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Expression of heat shock factor and heat shock protein 70 genes during maize pollen development

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Abstract

We have analysed the expression of heat shock protein 70 (HSP70) and heat shock factor (HSF) gene during maize pollen development, HSFs being the transcriptional activators of *hsp* genes. In order to eliminate the sporophytic tissues of anthers, we have isolated homogeneous cell populations corresponding to five stages of maize pollen development from microspores to mature pollen. We show that in the absence of heat stress, *hsp70* genes are highly expressed late-bicellular pollen as compared to other stages. HSP70 transcripts are significantly accumulated in response to a heat shock at the late microspore stage but to a much lower extent than in vegetative tissues. The latest stages of pollen development, i.e. mid-tricellular and mature pollen, do not exhibit heat-induced accumulation of HSP70 transcripts. Therefore, we analysed the expression of *hsf* genes throughout pollen development. We demonstrate that at least three *hsf* genes are expressed in maize and that transcripts corresponding to one *hsf* gene, whose expression is independent of temperature in somatic as well as in microgametophytic tissues, are present at similar levels throughout pollen development. In addition, we show that the expression of the two other *hsf* genes is heat-inducible in maize vegetative tissues and is not significantly increased after heat shock at any stage of pollen development. These results indicate that the loss of *hsp* gene expression at late stages of pollen development is not due to a modification of *hsf* gene expression at the mRNA level and that *hsf* gene expression is differentially regulated in vegetative and microgametophytic tissues.

Introduction

Heat shock proteins (HSPs) constitute a non-homogeneous group of proteins that have been originally defined by their increased expression after a temperature upshift [2, 51]. However, several members of the multigenic *hsp* gene families are also expressed during normal growth in the

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X82943.

absence of stress [2, 51]. During the past few years, transcription of members of *hsp90*, *hsp70* and low-molecular-weight *hsp* gene families have been reported during gametogenesis and embryogenesis in animals [26]. However, the precise role of these HSPs in developmental decisions is still unknown. Interestingly, the heat-induced expression of inducible *hsp* genes is often repressed or incomplete during developmental processes [26].

In plants, the developmentally regulated expression of *hsp* genes has been mostly studied during embryogenesis, flower and anther development [15, 17, 18, 30, 38, 49, 51, 52]. In particular, it has been shown that members of *hsp90* and *hsp18* gene families are specifically expressed at certain developmental stages of whole maize anthers [4, 10, 19, 33]. However, it remains to be determined whether these genes are expressed within the sporophytic tissues of the anther or in the microgametophytes per se.

The ability of the HSPs to be synthesized upon heat shock has been shown to be lost gradually during maize pollen development [23] and is completely lost in mature and germinating pollen grains [16, 22, 23]. Likewise, a reporter gene under the control of the *hsp70* promoter of *Drosophila* is induced by heat in vegetative tissues of transgenic tobacco plants whereas heat shock fails to stimulate its expression in pollen [46]. However, in transcriptionally active germinating pollen, *hsp70* and *hsp18* genes are transcribed in response to heat shock, but at a very low level not sufficient for an effective accumulation of HSPs [27]. These studies on both developmentally regulated and heat-induced expression of *hsp* genes during pollen development raise the problem of the control of *hsp* gene transcription.

In eukaryotes, transcriptional control of *hsp* genes is mediated by the heat shock factors (HSFs) [31, 36, 45]. HSF binds to the heat shock element (HSE), a conserved sequence present in multiple copies in the promoter regions of *hsp* genes. In animals and yeasts, a constitutively synthesized HSF is responsible for the activation of *hsp* genes upon heat shock, and *hsf* gene expression is not modified by the temperature increase. In contrast, in tomato, the transcription of two *hsf* genes is induced by heat shock while another one is constitutively expressed [42]. A *hsf* gene whose expression is increased upon heat-shock has also been recently characterized in *Arabidopsis thaliana* [28]. Hence, the expression of *hsf* genes seems to be regulated in a different manner in plants and animals. To date, the expression of *hsf* genes during differentiation or developmental processes has been investigated in mammals and chicken. Analysis of HSF activity in mammalian developmental systems have suggested that distinct HSFs are responsible for the stress-induced and the developmentally regulated expression of *hsp* genes [35, 36, 41]. No data are currently available concerning *hsf* gene expression during any plant development process.

In the present paper, we have investigated the developmentally regulated and heat-induced expression of *hsp70* and *hsf* genes during maize pollen development. To this end, we have isolated pollen at five stages of development in order to eliminate any contribution of the sporophytic tissues of the anthers. We show that *hsp70* genes exhibit a developmentally regulated expression pattern and that HSP70 transcripts accumulate in response to heat shock only at the beginning of pollen development but to a much lesser extent than in vegetative tissues. Our results also indicate that transcripts of one *hsf* gene are present throughout pollen development, including mature pollen and that the expression of two other *hsf* genes, which is heat-inducible in vegetative tissues, is not significantly enhanced upon heat shock at any stage of pollen development.

These data lead to the conclusion that although *hsf* genes appeared to be differentially regulated in vegetative and microgametophytic tissues, the loss of *hsp* gene activation during maize pollen development is not due to a modification of the expression of *hsf* genes.

Material and methods

Plant material

Maize (*Zea mays* L.) plants (genotype DH5 × DH7) were grown with a 16 h illumination period

(700–800 $\mu E\, m^{-2}\, s^{-1}$) at 24/19 ± 1 °C (day/night) and 80 ± 5% relative humidity. DH5 and DH7 are doubled haploid lines obtained by *in vitro* androgenesis [5]. For vegetative tissue analysis, 10-day old plantlets were incubated for 2 h at 28 °C or 40 °C in water-saturated atmosphere. Leaves and roots were then quickly excised and immediately frozen in liquid nitrogen.

Immature pollen isolation

Five different stages of pollen development were studied: late microspore (vacuolated), late-bicellular pollen (approximately half starch-filled), early-tricellular pollen, mid-tricellular pollen and mature pollen. Late microspores were isolated as described by Gaillard *et al.* [24]. Late-bicellular and early-tricellular pollen were isolated from spikelets located at the bottom of fully emerged tassels which did not bear any dehiscing anthers. These selected anthers would have produced mature pollen 7–8 days later. Fragments of tassel branches were submerged in 0.5% (w/v) sodium hypochlorite solution for 10 min at 4 °C. The whole isolation procedure was then performed at 4 °C under sterile conditions. After 5 rinses with cold sterile water, each fragment was cut into 1–2 mm pieces in MBKS12 buffer (20 mM MOPS pH 7.1, Brewbacker and Kwack's salts [12], 12% sucrose (w/v)). After gentle stirring, the slurry was filtered through a 100 μm pore size sieve to remove large anther debris. Immature pollen was then retained on a 50 μM pre size sieve. After resuspension in MBKS12, bicellular and early-tricellular pollen were loaded onto discontinuous Percoll (Pharmacia) gradients (25%, 35% and 50% (v/v) in MBKS12) and centrifuged at 250 × *g* for 4 min. Early-tricellular pollen pelleted to the bottom of the gradient. The bicellular pollen fraction was collected at the top of the 35% Percoll layer and, in order to eliminate some contaminating stages, recentrifuged at 250 × *g* for 4 min through a 25–35% Percoll gradient. Both late-bicellular and early-tricellular pollen fractions were collected and washed once with MBKS12 and incubated in this buffer as described below. Mid-tricellular pollen was isolated as detailed for early-tricellular pollen but from the bottom of tassels whose tops bore dehiscing anthers. The selected anthers would have produced pollen 3–4 days later. Mature pollen was collected at anthesis and it was checked that its water content, which is a good criterion for pollen quality [29], was at least 55% (w/w) of its fresh weight. Incubation buffer for mature pollen was 20 mM MOPS pH 7.1, Brewbaker and Kwack's salts [12], 15% sucrose (w/v). All microgametophyte populations were incubated for 2 h at 28 °C or 40 °C and then stored in liquid nitrogen until analysis.

