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Heat Shock Protein Synthesis and Thermal Tolerance in Wheat[1]

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ABSTRACT

Plants respond to high temperature stress by the synthesis of an assortment of heat shock proteins that have been correlated with an acquired thermal tolerance to otherwise lethal temperatures. This study was conducted to determine whether genotypic differences in acquired thermal tolerance were associated with changes in the pattern of heat shock protein synthesis. The pattern of heat shock protein synthesis was analyzed by ^{35}S-methionine incorporation in wheat (*Triticum aestivum* L.) varieties exhibiting distinct levels of acquired thermal tolerance. Significant quantitative differences between the cultivars Mustang and Sturdy were observed in the HSP exhibiting apparent molecular weights of 16, 17, 22, 26, 33, and 42 Kilodaltons. Genotypic differences in the synthesis of the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase were observed at 34°C. Two-dimensional electrophoretic analysis revealed unique proteins (16, 17, and 26 kilodaltons) in the thermal tolerant variety Mustang that were absent in the more thermal sensitive variety Sturdy. These results provide a correlation between the synthesis of specific low molecular weight heat shock proteins and the degree of thermal tolerance expressed following exposure to elevated temperatures.

Plants respond to high temperature stress by synthesizing an assortment of proteins, termed HSP,[3] which are usually undetectable at optimal growing temperatures (12, 22). These stress proteins can be arbitrarily divided into different size groups. A high mol wt group ranges from 68 to 110 kD and is ubiquitous among all organisms studied to date. A low mol wt group ranges from 15 to 27 kD and is most prominent in higher plants.

Although the kinetics of HSP synthesis, their electrophoretic profiles, and intracellular localization have been characterized in several crop plants, particularly soybean (1, 10, 11), corn (2, 7, 8), and tomato (18, 19), little is known about the role of these stress proteins in cellular thermal tolerance in plants.

Several studies involving mammalian, insect, bacteria, and yeast cells have shown a positive correlation between the HSP synthesis and the development of thermal tolerance or thermal protection (17). Thermal protection is defined as the ability of organisms to tolerate normally lethal high temperatures after an initial exposure to a sublethal, but elevated HSP-inducing temperature. The development of thermal tolerance in a crop cultivar was best demonstrated in "Wayne" soybean seedlings by Lin *et al.* (16). This report shows that the growth of seedlings was protected during a subsequent incubation at 45°C for 2 h (normally a lethal stress) after a heat shock at 40°C for 1 to 2 h. This has lead to the suggestions that HSP provide a foundation for thermal protection, which is an important component of overall cellular thermal tolerance.

Based on the reports that HSP accumulate in field grown, heat stressed plants (5, 13), we were interested in determining the role of heat shock genes in heritable thermal tolerance and the potential use of HSP as a biochemical selection criterion in improving plant germplasm for hot environments. Our initial investigations focus on the comparative analysis of HSP synthesis in cultivars exhibiting differential thermal tolerance. Significant genetic variability in cellular thermal tolerance in hexaploid winter wheat has been identified using TTC reduction as a viability test (6). Analysis of HSP synthesis using one-dimensional SDS-PAGE established quantitative differences in the leaves of a heat tolerance and a heat susceptible wheat cultivar (14). Genetic diversity in HSP synthesis and its relationship to the genetic diversity in thermal tolerance of wheat leaves was investigated in this study by the use of two-dimensional gel electrophoretic techniques in an attempt to better identify quantitative and qualitative genetic differences in HSP synthesis associated with differences in thermal tolerance.

MATERIALS AND METHODS

Plant Materials

Two winter wheat (*Triticum aestivum* L.) cultivars, Mustang and Sturdy, were used because of the previously identified genetic differences in cellular thermal tolerance (14). Seeds were planted in moist vermiculite in plastic trays and germinated at 22°C in the light. Green leaf tissue was used

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[3] Abbreviations: HSP, heat shock protein(s); TTC, 2,3,5-triphenyltetrazolium chloride.

