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Cytosolic heat-stress proteins Hsp17.7 class I and Hsp17.3 class II of tomato act as molecular chaperones in vivo

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Abstract. Small heat-stress proteins (sHsps) are the most abundant stress-induced proteins with up to 20 different members in higher plants. In the cytoplasm, two different classes can be distinguished. Two cDNA clones from tomato *Lycopersicon peruvianum* (L.) Mill., each coding for one of the cytoplasmic sHsp subfamilies, were analyzed with respect to their transcript and protein expression, genome organization and chaperone activity. Neither type was present under control conditions but both appeared upon heat stress and in mature fruits. Expression of the class II transcript was found to be induced at slightly lower temperatures than the class I transcript. Protein analysis using class-specific antibodies revealed an identical expression pattern of both corresponding proteins. Transient expression in an *Arabidopsis thaliana* (L.) Heynh. cell culture showed that, despite the difference in their amino acid sequence, both classes are functionally active as chaperones in vivo, as shown by their ability to prevent thermal inactivation of firefly luciferase in a cellular environment.

Key words: Chaperone activity – Heat stress – *Lycopersicon* – Small stress proteins

Introduction

All organisms synthesize heat-stress proteins (Hsps) in response to supraoptimal temperatures and other types of stress. Their main cellular task is to prevent irreversible protein damage and to normalize cellular functions during recovery (reviewed by Forreiter and Nover 1998). Heat-stress proteins can be assigned to eleven protein families conserved among bacteria, plants and animals. Most of them act as molecular chaperones aiding other proteins to maintain or regain their native conformation by stabilizing partially unfolded states (summarized by Beissinger and Buchner 1998). They do not contain specific information for correct folding, but rather prevent unproductive interactions (aggregation) between non-native proteins. Many data indicate that members of different Hsp families act together in multi-subunit complexes to generate a network for protein maturation, assembly and targeting (Frydman and Höhfeld 1997; Johnson and Craig 1997; Bukau and Horwich 1998).

One intriguing observation, especially in plants, is the multiplicity and massive accumulation of the small Hsp family upon heat stress. These proteins usually exist in oligomeric and functionally active complexes of 200–800 kDa, composed of individual sHsps having a size of 15–30 kDa. All of them, including α-B-crystallin, the homologous protein of the vertebrate eye lens, share a highly conserved C-terminal region called the α-crystallin domain. In plant cells under elevated temperatures sHsps form large cytosolic aggregates of approx. 40 nm diameter, termed heat-stress granules (Nover et al. 1983, 1989). This indicates a specific role for sHsps in stress-induced protection of cellular structures (Kimpel and Key 1985). In support of this, members of the sHsp family were shown to prevent aggregation of thermally inactivated reporter proteins in vitro (Horwitz 1992; Jakob et al. 1993; Lee et al. 1995, 1997; Ehrnsperger et al. 1997). This protective effect does not require ATP. The in-vitro effects were recently complemented and extended for a class I protein by in-vivo studies using plant cells and firefly luciferase (Luc) as reporter (Forreiter et al. 1997). All cytoplasmic sHsps in plants described so far belong to two different classes, class I and class II, according their amino acid sequence (Waters et al. 1996; Heckathorn et al. 1998). However, it is not clear whether there is a functional difference between members of the two classes. Recent reports analyzing class I and class II sHsps from pea indicate that they form complexes of similar size to class I sHsp

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Abbreviations: HSG = heat-stress granules; sHsp = small heat-stress protein; Luc = luciferase; UTR = untranslated region

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oligomers, do not interact with each other and that both of them are able to prevent protein denaturation in vitro (Lee et al. 1995).

Here we compare two different cytoplasmic small stress proteins from tomato, each representing one of the distinct classes with regard to their transcript and protein expression, genomic organization, and show that both of them act as molecular chaperones in vivo.

Materials and methods

Plant material, growth conditions and heat-stress regimes. *Lycopersicon peruvianum* (L.) Mill. (LpVII; Institut für Pflanzenbiochemie, Halle, Germany) was used for tomato cell cultures. For culture conditions and maintenance of the tomato cell-suspension culture we refer to Nover et al. (1983). The heat treatment consisted of a 15-min heat pulse treatment at 42 °C followed by 2 h of recovery at 25 °C and a second heat treatment at 42 °C for 2.5 h. Tomato (*Lycopersicon esculentum* Mill. cv. Harzfeuer) plants were grown at 23 °C in soil under a 16:8 h day/night-cycle at a photon flux density of approx. 300 μmol m^{-2} s^{-1}. For heat stress, plants were incubated for 3 h at the indicated temperatures. For protein and RNA analysis, different plant tissues or cell culture were harvested and frozen in liquid nitrogen prior to preparation.

