Plant Physiol. (1996) 111: 1085–1096

Complexity and Genetic Variability of Heat-Shock Protein Expression in Isolated Maize Microspores[1]

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The expression of heat-shock proteins (HSPs) in isolated maize (*Zea mays* L.) microspores has been investigated using high-resolution two-dimensional electrophoresis coupled to immunodetection and fluorography of in vivo synthesized proteins. To this end, homogeneous and viable populations of microspores have been purified in sufficient amounts for molecular analysis from plants grown in controlled conditions. Appropriate conditions for thermal stress application have been defined. The analysis revealed that isolated microspores from maize display a classical heat-shock response characterized by the repression of the normal protein synthesis and the expression of a set of HSPs. A high complexity of the response was demonstrated, with numerous different HSPs being resolved in each known major HSP molecular weight class. However, the extent of this heat-shock response is limited in that some of these HSPs do not accumulate at high levels following temperature elevation. Comparative analysis of the heat-shock responses of microspores isolated from five genotypes demonstrated high levels of genetic variability. Furthermore, many HSPs were detected in microspores at control temperature, indicating a possible involvement of these proteins in pollen development at stages close to first pollen mitosis.

The rapid synthesis and accumulation of a small set of HSPs upon heat shock is a highly conserved cellular response common to most organisms; it involves the redirection of both normal gene transcription and mRNA translation (Vierling, 1991; Morimoto et al., 1992). Yet, unlike female tissues, mature maize (*Zea mays* L.) pollen is unable to mount a heat-shock response when it is exposed to supraoptimal temperatures (Frova et al., 1989; Dupuis and Dumas, 1990). Given the potential role of HSPs in protecting against heat stress (Vierling, 1991), it is assumed that this inability of mature pollen to produce HSPs may account at least in part for the sensitivity of maize fertilization to heat and for the resulting depression of kernel production that occurs in the field in high-temperature conditions (Dupuis and Dumas, 1990; Hopf et al., 1992). However, several studies have revealed that genotypic differences in the sensitivity of maize pollen germination to high temperature do exist (Herrero and Johnson, 1980; Lyakh et al., 1991), and Frova et al. (1989) demonstrated that the loss of ability to synthesize HSPs occurs gradually during pollen development, with the uninucleate microspores displaying the highest response. Recent data indicate that this gradual loss seems to be due to a stage-dependent defect in accumulating HSP mRNA (Gagliardi et al., 1995). Consequently, one hypothesis to explain maize pollen thermosensitivity may be that the transition from uninucleate to binucleate pollen marks the onset of the progressive decrease in the pollen's ability to synthesize HSPs, eventually leading to the complete inhibition of HSP synthesis in mature pollen. It is interesting that HSP genes are not the only genes in which expression decreases or is turned off after the first pollen mitosis. Indeed, this stage of pollen development is an important developmental switch characterized by the decrease of "early" pollen gene expression and the onset of "late" pollen gene expression (McCormick, 1993, and refs. therein).

In addition, a growing body of data indicates that in a number of systems, members of the HSP families are also expressed at control temperature and are likely to be involved in differentiation and developmental processes (Hightower and Nover, 1991). During maize male development, such HSP gene expression has been reported at meiosis (Dietrich et al., 1991; Atkinson et al., 1993; Bouchard et al., 1993; Marrs et al., 1993). As with meiosis, first pollen mitosis is a key event of pollen formation (McCormick, 1993; Eady et al., 1995). Consequently, it may be interesting to investigate expression of HSPs at this stage at normal temperature in relation to their possible involvement in pollen development.

The present work was conducted to provide a detailed assessment of HSP expression in developing gametophytes around first pollen mitosis, the stage that immediately precedes the decrease in the maize pollen's ability to synthesize HSPs in response to heat (Frova et al., 1989). First, we investigated HSP inducibility upon heat shock and the extent of the genetic variability of this response. Also, we examined HSP expression at normal temperature in developing pollen at these stages, corresponding to first pollen mitosis transition.

For this purpose we used isolated staged microspore suspension cultures from five different maize genotypes.

[1] This work was supported in part by a grant from the Human Frontier Science Program Organization to C.D. and by the French Ministère de la Recherche et de la Technologie (Action Incitative Programmée "Microspores Isolées").

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Abbreviations: 2D, two-dimensional; HSP, heat-shock protein.

The expression of HSPs at control (28°C) or heat-shock (40°C) temperature in these microspore samples was analyzed by high-resolution 2D electrophoresis coupled to fluorography of [^{35}S]Met-labeled proteins and western blotting.

Our results show that isolated maize microspores display a complex and genetically variable HSP expression pattern. However, they do not accumulate massive amounts of HSPs upon heat shock. This suggests that this developmental stage of pollen already displays some deficiency in the extent of its heat-shock response.

In addition, we show that members of several major *hsp* gene families are expressed at the protein level close to first pollen mitosis at normal temperature.

MATERIALS AND METHODS

Five maize (*Zea mays* L.) genotypes were used in this study: the doubled haploid lines DH5 and DH7, the inbreds A188 and Mo17, and the hybrid line DH5 × DH7. DH5 and DH7 were derived through in vitro androgenesis from essentially Chinese stocks and were selected for their high androgenic capabilities. Their cross reveals considerable heterosis for this trait (Barloy et al., 1989).

Pairwise comparisons using restriction fragment length polymorphism markers between DH5, DH7, and A188 revealed that they display a high level of genetic divergence, in the range of the divergence between lines from different maize heterotic groups (Murigneux, 1993). Additionally, pedigree records indicate that Mo17 contains 50% Lancaster Surecrop germplasm and 25% Reid Yellow Dent germplasm, whereas A188 was developed from the cross 64 × 4–29. Line 64 was derived from a strain of Reid Yellow Dent, and 4–29 was derived from the variety Silver King (Gerdes and Tracy, 1993; Gerdes et al., 1993).

Seeds were sown in a mixture of peat moss:basalt (1:1, v/v) in pots 25 cm in diameter and 30 cm high. After 14 d, one plant was maintained per pot. Plants were grown with a photoperiod of 16 h in a strictly controlled growth chamber at a PPFD of 700 to 800 μmol m^{-2} s^{-1} PAR at the top of the plants provided by 1000-W high-pressure sodium vapor bulbs (Sodiclaude, Claude, Belgium). The temperature and the RH were 24 ± 1°C and 80% during the day, and 19 ± 1°C and 90% during the night, respectively. Plants were irrigated for 3 min 16 times a day with a nutritive solution.

Microspore Isolation

Tassels were harvested just before emergence from the leaf whorl and immediately transferred to the laboratory, where they were wrapped in aluminum foil and stored temporarily on ice. The developmental stages of the microspores in the tassel branches were quickly determined, using the staining method of Alexander (1969). Tassel segments with first floret anthers containing mid- to late uninucleate microspores and early binucleate pollen grains were then processed for microspore isolation according to the method of Gaillard et al. (1991). The original procedure was modified in that the Percoll gradient centrifugation step was simplified; the first microspore pellet was resuspended in 2.5 mL of isolation medium containing 1% (v/v) Percoll and directly loaded onto 5 mL of a mixture of isolation medium and Percoll (7:3, v/v) in a 15-mL tube (no. 2095; Falcon, Becton Dickinson). This gradient was centrifuged at 225*g* for 3 min. The band of staged microspores floating at the interface was then further purified as described by Gaillard et al. (1991), and the recovered fraction was finally suspended in culture medium (Gaillard et al., 1991; see "Results"). The number of microspores in the suspension and the cell viability were evaluated using a Malassez cell and fluorescein diacetate staining (Heslop-Harrisson and Heslop-Harrisson, 1970), respectively. The entire isolation procedure was performed aseptically at 0 to 4°C and required approximately 1 h.

