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Chromosomal location of genes controlling heat shock proteins in hexaploid wheat

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Summary. The low molecular weight heat shock protein (HSP) profiles of the hexaploid wheat cultivar "Chinese Spring" and its ditelosomic series were characterized by isoelectric focusing polyacrylamide gel electrophoresis of denatured in vivo radiolabeled proteins. Comparisons of the ditelosomics (DTs) to the euploid "Chinese Spring" enabled the assignment of genes controlling 9 of the 13 targeted HSPs to seven chromosome arms. There did not appear to be a genome-specific action in the regulation of expression of these HSPs. There did appear to be a higher frequency of controlling genes within homoeologous DT lines 3, 4 and 7. Significant variation in protein quantity was evident among the DT lines for some HSPs, while other HSPs were remarkably stable in their expression across all DTs examined. The results are useful in identifying specific DT lines for the investigation of HSP functions in hexaploid wheat.

Key words: Heat shock proteins – 2D electrophoresis – *Triticum aestivum* – Gene location – Gene regulation

Introduction

All organisms studied to date respond similarly to a high temperature stress or heat shock (HS) of 8°–10 °C above their normal growing temperatures by synthesizing a special set of proteins – heat shock proteins (HSPs). The synthesis and accumulation of HSPs is accompanied by a decrease in normal protein synthesis and has been correlated with the acquisition of thermal tolerance (Lin et al. 1984; Kimpel and Key 1985; Sachs and Ho 1986; Lindquist 1986; Krishnan et al. 1989). Although the specific physiological functions of HSPs have not been fully described to date, there is evidence that HSPs associated with the chloroplast, HSP 22 K in particular, prevent damage to the photosystem II reaction center during heat shock in the light (Schuster et al. 1988). There is also evidence that HSPs in the range of 15 K are associated with the plasma membrane and may be important in preventing HS-induced leakage from the cell (Lin et al. 1984).

Heat shock proteins in higher plants can been categorized into two major groups based on their molecular weight: high molecular weight (HMW) (65–110 K) and low molecular weight (LMW) (15–27 K) (Kimpel and Key 1985). Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) of total soluble protein from heat-shocked plant tissue reveals that HSPs from both groups are synthesized and accumulate, but the major group of HSPs in plants is the LMW group (Mansfield and Key 1987). Analysis of this group of proteins in several plant species reveals that they accumulate to significant levels after heat shock and are very diverse in their isoelectric point, molecular weight, stainability and radiolabel incorporation (Mansfield and Key 1987). A very prominent subset of the LMW HSPs, in the range of 15–18 K, were found to be unique to higher plants (Sachs and Ho 1986).

The ubiquitous nature of the HS response has in itself presented an obstacle in defining the physiological and biochemical functions of HSPs in the development of thermal tolerance. The lack of HSP mutant types in higher plants has prevented the classical approach of establishing a cause-and-effect relationship between the synthesis of HSPs and the development of thermal tolerance. The lack of mutants also complicates the study of the functions of HSPs in conferring thermal tolerance.

The obstacles caused by the lack of HSP mutants can be circumvented to a certain degree through the analysis of the HS response in aneuploid genetic stocks. In theory,

the loss of structural or regulatory genes located on a missing chromosome arm of a particular ditelosomic line would be manifested by the qualitative or quantitative reduction of the corresponding HSP controlled. The effect of the loss of this particular HSP on the plant's ability to acquire thermal tolerance could then be analyzed through physiological and biochemical assays of the level of thermal tolerance.

Analysis of the aneuploid genetic stocks in wheat is a common approach used to localize structural and regulatory genes of enzymes (Hart 1983) and endosperm proteins (Galili and Feldman 1983). The use of aneuploid genetic stocks of wheat combined with recent technical improvements in IEF-PAGE of denatured proteins (Damerval et al. 1986) enabled Colas des Francs and Thiellement (1985) to locate 35 structural genes on 17 chromosome arms of the ditelosomic lines of "Chinese Spring" hexaploid wheat.

In this study we have used IEF-PAGE to characterize the synthesis patterns of a major group of HSPs (the LMW HSPs) from the ditelosomic lines of "Chinese Spring". The purpose of this study is to identify the chromosomal location of HSP structural genes and their regulators.