Plasmid construction. The cDNAs encoding sHsp class-I (accession nos. AJ 225046 and AJ 225047) and class-II (accession no. AJ 225049) proteins were isolated from a λgt11 phage library obtained from a heat-stressed *L. peruvianum* cell culture (Scharf et al. 1990), excised with *Eco*RI and inserted into pBluescript II S/K (Stratagene) as described by Forreiter and Löw (1998a,b). For expression in cells of *Arabidopsis thaliana* (L.) Heynh., the coding regions of Hsp17.7 class I and Hsp17.3 class II were amplified by polymerase chain reaction (PCR). The primers contained restriction sites to produce an N-terminal *Nco*I and a C-terminal *Xba*I-site for Hsp17.7 and *Kpn*I/*Xba*I site for Hsp17.3, respectively. After digestion, the resulting fragments were inserted into a pRT vector under control of a modified 35S-cauliflower-mosaic-virus (CaMV) promoter (Reichel et al. 1996).

Generation of digoxigenin-labeled DNA probes. Probes were labeled by PCR-amplification of the corresponding cDNA fragments using either Hsp17.7 class I or Hsp17.3 class II sHsp cDNA as template in the presence of digoxigenin-labeled nucleotides according to the manufacturers' instructions (Boehringer).

Analysis of RNA blots. Total RNA was isolated using a modified guanidinium-isothiocyanate (GTC) method described earlier (Forreiter and Apel 1993). The RNA was separated, transferred to Hybond N^+ (Amersham) and fixed by UV-irradiation in a Stratalinker (Stratagene). Blots were hybridized with digoxigenin labeled probes according to the manufacturers' instructions (Boehringer) washed twice for 15 min with 0.1× saline sodium citrate buffer (SSC; 1× SSC = 0.15 M NaCl, 15 mM Na_3-citrate, pH 7.0) 0.1% (w/v) SDS at 55 °C and processed for detection with anti digoxigenin-alkaline phosphatase conjugate and disodium 3-[4-methoxyspiro (1,2 dioxetane-3,2′-(5′-chloro)-tricyclo-(3.3.1.1.^{3,7}) decan)-4-yl) phenylphosphate (CSPD; Boehringer).

Analysis of DNA blots. Genomic DNA was isolated according to standard techniques (Sambrook et al. 1989). Ten micrograms of DNA were digested overnight, separated on agarose gels and transferred onto a positively charged nylon membrane (Boehringer). The DNA was fixed to the membrane by UV-radiation (Stratagene). Membranes were either probed with full-length cDNAs coding for both sHsps or gene-specific probes using the 3′-untranslated region (UTR) as template for PCR-generated probes (see above). Filters were hybridized following the manufacturers' instructions and washed either under low-stringency conditions using 0.5× SSC, 0.1% SDS at 61 °C or under high-stringency conditions using 0.1× SSC, 0.1% SDS at 61 °C.

Antibody production. Immunserum against sHsp class I was generated by using a cDNA clone coding for *Pisum sativum* Hsp18.1 (obtained from E. Vierling, University of Arizona, Department of Biochemistry, Tuscon, Ariz., USA) introduced into the prokaryotic expression vector pJC20 using *Nde*I and *Apa*I restriction sites which placed the gene under the control of the T7 promoter (Clos and Brandau 1994), providing an N-terminal His6-Tag. Expression in *Escherichia coli* was induced by standard induction protocol with *iso*propyl-β-D-thiogalactopyranoside and verified by Western analysis with a specific antiserum raised against pea sHsp 18.1 (class I) provided by E. Vierling. Resulting proteins were purified according to standard procedure with Ni-Agarose beads (Pharmacia). Antibodies were raised in rabbit by Bioscience (Göttingen, Germany).

Protein analysis. Protein concentrations were measured according to Smith et al. (1985). After separation by SDS-PAGE (Laemmli 1970), proteins were blotted onto nitrocellulose (Schleicher & Schüll, Dassel, Germany). After blocking, membranes were incubated with antibodies raised against *Pisum sativum* Hsp18.1 class I or against *Arabidopsis thaliana* Hsp17.7 class II (obtained from E. Vierling). Detection was performed with anti-rabbit-alkaline phosphatase conjugate (Boehringer) using an enhanced chemoluminescence (ECL) system as substrate according to the manufacturers' instructions (Boehringer).

Transformation of Arabidopsis cell culture. Cultivation of the *A. thaliana* cell suspension culture and preparation of protoplasts were performed according to Forreiter et al. (1997). Ten micrograms of plasmid DNA and 10 μg carrier DNA (empty vector) were added to 10^6 protoplasts. Protoplasts were incubated for protein expression at 22 °C for 16 h.

In-vivo chaperone assay. Luciferase (Luc) activity was measured as described earlier (Forreiter et al. 1997). For measuring thermal inactivation of Luc, protoplasts were exposed to 41 °C. An aliquot was measured prior to denaturation, representing 100% activity. Every minute, one aliquot was measured for residual Luc activity, representing a total time of 20 min at 41 °C for the last sample.

