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Analysis of Chaperone Function and Formation of Hetero-oligomeric Complexes of Hsp18.1 and Hsp17.7, Representing Two Different Cytoplasmic sHSP Classes in *Pisum sativum*

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Abstract

Small heat shock proteins (sHSPs) are the most abundant stress proteins in plants. Usually not expressed under permissive conditions, they can accumulate to more than 2% of the total cellular protein content during heat stress. At present several points of evidence indicate that these proteins act as molecular chaperones by keeping partially denatured proteins in a folding-competent state. In plants sHSPs are encoded by a multigene family, which can be segregated into several classes according to their subcellular position and/or sequence homology. Curiously, two different classes appear in the cytoplasm. Their specific role during heat shock remains elusive. Here we present some evidence that both classes of sHSPs enhance recovery of reporter protein activity in the presence of HSP70. Applying peptide arrays prepared by SPOT synthesis and *in situ* analysis by confocal laser scanning microscopy, we could further show that the two classes of sHSP are attached to each other and are able to interact with non-native proteins both *in vivo* and *in vitro*. Although both of the sHSPs act similarly as molecular chaperones, immunohistochemistry experiments support the hypothesis that the two have different cellular functions in the development of heat-induced cytoplasmic heat shock granules under elevated temperatures.

Key words: SHSP; Chaperone; Plants

Introduction

Small heat shock proteins (sHSPs) are found in all eukaryotic organisms. In plants, they accumulate to

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form the preponderant protein group under heat shock conditions (Waters 1995; Van Montfort and others 2001; Haslbeck 2002). Despite several reports concerning their function as molecular chaperones, little is known about their *in vivo* function in preventing cellular damage as a result of stress.

In contrast to other chaperone systems, sHSPs are able to keep proteins under nonpermissive temperatures in a folding-competent state without consuming adenosine triphosphate (ATP). This prominent and powerful feature of sHSPs has been demonstrated *in vitro* using various reporter proteins (Horwitz 1992; Ehrnsperger and others 1997; Lee and others 1997; Haslbeck and others 1999; Lee and Vierling 2000). Although sHSPs are able to prevent reporter protein aggregation, their *in vitro* ability to prevent reporter protein inactivation is poor, leading to speculation that other partners, like the HSP70 chaperone system, might be necessary for this chaperone activity (Ehrnsperger and others 1997; Forreiter and others 1997; Lee and Vierling 1998). Based on data linking chaperone activity to cellular thermotolerance (Forreiter and others 1997), it was speculated that sHSPs act as chaperones in the first line of defense by preventing massive irreversible aggregation of cellular proteins under thermal stress, while active refolding is carried out later by other chaperone systems (Haslbeck and Buchner 2002). However, this would not necessarily explain the massive sHSP accumulation upon heat shock or during certain developmental stages in plants. It could be shown that, derived from dimers, many sHSPs form variable oligomeric structures, which can rapidly change their composition with changes in the environment. It is clear that this process is necessary to mediate cellular thermotolerance in yeast or mouse (Ehrnsperger and others 1999; van Montfort and others 2001; Horwitz 2003; Stromer and others 2003). Interestingly, these variable structures cannot be detected in plants, where sHSPs are organized in oligomeric complexes of 12 subunits of defined structure, shown for the crystallized plant sHSP oligomer from wheat (van Montfort and others 2001). In addition, sHSPs have other interesting properties peculiar to plants:

1. Based on their amino acid sequence, two classes of sHSPs, termed class I and class II, can be distinguished in the cytoplasm (Waters 1995). Meanwhile a third class has been defined, which was reported to be targeted to the nucleus (Siddique and others 2003). Though present in different organelles of the cell, like plastids (Vierling and others 1988; Chen and others 1994), mitochondria (Lenne and others 1995), or the lumen of the endoplasmic reticulum (Helm and others 1993), the appearance of the two different classes in the cytoplasm remains enigmatic.

2. Proteins of the plant sHSP family usually form oligomeric complexes of 120 kDa–2 MDa. In plant cells they undergo further changes, resulting in a tight assembly of sHSPs into detergent- and salt-resistant complexes, which subsequently form large cytosolic aggregates called *heat shock granules* or *heat stress granules* (Nover and others 1983; Neumann and others 1984). Formation of heat shock granules depends on the presence of both of the two different cytosolic sHSP classes (Kirschner and others 2000). Several functions have been attributed to this structure, like binding specifically housekeeping mRNA (Nover and others 1983, 1989; Stuger and others 1999; Low and others 2000; Smykal and others 2000). Recently it was shown that the formation of heat stress granules seems to be important not only for thermotolerance of the plant cell but also for the plant organism as a whole (Miroshnichenko and others 2005). Nevertheless, the biological function of heat stress granules is far from understood.

Using SPOT synthesis (Reineke and others 2001; Frank 2002), we prepared peptide arrays displaying overlapping scans of class I and II sHSP-derived peptides. After testing both arrays with either class I sHSP or class II sHSP, we could show that the two types of cytoplasmic sHSPs bind to each other *in vitro* and that both form complexes with denatured but not native luciferase in tobacco protoplasts. The presented *in vivo* data provide further evidence that the two forms differ in their ability to form heat shock granules. However, their direct interplay appears to be necessary to create heat shock granules, which act as transient cytoplasmatic compartments storing denatured proteins in a folding-competent state.

Materials and Methods

Plant Material

Stable luc-transformed *Arabidopsis* cell culture used for *in vivo* recovery experiments was described previously (Forreiter and others 1997). Thermotolerant cells were obtained by exposure of plant material to a moderate heat pulse of 15 min at 39°C 3 h prior to the experiment.

In vivo Chaperone Assay

In vivo chaperone activity of both classes of sHSPs in the presence of HSP70 was measured as described earlier (Forreiter and others 1997). Preparation of

the sHSP constructs used for transient expression and *in vivo* chaperone analysis were described by Low and others (2000). For the assay described here, the *Pisum sativum HSP 18.1* (Accession No. M33899) and *HSP17.7* (Accession No. M33901) were used.

