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Hsp70 heat shock protein cognate is expressed and stored in developing tomato pollen

Nicholas B. Duck [1,*] and William R. Folk
Department of Biochemistry, 117 Schweitzer Hall, University of Missouri, Columbia, MO 65211, USA;
[1] *Present address: Ciba Geigy Corporation, Agricultural Research Biotechnology Unit, NC 27709, USA*
(author for correspondence)*

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Abstract

Pollen of angiosperms lacks the ability to respond to heat stress by synthesizing heat shock proteins (hsps). In tomato developing microspores were found to have 70 kDa heat shock proteins (hsp70s) present throughout development, even in the absence of heat stress. Heat shock protein family members expressed in the absence of heat stress are called cognate (hsc70) genes. Antisense RNA and antibody probes were used for *in situ* hybridizations which detected hsc70 expression in developing pollen of immature buds. Hsc70 mRNA transcripts and proteins were detected in nonstressed sporogenous tissues, microspores and in pre-tapetal layers during early pollen development. While immunoblot analysis detected hsc70 proteins stored in mature pollen, heat stress could not induce the synthesis of new hsp70 protein as measured by ^{35}S-methionine labeling followed by immunoprecipitation.

Introduction

Upon elevating the temperature of an organism a few degrees above its normal temperature range a characteristic group of proteins are synthesized, termed heat shock proteins (hsps). This response is evolutionarily conserved [12]. The proteins produced can be grouped into several gene families ranging in molecular mass from 17 kDa to > 100 kDa. The 70 kDa hsps comprise a multigene family which is is highly conserved across species. Some family members are expressed in the absence of heat stress and are called cognate hsp70s, or hsc70s. hsp/hsc70 proteins have been shown to have a role in intracellular transport of proteins into organelles in eukaryotic cells and are sometimes called chaperonins [3, 10, 14].

Several reports have examined the expression of hsps in heat stressed pollen by examining changes in hsp mRNA or ^{35}S-methionine-labeled proteins following a heat stress. In angiosperms, such as lily, *Petunia*, *Tradescantia* and maize, mature pollen does not respond to heat stress by synthesizing hsps [2, 7, 15, 16]. Germinating *Tradescantia* pollen was able to survive heat stress conditions without inducing hsps [16]. During maize pollen development hsps are induced, with the strongest expression observed in uninucleate microspores [7]. In mature maize pollen the blocking of hsp expression occurs at the transcriptional level as hsp70 mRNA transcripts failed to accumulate upon heat stress [8]. No study has examined the expression of hsp cognate proteins during normal pollen development, without heat stress. Since the hsc70 proteins have been shown to have a role in intracellular trans-

port in plants [14], and are expressed during normal development in tomato [6], the present study was initiated to examine the expression hsc70 proteins during tomato pollen development. Our findings showed that hsc70 proteins were synthesized during early pollen development and stored in mature pollen and germinating pollen. We also confirmed the results of previous studies which have shown that mature pollen does not respond to heat stress with the synthesis of new hsp70.

Materials and methods

Plant maintenance and pollen collection

Tomato plants (*Lycopersicon esculentum* cv. VF36) were grown in growth chambers with 10 h light/14 h dark cycles at 22 °C. Flower buds and pollen from flowers at anthesis were collected at the end of the dark cycle. Pollen was vibrated free from dehiscing anthers, collected in microcentrifuge tubes and used immediately for immunoblot analysis or ^{35}S-methionine pulse labeling.

In situ *hybridizations*

In situ hybridizations were performed essentially as described by Cox and Goldberg [4]. Buds were fixed in 1% glutaraldehyde/0.05 M sodium phosphate buffer pH 7.0, dehydrated in an ethanol gradient series from 10% to 100%, infiltrated with xylenes and embedded in Paraplast-plus paraffin (Fisher). Then, 10 μm sections were treated as described by Cox and Goldberg [4] except that the tissue was hybridized with 2.5×10^5 CPM of a ^{35}S-substituted uridine 5′-(thio)triphosphate complementary riboprobe described previously [6]. Control *in situ* hybridizations were performed using a labeled RNA made from the transcription template supplied with the Promega SP6 *in vitro* transcription kit. Tissue sections were stained with 0.05% toluidine blue and visualized by dark-field microscopy and photographed with Kodak ASA 200 film. Bright-field photographs were also taken of adjacent sections from serial microtomy. These sections did not undergo hybridization so that the tissue integrity remained intact and are presented for comparison.

