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REVIEW ARTICLE

Evolution, structure and function of the small heat shock proteins in plants

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Abstract

The α-crystallin-related, small heat shock proteins (smHSPs) are ubiquitous in nature, but are unusually abundant and diverse in higher plants as opposed to other eukaryotes. The smHSPs range in size from ~17 to 30 kDa and share a conserved C-terminal domain common to all eukaryotic smHSPs and to the α-crystallin proteins of the vertebrate eye lens. In higher plants six nuclear gene families encoding smHSPs have been defined. Each gene family encodes proteins found in a distinct cellular compartment, including the cytosol, chloroplast, ER, and mitochondrion. Evolutionary analysis suggests that the smHSP gene families arose by gene duplication and divergence prior to the radiation of angiosperms. In general, the smHSPs are not found in normal vegetative tissues, but accumulate to high levels in response to heat stress. Specific smHSPs are also expressed during various phases of plant development as part of the endogenous developmental programme. Thus, although the smHSPs are apparently not essential for basal cell functions as are the high molecular weight HSPs such as HSP90, HSP70 and HSP60, their functions are likely to be critical for survival and recovery from heat stress as well as for specific developmental processes. Biochemical analysis indicates that smHSPs are found in high molecular weight complexes between 200–400 kDa that are most likely composed solely of multiple smHSP subunits. Purified recombinant plant smHSPs facilitate reactivation of chemically denatured enzymes in a nucleotide-independent fashion and also prevent heat-induced aggregation or reverse inactivation of protein substrates. Based on these data, it is suggested that smHSPs act *in vivo* as a type of molecular chaperone to bind partially denatured proteins preventing irreversible protein inactivation and aggregation, and that smHSP chaperone activity contributes to the development of thermotolerance.

Key words: Chaperone, heat stress, organelles, phylogeny, seed development.

Introduction

All organisms respond to elevated temperatures and many other stresses with the production of a defined set of proteins called heat shock proteins (HSPs) (Parsell and Lindquist, 1993; Nover, 1991; Vierling, 1991; Morimoto *et al.*, 1994). HSPs or highly homologous proteins are also expressed in some cells either constitutively or under cell cycle or developmental control. The major HSPs synthesized by eukaryotes, including plants, belong to five conserved classes: HSP100, HSP90, HSP70, HSP60, and small (sm) HSPs (~17 to 30 kDa). The evolutionary conservation of the heat shock response and the HSPs, along with the correlation of HSP expression with cellular resistance to high temperature, has led to the long-standing hypothesis that HSPs protect cells from the detrimental effects of high temperature, and that accumulation of HSPs leads to increased thermotolerance. The mechanism by which HSPs may effect such protection has not been determined in detail, but considerable recent data indicate that several HSPs function as 'molecular chaperones'. Molecular chaperones are proteins that bind to partially folded or denatured substrate proteins and thereby prevent irreversible aggregation or promote correct folding of substrate (Hartl *et al.*, 1992; Hendrick and Hartl, 1993; Landry and Gierasch, 1994). Binding of

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Abbreviations: HSP: heat shock protein; smHSP: small heat shock protein; HSF: heat shock transcription factor; Rubisco: ribulose bisphosphate carboxylase.

substrate to chaperones can also maintain the substrate in an unfolded conformation in order to facilitate protein translocation across membranes and other processes. It is proposed that during high temperature stress HSPs prevent accumulation of heat denatured protein aggregates or facilitate protein reactivation following stress. Evidence for both of these activities has been obtained in several systems (Parsell *et al.*, 1994; Parsell and Lindquist, 1993; Morimoto *et al.*, 1994).

There have been numerous excellent reviews of the heat shock response in plants and other organisms (Vierling, 1991; Neumann *et al.*, 1989; Howarth, 1991; Morimoto *et al.*, 1994; Lindquist and Craig, 1988), and the molecular chaperone activities of HSPs have been thoroughly discussed in many recent articles (Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Jakob and Buchner, 1994). Despite this extensive literature, there has been minimal review of the structure and function of the smHSPs (Arrigo and Landry, 1994; Jakob and Buchner, 1994; Jaenicke and Creighton, 1993), particularly smHSPs from plants. The smHSPs are those HSPs between ~17 to 30 kDa that share a carboxyl-terminal domain of ~100 amino acids with the α-crystallin proteins, structural proteins of the vertebrate eye lens (deJong *et al.*, 1993; Plesofsky-Vig *et al.*, 1992). A discussion of the smHSPs is particularly relevant to the heat stress response in higher plants for several reasons. Firstly, smHSPs dominate the protein synthesis profile of many plants during heat stress. In contrast, HSP70 is often the predominant HSP expressed in other eukaryotes. Secondly, particular smHSPs can accumulate to over 1.0% of total leaf or root cell protein under certain heat stress conditions (Hsieh *et al.*, 1992; DeRocher *et al.*, 1991). Thirdly, plants have at least six nuclear gene families encoding smHSPs, while non-plant eukaryotes typically have one to four single genes for smHSPs. Most importantly, proteins encoded by the different smHSP gene families are targeted to different cellular compartments, including the cytosol, chloroplasts, mitochondria, and endoplasmic reticulum (Vierling, 1991; Lenne, 1995). This diversification of the smHSPs is completely unique to plants, and plants are the only eukaryotes in which organelle-localized smHSPs have been described.

Whether the smHSPs are required for the development of thermotolerance in plants or are otherwise important for survival during exposure to high temperatures remains an open question. The goal of this review will be to summarize the molecular, physiological and biochemical data that indicate these HSPs have potentially important roles in higher plants, and to discuss a model for smHSP function.

Small HSP gene families and evolution

Plant smHSPs are represented in current gene databases by over 45 complete protein coding sequences including sequences from many different angiosperms, and a gymnosperm (*Pseudotsuga menziesii*) (Tranbarger and Misra, 1996). These sequences comprise four well-defined gene families encoding proteins localized to the cytosol (class I and II), the chloroplast and the endoplasmic reticulum (ER) (Vierling, 1991; Helm *et al.*, 1993, 1995). Recently, a fifth class of smHSP gene encoding a protein localized to mitochondria has been identified in several species (Lenne and Douce, 1994; Lenne, 1995; LaFayette *et al.*, 1996; Kloppstech, personal communication). A potential sixth class of smHSP is represented by a single cDNA from *Glycine max*, GmHSP22.3. The GmHSP22.3 protein most likely localizes to a membrane compartment as evidenced by the presence of a signal peptide at the amino terminus and data demonstrating that the mRNA is translated on membrane-bound polysomes (Lafayette *et al.*, 1996). However, it lacks the putative ER retention signal found in the ER-localized smHSPs. It will be interesting to determine the final intracellular location of this protein, as well as whether or not similar smHSP genes are present in other plant species.