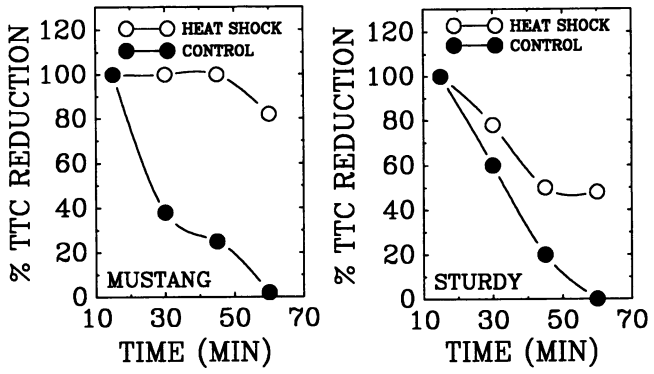

Figure 1. Time course of changes in TTC reduction of control and heat shocked wheat leaves exposed to a 50°C temperature for 1 h

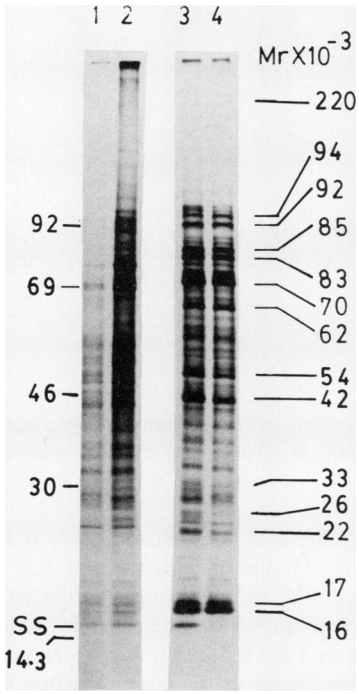

Figure 2. Fluorograph of the proteins synthesized by green leaf tissue at control (22°C) and heat shock (34°C) temperatures. The leaf samples were preincubated at 22°C (control) and 34°C (heat shock) for 1 h and labeled using [^{35}S]methionine for an additional hour at the respective temperature. The apparent mol wt of standards are shown on the left and the apparent mol wt of the HSP are shown on the right. Lane 1, Mustang control; lane 2, Sturdy control; lane 3, Mustang heat shocked at 34°C; lane 4, Sturdy heat shocked at 34°C.

exclusively in our experiments. Seedlings were heat shocked at the two leaf stage at 34 and 37°C.

Cell Viability Assay

The reduction of TTC by leaf tissues was measured as described by Chen *et al.* (6). Leaf segments were transferred to test tubes containing 3 mL of 0.8% (w/v) TTC in 50 mM sodium phosphate buffer (pH 7.4). The samples were vacuum infiltrated to ensure solution penetration into the tissue. Samples were incubated for 18 to 20 h in the dark. The leaf

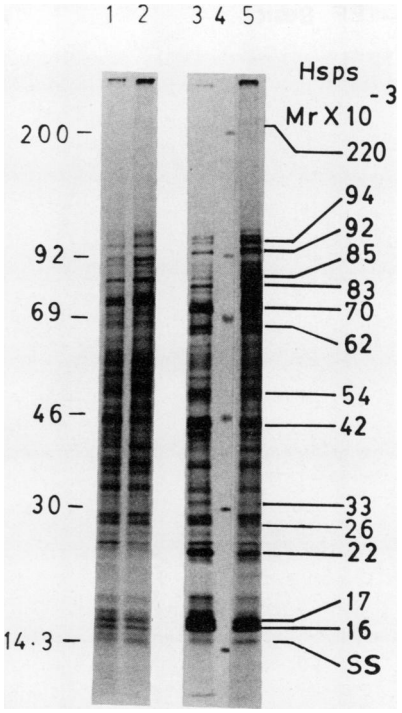

Figure 3. Fluorograph of the proteins synthesized at control (22°C) and heat shocked (37°C) temperatures in green leaf tissue. The leaf samples were heat shocked at 37°C for 1 h and labeled using [^{35}S]-methionine for another 1 h at the respective temperatures. Lane 1, Mustang control; lane 2, Sturdy control; lane 3, Mustang heat shocked at 37°C; lane 4, ^{14}C-labeled mol wt standards (Amersham); lane 5, Sturdy heat shocked at 37°C.

segments were removed after incubation, rinsed with distilled water and placed in a test tube containing 3 mL of 95% ethanol. The samples were boiled to dryness and resuspended in 3 mL of 95% ethanol. Absorbance was read at 485 nm.

