Sequence and expression of the mRNA encoding HSP22, the mitochondrial small heat-shock protein in pea leaves

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A 3 h treatment at 40 °C of pea (*Pisum sativum* var. Douce Provence) plants induces production and accumulation of a small heat-shock protein of 22 kDa apparent molecular mass, designated HSP22, in the matrix compartment of mitochondria [Lenne and Douce (1994) *Plant Physiol.* **105**, 1255–1261]. We show here that the HSP22 precursor (i.e. the mature protein plus the transit peptide) has an apparent molecular mass of 26 kDa after *in vitro* translation of mRNA extracted from heat-stressed pea plants and immunodetection. We have isolated, cloned and sequenced the full-length cDNA encoding the precursor of the mitochondrial HSP22. An analysis of the amino acid sequence of the mitochondrial HSP22 reveals that this protein is a representative member of the low-molecular-mass heat shock protein (HSP) superfamily, exhibiting the specific consensus regions that are typical of the small HSPs. Most importantly, comparison of the mitochondrial HSP22 sequence with that of chloroplast small

HSPs indicates that HSP22 does not contain the typical chloroplast consensus region III. We have also analysed the kinetics of HSP22 induction, and report results on the temporal expression of HSP22 at the transcriptional level. HSP22 mRNA was detected as soon as 10 min after the temperature was raised to a high temperature of 40 °C. Then the amount of HSP22 mRNA declined considerably even though pea plants were still submitted to the heat treatment. These results are discussed in light of the translation data previously published [Lenne and Douce (1994) *Plant Physiol.* **105**, 1255–1261], particularly concerning the physiological behaviour of mitochondria when plants are heat-stressed. Furthermore, we have studied the dependence of HSP22 accumulation with temperature and demonstrate that the pea mitochondrial heat-shock response is only developed under extreme environmental growth conditions.

INTRODUCTION

In response to elevation of environmental temperature, most living organisms, both prokaryotic and eukaryotic, exhibit a dramatic change in their pattern of gene expression which is called heat-shock response. Transcription of a small set of genes, referred as heat-shock genes, is enhanced and the new mRNAs are translated into heat-shock proteins (HSPs) (for a review, see [1]). In marked contrast to yeast and animal systems, the most abundant HSPs in higher plants are a group of small HSPs (smHSPs) of 15–30 kDa that belong to the HSP20 superfamily. Plant smHSPs exhibit a conserved C-terminal 'heat-shock domain' of approximately 80 amino acids, which is also found in all other members of the HSP20 superfamily, and this 'heat-shock domain' presents sequence similarity with the vertebrate eye lens protein α -crystallin [2,3]. Plant smHSPs are divided into four multigene families, being localized to different cellular compartments including the chloroplast, the cytosol and the endomembrane system [1,4,5].

Recently [6], we reported and analysed for the first time the accumulation of a 22 kDa polypeptide in pea (*Pisum sativum* L.) leaf mitochondria that is induced by exposure of pea plants to a high temperature of 40 °C, and which we called HSP22. This initial study demonstrated that HSP22 is internalized within heat-stressed pea leaf mitochondria in the soluble matrix compartment. Under heat-shock conditions close to those found in nature (40 °C for 3 h), the expression of nuclear-encoded HSP22 is markedly induced, up to final concentrations in the range of

1–2 % of total matrix proteins. Therefore, following heat-shock treatment, HSP22 is a major matrix protein. We have partially purified HSP22 and developed a polyclonal antibody directed against this protein. Use of the antiserum allowed us to show that the heat-shock response is a rapid process since HSP22 can be detected in total protein extracts as early as 30 min after the beginning of the stress treatment. The maximum level of HSP22 expression was achieved after 3 h at 40 °C, and this level was maintained for 3 days after shifting heat-stressed pea plants back to the control growth temperature of 25 °C. Since HSP22 was present within pea root and etiolated pea leaf mitochondria, it is clear that the protein accumulates in a light-independent manner. Finally, we have shown that HSP22 is included in a high-molecular-mass particle of 230 kDa.

The aim of the present study is 2-fold: (i) to determine whether or not mitochondrial HSP22 is a representative member of the well-documented low-molecular-mass HSP superfamily, and (ii) to analyse the kinetics and mechanism of HSP22 induction at the transcriptional level.

To this end, we have isolated, cloned and sequenced a pea leaf mitochondrial HSP22 cDNA. For this purpose, we have used a two-step strategy. First, a cDNA probe was quickly obtained by PCR using a method developed previously [7]; this was based on minimal knowledge of protein sequence, such as an N-terminal sequence. The obtained cDNA probe was then used to clone the full-length HSP22 cDNA by screening a pea-leaf cDNA library. The full-length cDNA encodes a precursor form of mature HSP22 containing a mitochondrial transit peptide. Analysis of

Abbreviations used: HSP(s), heat-shock protein(s); smHSP(s), small heat-shock protein(s); PVDF, polyvinylidene difluoride; SSPE, 0.15 M NaCl/10 mM NaH₂PO₄/1 mM EDTA.

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the primary sequence of HSP22 precursor displayed considerable similarity with members of the low-molecular-mass HSPs superfamily. We also report the temporal expression of HSP22 at the transcription level and discuss the results in light of the translation data reported previously [6].