In contrast to the highly conserved HSP70 proteins (50% identity between prokaryotes and eukaryotes (Lindquist and Craig, 1988)), smHSPs show much less sequence similarity. This applies not only to comparisons of smHSPs between divergent species, but also to comparisons between the different classes of plant smHSPs. The sequence identity between representatives of the different gene families of smHSPs from a single plant species is presented in Fig. 1. As mentioned above, high identity between sequences is limited to a carboxyl-terminal domain, which is sometimes termed the 'heat shock' domain. This domain spans approximately 100 amino acids and can be further divided into two subdomains, consensus I and II, separated by a variable length hydrophilic region. Within consensus I, the residues Pro- $X_{(14)}$-Gly-Val-Leu are a signature typical of almost all small HSPs. Interestingly, a similar motif also appears in the consensus II region, Pro-$X_{(14)}$-X-Val/Leu/Ile-Val/Leu/Ile. Furthermore, Caspers *et al.* (1995) have noted that the regions comprising consensus I or II have similar hydropathy profiles and predicted secondary structure. The significance of the heat shock domain and these conserved amino acid motifs to smHSP structure and function remains to be determined.

As can be seen in Fig. 1, the amino-terminal domains of the plant smHSPs are quite divergent between classes. For the chloroplast, ER and mitochondria-localized proteins amino-terminal sequences typical of organelle targeting peptides are present. Within the amino-terminal regions of the mature proteins, comparisons from multiple plant species identify consensus domains that are unique to each class of smHSP (Chen and Vierling, 1991; Waters, 1995). For the chloroplast proteins this consensus domain is 28 amino acids long and represents the most highly

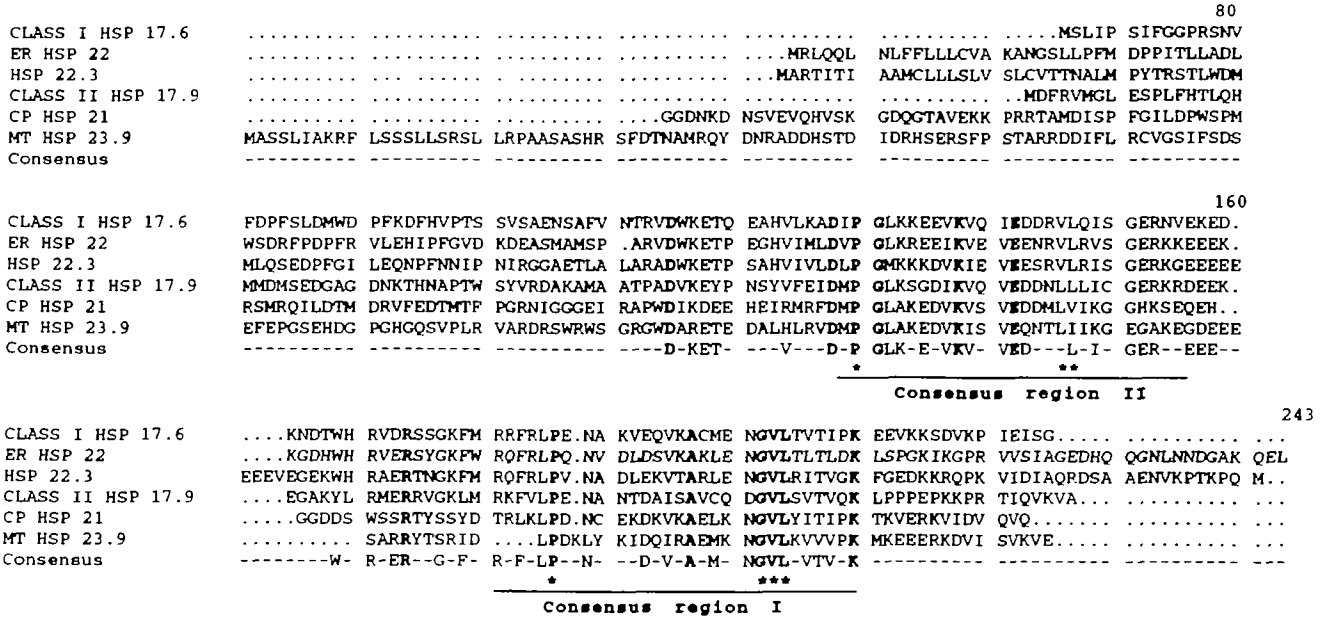

Fig. 1. Amino acid sequence alignment of *Glycine max* representatives from the six smHSP gene families. Consensus sequence appears below the alignment with those residues that are identical in all sequences typed in bold. The conserved heat shock domain comprises the entire region in which consensus residues appear. The defined consensus regions I and II are underlined and asterisks indicate important residues within these regions as discussed in the text. Within the alignment '.' indicates a gap introduced to optimize the alignment. CP = chloroplast-localized. MT = mitochondria-localized. HSP22.3 contains a signal peptide but no ER retention signal and its intracellular localization is unclear; see text for further details. Note that the CP GmHSP21 sequence is incomplete; the cDNA was truncated and did not include sequences encoding the amino-terminal transit peptide (Vierling *et al.*, 1988). However, the sequence includes essentially all of the predicted mature protein. Database accession numbers for sequences shown are: Hsp17.6: M11317; ER Hsp22: X63198; Hsp22.3: U21723; Hsp17.9: X07159; CP Hsp21: X07188; MT Hsp 23.9: U21722.

conserved domain in this smHSP with 22 identical residues and 5 conservative replacements comparing species as divergent as pea and maize. Structure predictions indicate that 14 of these residues would form ~3.5 turns of a strongly amphipathic alpha-helix with a striking 100% conservation of residues on the hydrophilic face across 5 species examined (Chen and Vierling, 1991). In the class II smHSPs a small region of 11 amino acids (corresponding to positions 104–115 in GmHSP17.9, Fig. 1) includes 9 residues that are identical in the majority of sequences available to date. Cytosolic class I proteins have a unique consensus region corresponding to positions 75 to 92 of GmHSP17.6 (Fig. 1). The presence of these highly conserved domains unique to different classes of smHSPs suggests that they serve important roles in the function of these proteins.

