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Differences in the heat-shock response between thermotolerant and thermosusceptible cultivars of hexaploid wheat

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Summary. Heat-shock protein (HSP) gene expression in two wheat lines cv 'Mustang' (heat-tolerant) and cv 'Sturdy' (heat-susceptible) were analyzed to determine if wheat genotypes differing in heat tolerance also differ in in-vitro HSP synthesis (translatable HSP mRNAs) and steady-state levels of HSP mRNA. Several sets of mRNA were isolated from seedling leaf tissues which had been heat-stressed at 37 °C for various time intervals. These mRNAs were hybridized with HSP cDNA or genomic DNA probes (HSP17, 26, 70, 98, and ubiquitin). Protein profiles were compared using in-vitro translation and 2-D gels. The Northern slot-blot data from the heat-stress treatment provide evidence that the heat-tolerant cv 'Mustang' synthesized low molecular weight (LMW) HSP mRNA earlier during exposure to heat shock and at a higher level than did the heat-susceptible cv 'Sturdy'. This was especially true for the chloroplast-localized HSP. The protein profiles shown by 2-D gel analysis revealed that there were not only quantitative differences of individual HSPs between the two wheat lines, but also some unique HSPs which were only found in the 'Mustang' HSP profiles. The high level of RFLP between the two wheat lines was revealed by Southern blot hybridization utilizing a HSP17 probe. These data provide a molecular basis for further genetic analysis of the role of HSP genes in thermal tolerance in wheat.

Key words: *Triticum aestivum* – Genetic differences – Heat-shock proteins – Heat-shock response – DNA polymorphism

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Introduction

Heat-shock protein (HSP) synthesis is a common response to heat stress in animal, microbe, and plant systems (Lindquist and Craig 1988; Vierling 1991). The hypothesis that HSPs contribute to thermal tolerance has been widely accepted by many investigators (Nover 1991). In comparison with other organisms, relatively limited knowledge of HSP function is available for higher plants because of several factors: (1) the HSPs are the product of multigene families and some cultivated crops are polyploid; therefore, recessive mutations will be difficult to isolate and mutation in one gene may be compensated for by other HSP genes within the family; and (2) there is a lack of effective transformation systems, especially in cereal species, for direct experimentation on HSP function. Therefore, utilization of natural genetic variation should be a useful approach in the study of HSP genes in cereal plants.

Genetic variability in the synthesis of HSPs in plants has been reported in wheat (Zivy 1987; Vierling and Nguyen 1990), sorghum (Ougham and Stoddart 1986), and maize (Frova et al. 1988). These results provide an avenue for the genetic analysis of HSP genes in plants.

Our previous work on in-vivo labelling of wheat HSPs demonstrated a positive correlation between genetic differences in cellular thermotolerance and the levels of LMW HSPs (Krishnan et al. 1989). Our objective in the present study was to provide molecular evidence of genetic variation in HSP synthesis in hexaploid wheat (*Triticum aestivum*. L.). To achieve this, heat-shock gene expression during a heat-stress time course in two wheat lines previously characterized as heat-tolerant and heat-susceptible was examined at the level of steady-state mRNA and in-vitro translatable HSP mRNA. Additionally the genetic difference between these two lines was exam-

ined by Southern blot hybridization with a HSP cDNA probe.

Materials and methods

Plant materials and heat stress conditions

Two hexaploid wheat genotypes cv 'Mustang' (tolerant) and cv 'Sturdy' (susceptible) were used, because of their previously identified genetic differences in cellular thermal tolerance and HSP synthesis (Krishnan et al. 1989). Seeds were planted in pots containing vermiculite and grown in a controlled environment chamber (Conviron Model E-15) which provided 600 μmols m^{-2} s^{-1} PPFD (photosynthetic photon flux density) during a 22/18 °C day/night regime with a photoperiod of 16 h as a normal growth condition. Relative humidity (RH) was 30% of saturation. Seven-day old green seedlings were used in characterizing the HSP profile after seedlings were exposed to a temperature treatment for different time periods. For heat stress, whole pots of seedlings were transferred into another controlled incubator with 100% RH and at a preset temperature of 38 °C for 15, 30, 60, and 120 min. High humidity was maintained in order to limit transpirational cooling of the leaves. Leaf temperatures reached 37 °C under these stress conditions, as monitored by an infrared thermometer. After heat shock, leaf tissue was excised, immediately frozen in liquid N_2, and held at −80 °C for poly(A)$^+$RNA isolation. Leaf tissues of seedlings from the normal growth condition were also collected as control samples.