Temperature Treatment and Protein Labeling

Generally, the isolation yield was sufficient to generate from one tassel both control and heat-shock samples of at least 2 × 10^5 microspores each. Cells were incubated in Petri dishes (60 × 15 mm; Falcon no. 3002) containing 2 mL of suspension at a density of 10^5 mL^{-1}. The dishes were sealed by a plastic film (Scell-O-Frais, Colgate-Palmolive, Courbevoie, France) and placed in darkness in incubators at 28°C (control) and 35 or 40°C (heat shock) for 1 h. For analysis by fluorography, 3.7 MBq (100 μCi) of L-[^{35}S]Met (41.7 TBq mmol^{-1}; NEN) were added per mL, and the dishes were returned to the control and heat-shock incubators. After 2 h the cell suspension was washed three times with culture medium and filtered on a nylon cloth (pore size 50 μm; ZBF, Rüschlikon, Switzerland) under light suction to collect the microspores. Excess liquid was quickly eliminated by blotting the filter onto a piece of tissue paper. The filter with the microspores was then placed into a cryotube and immediately frozen in liquid nitrogen. When the microspore samples were used only for immunoanalysis, the dishes were kept continuously at 28 or 40°C for 3 h. All of the samples were stored at −196°C until protein extraction.

Protein Extraction and Assays

Microspores were homogenized in 1.5-mL microfuge tubes with extraction buffer (0.6 μL per 10^3 microspores) containing 62.5 mM Tris-HCl (pH 6.8), 5% (w/v) SDS, 2% (w/v) DTT, 5% (w/v) Suc, and 1% (w/v) insoluble PVP (P 6755; Sigma). The homogenate was vortexed for 5 s, heated for 5 min at 100°C, and centrifuged for 15 min at 15,000*g* to remove cell debris and insoluble PVP. The supernatant was then recentrifuged for 5 min and stored at −80°C. Total incorporated counts were determined by scintillation counting on the TCA precipitate of a 5-μL aliquot of the crude extract, and the protein content was assessed by the method of Lowry et al. (1951), using the acetone precipitate of 10 μL of the extract with BSA as standard. Proteins contained in crude extracts were precipitated overnight at −20°C with 5 volumes of cold acetone and then washed three times with cold 80% (v/v) acetone. Finally, the resulting protein pellets were dissolved in urea-potassium

carbonate-SDS solution (Damerval et al., 1986) to a concentration of 6 to 10 $\mu g\ \mu L^{-1}$ and stored at −80°C until analysis by 2D electrophoresis.

2D Electrophoresis

2D electrophoresis was performed as described by Damerval et al. (1986), with some modifications. The IEF rod gels were 14.5 cm long and 1 mm in diameter and contained 2% (w/v) Ampholine (pH 3.5–9.5; Pharmacia LKB) and 2% (w/v) Pharmalyte (1.5%, pH 5–8, and 0.5%, pH 5–6; Pharmacia). They were loaded with 2×10^5 (28 and 35°C samples) or 6×10^4 (40°C extracts) cpm of TCA-precipitable radioactivity for fluorography analysis and 100 μg of 28 or 40°C nonlabeled protein extracts for immunodetection of HSPs. IEF was conducted at 25°C for 14,600 $V\ h^{-1}$ in a Bio-Rad 175 unit. Then, the gels were incubated for 5 min in 10 mL of equilibration buffer (Damerval et al., 1986), drained, and immediately stored at −80°C until the second dimension run. The pH gradient was determined by direct pH measurement of 1-cm pieces cut from non-equilibrated gels.

2D SDS electrophoresis was done with 12% (w/v) acrylamide slab gels (160 × 180 × 1 mm) without a stacking gel. The molecular masses were determined using the Pharmacia LMW electrophoresis calibration kit.

Fluorography

Following electrophoresis of labeled samples, gels were first stained with Coomassie blue to reveal size markers, destained, and subsequently impregnated for 30 min with Amplify (Amersham). They were then dried and exposed to Kodak X-Omat x-ray film at −80°C for 10 to 20 d.

All fluorography experiments were replicated at least three times (i.e. three tassels per cultivar and two fluorograms per protein sample), except for the inbred line Mo17, for which two plants were analyzed. Fluorograms were visually inspected, and only reproducible differences have been considered for the analysis.

Immunoblotting of Proteins

Following 2D electrophoresis, the gels were first equilibrated in transfer buffer (10 mM Caps, pH 11.0, 10% methanol) for 5 min. Proteins were then electrotransferred onto a nitrocellulose membrane using the Milliblot SDE semidry transfer system (Millipore). The quality of the transfer and the positions of the molecular weight markers were assessed by Ponceau red staining of the membrane. For immunodetection, the membrane was blocked for 1 h in TBST (20 mM Tris-HCl, pH 7.6; 137 mM NaCl; 0.1% Tween 20 [v/v]) plus 1% (w/v) BSA and rinsed several times with TBST. It was subsequently incubated for 1 h with the primary antibody diluted in TBST (Table I). The membrane was then rinsed with TBST and probed for 1 h with a peroxidase-linked secondary antibody (Amersham NA 931 or NA 934) diluted 1:5000 in TBST. Following incubation and rinsing with TBST, immune complexes were detected with an enhanced chemiluminescence (ECL) detection kit (Amersham) according to the manufacturer's directions. The immunodetection experiments were replicated with extracts from three different tassels.

RESULTS

Isolation of a Gametophyte Population Suitable for HSP Synthesis Studies

The isolation procedure used in the present study allowed the efficient purification of staged gametophytic fractions in sufficient amounts for molecular analysis. On average, one tassel from the hybrid line and the inbreds yielded 7×10^5 and 4×10^5 microspores, respectively. Approximately 1 μg protein 10^{-3} cells could be recovered. The final cell suspensions routinely displayed high viability rates (75–85%). As illustrated in Figure 1, the purified populations contained predominantly mid- to late uninucleate microspores, displaying a single large vacuole. A few early binucleate young pollen grains, with their vegetative and generative nuclei close to each other, generally co-purified with the microspores (Fig. 1). Thus, our purified fractions correspond to first pollen mitosis or to stages close to it. This stage is favorable for the analysis of HSP synthesis in that it is intermediary between the A and B stages studied by Frova et al. (1989) and described by them as responsive to heat shock. In the following sections, the isolated gametophytes will be referred to as "microspores" for simplification.