Nucleic acid extraction

Poly(A)$^+$ RNA was extracted using Dynabeads oligo(dT)$_{25}$ (Dynal) according to manufacturer's recommendations. Genomic DNA was extracted using the method of Rogers and Bendich [40] from 10-day old plantlet leaves.

Production of a maize hsf *probe*

Unstressed leaf poly(A)$^+$ RNA (0.5 μg) was treated by DNaseI (Boehringer-Mannheim) and extracted once with phenol-chloroform and twice with chloroform. After ethanol precipitation, single-strand cDNA was synthesized for 1.5 h at 42 °C in a 50 μl reaction of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 40 U RNasin, 10 mM DTT, 0.5 mM dNTP, 20 μg/ml oligo(dT)$_{16-18}$, 0.1 mg/ml BSA and 200 U M-MLV reverse transcriptase (Gibco BRL). Sense (P1) and antisense (P2) PCR primers were derived from the amino acid sequences P(KR)(YF)FKH and W(EQ)F(AE)NE which correspond to conserved motifs in the DNA binding domain of tomato, *Arabidopsis* and yeast HSFs (see Fig. 1 and Fig. 4 for primer sequences and motif positions). In a reaction volume of 100 μl, 2% of an unstressed leaf cDNA reaction was used for amplification in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μM of

each dNTP, 1 μM of each primer and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). PCR conditions were 94 °C/5 min followed by 30 cycles of 94 °C/45 s; 50 °C/45 s; 72 °C/45 s and a final step at 72 °C/5 min. Of the reaction 1% was subjected to a second round of PCR amplification under similar conditions with primers corresponding to P1 and P2 but containing *Pst* I and *Cla* I restriction sites, respectively. The resulting fragment was cloned into pBluescript and sequenced by the dideoxy chain termination method (Sequenase 2.0 kit, US Biochemical). This 101 bp fragment is further referred to as '*hsf* probe' (see Results).

Construction and screening of cDNA libraries

Two maize (inbred line A188) PCR-generated cDNA libraries obtained from male gametes (sperm cells) and transition-stage embryos were screened using the '*hsf* probe'. The construction of the maize transition-stage embryo cDNA library is described elsewhere [11]. Maize male gamete-enriched fractions were prepared from mature pollen according to Dupuis *et al.* [21]. Poly(a)$^+$ RNA was extracted from 4×10^6 isolated male gametes and were processed for cloning into λZAP (Stratagene) using the same strategy as for the embryo library. After *in vitro* encapsidation, approximately 2×10^5 recombinant bacteriophages were amplified to produce the working library. For each library, 10^6 phage were plated and transferred in duplicates onto Hybond-N membranes (Amersham). Filters were prehybridized at 60 °C for 3 h in 6× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate), 5× Denhardt's solution, 0.5% SDS and 0.2 mg/ml sheared and denatured herring sperm DNA. Hybridization was performed at 42 °C for 16 h in 6× SSC, 5× Denhardt's solution, 50% formamide, 0.5% SDS, 0.2 mg/ml sheared and denatured herring sperm DNA. The '*hsf* probe' was labelled by random priming (Boehringer-Mannheim) using [^{32}P]dCTP and added to a final concentration of 1.5×10^6 cpm/ml. Filters were washed for 2 × 30 min at 60 °C in 2× SSC, 0.5% SDS and for 2 × 30 min at 60 °C in 1× SSC, 0.25% SDS. Clone *Zmhsfa* was isolated from the transition-stage embryo cDNA library and clones *Zmhsfb* and *Zmhsfc* from the male gamete library by three rounds of plaque purification. Phagemids were *in vivo* excised according to Stratagene's protocol and sequenced as above.

Northern blot hybridization analysis

Poly(A)$^+$ RNA (ca. 0.5 μg) was electrophoresed on denaturing 1.5% agarose/2.2 M formaldehyde gels and blotted onto Hybond-N membranes (Amersham). We have determined by *in vitro* translation that actin mRNA level does not vary during heat shock at any stage of pollen development (data not shown). Filters were hybridized with an actin probe for comparing poly(A)$^+$ RNA amounts in unstressed and heat-shocked samples. We have also noticed by two-dimensional electrophoresis analysis of *in vitro* translated products that actin mRNA level is much lower in late microspores than in later stages of pollen development but its roughly constant from late-bicellular to mature pollen (data not shown). Thus, the actin control can be used to compare poly(A)$^+$ RNA amounts from late-bicellular to mature pollen. All probes were labelled by random priming (Boehringer-Mannheim) using [^{32}P]dCTP. Prehybridization and hybridization were as for library screening. The *hsp70* probe corresponds to a conserved 630 bp central part of cytoplasmic *hsp70* genes [6]. This probe was obtained by PCR amplification from maize genomic DNA using primers derived from the amino acid sequences GGEDFD and DNQPGV positioned at amino acids 233–238 and 435–440 with respect to the maize gene isolated by Rochester *et al.* [39]. When using the *hsp70* probe, actin probe and phagemid inserts, washes were for 2 × 30 min at 60 °C in 1× SSC, 0.5% SDS and for 2 × 30 min at 60 °C in 0.1× SSC, 0.25% SDS. When using the 101 bp '*hsf* probe', washes were performed at 55 °C.