In situ localization of hsc70 proteins was performed with rabbit polyclonal antisera and the Auroprobe LM (Amersham) gold-labeled secondary antibody and IntenSE M (Amersham) silver staining reagents. Procedures were according to the manufacturer's specifications except that the tissues were fixed in Hepes-buffered paraformaldehyde (30 mM Hepes pH 7.2, 70 mM NaCl, 2 mM $CaCl_2$, 2% paraformaldehyde) and incubated at 25 °C for 1 h using a 1:500 dilution of polyclonal antisera made against tomato hsc70 [14]. Controls were performed using pre-immune sera under the same conditions. Sections were visualized directly without staining by bright-field microscopy. Adjacent sections from serial microtomy were stained with toluidine blue and are presented to better visualize the tissues. The preparation of polyclonal antisera in described elsewhere [14].

Immunoblot analysis

Mature pollen (10 mg) was either germinated in media [13] then ground in 100 μl of Laemmli buffer [11] or ground directly in 150 μl of Laemmli buffer using a mortar and pestle. Extracts were cleared by microcentrifugation ($13000 \times g$ for 5 min) and aliquots containing 50 μg of soluble proteins were analyzed by SDS-PAGE [11]. The gels were either stained with Coomassie brilliant blue or proteins were transferred to nitrocellulose by electrophoretic transfer (Hoefer TE-42 Transfor unit) in 25 mM Tris pH 8.3, 192 mM glycine and 20% methanol. The nitrocellulose filter was blocked in 10 ml of PBS containing 10% dry milk for 30 min, followed by the addition of 20 μl of antibodies (1:500 dilution) directed against the hsc-1 protein. Filters were agitated at room temperature for 2 h and then washed in 200 ml of PBS for 15 min. The filter was then incubated in PBS containing 10% dry milk with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:1000 dilution) (BioRad). The al-

kaline phosphatase color reaction was performed in 50 ml of 50 mM sodium carbonate buffer pH 9.8, 100 mM NaCl and 5 mM $MgCl_2$ with 15 mg of *p*-nitroblue tetrazolium chloride (predissolved in 70% *N,N*-dimethylformamide) and 7 mg of 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine (dissolved in *N,N*-dimethylformamide).