Table I. *Characteristics of the primary antibodies used in this study*

Primary Antibody	Description	Working Dilution
Anti-cytoplasmic HSP70	Mouse monoclonal antibody directed against the ATP-binding region of a recombinant human HSP70 (MA3 007 5A5—Affinity Bioreagents)	1:2500
Anti-maize mitochondrial HSP70	Mouse monoclonal antibody directed against maize mitochondrial HSP70 (hybridoma culture supernatant)	1:25
Anti-potato mitochondrial HSP60	Rabbit antiserum raised against purified HSP60 from isolated potato mitochondria	1:5000
Anti-maize mitochondrial HSP60	Mouse monoclonal antibody directed against maize mitochondrial HSP60 (hybridoma culture supernatant)	1:25
Anti-low-molecular-mass HSP	Rabbit antiserum raised against a conserved maize HSP18 overexpressed in *Escherichia coli* (Greyson et al., 1996)	1:5000

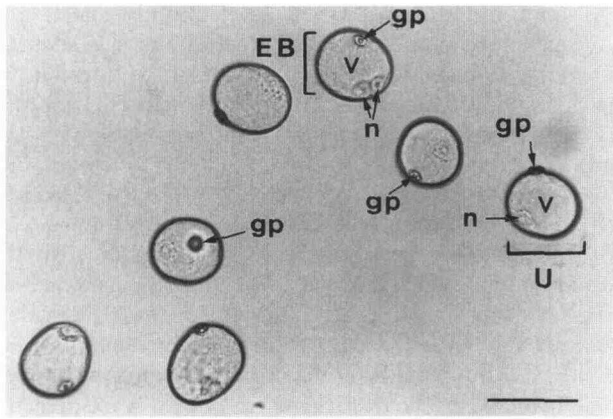

Figure 1. Bright-field microscopy observation of a representative portion of a microspore population issued from the isolation procedure. A uninucleate microspore (U) and an early binucleate pollen grain (EB) are indicated. The strongly contrasted rounded structures at the surface of the gametophytes are the germ pores (gp). Nuclei (n) and vacuoles (v) are also indicated. Bar = 100 μm.

Optimization of the Conditions for Heat-Stress Application

An essential prerequisite to the study of HSP synthesis in isolated microspores was the establishment of microspore culture conditions sustaining cellular viability during heat shock and incorporation of a radioactive label into proteins. During preliminary experiments, microspore incubation was conducted in a culture medium containing 0.15 M Suc. Because these conditions led to an important loss in cell viability (up to 50%) after 3 h at 40°C, we increased the Suc molarity to 0.35 M and found that this concentration limited the viability decrease during a 3-h heat shock to approximately 10% (data not shown). Sufficient labeling for subsequent analysis by 2D electrophoresis was obtained at both control and heat-shock temperatures in these incubation conditions (Fig. 2).

Characterization of the Microspore HSP Profile

The heat-shock response of the isolated microspores was first analyzed in the hybrid cv DH5 × DH7. Measurements of [^{35}S]Met incorporation into microspore proteins in the different temperature conditions revealed that heat shock primarily induces a decrease of the net global proteosynthesis in these cells (Fig. 2). In fact, whereas a mild treatment at 35°C resulted in only a 1.5-fold decrease of the number of TCA-precipitable counts per microgram of microspore protein, the more severe 40°C treatment caused a dramatic 8.8-fold reduction (Fig. 2).

At the qualitative level, the comparison of 28 and 40°C fluorograms showed that the 40°C treatment induces a drastic reduction in the number of visible spots. A decrease or disappearance of many of the control proteins was detected, whereas a set of HSPs was preferentially synthesized (Fig. 3, A and B). In this analysis, HSPs were first defined as proteins in which expression could be detected or those that could be reproducibly enhanced on fluorograms only at heat-shock temperature. Under our experimental conditions, DH5 × DH7 microspores reproducibly synthesized at detectable levels HSPs with molecular masses of 100, 73 to 70, 60, 25, 22 to 17 (referred to as low-molecular-mass HSPs), and 14 kD (Figs. 3B, 4, and 5). Two additional putative HSPs, HSP81 and HSP82, present on both 28 and 40°C fluorograms, have been tentatively identified (Fig. 4, A and B) on the basis of comparisons with fluorograms from heat-shocked roots (data not shown; see "Discussion").

HSPs of similar sizes were previously reported to be induced in maize vegetative tissues by heat shock (Baszczynski et al., 1982). Given the extensive conservation of the HSPs among molecular weight classes in plants (Vierling, 1991), most of these microspore heat-induced proteins are likely to be members of known HSP families. To ascertain their belonging to these conserved classes and to assess their accumulation at both control and heat-shock temperatures, antibodies directed against members of three major HSP families (HSP70, HSP60, and low-molecular-mass HSPs) were used to probe western blots of proteins from control and heat-shocked microspores. These experiments allowed us to identify additional proteins as HSPs, although their synthesis was not increased by heat shock.

In the HSP70 class, the synthesis of at least seven polypeptides with M_rs ranging from 70 to 73 and pIs from 5.3 to 5.8 was up-regulated during heat shock (Figs. 4, A and B, and 6, A and F). Close molecular species displaying fine differences in pI were clearly distinguishable in the 70- and 72-kD constellations. In addition, shorter exposures than those used for Figures 4B and 6F revealed that the composite appearance of the major spot 70a was due to the presence of several discrete polypeptide spots (data not shown). The less acidic forms of HSP70 and 72 as well as

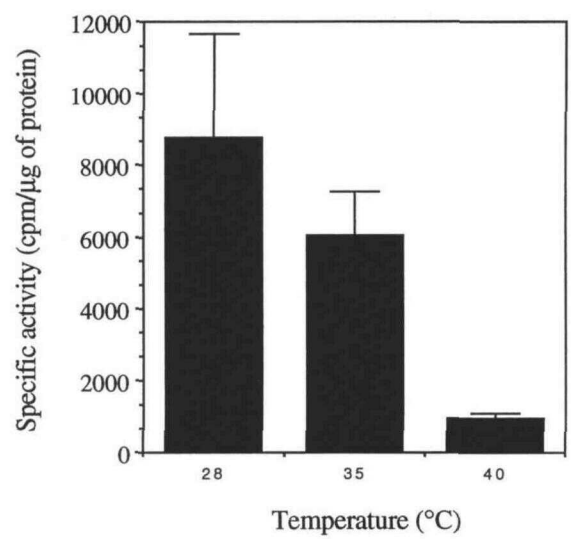

Figure 2. Incorporation of [^{35}S]Met into DH5 × DH7 microspore proteins in different temperature conditions. Protein content and TCA-precipitable counts were determined in DH5 × DH7 microspore extracts as described in "Materials and Methods," and the amount of precipitable radioactivity was then plotted against to the quantity of protein. The values are the means and SE of the determinations performed on the different extracts for each temperature condition.