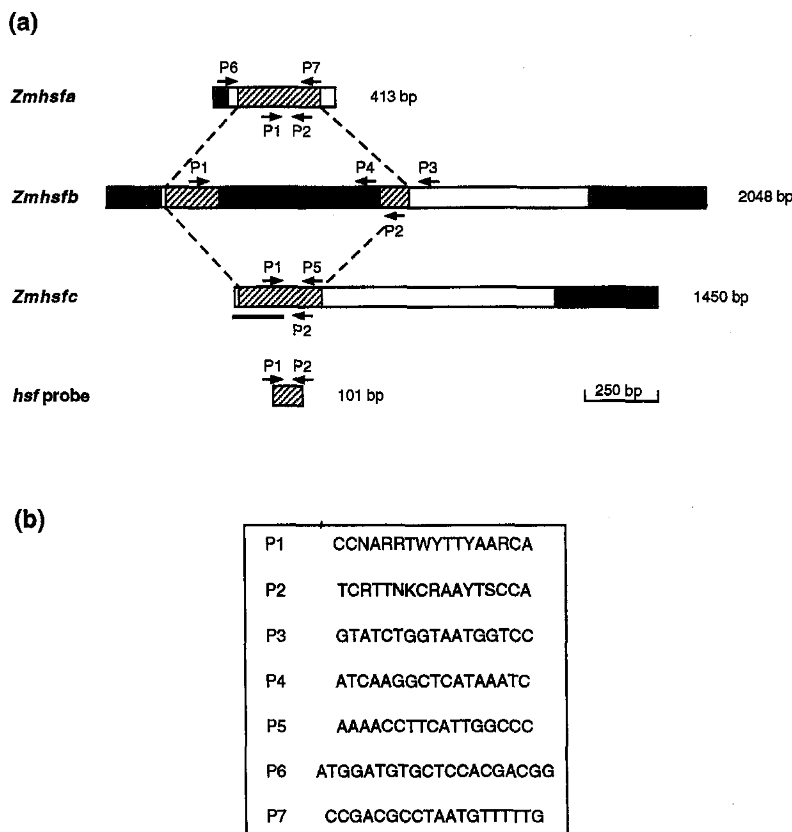
RT-PCR analysis

Three *hsf* gene-specific primer sets were designed from the sequences of three different maize *hsf* clones, *Zmhsfa*, *b* and *c* (see Fig. 1 for PCR primer positions and sequences, and Results for clone description). Fragments of 291, 206 and 108 bp were specifically amplified from clones *Zmhsfa*, *b* and *c* by the primer sets P6-P7, P1-P3 and P1-P5, respectively. RT-PCR experiments were conducted as described for the production of the 101 bp '*hsf* probe'. When using the P1-P5 primer set, the amplification efficiency was lower than that observed with the other primer couples and, thus, 1% of the first PCR reaction was subjected to a second round of PCR under the same conditions. Since the three primer couples amplify fragments which can hybridize to the 101 bp '*hsf* probe', amplification products could be unequivocally identified as part of *hsf* genes by subjecting aliquots of PCR reactions to Southern blot analysis. Hybridization conditions and washes were as for northern analysis.

Results

Isolation of microgametophyte populations at five different stages

We have isolated homogeneous microgametophyte populations corresponding to five different



P1	CCNARRTWYTTYAARCA
P2	TCRTTNKCRAAYTSCCA
P3	GTATCTGGTAATGGTCC
P4	ATCAAGGCTCATAAATC
P5	AAAACCTTCATTGGCCC
P6	ATGGATGTGCTCCACGACGG
P7	CCGACGCCTAATGTTTTTG

Fig. 1. Position and sequence of PCR primers. (a) Schematic representation of *Zmhsfa*, *b*, *c* clones and *hsf* probe. Position and orientation of PCR primers are indicated by arrows. Regions coding for DNA-binding domain are indicated by hatched rectangle, other coding regions by white rectangle, 5′ and 3′-untranslated regions by shaded rectangle, and intron by black rectangle. Note that the portion of *Zmhsfc* sequence which is underlined has been obtained by 5′ RACE-PCR. (b) Sequence of PCR primers. N = (G, A, T, or C); R = (A or G); W = (A or T); Y = (C or T); K = (G or T); S = (G or C).

stages of pollen development, i.e. late microspores, late-bicellular pollen, early- and mid-tricellular pollen and mature pollen (Fig. 2). These isolation procedures allowed the purification of 400000 to 1000000 gametophytes per tassel. Homogeneity was assessed by checking several cytological criteria that change during pollen development. Hence, starch accumulation as well as vacuole size were monitored according to Alexander's procedure [1], and the number and condensation state of nuclei were determined by DAPI staining (results not shown). Late microspores, late-bicellular or tricellular pollen never co-purified due to their distinct respective buoyant densities, which depend on the level of starch accumulation. However, a careful selection of spikelets before microspore isolation was necessary to avoid contamination with early-bicellular pollen, the density of which is identical to late microspore density. It should be noted that we never observed contamination by anther tissues. Cell viability was determined using fluorescein diacetate as an indicator of membrane integrity [25] and was 85–90% for late microspores and bicellular pollen and higher than 90% for tricellular pollen (results not shown). These isolated fractions correspond to defined developmental stages, display high cellular viability and

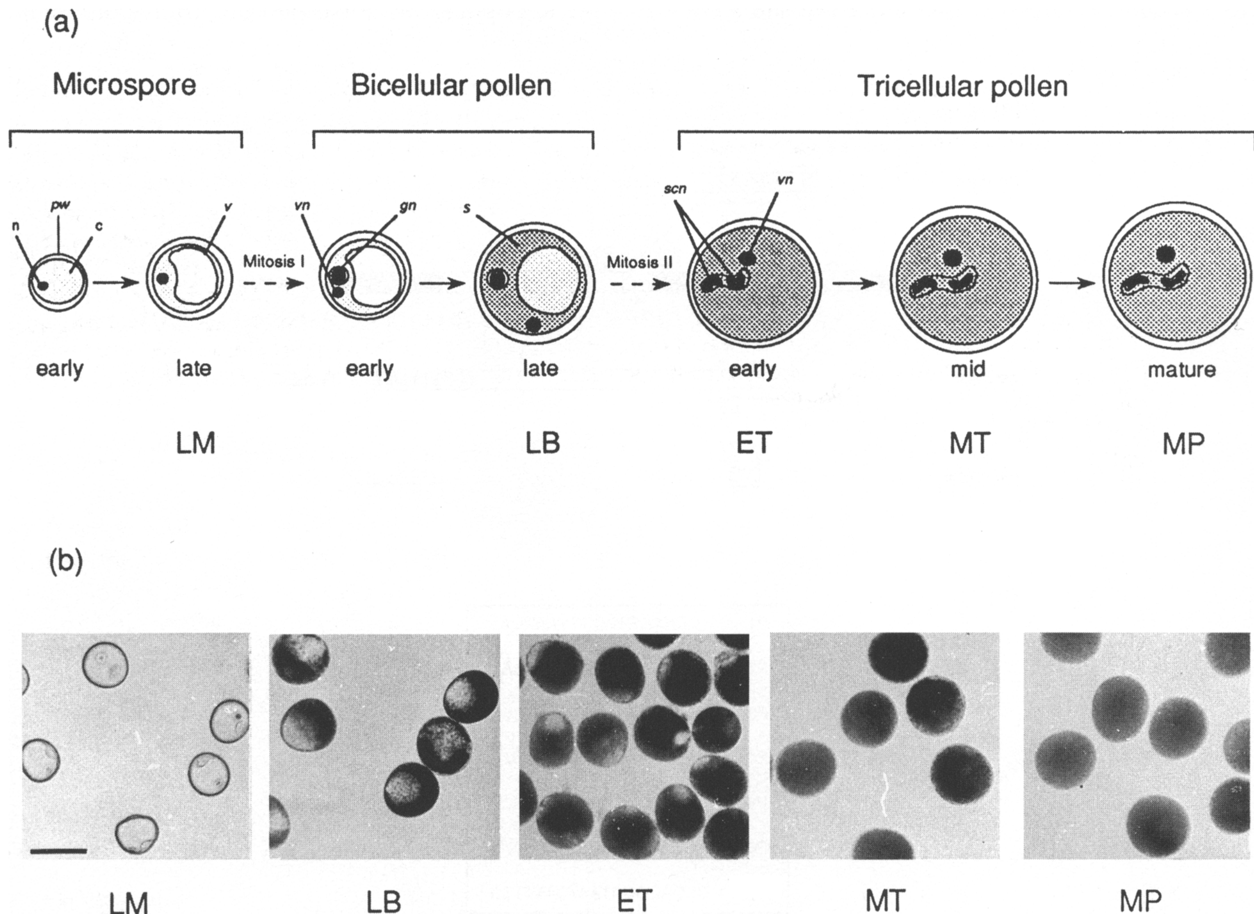


Fig. 2. Schematic representation of maize pollen development (a) and bright-field microscopy observation of isolated microgametophyte populations (b). LM, late microspore; LB, late-bicellular pollen; ET, early-tricellular pollen; MT, mid-tricellular pollen; MP, mature pollen. Cytological details are indicated: n, nucleus; pw, pollen wall; c, cytoplasm; v, vacuole; vn, vegetative nucleus; gn, generative nucleus; s, starch; scn, sperm cell nuclei. Note that the o-ring-shaped structure at the surface of late microspore (LM, b) corresponds to the germinative pore. Bar scale = 100 μm.

are devoid of any sporophytic contamination. They are then suitable for molecular investigations of pollen development.