Transient Expression of Heterologous sHSP in Tobacco Protoplasts

Preparation and transformation of tobacco (*Nicotiana plumbaginifolia*) mesophyll protoplasts was performed as described elsewhere (Lyck and others 1997). At a point 16 h prior to analysis, 1×10^5 tobacco protoplasts were transformed with 10 μg of the indicated expression plasmid containing a gene coding either for class-specific sHSPs or firefly luciferase (de Wet and others 1985), lacking the peroxisomal target sequence. All constructs were under the control of a CaMV 35S promoter.

Expression and Purification of Class I and Class II sHSP for SPOT Hybridization Analysis

The coding regions of *P. sativum* HSP18.1 and HSP17.7 were fused behind an N-terminal (His)6-element and introduced into a prokaryotic expression plasmid, pJC20, under control of a T7-promoter (Clos and Brandau 1994). After *E. coli* transformation, protein expression was induced with 100 mM IPTG for 2 h. Bacteria were lysed by ultrasonification for 3× 30 s, and the resulting crude protein fraction was applied to a nickel-Agarose column. Purified protein was eluted with 200 mM imidazole according to the manufacturer's instructions (Pharmacia, Freiburg, Germany).

Antibody Production

Polyclonal antibodies against cytoplasmic *P. sativum* class I and II sHSP were raised in rabbits and guinea pig immunized with recombinant protein as described by Low and others (2000).

Immunohistochemisty

Immunohistochemistry was performed essentially as described by Lyck and others (1997). For double fluorescence experiments, *P. sativum* HSP 18.1 class I sHSPs proteins were detected by applying a polyclonal rabbit antiserum (1:5000) followed by a commercially available FITC-labeled anti-rabbit IgG, whereas *P. sativum* HSP17.7 class II sHSPs were detected by a class II specific polyclonal serum raised in guinea pig (1:2000). Guinea pig serum was detected by using commercially available Cy3-labeled anti-guinea pig IgG, unless otherwise indicated. Confocal laserscan analysis was performed with a Leica TCS NT confocal microscope and software system (Leica, Wetzlar, Germany).

In vitro Interaction of sHSP Using Peptide Filters

The peptide sequences of *P. sativum* HSP18.1 class I and HSP17.7 class II were used to create peptide fragments. These oligopeptides were synthesized on cellulose membranes and consist of 13 amino acids overlapping in sequence by 11 amino acids (Kramer and Schneider-Mergener 1998; Frank 2002). After synthesis, oligopeptides were spotted onto a cellulose membrane (Jerini Peptide Technology, Berlin, Germany; www.jerini.de). The resulting peptides were synthesized and C-terminally linked to a cellulose membrane (Whatman, Maidstone, UK) *via* a (β-Ala)2 spacer (Kramer and Schneider-Mergener 1998). The dry membranes were incubated once with methanol and three times with wash buffer (100 mM KCl and 30 mM Tris/HCl, pH 7.6) at room temperature for 10 min. Both filters were subsequently incubated with the indicated proteins as described elsewhere (Reineke and others 1996). For denaturation, luciferase was treated for 1 h in 3 M guanidinium isothiocyanate prior to incubation (Herbst and others 1998). For analysis of protein binding activity, the membrane was incubated with 1 μg/ml of HSP18.1, HSP17.7, native luciferase (Sigma, München, Germany), and denatured luciferase in binding buffer (100 mM KCl, 5% (w/v); sucrose, 0.05% (v/v); Tween 20, 0.05% (w/v); bovine serum albumin; 30 mM Tris/HCl, pH 7.6) for 60 min at 25°C with gentle shaking. Nonspecifically bound protein was removed by washing the membrane with wash buffer for 3 min at room temperature. Peptide-bound protein was transferred onto polyvinylidenedifluoride (PVDF) membranes by blotting. The PVDF membrane and the cellulose membrane were laid on top of filter papers soaked in cathode buffer (75 mM Tris base, 120 mM 6-aminohexanoic acid, 0.01% (w/v) SDS) and overlaid with filter papers soaked in anode buffers AI (90 mM Tris base) and AII (300 mM Tris base) at 4°C. Blotting was performed for 30 min at a constant power of 0.8 mA/cm^2. The membrane was blocked with 5% (w/v) non-fat dry milk in phosphate buffered saline (PBS) and incubated with the indicated antibody. For detection of class I sHSP a polyclonal antiserum was used, which was raised in

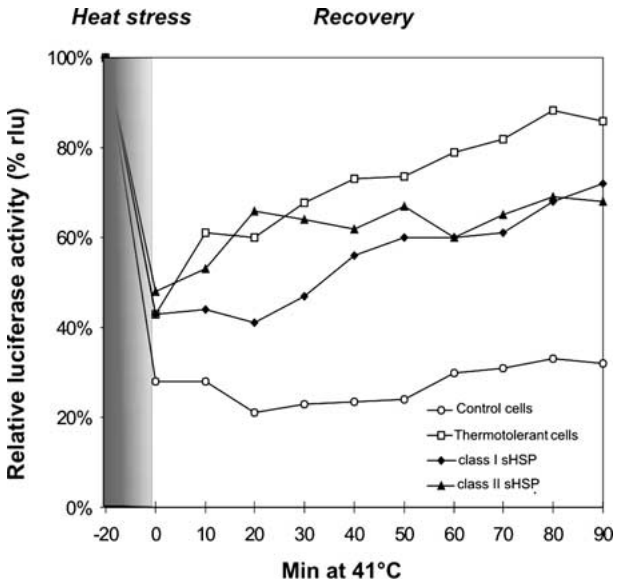

Figure 1. *In vivo* recovery of thermally denatured luciferase in the presence of transiently co-transformed class I and class II sHSPs. *Arabidopsis* cell culture was protoplasted and subsequently transformed with a plasmid coding for a Petunia spec. *hsp70*-gene (open square and open circle) or *hsp70* co-transformed with a cDNA coding for *P. sativum HSP18.1* class I (closed rhombus) or *P. sativum HSP17.7* class II sHSP (closed triangle). Relative luc activity was measured prior to stress and resulting relative light units (rlu) were set as 100%. At this time point 10 μg/ml cycloheximide was added. After that cells were exposed to 41°C for 20 min. Recovery was monitored over a time period of 90 min by removing an aliquot every 10 min. Thermotolerant cells were obtained by exposing cells to a moderate heat pulse of 15 min at 39°C 3 h prior denaturation.