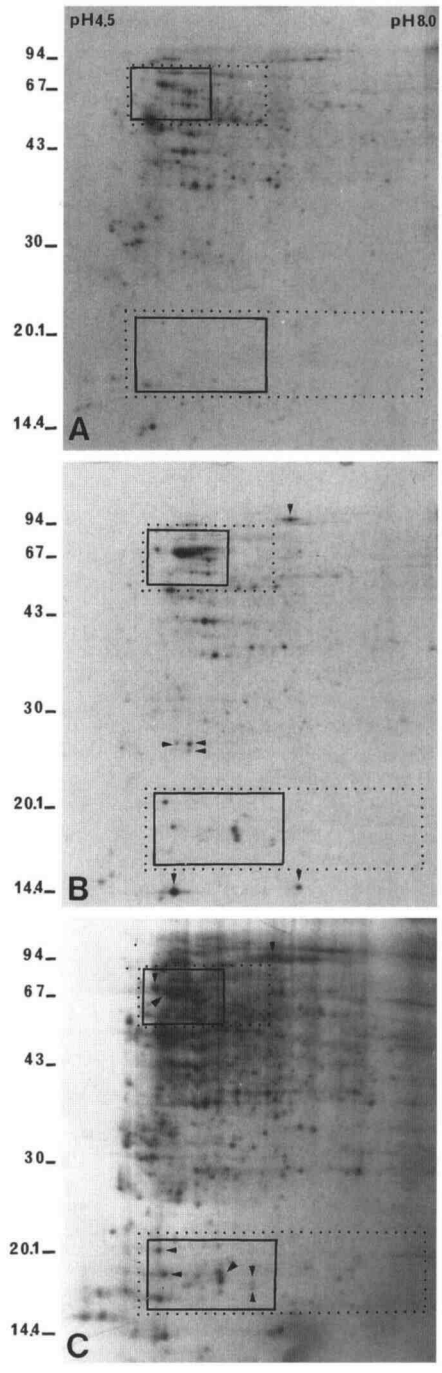

Figure 3. 2D fluorograms of in vivo labeled proteins expressed by DH5 × DH7 microspores in different temperature conditions. A, 28°C; B, 40°C; C, 35°C. The top and bottom boxes delineate the positions of the 70- and 60-kD HSP families on the one hand and the positions of the low-molecular-mass (17–22 kD) HSP family on the other hand. The areas delimited by dotted lines are detailed in Figures 4 and 5, whereas those bounded by continuous lines are presented in Figures 6 and 7. In B, arrowheads indicate the HSPs belonging to the 100-, 25-, and 14-kD groups. In C, arrowheads point to HSPs 100, 73, 70a, and major members of the low-molecular-mass HSP group (see text). Molecular mass scale is in kD.

HSP73 were mostly heat inducible. However, some faint spots could be visualized on control fluorograms at the relevant positions (Figs. 4, A and B, and 6, A and F). Low-level synthesis of the inducible forms at 28°C likely accounts for the detection of these spots. Conversely, spot 70a was already clearly expressed at the control temperature (Fig. 4A). Spot 70a and spot 70b were the only HSP70 family members detected by the anti-cytoplasmic HSP70 antibody (Fig. 4C). Notably, western blot analysis revealed that the proteins that constituted 70a and 70b were present at similar levels at 28°C (not shown) and 40°C (Fig. 4C), although their synthesis was markedly increased during heat shock (Fig. 4, A and B). The use of an anti-mitochondrial HSP70 antibody allowed us to identify several 66-kD proteins as additional HSP70 family members (Fig. 4D). Their molecular mass is consistent with that of previously characterized mature plant mitochondrial HSP70 (Neumann et al., 1993). Yet these proteins are immunodetected in identical amounts at 28°C (not shown) and 40°C (Fig. 4D), and their synthesis is not affected by heat shock (Fig. 4, A and B). Hence, they had not been classified at first as HSPs on the basis of fluorography analysis alone.

The comparison of 28 and 40°C fluorograms indicated that heat induced the synthesis of only one protein in the 60-kD class (referred to as spot 60, Fig. 4, A and B). This HSP was resolved as one spot of a cluster of 60-kD proteins synthesized at both 28 and 40°C (Fig. 4, box II). Spot 60 was not detected by the anti-potato HSP60 polyclonal antibody, yet the other proteins of the cluster were tentatively identified as members of the HSP60 family (Fig. 4, E and F). They were then assigned to mitochondrial HSP60 by the use of a more specific monoclonal antibody directed against maize mitochondrial HSP60 (data not shown). Neither their synthesis nor their abundance were markedly modified by heat shock (Fig. 4, A, B, E, and F). Surprisingly, the anti-potato HSP60 cross-reacted with a mitochondrial HSP70 (Fig. 4, E and F). It also detected additional HSP60 forms that displayed either constitutive expression or enhanced accumulation in response to heat shock (Fig. 4, E and F).

A combination of fluorography and western blotting data revealed a highly complex low-molecular-mass HSP expression pattern in microspores. The low-molecular-mass HSP group defined by [^{35}S]Met labeling comprised 10 different members with pIs ranging from 5.4 to 6.4, with some of them showing subtle variations between their molecular masses (Fig. 5, A and B). None of these HSPs was visible on 28°C fluorograms. Only six of these spots were detected by the anti-low-molecular-mass HSP antibody. Strikingly, they were also present at a high level at the control temperature (Fig. 5, C and D). In addition, some low-molecular-mass HSPs were immunodetected but did not display detectable labeling on fluorograms. In this latter group, four proteins were present at low basal levels at 28°C and accumulated upon heat shock (Fig. 5, C and D). By contrast, two other immunodetected proteins exhibited a surprising pattern of expression in that their levels were reduced during heat shock (Fig. 5, C and D). A last group of low-molecular-mass HSPs detected only on western

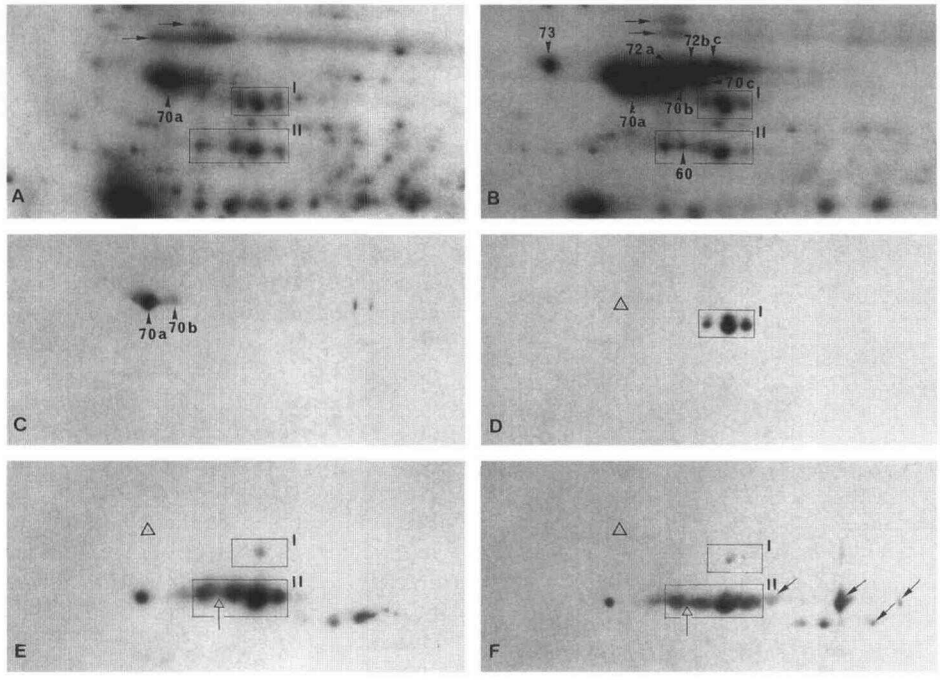

Figure 4. Expression of the 60- and 70-kD HSP families in DH5 × DH7 microspores. The area presented in this figure corresponds to the 60- to 80-kD area delimited by dotted lines in Figure 3. A and B, Fluorograms of [^{35}S]Met-labeled proteins expressed at 28 and 40°C, respectively. C and D, Immunodetection of HSP70 on blots of DH5 × DH7 40°C protein extracts. C, Cytoplasmic HSP70; D, mitochondrial HSP70; the 28°C extracts displayed identical profiles. E and F, Immunodetection of HSP60 on blots of 28 and 40°C DH5 × DH7 protein extracts, respectively, using the anti-potato HSP60 antibody. In A and B, the arrowheads point to the HSPs detected by fluorography. These HSPs are indexed according to their molecular masses; HSPs with identical molecular masses are distinguished by letters. In A and B, the arrows point to putative members of the HSP90 family (HSP81 and HSP82). Boxes I and II indicate the mitochondrial HSP70 and the HSP60 spots, respectively, that are detectable on fluorograms. The HSP60 spots contained in box II were also the only spots detected by the anti-maize mitochondrial HSP60 antibody (data not shown). In D, E, and F, the open triangle indicates the position of spot 70a. In E and F, the open arrow points to the position of spot 60, which was not immunodetected. In F, the black arrows point to the heat-inducible HSP60 detected by immunoblotting.

blots was included in a less accurately resolved area of the gel. Consequently, we could not precisely match up the three spots present at 28°C to the four species detected at 40°C (Fig. 5, C and D, boxed area).