Expression of hsp70 *genes during maize pollen development*

In order to determine the heat-induced and developmentally regulated expression of *hsp70* genes during maize pollen development, the abundance of HSP70 transcripts was investigated at the five stages of pollen development by northern blot analysis (Fig. 3). The probe we used can hybridize to both constitutive and inducible maize cytoplasmic *hsp70* genes [6]. Interestingly, in the absence of stress, *hsp70* genes were highly expressed at the late-bicellular stage as compared to leaves or other stages of pollen development (Fig. 3a, lanes c). As described earlier for this probe [6], a heat shock-induced accumulation of HSP70 transcripts in leaves (Fig. 3a, lanes L). This increase was also detectable in late microspores but in a significantly weaker manner (Fig. 3a, lanes LM). At the late bicellular stage, the high level of HSP70 mRNA remained constant upon heat shock (Fig. 3a, lanes LB). The signal increase for the heat-shocked early tricellular stage is overestimated due to a larger amount of poly(A)$^+$ RNA (Fig. 3a and b). The level of HSP70 mRNA remained constant in case of a heat shock at mild tricellular stage and, for comparable amounts of poly(A)$^+$ RNA, no signal was detected in control or heat-stressed mature pollen (Fig. 3a, lanes MP).

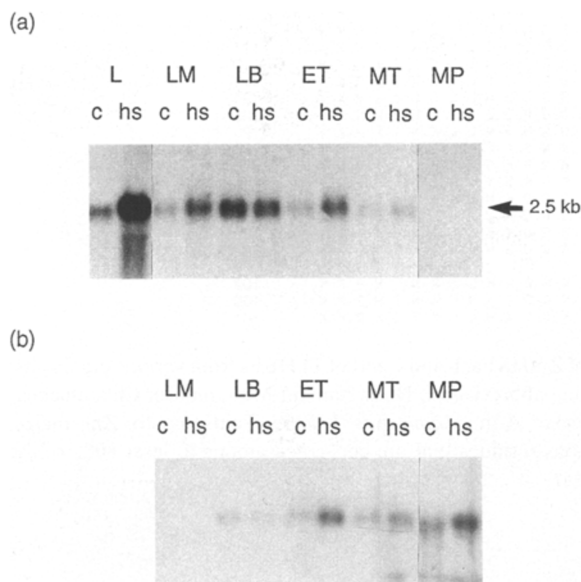


Fig. 3. Northern analysis of HSP70 and actin mRNA expressed during pollen development. Poly(A)$^+$ RNA (0.5 μg) was extracted from leaves (L), isolated late microspores (LM), late-bicellular (LB), early-tricellular (ET), mid-tricellular (MT) and mature pollen (MP) incubated 2 h either at control (c) or at heat-shock (hs) temperature. (a) Hybridization with the *hsp70* probe. A band of 2.5 kb is indicated on the right. (b) Hybridization with the actin probe (see Materials and methods).

Thus, in the absence of heat shock, the level of HSP70 transcripts is developmentally regulated in maize microgametophytes, the late-bicellular stage exhibiting a massive accumulation of HSP70 mRNA. In addition, the HSP70 transcript level can be clearly enhanced by a heat shock at the late microspore stage but to a much lesser extent than in vegetative tissues. During the final stages of pollen development, HSP70 mRNA cannot be accumulated in response to a heat shock.

Production and characterization of a maize hsf *probe*

As we observed an intricate regulation of the expression of *hsp70* genes during pollen development, we were interested in analysing the expression of *hsf* genes during this developmental process. To this end, we produced a probe for detection of maize *hsf* genes. All known *hsf* genes exhibit remarkable homology in the region encoding their DNA binding domains ([36], Fig. 4). Two primers, P1 and P2 (Fig. 1 and Fig. 4), were designed in order to amplify the most conserved part of this region. A 101 bp fragment was amplified by PCR from maize unstressed leaf cDNA. Its deduced amino acid sequence is identical to the residues present at position 82–115 of the predicted protein sequence of tomato HSF8 [42] and most of these amino acids are conserved in the deduced protein sequences of 14 HSFs

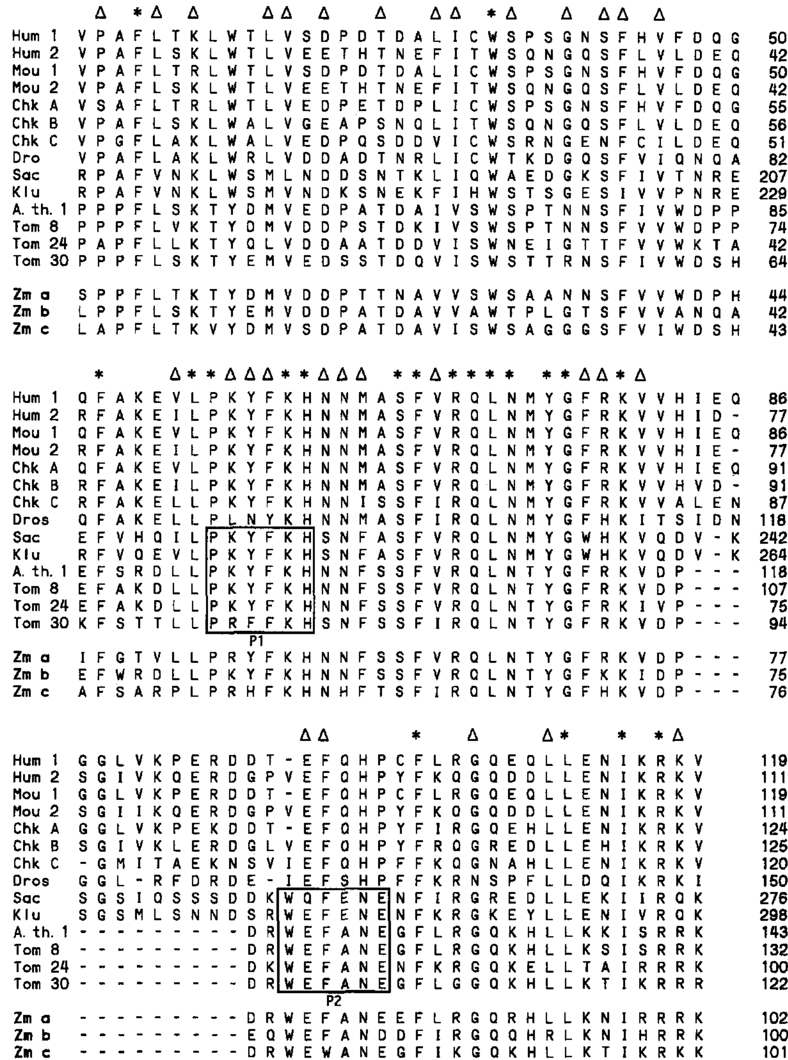


Fig. 4. Amino acid sequence alignment of the DNA binding domains of ZmHSFa, b and c and of 14 HSFs from various organisms. The respective origin of the different HSFs is indicated by the following abbreviation: Hum, human; Mou, mouse; Chk, chicken; Dro, *Drosophila*; Sac, *Saccharomyces cerevisae*; Klu, *Kluyveromyces lactis*; A.th., *Arabidopsis thaliana*; Tom, tomato, Zm, maize. Asterisks indicate identical amino acids and open triangles homologous residues that are conserved among at least 80% of the sequences. Motifs corresponding to P1 and P2 primers are boxed.

