the level of heat tolerance (Table IV). More F_1 progeny (35) with the HSP25 polypeptides were in the low damage score class than F_1 progeny (5) without the HSP25 polypeptides ($\chi^2 = 22.45$, $P < 0.001$). This indicates that there is a significant relationship between the presence of the extra HSP25s and thermotolerance. There was no apparent relationship between the synthesis of the additional HSP18 polypeptides in NSB and thermotolerance.

DISCUSSION

Since acquired thermotolerance has been positively correlated with the synthesis of HSPs, we initially analyzed NSB and SB for differences in the patterns of HSPs synthesized. We did not compare the pattern of HSP synthesis of the parent cultivar, Penncross, with those of NSB and SB, because Penncross results from the open pollination of three genotypes (Hanson, 1959). Therefore, each seed of Penncross is a different genotype. The advantage of using SB and NSB for this study is their close genetic backgrounds. Like most plants, both SB and NSB synthesized two groups of HSPs, the high-molecular-mass HSP (60–110 kD) and the low-molecular-mass HSP (16–32 kD) (Vierling, 1991). There were no major differences in the time or temperature required to induce HSP synthesis in the two variants.

In addition to the low- and high-molecular-mass HSPs, a 40-kD HSP was apparent when the proteins were analyzed on 1-D gels (SDS-PAGE) (Fig. 3). This 40-kD HSP was not present on the 2-D gel shown in Figure 5, but it was observed on other 2-D gels (data not shown). The reason for its inconsistent expression is unknown. HSPs of approximately 40 kD have been identified in several other species, including wheat (Krishnan et al., 1989; Helm and Abernethy, 1990), barley (Necchi et al., 1987), and carrot (Hwang and Zimmerman, 1989). The molecular mass of these proteins (approximately 40 kD) is similar to that of the bacterial HSP, DnaJ (Zhu et al., 1993). Analogs of DnaJ have been found in *Atriplex* and other plant species (Zhu et al., 1993).

Although differences in HSP synthesis were not evident on 1-D gels, analysis of the HSPs by 2-D gel electrophoresis did reveal two qualitative differences between NSB and SB. In NSB, two additional members of the HSP18 family were synthesized. In SB, two to three additional members of the HSP27 family were synthesized. In preparation for 2-D gel analysis, proteins were isolated from leaf tissue using a phenol extraction procedure, which was developed for the efficient isolation of membrane-associated proteins (Hurkman and Tanaka, 1986). This may account for the visibility of the HSP25 polypeptides on the 2-D but not the 1-D gels. When proteins isolated using the phenol extraction procedure were analyzed on 1-D gels (SDS-PAGE), the additional HSP25 polypeptides were evident (data not shown). Alternatively, when proteins extracted using the SDS-PAGE procedure (Laemmli, 1970) were analyzed on 2-D gels, the additional HSP25 polypeptides were absent. The extraction of the additional HSP25 polypeptides using the phenol method provides indirect evidence for their association with membranes or organelles.

We do not know why these additional HSP25 polypeptides are synthesized in SB and not in NSB. Karyotyping did not provide any evidence for chromosome duplication in SB. It is possible that the genes encoding the additional HSP25 polypeptides are present in NSB but not expressed. A regulatory mutation may have occurred during cell culture that allowed the HSP25 polypeptides to be expressed in SB but not NSB.