Poly(A)$^+$RNA isolation

Total RNA was isolated from each sample of 8–10 g fresh weight of leaf tissue and poly(A)$^+$RNA was purified using oligo (dT)-cellulose (New England BioLabs, Beverly, Mass.) chromatography (Jacobsen 1987). Buffers were the same as those previously described except that LiCl was substituted for NaCl and the wash buffer contained 0.05% SDS (w:v).

In-vitro translation and 2-D gel electrophoresis

In-vitro translation of poly (A)$^+$RNA was carried out using a rabbit reticulocyte lysate translation system (Promega, Madison, WIS.) in accordance with the manufacturer's instruction. Translated peptides were labelled by supplementing each reaction with 2.0 MBq of ^{35}S-methionine (ICN, Costa Mesa, Calif.). The protein products were separated using 2-D gel electrophoresis (Damerval et al. 1986). In isoelectric-focusing gels, the mixture of ampholytes was 80%(v/v) Servalyt pH 5–7, 20%(v/v) Servalyt pH 3–10. The IEF dimension effectively separated peptides of pI range about 3.5 to 8.0. The acrylamide concentration in the second dimension gel was 12%(w/v). Samples for fluorographic analysis were loaded on the basis of an equal number of counts, usually 20 kBq of labelled peptides for IEF gels, and visualized by fluorography according to Krishnan and Nguyen (1990). Kodak XAR-5 film was used for fluorography at −80 °C.

Northern slot-blot analysis

Northern slot-blot analyses were conducted by hybridization with different HSP gene probes. For Northern slot blots, the minimum amount of detectable poly(A)$^+$RNA was 0.05 μg/slot under our experimental conditions. For best results, an equal amount of poly(A)$^+$RNA (0.25 μg/slot) was used in our experiments and transferred onto a Zeta-Probe nylon membrane (Bio-Rad, Richmond, Calif.) using a Bio-Dot SF microfiltration unit (Bio-Rad) with 10 × SSPE (1.8 M NaCl/100 mM NaH_2PO_4/10 mM EDTA, pH 7.4). The DNA fragments of the gene-coding region were isolated from a wheat HSP16.9 cDNA clone, pWHSP16.9 (McElwain and Spiker 1989), three maize HSP genes [HSP26 cDNA clone, pMHSP26 (Nieto-Sotelo et al. 1990); HSP70 genomic clone, pMON9502 (Rochester et al. 1986); and HSP98 cDNA clone, pMHSP98 (kindly provided by Dr. Tuan-Hua David Ho, Washington University, St. Louis)], and a chicken ubiquitin cDNA clone, pUB1.6 (Agell et al. 1988). These fragments were used to make ^{32}P-labelled probes by random primer DNA labellig (Sambrook et al. 1989). Northern slot-blot hybridizations were performed according to the protocols of Sambrook et al. (1989), except that, for the heterologous probes from maize and chicken, the stringency of washing condition (i.e., twice with 2 × SSPE and twice with 1 × SSPE, each time at 50 °C for 20 min) was reduced.

DNA isolation and southern blot analysis

Genomic DNA was extracted from leaf tissue of 7-day old green wheat seedlings as described by Sambrook et al. (1989). Southern blot analysis was conducted by hybridization with a wheat HSP16.9 cDNA clone as probe (McElwain and Spiker 1989). For Southern blots, an equal amount of genomic DNA (10 μg/sample) was digested with *Bam*HI, *Eco*RI, and *Hin*dIII, respectively; then separated on an 0.8% agarose gel by electrophoresis and transferred onto a Zeta-Probe nylon membrane (Bio-Rad) utilizing a VacuGene vacuum blotting system (Pharmacia LKB, Piscataway, N.J.) with alkaline solution (0.4 M Na OH/1 M NaCl) in accordance with the manufacturer's instruction. The blot was hybridized with a random primer ^{32}P-labelled probe and carried out as described above under the Northern slot-blot procedure.

HSP identification and hybridization signal intensity quantitation

All experiments were replicated at least twice and the data obtained were consistent in each experiment. HSPs were identified by overlaying HSP gels on control gels. HSPs were defined as protein spots present on HSP gels that were not detectable on control gels. The hybridization signal intensities of mRNA blots were quantified and analyzed on a Bioimage Visage 2,000 densitometer (Kodak, Rochester, N.Y.) according to 1-D analysis procedure.