MATERIALS AND METHODS

Plant material, growth and heat-shock conditions

Plants were grown from mature pea seeds (*Pisum sativum* L., var. Douce Provence) in vermiculite for 14 days at 25 °C, either under a 12-h photoperiod of white light from fluorescent tubes (500 mmol·m⁻²·s⁻²) or in the dark for etiolated plants. Plants were watered every day with tap water. Heat stress was administered by incubating light-grown or etiolated 14-day-old intact plants at 40 °C for 0–3 h, in a growth chamber (Bioclim 1600 sp, Weiss technik, Lindenstruh, Germany). A relative humidity of 70 % was maintained throughout the stress period to minimize plant dehydration. Leaves were harvested immediately after the stress period.

HSP22 N-terminal and internal sequence determinations by microsequencing

Mitochondria and matrix proteins were prepared as described previously [6]. Two-dimensional gel electrophoresis was performed [8]. In the first dimension, soluble mitochondrial proteins were separated by isoelectric focusing from pH 3.5 to 9 (1.6 % Pharmalytes pH 3 to 10, 3.4 % ampholines pH 5 to 7, Pharmacia). Separation in the second dimension was carried out on a 7.5–15 % polyacrylamide gel under denaturing conditions (SDS/PAGE). For N-terminal microsequencing, proteins on the gel were electrophoretically transferred on to polyvinylidene difluoride (PVDF) membrane (Problott, Applied Biosystems), in 10 mM CAPS (3-cyclohexylamino-1-propanesulphonic acid), pH 11, prepared in 10 % methanol, using a Bio-Rad electrotransfer apparatus. PVDF membrane was stained with Coomassie Blue R-250 and destained in 50 % methanol. The HSP22 spot was

visualized and excised. HSP22 N-terminal sequencing was performed on an automated Applied Biosystems sequenator (477A) equipped with an on-line phenylthiohydantoin (PTH)-amino acid analyser (120A). The N-terminal sequence of mitochondrial HSP22 obtained is presented in Figure 1. For internal microsequencing, the gel was stained for 20 min with 0.1 % Coomassie Blue R-250 in 20 % methanol/0.5 % acetic acid; the HSP22 spot was then visualized and cut out from the gel. The stained protein spot was then destained with 30 % ethanol for 1 h at room temperature, washed twice with 20 ml of 50 % acetonitrile/100 mM ammonium bicarbonate for 15 min at 30 °C, equilibrated twice with 20 ml of cleavage buffer (25 mM Tris/HCl, 1 mM EDTA, 10 % acetonitrile, pH 8.5), and partially dehydrated in a Speed Vac concentrator (Savant). The gel slice was then rehydrated in 10 µl of cleavage buffer. Then trypsin (EC 3.4.21.4; Boehringer) was added at an enzyme to protein ratio of 1:50 (w/w). The digestion was carried out for 36 h at 37 °C. Peptides were extracted [9] and purified by reverse-phase HPLC using a VYDAC TP C18 column (5 µm, 300 Å, 250 mm × 2.1 mm; Pharmacia) according to Bourmeyster et al. [10]. Peptide-containing fractions were spotted on to a glass-fibre disk coated with polybrene, and amino acid sequencing was performed as described above. Internal sequences of mitochondrial HSP22 obtained are presented in Figure 1.

Total RNA and poly(A)⁺ mRNA isolation: preparation of cDNA

Total RNA was isolated from 14-day-old etiolated pea plants, grown at 25 °C in the dark and heat-stressed at 40 °C for 3 h. Pea leaves (7 g) were ground to a fine powder in liquid nitrogen with a pestle and mortar. The resultant powder was suspended in a mixture consisting of 1 vol. of 50 mM Tris/HCl (pH 9)/1 % SDS and 1 vol. of phenol. The mixture was completely thawed at room temperature and the two phases were separated by centrifugation. The upper aqueous phase was collected, washed with a phenol/chloroform extraction, and RNA was specifically precipitated with 2 M LiCl at 0 °C for 2 h. After centrifugation, the pellet was solubilized in sterile distilled water and stored at –20 °C. Poly(A)⁺ mRNA was selected twice by oligo(dT) cellulose chromatography (Pharmacia) [11]. Synthesis of single-stranded cDNA was realized with M-MuLV reverse transcriptase (BioLabs). The primer used in the reverse transcriptase reaction, named RT-primer (Figure 1), is a 38-mer oligonucleotide consisting of 21 nucleotides and containing *Eco*RI, *Bam*HI and *Hind*III sites, followed at the 3'-end by a stretch of (dT)₁₇.

In vitro translation

Poly(A)⁺ mRNA extracted from control or heat-stressed (40 °C, 1.5 h) pea leaves was translated using a wheat germ translation kit (Boehringer) in the presence of the full set of 20 amino acids. After RNase (0.1 mg/ml) treatment in 50 mM EDTA for 15 min at 37 °C, synthesized proteins were purified on SDS/PAGE and immunoblotted with anti-HSP22 serum as described previously [6].