(Fig. 4). Therefore, this 101 bp sequence was unambiguously identified as part of a *hsf* gene. Its nucleotide homology with the three tomato corresponding sequences ranges from 59 to 69%. Thus, it is likely that this fragment hybridizes to most if not all maize *hsf* genes and is referred to as '*hsf* probe'.

Cloning of three maize hsf *cDNAs*

In order to isolate *hsf* genes expressed during maize developmental processes, two cDNA libraries constructed from transition-stage embryos or isolated male gametes were screened with the '*hsf* probe'. Fourteen positive clones were isolated and sequence analysis indicated that these

clones represented three distinct sequences (*a*, *b*, *c*). Clone *a* was isolated from the transition-stage embryo library and clones *b* and *c* from the male gamete library. The sizes of the longest cDNA of each type obtained were 413 bp (*a*), 2048 bp (*b*) and 1290 bp (*c*), respectively. The length of clone *c* sequence was further brought to 1450 bp by 5′ RACE-PCR experiments. When translated, a region of these cDNAs showed high homology to the DNA binding domain of 14 previously characterized HSFs (Fig. 4). The three clones were consequently designated *Zmhsfa*, *b* and *c* and the predicted proteins ZmHSFa, b and c.

When compared to HSFs characterized in yeasts and animals, the putative DNA binding domains of the three maize HSFs exhibit a gap of 11 to 12 residues (Fig. 4). This gap seems particular to higher plant HSFs as it is also found in the tomato and *Arabidopsis* HSFs ([28, 42], see Fig. 4). As expected, sequence comparison (Clustal method) indicates that the DNA binding domains of ZmHSFa, b and c are more related to their tomato counterparts (57.4 to 80.9% similarity) than to animal and yeast corresponding motifs (37.2 to 52.1% similarity). It should be noted that the maize motifs are more similar to mammals and avian HSF1 or HSFA (46.8 to 52.1%) than to HSF2 or HSFB (37.2 to 44.7%). The highest identity scores for the DNA binding domain of ZmHSFa, b and c are 80.9% with tomato HSF8, 70.2% with tomato HSF8 and 69.1% with tomato HSF30, respectively. On the other hand, phylogenetic analysis (based on Clustal method) revealed that the DNA binding domain of ZmHSFb is more related to that of tomato HSF24.

The nucleotide and deduced protein sequences of the longest clone, *Zmhsfb*, are presented in Fig. 5. A presumptive start codon is present at position 177. It should be noted that no in frame stop codon was found upstream of this ATG codon and thus, we cannot certify that no additional start codon is used *in vivo*. However, the bases present in 5′ and 3′ of this putative start codon correspond well to the ATG context consensus sequence (G, A(C,A)(C, G)ATGG(C,A)G described for maize genes [32]. A stop codon and a poly(A) tail are present at position 1644 and 2039, respectively. Thus, it is very likely that *Zmhsfb* represents a full-length clone.

Surprisingly, the open reading frame of the clone *Zmhsfb* is interrupted at position 381 by a 543 bp region (Fig. 5). Several arguments indicate that this region corresponds to an intron. First, consensus 5′ and 3′ splice junction sequences [13, 43] were identified and 6 inframe stop codons are present in this 543 bp region (Fig. 5). Second, as shown in Fig. 6a, fragments with the expected size difference were obtained in PCR experiments using genomic DNA or unstressed leaf cDNA as templates, and primers P1 and P3 flanking the 543 bp region (see Fig. 1 and Fig. 5 for primer sequences and positions). In addition, an intron was reported at the same position in tomato *hsf8* (EMBL accession number X67599) and *Arabidopsis Athsf1* [28] genes. We have also detected the presence of an additional sequence in the same region of *Zmhsfa* and *Zmhsfc* genes by using genomic DNA or cDNA in PCR experiments (results not shown). In addition to the presence of a poly(A) tail, the length of the clone *Zmhsfb*, minus that of the intron, corresponds to the size estimated from northern experiment (see below). Thus, the clone *Zmhsfb* could represent a pre-messenger RNA. Indeed, this pre-messenger RNA was detected in stressed leaves by PCR (Fig. 6b) using primers P1 and P4 (see Fig. 1 and Fig. 5 for primer sequences and positions). As expected, a 550 bp fragment was amplified only when poly(A)$^+$ RNA was reverse transcribed indicating that this pre-messenger RNA is present in heat-shocked leaves and that no genomic DNA contamination occured during RT-PCR experiments.

The open reading frame determined for the clone *Zmhsfb* is 927 nucleotides in length and encodes a protein of 308 amino acids with a predicted molecular mass of 35 370 Da. The putative protein contains both the conserved DNA-binding domain and arrays of hydrophobic amino acid heptad repeats of the leucine zipper type that are characteristic of HSFs (Fig. 5) [36]. The first leucine zipper region is located immediately after the DNA binding domain and the second one,