Statistical analysis. To check whether there were significant differences between the Luc denaturation curves several statistical tests were performed. Analyzing the inactivation kinetics revealed a significant deviation from linearity for each curve, even after logarithmic transformation ($P < 0.01$). Therefore, we fitted the four sets of measurements to a non-linear function in the following way: Under the assumption that for all kinetics the three repeated measurements y_i follow the normal distribution curve at each time step t_i, we calculated the mean value and the standard deviation $y'_i = \bar{y}_i \pm SD_{yi}$. Replacing the original y'_i by these new values, we performed for all kinetic curves a calculus of observations according to the method of least error squares after Gauss (for details, see Brandt 1992). Because of the non-linearity of the measurement points, a Taylor series was established, which was discontinued after the first member. All kinetics fitted best to the function

$$y_i = A0 + A1 \times e^{bxi}$$

Given first guesses for the function parameters, the iterative solution of the Taylor series yielded better approximations for the function parameters as well as for the mean values of the kinetic curves. Since fitting by calculus of observations provided mean value and standard deviation for each estimated parameter, it was possible to compare function parameters A0, A1 and b for all kinetics by a chi-square test for homogeneity, proposed by Brandt (1992) and originally used for fit weighted means to a constant. All

calculations were performed using the statistical software package STATw 1.1 (1998), developed for Windows 3.1x and Windows 95 by one of the authors (developed by K. Brändle).

Results

Sequence comparison of cytoplasmic tomato sHsps. Using probes coding for cytoplasmic class I (Helm and Vierling 1989) and class II (Bartling et al. 1992) *shsp* genes from *Arabidopsis thaliana*, three different clones coding for cytoplasmic sHsps (GenBank Accession Nos. AJ225046, AJ225047 and AJ225049) were isolated from a cDNA library obtained from a previously described heat-stressed cell-suspension culture of *Lycopersicon peruvianum* (Scharf et al. 1990). Comparison with other plant sHsps representing the two cytoplasmic subfamilies revealed that two cDNAs coded for cytoplasmic class I sHsp subfamily members, the other for class II (Fig. 1). While the two class I proteins have a very high overall homology if compared with each other, they differ slightly within their N-terminal regions from other class I proteins. However, both share the typical consensus elements of plant sHsps defined by Waters et al. (1996), including the Pro-$X_{(14)}$-Gly-Val-Leu motif of consensus region I and the Pro-$X_{(14)}$-Val-Leu motif of consensus region II. These elements are common for all eukaryotic sHsps described so far and are also present in archaebacteria (Kim et al. 1998).