Cloning strategy of HSP22 cDNA

(I) PCR production of a 548 bp fragment of HSP22 cDNA

The first part of the strategy was based on amplification of cDNA by PCR [7] (Figure 2a). This method requires the identification of 18 consecutive residues in the protein N-terminal sequence. Single-stranded cDNA prepared from etiolated heat-stressed pea leaves was used as a template for each PCR

N-terminal	NH ₂ -NTNAMRQYDQHSDDRNVDVY-COOH
Internal sequences	NH ₂ -XTEDALFLX-COOH NH ₂ -LYK-COOH

Primer 1 (P1)	5' GGGAAATTC CAACACGACGCGATGAG 3' <i>Eco</i> RI T A T A C T T C C
Primer 2 (P2)	5' GGAAGCTT TACGTCGACGTTGCTGTCGTC 3' <i>Hind</i> III A A A A GA A T T C C
Primer 3 (P3)	5' GGGAAATTC CCAGTACGATCAACACTCC 3' <i>Eco</i> RI
Primer 4 (P4) or RT primer	5' ACGGGATCCCTCGAGTCTAGATTTTTTTTTTTTTTTT 3' <i>Bam</i> H1 <i>Xho</i> 1 <i>Xba</i> 1

Figure 1 N-terminal and internal sequences of pea mitochondrial HSP22, and the nucleotidic sequences of primers used for PCR cloning strategy

Amino acid sequences were determined as described in the Materials and methods section. Annealing regions of primers are shown in bold and restriction enzyme sites in italic typeface. The primers P1 and P2 are based on the two extremities of the known HSP22 N-terminal sequence and each primer mixture is a degenerate sequence containing all possibilities predicted by the genetic code.

Figure 2 Cloning strategy to obtain the HSP22 full-length cDNA

(a) PCR method for obtaining a 700 bp fragment of the HSP22 cDNA. Oligonucleotide primers are indicated as P1, P2, P3 and P4, and restriction enzymes sites as *E*, *H*, *A*, *O* and *B* for *Eco*RI, *Hind*III, *Xba*I, *Xho*I and *Bam*HI respectively. SS and DS cDNA stand for single-stranded and double-stranded cDNA. The PCR conditions are described in the Materials and methods section. (b) Nucleic screening of a pea leaf cDNA library. ATG and TAG are respectively the initiation translation codon and the stop translation codon of the HSP22 cDNA. Abbreviations: M, marker DNA ladder in base pairs (bp); I, first PCR; II, second PCR; nt, nucleotides.

amplification. Two 25-mer degenerate oligonucleotide mixtures (17 nt corresponding to HSP22 plus 8 nt for linker sequences), P1 and P2 (Figures 1 and 2), corresponding to both extremities of the known N-terminal sequence of HSP22, were first used to amplify by PCR a 70 bp DNA fragment to be subcloned in pUC18 vector and sequenced. A two-stage amplification, known as a 'booster' PCR [12], was done for this reaction. The reaction was performed in a solution containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol (DTT), the four dNTP (500 μ M each), cDNA (2 ng) and *Thermus aquaticus* DNA polymerase (0.5 unit; Appligene). A different primer concentration was employed at each stage. The first 30 cycles were carried out at a primer concentration of 50 nM each (reaction volume 70 μ l) and then, for the 30 subsequent cycles, the primer concentration was increased to 2 μ M each (final reaction volume 100 μ l). The reaction mixture was first incubated for 5 min at 94 °C to denature the DNA and then cooled to 35 °C. Then, each step of the 30 cycles was composed of the following: denaturation for 1 min at 95 °C, annealing for 2 min at 5 °C below the minimum dissociation temperature of the oligonucleotide mixture, and DNA synthesis for 2 min at 70 °C. The reaction was terminated by a 7-min-long incubation at 70 °C. The 70 bp DNA fragment produced by PCR encodes the N-terminal part of HSP22, but because its 5'- and 3'-ends arose from mixed primers, only the central part of the sequence corresponds exactly to the HSP22 cDNA. An oligonucleotide (19-mer plus 8-mer of linker sequence), P3 (Figure 1), was synthesized from this unique sequence in order to extend DNA synthesis towards the 3'-end of the coding strand. A second PCR amplification of cDNA was then done using P3 primer and a 23-

mer oligo(dT) reverse primer, P4 (Figure 1). This method allowed us to obtain a 700 bp DNA fragment which was subcloned in pUC18 and sequenced.

(II) Nucleic screening of a pea-leaf cDNA library

In order to clone the full-length HSP22 cDNA, a 548 bp fragment of HSP22 cDNA obtained by *Eco*RI-*Xba*I digestion of the pUC18-HSP22 clone was used to screen a pea cDNA library (Figure 2b). The λ gt11 etiolated-pea-leaf (10-day-old) cDNA amplified library was kindly provided by Dr. David Macherel (Grenoble, France). Before screening, the library was checked for the presence of HSP22 cDNA by PCR amplification with specific HSP22 primers. A total of 135000 plaques were grown on *Escherichia coli* Y1090 and transferred to Hybond-N⁺ membranes (Amersham). The membranes were prehybridized for 1 h at 65 °C in 5 $\times$ SSPE (0.75 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA)/5 $\times$ Denhardt's solution/0.5% SDS/20 μ g/ml of denatured salmon sperm DNA. Hybridization was for 16 h at 65 °C in the presence of the same reagent and 20 ng of HSP22 cDNA fragment labelled with [α -³²P]dCTP by the Rediprime DNA labelling system (Amersham). The membranes were washed twice in 2 $\times$ SSPE/0.1% SDS, at 25 °C, and three times in 1 $\times$ SSPE/0.1% SDS, at 65 °C, for 15 min each time. Three positive clones were purified by two rounds of screening. Phage DNA was extracted and digested with *Eco*RI. Hybridization and restriction-fragment analysis showed that the three clones were identical. One of the inserts that was 883 bp long was subcloned into pUC18 for sequencing.