rabbits (Bioscience, Göttingen, Germany) immunized with purified class I protein obtained as described above (1:10,000). For detection of class II protein, we used a polyclonal antiserum (1:2000) obtained from E. Vierling (Tucson, AZ), and for luciferase detection, we used commercially available antibodies (Promega, Mannheim Germany; 1:10,000). Color detection was performed using an (anti-rabbit)-antibody conjugated to alkaline phosphatase according to the manufacturer's manual (Roche, Mannheim, Germany).

Results

Both Types of Cytoplasmic sHSPs Act as Molecular Chaperones *In Vivo*

As a first step in understanding the different roles of cytoplasmic sHSPs, we analyzed their ability to act as molecular chaperones *in vivo*. We examined their capability to restore reporter enzyme activity in an *Arabidopsis* cell culture after a given heat pulse. This culture expresses the *Photinus pyralis* luciferase gene (*luc*) under control of a CaMV 35S promoter. The cell culture was transiently co-transformed with two sHSP members from *P. sativum* HSP18.1 (Helm and Vierling 1989) and HSP17.7 (Lauzon and others 1990), representing both main cytoplasmic classes. The cells were exposed to 42°C for 20 min. During that time period luc activity is dramatically reduced in control cells. As described earlier (Low and others 2000), and in line with observations reported by Smýkal and co-workers (2000), both sHSPs are able to reduce the amount of thermal luc inactivation significantly, extending the half-life of enzymatic luc activity from 5 to 10 min. As a result, remaining luc activity after 20 min is roughly twice as high as in cells expressing exogenously applied sHSPs. Using this residual activity as a starting point, we analyzed the ability of both sHSP classes to accelerate recovery of luc activity after a given heat stress in the presence of HSP70 under conditions in which new luc translation was blocked by cycloheximide (Forreiter and others 1997). Summarized in Figure 1 and in line with our previous observations that both types of sHSPs are able to stabilize luc activity under high-temperature stress, it was evident that both classes of cytoplasmic sHSPs are also able to enhance luc recovery after a given stress period. Although the recovery rates obtained with the two overexpressed sHSPs did not exceed 60% of that obtained in thermotolerant cells, it was obvious that in the presence of HSP70 both cytoplasmic stress proteins enhanced recovery to a similar extent, and approximately 10-fold more than observed in control cells.

The Two Classes Differ in Their Ability to Form Heat Shock Granules *In Vivo*

In a next step, sHSP plasmids encoding either one (Figure 2) or both (Figure 3) cytoplasmic classes were transiently transformed into tobacco mesophyll protoplasts. Tobacco cells were chosen because of their much bigger size compared to the small cultured *Arabidopsis* cells used in the *in vivo* assay. After overnight protein synthesis, cells were harvested, fixed, and subsequently incubated with a corresponding class-specific antibody raised either in rabbits (for class II) or guinea pig (for class I). For detection of the resulting antigen–antibody complex, cells were treated with a secondary antibody directed against rabbit IgG labeled with FITC or

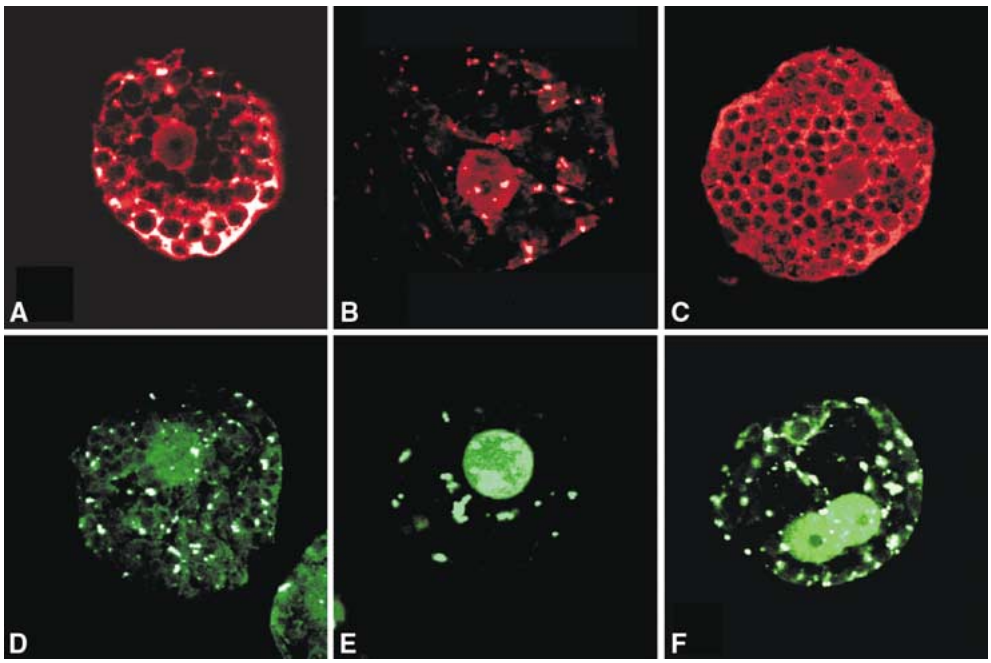

Figure 2. Tobacco protoplasts transiently transformed with plasmids encoding class I (*PsHSP18.1*) and class II (*PsHSP17.7*)-specific genes. After transformation and overnight incubation to allow protein synthesis, cells were kept either under control conditions (**A, D**) or exposed to a 3 h heat shock at 41°C (**B, E**). Additionally, they were exposed to a heat shock in the presence of cycloheximide (**C, F**). Class I protein (shown in **A, B & C**) was detected by a class I antiserum. Subsequently cells were incubated with an anti-rabbit TRIC-labeled antibody. Class II proteins were detected by a class specific antiserum and subsequent incubation with FITC-labelled anti rabbit antibodies (**D–F**).