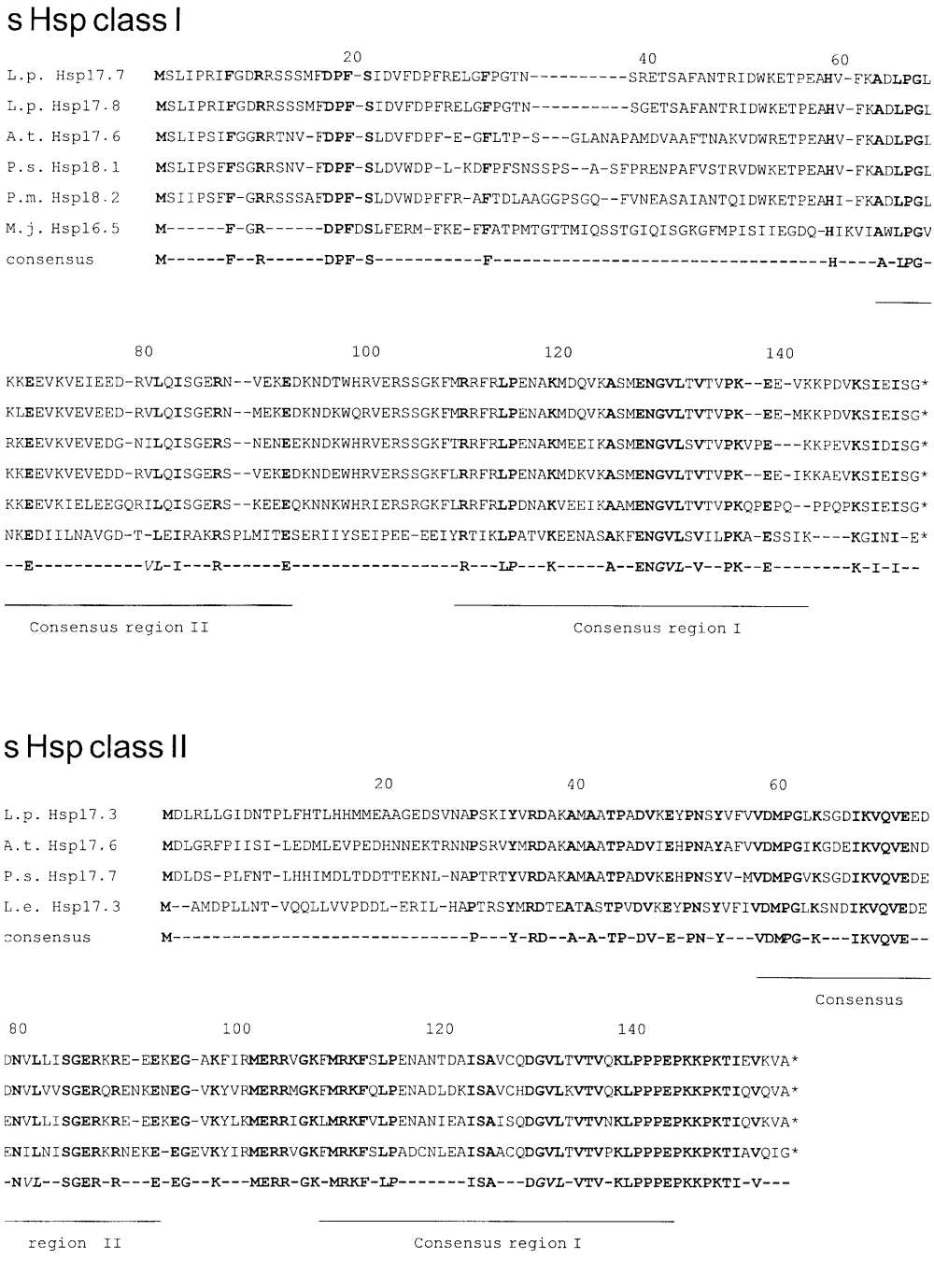

Fig. 1. Amino acid sequence alignment of class I and class II sHsps. Alignment of deduced amino acid sequences of the isolated class I and class II sHsp cDNAs from *Lycopersicon peruvianum* (*L.p.*) with the corresponding class I proteins from *Arabidopsis* (*A.t.*; X16076; Helm and Vierling 1989) pea (*P.s.*; M33899; Lauzon et al. 1990), douglas fir (*P.m.*; accession no. 92983; Kaukinen et al. 1996) and *Methanococcus jannaschii* (*M.j.*; 1SHSA; Kim et al. 1998) and the corresponding class II proteins from *Arabidopsis* (X6344; Bartling et al. 1992), pea (M33901; Lauzon et al. 1990) and *Lycopersicon esculentum* (*L.e.*; U72396; Kadyrzhanova et al. 1998). Identical amino acids are marked in *bold* and summarized in the *bottom line*. The proposed domain motifs for consensus region I and consensus region II are *underlined*. The highly conserved P-X_{14}-GVL of consensus region I and the P-X_{14-16}-VL motif of consensus element II are indicated in *italics*

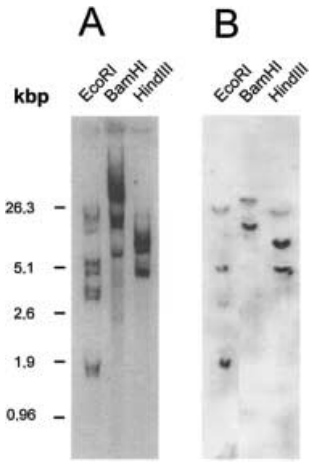

Fig. 2A,B. Analysis of DNA gel blots for sHsp class I genes. DNA gel blots were obtained by digesting 10 μg of total genomic DNA of *L. esculentum* with the indicated enzymes, hybridizing with digoxigenin-labeled probes coding for the full-length **A** *L. peruvianum* Hsp17.7 class I or its sequence-specific 3′UTR **B** and autoradiographing for 10 min

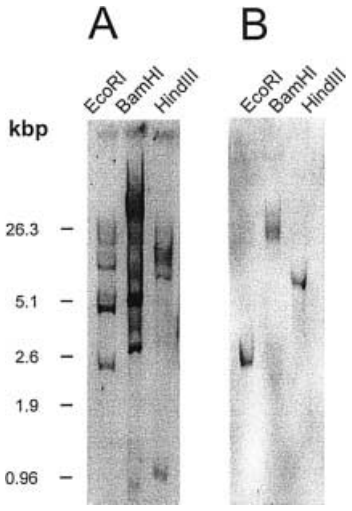

Fig. 3A,B. Analysis of DNA gel blots for sHsp class II genes of genomic *L. esculentum* DNA. Blots were prepared as in Fig. 2 and incubated with digoxigenin-labeled full-length **A** or 3′UTR **B** cDNA probes coding for *L. peruvianum* Hsp17.3 class II

Comparing the deduced amino acid sequences of the isolated tomato class I and class II cDNAs showed that both classes have a homology of 41% only. As shown in Fig. 1, each class was more similar to proteins belonging to the same class of other organisms than to each other. Even in plants not closely related to tomato, such as the gymnosperm *Pseudotsuga menziesii* (Kaukinen et al. 1996), class I proteins revealed 67% homology. In addition, the isolated tomato class II protein showed 62% homology to a class II protein from *Picea glauca*. Since conifers separated from angiosperms more than 290 million years ago, our data support previous speculations that diversification of the two sHsp classes occurred before this time (Doyle and Donoghue 1993). Higher homology of tomato class I than class II proteins to an sHsp from *Methanococcus jannaschii* (30%; Kim et al. 1998), supports the hypothesis that class I-like proteins were already established in archaebacteria.