DNA sequencing and sequence analysis

The subcloned cDNA insert was sequenced by the dideoxy-chain-termination method [13], using the Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH, U.S.A.). Both strands were sequenced using reverse and forward primers complementary to either pUC18 or the HSP22 cDNA sequence. Reaction products were run on polyacrylamide gels and DNA sequence analyses were done with the PC/Gene Release 6.8 program (IntelliGenetics).

Northern blot hybridization

RNA was extracted, as described above, from control or heat-stressed pea leaves grown in the controlled atmosphere of the growth chamber. After denaturation with glyoxal, RNA was transferred to Hybond-N⁺ membranes (Amersham) and hybridized with a ³²P-labelled HSP22 cDNA probe, identical to that used for cDNA library screening. Northern blot analysis was done as described previously [14].

RESULTS

Identification of HSP22 precursor

To identify the HSP22 precursor, we performed the *in vitro* translation of poly(A)⁺ mRNA of heat-shocked or control pea leaves (Figure 3), using the wheat-germ system described in the Materials and methods section. The translated products were then separated by electrophoresis on a polyacrylamide gel under denaturing conditions. Polypeptides were transferred on to a nitrocellulose membrane and exposed to the anti-HSP22 antibodies [6]. This analysis revealed a polypeptide with an apparent molecular mass of about 26 kDa, a value that is substantially higher than that estimated for mature HSP22 as isolated from pea leaf mitochondria. The latter value is of the order of 22 kDa

Figure 3 *In vitro* translation of poly(A)⁺ mRNA extracted from heat-stressed pea leaves

mRNA (1 µg) was prepared from heat-stressed pea plants at 40 °C for 1.5 h. mRNA was submitted to *in vitro* translation in a wheat germ system (Boehringer) with the full set of 20 amino acids. *In vitro* translation products were analysed by one-dimensional electrophoresis under denaturing conditions, and immunoblotted with the antiserum directed against mitochondrial HSP22 (dilution 1/10 000). Lane C, immunoblotting analysis of *in vitro* translation products obtained with poly(A)⁺ mRNA extracted from control pea plants (25 °C); lane S, immunoblotting analysis of *in vitro* translation products obtained with poly(A)⁺ mRNA extracted from heat-stressed pea plants at 40 °C for 1.5 h.

[6]. These results suggest therefore that HSP22 is synthesized in a precursor form, presumably containing a transit peptide.

Isolation and characterization of HSP22 cDNA clone

A HSP22 cDNA probe was obtained by PCR (see the Materials and methods section and Figure 2). The specificity of the probe was established by comparison of the deduced amino acid sequence of the encoded protein with the N-terminal and internal sequences of mature HSP22. The N-terminal sequence of mature mitochondrial HSP22, determined by microsequencing, is effectively present in the predicted sequence, as well as the two internal sequences obtained from trypsin digestion (Figure 4). A pea leaf cDNA library, constructed in λgt11, was screened using this HSP22 cDNA probe as described in the Materials and methods section. One positive clone was fully analysed. The insert was completely identical to the HSP22 cDNA previously obtained by PCR, except it was 245 bp longer at the 5'-end (Figure 4). The insert is approximately 900 bp long and hybridizes to a 0.9 kb mRNA detected in large amounts specifically in heat-shocked pea leaves (see later, Northern-blot experiments). The size of the detected mRNA and the length of the cDNA insert suggested that the cDNA sequence is close to full length. This insert is exactly 883 bp long and contains only one uninterrupted open reading frame of 606 bp, comprising of the sequence between the ATG translation initiation start site at position 133 and the TAG stop codon at position 739. The cDNA presents a 132 bp 5'-untranslated region before the putative translation initiation ATG triplet. The sequence around the first methionine, CTCAATGGC, agrees with the -1, +4 and +5 positions of the consensus sequence for translation initiation sites in plant nuclear genes, AACAATGGC [15]. The synthesis of HSP22 will therefore begin with the dipeptide methionyl-alanine as do the majority of plant proteins [15]. A 139 bp 3'-untranslated region starts with the TAG translation stop codon at position 739 and is followed by a poly(A)⁺ tail. This region does not exhibit any consensus polyadenylation signal [16]. This feature is shared by other heat-shock genes in plants [17]. Compared with other cytosolic HSP genes in maize, the GC nucleotide content of the open reading frame of HSP22 is low (48 % versus 70 %) [18]. Nevertheless, this GC nucleotide content is clearly different from that of its 3'-flanking region that is only 30 %, without considering the poly(A)⁺ tail, which is common among most genes and probably prevents formation of stable secondary structures [16].

Starting with the first in-frame methionine residue, the open reading frame encodes a protein of 202 amino acids (Figure 4), with a molecular mass of 22.9 kDa. The N-terminus of the

Figure 4 Nucleotide sequence of the HSP22 cDNA and deduced amino acid primary sequence of HSP22

The full-length HSP22 cDNA is 883 nt long, with an uninterrupted open reading frame of 606 bp. The HSP22 protein primary sequence is indicated below the HSP22 cDNA sequence and represents a 202-amino-acid precursor protein. Initiation (ATG) and stop (TAG) translation codons are indicated in bold at the beginning and the end of the HSP22 cDNA. Annealing regions of primers P1, P2, P3 and P4 are underlined, and amino acid sequences determined by microsequencing (N-terminal and internal sequences of the mature protein) are presented in bold within the HSP22 predicted protein sequence. The beginning of the mature protein, present in the matrix space after import and maturation of the cytosolic HSP22 precursor, is indicated. The HSP22 probe, obtained by the PCR part of the cloning strategy, is limited by brackets. The *Xba*I restriction site, localized in the non-coding flanking 3'-region, is indicated in both bold and italic type. The nucleotide sequence reported here will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X86222.