In Vivo Labeling and Protein Extraction

Leaf segments (25 mg) were placed in 8 × 75 mm glass tubes containing 100 μL of 20 mM Tris-HCl buffer (pH 7.5) and 50 μg chloramphenicol/mL. The samples were placed in a waterbath shaker for 1 h at either control (22°C) or heat shock temperatures. Following this preincubation, 100 μCi of [^{35}S]methionine (>1000 Ci mmol^{-1}; Trans S-35 label, ICN) was added, vacuum infiltrated for about 1 min, and incubated for an additional hour. Leaf segments were washed with 1 mM methionine (nonradioactive) and the proteins were extracted as described by Damerval *et al.* (9).

SDS-PAGE and Two-Dimensional Gel Electrophoresis

One-dimensional gel electrophoresis was performed as described by Laemmli (15) using a 7.5 to 12.5% linear acrylamide gradient gel. Two-dimensional gel electrophoresis was performed as described by Damerval *et al.* (9). The isoelectric focusing gels were 14 cm long and the gel mixture contained 3.78% (w/v) acrylamide, 0.22% (w/v) *N,N'*,-methylenebis-acrylamide, 9.2 M urea, 3% (v/v) Triton X-100, 4% (v/v) carrier ampholytes consisting of a mixture of 4/5 Pharmalyte

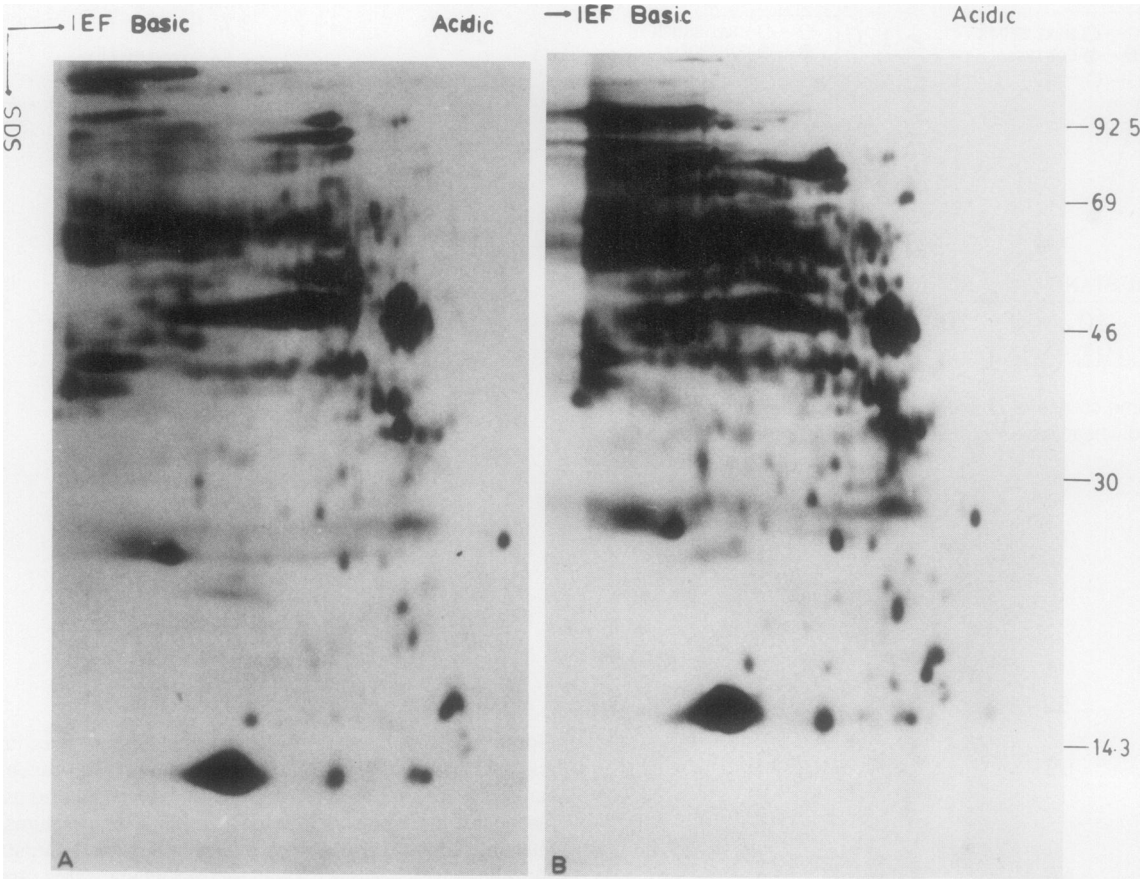

Figure 4. Two-dimensional separation of total proteins from the green leaf tissue. Total proteins extracted from the green leaf tissue labeled at 22°C using [^{35}S]methionine were resolved by two-dimensional IEF/SDS-PAGE. A, Mustang; B, Sturdy. The samples were loaded with equal number of counts.