Evolutionary analysis reveals that the different classes of smHSPs arose prior to the divergence of the major groups of angiosperms. Parsimony analysis using PAUP (Swofford, 1993) of sequences from representative dicots (*Glycine max* and *Arabidopsis thaliana*) and a monocot (*Triticum aestivum*) shows that the smHSPs are more closely related to members of the same protein class from divergent species than they are to other smHSPs from the same species (Fig. 2). In this analysis, the branches leading to the cytosolic class I and II, the chloroplast (CP), ER, and mitochondrial (MT) families are highly supported by Bootstrap analysis. Analysis of an additional 32 smHSP sequences using PAUP does not alter the relationships of proteins within these families. The placement of the *G. max* HSP22.3 sequence with the ER proteins is not highly supported (59 out of 100 trees). As mentioned above, GmHSP22.3 is not likely to be a resident ER protein and sequence analysis clearly indicates that GmHSP22.3 represents a sixth gene family. Its placement in the tree may reflect either a more recent duplication of the gene encoding the ER protein or the result of selection maintaining sequence similarity between GmHSP22.3 and the ER proteins. Overall, the pattern of sequence relatedness shown in Fig. 2 is consistent with the hypothesis that the smHSP families arose from a relatively ancient gene duplication(s) followed by sequence divergence.

The gene duplications that gave rise to the smHSP gene families certainly occurred before the divergence of the monocots and dicots, a minimum of 150 million years ago (Doyle and Donoghue, 1993). Identification of a cytosolic class I gene in a gymnosperm (Tranbarger and Misra, 1995) suggests these families are even older. There is a complete lack of information on smHSPs in earlier plant groups that might help further define the timing of such proposed duplications. Only one algal smHSP has been characterized, HSP22 from the Chlorophyte alga *Chlamydomonas*, and this protein does not group with

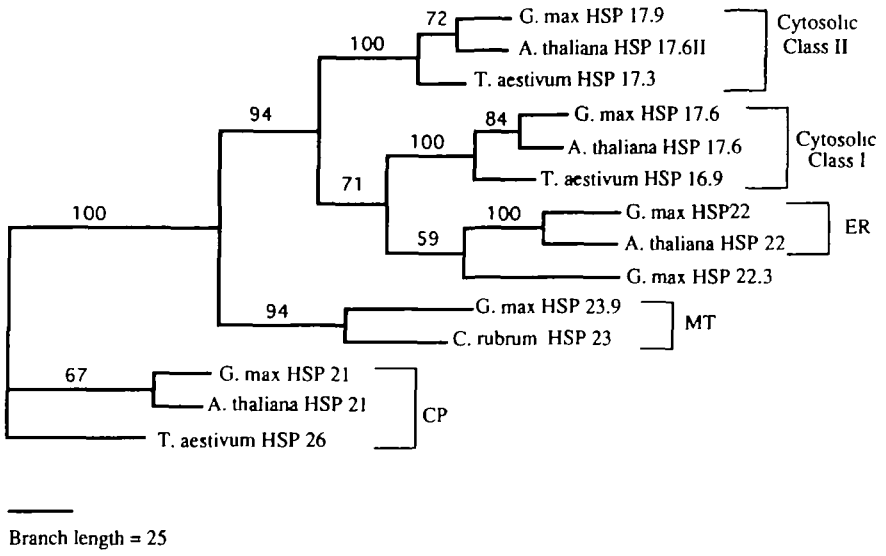

Fig. 2. Evolutionary analysis of smHSP sequences The most parsimonious phylogenetic tree of the smHSP sequences was generated with PAUP (Phylogenetic Analysis Using Parsimony). The level of support for the tree was evaluated with Bootstrap analysis. The number of times out of 100 bootstrap replicates that a branch was found is noted above the branch. The tree has 964 steps and a consistency index of 0.882. It is arbitrarily rooted with the sequences of the chloroplast localized proteins. Alignment is available upon request from the authors.

any of the six angiosperm classes (E. Waters, unpublished data). The absence of a similar gene family structure for smHSPs in non-plant eukaryotes, and analyses of the evolutionary relatedness of the plant and animal sequences (deJong *et al.*, 1993; Plesofsky-Vig *et al.*, 1992) suggest that the duplications which gave rise to the many smHSPs in plants occurred only in the lineage leading to the higher plants. Gene duplication followed by gene diversification is both a common and extremely important force in gene and genome evolution (Ohta, 1988, 1991). The evolution of the smHSPs in plants are a clear example of this pattern of gene evolution. After duplication, Darwinian selection for a new function may drive the sequence evolution of newly duplicated genes, and differences in rates of evolution among members of a gene family may reflect different selective constraints. As suggested by the variation in branch lengths leading to the smHSP classes (Fig. 2), analysis of rates of evolution reveal that the smHSP gene families are evolving at unequal rates (Waters, 1995). The cytosolic class I and chloroplast localized proteins are evolving more slowly than the class II and ER proteins. The high level of sequence divergence among smHSP classes, coupled with apparent differences in their rates of evolution, may indicate functional divergence among the smHSP classes that extends beyond simple differences in intracellular localization.

Homology of the plant smHSPs to smHSPs from evolutionarily distant organisms is shown in Fig. 3, comparing representatives of the plant class I and II cytosolic smHSPs (from pea), to yeast HSP26 and human HSP27. Similarity is restricted to the carboxyl-terminal domain; alignments amino-terminal from position 109 in the comparison are essentially random. Within the heat shock domain (~positions 110 to 208), residues that are conserved across these and the majority of smHSPs from diverse organisms include the Gly-Val-Leu motif (more generally Gly-Val/Thr/Ile-Leu at positions 192 to 194) and the Pro residue amino-terminal to this motif (position 174), as discussed above. The comparison also reveals that the plant and yeast proteins are much more similar to each other than to the human smHSP. Yeast HSP26 is more closely related to the plant class I cytosolic proteins (33.5% identical and 54% similar) than to smHSPs from any other organism. For comparison, yeast HSP26 and plant class I proteins are only 19.6% identical and 42.6% similar to mammalian smHSPs. It is reasonable to hypothesize that, unlike the HSP70 proteins, smHSPs have divergent functions in vertebrates as opposed to plants or yeast.

SmHSP expression during heat stress

Examination of the expression pattern of the smHSPs provides important insight into potential roles of these proteins. With few exceptions, the smHSPs are not expressed by vegetative tissues in the absence of heat stress. Sensitive Western analysis fails to detect smHSP proteins in leaf or root tissues of plants grown under controlled, non-stress conditions. The general pattern of smHSP expression in leaves during heat stress and recovery, as compared with the expression of the α and β subunits of the Rubisco binding protein, is illustrated in Fig. 4. The latter protein, which is a member of the HSP60 protein family, is present at high levels constitutively and shows at most a 2-fold increase under the same