guinea pig IgG labeled with Cy3. After immunodecoration, cells were analyzed by confocal laser scanning microscopy. As shown in Figure 2A, the transiently expressed class I protein revealed a consistent distribution in the cytoplasm, which was in line with observations by Kirschner and others (2000). This was different if a class II sHSP was expressed instead (Figure 2D). Yet, in contrast with other observations (Kirschner and others 2000), we found that this sHSP class alone tends to form cytoplasmic aggregates even under permissive temperature conditions. A more detailed analysis revealed that if transformed cells were exposed to a severe heat shock prior to fixation (2 h at 40°C), which allows synthesis of endogenous HSPs, the class-specific antiserum detected both cytoplasmic classes in clusters located within the cytoplasm (Figure 2B and 2E), indicating that newly synthesized proteins may be necessary for class I cluster formation. This hypothesis was supported by a parallel experiment in the presence of 10 μg/ml cycloheximide to block *de novo* synthesis of other heat-induced proteins. As expected, the distribution of class II was not affected, but class I proteins showed a similar distribution as under permissive conditions, indicating that endogenous heat shock protein synthesis is required to drive at least class I sHSP proteins into cluster formation, which supports the main conclusions of Kirschner and others (2000).

Class II sHSPs Recruit Class I sHSPs for the Formation of Heat Shock Granules

It was tempting to speculate that newly synthesized endogenous class II sHSPs were responsible for this effect. For this reason, we transformed class I sHSP and class II sHSP simultaneously into tobacco protoplasts and analyzed their distribution under permissive temperatures. Indeed, class I proteins were not evenly distributed among the cytoplasm under these conditions but clustered within discrete dense areas (Figure 3A). If the class II sHSP distribution was analyzed in the same cell using a class II-specific serum allowing class specific fluorescence labeling, a similar distribution of the two proteins in the cytoplasm was perceived (Figure 3B). This became evident when the two channels were merged and their detected fluorescence signals coincided (Figure 3C). Both results were unanticipated, be-

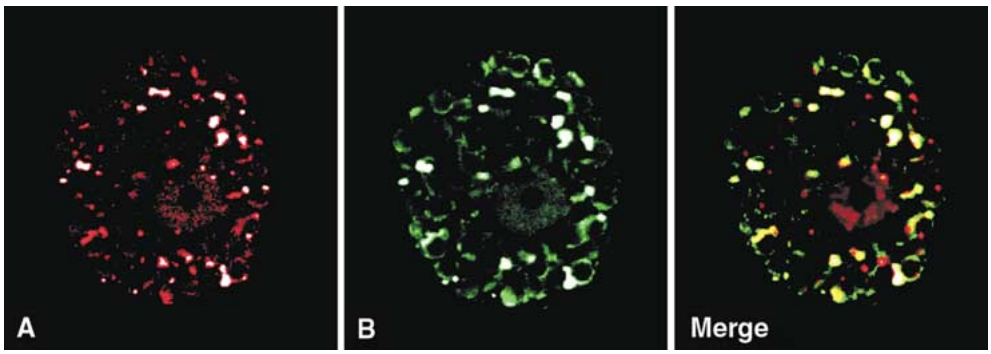

Figure 3. Co-localization of class I and class II sHSPs in tobacco cells. Plasmids containing *PsHSP18.1* (class I) and *PsHSP17.7* (class II) were transformed into protoplasts. After protein synthesis, cells were analyzed by a class I-specific antiserum developed in rabbit. Simultaneously cells were incubated with an antiserum against class II sHSPs developed in guinea pig. After washing, cells were subsequently incubated with FITC-labeled anti-rabbit IgG and Cy3-labeled anti-guinea pig IgG. Green (**A**) and red (**B**) fluorescence resulting from laser excitation was analyzed. C represents a merged picture of both channels.

cause interaction among cytoplasmic class I and class II proteins had not been observed *in vitro* (Lee and others 1995).

Peptide Library Analysis Revealed Interaction between the Two Different Classes of Cytoplasmic sHSPs

To analyze the interaction of the two proteins *in vitro*, we used the SPOT synthesis approach (Reineke and others 2001) to test binding to overlapping peptides derived from the two sHSP classes. This has already been successfully applied for analyzing antigenic domains of antibodies (Reineke and others 1999) and other protein/protein interactions like binding of HSP70 to certain domains of different substrate proteins (Rudiger and others 1997, 2001). For peptide analysis, pea sHSPs coding either for HSP18.1, a class I protein (Helm and Vierling 1989) or HSP17.7, a class II protein (Lauzon and others 1990), were dissected into 50 overlapping peptide fragments. These oligopeptides were synthesized on cellulose membranes and consist of 13 amino acids that overlap in sequence by 11 amino acids each. The SPOT membranes were incubated either with purified class I or class II sHSP. After gently removing excess protein by washing, bound sHSPs were transferred from the cellulose filter onto a PVDF membrane by semi-dry blotting. Following protein transfer, the PVDF membrane was subsequently incubated with class-specific antiserum directed against either sHSP class I or class II. Only when a direct interaction between oligopeptides and the purified protein was strong enough to resist the washing procedure could these proteins be transferred from the cellulose support to the PVDF membrane and subsequently traced by antibodies directed against the purified protein. Figure 4 displays the results. The two filters comprising either oligopeptides of a class I sHSPs or class II sHSP interact with the purified sHSP of the same class (Figure 4 A & B). This could be expected because different approaches led to a similar conclusion (Lee and others 1997; Kirschner and others 2000). The filters revealed that homologous interaction for both classes is mediated by the C-terminus comprising parts of the α-crystallin consensus domain on the one hand, and the short C-terminal arm at the very end of the polypeptide chain, on the other. The interacting regions in pea comprised the conserved Arg-residue as well as the two Ile-residues at the C-terminus described for wheat sHSP class I interaction (van Montfort and others 2001). In addition, a second area of interaction could be detected for class I homooligomers approximately between amino acid P30 and V49 located in the more N-terminal region of the protein, which was found to be involved in substrate binding too (Figure 4 A; see also Figure 6). We found no interaction with the area of the α1-helix and no interaction within the β4-strand, as has been described for wheat class I proteins. Not expected, yet clearly visible was that the class II sHSP was also able to interact, albeit weakly, with oligopeptides on the class I filter. Only small amounts of class II protein were bound to the filter revealing a signal between residue E43 and F64. Binding of the heterologous protein apparently occurred further downstream of homooligomer binding.