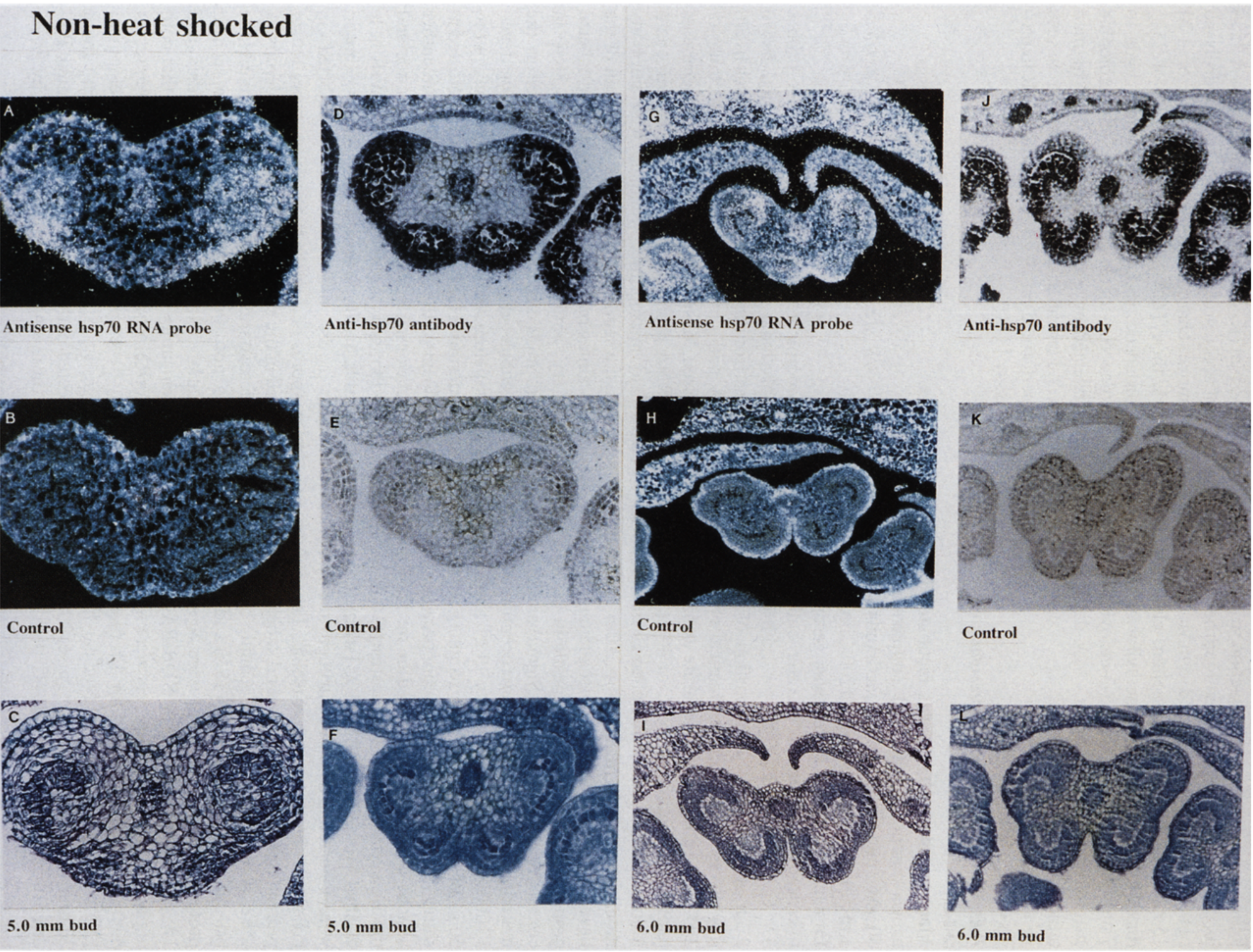
Pulse labeling and immunoprecipitation

Mature pollen (10 mg) was incubated in 200 μl in germination media [9] supplemented with 50 μM amino acids minus methionine and using 1 mCi/ml ^{35}S-methionine (1200 Ci/mmol) (New England Nuclear). Pollen was germinated in a microcentrifuge tube in a water bath at 25 °C, 37 °C, 39 °C, and 42 °C, for 1.5 h followed by 2.5 h at 25 °C. A 10 mg portion of 0.5 cm leaf tissue was treated under identical conditions at 39 °C as a control. In a separate experiment, pollen was germinated at 25 °C or 40 °C for 45 min followed by 1 h at 25 °C. Pollen germination was monitored by light microscopy. After labeling the tissue was freeze-thawed twice in liquid nitrogen, ground with a mortar and pestle in 0.5 ml PBS containing 0.1% Tween 20 (Sigma) and insoluble material was removed by microcentrifugation at $13000 \times g$ for 5 min. Twenty μl of antisera and 50 μl of protein G agarose (Sigma), in a water slurry, were used in the immunoprecipitation reactions which were incubated on ice for 2 h with mixing every 15 min. The protein G beads were washed 5 times with PBS 0.1% Tween. The beads were collected in a microcentrifuge tube by a brief (15 s) spin between each washing. The immunoprecipitates and 10 μg of total labeled proteins were boiled in 50 μl of Laemmli buffer, spun briefly in a microcentrifuge (15 s) and the soluble proteins in the supernatant were separated on a 10% polyacrylamide gel by SDS-PAGE according to Laemmli [11]. The gels were fixed in 40% methanol, 7% acetic acid for 1 h then soaked in Enhance (New England Nuclear-Dupont) for 30 min followed by a water rinse. The gels were dried under vacuum at 60 °C then exposed to XAR-5 film (Kodak) for autoradiography. A 25 μl aliquot of the protein extract was base hydrolysed to remove charged tRNAs in 250 μl of 1 M NaOH, then TCA-precipitated in 1 ml of 25% trichloroacetic acid with 2% casamino acids to determine the incorporation of ^{35}S-methionine and collected by vacuum filtration on Whatman GF/A 2.4 cm filter disks. Bradford microassays were performed on all samples using BioRad protein assay [1].