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          1                                                                  60
Pea17.7   -d-dsp--.. .......... .......... .......... .......... .....-t-hh
Pea18.1   -s-ips--s. .......... .......... .......... .......... .grrs-v-dp
Yeast26   -s-nsp--df fdninnevda fnrllgeggl rgyaprrqla ntpakdstgk evarp-n-ag
Human27   .......... .......... .........m terrvpfsll rgpswdpfrd wyphsrl-dq
Consensus M-L---FF-- ---------- ---------- ---------- ---------- -----N-F--
          61                                                                 120
Pea17.7   i.m-lt-dtt -kn-..napt rt-v-dakam aatpa--k-h pnsyv-mvd- --v.......
Pea18.1   fsl-v--plk -fp-snssps as-p-en-af vstrv-wk-t peahv-kad- --l.......
Yeast26   aly-p--etl -dw-dndlsl fpsgfgf-rs vavpv--l-h dnnye-kvvv --v.......
Human27   afglp-lpee wsq-lggssw pg-v-pl-pa aiespa-aap aysra-srq- ssgvseirht
Consensus ---D-RD--- D--F------ --Y-R--P-- -----DV-E- ---------L PG--------
          121                                                                180
Pea17.7   ..-.sg--k- qv.ed-nvll ----rkr-ee kegvk-lkm. -rri---m-k -v---nan--
Pea18.1   ..-.ke--k- ev.ed-rvlq ----rsv-ke dknd--hrv. -rs----l-r -r---nakm-
Yeast26   ..-skk--d- eyhqnknqil ----ipstln eesk-kvkvk -ss----k-v it---ypg--
Human27   adrwrvsld- nhfap-eltv ktk-gvv-it gkhe--qdeh gyi-rc-t-k -t--..pg--
Consensus --K---DI-V -----D---- ISGE---E-- ----E----- E--SGKF-R- F-LPE---VD
          181                                                        230
Pea17.7   a. is-is.q d-------nk lpppepk-pk tiqvkva... ..........
Pea18.1   k..vk-sm.e n-------pk .  eeik-ae vksieisg.. ..........
Yeast26   adnik-dy.a n----l--pk lkpqkdg-nh vkkievssqe swgn......
Human27   ptqvssslsp e-t---eapm pklatqsnei tipvtfesra qlggrsckir
Consensus -----A---- -GVLTVTVPK -------K-- ---------- ----------
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Fig. 3. Amino acid sequence alignments of smHSPs from diverse eukaryotes. Class I and II cytosolic smHSPs from pea (PsHSP18.1 and PsHSP17.7, respectively), yeast ScHSP26, and human HSP27 were aligned with the Wisconsin GCG multisequence comparison programs. A consensus sequence is shown at the bottom for residues appearing in three of the four sequences. Within the alignment '-' indicates identical residues and '.' indicates a gap introduced to optimize the alignment. Database accession numbers for the sequences shown are: PsHSP18.1: P19243; PsHSP17.7: M33901; yeast26: P15992; human27:P04792.

conditions that induce the smHSPs greater than 200-fold. The smHSPs accumulate rapidly during temperature stress and accumulation is proportional to the temperature and duration of the stress. In mesophytic plant species, such as *Pisum sativum*, accumulation of the class I proteins can be detected at temperatures as low as 30 °C. Maximum synthesis and accumulation of smHSPs is observed at temperatures just below lethal temperatures (Howarth, 1991). Quantitative analysis indicates that class I proteins accumulate to over 1% of total leaf protein (Hsieh *et al.*, 1992; DeRocher *et al.*, 1991), and in the majority of plant species they are probably the most abundant group of smHSPs. In comparison, the chloroplast protein has been estimated to comprise only 0.02% of total leaf protein even after maximum induction (Chen *et al.*, 1990). The smHSPs are also quite stable following stress, with half-lives of 30–50 h (Chen *et al.*, 1990; DeRocher *et al.*, 1991), indicating their function may be critical for the recovery period.

As has been shown for many HSPs, plant smHSPs are regulated at the transcriptional level in response to heat stress. Gurley and Key (1991) have comprehensively reviewed data on transcriptional regulation by heat shock in plant systems. Considerable data indicate that the basic mechanism of HSP gene activation is conserved among eukaryotes. The core *cis*-acting element in the 5′ promoter sequences of plant HSP genes is identical to that found in other organisms. To date some of the best studied plant heat shock promoters have been derived from smHSP genes (Baumann *et al.*, 1987; Czarnecka *et al.*, 1992; Takahashi *et al.*, 1992). The class of transacting factor that binds to the heat shock element (heat shock transcription factor or HSF) has a defined DNA binding domain common to the HSFs identified in divergent eukaryotes. One unique feature of heat shock transcriptional regulation in plants is the presence of multiple genes encoding HSFs, and these HSF genes are differentially regulated by heat shock. Three HSF genes have been cloned from tomato (Scharf *et al.*, 1990), and both soybean and *Arabidopsis* have more than four HSF genes (W. Gurley, personal communication). Outside of the common DNA binding domain and a coiled-coil trimerization domain, the different plant HSFs are quite divergent. The presence of multiple HSFs, as well as their differential regulation, suggests these transcription factors may regulate genes in response to signals other than heat stress. As discussed below, genes encoding HSPs are transcribed in response to other environmental and endogenous signals. It will be interesting to determine how this multiplicity of transcription factors may control HSP gene activation both during and in the absence of heat stress.

SmHSP expression in the absence of heat stress

Although like other HSPs the smHSPs were first identified as proteins whose expression is highly induced by elevated temperatures, many recent studies indicate that these

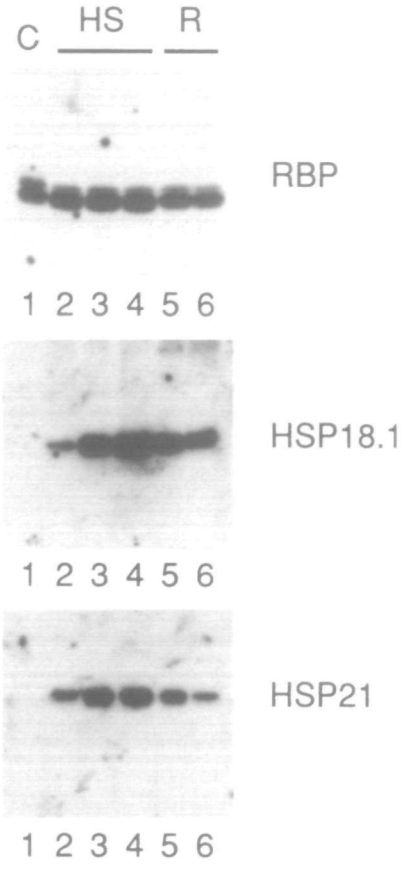

Fig. 4. Immunoblot analysis of HSP levels during heat stress and recovery in intact pea plants. Total proteins were isolated from pea leaves removed from intact 10-d-old plants before (lane 1), during (lanes 2–4) or after (lanes 5 and 6) a 38 °C heat stress. Plant temperature was increased 4 °C h^{-1} to 38 °C and held at 38 °C for 4 h and then decreased to 22 °C at 4 °C h^{-1}. Lane 1: control. Lane 2: beginning of 38 °C treatment. Lane 3: after 2 h at 38 °C. Lane 4: after 4 h at 38 °C. Lane 5: when temperature had returned to 22 °C after the stress treatment. Lane 6: 72 h after stress treatment. Equal quantities of protein from each sample were separated by SDS–PAGE, blotted to nitrocellulose and reacted with anti-Rubisco binding protein (gift of Dr S Hemmingson), anti-cytosolic HSP18.1 (DeRocher *et al.*, 1991) or anti-chloroplast HSP21 antibodies (Vierling *et al.*, 1989). See cited references for further details concerning antibody specificity as well as antigen quantification discussed in the text