Comparable to class I homo-oligomerization, on class II filters, interaction with class II proteins was

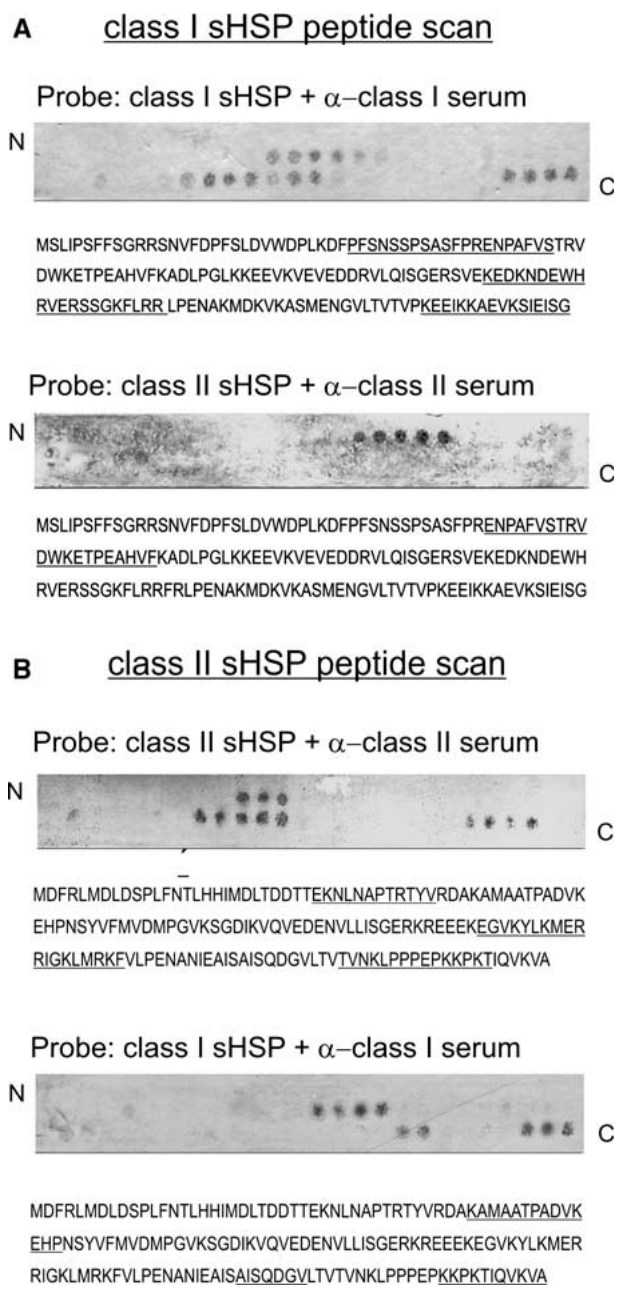

Figure 4. Analysis of class I and class II sHSP domain interaction using peptide libraries. (**A**) Peptide fragments (13mers, 11 amino acids overlapping) derived from *P. sativum* sHSP class I protein (PsHSP18.1-CI) were synthesized on cellulose membranes from left to right. N- and C-termini are indicated. Filters were subsequently incubated with either 1 μg/ml PsHSP18.1-CI or 1 μg/ml PsHSP17.7-CII. After washing and transfer to PVDF membranes, bound proteins were detected with the indicated class-specific antiserum against sHSP class I or class II. **B**. As shown in **A**, oligopeptides of PsHSP17.7.-CII were applied to a cellulose filter and subsequently incubated with class II or class I sHSP. After transfer to solid support, bound proteins were detected with a class-specific anti-serum. Protein sequence information is outlined below each filter. Interacting amino acids are underlined.

perceptible not only at the C-terminus, but also in a second more N-terminal area between amino acids E27 and R39 (Figure 4B). Heterologous interaction with class I sHSPs could also be detected and was stronger than *vice versa*. In addition to an N-terminal region between residues R48 and H63, class I interaction was also seen in the C-terminal region, resulting in an overlap between homo- and hetero-oligomerization. This may be the reason why class I proteins can be recruited to cluster formation by class II proteins and not *vice versa*. Considering these results and comparing them with the binding of denatured luciferase (see also Figure 6), it became evident that some regions overlap to a certain extent, which might be simply explained by the fact that domains may bind different polypeptides facing different sides of their structure.