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gcccacgcagcaatctggtcttctggaaatctccatcaatccgattccgaacggcagaaattttcccttcccccggcca   80
ccaaccaaccaaacccgagcaaccagcctgagccacctcccatgcgccgcgccgcagcctgaggaggaggaggaggacgc  160
ggtggtctggagagggATGGAGGGCGCGTCCTCGCTGCCGCCCTTCCTGAGCAAGACGTACGAGATGGTGGACGACCCGG  240
                 M  E  G  A  S  S  L [P  P  F  L  S  K  T  Y  E  M  V  D  D  P
CCACGGACGCCGTGGTGGCGTGGACGCCGCTGGGGACCAGCTTCGTCGTCGCGAACCAGGCCGAGTTCTGGAGGGATCTG  320
A  T  D  A  V  V  A  W  T  P  L  G  T  S  F  V  V  A  N  Q  A  E  F  W  R  D  L
  ------ P1 ------>
CTCCCCAAGTACTTCAAGCACAACAACTTCTCCAGCTTCGTGCGGCAACTGAACACCTACgtaagcccccaccgcacagc  400
 L  P  K  Y  F  K  H  N  N  F  S  S  F  V  R  Q  L  N  T  Y
acgatcattcattttcttcgccccatctccctctctgtccacctgtggttttgatcaatcggggtgcgtggcggcgtgct  480
actgtcagctatgctgttgcccctttgcaaaaaactccacaagttggcatttgggggcgaatcttgttagccttgtag  560
tagaacaattgtttagggtatcgtcttcggtcgaatgaactgtttccttgttatagcatagcgtgttgacaacttggtgg  640
cgataactttaaattacatgtgtccactatatatctagtatatatgctcaattatcttgttgagatgtttttccttttct  720
aaatccaactagagttagtgtaccttttgacaaatgaagtgctgctaacggtaaacactgttgaattggcatctttgttg  800
                                                          <------ P4 ------
acacccaaattaaatccaatagaaaccataatgatttcggatggttactttgatttatgagccttgatgggtcaaattt  880
                                                                     <----- P2
ctgtgatgttgtgttaatcgttttcctgcttcttcttatgtagGGCTTTAAGAAAATTGATCCTGAACAATGGGAGTTTG  960
                                            G  F  K  K  I  D  P  E  Q  W  E  F
CAAATGATGATTTCATTAGGGGACAACAGCACCGACTGAAAAATATACACAGGCGTAAGCCTATATTCAGCCATTCATCG 1040
A  N  D  D  F  I  R  G  Q  Q  H  R  L  K  N  I  H  R  R  K] P  I  F  S  H  S  S
                <------ P3 ------
CATACTCAGGGTTCTGGACCATTACCAGATACCGAAAGGAGGGATTATGAGGAGGAAATCGAAAGGCTTAAGTGTGACAA 1120
 H  T  Q  G  S  G  P  L  P  D  T  E  R  R  D  Y  E  E  E (I) E  R (L) K  C  D  N
TGCAGCTCTGACCTCAGAGCTTGAAAAGAATGCACAGAAGAAACTTGTTACAGAGAAACGAATGCAGGATCTAGAAGATA 1200
  A  A (L) T  S  E (L) E  K  N  A  Q  K  K  L  V  T  E  K  R (M) Q  D (L) E  D
AGTTGATCTTTTTGGAGGATCGGCAGAAGAATCTGATGGCGTATGTCAGGGATATTGTACAGGCACCAGGATCTTTCTCT 1280
K (L) I  F (L) E  D  R  Q  K  N (L) M  A  Y (V) R  D (I) V  Q  A  P  G  S  F  S
AGCTTTGTGCAGCAACCTGATCATCACGGAAAGAAAAGGAGACTACCAGTACCTATCTCTCTCTACCAAGATTCTAATGC 1360
 S  F  V  Q  Q  P  D  H  H  G  K  K  R  R  L  P  V  P  I  S  L  Y  Q  D  S  N  A
TAAGGGGAACCAGGTTGTGCATGGGAGCTTCATCACCAACCCACCAGCTTGCAGGGAATCATTTGACAAGACGGAATCTT 1440
 K  G  N  Q  V  V  H  G  S  F  I  T  N  P  P  A  C  R  E  S (F) D  K  T  E  S
CATTGAACTCGTTGGAGAATTTCCTTCGGGAAGCGAGTGAAGCGTTCAATATTTCATATGATGATGGCCTCCCTGGCCTT 1520
S (L) N  S (L) E  N  F (L) R  E (A) S  E  A (F) N  I  S  Y  D  D  G  L  P  G  L
CATCTGCTGTCGTTATCACAGAGCTCCATTCGTCCGGAGAAAGTGATCCCCATGTGCCATCACCTGTCTCAAGAATGCAT 1600
 H  L  L  S  L  S  Q  S  S  I  R  P  E  K  V  I  P  M  C  H  H  L  S  Q  E  C  I
ACATCTTCGGCTGGTGCGGGAGATTCGCTCTCTTCCCGCGATTtagcagagtcaactagctgcgctgagagccctcgcct 1680
 H  L  R  L  V  R  E  I  R  S  L  P  A  I
ccctcagatccaaccctgtacagattcacgaactaaggtgtctgagatcgatgtcaatcgggagcctgctgtcacagaaa 1760
ctggtccgacaagggaccaacctgccgaggagcctccccacgtagcagctggggtgaacgatggcttctggcagcagttc 1840
ctcaccgagcagcccggtcctgatgtacaccaggaggcccaatcagaaaggagagatggcgatgatgataagggcgatca 1920
gacgaggataggggaccgagaaattttggtgggggaagaagaatgttgagcagatgagagagaagctggggcgtctca 2000
cctcggtggagaaaacctgactgtatttcttgtagctgaaaaaaaaaa                                 2048
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Fig. 5. Nucleotide and deduced amino acid sequences of *Zmhsfb*. The 5′- and 3′-untranslated regions, as well as the intron sequence, are in lower case letters. The open reading frame is in upper-case letters. The limits of the putative DNA-binding domain are indicated by square brackets. Bases corresponding to 5′ and 3′ splicing consensus sequences are underlined. Stop codons, including those present within the intron, are boxed. Circles indicate amino acids potentially implicated in leucine zippers. Position and orientation of primers P1, P2, P3 and P4 are indicated by arrows.

near the carboxy terminus of the predicted protein (Fig. 5).

Expression of hsf *genes in maize leaves*

We first studied the expression of *hsf* genes in control and heat-shocked leaves. Northern blot analysis was performed using the 101 bp '*hsf* probe' (Fig. 7a). A transcript of ca. 2.3 kb was detected both under control and heat-shock conditions while two other transcripts of 1.6 and 1.5 kb were only revealed in heat-shocked samples. To identify these bands, complementary northern blot analysis was performed with the inserts of clones *Zmhsfa*, *b* and *c* (Fig. 7b). *Zmhsfa* revealed the constitutively expressed *hsf*

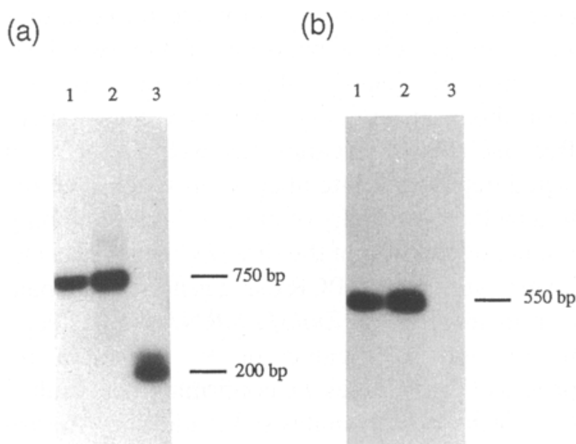

Fig. 6. Detection of an intron in *Zmhsfb* and presence of the pre-messenger RNA in leaves. (a) PCR experiments were conducted using primers P1 and P3, flanking the intron (see Fig. 1 and Fig. 5 for primer positions), and *Zmhsfb* plasmid (1), genomic DNA (2) and unstressed leaf cDNA (3). (b) PCR experiments were conducted using primers P1 and P4, the latter one being located within the intron (see Fig. 1 and Fig. 5 for primer positions), and: *Zmhsfb* plasmid (1), heat-shocked leaf cDNA (2), poly(A)$^+$ RNA, isolated from heat-shocked leaves without reverse transcription (3). Ten μl of PCR reactions were analyzed by Southern blot using the conserved '*hsf* probe'. Filters were exposed for 30 min.

transcript (2.3 kb) whereas *Zmhsfb* and *Zmhsfc* hybridized with the inducible 1.6 kb and 1.5 kb transcripts, respectively.

The expression of the three *hsf* genes was further investigated by RT-PCR using gene-specific primer sets (Fig. 7c). As expected from the previous northern experiments, *Zmhsfa* mRNA was detected in roughly equal amounts both in control and heat-shocked leaves. Interestingly, *Zmhsfb* and *Zmhsfc* mRNAs were revealed in unstressed leaves and the signals were slightly enhanced upon heat shock (Fig. 7c). This signal increase was reproducibly observed in several experiments, the constant signal for *Zmhsfa* gene being used as internal standard.

