Planta (1993) 190:32–43

Planta

HSP68 – a DnaK-like heat-stress protein of plant mitochondria

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Received: 7 September 1992 / Accepted: 3 November 1992

Abstract. A 68-kDa heat-stress protein (HSP68) has been purified from cell-suspension cultures of tomato (*Lycopersicon peruvianum* L.). Antibodies raised against HSP68 cross-react with the *Escherichia coli* heat-stress protein DnaK. HSP68 was found to be a hydrophilic, ATP-binding protein. Immunological analysis of subcellular fractions and immunogold-labelling of ultrathin sections showed consistently that HSP68 is localized in the mitochondrial matrix. In-vitro translation experiments indicated that HSP68 is synthesized as a precursor protein. Immunoscreening of cDNA libraries from tomato and potato (*Solanum tuberosum* L.) led to the isolation of corresponding cDNA clones. The deduced amino-acid sequences show strong relationships to the DnaK-like proteins from bacteria and organelles of eukaryotic cells. The protein HSP68 is constitutively expressed, but its synthesis is increased during heat stress in all cells of higher plantes investigated so far.

Key words: Heat stress – Chaperonin – *Lycopersicon* – Mitochondrion – *Solanum*

Introduction

A typical response of all living cells to elevated temperatures is the synthesis of a specific subset of heat-stress proteins (HSPs). Among them, proteins of the HSP70-family are the most prominent and best studied. Different types of HSP70 coexist in eukaryotic cells. In spite of basic similarities of conserved domains they can be classified into three subfamilies with characteristic intracellular localizations (for review, see Nover 1991): (i) nuclear/cytoplasmic-localized proteins (HSC70/HSP70), (ii) ER/Golgi-localized proteins (GRP78) and (iii) DnaK-like proteins of chloroplasts and mitochondria. All proteins of the HSP70-family share the ATP-binding sites and other conserved domains (Nover 1991). Nevertheless, typical differences exist among these three groups: (i) The nuclear/cytoplasmic HSC70/HSP70 types have no N-terminal leader or transit peptide. The C-terminus is rich in PGG-motifs and ends with –EEVD. In contrast to the other two subfamilies they show a putative nuclear-targeting sequence (–KRKHK– around position 250). Some representatives are constitutively expressed (HSC70), while others are induced by heat stress (HSP70). All are subjected to a typical heat-stress-dependent translocation to the nuclear compartment (for review, see Nover 1991). (ii) Representatives of the ER/Golgi proteins (GRP78 = BiP) have a cleavable leader sequence and end with an ER retention signal –KDEL (Munro and Pelham 1987; Pelham 1989, 1989a). They are constitutively expressed mass proteins of the ER. Their synthesis increases under conditions of aberrant protein accumulation in the ER, e.g. under glucose deficiency (Lee 1987). (iii) The organellar members of the HSP70 family belong to the prokaryotic DnaK-like proteins. They are constitutively expressed, but their synthesis is enhanced upon heat stress. They have an N-terminal transit peptide, necessary for their proper localization in choroplasts and mitochondria.

While the work reported here was in progress, Watts et al. (1992) reported for the first time the sequence of a mitochondrial DnaK-like protein from a plant source (pea). Our paper reports on the purification, immunological localization and sequence analysis of HSP68 from tomato and potato.

Materials and methods

Plant material. The origin and culture conditions of the cell-suspension cultures of *Lycopersicon peruvianum* L., *Catharanthus roseus* L., *Digitalis lanata* Ehrh., *Peganum harmala* L., and *Petroselinum crispum* Mill. were the same as described in Nover et al. (1982) for *Lycopersicon*. Seedlings of *L. peruvianum* L., *Nicotiana rustica* L., *Solanum tuberosum* L., and *Zea mays* L. were grown at 25° C under greenhouse conditions. The following heat-stress conditions were used: preincubation for 15 min at 40° C followed by 2 h at 25° C

Abbreviations: HSP = heat-stress protein; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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and a second heat stress at 38° C *(L. peruvianum, S. tuberosum)*, 40° C *(N. rustica)*, or 43° C *(Z. mays)*. Heat-stress treatment of *Escherichia coli* was for 15 min at 41° C.

Preparation of antibodies and mitochondrial proteins. Spots of HSP68 were cut out from Coomassie-stained two-dimensional polyacrylamide gels. After thorough washing with distilled water the gel pieces were homogenized in complete Freund's adjuvant (Serva, Heidelberg, FRG) for subcutanous injection into rabbits. After six weeks the rabbits were given a booster injection, and bled 2 d later. The crude sera were characterized by dot-blotting and Western blot analysis as described earlier (Neumann et al. 1987). The preparation and subfractionation of potato mitochondria was carried out as outlined previously (Braun et al. 1992a).

Electron-microscopic immunocytochemistry. As shown earlier, HSPs are sensitive compounds, which loose their immunoreactivity after glutaraldehyde fixation and embedding in epoxy resins (Neumann et al. 1987). Therefore, the plant material was fixed for 2 h at 4° C with 3% formaldehyde (w/v), dehydrated at low temperature with ethanol, and embedded at −20° C in Lowicryl 4KM (Serva) (Carlemalm et al. 1981). Ultrathin sections on Ni-grids were preincubated in modified phosphate-buffered saline (mPBS: 10 mM K-phosphate buffer pH 7.4, 0.5 M NaCl, 1% Tween 20) for 15 min at 25° C and incubated in the monospecific antiserum (dilution 1:50 to 1:100, 12 h, 4° C). Immunstaining was carried out with protein A-Au_{16} (Neumann et al. 1987) for 2 h at 25° C. All washing solutions contained mPBS. Grids were finely stained with 5% aqueous uranyl acetate (20 min, 60° C). Preimmune rabbit sera or protein A-Au alone were used in controls.

Purification of HSP68 on ATP-Sepharose. Heat-treated cells of a cell-suspension culture of *L. peruvianum* were sonified in buffer M [50 mM Tris-HCl, pH 7.5; 10% glycerol; 25 mM NaCl; 10 mM $MgCl_2$; 0.1% Triton X–100; 14 mM mercaptoethanol; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (Sigma, Deisenhofen, FRG)]. The 15 000 · *g* supernatant was applied to a column of diethylaminoethylcellulose (DEAE) 23SS equilibrated with the same buffer. The HSP68/HSP70-containing fractions were detected by dot-blot analysis with the corresponding antibodies. After adjusting to 500 mM NaCl they were applied to an ATP-agarose column equilibrated with buffer M containing 500 mM NaCl. After carefully washing, the bound proteins were eluted with buffer M supplemented with 3 mM ATP. Fractions were precipitated with trichloroacetic acid (8% final concentration), separated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto nitrocellulose for Western analysis.