Results

Heat-shock response of wheat seedlings during heat stress

Analysis of the in-vitro translation products separated on 2-D gels revealed that there are qualitative and quantitative HSP differences between 'Sturdy' and 'Mustang' at all time points of the HS time course (data illustrated in Fig. 1, 2-D profile of HS for 30 min). The data indicate that all four major classes of HSP genes in wheat, HSP15-30, HSP60, HSP70, and HSP90-110, were expressed within 15 min of heat shock at 37 °C and also indicate that 'Mustang' not only has some stronger HSP signals than 'Sturdy', but also has some individual HSP spots, five (nos. 1–5) in the 15–18 kDa range and four (nos. 9–12) in the 33–40 kDa range, which were not found in 'Sturdy'. After 30 min of HS treatment, the data indicate that the translatable mRNA species were continually increased and accumulated, especially in the 30 kDa and 70 kDa range. Furthermore, one new spot

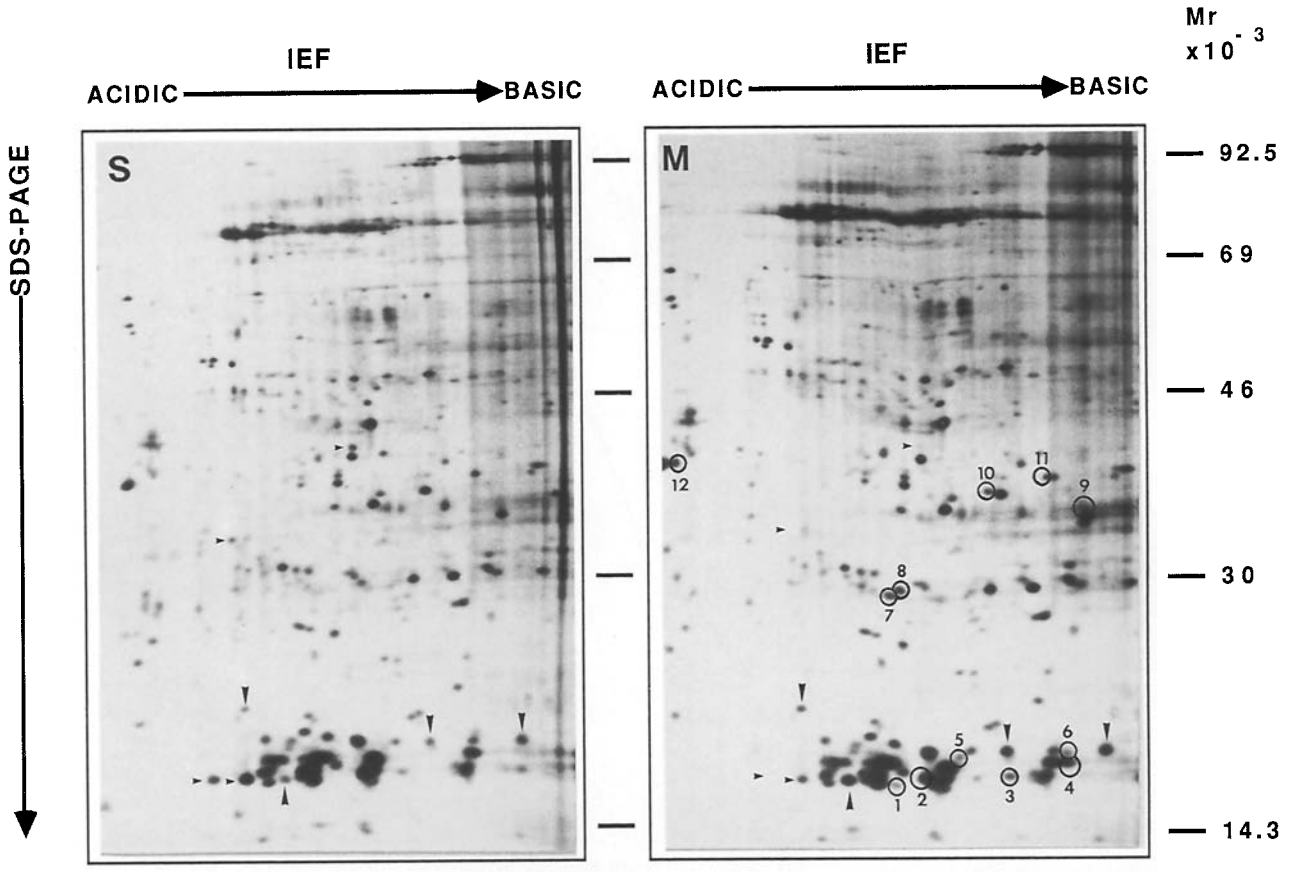

Fig. 1. Comparison of the HS response during heat stress between the heat-susceptible line 'Sturdy' (S) and heat-tolerant line 'Mustang' (M) by two-dimensional gel analysis. Figures exemplified correspond to 30 min in heat stress at 37 °C. For each sample, equal Bq of RNA-dependent incorporation were electrophoresed and equal exposure times of autoradiogramms were performed. Translation products whose quantitative levels changed consistently across all replicate experiments are indicated by *arrows; vertical direction of arrow* shows that the signals of HSP spots in M are stronger than S while *arrows in horizontal direction* indicate that signals of HSP spots in S are stronger than M. Qualitative difference of unique HSP spots are *circled and numbered*