Results

Hsp/hsc70S are expressed during pollen development

Both complementary ^{35}S-labeled RNA probes and polyclonal antibodies made against hsc70 proteins from tomato were used to detect hsc70 mRNA and proteins in histological sections of unstressed, developing anthers. In experiments using RNA probes, the signal appears white in a dark-field micrograph, whereas, with antibody probes, the signal appears black as a result of the silver staining reaction carried out on gold-labeled secondary antibody. Figure 1 shows that in 5–6 mm buds hsc70 protein is localized in the sporogenous cells, and in the surrounding developing tapetum and endothecium extending out to the epidermis. Hsc70 protein was also detected in the phloem of the vascular bundles in the anther and developing petal. The connective tissue and the epidermis in contact with it has relatively little detectable hsc70 protein. Figure 2 shows that in 10 mm buds, hsc70 protein is detected in microspores and in the tapetum but is no longer detected in the endothecium and the epidermis. A few microspores were not cut by the knife blade and do not stain. Identical results are seen in localization experiments using antibodies and complementary RNA probes indicating that both hsc70 mRNA transcripts and proteins are present in early-developing pollen. At later developmental stages (> 10 mm buds) hsp70 protein cannot be detected in the tapetum as it degenerates. Pollen at these stages cannot be analyzed by *in situ*


Non-heat shocked
A
Antisense hsp70 RNA probe
D
Anti-hsp70 antibody
G
Antisense hsp70 RNA probe
J
Anti-hsp70 antibody
B
Control
E
Control
H
Control
K
Control
C
5.0 mm bud
F
5.0 mm bud
I
6.0 mm bud
L
6.0 mm bud

hybridization or with antibodies in histological sections, as a thick exine wall makes microscopic sectioning difficult in paraffin-embedded tissues. However, in mature pollen and pollen germinated for 2 h, hsc70 protein can be detected by immunoblot analysis (Fig. 3). In previous studies, it was determined that no hsc/hsp70-like mRNA transcripts are present in mature pollen [6]. To confirm this, germinating pollen was labeled with ^{35}S-methionine followed by immunoprecipitation with anti-hsc/hsp70 antibodies. Results in Fig. 4A (lane 1) show no newly synthesized hsc70 protein could be detected in pollen incubated in germination media for 4 h. Therefore, hsc70 protein present in germinating pollen, as indicated by immunoblot analysis, is most likely synthesized during earlier pollen development and then stored in mature pollen.

Hsp70 proteins cannot be induced with heat stress in pollen

Since it appeared that no new hsc70 proteins were being synthesized in germinating pollen under nonstressed conditions, pulse chase experiments were performed at elevated temperatures to determine if hsp70 could be induced with a heat stress. Pollen was imbibed in germination medium containing ^{35}S-methionine for 15 min, then heat-shocked for 1.5 h at various temperatures, followed by a 2.5 h recovery at 25 °C to allow for protein synthesis (Fig. 4A, lanes 1–4). ^{35}S-methionine was present in the germination media throughout these treatments. Leaf tissue was treated under identical conditions as a positive control (Fig. 4A, lane 7). To test if germinating pollen could be induced to synthesize hsp70s another sample of pollen was incubated in medium at 25 °C for 2.5 h, shifted to 39 °C for a 1.5 h heat shock, followed by a 1.5 h recovery for protein synthesis (Fig. 4A, lane 5). Proteins were extracted from the samples followed by immunoprecipitation with mild washing conditions (PBS, 0.1% triton X-100) and analyzed by SDS-PAGE followed by autoradiography. Neither mature pollen nor germinating pollen responded to a heat stress by producing hsp70 proteins under any of these treatment conditions. Also, no hsp70 proteins could be detected in similar labeling and immunoprecipitation experiments when pollen was heat shocked at 40 °C for 45 min followed by a 1 h recovery to allow for protein synthesis (Fig. 4B). However, in leaf samples, elevated levels of hsp70 proteins were always detected under identical treatment conditions at 39 °C or 40 °C (Fig. 4A, lane 7). While heat stress decreases the overall ^{35}S-methionine incorporation in both pollen and leaf about 2–4-fold at 39 °C, both the pollen and leaf samples heat stressed at this temperature (Fig. 4A, lanes 3 and 7 respectively) exhibited identical ^{35}S-methionine incorporation (1×10^5 CPM/mg tissue). To eliminate the possibility that pre-existing, unlabeled hsp70 could be competing for the anti-hsc/hsp70 antibody, in one sample equal amounts of the labeled leaf protein as in the positive control (Fig. 4A, lane 7) was mixed with the same amount of unlabeled pollen protein used in samples in lanes 1–5 and an immunoprecipitation reaction was performed under identical conditions described previously for all other samples. Figure 4A (lane 6) shows that an equal amount of labeled leaf hsp70 proteins was immunoprecipitated in the mixed sample as in the sample containing only labeled leaf proteins (Fig. 4A, lane 7). Therefore,