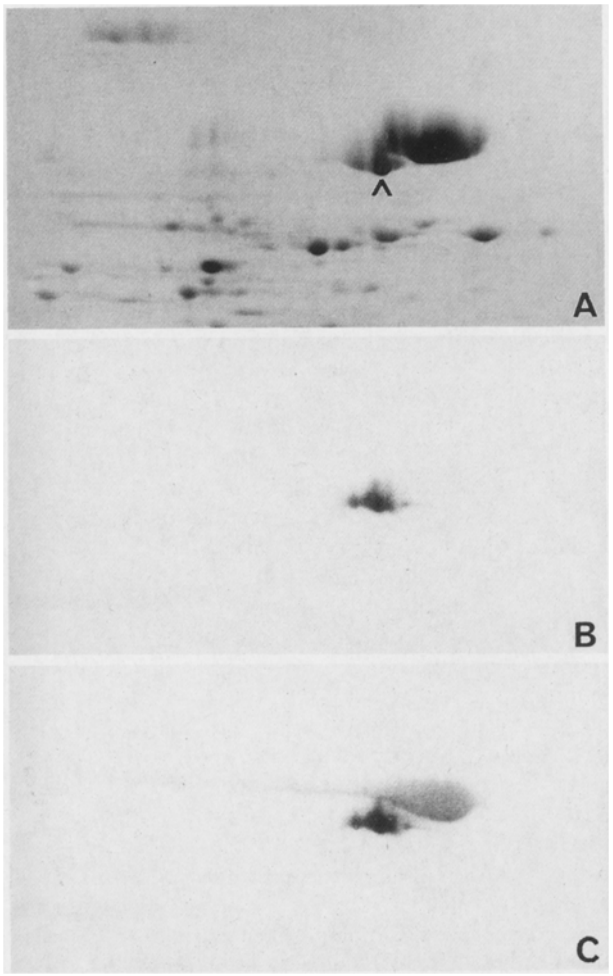

Fig. 1A–C. Specificity of anti-HSP68 antibodies raised against *Lycopersicon peruvianum* HSP68. **A** Part of a Coomassie-stained two-dimensional gel of crude heat-shock proteins from tomato. The spot cut out for immunization (HSP68) is marked by an *arrowhead*. **B** Two-dimensional immunoblot with HSP68 antibodies. **C** Two-dimensional immunoblot successively treated with HSP68 and HSP70 antibodies. No cross-reaction between HSP68 and the cytoplasmic HSP70 can be observed with the HSP68 antibodies

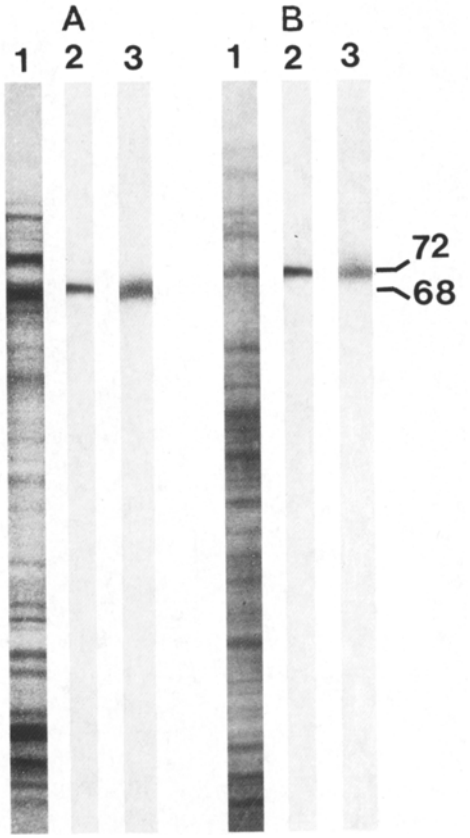

Fig. 2A, B. Immunological characterization of *Lycopersicon peruvianum* HSP68. **A** Sodium dodecyl sulfate-polyacrylamide gel of tomato proteins after heat stress: *lane 1*, stained wih Coomassie Blue; *lane 2*, Western blot after incubation with HSP68 antibodies; *lane 3*, Western blot after incubation with DnaK antibodies. **B** Sodium dodecyl sulfate-polyacrylamide gel of *E. coli* proteins after heat stress: *lane 1*, stained with Coomassie Blue; *lane 2*, Western blot after incubation with HSP68 antibodies; *lane 3*, Western blot after incubation with DnaK antibodies