It is interesting that microspores incubated at the intermediate temperature of 35°C displayed a composite protein profile: the bulk of the 28°C proteins was still synthesized, yet the major classes of HSPs were already induced (Fig. 3C). However, HSP synthesis appeared to proceed at a lower intensity at 35 than at 40°C, and consistent HSP spot visualization was only achieved on overexposed fluorograms (Fig. 3C). Conversely, overexposure of 28°C fluorograms did not result in the detection of heat-inducible HSPs.

Genetic Variability of the Microspore Heat-Shock Response

The heat-shock response of microspores isolated from the doubled haploid lines DH5 and DH7 and from the inbreds A188 and Mo17 was also evaluated using fluorography of [^{35}S]Met-labeled proteins. Inspection of the resulting control and heat-shock fluorograms revealed that these microspores exhibited the same type of highly reproducible protein synthesis alterations as DH5 × DH7 microspores, i.e. the repression of the global proteosynthesis and the induction of a set of HSPs (whole fluorograms not shown).

A considerable amount of qualitative and quantitative variability of the heat-shock response was found among the five genotypes (Figs. 6 and 7). In the HSP70 group, both quantitative and qualitative variations occurred so that each genotype displayed a unique HSP70 pattern (Fig. 6). Among the clear-cut quantitative differences, HSP73 was expressed at higher levels at 40°C in DH5, DH5 × DH7, and Mo17 than in A188, whereas it hardly displayed heat inducibility in DH7 (Fig. 6). More subtle and yet reproducible variations of the relative spot intensities were also found among cultivars. For example, DH5 displayed a specific HSP72b predominant labeling among the 72-kD forms, which was not seen in those genotypes also expressing 72b (Fig. 6G). At the qualitative level, HSP72 forms were detected in a genotype-dependent manner. For instance, 72d and 72e were found to be synthesized by only Mo17 (Fig. 6J). The major cluster 70a also clearly displayed genetic variability, and according to its global shape on the 40°C fluorograms, three groups could be distinguished: (a) DH5 × DH7 and A188; (b) DH5 and Mo17, which displayed a

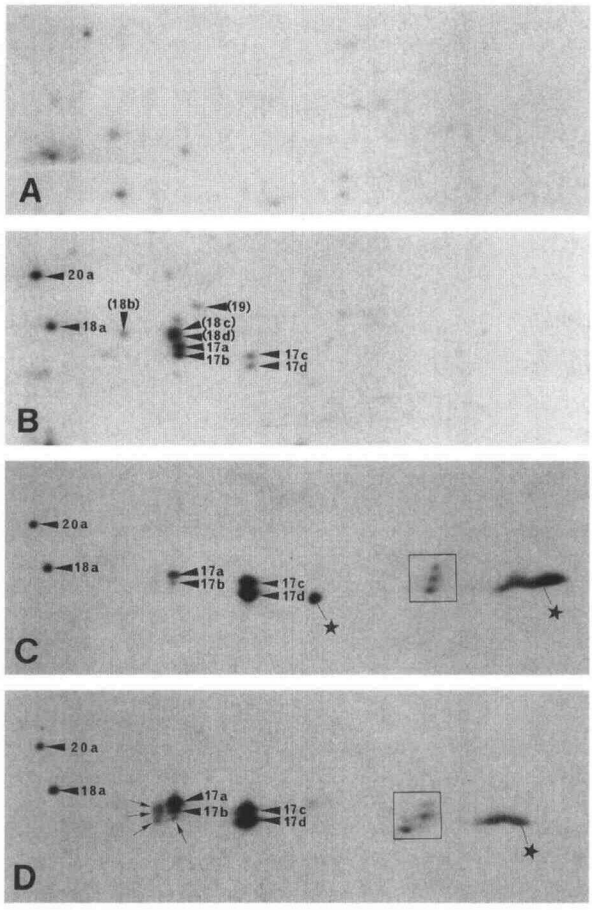

Figure 5. Expression of the low-molecular-mass HSP family in DH5 × DH7 microspores. The area corresponds to the 17- to 22-kD area delineated by dotted lines in Figure 3. A and B, Fluorograms of [^{35}S]Met-labeled proteins. A, 28°C extract; B, 40°C extract. C and D, Immunodetection of low-molecular-mass HSPs in 28°C (C) and 40°C (D) extracts. The low-molecular-mass HSPs characterized by fluorography are designated by arrowheads and labeled as in Figure 4B. Those that are not immunodetected are indicated by parentheses. Among the HSPs specifically detected on immunoblots, those that are heat inducible are designated with arrows, whereas those presenting a higher expression at 28°C relative to 40°C are marked with stars. The boxed area encompasses three spots present on the 28°C immunoblots, which do not precisely match up to the four species detected at 40°C (see text).

characteristic prominent extension of the bottom part of the spot (this pattern is already observed at 28°C); and (c) DH7, in which the 70a cluster expressed at 40°C was primarily resolved as a streak showing four distinct broad areas.

As in the hybrid, the low-molecular-mass HSPs were not present on control fluorograms in the four inbreds, and they were globally synthesized at approximately similar levels in the five genotypes in response to heat shock (Fig. 7). Their detailed patterns of expression appeared even more polymorphic than those found for the 70- to 73-kD HSPs. A188 and Mo17 displayed rather similar expression, although Mo17 lacked 18a, which is a major spot in A188 (Fig. 7, H and J). Also, DH5 × DH7 and its male parent DH7 synthesized the same low-molecular-mass HSPs except for the three species 17a, 17d, and 18a (Fig. 7, B and F). In contrast, the DH5 pattern appeared quite different (Fig. 7, B, D, and F). This contrasts with the similarities observed between the DH5 and DH5 × DH7 patterns for the HSP70 family.

Apart from the two main classes, genetic variability was also found for the other HSPs. Among the four inbreds, only DH5 and Mo17 displayed detectable HSP100 expression (data not shown). The cluster of mitochondrial HSP60 was expressed at both control and heat-shock temperatures in all of the five genotypes (Fig. 6). However, spot 60 synthesis was not induced by heat in DH5, whereas DH7 displayed concomitant induction of this spot and decreased synthesis of the others members of the cluster (Fig. 6, G and H).

Finally, it is noteworthy that the heat-shock profile of DH5 × DH7 microspores represented a combination of DH5 and DH7 microspore HSP patterns (Figs. 3, 6, and 7). However, two exceptions were found: one of the 25-kD HSPs and spot 17d were induced in DH5 × DH7 microspores but were not detected in the parent strains.

DISCUSSION

High-Resolution Analysis of the HSP Expression in Maize Microspores

Several previous reports have emphasized that the strength of the heat-shock response of maize pollen is highly dependent on the developmental stage; whereas mature and germinating pollen is unable to detectably produce typical HSPs (Cooper et al., 1984; Dupuis and Dumas, 1990; Hopf et al., 1992), microspores and young developing pollen grains display the synthesis of a set of HSPs in response to a temperature increase (Frova et al., 1989). However, the scope of these studies has been limited by the use of field-grown material and one-dimensional protein electrophoresis techniques. In this way, it has been demonstrated that field-cultivated plants synthesize heat-inducible HSPs during a normal growing season (Hernandez and Vierling, 1993). Because the quality of the starting material might represent a critical parameter in stress physiology and HSP expression studies, it is important to emphasize that in the present work constant optimal growing conditions prevented any possible influence of stress experienced by the plants before the experiments.