Denatured luc Protein Clusters in the Presence of Cytoplasmic sHSPs

Because sHSPs have frequently been reported to keep proteins in a folding-competent state (Jakob and others 1993; Lee and others 1995, 1997; Ehrnsperger and others 2000), we expected that partially denatured proteins would not only interact with sHSP oligomers to avoid further irreversible denaturation but would be driven—at least in the cytoplasm of plant cells—under heat stress conditions into heat shock granules to ride out the stress period and to conserve a refoldable constitution. This transient compartment, which is only present under elevated temperatures, may provide partially denatured proteins efficient protection from further damage, because the dense packing and the presence of abundant sHSP in these structures may be additionally beneficial for stabilization and hence better refolding during recovery. To test this possibility, we transformed firefly luciferase as a thermolabile reporter protein into tobacco protoplasts and analyzed its distribution under permissive and non-permissive temperatures. We monitored luc distribution in fixed cells by using a luc-specific antiserum conjugated to FITC. The results are shown in Figure 5. At 25°C the luc protein is still stable and functionally active as proven by standard luc activity assays (not shown). In this form, it showed an even distribution in the cytoplasm (Figure 5A). However, if the cells were incubated for 3 h at elevated temperatures (30 min 38°C, 30 min 39°C, 2 h 40°C), luc protein was locally concentrated in dense clusters (Figure 5B). These clusters could

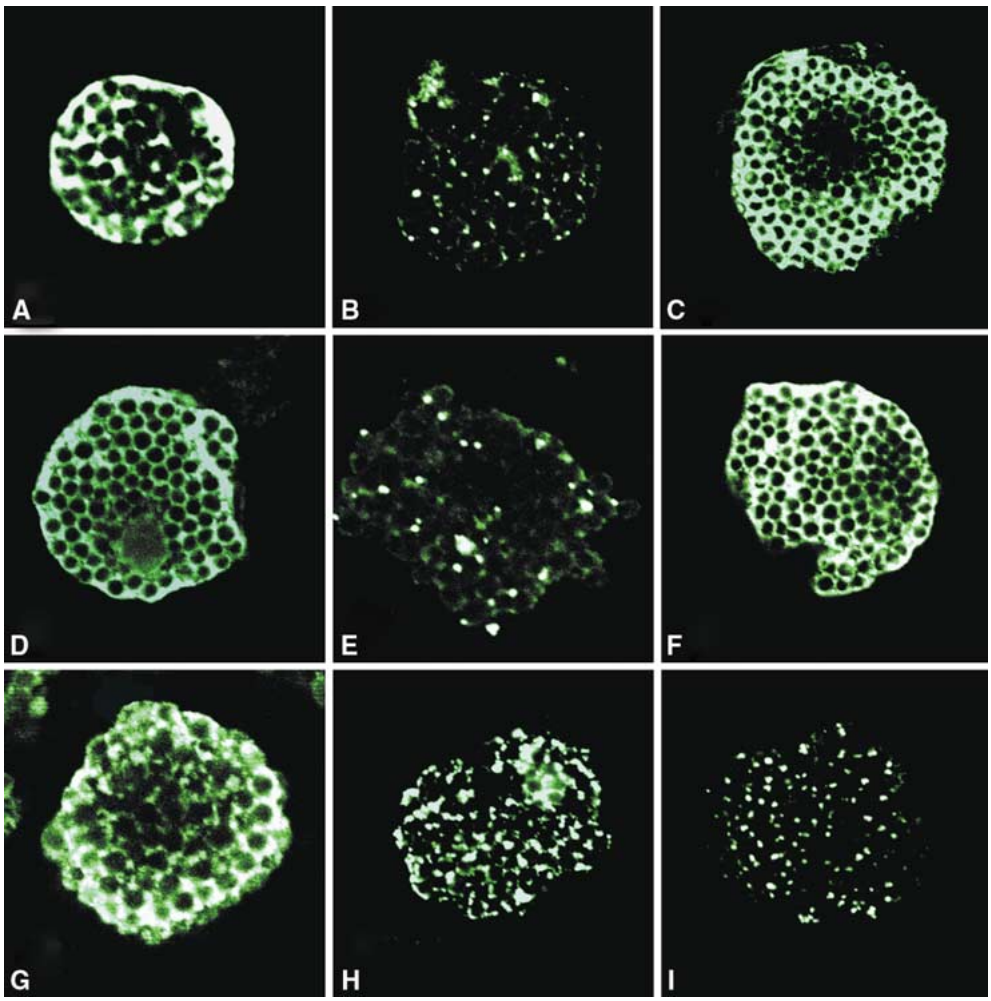

Figure 5. Cluster formation of luc reporter proteins in the presence of different sHSPs in tobacco cells. Plasmids containing either the coding region of *luc, PsHSP18.1-CI,* or *PsHSP17.7-CII* were transformed into tobacco protoplasts. After protein formation, cells were analyzed for luc distribution using a luc-specific antiserum. Prior to fixation, cells were kept for 3 h at 25°C (**A, D, G**) under nonpermissive conditions (30 min 38°C, 30 min 40°C, and 2 h at 40°C) either without (**B, E, H**) or with 10 μg/ml cycloheximide (*C, F, I*). Transient transformation was performed either with luc alone (**A–C**) or mutually with PsHSP18.1-CI (**D–F)** and PsHSP17.7-CII (*G–I*), respectively.

only be observed if no cycloheximide was added, indicating that newly synthesized endogenous proteins were necessary to force luc cluster formation. Thus, in the absence of any sHSPs, even denatured luc was equally distributed within the cytoplasm (Figure 5C). A similar distribution was observed if in parallel a cytoplasmic class I protein was co-transformed (Figure 5 D–F). Evenly distributed in the cytoplasm under permissive temperatures (Figure 5D), luc protein clusters became visible under luc-denaturing conditions (Figure 5E), if cycloheximide was present, as shown in Figure 5F. This observation was in line with the previously described inability of class I sHSP to form heat shock granules alone, which implies once again that, although class I oligomers interact with denatured luc, further cluster formation requires class II sHSPs. This possibility could be confirmed if a class II sHSP was co-transformed with luc (Figure 5 G–I). Although class II proteins had been found in clusters under control conditions (see Figure 2B), native luc was evenly distributed in the cytoplasm (Figure 5G), indicating that native luc protein is no substrate for class II sHSP under permissive temperature conditions. However, under denaturing conditions and in the presence of class II protein, luc protein was sequestered into clusters. This occurred regardless of cycloheximide treatments (Figures 5 F, E). We conclude from these data that cytoplasmic sHSPs