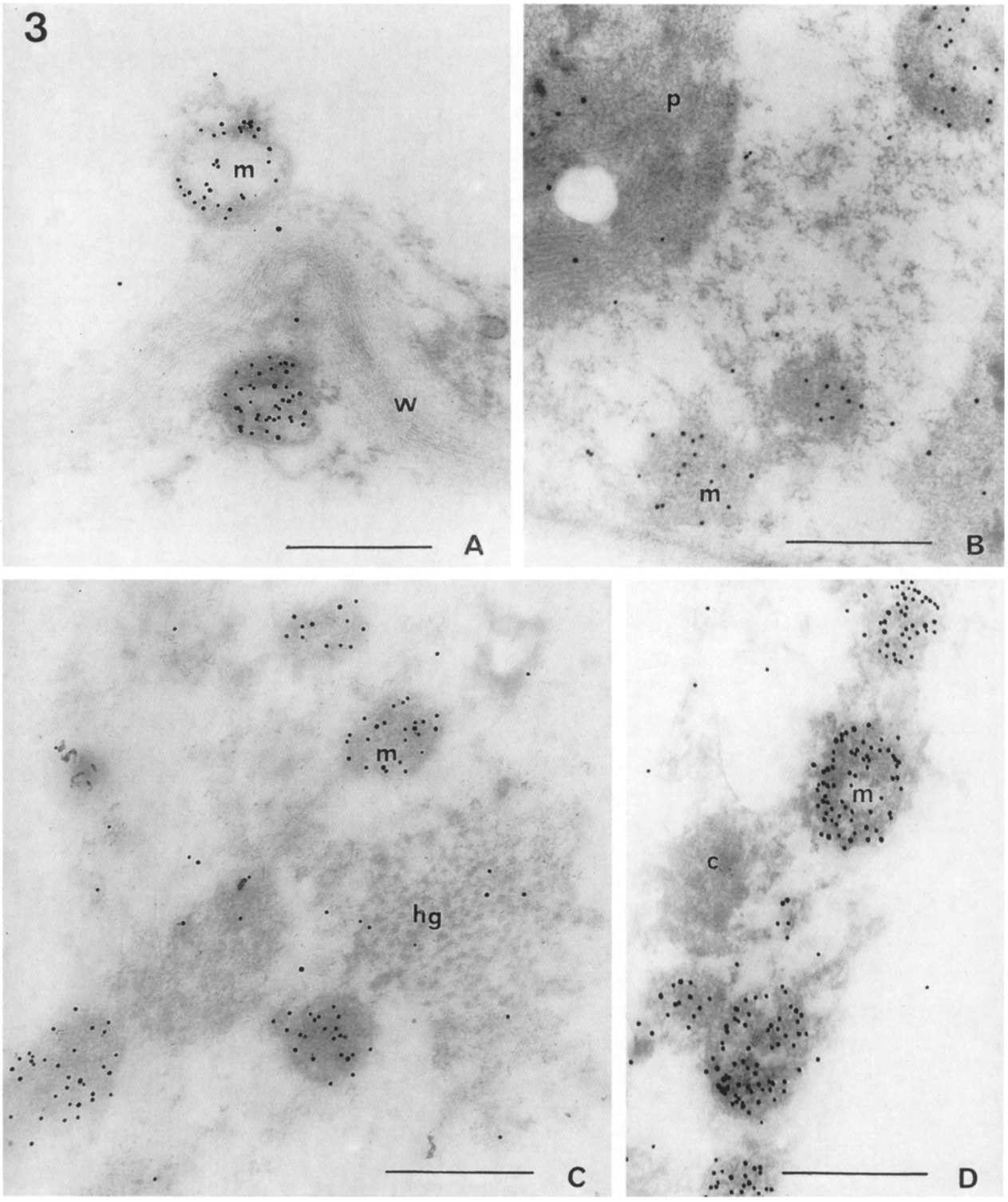

Fig. 3A–H. Subcellular localization of HSP68 in different plant species. Ultrathin sections of heat-stressed tissues and bacteria were incubated with HSP68 antibodies. **A** *Lycopersicon peruvianum* cell-suspension culture. **B** *Nicotiana rustica* leaf. **C** *Zea mays* leaf. **D** *Lycopersicon peruvianum* root. **E** *Solanum tuberosum* leaf. **F** Soil bacteria found in root preparations. **G** *E. coli*. **H** *Lycopersicon peruvianum* cell culture – control (preimmune serum). The labelled protein is nearly exclusively localized in the mitochondria (*m*). In *E. coli* and soil bacteria the HSP68 antibodies recognize the bacterial heat-shock protein DnaK. *n*, nucleus; *p*, plastid; *w*, cell wall; *hg*, heat-shock granules. **A–G** ×24000, **H** ×20000; bars = 1 μm

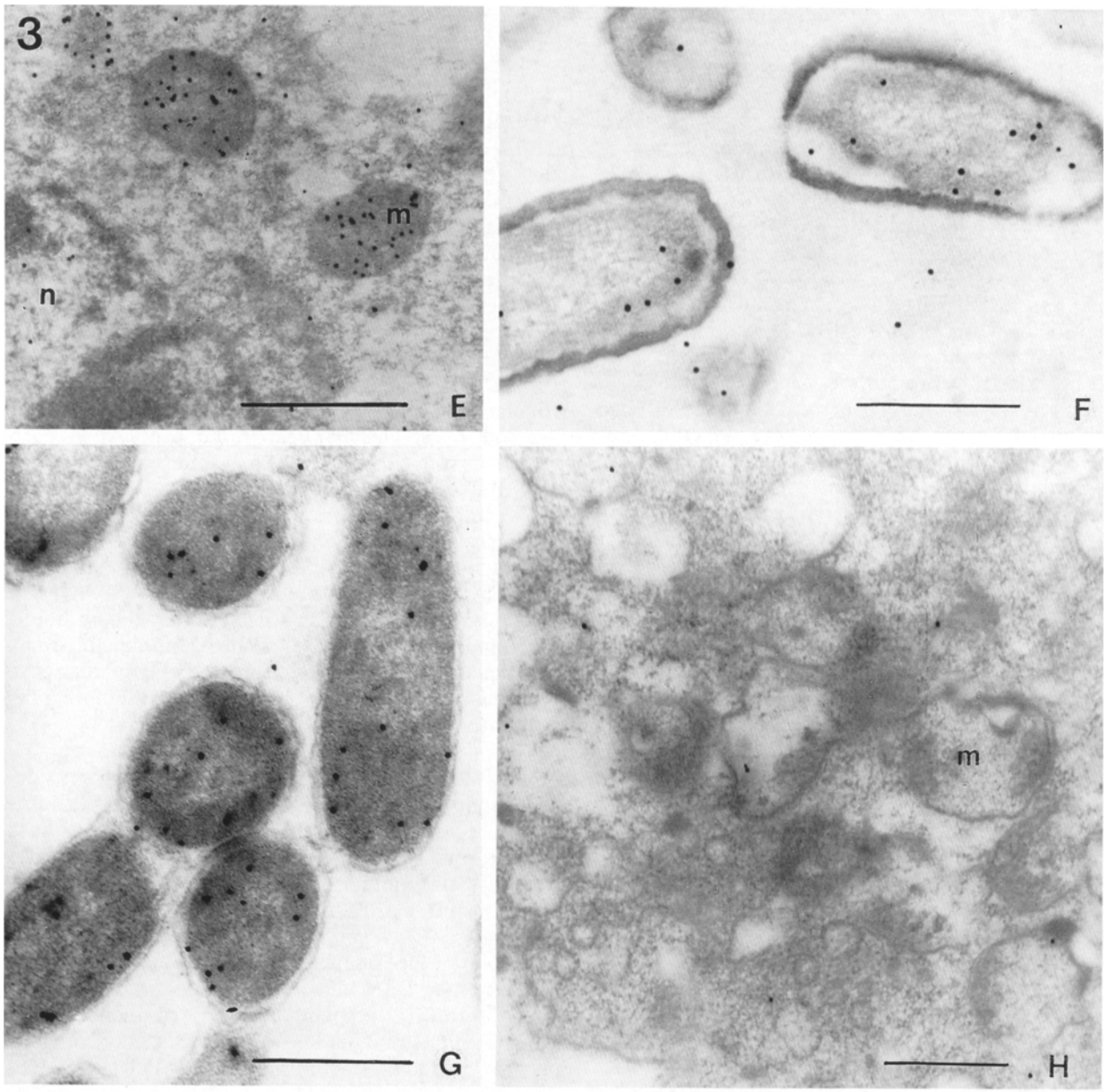

Fig. 3E–H

In-vitro translation and immunprecipitation. Total RNA from heat-stressed cell cultures of *L. peruvianum* was isolated by standard methods. The in-vitro translation was carried out in a wheat-germ system (Nover et al. 1989). For immunprecipitation cell wall preparations of *Staphylococcus aureus* in TNET buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X–100) were loaded with HSP68 antibodies (2 h, 25° C) and then washed carefully with TNET buffer. A 100-µl aliquot of the cell-wall preparation was added to the in-vitro translation mixture and incubated for 12 h at 4° C. After sedimentation and washing with TNET buffer the proteins were removed from the cell walls with 100 µl of glycine buffer (200 mM glycine-HCl, pH 2.3; 500 mM NaCl; 0.5% Tween 20) and analysed by SDS–PAGE and autoradiography.