Materials and methods

Plant material and treatments

The hexaploid wheat (*Triticum aestivum* L., 2 n = 6 x = 42) cultivar "Chinese Spring" and the ditelosomic (DT) series derived from "Chinese Spring" used in this study were generously provided by Dr. E. Sears (University of Missouri, Columbia, USA) (Sears 1954). Of the 42 total DT lines, 33 were analyzed. DT lines are designated by their homoeologous group (1–7), genome (A, B or D) and the chromosome arm length (L = long, S = short, if known, otherwise they are α and β). The chromosome arms represented by the nine missing lines (2AL, 2BS, 4Aβ, 4BS, 4DL, 5AS, 5BS, 5DS and 7DL are missing due to infertility problems) were complimented through the use of their corresponding nullisomic-tetrasomic lines.

Seeds of each DT line were germinated in 50-ml beakers of moist Vermiculite at 22 °C under a fluorescent light bank (300 µmol photon m^{-2} s^{-1}) with a 12 h day/night cycle. The procedure for radiolabeling of green leaf tissue was modified from Marder et al. (1986). At 10–14 days after emergence, 30 mg of green leaf tissue was cut and rinsed with distilled H_2O. This tissue was cut into 5-mm long sections and placed in a 10 × 75 mm glass tube with 150 µl of incubation buffer (20 m*M* TRIS-HCl, pH 7.5; 5 mg/ml chloramphenicol). This tube was submerged in a controlled-temperature water bath and incubated with gentle shaking at either the heat shock temperature (37 °C) or the control temperature (22 °C). After 1 h of incubation, the buffer was replaced with buffer containing 100 µCi of Tran^{35}S-label (ICN Biomedicals) and vacuum infiltrated approximately 5 min. Samples were returned to the water baths and protein synthesis was radiolabeled for 3 h at the corresponding heat shock or control temperature. After radiolabeling, samples were rinsed three times with 1 m*M* methionine to remove all residual isotope, and proteins were extracted.

Protein extraction and electrophoresis

Proteins were extracted and denatured according to the method described by Damerval et al. (1986), with the following modification made to the "UKS" solution: the 2.0% LKB Ampholine, pH 3.5–10, was replaced with 2.0% Servalyt 3–10. One mg of dried protein pellet was solubilized with 70 µl of "UKS" instead of 50 µl (Zivy 1987). The IEF gels were 14 cm long with a diameter of 1 mm. The gel mixture was 3.78% acrylamide, 3.2% Servalyt 5–7, 0.8% Servalyt 3–10, 3.0% Triton X-100, 9.2 *M* Urea, and 0.22%, N,N'-methylenebisacrylamide. Protein samples of known cpm were loaded on top of the gels and overlayed with 5 µl of twice diluted "UKS" solution. IEF was performed for 21,000 Volt × Hours in 0.1 *N* NaOH and 0.06% H_3PO_4 electrode solutions. IEF gels were equilibrated and SDS-PAGE was performed as described by Damerval et al. (1986), except the slab gel used was 16 cm × 20 cm × 1 mm and the run was at 24 mA per gel. The migration buffer used is described by Laemmli (1970).

Gel processing and comparison

After completion of the run, SDS slab gels were immediately fixed in 40% methanol and 10% acetic acid for 1 h. Fluorographic processing was performed using the method described by Skinner and Griswold (1983) with the following modification: instead of drying under 1.33 kPa vacumn at 70 °C, the gel was placed on a wet sheet of cellophane, covered with a sheet of plastic wrap, topped with another sheet of wet cellophane and dried overnight sandwiched in an acrylic frame. The dried gel was exposed to Kodak X-Omat X-ray film at −70 °C for the desired period of time.

Results reported here are based on the qualitative and quantitative differences observed between radiolabeled HSP synthesis profiles of each DT line (duplicate gels were run for each DT) and the profile of the euploid which serves as the control. The efficiency of incorporation of the radiolabel (^{35}S-methionine) during HSP synthesis varied among the individual diteloscomics due to their individual level of metabolic fitness. Given the constraint of a limit on the amount of protein that can be loaded per IEF gel, it was not always possible to load equal cpms per gel when performing IEF. To compensate for this, the maximum cpms were loaded, within the limit of protein that can be loaded per gel, and the exposure time of the fluorograph at −70 °C was adjusted to a standard of 1×10^6 cpms per day per gel.