(pH 5–7), 1/10 Pharmalyte (pH 3–10), and 1/10 Servalyte (pH 3–10). The sample was solubilized in 9.5 M urea, 1.25% (w/v) SDS, 0.5% (w/v) dithiothreitol, 2% (v/v) Ampholytes consisting of a mixture of 1.8% (v/v) Pharmalyte (pH 5–7), 0.1% (v/v) Pharmalyte (pH 3–10), 0.1% (v/v) Servalyte (pH 3–10), 6% (v/v) Triton X-100. Samples for fluorographic analysis were loaded on the basis of an equal number of counts, and samples for protein staining contained 60 μg of protein in a 20 μL volume. Samples routinely had 0.5 to 1.0 $\times 10^6$ cpm/100 μg of protein for IEF gels and 1.0×10^5 cpm/60 μg of protein for one-dimensional gels. IEF was performed for 8000 Vh with 0.1 M NaOH as the cathodic solution and 0.1 M phosphoric acid as the anodic solution as described by O'Farrell (20). The gels were equilibrated for 15 min in 2.3% (w/v) SDS, 10% (v/v) glycerol, 62.5 mM Tris-HCl (pH 6.8), and 0.1% (v/v) 2-mercaptoethanol. Slab gels (1 mm thick) contained 11% (w/v) Acrylamide, 0.29% (w/v) *N,N'*-methylenebiscarylamide, 0.2% (w/v) SDS, 0.375 M Tris-HCl (pH 8.8). Fluorography was carried out with Kodak X-Omat AR5 film at −90°C for 24 h as described by Skinner and Griswold (23). Silver staining was done as described by Blum *et al.* (4).

RESULTS

Genotypic differences in cell viability were observed between the cultivars Mustang and Sturdy upon exposure to a lethal temperature of 50°C, following a 17 h 37°C heat shock treatment (Fig. 1). Leaf segments that had not been heat shocked prior to the 50°C treatment exhibited a rapid decline in cell viability as monitored by the TTC reduction assay. A complete loss in TTC reduction was observed within 60 min of the 50°C treatment in the control tissue, while reduction levels of 80 and 50% were retained in the leaf segments of the heat shocked Mustang and Sturdy, respectively. Genotypic differences in cell viability between Mustang and Sturdy over a time course at 37°C were reported earlier (14). Our results indicated that there is a positive correlation between cell survival at the HSP-inducing temperature and the acquisition of thermal tolerance.

Wheat leaf tissue exhibited an alteration in the pattern of protein synthesis when heat shocked either at 34°C (Fig. 2) or 37°C for 2 h (Fig. 3). The appearance of 13 HSP in the wheat leaf tissue were observed by one-dimensional SDS-PAGE analysis after a heat shock of 34°C for 2 h. Significant quantitative differences between the cultivars Mustang and Sturdy were observed in the HSPs 22, 26, 33, and 42 (Fig. 2). The synthesis of the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) was reduced at 34°C in the cultivar Sturdy compared to Mustang. No significant differences were observed in the polypeptide pattern of the high mol wt HSP at 34°C. Many of the differences in the synthesis