proteins are also regulated by a variety of other environmental and developmental cues in animals (Arrigo and Landry, 1994) and in plants (Table 1). The HSP90 and HSP70 proteins are also responsive to other stresses and endogenous signals (Winter and Sinibaldi, 1991; DeRocher and Vierling, 1995; Yabe *et al.* 1994). However, these high molecular weight HSPs, as well as HSP60, are also abundant components of most unstressed cells, and their induction represents increased synthesis of one or more HSP isoforms. In contrast, as discussed above, there is no evidence that smHSPs are major constitutive cellular components. This fact suggests that smHSP function is restricted to specialized cellular conditions shared by different developmental and stressed states.

As seen from Table 1, smHSPs appear during an intriguing array of developmental stages in a variety of plant species. An important generality is that developmental induction typically involves only a specific subset of smHSPs, either only certain classes of smHSPs, and/or only specific members of a smHSP class. This further supports the idea of functional distinction between classes, and indicates there may even be functional distinctions between proteins of the same class.

The best characterized cases of developmental regulation are expression during pollen development and during seed maturation. The expression of cytosolic class II smHSPs during pollen development was discovered during differential screening of cDNA libraries designed to identify transcripts specific to meiosis (Bouchard, 1990; Kobayashi *et al.*, 1994). Studies in both lily and maize indicate cytosolic class II mRNAs are absent prior to meiosis and accumulate significantly during meiotic prophase and are present in tetrad microsporocytes. Class II mRNAs are notably absent from mature pollen (Kobayashi *et al.*, 1994; Atkinson *et al.*, 1993; Hopf *et al.*, 1992; Dietrich *et al.*, 1991) and are minimally inducible during pollen germination (Hopf *et al.*, 1992). In contrast, cytosolic class I mRNAs are detected during maturation of the pollen from the bicellular stage to the mature pollen grain. To date there is no published information about the relative levels of the corresponding proteins in developing pollen. It will be interesting to detemine the intracellular localization of the smHSPs during these developmental transitions.

Several groups have reported smHSP expression during seed maturation (Table 1), and recent experiments perfomed with seeds matured under controlled environments have demonstrated that expression is due to endogenous, rather than environmental signals (Coca *et al.*, 1994; DeRocher and Vierling, 1994). Seeds are also capable of mounting a full heat shock response at all but the earliest stages of development (Altschuler and Mascarenhas, 1982; Apuya and Zimmerman, 1992; DeRocher and Vierling, 1994). SmHSPs are both temporally and spatially regulated during seed maturation and only a subset of smHSPs (cytosolic class I and II) respond to these developmental cues. The specificity of their regulation suggests that they may have distinctive functions in the maturing seed. Initially, several groups proposed that smHSPs were critical for protection of cellular components during seed desiccation and/or rehydration. Consistent with this hypothesis, Jordano and colleagues (Almoguera *et al.*, 1993; Coca *et al.*, 1994) have reported certain smHSPs in sunflower are also regulated in response to water/desiccation stress in leaves. However, this does not appear to be true in *Arabidopsis* leaves (N.Wehmeyer and E. Vierling, unpublished observations). Furthermore, it has been found that a seed development mutant of *Arabidopsis*, *abi3–1*, has 10-fold lower levels of class I smHSPs than wild-type seeds, but is still

Table 1. *Expression of smHSPs in cases other than heat stress*

Plant species	Reference	SmHSP class	Observations
Embryo development			
Helianthus annuus	(Almoguera and Jordano, 1992)	Cytosolic I	mRNA accumulates during mid-maturation and present in dry seeds
	(Coca *et al.*, 1994)	Cytosolic I, II	mRNA and protein expression during embryogenesis
Pisum sativum	(Vierling and Sun, 1989)	Cytosolic I	mRNA present in mature seeds
	(DeRocher and Vierling, 1994)	Cytosolic I, II	mRNA and protein expression during embryogenesis and germination
Arabidopsis thaliana	[a]	Cytosolic I	A specific subset of class I proteins accumulate in mid-maturation and are present in the dry seed
Sorghum bicolor	(Howarth, 1990)	ND	mRNA from mature dry seeds produced smHSPs when translated *in vitro*; mRNA gone by 24 h imbibition
Triticum aestivum	(Helm and Abernethy, 1990)	ND	Quiescent embryo mRNA produced smHSPs when translated *in vitro*; mRNAs decayed on imbibition
Zea mays	(Shen *et al.*, 1994)	Cytosolic I	Random sequencing of cDNAs from mature maize endosperm
Germination			
Pisum sativum	(Vierling and Sun, 1989)	Cytosolic I	Proteins present in dry seed and decline during germination
Arabidopsis thaliana	[a]	Cytosolic I	Proteins present in dry seed and decline during germination and early seedling growth
Pseudotsuga menziesii	(Tranbarger and Misra, 1996)	Cytosolic I	mRNA absent during germination, peaks directly following germination in young seedlings
Hordeum vulgare	(Kruse *et al.*, 1993)	Chloroplast	mRNA and protein in 2-d-old seedlings; growth condition of seeds unknown
Somatic embryogenesis			
Medicago sativum	(Györgyey *et al.*, 1991)	Cytosolic I	mRNA accumulated in globular and heart stage embryos
Nicotiana tabacum	(Zarsky *et al.*, 1995)	Cytosolic I	mRNA expressed during sucrose starvation induced pollen embryogenesis
Pollen development			
Lilium	(Bouchard, 1990)	Cytosolic II	cDNAs isolated from microsporocytes; expressed during meiotic prophase and in tetrad microsporocytes
	(Kobayashi *et al.*, 1994)	Cytosolic II	cDNAs isolated from microsporocytes; expressed as above; not present in mature pollen
Zea mays	(Dietrich *et al.*, 1991)	Cytosolic II	mRNA detected during prophase of pollen meiosis; absent in mature pollen
	(Atkinson *et al.*, 1993)	Cytosolic II	mRNAs encoding two HSPs accumulate independently in a stage-specific manner during microsporogenesis absent in mature pollen
Nicotiana tabacum	(Zarsky *et al.*, 1995)	Cytosolic I	mRNA present in late bicellular to mature pollen; smHSP promoter-GUS fusion directs expression in pollen
Fruit maturation			
Lycopersicon esculentum	(Fray *et al.*, 1990)	Cytosolic I	cDNA isolated in differential screen of maturing fruit
	(Lawrence, 1993)	Chloroplast	mRNA expressed in green and maturing fruit
Water stress			
Helianthus annuus	(Almoguera *et al.*, 1993)	Cytosolic I, II	mRNA and protein accumulate in specific tissues of water-stressed plants in the absence of heat stress
Phaseolus vulgaris	[b]	Cytosolic II	cDNA isolated in differential screen of beans subjected to water deficit conditions
Cold storage			
Solanum tuberosum	(Van Berkel *et al.*, 1994)	ER	mRNA accumulates during cold storage
Photoperiod			
Pharbitis nil	(Krishna *et al.*, 1992)	Cytosolic II	Specific class II gene mRNA accumulates following light treatment of dark-grown seedlings and specific photoperiod
Other			
Glycine max	(Czarnecka *et al.*, 1984)	Cytosolic I, II ER, Chloroplast	mRNAs detected under various stress conditions
Helianthus annuus	(Almoguera and Jordano, 1992)	Cytosolic I	mRNA increased in seedlings exposed to 0.3 M mannitol, or 0.1 mM ABA
Papaver somniferum	(Facchini and De Luca, 1994)	Cytosolic I	mRNA detected in mature unstressed roots and callus cultures