Table 1. Unique heat shock proteins (HSPs) found in 'Mustang' winter wheat seedlings during exposure to heat stress for the indicated time periods. Individual HSP nos. correspond to those circled in Fig. 1

HSP groups	Individual HSP no.	Time of heat shock treatment (min)[a]				
		0	15	30	60	120
16–18 kDa	1	–	±	+	+	+
	2	–	++	+++	+++	+++
	3	–	±	+	+	+
	4	–	+	++	++	++
	5	–	±	+	+	+
	6	–	–	+	–	–
26–30 kDa	7	–	–	++	+	+
	8	–	–	++	+	+
33–40 kDa	9	–	++	++	++	++
	10	–	+	+	–	–
	11	–	+	+	+	+
	12	–	+	+	+	+

[a] Heat shock treatments were performed at 37 °C with high humidity; no signal, –; signal strength, +++ > ++ > + > ±

(no. 6) appeared in the 16–18 kDa range and another two (nos. 7 and 8) in the 26–30 kDa range of 'Mustang' but not in 'Sturdy' (Fig. 1). After HS treatment for 60 min, the products of HSP-gene expression were intensified in the area of LMW HSP, especially in the 16–18 kDa and 30–34 kDa range. Meanwhile, one unique spot (no. 6) in the 16–18 kDa and another unique spot (no. 10) in the 33–40 kDa disappeared. The profile of HS treatment for 120 min had the same pattern as the HS treatment for 60 min, but in the area of high molecular weight (HMW) HSP, 'Mustang' had much stronger signals than 'Sturdy'. The change of unique proteins which was found in 'Mustang' but not in 'Sturdy' under heat stress during the time course is summarized in Table 1.

Northern slot-blot analysis of poly(A)$^+$RNA

Further comparison of the expression of HS genes of these two cultivars utilized different HSP genes as probes to hybridize with poly(A)$^+$RNA obtained from both control and heat-stressed samples. Due to the high ho-