Results

The characteristic profile of euploid "Chinese Spring" showing the 13 LMW HSPs targeted for this study is presented in Fig. 1 a. These 13 HSPs were targeted because they are the most prominent of the LMW HSPs synthesized in "Chinese Spring". These 13 LMW HSPs are very similar electrophoretically to the 12 LMW HSPs detected in wheat by Mansfield and Key (1987). A diagram, with the 13 LMW HSPs numbered for ease of identification, is presented in Fig. 1 b. HSPs 1, 2, and 3 are in the range of pH 5–6 with a molecular weight of 27 K. The complex of spots 4–8 and 9–13 are in the range of pH 5.5–6.5 with molecular weights ranging from 16 to 18 K. One group of HSPs in the range of 22 K that is commonly expressed in other wheat cultivars is not prominent in "Chinese Spring". Levels of expression

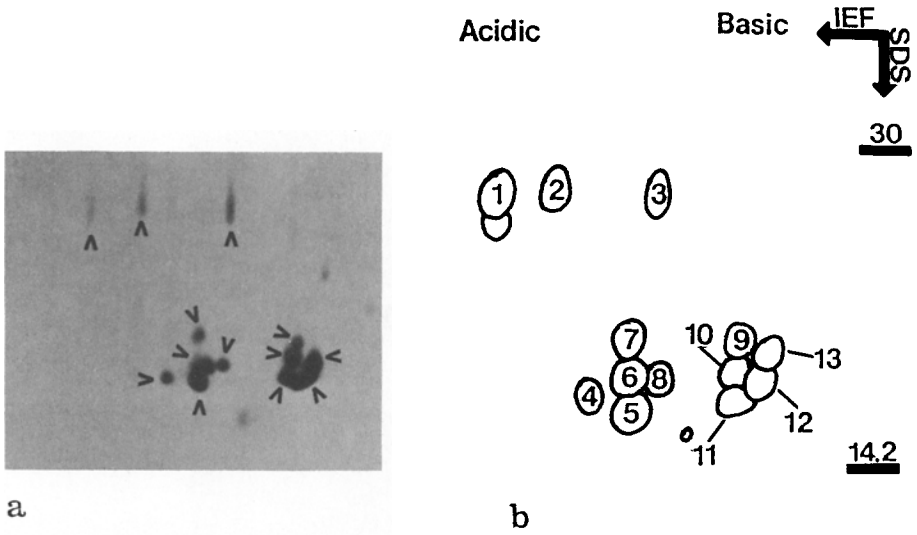

Fig. 1. a IEF-PAGE fluorograph of euploid "Chinese Spring" LMW HSPs. **b** Diagram of 13 LMW HSPs of euploid "Chinese Spring" analyzed for chromosomal location of controlling genes. Molecular weight standards are indicated in kilodaltons on the right

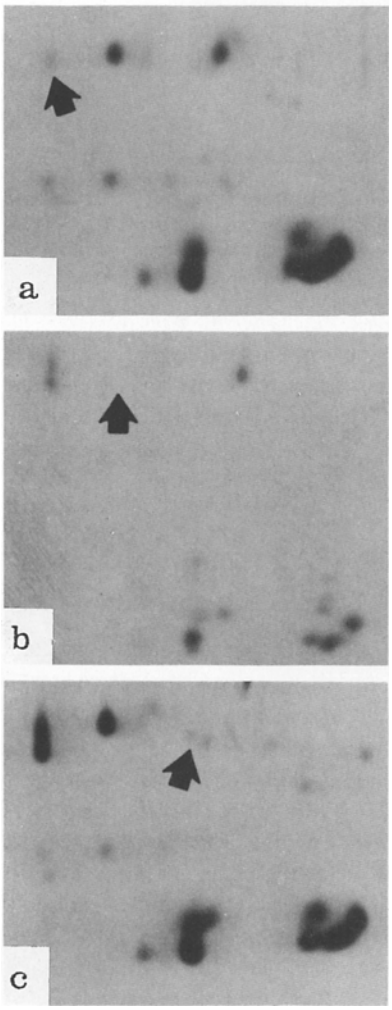

Fig. 2a–c. Fluorographs of heat-shocked ditelosomics. **a** DT4BL (*arrow* indicates reduced HSP 1); **b** DT4Aα (*arrow* indicates reduced HSP 2); **c** DT4DS (*arrow* indicates reduced HSP 3)

for this group of HSPs have been shown to be genotype-specific (Krishnan et al. 1989).