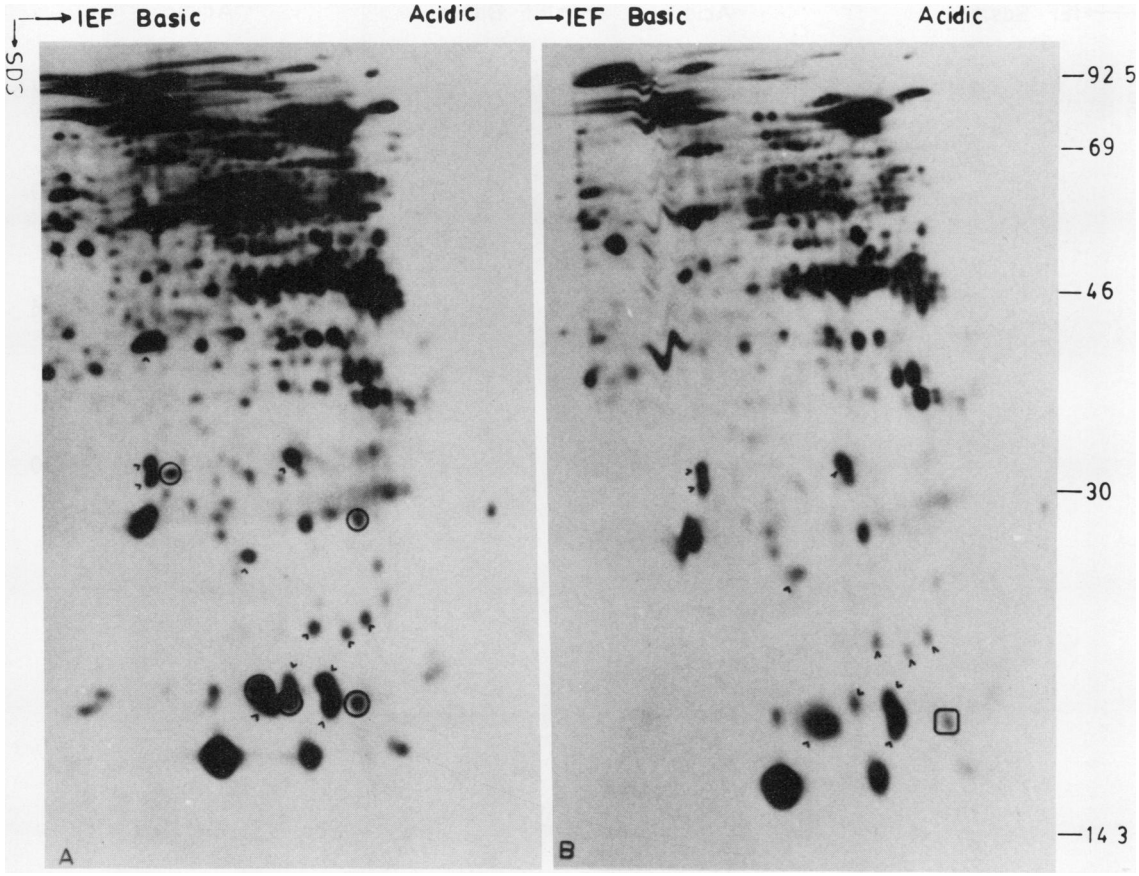

Figure 5. Two-dimensional separation of total leaf proteins from the green leaf tissue heat shocked at 34°C for 2 h. A, Mustang; B, Sturdy. Arrows indicate the HSP. HSP in the circles are unique to Mustang and in the square unique to Sturdy.

of HSP between Mustang and Sturdy became more apparent when the leaves were heat shocked at 37°C (Fig. 3). Quantitative differences were observed in the synthesis of HSP 22 and more subtle differences detected in the synthesis of major HSP 16 and 17. Increased synthesis of HSP 33, 42, and 62 was observed in the heat tolerant Mustang. Several high molecular wt HSP (83, 85, 92, 94 kD) were synthesized at elevated levels in the heat susceptible variety Sturdy. The small subunit of Rubisco which showed a differential synthesis at 34°C, showed no significant differences in synthesis at 37°C. Although the synthesis of several normal proteins were inhibited by the heat shock treatments, the synthesis of some normal proteins was still observed both at 34 and 37°C.