Cloning and analysis of nucleic acids. A cDNA library from poly(A)$^+$ RNA of heat-stressed *L. peruvianum* cell cultures (Scharf et al. 1990) was screened with HSP68 antibodies. An HSP68 cDNA fragment from tomato was used to schreen a cDNA library from poly(A)$^+$-enriched RNA of potato tubers and leaves (Emmermann et al. 1991). The inserts of positively reacting plaques of tomato and potato were cloned into "Bluescript" vectors (Stratagene, La Jolla, Calif. USA) and sequenced from both ends following the dideoxy-nucleotide method (Sanger et al. 1977). Overlapping subclones were produced using the exonuclease-III deletion procedure (Dale et al. 1985).

Results

Immunological characterization of a 68-kDa heat-stress protein (HSP68) from Lycopersicon peruvianum. Among the heat-stress proteins of tomato cell cultures, one abundant protein was detected, which was slightly more basic and smaller than the cytoplasmic HSP70. It was designated HSP68 (Nover and Scharf 1984). To generate antibodies in rabbits, heat-stress proteins were isolated from

L. peruvianum. The specifity of the rabbit antisera against tomato HSP68 was tested by Western blot analysis of two-dimensional gels (Fig. 1). No cross-reaction with other tomato proteins, including the cytoplasmic HSP70 could be observed. Interestingly, antisera against the *E. coli* DnaK cross-reacted with HSP68 and, vice versa, antibodies against tomato HSP68 recognized the 72-kDA DnaK protein of *E. coli* (Fig. 2). Thus, HSP68 is immunologically related to the prokaryotic type of HSP70 proteins (DnaK-like).

Intracellular localisation of HSP68. The cross-reaction between antibodies directed against HSP68 and the bacterial DnaK protein would argue for an organellar localization of the heat-stress protein. To test this hypothesis, ultrathin sections of normally grown (see below, Fig. 7) and heat-stressed tomato cells (Fig. 3) were incubated with the HSP68 antiserum and afterwards labeled with protein A-gold. Immunocytochemical analysis of HSP68 showed a prominent labeling of the mitochondria. In all cell cultures additionally investigated (*Digitalis lanata* Ehrh., *Peganum harmala* L., *Petroselinum crispum* Mill., *Catharanthus roseus* L.: data not shown), and in different organs of whole plants (*L. peruvianum, N. rustica, S. tuberosum,* and *Z. mays:* Fig. 3; *L. esculentum* Mill., *Glycine max* L., *Pisum sativum* L. and *Hordeum vulgare* L.: data not shown), a high density of specific labeling of the mitochondria could be shown. In addition, a small fraction of gold particles was detected in other compartments (cytoplasm, cell wall, nucleus and plastids, see Fig. 3). With the exception of the cytoplasm, where the synthesis of HSP68 proceeds, the source of this labeling presumably represents unspecific binding of the antibodies. This view is substantiated by control experiments with preimmune sera, which showed a very weak labeling of the cell wall and the nucleus. However, we cannot completely exclude a weak interaction of the antibodies with a DnaK-like protein of the plastids. In accordance with the Western blots (Fig. 2), the HSP68 antibodies raised in *L. peruvianum* recognized proteins in heat-stressed *E. coli* cells (Fig. 3G) as well as in soil bacteria (Fig. 3F).

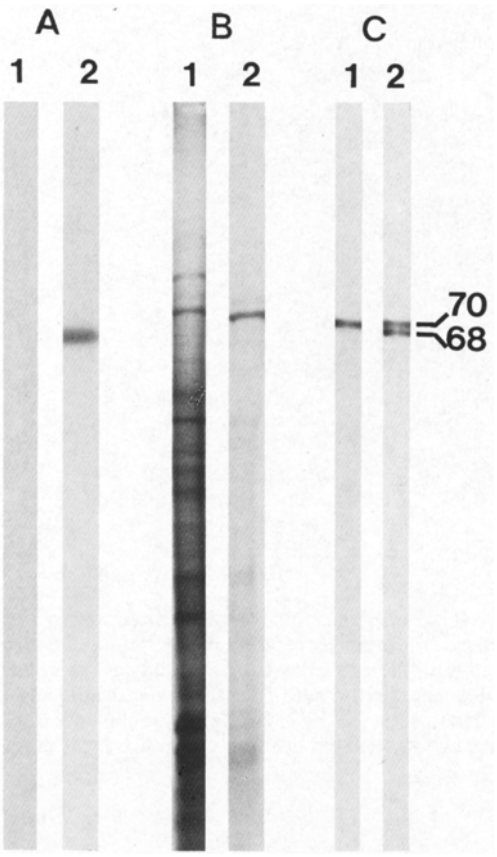

Fig. 4A–C. Intramitochondrial localization and properties of *Lycopersicon peruvianum* HSP68. **A** Western blot of SDS-denatured proteins from potato mitochondria after incubation with HSP68 antibodies: *lane 1*, membranes; *lane 2*, matrix. **B** In-vitro translation products of tomato heat-stress RNA analysed by SDS–PAGE *(lane 1)* and after immunoprecipitation with HSP68 antibodies *(lane 2)*. **C** Western blot of ATP-agarose-purified tomato proteins after incubation with HSP70 (cytoplasmic) antibodies *(lane 1)* and successive treatment with HSP70 and HSP68 antibodies *(lane 2)*

To establish the intramitochondrial localization of HSP68, isolated mitochondria from potato were subfractionated into matrix and membranes, as described previously (Braun et al. 1992a). After SDS–PAGE the purity of the subfractions was tested with antibodies against marker enzymes of the matrix and the mitochondrial membranes (Braun et al. 1992b). Immunoblots show that HSP68 is found exclusively in the mitochondrial matrix (Fig. 4A).

In-vitro translation. If HSP68 is a nuclear-encoded mitochondrial protein it should be synthezized as a precursor. To test this hypothesis, total RNA from heat-stressed tomato cells was translated in vitro in a wheat-germ system. Immunoprecipitation of the translation products with HSP68 antibodies yielded a protein of 72kDa (Fig. 4B), which is likely to represent the precursor protein of HSP68. The increase of the apparent molecular weight by 4 kDa corresponds to the putative length of the transit peptide. The presequence of the tomato HSP68 is not yet known; however, the sequenced part has 95% identity with the potato HSP68 (see Fig. 6).