Genomic organization of cytoplasmic class I and class II shsp genes from tomato. Both cDNAs representing sHsps of class I are members of a small gene family, as revealed by Southern analysis using a full-length sequence of a class I sHsp cDNA. Up to nine different bands could be detected under low-stringency conditions (Fig. 2A). A similar observation was made with a class II sHsp cDNA probe (Fig. 3A). This observation is in accordance with the genomic organization of sHsps found in other plant organisms where up to eight different members have been reported in a single organism (Waters et al. 1996). Because of the high overall homology of the isolated class I sHsp cDNAs (see Fig. 1) we used probes for the gene-specific 3′-UTR of the class I and class II cDNA, which showed no cross-hybridization, as verified by Southern analysis of the UTR probes with the different sHsp-coding plasmids (data not shown). While the class II probe hybridized only with a single gene fragment (Fig. 3B), the corresponding class I probe hybridized with four fragments derived from genomic DNA (Fig. 2B).

Expression of class I and class II sHsps in tomato plants and cell culture. Since sHsps were reported to be expressed mainly under elevated temperatures and certain developmental conditions (DeRocher and Vierling 1994) in seedlings (Coca et al. 1994) or ripening fruits (Fray et al. 1990), we analyzed the distribution of class I and class II transcripts in different tissues and after exposure to elevated temperatures (Fig. 4). Using a gene-specific probe for tomato class I and class II genes it could be shown that in tomato cell cultures and in leaves the appearance of the transcript was strictly temperature dependent. In tomato cell cultures transcripts were not present at normal temperatures and appeared only after heat stress. Lower temperatures (< 30 °C) were not sufficient to induce transcription or protein expression, as revealed by corresponding Western blots, using sHsp-class-specific antisera (Fig. 5). When different tissues were analyzed, mRNA and protein of both classes were almost absent in all tissues (data not shown) except in fruit flesh and immature seeds after tomato fruits had passed the climacteric ripening stage (Figs. 4, 5). After seeds had turned dry, the class I coding transcript was no longer found and sHsp protein was not detectable. However, a considerable amount of class II transcript was still present in dry seeds (Fig. 4A), although the corresponding protein could not be detected.

In-vivo chaperone activity of different cytoplasmic sHsp proteins. To learn more about the function of small stress proteins, we investigated their chaperone activity in vivo. There are several lines of evidence that one important feature of sHsps is the binding of non-native proteins in order to keep them in a folding-competent

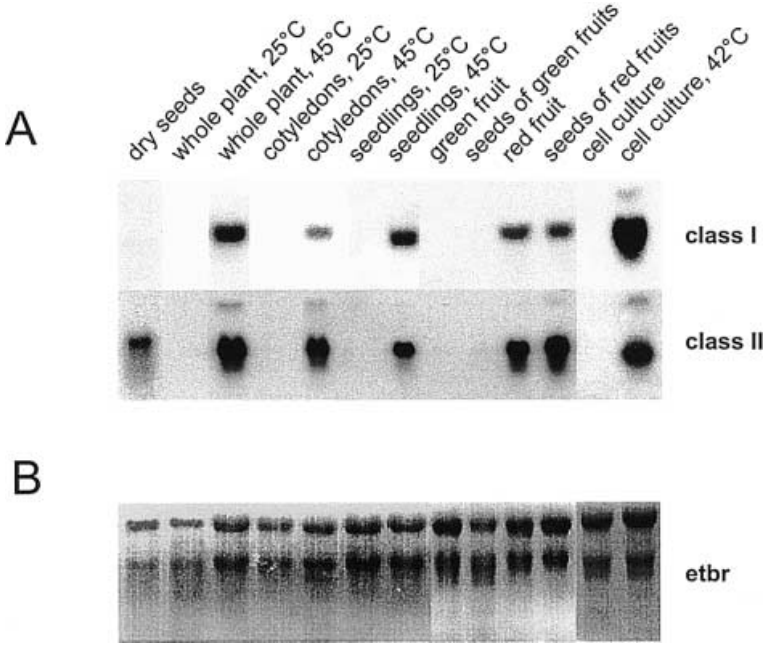

Fig. 4A,B. Gel blot analysis of RNA from different *L. esculentum* tissues and suspension-cultured cells of *L. peruvianum*. **A** Ten micrograms of total RNA was separated on a denaturing gel containing formaldehyde, blotted and probed with a digoxigenin-labeled 3′UTR-specific probe derived from a *L. peruvianum* Hsp17.7 class I cDNA or a *L. peruvianum* Hsp17.3 class II cDNA. Note: *lanes 2*, *3*, 21-d-old whole plant without cotyledons: *lanes 4*, 5, cotyledons only. **B** Ethidium bromide-stained agarose gel representing corresponding amounts of blotted RNA