Fig. 1. Localization of hsp/hsc70 expression in unstressed developing pollen. Floral buds were fixed, imbedded in paraffin, and 10 μm sections were hybridized with either ^{35}S-RNA probes or antibody probes. After post-hybridization washes, RNA-probed slides were dipped in emulsion film and exposed for 7 days in total darkness, developed and visualized by dark-field microscopy where silver grains appear white. Antibody probed slides were hybridized with gold-labeled secondary antibody, washed, silver-stained and visualized by bright-field microscopy so that silver grains appear black. A and G. Anther cross section through a 5 mm (A) or 6 mm (G) bud hybridized with antisense ^{35}S-RNA. B and H. Anther cross sections as in A and G respectively, hybridized with a control ^{35}S-RNA. D and J. Anther cross section through a 5 mm (D) or 6 mm (J) bud probed with anti-hsp/hsc70 antibody. E and K. Anther cross section as in D and J respectively probed with pre-immune sera. C, F, I and L. Anther cross sections stained with toluidine blue.

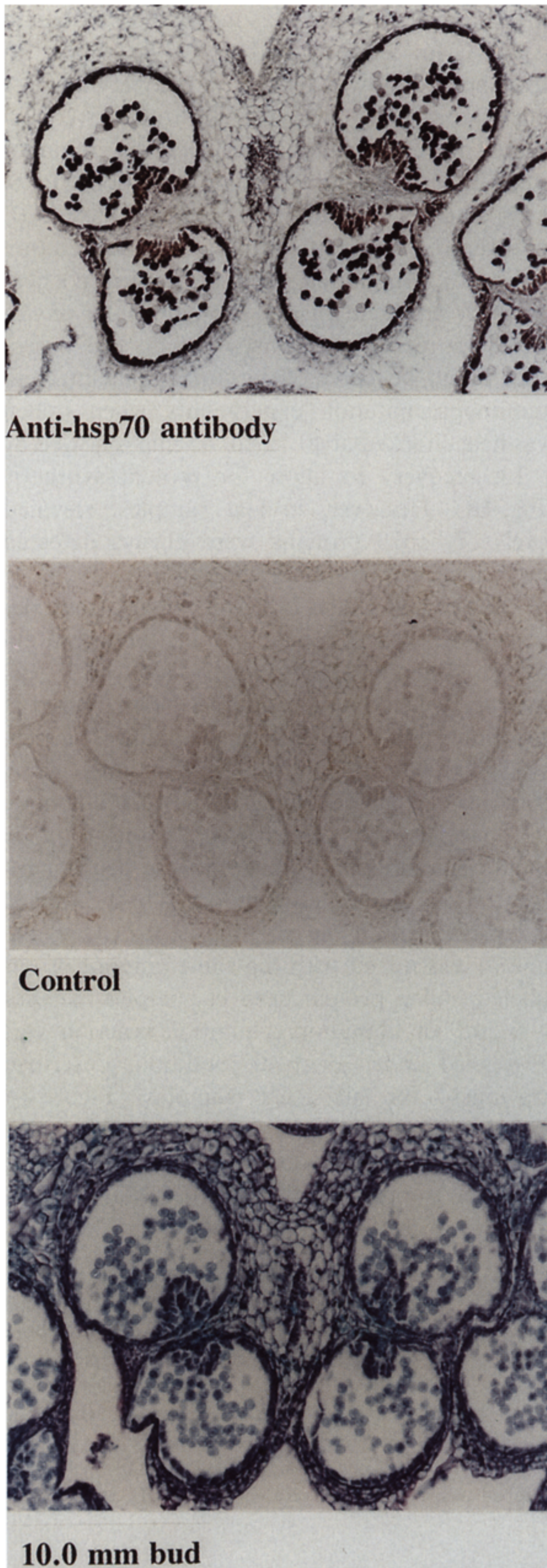


Fig. 2. Localization of hsp/hsc70 expression in unstressed developing pollen from a 10 mm floral bud. A 10 mm floral bud was fixed and treated with antibody probe as in Fig. 1. The anther cross section was probed with anti-hsp/hsc70 antibody (top), with pre-immune sera (middle), or stained with toluidine blue (bottom).