ND, not determined. (a) N Wehmeyer and E Vierling, in preparation. (b) AA Covarrubias, personal communication.

desiccation tolerant (Wehmeyer *et al.*, 1995). Thus, either smHSPs are not required for desiccation tolerance, or they can function at significantly reduced levels. Whether the reduced level of smHSPs in the *abi3–1* mutant is causally related to other phenotypes of the mutant, such as reduced seed longevity or reduced deposition of storage materials, remains to be investigated.

Biochemistry of the smHSPs

The smHSPs from many different organisms have all been found in high molecular weight complexes *in vivo*, between 200–800 kDa. As determined by non-denaturing gel electrophoresis of plant extracts, the class I smHSP complexes are approximately 200–300 kDa in size (Helm *et al.*, 1993; Jinn *et al.*, 1995), and similar sizes have been observed for the class II (Helm and Vierling, unpublished), the chloroplast (Clarke and Critchley, 1994; Chen *et al.*, 1994; Osteryoung and Vierling, 1994) and the mitochondrial (Lenne and Douce, 1994) smHSPs. Considerable recent data indicate that these complexes are homo-oligomers of smHSPs. Human HSP27, avian HSP25, and murine HSP25 have been purified from their native sources and shown to be homo-oligomers (Arrigo and Welch, 1987; Collier *et al.*, 1988). Plant smHSPs expressed in *E.coli* form soluble high molecular weight complexes similar in size to smHSP structures observed *in vivo* (Fig. 5) (Lee *et al.*, 1995; Suzuki and Vierling, unpublished). Analysis of recombinant pea PsHSP18.1 (a class I cytosolic smHSP) purified from *E. coli* reveals that the protein forms a globular homo-oligomer of 12 subunits and PsHSP17.7 (a class II protein) is also most likely a dodecamer. The subunit structure of smHSP complexes from other organisms has not yet been defined.

Interestingly, although both the class I and II proteins are thought to accumulate in the cytoplasm, *in vivo* and *in vitro* data indicate these proteins do not form hetero-oligomers. By several criteria, class I and II oligomers are separable in plant extracts (Helm and Vierling, unpublished observations), and although recombinant proteins of both classes can be disassembled and assembled *in vitro*, they will not co-assemble (Lee, Krawitz and Vierling, unpublished observations). These results again support functional distinctions between these highly conserved classes of proteins. As the α-crystallin proteins also occur in high molecular weight structures, it has long been suggested that common features of the carboxyl-terminal domain are responsible for complex formation. However, this hypothesis has not yet been critically tested and formation of distinct class I and II smHSPs oligomers argues there are additional determinants involved in formation of these structures.

A curious property of the smHSP complexes is their ability to associate into insoluble structures larger than 1 MDa, which have been referred to as 'heat shock granules'

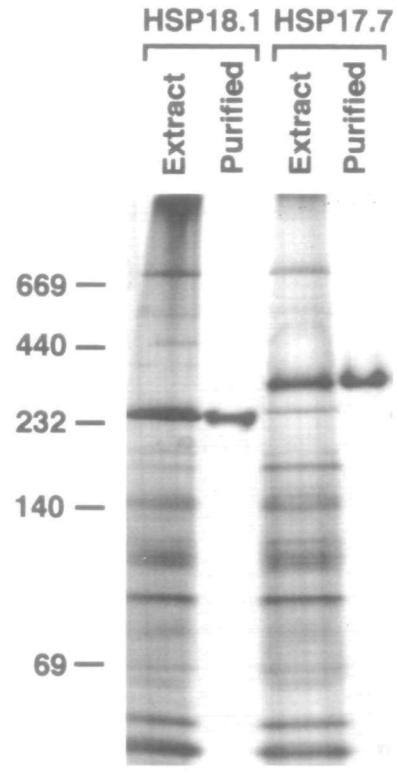

Fig. 5. Recombinant PsHSP18.1 (class I) and PsHSP17.7 (class II) migrate as discrete high molecular weight complexes on non-denaturing pore exclusion gel electrophoresis. Soluble protein extracts from *E. coli* cells expressing plasmid-borne copies of either pea HSP18.1 or pea HSP17.7, and purified HSP18.1 and HSP17.7 complexes were separated on non-denaturing pore exclusion gels (see Lee *et al.*, 1995, for details). Gel was stained with Coomassie Blue. Positions of molecular weight markers are shown at the left.

(Nover, 1991). Formation of these large structures appears to be reversible and occurs primarily at more severe temperatures. Evidence suggests formation of these structures is common to all smHSPs, including the organelle-localized forms (Osteryoung and Vierling, 1994). Studies of heat shock granule composition and possible functional significance have been greatly hampered by their insolubility, and their functional significance remains obscure.