The two-dimensional IEF-SDS-PAGE electrophoretic analysis of total leaf proteins from the control (Fig. 4, A and B) and 34°C heat shocked leaf tissue (Fig. 5, A and B) revealed substantial qualitative and quantitative differences in HSP synthesis between Mustang and Sturdy. At least 16 low mol wt HSP were visible in the fluorograms. Mustang (Fig. 5A) showed three unique protein spots (circles) in the 16 to 17 kD range, one in the 26 kD range, and one at 33 kD. The cultivar Sturdy on the other hand (Fig. 5B) had one unique spot in the 16 to 17 kD range (square). Quantitative differences were observed for three HSP in the 22 kD range with a higher level of synthesis in Mustang. Similar differences in the synthesis of low mol wt HSP were also observed in two-dimensional gel analysis when the leaf tissue from Mustang and Sturdy were heat shocked at 37°C (Fig. 6, A and B). At this temperature, Mustang had two unique spots in the 16 to 17 kD range and one unique spot in the 26 kD range (circules). Quantitative differences were also detected between the two cultivars in the 33 kD. Heat shock proteins accumulated to detectable levels using silver staining after a 40°C treatment for 5 h (Fig. 7, A and B). As many as 9 HSP in the low mol wt region alone were detected by silver staining in Mustang cultivar.

DISCUSSION

The ability of crop plants to adapt to heat stress is an important component of tolerance to temperature stress. TTC reduction has been used as a viability assay for many years (3) and represents a simple, rapid means of assessing viability when dealing with large numbers of samples. Steponkus (24) has concluded that the TTC procedure gives a reasonably accurate estimate of viability for leaf discs, stem sections, and tissue cultures. The results in our cell viability studies indicate that genetic differences in the ability to induce a cellular acquired thermal tolerance mechanism exist between the wheat cultivars Mustang and Sturdy (Fig. 1). The differences in TTC reduction observed in our study are similar to the differences previously reported by Chen *et al.* (6) for genetic diversity in the thermal tolerance of beans and tomatoes.

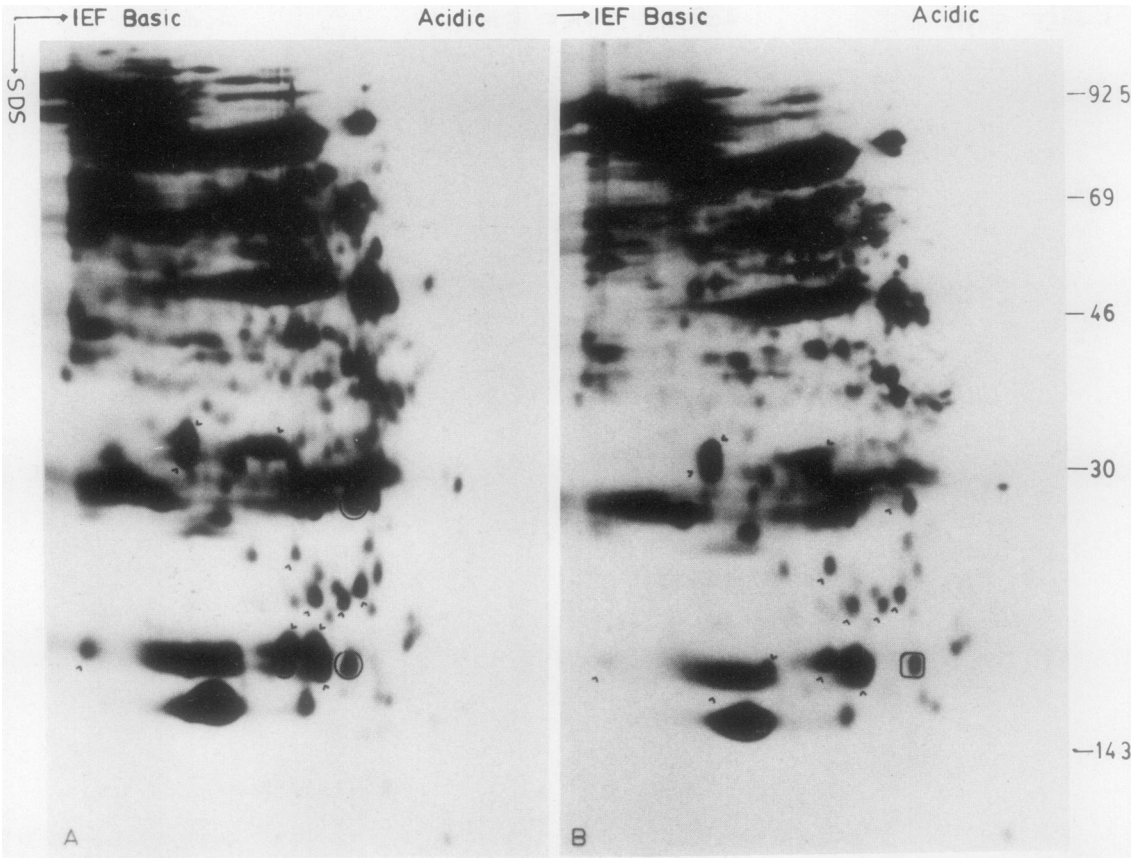

Figure 6. Two-dimensional separation of total leaf proteins from the green leaf tissue, heat shocked at 37°C for 2 h. A, Mustang; B, Sturdy.