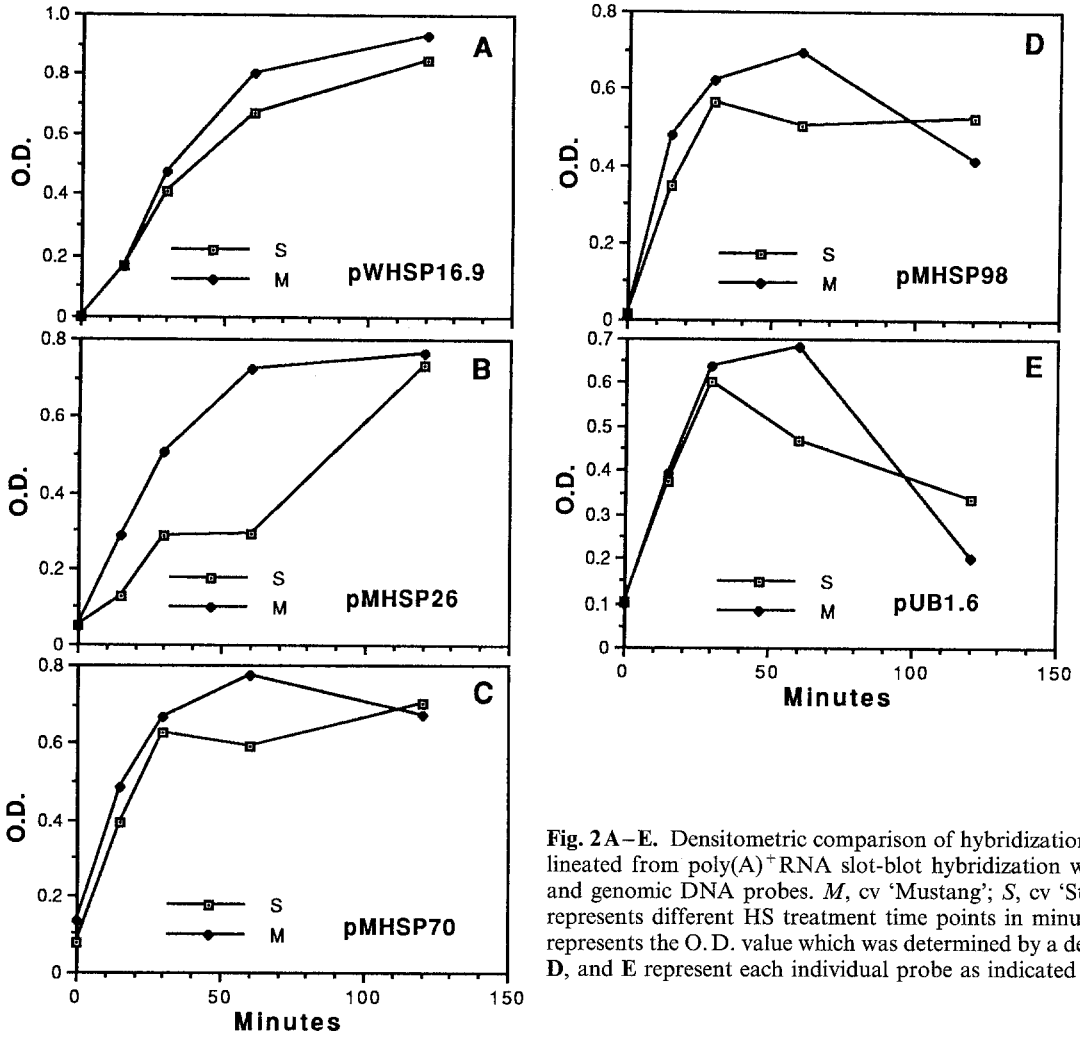

Fig. 2A–E. Densitometric comparison of hybridization signal intensity delineated from poly(A)$^+$RNA slot-blot hybridization with different cDNA and genomic DNA probes. *M*, cv 'Mustang'; *S*, cv 'Sturdy'. The ordinate represents different HS treatment time points in minutes and the abscissa represents the O.D. value which was determined by a densitometer. **A**, **B**, **C**, **D**, and **E** represent each individual probe as indicated

mology of HSPs between the different species and also due to the lack of availability of wheat HSP genes [except LMW HSP16.9 (pWHSP16.9)], the maize HSP26 cDNA clone, pMHSP26, the maize HSP70 genomic clone, pMON9502, the maize HSP98 cDNA clone, pMHSP98, and the chicken ubiquitin cDNA clone, pUB1.6, were used as heterologous probes; the wheat HSP16.9 cDNA clone, pWHSP16.9, was used as a homologous probe to monitor related gene expression in wheat.

The O.D. values of slot blots hybridized with pWHSP16.9, pMHSP26, pMHSP70, pMHSP98, and pUB1.6, which represent each major HSP group, are presented in Fig. 2A–E. As with the 2-D gel analysis, 'Mustang' generally had a higher signal on steady-state mRNA level than 'Sturdy'. 'Mustang' also responded faster to HS as exemplified when probed with a chloroplast-localized HSP gene, pMHSP26 (Fig. 2B). In 'Mustang', the gene expression level of ubiquitin, which represents a superfamily of small heat-shock proteins that participate in the process of degradation of abnormal proteins in the cell, was also higher than that of 'Sturdy'. In 'Mustang', the maximum level of ubiquitin mRNA increased approximately seven-fold during HS compared to an approximately six-fold increase in 'Sturdy' (Fig. 2E).

RFLP of multigene family HSP17 between 'Mustang' and 'Sturdy'

Southern blot analysis of genomic DNA from 'Mustang' and 'Sturdy' was performed by hybridization with pWHSP16.9. Because there are no *Bam*HI, *Eco*RI, and *Hin*dIII restriction enzyme cut sites in the wheat HSP16.9 coding region (McElwain and Spiker 1989), the blot data indicates that the hexaploid wheat genome contains a HSP17 family of at least ten genes and there is a high level of RFLP between the two wheat lines (Fig. 3).