Mammalian smHSPs are phosphorylated on three Ser residues (Ser 15, 78 and 82 in human HSP27; see Fig. 3) in response to stress and different growth promoting agents (Freshney *et al.*, 1994; Rouse *et al.*, 1994). The phosphorylated serine residues are in the N-terminal part of the protein and are conserved among mammalian smHSPs (Gaestel *et al.*, 1991). Recently, a specific MAP kinase activated protein kinase (MAPKAP kinase 2), which phosphorylates the smHSPs has been identified (Stokoe *et al.*, 1992). To date, conflicting results as to the significance of phosphorylation have been obtained, with some reports indicating phosphorylation is required for smHSP effects on thermotolerance and other reports indicating it is not important (Benndorf *et al.*, 1994; Kato

et al., 1994; Knauf *et al.*, 1994; Lavoie *et al.*, 1993). However, the regulation of smHSP phosphorylation by a specific MAP kinase cascade suggests that this modification influences some aspect of smHSP function and has generated considerable excitement in the field. These observations also lead to the question of whether or not plant smHSPs are similarly phosphorylated, as MAP kinases have also been identified in plants (Stone and Walker, 1995). In early studies, Nover and Scharf (Nover and Scharf, 1984) failed to detect smHSP phosphorylation in cultured tomato cells. Significantly, the plant smHSPs also lack those residues phosphorylated in mammalian smHSPs and do not have the consensus Arg-X-X-Ser phosphorylation motif at any other positions. Re-examination of smHSP phosphorylation in plants indeed indicates that it does not occur (Suzuki and Vierling, unpublished). These observations further distinguish the plant and animal smHSPs.

In vitro chaperone activity of smHSPs

Several different hypotheses have been put forward as to the function of the smHSPs. In mammalian cells, Landry and colleagues (Arrigo and Landry, 1994) support a model in which smHSPs interact with the actin cytoskeleton to protect and restore cellular structure. There is currently no evidence that cytosolic plant smHSPs interact with actin. Nover *et al.* (1989) have proposed that smHSPs sequester and protect normal cellular mRNAs which are not translated during heat stress. However, this hypothesis is based on cofractionation experiments and has not been further tested. The chloroplast smHSP has been implicated in protection of PSII (Kruse and Kloppstech, 1992), but proof of functional association with thylakoid membranes is lacking (Osteryoung and Vierling, 1994). The extensive data indicating that HSP70 and HSP60 are molecular chaperones, along with data indicating HSP90 and HSP100 also have chaperone activities, led to the hypothesis that smHSPs might also act in this fashion. The idea that smHSPs are chaperones is consistent with the fact that they are found in multiple plant cell compartments, and could also explain a diversity of protective effects as resulting from interaction with multiple substrates. Thus, this hypothesis of smHSP function is currently favoured and supporting data are summarized below.

In vitro studies have provided evidence that both α-crystallins and smHSPs have molecular chaperone activity (Horwitz, 1992; Jakob *et al.*, 1993). Thermal aggregation of several proteins was prevented in the presence of α-crystallin at a ratio of about 20 substrate molecules per crystallin oligomer. Recombinant mammalian smHSPs prevented heat-induced aggregation (>45 °C) of proteins *in vitro* as assessed by light scattering, and increased the half-time of α-glucosidase heat inactivation. They also increased the yield of active citrate synthase and α-glucosidase after dilution from denaturant. These effects were ATP independent. A model consistent with these limited data is that smHSPs provide a binding surface for denatured substrates, decreasing their free concentration and, consequently, preventing aggregation.

The idea that plant smHSPs are involved in protection of proteins from heat-induced aggregation was first proposed by Lin and colleagues (Jinn *et al.*, 1989, 1993). In their experiments ammonium sulphate fractionation of heat-shocked soybean extracts was used to prepare protein samples highly enriched in the smHSPs. When mixed with total cell proteins and then heated at 55 °C, this fraction protected 50% of cell proteins from aggregation.

Recently, purified recombinant plant cytosolic class I and II smHSPs have been used in several assays to demonstrate these proteins can act as molecular chaperones *in vitro* (Lee *et al.*, 1995). Similar to the results obtained for recombinant mammalian smHSPs, stoichiometric levels of PsHSP18.1 and PsHSP17.7 (class I and II proteins, respectively, from *Pisum sativum*) enhanced the refolding of chemically denatured citrate synthase approximately 2-fold regardless of the presence or absence of nucleotide triphosphates. A mixture of PsHSP18.1 and PsHSP17.7 enhanced refolding of chemically denatured lactate dehydrogenase to a similar extent as was observed for equivalent amounts of the individual proteins, providing evidence that class I and II proteins function independently. However, refolding of citrate synthase by both plant and mammalian smHSPs appears to be less efficient than refolding obtained with the ATP-dependent GroEL/GroES system (Buchner *et al.*, 1991). Such differences imply that the mechanism of refolding by smHSPs lacks the precise regulation of substrate binding and release that occurs during the GroE reaction cycle. Alternatively, the *in vitro* system may lack one or more components that regulate smHSP activity *in vivo*.

Using *in vitro* conditions that simulate physiological heat stress and recovery temperatures for plants, it has been shown that stoichiometric levels of PsHSP18.1 and PsHSP17.7 prevent irreversible thermal inactivation of citrate synthase (Fig. 6). For example, when citrate synthase is denatured at 38 °C, then allowed to refold at the permissive temperature of 22 °C, significant recovery of citrate synthase activity is observed only when the enzyme is incubated with smHSPs from the beginning of the heat treatment. These results suggest that in plants, smHSPs function during heat stress and subsequent recovery to prevent irreversible protein denaturation. Other *in vitro* results demonstrate that substoichiometric to stoichiometric amounts of PsHSP18.1 prevent aggregation of model substrates such as citrate synthase (Lee *et al.*, 1995), malate dehydrogenase, and glyceraldehyde 3-phosphate dehydrogenase (Lee and Vierling, unpublished) at temperatures above 40 °C. Unlike PsHSP18.1,

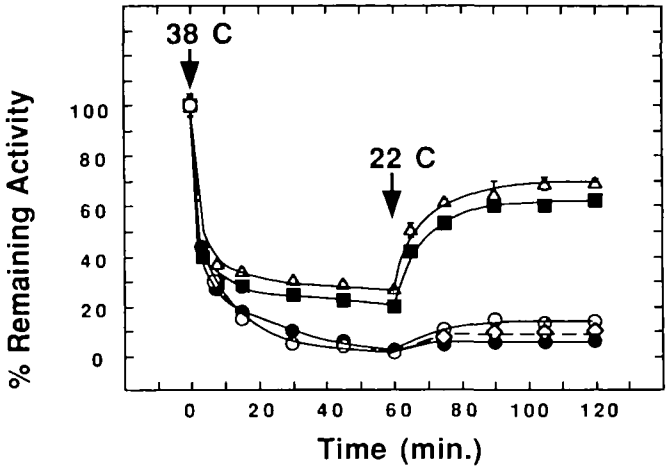

Fig. 6. Purified recombinant PsHSP18.1 and PsHSP17.7 prevent irreversible thermal inactivation of citrate synthase at 38 °C. 150 nM citrate synthase monomers were incubated at 38 °C in the absence (●) or presence of 32 μg ml^{-1} catalase (○), 150 nM PsHSP18.1 (△) or 150 nM PsHSP17.7 (■) complexes. Where indicated, samples were shifted to 22 °C. (◇) indicates samples in which 150 nM citrate synthase monomers were incubated alone for 60 min at 38 °C, then supplemented with 150 nM PsHSP18.1 or PsHSP17.7 at the time of temperature shift to 22 °C. Citrate synthase enzymatic activity was determined at the times indicated at 22 °C. Reprinted by permission from Lee *et al.* (1995).