Furthermore, reports focusing on HSP gene expression during maize pollen development arose from experiments using either whole tassels (Hopf et al., 1992; Marrs et al., 1993) or whole anthers (Atkinson et al., 1993) as sources for RNA or protein extraction; thus, they do not depict the exact pattern of HSP gene expression and inducibility in the gametophytic cells per se. In contrast, we have analyzed the synthesis of HSPs in staged isolated microspores.

In addition, 2D gel analysis of proteins is a necessary tool in the evaluation of HSP expression, because in higher plants most HSPs are encoded by multigenic families in which highly homologous members are found (Vierling, 1991). This is especially true for the HSP70 and low-

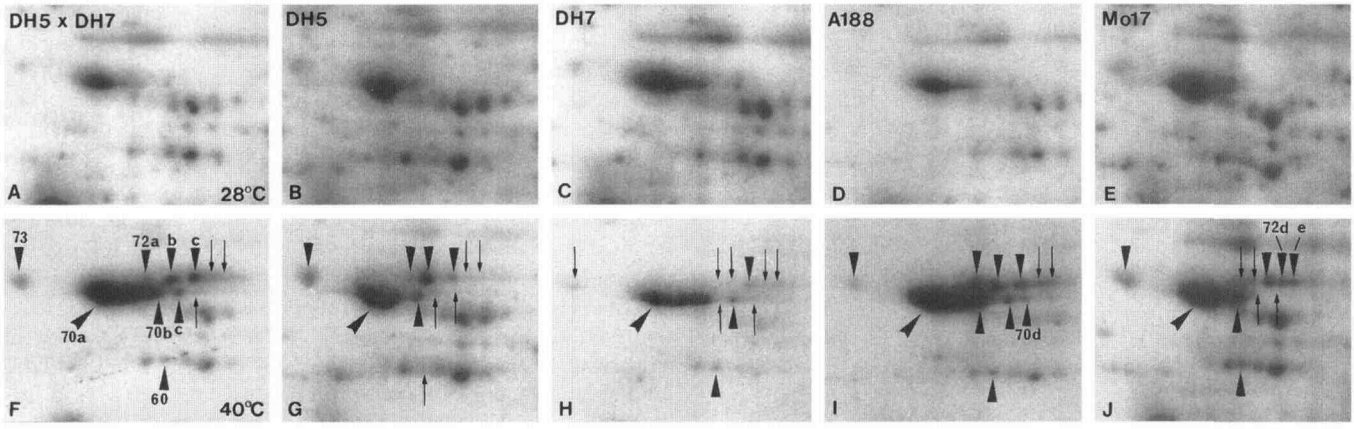

Figure 6. Expression of the 60- and 70-kD HSP families analyzed by fluorography of [^{35}S]Met-labeled proteins in the five genotypes. Details of the 60- to 80-kD area of the control (A–E) and heat-shock (F–J) fluorograms are shown. This area corresponds to the top box delineated by continuous lines in Figure 3. A and F, DH5 × DH7; B and G, DH5; C and H, DH7; D and I, A188; E and J, Mo17. Arrowheads point to HSPs actually expressed in the considered genotype, whereas arrows indicate the positions of HSPs detected in one or several other lines but not visible in the considered genotype. For instance, HSP 73 synthesis is increased upon heat shock in all of the genotypes but DH7.

molecular-mass HSP families and also appears to be also the case for maize (Atkinson et al., 1989, 1993; Jorgensen et al., 1992; Bates et al., 1994; Jorgensen and Nguyen, 1995). In this first characterization of maize microspore HSPs by 2D techniques, the electrophoresis conditions, i.e. wide pH gradient IEF and 12% acrylamide 2D gels, were chosen to maximize the range of HSP detection while attaining a sufficient resolution of both high- and low-molecular-mass HSPs.

To characterize the pattern of microspore HSP expression, we have studied two separate parameters. The net synthesis of HSPs after incubation at 28 or 40°C was evaluated by [^{35}S]Met incorporation, whereas their amount at the termination of the incubation was assessed by immunodetection. In their pioneering study of HSP inducibility during maize pollen development, Frova et al. (1989) were unable to obtain [^{35}S]Met incorporation into isolated microspores and thus incubated whole anthers to label microspore proteins. This latter procedure provided low and somewhat inconsistent labeling. In contrast, in this study we used refined procedures to isolate and culture highly viable gametophytic populations in suitable amounts and under appropriate conditions for the detailed study of HSP synthesis and accumulation.

Complexity of the Maize Microspore HSP Profile

Using this combination of immunodetection and fluorography studies, we have characterized the patterns of HSP expression in maize microspores at both control and heat-shock temperatures. Some HSPs were found to be constitutively expressed, whereas others were specifically induced upon heat shock. Consequently, we have classified proteins as HSPs either because they were detected by an anti-HSP antibody at either temperature or because they presented an enhanced expression on 40°C fluorograms.

Our data extend the complexity of the maize microspore HSP profile far beyond what has already been described (Frova et al., 1989), especially concerning the large number of species detected in the different HSP classes.

For instance, in the DH5 × DH7 HSP70 family, high-resolution 2D electrophoresis resolved at least 11 different spots, including mitochondrial HSP70. However, it is probable that different HSP70 forms may actually be issued from posttranslational modifications of a polypeptide encoded by a single gene. Among the HSP70 family members, the use of specific antibodies allowed us to discriminate among subclasses of HSP70 proteins. Whereas the antibody directed against mitochondrial HSP70 detected a group of at least four spots, spots 70a and 70b were the only ones recognized by the anti-cytoplasmic HSP70 antibody. However, this antibody was raised against a human HSP70, and although it presumably recognizes an epitope located in the highly conserved ATP-binding domain of cytoplasmic HSP70, it may not recognize all plant cytoplasmic HSP70s. Microspore cytoplasmic HSP70 could also be present at levels not sufficient for consistent immunodetection. Alternatively, some microspore HSP70 species (e.g. HSP72 and HSP73) may correspond to chloroplast- or ER-localized (DeRocher and Vierling, 1995) HSP70 members.

The information available concerning plant HSP60 indicates that this HSP family comprises both mitochondrial and chloroplastic members. In the species studied so far, only one single mitochondrial HSP60 gene and a small number of genes coding for chloroplastic α and β forms have been characterized (Prasad and Stewart, 1992; Tsugeki et al., 1992; Zabaleta et al., 1994). However, in our study the anti-potato HSP60 polyclonal antibody detected numerous species, and, strikingly, four of these spots were also recognized by a monoclonal antibody directed against maize mitochondrial HSP60. This raises the possibility that complex posttranslational regulations result in the synthesis of several isoforms of single HSP60 translation products.