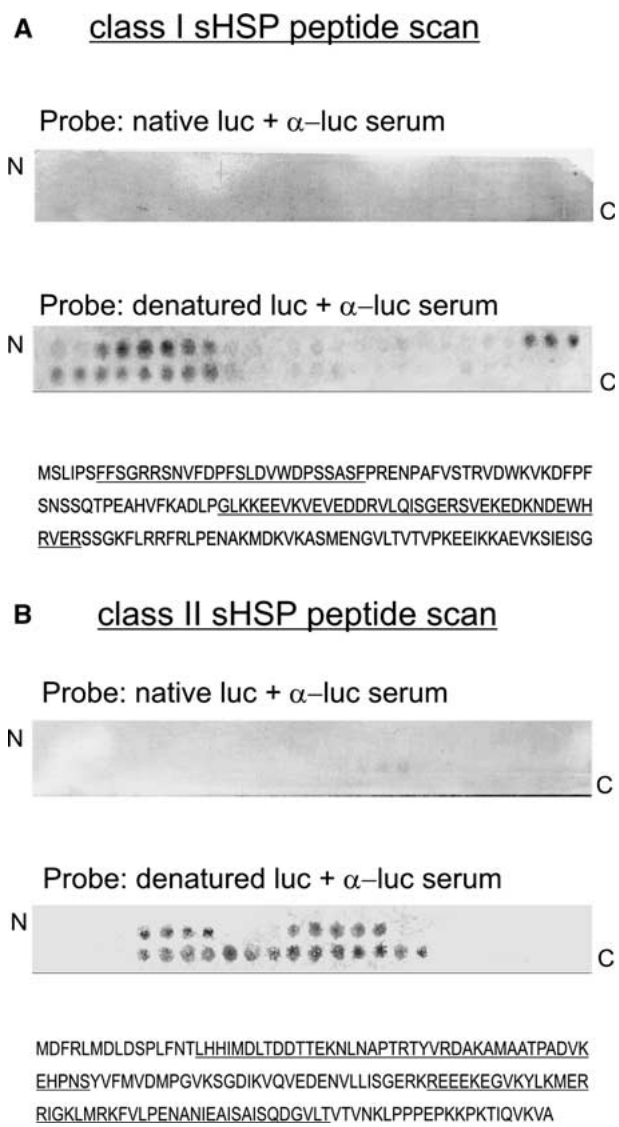

Figure 6. Analysis of class I and class II sHSP domains with native and denatured reporter proteins using peptide libraries. **A**. A cellulose filter comprising class I sHSP-derived overlapping 13mers peptide fragments (see Figure 4) was incubated with 1 μg/ml native luc protein or 1 μg/ml luc denatured with 3 M guanidinium isothiocyanate prior to incubation. After washing and transfer to PVDF membranes, bound luc was detected with an antiserum directed against firefly luciferase. **B**. As described in **A**, native (upper filter) and denatured luc protein (lower filter) was applied to a class II sHSP cellulose filter. Bound luc protein was transferred to PVDF membrane and detected by an antiserum directed against luc protein.

not only have the capability to interact with denatured luc, but that class II proteins were directly capable of driving denatured luc protein into clusters and are therefore the most important element for cellular heat shock granule formation in plants.

Only Denatured luc Protein Binds to sHSP Peptide Fragments

These data could be confirmed by *in vitro* analysis using class-specific sHSP peptide libraries. Two filters containing class I and class II sHSP sequences were incubated with either native luc protein or chemically denatured protein (Figure 6). Obviously, native luc protein does not interact with either filter, whereas chemically denatured luciferase interacted with both. Interaction occurred preferentially with the N-terminal part of the protein. This part of the protein is the most heterologous (Waters 1995). For this reason it was suspected that this part would be involved in binding of the substrate protein. Class I filter interaction occurred between amino acid residue F7 and F31, as well as between G71 and H104. This area has very little overlap with protein domains involved in homo- and hetero-oligomer binding. Using a class II sHSP oligopeptide cellulose filter, interaction could be detected between L16 and S57, comprising two oligopeptides that show no interaction and a domain flanked by amino acid R91 and T115. These parts show a considerable overlap with regions involved in class-specific interaction as well as with hetero-oligomerisation. This is not necessarily a contradictory result, because one might expect that protein domains represented partially by the spotted oligopeptides provide room for different interactions on different sides of its native structure.

Discussion

The ability of small heat shock proteins to prevent irreversible denaturation of thermally destabilized proteins has been recurrently reported (van Montfort and others 2001). However, these proteins do not serve simply as an unspecific surface for partially denatured proteins but are also a main structural element of highly dynamic protein complexes formed in several organisms during heat shock. Although they are not able to promote refolding directly, they are reported to enhance cellular thermotolerance in many organisms. This effect is obviously linked to their facility to form oligomers (Giese and Vierling 2002; Stromer and others 2003). In our approach we used peptide library analysis, which not only provides evidence for direct interaction of both different cytoplasmic sHSP classes but also gives additional information about the domains involved. However, it might be argued that this approach is highly artificial. Therefore data obtained by this method need to be corroborated by

other approaches. We could show, both by peptide analysis and *in situ* immunohistochemistry, that the two different cytosolic sHSP classes not only form homo-oligomers but are also able to interact with each other. This was unexpected, because direct interaction between class-specific cytoplasmic sHSPs did not occur when the two classes were mixed *in vitro* (Lee and others 1997). Peptide library analysis revealed that class II could bind to a more N-terminally located area of a class I sHSP, whereas the class I protein recognized two different domains of the class II protein. These data were confirmed by immunohistochemical analysis, where class II proteins are not only able to recruit class I proteins to cytoplasmic clusters—in line with observations by others (Kirschner and others 2000)—and as shown in a double fluorescence experiment, colocalized in the cytoplasm.