Discussion

In this study, a time-course analysis of steady-state mRNA levels in seedlings of two wheat lines differing in heat

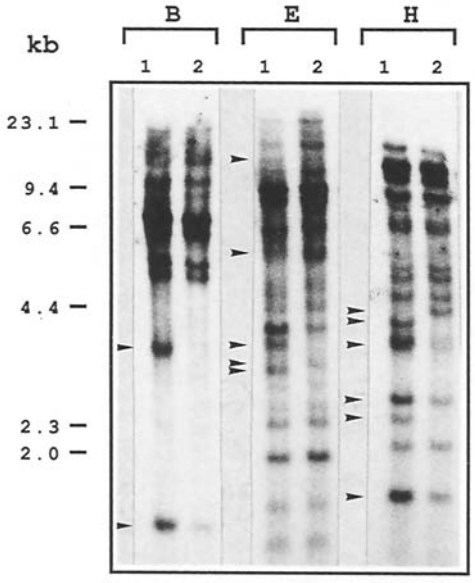

Fig. 3. Southern-blot hybridization profiles with the wheat HSP16.9 cDNA probe. For Southern-blots, 10 µg genomic DNA was digested with *Bam*HI (*B*), *Eco*RI (*E*), and *Hin*dIII (*H*), respectively. *Lane 1* represents 'Mustang', and *lane 2* is 'Sturdy'. The restriction fragment length polymorphism among the genotypes are indicated by *arrows*

tolerance was performed and the molecular basis of HSP gene expression was examined. The results presented here indicate that there are qualitative and quantitative HSP mRNA differences between 'Sturdy' and 'Mustang' at all time points during HS treatment. Studies on the regulation of heat-shock response in soybean seedlings (Kimpel et al. 1990) and other higher plants (Schoffl et al. 1986) indicate that heat-shock gene expression involves both transcriptional and post-transcriptional regulation. The data obtained by 2-D gel analysis (Fig. 1) demonstrate that when wheat seedlings were treated at 37 °C, an optimal HSP-inducing temperature for wheat (Hendershot et al. 1992), most of the HSP-gene expression occurred within 15 min. The heat-tolerant wheat line 'Mustang' not only had higher relative amounts of translatable HSP mRNAs than the heat-susceptible line 'Sturdy', but also had more unique mRNA species (Table 1). If HSPs themselves are involved in regulation of HS-gene expression (Nagao et al. 1990), some unique HSPs in 'Mustang' but not in 'Sturdy', especially those transcripts which can only be detected within several minutes of HS, may play an important role in the regulation of HS-gene expression and may result in differences of thermosensitivity between these wheat lines when under heat stress.

The Northern slot-blot hybridization data obtained by utilization of specific HSP-gene probes further confirmed that 'Mustang' had a higher steady-state level of LMW HSP mRNAs and faster transcript accumulation in response to heat stress than 'Strudy' (Figs. 2). LMW HSP is most prominent in higher plants in comparison to microbes, insects, and animals. The earlier response to heat stress, along with a higher level of HS-gene expression, may be important for the increased level of acquired cellular thermal-tolerance in wheat.

One interesting observation in this study is that of ubiquitin gene expression during heat stress (Fig. 2E). 'Mustang' had higher levels of ubiquitin mRNA than 'Sturdy' during the time course of heat stress. Ubiquitin is one kind of HSP that participates in an ATP-dependent proteolytic pathway under high temperature stress and has been widely investigated (Monia et al. 1990). Recent studies of the physiological function of ubiquitin in wheat roots during HS (Ferguson et al. 1990) indicate that high temperatures increase the breakdown of root proteins which are degraded via the ubiquitin proteolytic pathway. The higher level of ubiquitin steady-state mRNA in 'Mustang' compared with 'Sturdy' may provide further molecular evidence that increased HSP levels are accompanied by greater acquired cellular thermal-tolerance in wheat.

The data presented here, combined with previous data from our laboratory (Krishnan et al. 1989), has demonstrated a positive correlation between increased HSP gene expression and genetic differences in cellular thermal-tolerance in winter wheat. Currently, gene-specific DNA probes are being isolated (Joshi et al. 1991; Weng et al. 1991 a, b), and recombinant inbred lines of wheat are being developed in our laboratory, for further genetic analysis of the role of unique HSP genes in heritable thermal-tolerance in wheat.

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