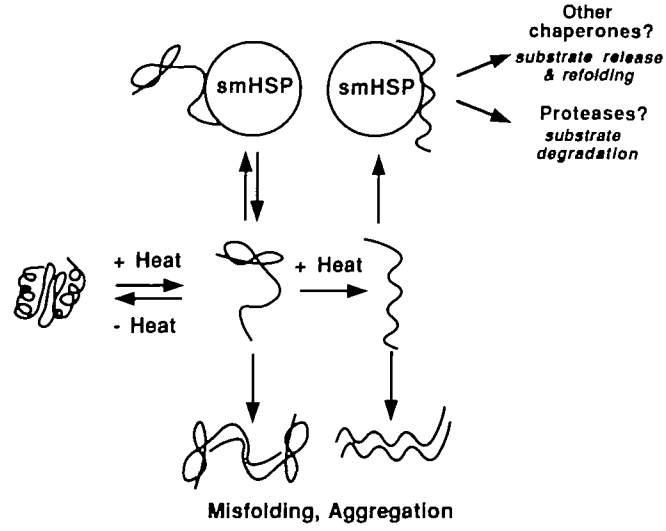

Fig. 7. Working model for smHSP interactions with protein substrates. The model is based on *in vitro* studies discussed in the text. See text for further details.

PsHSP17.7 confers less protection to these substrates, again suggesting class I and II proteins have functional differences, although differences between the recombinant proteins and *in vivo* complexes might also contribute to these distinctions. Importantly, under the high temperature conditions at which PsHSP18.1 prevents aggregation of target proteins, the target proteins themselves bind irreversibly to the smHSP and are stably bound in the presence of ATP, high ionic strength, or low temperature. In fact, stable complexes between glyceraldehyde 3-phosphate dehydrogenase and PsHSP18.1 are formed at temperatures as low as 34 °C (Lee and Vierling, unpublished). Lin and colleagues (Jinn *et al.*, 1995) also observe binding of smHSP complexes to other soluble proteins at high temperatures.

Based on the above *in vitro* data using model substrates, a working model of smHSP activity as depicted in Fig. 7. is proposed. The present findings suggest that PsHSP18.1 interacts selectively with non-native proteins in two modes. At lower temperatures such as 34–38 °C, target proteins interact reversibly with the smHSP to limit misfolding and/or aggregation, leading to increased reactivation upon removal of heat. At higher temperatures such as 40 °C or above, the smHSP binds target proteins irreversibly to decrease the concentration of aggregation-prone intermediates, thereby preventing accumulation of insoluble protein aggregates within the cell. The strength of substrate-smHSP binding is most likely determined by the extent of substrate denaturation and the unmasking of interactive surfaces. Substrate binding would, therefore, be temperature-dependent and unique for each substrate as has been observed (Lee and Vierling, in preparation). Temperature-dependent structural changes in the smHSP, which increase the binding affinity for substrates, may also occur. Since irreversible binding of heat-denatured proteins appears to be a general phenomenon *in vitro*, it is likely that stable complexes of smHSPs with substrates are also formed *in vivo*. The potential *in vivo* role of irreversible substrate binding to smHSPs is unclear. It is speculated that unfolded substrates bound to smHSPs *in vivo* interact with other chaperones which can release and refold the substrates, or alternatively that the unfolded substrates are presented to the cellular proteolytic machinery for degradation. This proposal is consistent with data indicating multiple chaperones often act on a single substrate, as well as growing evidence that chaperones participate in proteolytic processes (Parsell and Lindquist, 1993). Further *in vitro* as well as *in vivo* studies are needed to test these ideas.

Future prospects

Studies of the smHSPs in plants have only just begun to define the complexity of their structure, regulation and possible functions. Many areas are in need of additional research. First and foremost is the question of whether smHSPs actually function as chaperones *in vivo*, and if so, what are their critical substrates and do their activities contribute significantly to thermotolerance? Experiments are currently in progress to trap proteins bound to smHSP complexes *in vivo*. Although several experiments in which mammalian smHSP levels were manipulated indicate that these proteins contribute to cellular thermotolerance (Arrigo and Landry, 1994), no comparable data are available in plants. SmHSP mutants or transgenic plant

models in which to test critically the *in vivo* role of plant smHSPs are still lacking. Even *in vitro*, the mechanism of smHSP chaperone activity is still far from being defined.

Considerable information is also still lacking at a very descriptive level. For example, it has mostly been assumed that expression of the smHSP genes shows little tissue specificity. However, this is clearly an over-simplification. Data indicate that the organellar smHSPs accumulate in proportion to the number of target organelles present in different cell types (Helm *et al.*, 1993; Chen *et al.*, 1990). Perhaps different members of the cytosolic smHSP gene families are also specialized for different cell types, particularly when they are expressed in the absence of heat stress. Tissue specificity has been suggested by the studies of Jordano and collegues on smHSP expression during dessication (Almoguera *et al.*, 1993). It also remains unclear exactly where the cytosolic smHSPs are localized within the cell. Although they are referred to here as cytosolic, studies have suggested that they move between the nucleus and cytoplasm in a stress-dependent fashion (Wollgiehn *et al.*, 1994). This movement would clearly have ramifications as to possible substrate interactions.

The evolution of smHSP gene complexity in higher plants is also intriguing. The simplicity of the smHSP genes in non-plant eukaryotes along with the complete absence of organelle-localized smHSPs poses questions about the selective forces which may have driven smHSP diversification in plants. One hypothesis is that smHSP genes diversified in plants due to stresses encountered during the transition to growth on land, an environment in which much greater fluctuations and extremes of temperature, light, and water availability were encountered. Isolation of smHSP genes from the precursors to land plants should begin to address this hypothesis. It is also interesting to speculate that smHSP expression in tissues that can not thermoregulate, such as reproductive structures, has contributed more significantly to plant fitness than has expression in vegetative tissues. Leaves very efficiently thermoregulate when plants are growing in environments to which they are adapted, thereby infrequently achieving temperatures required to induce HSPs. In contrast, in the same 'optimal' growth environments reproductive structures often reach high temperatures sufficient to induce smHSPs (Hernandez and Vierling, 1993). There is still virtually a complete lack of data on the expression of smHSPs (or any other HSP) in plants growing in their natural environment that would provide general information about the possible importance of HSPs to plant survival and fitness.

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