Binding of ATP by HSP68. Prokaryotic and eukaryotic HSP70 are known as ATP-binding proteins (Lewis and Pelham 1985). The ATP-binding site was identified by our group (Neumann et al. 1989) and later on confirmed by crystallization of the N-terminal domain of the ATPase fragment of HSC70 (Flaherty et al. 1991). HSP68 from *L. peruvianum* was prepurified using DEAE-cellulose and ATP-binding was checked by absorption on ATP-agarose. The fractions were tested by dot-blot and Western analysis (Fig. 4C). Two different ATP-binding proteins could be identified. One of them was identical to the cytoplasmic/nuclear HSC70/HSP70 (Fig. 4C, lane 1), while the other reacted with HSP68 antibodies (Fig. 4C, lane 2).

Sequence analysis. Using the HSP68 antibodies, a cDNA fragment encoding a part of tomato HSP68 was isolated. The labelled DNA fragment was applied to rescreen the library and to screen a potato cDNA library (Emmer-

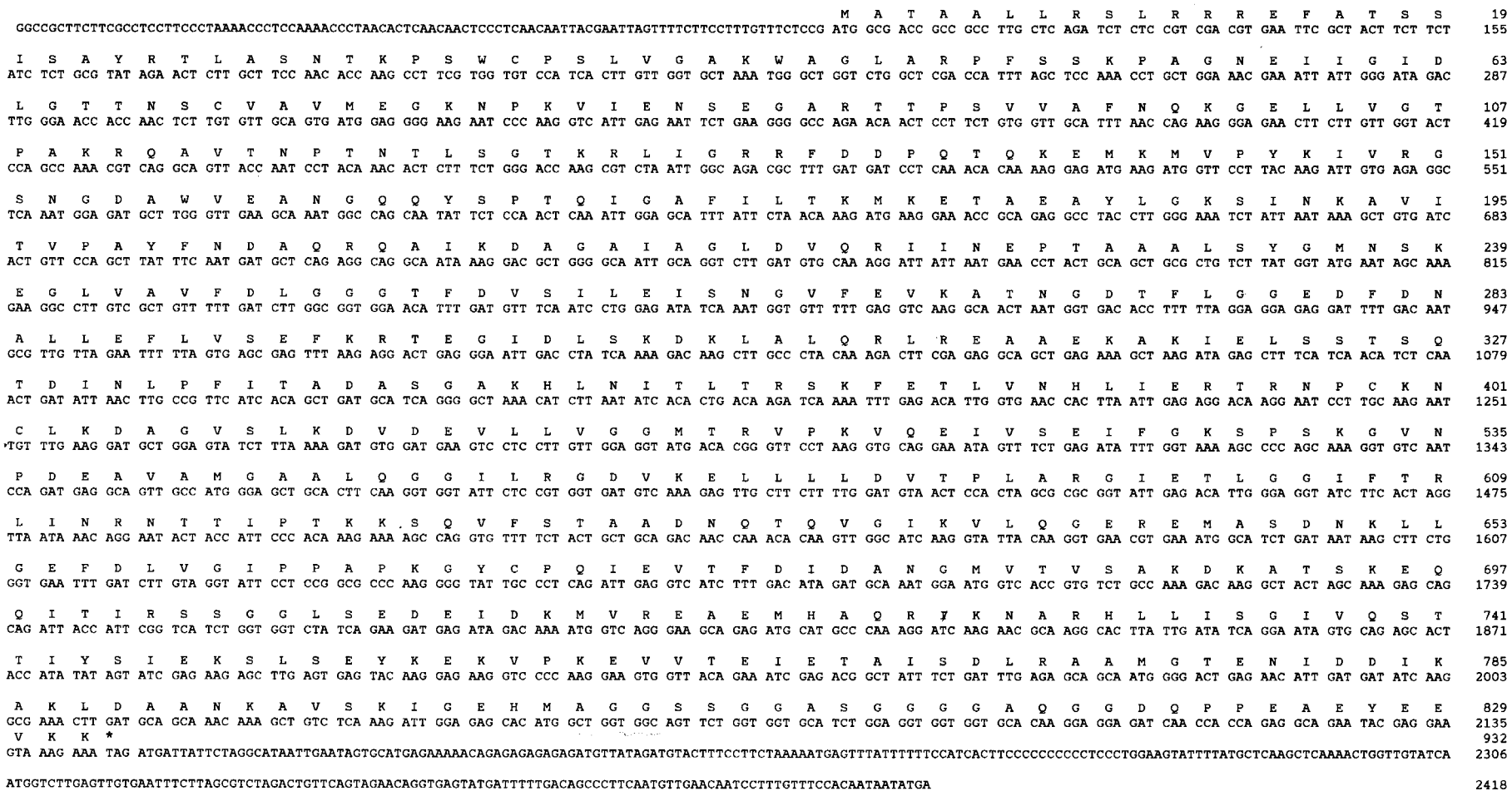

Fig. 5. Nucleotide and deduced amino-acid sequence of the insert of clone pHSP1 from potato. Amino acids are indicated above the codons using a single letter code