Most HSPs were stable in the intensity of their expression across the DTs studied and as compared to the euploid control. HSP 1 was drastically reduced in its expression in DT4BL (Fig. 2a). HSP 2 and HSP 3 disappear in DT4Aα and DT4DS (Fig. 2b and c, respectively). HSP 4 was synthesized at a low level in all DTs examined. HSP 5 was remarkably stable in its expression across all DT lines analyzed. Interpretation of results for HSPs 6 and 8 were complicated by their similarity in molecular weight and isoelectric point. In euploid "Chinese Spring", HSPs 6 and 8 were separate, well-resolved spots. In the majority of DTs examined, HSPs 6 and 8 appeared to co-migrate and these products were perceived to be HSP 6 (Fig. 3a). There was significant variability in the level of expression detected for HSPs 6 and 8 that co-migrated (Fig. 3a and b). An approximate two-fold increase was detected in DT3DL as compared to DT1BL (Fig. 3a and b, respectively). There was also a significant decrease in expression of the co-migrated HSPs in DT4BL (Fig. 2a). There were clear cases where both HSP 6 and 8 were present (e.g. DT5AL, Fig. 6a), but the majority of gels had both products focused at the same spot. Expression of HSP 7 was low in all DTs examined but completely disappeared from DT4DS (Fig. 2c). HSP 9 is similar to HSP 7 in that its expression level was low, but was non-detectable in DT4BL and DT4DS (Fig. 2a and c). Interpretation of results for HSPs in the complex 10–13 was also complicated by their similarity in isoelectric points and molecular weight. Levels of expression for HSP 10 varied considerably, dependent on the individual DT, but the product was always synthesized. The only clear case of a missing spot from this complex of spots was HSP 11 in DT3BL (Fig. 4c). HSP 11 and 12 do not co-migrate,

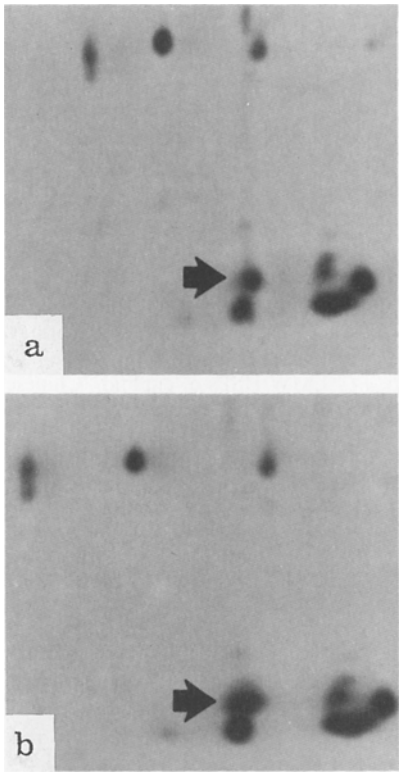

Fig. 3a and b. Fluorographs of heat-shocked ditelosomics. **a** DT1BL (*arrow* indicates normal HSP 6); **b** DT3DL (*arrow* indicates increased HSP 6)

but they are isomorphic and overlap in DT1BL and DT3DL (Fig. 3a and b), which makes them difficult to distinguish electrophoretically. The level of expression for HSP 12 was the most variable of the HSPs in the complex 10–13. In DT3AS (Fig. 4a) there appear to be two protein spots focused closely together at HSP 12. In the remaining DTs there appears to be one HSP focused at this spot (e.g. DT3AL, Fig. 4b). HSP 13 is similar to HSP 5 in that its level of expression was very stable across at DTs.

In addition to the observed qualitative and quantitative reductions in expression of single HSPs, there were also examples detected of increase in expression of HSPs compared to normal levels (e.g. increase of HSP 6 in DT3DL as compared to DT1BL, Fig. 3b and a, respectively). There was also an example detected of the quantitative reduction of multiple HSPs within a single DT (e.g. HSP 1 and 3 in DT7AL as compared to DT7AS, Fig. 5a and b).

There did not appear to be genome-specific action in the control of these 13 HSPs. The A, B and D genome were represented equally in the DTs that exerted control over the synthesis of these HSPs. While some homoeologous DTs (e.g. DT5AL, DT5BL and DT5DL) did not appear to have any impact on the synthesis pattern