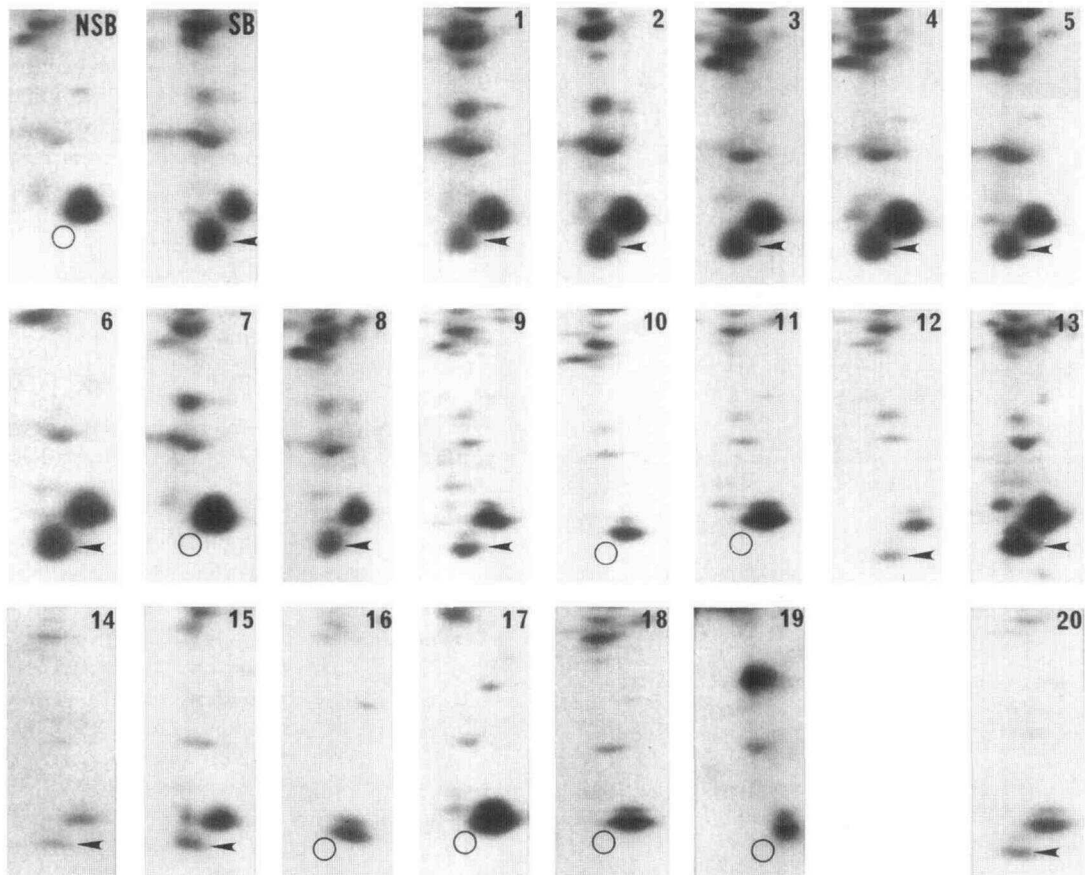

Figure 7. HSP25 polypeptides synthesized by NSB, SB, and F_1 progeny of NSB × SB (progeny 1–19) and SB × NSB (progeny 20). The region of the 2-D gels showing the HSP25 region has been enlarged. Arrowheads indicate the presence and open circles indicate the absence of additional HSP25 polypeptides.

The low-molecular-mass HSPs, including the HSP18 and HSP25 groups, are the most abundant proteins induced by heat stress in higher plants (Vierling, 1991). Sequence analysis of members of these families indicates that they can be classified as four multigene families (Vierling, 1991). Two families encode HSPs localized in the cytoplasm, one family encodes HSPs localized in the chloroplast, and the other encodes HSPs found in the endomembrane regions (Vierling, 1991). Other reports indicate that members of the HSP25 family are found in the chloroplast (Vierling, 1991). It has been shown that they form high-molecular-weight aggregates during heat shock (Clarke and Critchley, 1994; Osteryoung and Vierling, 1994). The data presented here suggest that two of the HSP27 polypeptides found in both SB and NSB and at least one of the additional HSP25 polypeptides found in SB are localized in the chloroplast.

The functions of the low-molecular-mass HSPs in plants are unknown (Vierling, 1991). It has been demonstrated that two cytosolic HSPs from plants (HSP18.1 and HSP17.7) acted as molecular chaperones in vitro (Lee et al., 1995a). In addition, murine HSP25, human HSP27, and bovine α-β-crystallin also act as molecular chaperones and assist in the folding of "model substrates" (Jakob et al., 1993). Because of the abundance of the low-molecular-mass HSPs in plants, it is assumed that they play some role in acquired thermotolerance. The accumulation of low-molecular-mass HSPs in etiolated soybean parallels the acquisition of thermotolerance (Vierling, 1991) and suggests that they may be involved in protection from thermal stress.

Transgenic Arabidopsis expressing chimeric heat-shock factors were insensitive to the negative regulation of HSP gene expression that occurs at normal temperatures (Lee et al., 1995b). This resulted in the constitutive expression (approximately 20% of the maximum heat-inducible level) of HSP18 at the normal temperature. These plants were tolerant to suddenly elevated temperatures (50°C for 1 h) (Lee et al., 1995b). Plants that did not express the chimeric heat-shock factor were killed above 46°C. This provides evidence that expression of HSP18 at the normal temperature improves the basic thermotolerance of the plant.

There have been a number of studies showing qualitative polymorphisms in low-molecular-weight HSPs synthesized by cultivars or variants differing in thermotolerance (Ougham and Stoddard, 1986; Hwang and Zimmerman, 1989; Krishman et al., 1989; O'Connell, 1994). For example, there is an extra member of the HSP25 family present in a thermotolerant cultivar of wheat (Krishnan et al., 1989). However, no genetic studies were conducted to determine whether the presence of the additional HSP26 polypeptide co-segregrated with thermotolerance. Genetic studies in

Table II. *Leaf damage scores[a] of F_1 progeny of NSB × SB (nos. 1–19) and SB × NSB (no. 20) after 9 d of hydroponic heat stress at 40°C*