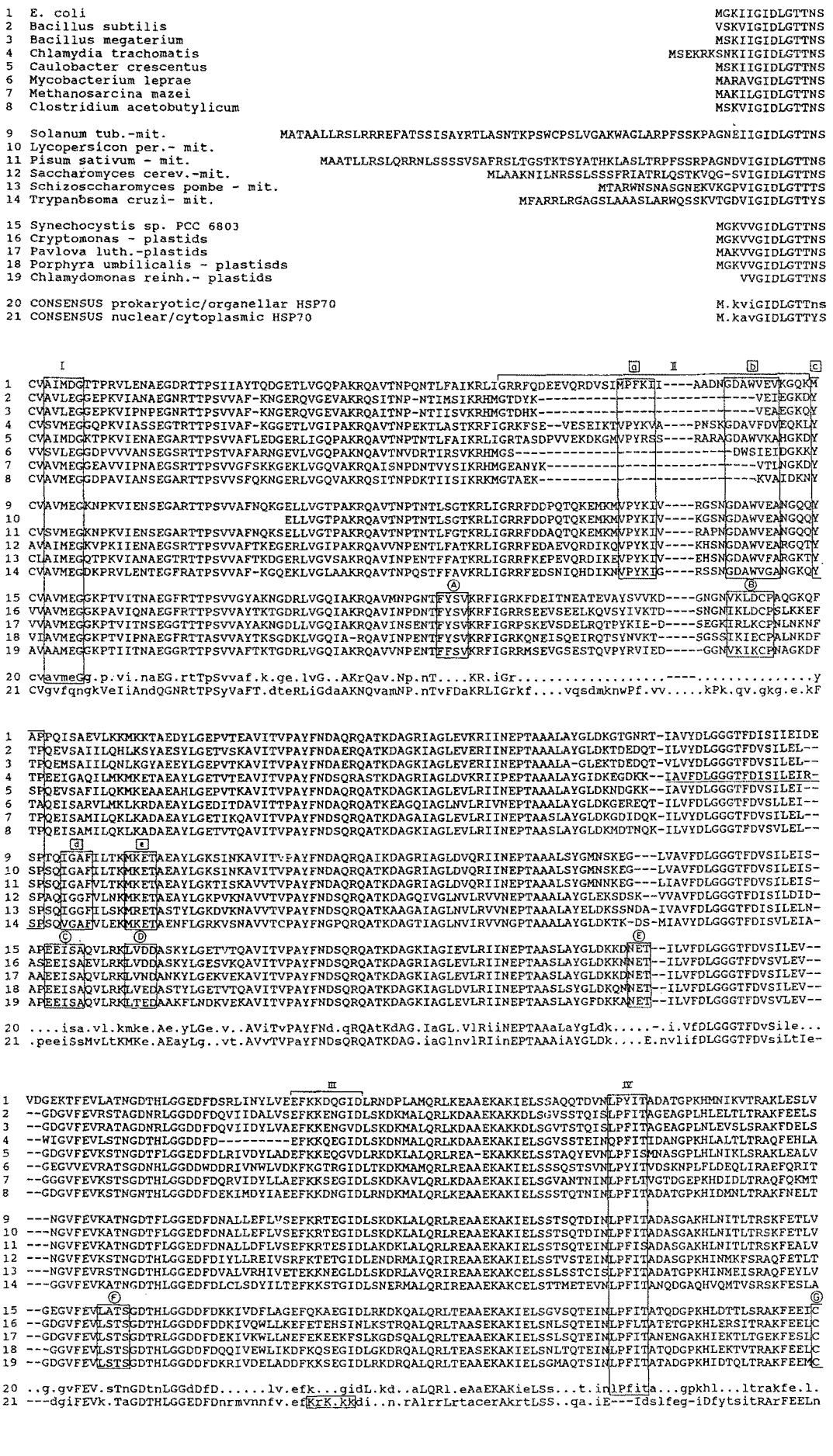
6
E. coli
Bacillus subtilis
Bacillus megaterium
Chlamydia trachomatis
Caulobacter crescentus
Mycobacterium leprae
Methanosarcina mazei
Clostridium acetobutylicum
Solanum tub.-mit.
Lycopersicon per.- mit.
Pisum sativum - mit.
Saccharomyces cerev.-mit.
Schizosccharomyces pombe - mit.
Trypanbsoma cruzi- mit.
Synechocystis sp. PCC 6803
Cryptomonas - plastids
Pavlova luth.-plastids
Porphyra umbilicalis - plastisds
Chlamydomonas reinh.- plastids
CONSENSUS prokaryotic/organellar HSP70
CONSENSUS nuclear/cytoplasmic HSP70

Fig. 6

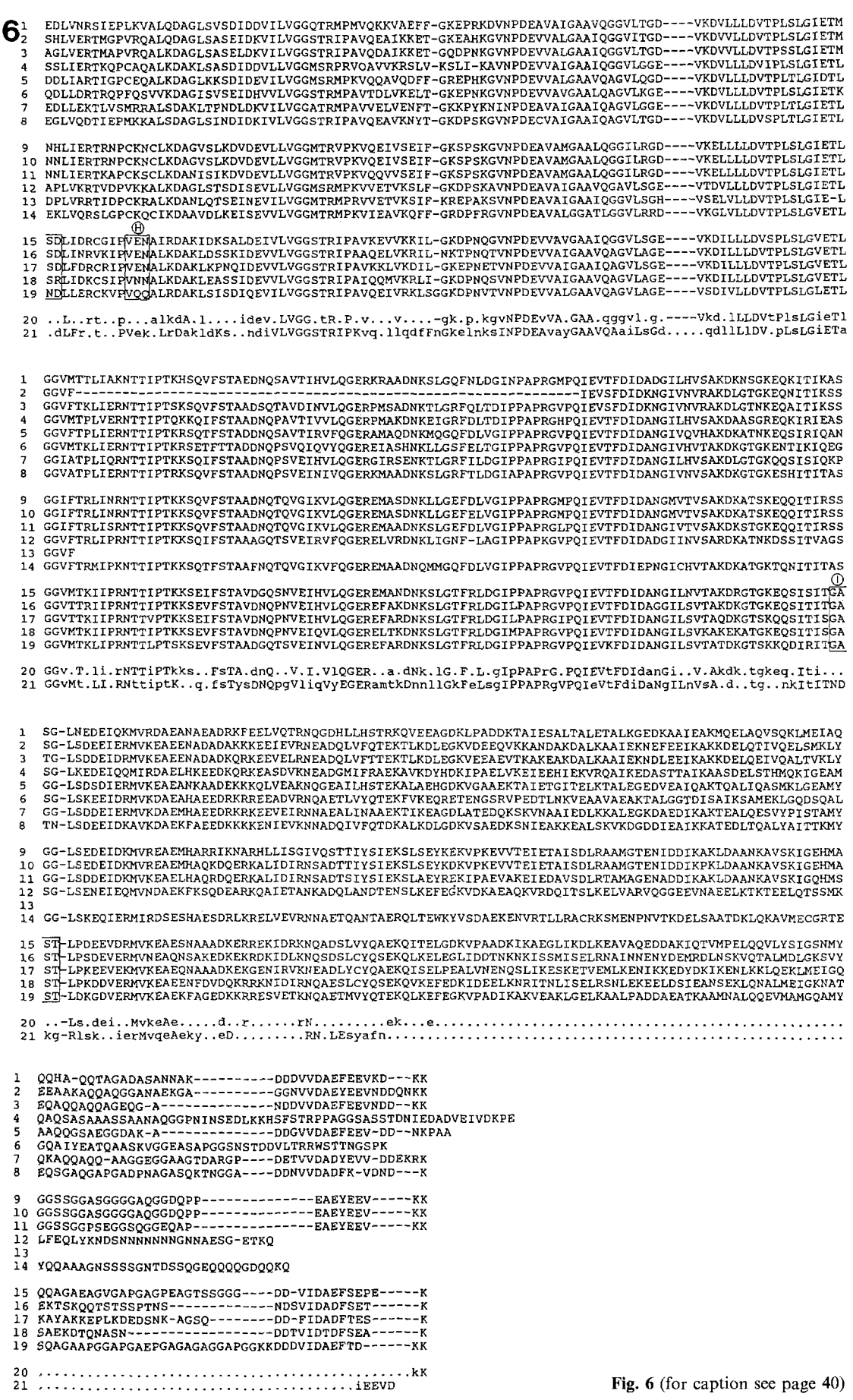

Fig. 6 (for caption see page 40)