2D analysis also allowed the detection of numerous low-molecular-mass HSPs, whereas Frova et al. (1989) detected

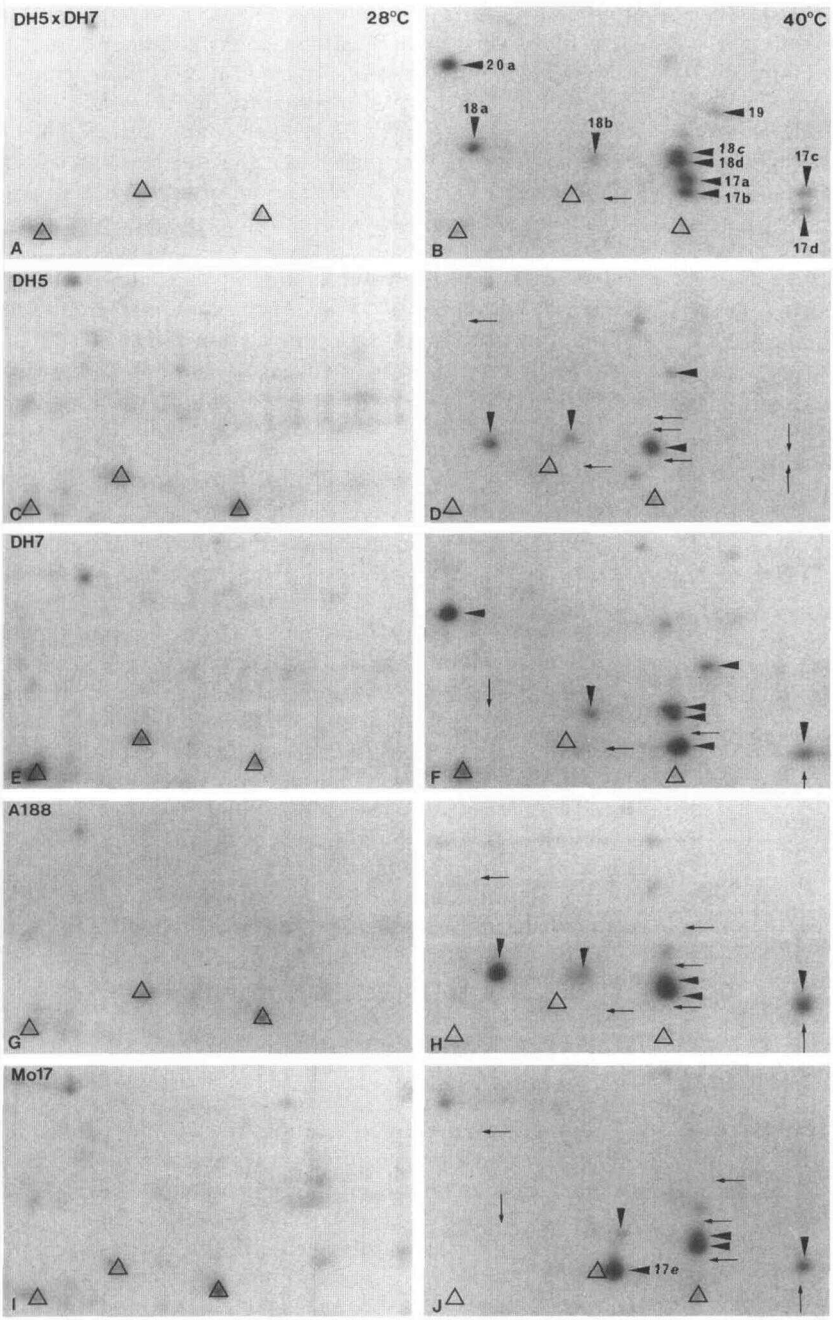

Figure 7. Expression of the low-molecular-mass HSP family analyzed by fluorography of [^{35}S]Met-labeled proteins in the five genotypes. Details of the 17- to 22-kD areas of the control (A, C, E, G, and I) and heat-shock (B, D, F, H, and J) fluorograms are shown. This area corresponds to the bottom box delineated by continuous line in Figure 3. A and B, DH5 × DH7; C and D, DH5; E and F, DH7; G and H, A188; I and J, Mo17. The use of arrowheads and arrows is the same as in Figure 6. The open triangles indicate the positions of three spots detected at 28°C in all the five genotypes and are used as reference marks to compare the 40°C fluorograms with their 28°C counterparts.

only one HSP band with the same M_r in maize microspores. Our results are supported by the comparison of our fluorograms with those depicted for maize seedlings in previous reports (Atkinson et al., 1989; Goping et al., 1991; Jorgensen and Nguyen, 1995). The anti-low-molecular-mass HSP antibody did not detect the totality of the low-molecular-mass HSPs revealed by fluorography. However, since this antibody is directed against cytoplasmic class II low-molecular-mass HSPs (Goping et al., 1991), it may not recognize the low-molecular-mass HSPs belonging to the other classes (cytoplasmic class I, endomembrane-localized or chloroplast-localized low-molecular-mass HSPs), which present only limited homologies with class II low-molecular-mass HSPs (Vierling, 1991). Alternatively, some proteins may not be detected because they present a high turnover, preventing their accumulation in an immunodetectable amount.

In conclusion, our results emphasize that maize microspores isolated close to first pollen mitosis are able to synthesize a complete array of HSPs.

Expression of Microspore HSPs at Non-Heat-Shock Temperature

Strikingly, maize microspores show a marked developmental HSP expression, and they display an intricate HSP profile

at normal temperature. Indeed, numerous members of at least three major HSP families (70 kD, 60 kD, and low-molecular-mass HSP) were already expressed at this temperature. Although such expression at control temperature might also be partly induced or enhanced by the isolation procedure and (or) culture of the cells in artificial conditions, these results are in agreement with reports describing the expression of HSPs at normal growth temperature in several systems, including plant systems (reviewed by Winter and Sinibaldi, 1991). For instance, the constitutive forms of HSP70 and HSP60 appear to be involved in normal cellular processes such as protein folding and intracellular targeting. In particular, mitochondrial HSP70 and HSP60, as well as chloroplastic HSP60, assist the folding of proteins after their import in the mitochondrial or chloroplastic matrices (Gething and Sambrook, 1992). Indeed, our data that show a constitutive expression of the mitochondrial HSP70 and of most of the HSP60 species in maize microspores are consistent with such functions. However, two separate regulatory mechanisms may explain the expression of these members of the HSP60 family at 28°C: some proteins were detected on both fluorograms and western blots of 28°C protein extracts, whereas the others were present only on immunoblots. The proteins of the former group may not be very stable and their constant level is likely to be maintained through constant synthesis, whereas in the latter group, the proteins are presumably more stable.

In addition to their implication in general cellular processes, a growing amount of evidence suggests more specific roles for certain HSPs during cell proliferation processes and development in a variety of eukaryotic organisms (Hightower and Nover, 1991; Winter and Sinibaldi, 1991; Lane et al., 1993). For instance, HSP70 expression has been reported during meiosis of mammalian spermatogenesis (Wolgemuth and Gruppi, 1991). Our results emphasize the developmental expression of HSP70 family members during male gametogenesis in maize and are in accordance with the data of Duck and Folk (1994) and Gagliardi et al. (1995) on HSP70 gene expression during pollen development in tomato and maize. Taken together, these data lead to the conclusion that certain HSP70s may have important functions during male gametogenesis in both plants and animals.

Similarly, low-molecular-mass HSP expression correlates with animal developmental processes. For instance, expression of low-molecular-mass HSPs at non-heat-shock temperature has been described at specific stages of *Drosophila* development and in differentiating tissue culture mammalian cells. In particular, mammalian HSP27 may be involved in regulating the dynamics of actin filaments in response to growth factor stimulation (Arrigo and Landry, 1994).