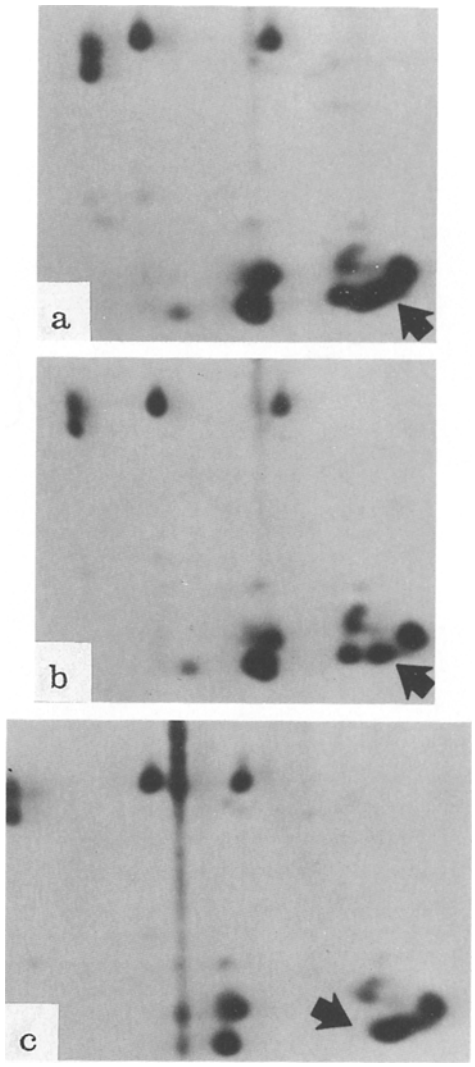

Fig. 4a–c. Fluorographs of heat-shocked ditelosomics. **a** DT3AS (*arrow* indicates two HSP 12 spots); **b** DT3AL (*arrow* indicates normal HSP 12); **c** DT3BL (*arrow* indicates missing HSP 11)

Table 1. Chromosome arms of "Chinese Spring" and the HSPs they control

DT line	HSP spot no.	Apparent molecular weight (K d)	Chromosome arm location of gene controlling HSP
4BL	1	27	4BS
7AS	1	27	7AL
4Aα	2	27	4Aβ
4DS	3	27	4DL
7AS	3	27	7AL
3DL	6	18	3DS
4DS	7	18	4DL
4BL	8	17	4BS
4BL	9	18	4BS
4DS	9	18	4DL
3BL	11	17	3BS
3AS	12	17	3AL

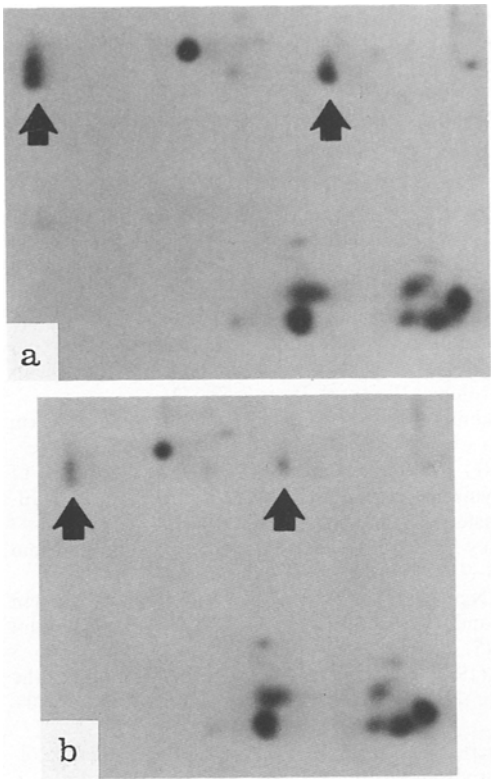

Fig. 5a and b. Fluorographs of heat-shocked ditelosomics. **a** DT7AL (*arrows* indicate normal HSPs 1 and 3); DT7AS (*arrows* indicate reeuded HSPs 1 and 3

(Fig. 6a–c, respectively), there did appear to be a higher frequency of controlling genes located within homoeologous DTs 3, 4 and 7 (Table 1).