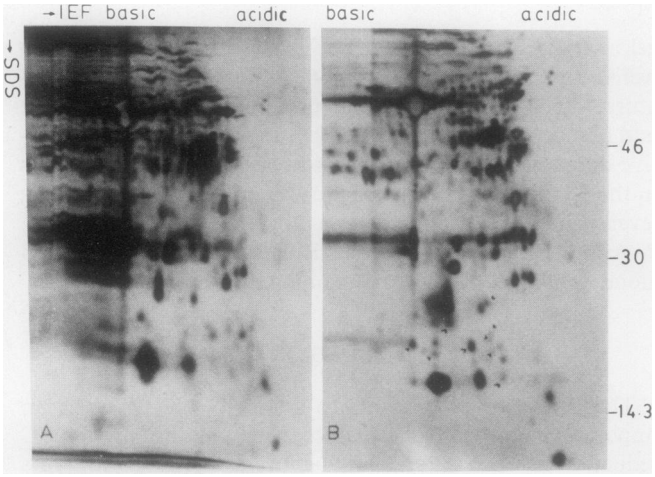

Figure 7. Two-dimensional separation of total proteins from the green leaf tissue heat shocked at 40°C for 5 h. The gel was visualized by silver staining. The arrows indicate HSP. A, Mustang control; B, Mustang heat shocked at 40°C for 5 h.

Concomitant with the difference in the development of thermal tolerance, the two winter wheat lines showed substantial genetic variation in the synthesis of HSP. Elevated synthesis of HSP 22 in the heat tolerant cultivar Mustang was observed at 34°C. Substantial difference in the synthesis of HSP 16 and 17 also was observed at 37°C. Two-dimensional analysis of the total leaf proteins indicated that not only the quantitative changes but also the qualitative changes may influence the difference in the levels of thermal tolerance between Mustang and Sturdy. Genotypic differences in the level of HSP synthesis was reported in grain sorghum (21), and these differences were related to heat tolerance in some lines during germination. It was suggested that the genetic differences in high temperature susceptibility may be correlated with variations in the temporal development of the capacity to synthesize HSP and acquire thermal tolerance (21). Another interesting observation made during the HSP analysis in Mustang and Sturdy was the higher level of synthesis of small subunit of Rubisco in Mustang compared to Sturdy at 34°C (Fig. 2). The more sensitive variety again showed a reduction in small subunit synthesis. These observations support the earlier report of reduced mRNA for the small subunit of Rubisco from dryland soybeans (13). In that study, however, the reduction in small subunit could have been associated with either water stress or thermal stress. In the present study, the reduction is in response to the thermal stress. The sensitivity of small subunit mRNA to heat shock suggests that decreased synthesis of chloroplast proteins produced in the cytoplasm may be an important causal factor of heat damage to plants (25). At 37°C, the synthesis of the small subunit of Rubisco was found to be similar in both the Mustang and Sturdy cultivars (Fig. 3). Although the mechanism for this response is not known, it may be that sufficient

cellular protection had occurred during the first hour at 37°C in the absence of radioactive label to overcome the inhibition of small subunit synthesis. This may be related to previous reports of increased HSP mRNA and heat shock protein synthesis at 37°C compared to 34°C (13).

The results with Mustang and Sturdy suggest that the level of synthesis of low mol wt HSP is positively correlated to genetic difference in thermal tolerance. The data do not allow a conclusion on cause and effect relationship, but support the hypothesis that low mol wt HSP have an adaptive role in thermal tolerance. Further research using a physiological genetic approach will help elucidate the cause and effect relationship. We are currently characterizing the genetic variability in specific HSP synthesis in a wide range of wheat germplasm and intend to develop isopopulations and near-isogenic lines selected for low and high levels of synthesis of small HSP (HSP 16-17 and HSP 22).

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