These results clearly show that at least three *hsf* genes are expressed in maize leaves. The level of *Zmhsfa* mRNA is independent of temperature whereas the levels of *Zmhsfb* and *Zmhsfc* RNA are increased upon heat shock.

Expression of HSF mRNA during pollen development

The developmentally regulated and heat-induced expression of *hsf* genes was then analysed at five

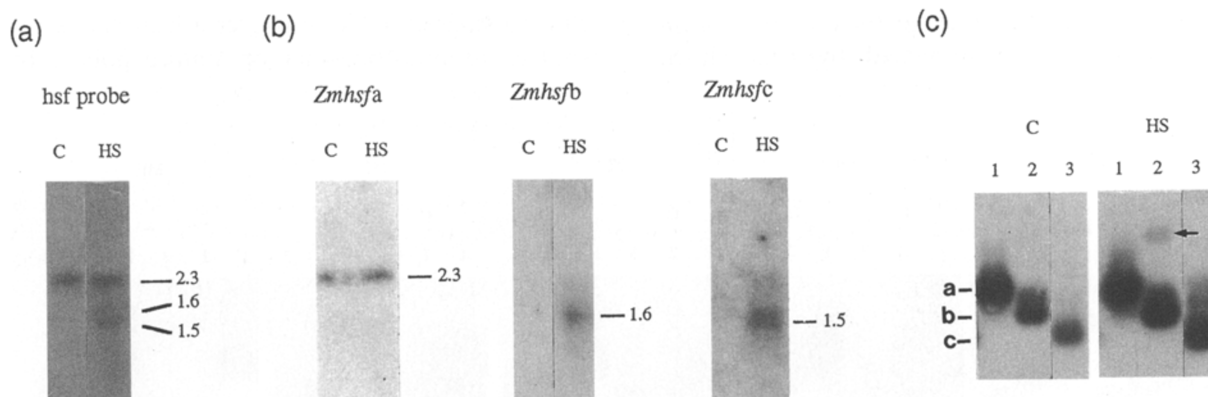

Fig. 7. HSF mRNA expression in unstressed and heat-shocked maize leaves. Poly(A)$^+$ RNA was extracted from leaves of 10-day old seedlings incubated either 2 h at control (C) or at heat shock (HS) temperature. (a) Northern analysis was performed using 0.5 μg of poly(A)$^+$ RNA and the 101 bp '*hsf* probe'. Transcript sizes are indicated in kb on the right. (b) Northern analysis was performed using the three clones *Zmhsfa*, *b* and *c* as probes. (c) RT-PCR analysis was conducted using primers P6 and P7 specific for *Zmhsfa* (lanes 1, band labelled with a), primers P1 and P3 for *Zmhsfb* (lanes 2, band labelled with b) and primers P1 and P5 for *Zmhsfc* (lanes 3, band labelled with c). In the latter case, 1% of the reaction was subjected to a second round of PCR. Ten μl of PCR reactions were subjected to Southern analysis using the conserved '*hsf* probe'. Filters were exposed for 30 min. Note that in lane HS, 2, a signal which size corresponds to the unspliced form of *Zmhsfb* mRNA is indicated by an arrow.

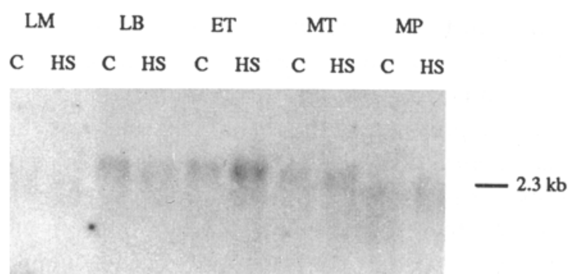

Fig. 8. Northern analysis of HSF mRNA expression during maize pollen development. Late microspores (LM), late-bicellular pollen (LB), early- and mid-tricellular pollen (ET and MT, respectively) and mature pollen (MP) were incubated either 2 h at control (C) or at heat-shock (HS) temperature. About 0.5 μg poly(A)$^+$ RNA were subjected to northern analysis using the conserved 101 bp '*hsf* probe' and the filter was exposed for 2 weeks. A band of 2.3 kb is indicated on the right. A faint band present in LM lanes may not be clearly visible on the photograph. Lanes LM to MT are from the same filter as in Fig. 3. See Fig. 3b for control of poly(A)$^+$ RNA amounts after hybridization with an actin probe for these lanes.

stages of maize pollen development, i.e. late microspore, late-bicellular pollen, early- and mid-tricellular pollen and mature pollen. The presence of HSF mRNA was first investigated by northern blot analysis using the '*hsf* probe' which recognises the three maize *hsf* genes (Fig. 8). A faint band corresponding to *Zmhsfa* mRNA (2.3 kb transcripts) was detected at all stages of pollen development. For each stage, the level of *Zmhsfa* transcripts was not modified by heat shock (Fig. 8), as it was observed for vegetative tissues. Interestingly, the expression of the two heat-inducible *hsf* genes could not be detected after heat shock at any stage of pollen development. Because this observation, as well as the weak signal detected in late microspores, could be due to a lack of sensitivity of our northern blot analysis, the expression of the three *hsf* genes was also investigated by RT-PCR using gene-specific pairs of primers (Fig. 9). *Zmhsfa* mRNA was detected throughout microgametophyte developmental process (Fig. 9, lanes 1), confirming the result of the northern blot analysis. In addition, *Zmhsfb* and *Zmhsfc* mRNAs were detected at the different stages of immature pollen. However, in contrast with leaves, their signals were not increased upon heat shock (Fig. 9, lanes 2 and 3). In mature pollen, the signal corresponding to *Zmhsfb* was no longer detectable. This result was confirmed by four independant preparations of cDNA. However, this phenomenon is probably not related to the loss of *hsp* gene inducibility since *Zmhsfb* transcripts can be detected at the mid tricellular stage, a stage at which no HSP mRNA synthesis can be induced.

In conclusion, both northern and RT-PCR analyses show that *Zmhsfa* mRNA is constitutively present throughout pollen development including stages unable to trigger a heat shock response, as mid-tricellular or mature pollen. In

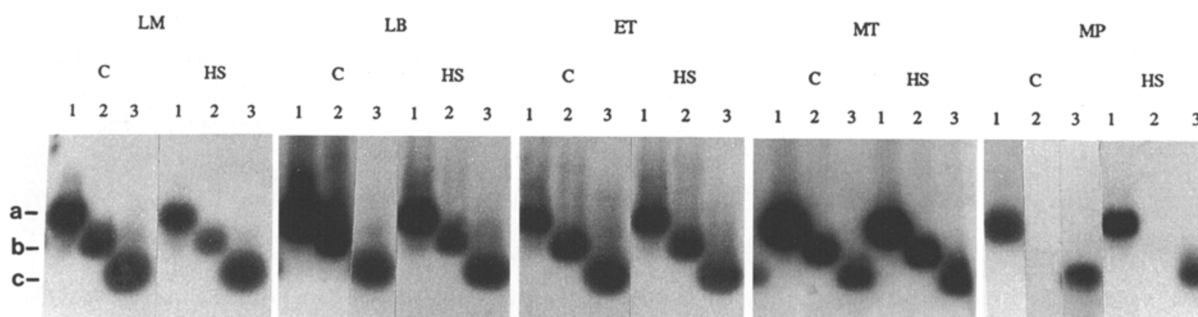

Fig. 9. RT-PCR analysis of HSF mRNA expression during pollen development. Late microspores (lm), late-bicellular pollen (LB), early- and mid-tricellular pollen (ET and MT, respectively) and mature pollen (MP) were incubated either 2 h at control (C) or at heat-shock (HS) temperature. PCR experiments were conducted using primers P6 and P7 specific for *Zmhsfa* (lanes 1, bands labelled with a), primers P1 and P3 for *Zmhsfb* (lanes 2, bands labelled with b) and primers P1 and P5 for *Zmhsfc* (lanes 3, bands labelled with c). When using this latter couple of primers, a second round of PCR was performed. Ten μl of PCR reactions were subjected to Southern analysis using the conserved 101 bp '*hsf* probe'. Filters were exposed for 30 min.