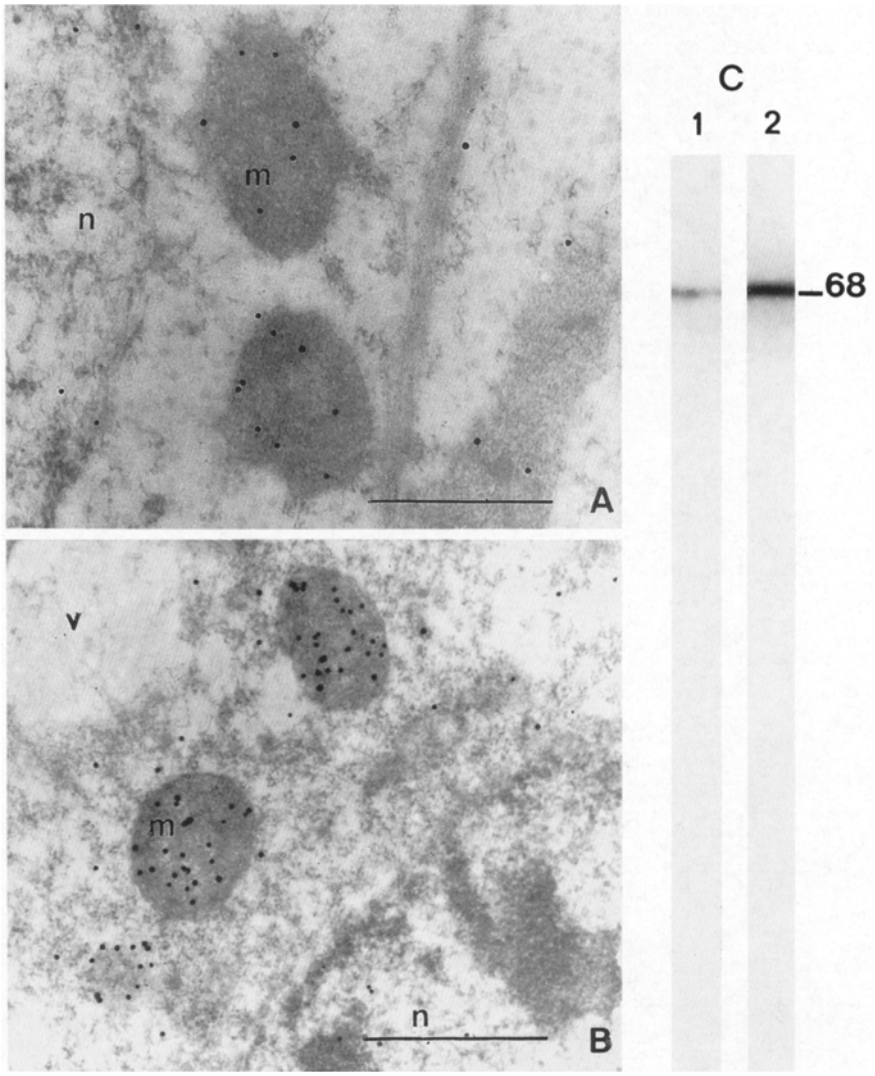

Fig. 7A–C. Expression of HSP68 *in L. peruvianum*. Ultrathin sections of tomato leaves after incubation with HSP68 antibodies (**A, B**) and Western blots of total proteins isolated from the same material (**C**). The labelled protein is nearly exclusively localized in the mitochondria (*m*). The protein is constitutively expressed (25° C; **A; C,** *lane 1*); its synthesis is increased during heat stress (40° C; **B; C** *lane 2*). *n*, nucleus; *v*, vacuole. × 24000; bars = 1 μm

Fig. 6. Alignment of HSP68 from potato (*9*) and tomato (*10*) with members of the prokaryotic/organellar HSP70 family grouped into bacterial (*1–8*), mitochondrial (*9–14*) and plastid (*15–19*) representatives. Sequences are: *1, E. coli* DnaK (Bardwell et al. 1984); *2, Bacillus subtilis* DnaK (Wetzstein et al. 1990); *3, Bacillus megaterium* DnaK (Sussman et al. 1987); *4, Chlamydia trachomatis* HSP75 (Danilition et al. 1990); *5, Caulobacter crescentus* DnaK (Gomes et al. 1990); *6, Mycobacterium leprae* HSP70 (McKenzie et al. 1991); *7, Methanosarcina mazei* S6 DnaK (Macario et al. 1991); *8, Clostridium acetobutylicum* DnaK (Narberhaus et al. 1992); *9, Solanum tuberosum* mitochondrial HSP68; *10, Lycopesicon peruvianum* mitochondrial HSP68; *11, Pisum sativum* mitochondrial PHSP1 (Watts et al. 1992); *12, Saccharomyces cerevisiae* mitochondrial SSC1 (Craig et al. 1989); *13, Schizosaccharomyces pombe* mitochondrial SSP1 (Powell et al. 1990); *14, Trypanosoma cruzi* mitochondrial Mtp70 (Engman et al. 1989); *15, Synechocystis sp.* DnaK (Chitnis et al. 1991); *16, Cryptomonas* plastid pDnaK (Wang et al. 1991); *17, Pavlova lutherii* plastid HSP70 (Scaramuzi et al. 1992); *18, Porphyra umbilicalis* DnaK plastid (Reith and Munholland 1991); *19, Chlamydomonas reinhardii* HSP80 (C. Beck, Institut für Biologie III, Freiburg, FRG, personal communication); *20*, consensus sequence of prokaryotic/organellar HSP70; *21*, consensus sequence of nuclear/cytoplasmic HSP70 (Nover 1991). A prokaryotic/organellar (this report) and a nuclear/cytoplasmic (Nover 1991) consensus are shown in 20 and 21, respectively. Invariant amino acids of all proteins of one group are given in uppercase letters and residues conserved in at least 50% of the organisms in lower-case letters. *Dots* indicate positions with lower conservation. The *underlined sequence* of *Clamydia trachomatis* (4) was corrected after consultation with the authors. All regions suitable to discriminate between families and subfamilies of HSP70 proteins are boxed. *Boxes I–IV* allow discrimination between prokaryotic/organellar and nuclear/cytoplasmic HSP70 proteins, *boxes a, b, c* distinguish prokaryotic and mitochondrial from chloroplastic HSPs, and *boxes A–I* delimit regions specific for the chloroplast proteins

mann et al. 1991) for full-length cDNA clones. Several potato cDNA clones with insert sizes around 2.3 kb were isolated. The complete primary sequence of the cDNA insert of one of the clones (pHSP1) was determined. The entire sequence is 2418 nucleotides long and contains an open reading frame of 2046 nucleotides (Fig. 5). Since the ATG of the open reading frame is preceded by an in-frame stop codon 21 bp upstream, we assume that this open reading frame encodes the entire protein. The HSP68 from potato is composed of 677 amino acids. It has a calculated molecular weight of 72 741 kDa, corresponding well with the estimated size of the in-vitro-synthesized HSP68 precursor protein on SDS polyacrylamide gels (see Fig. 4B). Sequence alignment of the precursor protein with prokaryotic DnaK proteins (Fig. 6) also indicates the presence of a presequence which may be about 40 amino acids long.