mature protein corresponds to Asn-33 in the deduced sequence, indicating that the protein is synthesized with a 32-amino-acid transit peptide. The mature protein is predicted to consist of 170 amino acids, giving a protein with a molecular mass of 19.5 kDa. Also, using the computer analysis, a potential mitochondrial transit peptide is predicted within the first 32 amino acids. These results are compatible with the HSP22 localization to the mitochondrial compartment which we have demonstrated in previous work [6]. This transit peptide region exhibits characteristics of mitochondrial transit peptides, such as a high content of serine (Ser) and basic residues (Arg, Lys), but no acidic residues (Asp, Glu) [19]. Moreover, a transmembrane helix is predicted [20] from residue 2 to 21. The hydropathy profile of HSP22 precursor reveals that, for the most part, the protein is hydrophilic and has no apparent membrane-spanning region [21]. We have already demonstrated that HSP22 is a protein localized in the soluble fraction of mitochondria [6]. The iso-

Figure 5 Alignment of the pea mitochondrial HSP22 primary sequence with pea smHSP primary sequences (a) and identification of the smHSP motif GVLKVTVP by comparison of the C-terminal region with those of smHSPs from several organisms (b)

The alignment of the proteins is done according to Higgins and Sharp [22]. The accession numbers in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases are indicated for each protein. (a) Alignment of the pea mitochondrial HSP22 and pea smHSP primary sequences: HSP22 (mit), mitochondrial protein (see the present work); HSP21 (chl), chloroplast protein (P09886) [4]; HSP18.1 (cyt), cytoplasmic protein of class I (P19243) [23]; HSP17.7 (cyt), cytoplasmic protein of class II (P19242) [23]; HSP22.7 (end), endomembrane protein (P19244) [5]. (b) Comparison of C-terminal regions between members of the smHSP superfamily and pea mitochondrial HSP22. HSP22 (pea), pea mitochondrial HSP22 (amino acids 141–189; the present work); HSP26 (yeast), *Saccharomyces cerevisiae* HSP26 (amino acids 141–191) (P15992) [38]; HSP18 (M.I.), *Mycobacterium leprae* HSP18 (amino acids 71–119) (P12809) [39]; α B-crist (bovin), chain B of the α -crystallin protein from the vertebrate eye lens (amino acids 100–151) (P02510) [40]. Key to symbols: *, identical amino acids; ., conservative replacements; —, gaps introduced to optimize alignments. Consensus I and II regions, as defined in [31], are underlined.

electric focusing point (pI) is 5.36 for the mature HSP22, i.e. very close to 6, as measured previously by two-dimensional electrophoresis experiments [6]. Therefore HSP22 is a relatively acidic protein at the matrix pH of 7.4. It is interesting to note that mature mitochondrial HSP22 presents two half-parts opposed in their electric behaviours. The N-terminus (amino acid 33 to first lysine of the sequence in position 123) exhibits an acidic pI of 4.56 and a global negative electric charge. In contrast, the C-terminus (amino acids 124 to 202) exhibits a very basic pI of 9.21 and a global positive electric charge.

Comparison of mitochondrial HSP22 precursor to other low-molecular-mass HSPs

The predicted amino acid sequence of mitochondrial HSP22 precursor was compared with four pea low-molecular-mass HSPs, according to Higgins and Sharp [22]. These four HSPs are localized to three subcellular compartments of the plant cell, including the cytosol (class I and II [23]), the chloroplasts [4] and the endomembrane system [5]. As Figure 5(a) shows, the similarity at the amino acid level between the five pea low-molecular-mass HSPs is low near the N-terminus (from amino acid 1 to 105 in HSP22, 35% similarity) and increases as the comparison

progresses toward the C-terminus (from amino acid 106 to 202 in HSP22, 56% similarity). All eukaryotic smHSPs present two similar consensus regions, I and II, in their amino acid sequences [1]. Consensus I (residues 162 to 187 in HSP22) and II (residues 117 to 145 in HSP22) are present in the mitochondrial HSP22 C-terminus (underlined in Figure 5a). A further sequence analysis revealed the presence, in the mitochondrial HSP22 C-terminus (Figure 5b), of a HSP motif GVLKVTVP (between amino acids 179 and 186) that is conserved in all smHSPs from prokaryotes and eukaryotes [3]. In addition, well-conserved residues are generally polar residues such as His, Lys, Arg, Asp or Glu. These amino acids could be involved in electrostatic interactions between smHSPs and/or other proteins, considering that the C-terminus in smHSPs is presumed to play a role in protein aggregate formation [1]. In support of this suggestion, we have demonstrated previously that pea leaf mitochondrial HSP22 is included in a high-molecular-mass particle of 230 kDa [6]. From this sequence analysis, we conclude that pea mitochondrial HSP22 belongs to the low-molecular-mass HSPs superfamily.