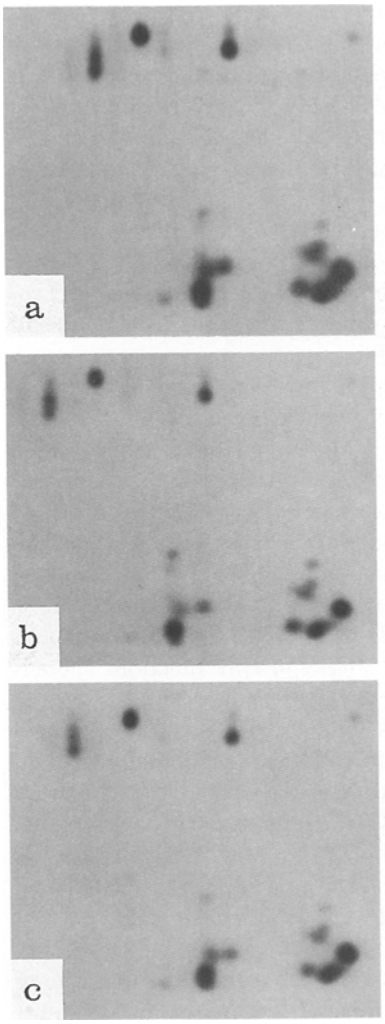

Fig. 6a–c. Fluorographs of heat-shocked ditelosomics. **a** DT5AL; **b** DT5BL; **c** DT5DL (normal HSP synthesis pattern shown by homeologous DTs)

Discussion

The significant variation observed in levels of expression among the DTs for the 13 LMW HSPs investigated in this study can be interpreted in most cases to be the result of regulatory effects. There are several factors to consider when comparisons are made of variations in intensity of a particular protein spot between fluorographs of proteins radiolabeled in vivo. At the level of radiolabeling of protein synthesis we must assume that the polypeptides being compared from one DT to another contain equal amounts of methionine and cysteine, otherwise there will be differential intensities detected. We don't consider this to be an important source of variation because of the conserved nature of the isoelectric points and molecular weights of the LMW HSPs among the DTs examined. Another important consideration is the allohexaploid nature of wheat. Variation in protein quantity can be interpreted as a result of the effect of a regulator, or the effect of varying amounts of homoeoallelic products with identical electrophoretic properties (Colas des Francs and Thiellement 1985). These variations in protein quantity can be manifested by dramatic decreases in HSP intensity that can be detected visually from the fluorographs (e.g. Fig. 2a–c). A comparison of intensity levels for a particular HSP across homoeologous DT lines may provide insight into regulatory effects being exerted on that HSP. If a given HSP spot is the result of one, two or three electrophoretically identical homoeoallelic products, then the absence of one or more products in a particular DT line could be detected by comparing the decrease in intensity of that HSP relative to other homoeologous DTs. Several examples of this variation can be found for a given HSP among homoeologous DTs, but interpretation of this type of regulation may be complicated by the possibility of gene dosage compensation effects among homoeologous DTs. This approach will, however, pinpoint DTs of interest for further study.

In general, there appear to be few clear cases of the loss of LMW HSPs, which may be caused by the loss of structural genes from individual DTs. In cases where there is a loss of a LMW HSP, the missing chromosome arm pair may contain either the structural gene that codes for the particular HSP or the regulatory gene needed for its expression. Further work with cloning and DNA hybridization techniques should prove useful in resolving these questions (Loi et al. 1988).

The objective of this study was to determine the chromosomal location of genes controlling HSPs in wheat. The rationale behind this objective was to identify genotypes that were deficient in the genes encoding HSPs and genotypes missing major regulatory genes that drastically suppressed the expression of the HSP structural genes. The identification of these genotypes provides the needed HSP mutant substitutes necessary to begin the study of the molecular and physiological role of these proteins in the development of thermal tolerance. We have targeted several DTs (Table 1) whose expression of certain HSPs is suppressed to a level low enough to be useful in molecular and physiological studies of HSP function. There is enough variability for the expression of HSPs 5–8 and 10–13 among the DTs highlighted in this study to begin the analysis of their association with differential cell viability following heat shock. If, e.g., these HSPs are analogous to the 15-K HSPs that have been associated with the plasma membrane (Lin et al. 1984), their levels of expression among the DTs are diverse enough to result in differential levels of cell membrane stability.

The synthesis of HSPs is one of the most highly conserved genetic systems known, yet the molecular role of the LMW proteins is a "complete mystery" (Lindquist and Craig 1988). Given the highly conserved nature of the heat shock response in higher plants, one would generally not anticipate diverse levels of HSP expression, especially considering the allohexaploid nature of wheat. In addition, one progenitor of hexaploid wheat, the diploid A genome contributor *Triticum urartu* L., synthesizes a similar number of LMW HSPs in the same pH range and molecular weight (R. Vierling, personal communication). Considering these factors, the effects of the loss of a chromosome arm pair on HSP synthesis reported here are dramatic. The intergenomic variation presented in this study and the intraspecific genetic variability in the synthesis of HSPs previously reported (Krishnan et al. 1989) provide a unique, valuable database for further study into the HS response in hexaploid wheat and the physiological functions of HSPs in plants.

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