state. So far, these conclusions have mainly been reached by in-vitro assays using murine Hsp25 (Ehrnsperger et al. 1997) or pea Hsp18.1 (Lee et al. 1995) and citrate synthase as reporter. In both cases it was observed that transient formation of a complex containing chaperone and reporter protein prevented irreversible aggregation. Although, in these systems, inactivation of the reporter during stress could not be prevented, Hsp70 had a positive effect on its reactivation by aiding its release from the chaperone, as demonstrated for Hsp25 (Ehrnsperger et al. 1997). It is therefore likely that other cellular components are required for full chaperone activity of sHsps. This was demonstrated by thermal denaturation experiments in a cellular environment using firefly Luc in the presence of sHsps (Forreiter et al. 1997). Since it is still not known if the two sHsp classes have different functions in the cytoplasm during stress, we tested them for their influence on Luc denaturation in vivo. As shown in Fig. 6, both sHsp classes tested seemed to stabilize Luc activity under heat-stress conditions to a similar extent as observed in thermotolerant cells containing the whole set of endogenous Hsps. Statistical analysis supports this hypothesis. We have shown that all kinetics were definitely non-linear and fitted best to the exponential function

$$y_i = A0 + A1 \times e^{bx_i}$$

Figure 6C presents the fitted curves together with the corrected measurements estimated by the calculus of observations, and Table 1 shows the corresponding parameters. The most important function parameter is exponent b, because it represents the curvature of the functions. We could prove that the absolute value of exponent b is significantly higher ($P < 0.01$) for the control curve compared to the rest of kinetics, which means that Luc activity decreases faster in control cells. For all other kinetics, the differences between the values of exponent b were not significant ($P > 0.05$). Terms A0 and A1 did not differ significantly for control cells, thermotolerant cells and sHsp17.3 class II cells ($P > 0.05$). Only for sHsp17.7 class I cells were

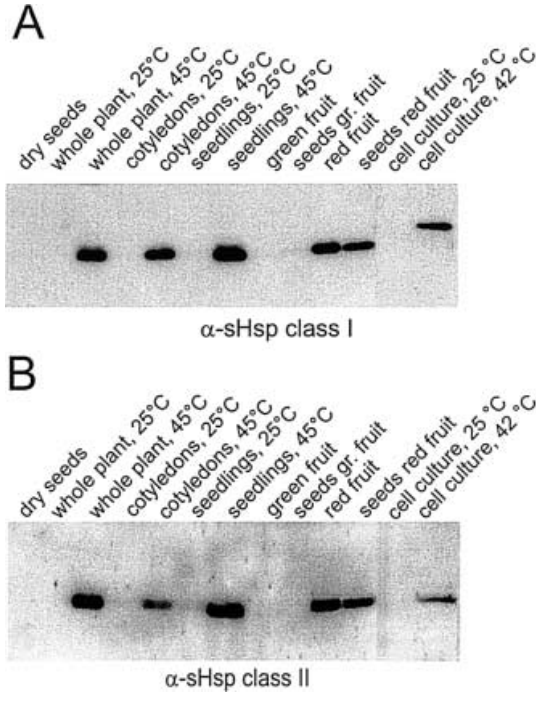

Fig. 5A,B. Western blot analysis of total protein extracts from different *L. esculentum* plant tissues under control and heat-stress conditions. Protein extracts were separated using SDS-PAGE, blotted and incubated with an antiserum against *Pisum sativum* Hsp18.1 class I **A** or an antiserum against *Arabidopsis thaliana* Hsp17.7 class II **B**. Note: *lanes 2*, 3, 21-d-old whole plant without cotyledons; *lanes 4*, *5*, cotyledons only