A similarity scan, according to Lipman and Pearson [24], of the entire HSP22 sequence through the Swiss-Prot databank disclosed a high similarity (67% similarity including 57% of identical amino acids and 10% of conservative replacements)

Figure 6 Alignment of the pea mitochondrial HSP22 primary sequence with the *Chenopodium rubrum* chloroplast HSP23 primary sequence

The accession numbers in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases are indicated for each protein. **(a)** Alignment of the two entire protein sequences according to Lipman and Pearson [24]. HSP22 (P.s), pea (*Pisum sativum*) HSP22 (the present work); HSP23 (C.r), *Chenopodium rubrum* chloroplast HSP23 (P11890) [28]. Upright lines indicate identical amino acids, dots represent conservative replacements and dashes indicate gaps introduced to optimize alignments. **(b)** Alignment of regions localized in the N-terminal portions of several smHSPs, around the chloroplast consensus region III domain. HSP22 (P.s), pea (*Pisum sativum*) mitochondrial HSP22 (amino acids 66–94) (the present work); HSP23 (C.r), *Chenopodium rubrum* chloroplast HSP23 (amino acids 64–92) (P11890) [28]; HSP21 (P.s), pea (*Pisum sativum*) chloroplast HSP21 (amino acids 89–116) (P09886) [4]; HSP22 (G.m), soybean (*Glycine max*) chloroplast HSP22 (amino acids 36–64) (P09887) [4]; HSP22 (P.h), petunia (*Petunia hybrida*) chloroplast HSP22 (amino acids 97–125) (P30222) [26]; HSP21 (A.t), *Arabidopsis thaliana* chloroplast HSP21 (amino acids 83–110) (P33170) [26]; HSP26 (Z.m), maize (*Zea mays*) chloroplast HSP26 (amino acids 85–112) [27]. Upright lines and asterisks indicate identical amino acids and dots indicate conservative replacements. The pea mitochondrial HSP22 sequence is written in bold, that of *Chenopodium rubrum* chloroplast HSP23 is underlined. Sequence similarity scores (%) between pea mitochondrial HSP22/C.r chloroplast HSP23 and C.r chloroplast HSP23/other chloroplast smHSPs are presented on the right.

with *Chenopodium rubrum* chloroplast HSP23 [25]. In addition, HSP23 is 204 amino acids long which is close to the HSP22 size (202 amino acids). The C-terminal regions are particularly conserved between the two proteins (Figure 6a) especially the two consensus regions I and II, typical of all eukaryotic smHSPs. Furthermore, the N-terminal parts of the two proteins are strictly identical at seven residues (in mitochondrial HSP22 primary sequence, amino acids 29 to 36). Interestingly, none of the proteins shows the highly conserved sequence referred to as consensus III which is typically located in the N-terminal part of chloroplast smHSPs [26]. Indeed, considering the chloroplast

Figure 7 Time-course of HSP22 mRNA accumulation

(a) Northern-blot analysis of the time-course of HSP22 mRNA accumulation. Total RNA extracts from control or heat-stressed pea plants (20 μ g for each sample) were separated by electrophoresis and transferred on to Hybond-N⁺ membrane. The membrane was hybridized with the PCR probe, as described in the Materials and methods section. The amount of RNA in each lane was monitored by Methylene Blue staining and each experiment was done twice. A marker ladder is indicated at the left of the membranes. (A) The 3 h heat shock at 40 °C applied to pea plants is followed by a recovery period at 25 °C. (B) The 3 h heat shock at 40 °C applied to pea plants is continued for an additional couple of hours. (b) Quantification of Northern-blot data obtained by scanning each band, and quantification of Western-blot data previously published [6]. For HSP22 mRNA, band areas are expressed as percentage values of the maximal induction in pea plants obtained after 60 min at 40 °C. For HSP22 protein, the data are normalized to the maximal expression, obtained after a 3 h heat shock at 40 °C [6]. □, HSP22 mRNA time-course accumulation during a 3-h-long 40 °C heat shock followed by a recovery period at 25 °C; ▽, HSP22 mRNA time-course accumulation during a 3-h-long 40 °C heat shock followed by an additional 2-h-long heat shock at 40 °C; ○, HSP22 protein time-course accumulation (see previous work [6]), during a 3-h-long 40 °C heat shock followed by a recovery period at 25 °C.

smHSPs from pea (HSP21) [4], soybean (HSP22) [4], petunia (HSP22) [26], *Arabidopsis thaliana* (HSP21) [26] and maize (HSP26) [27], there is as much as 90 % sequence similarity in this consensus III region (Figure 6b). We calculated that *C. rubrum* chloroplast HSP23 only exhibits, within this region, a 48 % similarity with chloroplast small HSPs, but a 69 % similarity with the pea mitochondrial HSP22 region. Finally, pea HSP22 is comparable with *C. rubrum* HSP23 in that both proteins can not form a methionine-rich amphipathic α -helix in this consensus III region, as opposed to chloroplast smHSPs [26].