The developmental role of the plant low-molecular-mass HSPs is far from being so extensively documented. Our data at the protein level extend those of Dietrich et al. (1991) and Atkinson et al. (1993) who reported developmental regulation of specific HSP18 mRNAs during maize male development and particularly their accumulation in anthers near meiosis. In addition, our data indicate that most of the members of the maize microspore low-molecular-mass HSP family are likely to be very stable, since they were present at the control temperature on immunoblots and were not detected on 28°C fluorograms. Such stability of the plant low-molecular-mass HSPs has been reported previously (DeRocher et al., 1991).

Hernandez and Vierling (1993), Coca et al. (1994), and DeRocher and Vierling (1994) also reported the accumulation of low-molecular-mass HSPs at non-heat-shock temperature during seed development in several species. It is interesting that these authors suggest that such HSP expression could contribute to the protection of the embryo during the process of seed desiccation. Since a similar desiccation process also occurs at the terminal phase of pollen development, this raises the possibility that low-molecular-mass HSPs may have a common function in both processes.

In maize, the HSP90 family comprises two members, HSP81 and HSP82 (Marrs et al., 1993), and similarly to HSP18 mRNAs, HSP81 mRNA accumulates in anthers at a microspore stage of male development (Marrs et al., 1993). The comparison in DH5 × DH7 of fluorograms from heat-shocked roots, from control roots, and from different stages of pollen development (D. Gagliardi, P. Vergne, and C. Dumas, unpublished data) suggests that two streaks visible on our control and heat-shock microspore fluorograms (Fig. 4, A and B) may correspond to the expression of HSP90 forms. However, analysis with specific antibodies would be necessary to clarify this point.

Such developmental expression of members of different HSP families in maize microspores is in accordance with a possible implication of these proteins in pollen development. Furthermore, this expression near first pollen mitosis could be linked to the important developmental changes occurring at this key stage of pollen formation.

Modification of HSP Expression following Temperature Elevation

Following temperature elevation to 40°C, the pattern of HSP expression displayed important alterations to the 28°C profile. These heat-induced modifications were especially striking in the low-molecular-mass and 70-kD families.

Although numerous members of the low-molecular-mass HSP family were already immunodetected at 28°C, their synthesis was not detected by fluorography at this temperature. The detection of most of these proteins on fluorograms following heat shock indicates an increase in their rate of synthesis. However, because the intensity of the signal was approximately equivalent on 28 and 40°C immunoblots, the global amount of these proteins was probably not extensively modified by heat shock, indicating a strong turnover of these proteins at 40°C and their nonaccumulation in response to heat shock.

The nondetection of several HSP70 species by the anti-HSP70 antibodies used in this study does not allow such a precise analysis for the HSP70 family members. However, although comparison of the 28 and 40°C fluorograms indicates that heat shock resulted in an increase in the rate of synthesis of several HSP70 family members, including HSP70a and HSP70b, the comparison of control and heat-

shock immunoblots for these two spots shows that they did not accumulate in response to heat.

Thus, this analysis reveals that, although the heat-shock response of maize microspores is characterized by the increased synthesis of numerous HSPs belonging to all known HSP families (Frova et al., 1989; this study), the response is also characterized by a nonaccumulation of some of these proteins in at least two of the major HSP families. These results parallel the data of Hopf et al. (1992), which showed that in heat-shocked germinating pollen, HSP70 and HSP18 mRNAs were synthesized but accumulated to only very low levels. These data lead to the hypothesis that the inability of mature pollen to survive high-temperature stress is at least partly due to its inability to accumulate HSPs in sufficient amounts upon heat shock, and that this characteristic may already occur in younger stages of pollen development.

It is also important to notice that some proteins not detected at control temperature either on immunoblots or fluorograms were revealed by the anti-low-molecular-mass HSP antibody on 40°C immunoblots only. Thus, the expression of these proteins following heat shock does not seem to depend on an increase in their rate of synthesis, since they were not detected on 40°C fluorograms. One explanation may be that they result from posttranslational modifications of preexisting proteins such as those detected on 28°C immunoblots.

Strikingly, heat shock had an opposite effect on some low-molecular-mass HSPs, which were immunodetected at 28°C but not on 40°C blots. Although we cannot exclude the possibility that the antibody directed against low-molecular-mass HSPs cross-reacted with some unrelated proteins, these species may also be HSPs implicated in developmental events or normal cellular processes whose function would not be essential during heat shock.

Genetic Variability of Microspore HSP Expression

In the present work, we also showed that the maize microspore heat-shock response is characterized by a high level of both qualitative and quantitative genetic variability. In this respect, the microgametophytic phase is thus similar in maize to the sporophytic phase (Jorgensen and Nguyen, 1995). Our data point to the possibility that differences in HSP expression between genotypes might result in specific abilities to withstand stress. For instance, the capacity of certain genotypes to express HSP100 may correlate with a better thermotolerance, as previously described for yeast (Sanchez et al., 1992). Complex and subtle differences of HSP expression between lines of maize might reflect putative differential specificities of function and activity among the members of a given HSP family. In particular, allelic variation may partly account for the variability observed. For instance, spots 72d and 72e, which are specifically expressed in Mo17, might be the allelic counterparts of 72a and 72b. On the other hand, it is likely that our data recorded at a single heat-shock time overestimate the overall qualitative variability of microspore HSPs among the genotypes studied. Indeed, Jorgensen et al. (1992) showed that several HSP mRNAs common to two maize lines were induced in leaves at different times of heat shock. Also, the synthesis of some HSPs might be more transient than that of other HSPs or the maximal expression of some HSPs might occur at more moderate stress temperature (Vierling, 1991). Therefore, we cannot rule out that similar differential gene regulations operate in the microspores of the five genotypes we studied and that they account in part for the genetic variability detected in the present work. Such differential regulations, as well as recombination events, might also explain the few discrepancies between the actual HSP pattern displayed by the microspores of the hybrid DH5 × DH7 and the one expected from the profiles shown by the microspores of the parent lines. In addition, given the complementary information revealed by the use of specific antibodies in DH5 × DH7, it is likely that a similar study performed on the other genotypes could greatly modify our appreciation of the complexity of their HSP profiles.

In conclusion, we have designed an experimental system, i.e. immature maize pollen isolation from high-quality mother plants and subsequent protein analysis by high-resolution 2D electrophoresis, that will serve as a basis for the molecular analysis of HSP expression during pollen development. Other experiments have shown that these microspore fractions are equally suitable for mRNA extraction and analysis (Gagliardi et al., 1995). As a first output of this experimental system, we characterized the highly complex microspore HSP profiles expressed both in response to heat shock and during normal development. We also provided insights into both the specificity and the genetic variability of the microspore heat-shock response.

ACKNOWLEDGMENTS

We acknowledge Dr. M. Beckert (Institut National de la Recherche Agronomique, Station d'Amélioration des Plantes, Clermont-Ferrand, France) for providing the maize seeds and Professor C.J. Leaver and Dr. A. Liddell (University of Oxford, Department of Plant Sciences, Oxford, UK) for the gift of anti-potato HSP60 antibody. We are grateful to Professor T.E. Elthon (University of Nebraska-Lincoln, School of Biological Sciences, Lincoln, NE) for the gift of anti-maize mitochondrial HSP60 and anti-maize mitochondrial HSP70 antibodies. We acknowledge Professor D.B. Walden (University of Western Ontario, Department of Plant Sciences, London, Ontario, Canada) for the gift of the anti-low-molecular-mass HSP antibody. We thank Richard Blanc for taking care of the plants and Pierre Audenis for photographic work. We acknowledge Drs. Elizabeth Bates, Dominique Gagliardi, and Peter Rogowsky for critical reading of the manuscript and for helpful discussions.

Received December 6, 1995; accepted April 2, 1996.

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