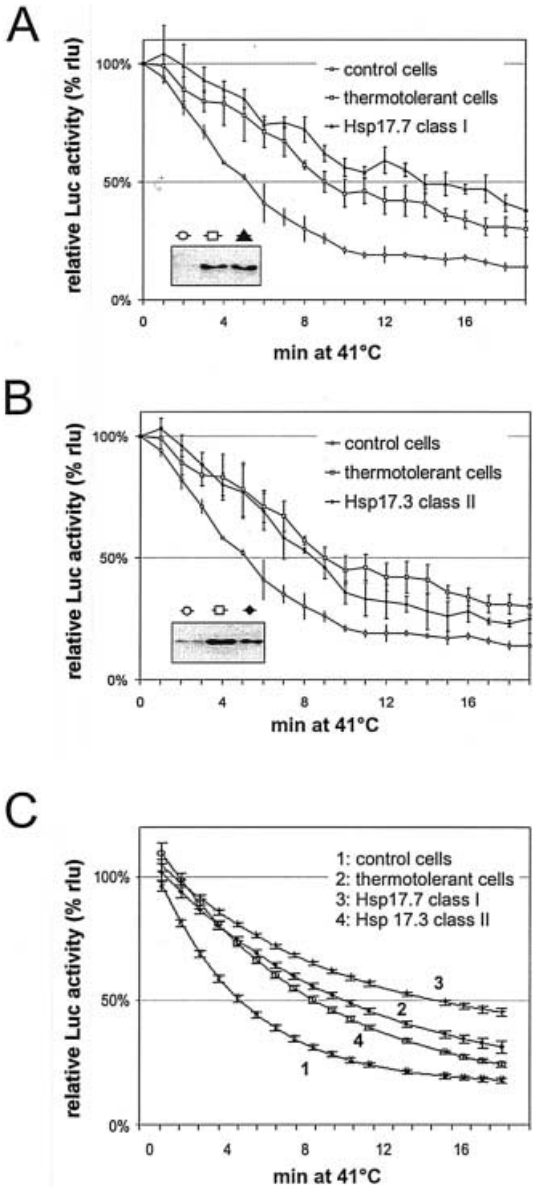

Fig. 6A,B. In-vivo protection of firefly luciferase in the presence of *L. peruvianum* class I (**A**) and class II (**B**) sHsP. Protoplasts were incubated at 41 °C for 20 min. Every minute an aliquot was removed. Luciferase activity was measured and compared to basal activity at room temperature (100%). Control protoplasts (*circles*) were kept at room temperature, thermotolerant protoplasts (*squares*) were given a 15-min heat pulse at 39 °C plus 3 h of recovery to switch on the endogenous set of chaperones. Plasmids coding for Hsp17.7 class I (*triangles*) and for Hsp17.3 class II (*rhombi*) were transiently introduced into protoplasts 16 h before denaturation. In parallel, control and thermotolerant protoplasts were mock-transformed with an empty vector. The mean values of three experiments ± SD are presented. Expression of chaperones was verified by western blot analysis. Small stress proteins were detected by using the above-mentioned class-specific antisera. *Lane 1*, proteins from control cells; *lane 2*, proteins from thermotolerant cells; *lane 3*, proteins from protoplasts transiently co-transformed with sHsp17.7 class I or sHsp17.3 class II. **C** Fitted curves and measurements after statistical evaluation. Comparison of the estimated function parameters indicate a reduced Luc inactivation for all kinetics compared to control cells, whereas no difference could be found between thermotolerant cells and cells after class II transformation. Cells after class I transformation, however, showed a significantly better thermoprotection than other cells

both parameters altered in such a way that the relative Luc activity was shifted to higher values for the time period regarded ($P < 0.01$). These findings support our view that even in vivo there is no fundamental difference in the mode of action of these two sHsp types. Interestingly, it became evident that a considerable amount of endogenous class II sHsp was already present under control conditions (Fig. 6B). This may explain the previously described faster inactivation of purified Luc compared to Luc inactivation within intact cells (Forreiter et al. 1997).

Discussion

Plants contain at least five different types of small stress proteins with representatives in mitochondria (e.g. Lenne 1995; Debel et al. 1997), plastids (Vierling et al. 1986, 1988), the endomembrane system (Helm et al. 1993) and two different classes in the cytosol (Waters et al. 1996). It is tempting to speculate that plants, as mainly sessile organisms lacking the ability to escape unfavorable environmental situations, need a more effective stress-response system than e.g. animals. From this point of view, the massive accumulation of sHsps in plants may indicate a special role for survival under stressful situations and recovery after stress. As a step towards a better understanding of this complexity we report the analysis of two different cytosolic classes of tomato sHsps.

The genomic organization and expression pattern of the different tomato sHsps presented here was in line with other observations about plant sHsps (reviewed by Waters et al. 1996). All of them are strongly induced by heat but are also expressed in fruit flesh and seeds as soon as fruits turn red. At this time, fruit cells undergo a drastic physiological rearrangement, evoking the increased presence of chaperones. The main goal of our analysis, however, was to gain a more detailed picture of the cellular function of sHsps during stress. A key to understanding sHsp function is the observation that their activity is intimately connected with other members of the Hsp-network, especially Hsp70 (Ehrnsperger et al. 1997; Forreiter et al. 1997; see review by Bukau and Horwich 1998; summarized in Fig. 7). Evidently, sHsps form oligomers which can bind partially unfolded polypeptides (Chen and Vierling 1991; Chen et al. 1994; Lee et al. 1995; Helm et al. 1997), thus preventing further aggregation. To regain native structure and activity, these proteins have to undergo refolding and release from the sHsp matrix, probably mediated by the Hsp70 chaperone system and ATP.