Time-course induction of mitochondrial HSP22 mRNA

Total RNA was isolated from 14-day-old pea leaves that had been exposed for various times at 40 °C, and also during the recovery period in which pea plants that had been treated for 3 h at 40 °C were returned for various times to 25 °C. Northern-blot analysis using the HSP22 cDNA probe (see the Materials and methods section) are presented in Figure 7(a) and quantification

Figure 8 Temperature-dependence of accumulation of mitochondrial HSP22 mRNA

Northern-blot analysis was performed with RNA extracted from control and heat-stressed pea plants at 32 °C, 36 °C or 40 °C for 1.5 h. Quantification of these results is presented below the Northern-blot and band areas determined by scanning are expressed as percentage values of the maximal induction obtained at 40 °C. Loading of equal amounts of RNA in each lane was monitored by Methylene Blue staining. Each experiment was done twice. A marker ladder, in kb, is indicated on the left-hand side of the membrane.

of these data in Figure 7(b). When plants were grown under control conditions (25 °C), the HSP22 transcript was not detectable (time 0 in Figures 7a and 7b). When plants were submitted to the 3-h-long treatment at 40 °C, the HSP22 transcript was easily detected, and the kinetics of accumulation of this transcript exhibited a complex behaviour. Following a lag period of about 10 min, the transcript accumulated in an exponential manner, reaching a maximum level after about 60 min at 40 °C. Half-maximal induction was achieved in less than 30 min. Surprisingly, although the heat treatment was maintained, the HSP22 mRNA level declined during the next couple of hours (Figure 7a), to reach a steady-state level accounting for 30% of the maximum level obtained after treatment for 60 min. Moreover, if heat treatment is maintained, the HSP22 mRNA remains at this constant level during the first additional hour at 40 °C (Figure 7a). On the other hand, upon transfer of the heat-stressed pea plants to control temperatures (25 °C), the total amount of HSP22 mRNA declined rapidly, to reach undetectable amounts after 4 h at 25 °C (Figure 7a, time 420 min). Finally, Figure 7(b) also presents, for comparison, the quantification of the HSP22 protein time-course accumulation within mitochondria, as followed by Western-blot analysis [6].

Effect of temperature on induction of mitochondrial HSP22 mRNA

Pea plants were maintained for 90 min at 25 °C, 32 °C, 36 °C or 40 °C, and total RNA was isolated from pea leaves. Northern-blot analysis of mitochondrial HSP22 mRNA temperature-dependent accumulation is shown in Figure 8(a) and quanti-

fication of these data in Figure 8(b). The HSP22 mRNA was undetectable at 25 °C, weakly detectable at 32 °C or 36 °C, and its accumulation level dramatically increased at 40 °C. Thus, the HSP22 mRNA accumulates in a strongly temperature-dependent manner. But the HSP22 transcript level only reached a high level for temperatures higher than 36 °C: for example, during the 90-min stress period 10-fold more transcript accumulates at 40 °C compared with 36 °C.

DISCUSSION

We have isolated and characterized the cDNA clone for the pea mitochondrial smHSP22. This cDNA encodes the amino acid sequence of HSP22, since the predicted amino acid sequence contains the N-terminal and two internal sequences determined by microsequencing of the purified mitochondrial HSP22. In addition, this cDNA recognizes an mRNA which is expressed in pea leaves only after heat stress. Several results indicate that the cDNA encodes the entire HSP22 precursor: the cDNA has the same size as the mRNA, and, furthermore, a classical mitochondrial transit peptide sequence is found between residues 1 and 32, before the N-terminal sequence of the mature protein. Thus, the HSP22 is nuclear-encoded [6] and the transit peptide is cleaved to yield the mature form of the protein during internalization into mitochondria. The calculated molecular mass of the HSP22 precursor (23 kDa) and of the processed HSP22 (19.5 kDa) are close to those determined by *in vitro* translation and SDS/PAGE (26 kDa and 22 kDa respectively). As is typical of organelle smHSPs [1,4,5,23], the nuclear-encoded pea mitochondrial HSP22 is synthesized as a precursor protein 4–5 kDa larger than the mature protein. The differences observed between the calculated molecular masses of the precursor and mature forms and their predicted molecular masses have frequently been noted for other HSPs [28].

Sequence analysis leads to the conclusion that HSP22 belongs to the HSP20 superfamily [1]. In plants, four multigenic classes are identified among the HSP20 superfamily, protein members of which have so far been localized within the three subcellular compartments cytosol, chloroplasts and endomembrane system [1]. Our data demonstrate for the first time that an smHSP belonging to this family is localized to the plant mitochondria compartment. This contention is supported both by the present characterization of the cDNA encoding this protein and our previous biochemical data establishing the subcellular localization of pea HSP22 [6].

The strong similarity between the mitochondrial HSP22 and the chloroplast HSP of 23 kDa from *Chenopodium rubrum* [25,28] is surprising, considering their different subcellular localization. The fact that neither of these two proteins exhibits the highly conserved domain III, typical of chloroplast smHSPs, may suggest that consensus region III is not required for functional activity of smHSPs in the chloroplast and in the mitochondrial compartments. We do not favour this interpretation since, indeed, this consensus III sequence is the most conserved region in chloroplast smHSP primary sequences [26]. Alternatively, our results may raise the question of the exact intracellular localization of the *C. rubrum* HSP23. We note that this protein has never been purified from purified chloroplasts. Furthermore, the main arguments of Knack et al. [28] which conclude that the 23 kDa protein is a precursor form of a chloroplast HSP are based upon structural and sequence analysis of the HSP23 transit peptide and the entire HSP23 primary sequence, and upon the results of *in vitro* import experiments. However, several characteristics typical of chloroplast transit peptides, such as serine and basic residue content and lack of acidic residues, are

also identified in mitochondrial transit peptides [19]. Also, the potential existence of a transmembrane helix within the amino acid sequence of the HSP23 transit peptide [28] is not itself a characteristic of chloroplast transit peptides, since such a structure is predicted by the sequence of the mitochondrial HSP22 transit peptide (residues 2 to 21). Finally, it is known that the transit peptide is not, by itself, sufficient to address polypeptide chains to either the chloroplasts or the mitochondria (see for example [29]). In contrast, we note that the four amino acids at the beginning of the two mature proteins, pea mitochondrial HSP22 and *C. rubrum* chloroplast HSP23, are identical. These amino acids are presumed to play a role in the addressing of proteins into cellular organelles [19]. In conclusion, we think that the exact intracellular localization of *C. rubrum* HSP23 requires further analysis.