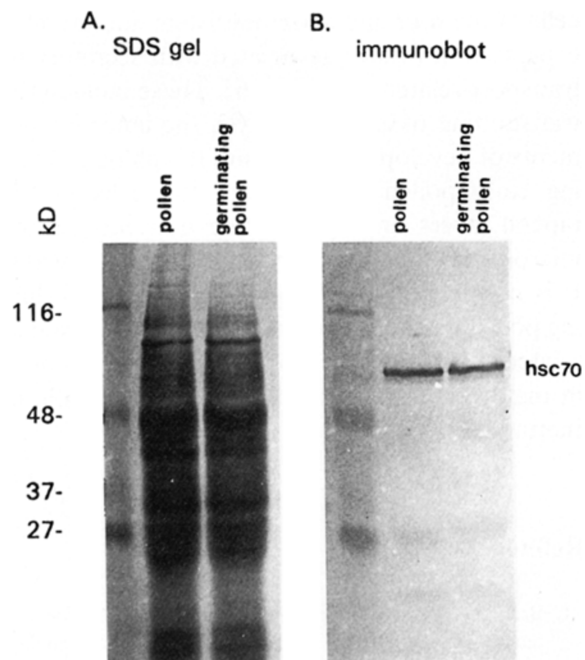


Fig. 3. Western analysis of pollen and germinating pollen. Pollen from flowers at anthesis was vibrated free from the anther and ground in Laemmli buffer or germinated for 2 h then ground in Laemmli. Total protein was separated by SDS-PAGE and either stained with Coommassie blue (A) or immunoblotted using anti-hsp/hsc70 antibody (B).

the presence of unlabeled hsc70 proteins in the pollen cannot account for the lack of detectable labeled hsp70 proteins since the antibody is in excess. The antibody used in these experiments binds several hsc/hsp70 family members under these same conditions, and accounts for the broad bands seen in Fig. 4 (lanes 6 and 7). The antibody reacts with tomato cognate and heat-inducible family members and with hsc/hsp70 proteins from maize and wheat [14].

Discussion

Few studies have addressed the expression of hsps in developing pollen. Frova *et al.* [7] reported that in uninucleate microspores of maize, hsps of 18, 72, 84 and 102 kDa can be heat induced. Also two proteins of 72 and 84 kDa are expressed throughout pollen development in the absence of heat stress. It was not determined if these were related to the heat inducible proteins. In mature pollen and germinating pollen, hsp synthesis was not able to be induced in maize and expression is blocked at the transcriptional level [8]. Germinating pollen in lily, *Petunia* and *Tradescantia* also do not synthesize hsps in response to heat stress [15, 16]. In *Tradescantia* there does appear to be a mechanism for acquired thermotolerance, which does not require the synthesis of hsps. Mature and germinating pollen of tomato do not respond to heat stress with the synthesis of new hsp70 proteins, as occurs in other plant species. In tomato there is expression of cognate hsp70 proteins in early pollen development with the protein retained in mature pollen. The possibility of stored hsp cognate proteins in pollen of other species has not been tested. In unstressed, germinating pollen a prominent, newly synthesized 70 kDa protein was observed. It was not recognized by anti-hsc/hsp70 antibody, and its synthesis was reduced by heat stress (Fig. 4B) making it unlikely that this protein is related to the hsp70 protein family. The antibody used in these studies recognizes both cognate and heat inducible hsp70 proteins and recognized hsp70s from maize and wheat [14] which is typical for polyclonal antisera directed against a highly conserved family of proteins.