Using these two different approaches, we could further show that binding of other proteins occurred only if these proteins were denatured. As shown in Figures 5 and 6, native luciferase was not able to bind to sHSPs, either *in vitro* or *in vivo*. After denaturation *in vitro*, either chemically or thermally, luc protein strongly interacted with both sHSPs classes. In both cases the N-terminal region of the sHSPs, which is thought to be involved in substrate binding, was responsible for the interaction (van Montfort and others 2001; Giese and Vierling 2002). It seems that in both sHSP classes, two distinct areas were able to bind the luc protein. Yet, comparisons of *P. sativum* data presented here with the structure information obtained by crystallizing wheat HSP16.9 homooligomers have to be done very carefully because different HSP, even if they belong to the same class, form oligomers containing different numbers of sHSP momomers. This may point to a variable feasibility of these proteins to interact with each other. In addition to these experiments, we tested the ability of both classes to facilitate luc refolding in a cellular environment. In previous experiments luc refolding was enhanced if HSP70 and sHSP were simultaneously expressed in protoplasts. Using this approach, we could show that both classes promote luc refolding to a similar extent. This was to be expected because both classes are also able to prevent luc inactivation during heat shock *in vivo* (Low and others 2000).

However, our main conclusions relate to the cluster formation mediated by class II sHSPs and their ability to interact with class I proteins. This is a prerequisite in plants for the transient formation of cytoplasmic structures, termed heat shock granules or heat stress granules (Nover and others 1983). It is becoming increasingly evident that the highly dynamic oligomeric structure of sHSPs is important for their ability to bind non-native protein and subsequently to prevent them from further, irreversible aggregation. This was shown for yeast HSP26 and mouse HSP25 (Stromer and others 2003), for HSP16.6 from *Synechocystis* (Giese and Vierling 2002) and wheat (Sobott and others 2002). All these data indicate that the dynamic of the quaternary structure is an important feature of the sHSP

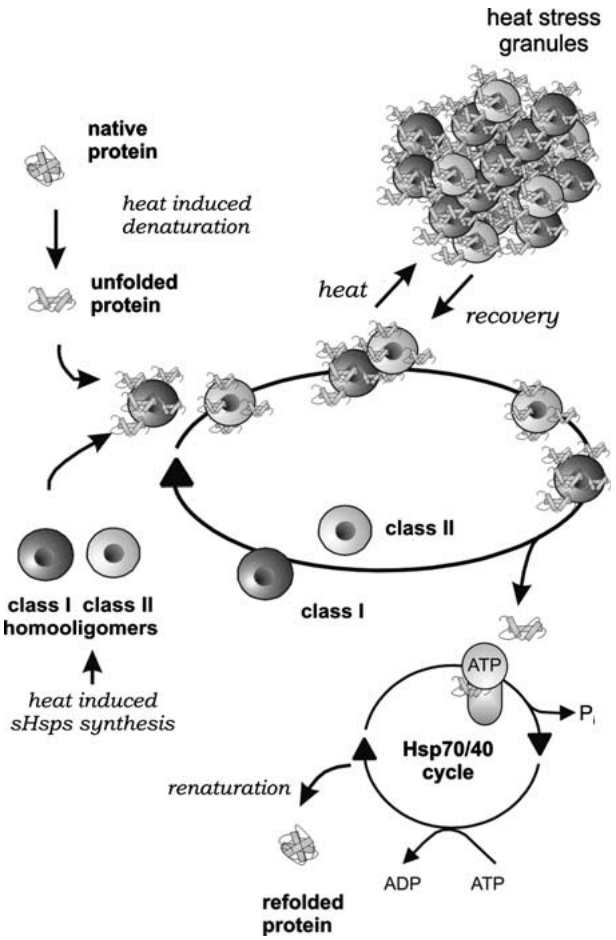

Figure 7. Model for cytoplasmic action of sHSPs in plants. After exposure of cells to elevated temperatures, unfolded proteins accumulate in the cytoplasm. Upon heat shock, synthesis of both cytoplasmic classes of sHSP is initiated. These proteins form homooligomers. Denatured proteins are bound by both sHSPs class I and II homooligomers, keeping the denatured proteins in a folding-competent state. Driven by class II proteins, class I and class II sHSPs form cytoplasmic granules with an average size of 40 nm. This complex consists of densely packed sHSPs and their bound, partially denatured proteins. These structures disintegrate during recovery, releasing class-specific homo-oligomers still covered with denatured proteins, which are then refolded by the HSP70/HSP40 chaperone machine.

cellular chaperone function. In plants, these quaternary structures can be huge, reaching approximately 40 nm in meristematic cells. It is obvious that organisms bound to their local environment would have to develop more sophisticated ways of withstanding stressful situations. For that reason, one might expect cellular stress responses in plants to be more effective than those in other organisms. One such difference might be the massive appearance of these heat-induced granule structures. Taken together, we would propose to extend our current models of cellular sHSP function and granule formation in plants (Figure 7): Following high temperatures, many denatured proteins accumulate in a very short time. As soon as the folding capacity of the cellular chaperone network is exceeded, proteins become entrapped by newly synthesized class I and class II sHSPs. For the sake of normal biochemical maintenance even under stressful conditions, class I and class II proteins loaded with substrate proteins bind to each other and heat shock granules are formed, and these cover and protect the bound proteins. This special trait is characteristic only of the cytoplasm of plant cells and is dependent on the presence of class II sHSPs. During recovery, these complexes disintegrate, slowly releasing their substrate proteins, which become refolded by the HSP70/HSP40 chaperone system. We think that this scheme is in line with other observations, for example those of Smykal and others (2000) or Haslbeck and others (2004). Further investigation will focus on the particular domains within the class II sHSP to understand which parts of the protein mediate the cluster formation. In doing so, we expect to gain further insight into the particularities of the plant specific cellular heat shock response.

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