contrast to leaves, the basal transcript levels of *Zmhsfb* and *Zmhsfc* genes are not significantly enhanced upon heat shock, at any stage of pollen development. These results provide evidence that the loss of *hsp* gene activation at the final stages of pollen development is not due to a modification of expression of these three *hsf* genes at the mRNA level.

Discussion

Expression of three hsf *genes in maize vegetative tissues*

We report here the expression of three *hsf* genes in maize leaves; *Zmhsfa* gene is constitutively expressed whereas the expression of *Zmhsfb* and *Zmhsfc* genes is strongly enhanced upon heat shock. Similar features have been previously described for tomato *hsf* genes [42]. From the phylogenetic analysis of the DNA-binding domain sequences, the sizes of the transcripts and the type of expression, it is likely that *Zmhsfa* is the maize counterpart of tomato *hsf8* gene and, *Zmhsfb* and *Zmhsfc* that of *hsf24* and *hsf30* genes, respectively. Recently, a *hsf* gene, *Athsf1*, has been characterized in *Arabidopsis* [28]. Its expression is enhanced upon heat shock. Hence, heat-induced expression of *hsf* genes has been characterized to date in three plant species and seems to be specific to higher plants. The three tomato HSFs are able, independently, to stimulate upon heat shock the transcription of a reporter gene fused with various promoters conferring heat inducibility [48]. However, the activation by each HSF is dependent on the position and numbers of target sequences, i.e. HSEs, in the promoter of the reporter gene but also on the stress regime [48]. Hence, the different plant HSFs could be required to modulate the expression of *hsp* genes depending on the stress conditions.

Developmentally regulated expression of hsp70 *and* hsf *genes during pollen development*

In plants, several reports have focused on the expression of *hsp18* and *hsp90* gene during development of the whole anther, i.e. a combination of sporophytic and gametophytic tissues [4, 9, 10, 19, 33]. In the present work, the use of isolated microgametophyte populations allowed us to characterize the expression of *hsp70* genes at the mRNA level during pollen development without taking into account the sporophytic tissues of the anthers. Using an *in situ* hybridization approach Duck and Folk [20] have also detected recently HSP70 mRNA at non-heat shock temperatures in developing tomato pollen (10 mm buds). Similarly, the expression of *hsp70* genes has been characterized during embryogenesis and gametogenesis in amphibians and mammals [8, 14, 34, 53] and during seed development in plants [18, 52]. Hence, members of *hsp70* family are expressed in plant and animal developmental processes but their precise biological role is still largely unknown.

In mammals and chicken, the developmental regulation of *hsp* genes appears to be mediated by specific HSFs [36]. Indeed, the DNA binding activity of mammalian HSF1 and avian HSFA is activated by various stresses, whereas HSF2 is activated during hemin-induced differentiation of erythroleukemia cells [44]. Strikingly, a HSE-binding activity was detected in non-stressed mouse embryonal carcinoma cells but this activity disappeared after *in vitro* differentiation into fibroblasts [34]. Also, a HSF2 activity is found in the absence of heat stress in the mouse testis [41] and in mouse blastocysts [35]. Thus, HSFs might be implicated in *hsp* gene regulation during developmental processes. Further investigations are needed to determine whether the stress-independent regulation of *hsp70* genes during maize pollen development is mediated by the HSF encoded by the constitutively expressed *Zmhsfa* gene. We did not identify a fourth kind of HSF mRNA (in addition to the three HSF mRNA species that we have detected in leaves) by screening two cDNA libraries of developing embryos and mole gametes or by northern blot analysis of developing pollen. However, in the latter case, a fourth type of HSF mRNA would have been revealed only if its size was different and if it was sufficiently expressed to be detected by this technique.

Expression of hsp70 *and* hsf *genes during pollen development under heat stress conditions*

In the present report, we show that HSP70 transcripts do not accumulate significantly in response to a heat shock after the late microspore stage. We have also observed by RT-PCR and *in vitro* translation that *hsp18* genes can be heat-induced only until the early-tricellular stage (unpublished results). Thus, the gradual loss of HSP synthesis in response to a heat shock, that was first reported by Frova *et al.* [23] during pollen development, seems to be due to a stage-dependent defect in accumulating HSP mRNA. Our results show that the defect in *hsp* gene expression during the final stages of maize pollen development is not due to a lack of *Zmhsfa* mRNA. Interestingly, a HSE-binding activity can be induced by a heat shock in *Xenopus* unfertilized eggs and cleavage stage embryos, stages at which *hsp* gene expression is inhibited [37]. A similar phenomenon might occur at the final stages of pollen development.

In contrast to leaves, the low basal expression of *Zmhsfb* and *Zmhsfc* genes is not significantly enhanced under heat stress during pollen development, although we have shown that *hsp* genes can be expressed in immature pollen. Therefore, it appears that the immediate heat-induced *hsp* gene transcription observed in immature pollen can occur without an increase of expression of the two heat inducible *hsf* genes, *Zmhsfb* and *Zmhsfc*. Nevertheless, we cannot exclude a very effective translation of the minute amounts of *Zmhsfb* and *Zmhsfc* mRNA that we have detected and which could lead to the accumulation of the corresponding proteins. Indeed, such a regulation was described in carrot globular embryos for HSP mRNAs which are very efficiently translated upon heat shock [3]. On the other hand, the heat-induced accumulation of HSP transcripts is much weaker during pollen development than in vegetative tissues. This phenomenon could be correlated to the lack of heat inducible *hsf* gene expression in immature pollen.

Promoter accessibility is likely to play an important role in regulating *hsp* gene expression in eukaryotes. Both human and *Drosophila* HSFs fail to bind *in vitro* to HSEs packaged in nucleosomes [7, 47] and specific factors, the GAGA factors, have been shown to disrupt nucleosomes present on *hsp70* promoters [50]. Thus, it could be interesting to investigate *hsp* gene promoter accessibility in developing pollen and in other tissues which exhibit a complete heat shock response. This might provide further insight into the gradual loss of *hsp* gene activation during pollen development.

In conclusion, we show that the defect in accumulating HSP70 transcripts during pollen development is not correlated with a modification of *Zmhsfa*, *b* or *c* gene expression. Our results suggest that the weak, immediate accumulation of HSP mRNA in response to a heat shock could be mediated in immature pollen by the HSF encoded by the constitutively expressed *hsf* gene, *Zmhsfa*.

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