F_1 Progeny	Extra HSP25	Damage Score[b]
7	−	9.4 A
16	−	9.0 A
17	−	8.3 A
11	−	7.2 A B
18	−	6.7 A B
19	−	5.4 B C D
8	+	5.4 B C D E
14	+	5.4 B C D E
1	+	4.9 B C D E F
6	+	4.9 B C D E F G
3	+	4.8 B C D E F G
2	+	4.5 B C D E F G
5	+	4.4 C D E F G
13	+	4.2 C D E F G
10	−	4.2 C D E F G
9	+	3.3 D E F G
12	+	3.3 D E F G
15	+	2.7 E F G
4	+	2.4 F G
20	+	2.1 G

[a] A damage score of 1 was given when less than one-half of the leaf blade contained necrotic regions. A damage score of 5 was given when more than one-half of the leaf blade contained necrotic regions, but green areas were still apparent. A damage score of 10 was given when the leaf blade was totally necrotic and desiccated. The scores for each leaf blade of the plant were added and divided by the number of leaf blades. [b] Means with the same letter are not significantly different (LSD = 2.74). Statistical analysis was done using Fisher's protected LSD.

other plant species (Fender and O'Connell, 1989, 1990; Marmiroli et al., 1989; Fova and Gorla, 1993; O'Connell, 1994) have not demonstrated a link between HSP polymorphisms and thermotolerance. Frova and Gorla (1993) used restriction fragment length polymorphism analysis to evaluate maize recombinant inbreds from the F_1 hybrid of thermotolerant × thermosensitive inbreds. They found a positive correlation between the quantitative expression of the low-molecular-weight HSP classes and thermotolerance. Qualitative polymorphisms in the low-molecular-weight HSPs have been identified in two corn inbreds, the F_1 hybrid and the F_2 progeny (Jorgensen and Nguyen, 1995). Alleles for six of the low-molecular-weight HSPs assorted in a 3:1 Mendelian ratio in the F_2 progeny. The inheritance of other HSPs may be more complicated. There was no attempt to link thermotolerance with low-molecular-weight HSP polymorphisms in this study (Jorgensen and Nguyen, 1995).

Table III. *Mean heat damage scores for F_1 progeny of NSB × SB and SB × NSB with and without the extra HSP25 polypeptides (LSD = 0.9848)*

Extra HSP25	Mean Damage Score[a]
−	7.17
+	4.02

[a] A damage score of 1 was given when less than one-half of the leaf blade contained necrotic regions. A damage score of 5 was given when more than one-half of the leaf blade contained necrotic regions, but green areas were still apparent. A damage score of 10 was given when the leaf blade was totally necrotic and desiccated. The scores for each leaf blade of the plant were added and divided by the number of leaf blades.

Table IV. *Distribution of damage scores for 120 F_1 progeny with or without the additional HSP25 polypeptides* ($\chi^2 = 22.45$ [$P < 0.001$]).

Heat Tolerance[a]	No. of F_1 Plants	
	+HSP25	−HSP25
High damage	14	24
Intermediate damage	29	13
Low damage	35	5

[a] A damage score of 1 was given when less than one-half of the leaf blade contained necrotic regions. A damage score of 5 was given when more than one-half of the leaf blade contained necrotic regions, but green areas were still apparent. A damage score of 10 was given when the leaf blade was totally necrotic and desiccated. The scores for each leaf blade of the plant were added and divided by the number of leaf blades. The higher the score, the greater the damage. High damage was a score of ≥7.5; intermediate damage was a score of >2.5 ≤ 7.5; low damage was a score of ≤2.5.

In this study we have determined that there is a significant positive correlation between the presence of the additional HSP25 polypeptides and thermotolerance in the F_1 progeny of NSB × SB. The χ^2 test of independence indicates that the presence of the additional HSP25 polypeptides and heat tolerance are linked. However, it seems unlikely that slightly increased amounts of HSPs resulting from the presence of two to three additional HSPs in the already large low-molecular-mass HSP group would account for the increased heat tolerance. There are several possible reasons why the presence of the additional HSP25 polypeptides may be involved with this phenomenon. If the additional HSP25 polypeptides are localized in the chloroplast, it would increase their concentration in the organelle and potentially provide more protection for the photosynthetic apparatus during heat shock. Since the additional HSP25 polypeptides are smaller than the HSP27s, they may have structural differences that enhance their function in vivo regardless of location. Alternatively, the HSP25 polypeptides may be indicative but not causal of increased thermotolerance. Increased thermotolerance in SB may be the result of a regulatory mutation that has altered the heat-shock response pathway. For example, we have determined that SB recovers normal levels of protein synthesis following heat shock about 2 h earlier than NSB. The putative regulatory mutation that results in the expression of the additional HSP25 polypeptides may also alter recovery from heat shock and other biochemical or physiological processes involved in the response to high-temperature stress.

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