Expression of HSP68. The expression of HSP68 was analysed at the protein level using specific antibodies. Ultrathin sections from normally grown *L. peruvianum* plants and from plants which had been exposed to a heat stress at 40° C were immunolabelled with anti-HSP68 antibodies. As shown in Fig. 7, heat stress enhanced the density of gold particles specifically in mitochondria, indicating the synthesis of elevated levels of HSP68 in these organelles. Mitochondria from control cells grown at 25° C contained at least two or three times less HSP68. Western analysis of total protein extracts probed with HSP68 antibodies confirmed these results, indicating that HSP68 belongs to the constitutively expressed heat-stress proteins, whose synthesis is increased during heat stress. Western analysis of cell cultures from different higher plants and different organs (roots, leaves, cotyledons, filaments and petals; data not shown) consistently showed that HSP68 is a common, constitutively expressed but heat-inducible protein of higher plants.

In contrast to our data, Watts et al. (1992) reported that HSP68 is not heat-inducible in pea. This discrepancy possibly results from application of a rather low temperature (30 min, 37° C) for induction of a heat-shock response. At least in our hands, pea seedlings, like all other plants investigated so far, had increased levels of mitochondrial HSP68 after induction by heat stress.

Discussion

Heat-stress proteins belonging to the prokaryotic subfamily of HSP70 have been found in protozoans, fungi and mammals (Craig et al. 1989; Engman et al. 1989; Leustek et al. 1989; Powell et al. 1990). They are localized in the mitochondria and have an amino-acid composition similar to that of the DnaK protein from *E. coli*. In plants, both plastids (Reith and Munhold 1991; Wang et al. 1991; Scaramuzi et al. 1992) and mitochondria (Watts et al. 1992) have been shown to contain related proteins. For the first time we have isolated HSP68, a member of this protein family, from a higher plant and raised antibodies against it. We demonstrate by immunoblotting of proteins from fractionated organelles and by immunogold labelling of ultrathin section that this protein is located in the mitochondria. Consistent with its hydrophilic properties, HSP68 is confined to the matrix space of the mitochondrial compartment. As a nuclear-encoded protein, HSP68 is synthesized with a cleavable presequence. The putative mature polypeptide shares nearly 60% sequence identity with members of the prokaryotic subfamily of HSP70. The fact that HSP68 is more similar to the DnaK protein from *E. coli* than to nuclear/cytoplasmic HSP70 from eukaryotes is consistent with the postulated bacterial origin of mitochondria. During evolution most of the genes of the prokaryotic ancestor of mitochondria and chloroplasts, including those that encode heat-stress proteins, were transferred to the nucleus, except in the case of chloroplasts from some algae, where a DnaK-like protein is encoded in the organelles (Chitnis et al. 1991; Reith and Munholland 1991; Wang et al. 1991; Scaramuzi et al. 1992).

We have tried to identify sequence motifs suitable for discriminating between the prokaryotic/organellar and the nuclear/cytoplasmic groups of HSP70 and to further subdivide the prokaryotic/organellar group as outlined in Fig. 6. Diagnostic regions discriminating prokaryotic and eukaryotic members of the HSP70 family are marked by boxes I–IV. Examples are the missing cluster of basic amino acids (box III), which represents a putative nuclear targeting signal of the cytoplasmic members, the variable region II, and also C-terminal parts. The characteristic –MPGG– motifs of the nuclear/cytoplasmic HSP70 and the conserved –EEVD– at the C-terminus are lacking in the prokaryotic members. Instead, part of a sequence motif found at the C-terminus of the *E. coli* DnaK are retained in practically all representatives of the prokaryotic subfamily (see Fig. 6).

From the evolutionary point of view, it is worth noticing that there are a number of chloroplast-specific (Fig. 6, boxes A–I) and mitochondria-specific motifs (Fig. 6, boxes a–e). In accordance with the endosymbiont hypothesis, the *Synechocystis* DnaK fits excellently into the plastid group, whereas the bacterial sequences are closer to the mitochondrial HSP70.

Regarding the remarkable degree of sequence conservation between the members of the HSP70 family, it is surprising that the HSP68 antibodies do not cross-react with the dominant cytoplasmic HSP70 and, vice versa, that the antibodies against the latter do not recognize HSP68. This provides the experimental basis for the unequivocal localization of HSP68 in plant mitochondria using Western blot analysis (Figs. 2, 4, 7) and immunoelectronmicroscopy (Fig. 3). These results provide a valuable confirmation of the earlier results using cell-fractionation procedures (Watts et al. 1992).

Our knowledge about the role of DnaK-related proteins derives mainly from the detailed investigations with yeast (Hartl and Neupert 1990; Kang et al. 1990; Baker and Schatz 1991). As chaperones (Laskey et al. 1978; for review, see Ellis and van de Vies 1991), they are essential components of the protein-import machinery and seem to be directly involved in translocation of proteins across the mitochondrial membrane, and possibly in refolding thereafter (Hartl and Neupert 1990; Kang et al. 1990;

Baker and Schatz 1991). Since mitochondrial HSPs are synthezised in the cytoplasm and imported into the organelle, it would be of interest to ascertain how much assistance they need by other chaperones of the same family. Though details of these ATP-dependent activities remain to be elaborated, it is evident that all types of plant HSP70 proteins, have similar functions.

Finally, it is worth recalling that the genetic term for the HSP70 from *E. coli* (DnaK) originally derives from its role in DNA synthesis. Together with DnaJ, and a group of other proteins, it is involved in the reversible assembly of the primosome complex initiating DNA replication (Alfano and McMacken 1989; Dodson et al. 1989; Wickner et al. 1991). Though the biochemistry of this chaperone function is probably very similar to the former one, it concerns a fundamentally different part of cell metabolism. Very little is known about plant mitochondrial DNA replication, and nothing is known about the role of HSP68 in this process. The immunological and molecular characterization of HSP68 from tomato and potato will be a first step towards a more complete understanding of the function of HSP68 in plant mitochondria.

This work was supported by the Deutsche Forschungsgemeinschaft.

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