One paradox that arises from these observations is that the pollen, one of the most sensitive tissues of the plant, does not have the ability to express heat shock proteins, which may contribute to protection against heat stress. The plant life cycle is dependent on the survival of pollen, but has no mechanism for producing new heat shock proteins to cope with one of nature's most common environmental assaults. Our results show that cognate hsp70 proteins are stored in mature pollen, and this may be in anticipation of potential heat stress.

hsp70 proteins have been implicated in transport-related activities. In eukaryotic cells, hsc/hsp70 proteins are involved in transporting precursor proteins into the mitochondria and endoplasmic reticulum [3, 5, 10, 14]. In tomato, hsc70 mRNAs are expressed in rapidly dividing

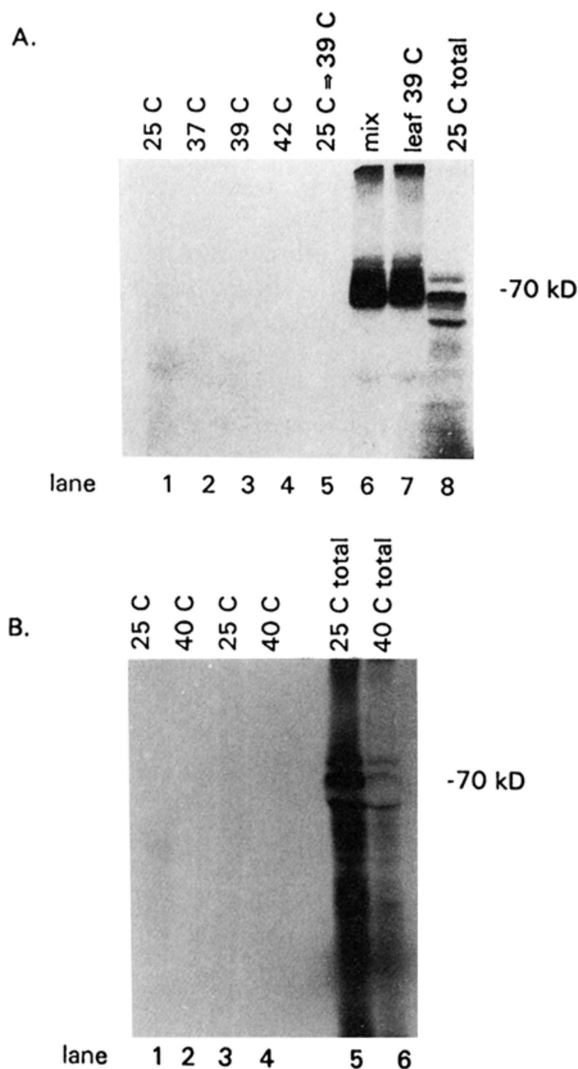


Fig. 4. Immunoprecipitations from ^{35}S-methionine-labeled pollen proteins. Ten mg of pollen from flowers at anthesis were germinated at various temperatures in media containing ^{35}S-methionine for 1.5 h followed by a 2.5 h recovery at 25 °C for protein synthesis (A). Pollen was also heat shocked at 40 °C for 45 min, followed by a 1 h recovery for protein synthesis (B). Labeled proteins were extracted and immunoprecipitates were separated by SDS-PAGE followed by autoradiography. A. Lanes 1–4 are immunoprecipitates from labeled pollen protein extracts germinated at 25, 37, 39, and 42 °C respectively. Lane 5 is an immunoprecipitate from protein extracts of pollen germinated for 2.5 h at 25 °C then shifted to 39 °C for 1.5 h followed by a 1.5 h recovery for protein synthesis. Lane 6 is an immunoprecipitate from labeled leaf protein extracts mixed with unlabeled pollen protein extracts to demonstrate that the antibody is in excess. Lane 7 is an immunoprecipitation from labeled leaf protein extracts as a positive control. Lane 8 is total labeled pollen proteins from pollen germinated at 25 °C. B. Lanes 1 and 2 are immunoprecipitates from extracts of pollen labeled for 45 min at 25 °C or 40 °C, respectively, followed by a 1 h recovery at 25 °C for protein synthesis. Lanes 3 and 4 are control immunoprecipitates performed using preimmune sera. Lanes 5 and 6 are total labeled proteins from pollen germinated at 25 °C and 40 °C respectively.

cells of the root and shoot meristem and are also expressed in tissues associated with secretory or transport-related activities [6]. These include the transmitting tissue of the style, the inner integuments of developing seeds, and the phloem. During early pollen development the cells in the tapetal layers are transporting nutrients to the microspore mother cells via secretory pathways. It is possible that hsc70 proteins expressed during pollen development are involved in transport-related activities of the tapetum and may be stored in mature pollen where they may have a role in thermotolerance.

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