We have shown that the mitochondrial HSP22 mRNA is undetectable when pea plants are grown at control temperatures (25 °C) and that the transcription of this mRNA is rapidly induced by heat-shock. This process, therefore, is a *de novo* transcription, as it was demonstrated for other smHSPs (pea chloroplast HSP21 [4]). In addition, since the accumulation of the protein HSP22 is also induced by heat treatment, and since the translation inhibitor cycloheximide can block HSP22 accumulation in heat-shocked pea leaves [6], mitochondrial HSP22 synthesis very likely results from a complete *de novo* synthesis, from transcription to translation, following the heat shock. However, our cDNA library screening strategy suggests that the mRNA for HSP22 was present, at least to a basal level, in unstressed plants (see the Materials and methods section). Two different hypotheses could account for this finding. In the first, the HSP22 mRNA is constitutively present in control pea leaf, albeit to such a low level that it escapes detection by Northern analysis. Indeed, when autoradiographic films are exposed to the hybridized Northern-blot membrane for a long time (1 week), we are able to detect a very slight, but specific, signal in control tissues. Thus, a very low amount of transcript could escape regulation and be present in non-stressed plants. In the second hypothesis, the cDNA library we have used may have already contained some heat-shock-regulated mRNA. This pea leaf library has been constructed from pea plants grown at temperatures close to 30 °C, and indeed, we have shown that a low level of HSP22 mRNA accumulation can already be induced at 32 °C.

The rate of HSP synthesis in plants or other eukaryotes has been shown to be directly proportional to the temperature applied during the stress [3,30,31]. Surprisingly, our observations do not confirm these results, because it seems that the pea mitochondrial heat-shock response is only developed under extreme environmental growth conditions. During the linear increase of temperature, the HSP22 mRNA does not show a linear proportional accumulation, but presents a strong accumulation only if the temperature becomes higher than 36 °C. Consequently, the stress protein seems to be a very sensitive stress signal, only developed in response to very dramatic conditions of temperature. Moreover, the HSP22 mRNA accumulation was extremely rapid (10 min at 40 °C). Induction of the transcription of the HSP22 gene may even be more rapid, because a substantial part of the 10-min lag period could be accounted for by the time needed for equilibration of pea leaf temperature to that of the growth chamber. In agreement with this finding, transcription induction of HSPs in salivary glands of *Drosophila* has been shown to occur in less than 5 min [32,33]. Also, Schöffl and Key [34] showed that soybean smHSP mRNA becomes detectable 3–5 min after heat-shock. In our case, after the rapid induction and the exponential accumulation of the

transcript, the HSP22 mRNA amount declined considerably before the end of the heat stress and 2 h before the HSP22 protein reaches its maximum level ([6] and Figure 7). At this moment, the synthesis rate of the mRNA became lower than its rate of degradation. Similar kinetic behaviour has been reported for pea cytoplasmic smHSP mRNA [35], and for smHSP mRNA from soybean seedlings [36]. Taken together, our results strongly suggest that temperature is not the unique factor regulating HSP22 expression. Finally, the life-time of the HSP22 transcript is quite short (4 h at 25 °C), as it is for other smHSP mRNA (for example, the turnover for class I cytosolic smHSPs in soybean is 6 h at 40 °C [36]). In contrast, the HSP22 mRNA level only declines slightly during the first additional hour at 40 °C following our usual 3 h stress period. This suggests that, after a 3 h treatment at 40 °C, the synthesis and degradation rates of the mRNA HSP22 are in equilibration, thus leading to an mRNA steady-state level.

Another interesting point emerging from the present study and our previous work [6] deals with the 20 min delay between the appearance of the HSP22 mRNA and protein (see Figure 7 for comparison between the two time courses). Presumably, this corresponds to the time needed for synthesis of the 26 kDa precursor protein in the cytosolic compartment, mitochondrial import of the 26 kDa precursor, and transit peptide processing. Furthermore, contrasting with the rapid disappearance of the HSP22 mRNA when pea plants, heat stressed for 3 h, are shifted back to the control temperature of 25 °C, the protein is still present at its maximum level for 3 days after the end of the stress period. Since the HSP22 mRNA has completely disappeared 4 h after the end of the heat stress, we are therefore able to affirm that new synthesis of the HSP22 protein is stopped 4 h after the end of the stress and that HSP22 protein is extremely stable under control temperature conditions. Such a persistence of smHSPs following heat stress has been observed in several organisms, including plants [30] and mammals [37]. The half-life of pea chloroplast HSP21 is 52 ± 12 h [31], and that of pea class I cytosolic HSP18.1 is 37.7 ± 8 h [35]. The astonishing stability of the mitochondrial HSP22 during the recovery period at 25 °C suggests that HSP22 function may be as critical during the recovery period as during the stress period. This may be of physiological significance. For example, HSP22 could be maintained within mitochondria for several days following a stress period in order to develop a thermotolerance to protect against subsequent heat-shocks.

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