The existence of specific sets of chaperones in all cellular compartments involved in processing of newly formed or accidentally unfolded proteins is well established. Yet, this would not explain the appearance of two different classes of sHsps in the cytoplasm. Compared to the very high sequence conservation between members of the same class, the divergence of class I vs. class II is remarkable (see Fig. 1). However, both can form

Table 1. Means and standard deviations of the estimated parameters for function $y_i = A0 + A1\ e^{bx_i}$ and correlation coefficient of the fitted curves (r)

	A0	A1	b	r
Control cells	12.217 ± 1.583	104.413 ± 3.556	−0.210 ± 0.017	0.996
Thermotolerant cells	12.113 ± 9.144	99.332 ± 6.8	−0.096 ± 0.021	0.991
sHsp class I	31.781 ± 4.994	81.578 ± 3.731	−0.105 ± 0.016	0.988
sHsp class II	8.928 ± 4.413	114.493 ± 3.92	−0.119 ± 0.016	0.989

oligomers (Helm et al. 1997; Lee et al. 1997) with an outstanding heat stability and no tendency to aggregate even under boiling conditions and both can bind non-native proteins in vitro. For this reason, we analyzed whether class II sHsps and other sHsps are able to affect the in-vivo inactivation of Luc as previously described for a class I sHsp (Forreiter et al. 1997). Summarizing the data, it is obvious that both class I and class II sHsps alone can stabilize Luc to almost the same extent as a mixture of the endogenous chaperones expressed in thermotolerant cells.

One important finding in this context was that, regardless of the sHsp expressed, the effect of thermal protection was always in the same range as observed in thermotolerant cells, even though these cells can accumulate massive amounts of sHsps under ongoing heat stress. This has been demonstrated by comparing class I-expressing cells to thermotolerant cells (Forreiter et al. 1997). Additionally, we found that, regardless of how much sHsps were expressed in transformed cells, it never resulted in a significantly improved thermoprotection of Luc compared with thermotolerant cells. On the contrary, transformation of increased amount of plasmid DNA beyond an optimal DNA/cell ratio resulted in a reduced level of expressed sHsps and coincided with reduced thermoprotection (data not shown). One explanation for this phenomenon could be that the limiting step for preventing Luc inactivation is not the amount of sHsps but of their cellular partners in the chaperone network, such as Hsp70/Hsp40. However, even if Hsp70 or any other member of the stress-response network is limiting for the in-vivo chaperone action of sHsps, the results cannot explain why plant cells accumulate these proteins to such a tremendous excess during stress. For this reason, we propose that sHsps may have an additional function in the formation of heat stress granules (summarized in Fig. 7). This transient compartment appears in plant cells under heat stress and disintegrates upon recovery (Nover et al. 1983; Neumann et al. 1984). Heat-stress granules contain most of the cytoplasmic sHsps of both classes. Forming this compartment may reflect a mechanism to trap the mass of partially unfolded housekeeping proteins occurring in a stress situation, if other components of the chaperone network are limiting. Formation of heat-stress granules in order to store different proteins in different stages of denaturation may also explain the presence of a broad N-terminal variability within the sHsps, as this part of the protein is proposed to be involved in substrate binding (Kim et al. 1998). This would not explain, however, the presence of a second well defined and differentiated class of sHsps in the plant cytoplasm. The role of a second cytosolic class of sHsps and biological significance in this context remain unclear and require further investigation.

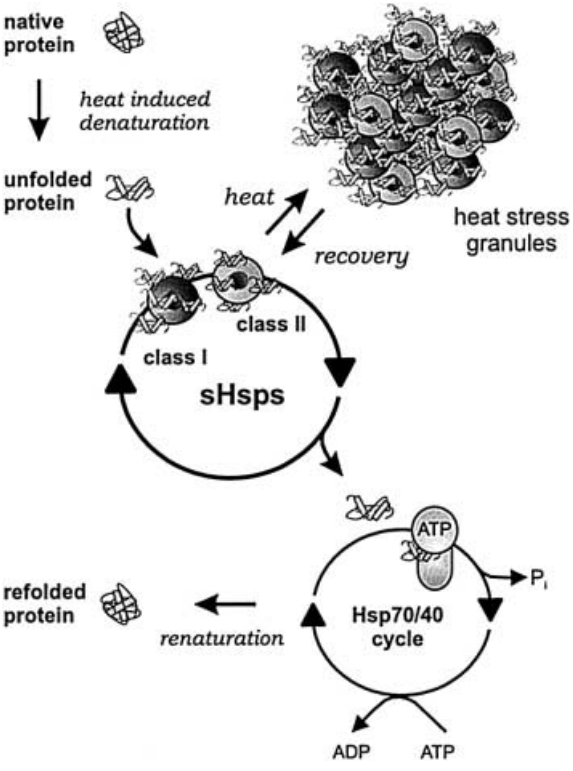

Fig. 7. Model for cytoplasmic action of sHsps in plants. After exposure of cells to elevated temperatures, unfolded proteins accumulate in the cytoplasm. These proteins are bound by both sHsp class I and class II oligomers and can subsequently interact with Hsp70/Hsp40 for renaturation. With ongoing heat stress the total of denatured proteins is above the refolding capacity of Hsp70/Hsp40 but still bound in a folding-competent state to sHsps. These complexes can be transiently stored in heat-stress granules, which consist of both types of sHsp and disintegrate during the recovery period

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