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BRIEF COMMUNICATION

Analysis of *Lupinus albus* heat-shock granule proteins in response to high temperature stress

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Abstract

An important aspect of heat-shock response of lupin (*Lupinus albus* cv. Rio Maior) is the formation of cytoplasmic granular aggregates, called heat-shock granules (HSGs). In this study, two-dimensional electrophoresis (2-DE) was used to detect the component proteins of HSG complexes formed *in vivo*. Evaluation of 2-DE revealed differential expression of several proteins under heat shock conditions when compared with control. Among them, small heat-shock proteins (sHSPs) of 15 to 30 kDa were found to be the major representative proteins along with other proteins of relative molecular mass ranging from 36 to 45 kDa and above.

Additional key words: molecular chaperone, small heat-shock proteins, thermotolerance.

Being sessile, plants are constantly exposed to various environmental stresses such as drought, salinity and high temperature (Cherian and Reddy 2003, Pinheiro *et al*. 2004, Cherian *et al*. 2006, Lin *et al.* 2008). Among them, high temperature stress causes reduction in seed yield, oil and protein content in many crop plants. Plants respond to high temperatures by repressing the synthesis of most proteins and by inducing the synthesis of a small group of specific proteins, widely known as heat-shock proteins (HSPs). Five major families of HSPs have been reported (Buchner 1996). Of these, small heat-shock proteins (sHSPs) are ubiquitous in all types of organisms, and under stress like heat, their expression is highly up-regulated (Horwitz 2000, Laksanalamai *et al*. 2001, Narberhaus 2002, Usui *et al*. 2004). However, in contrast to other eukaryotes, higher plants synthesize predominantly heat-shock proteins with masses of approximately 15 to 30 kDa (Vierling 1991, Waters *et al*. 1996, Sun *et al*. 2002). sHSPs are reported to be associated with the cytosolic formation of heat-shock/heat-stress granules (HSGs) and are evidently part of a complex structure including a defined subset of total mRNA population (Nover *et al*. 1989). Analogous structures in mammalian cells were termed as stress granules (Anderson and Kedersha 2006). In plants, although sHSPs are the core component of HSGs, their individual function and identity were not completely understood (Nover *et al*. 1989). Considerable variation exists in HSP production among different plant species, and even among individuals of the same species (Araujo *et al*. 2003, Caeiro *et al*. 2008).

Lupins are an important class of leguminous crops for both animal and human nutrition because of their high seed protein and oil content (Pinheiro *et al*. 2004, 2005). The study of changes in gene activation, transcription, translation and expression induced by high temperature may help to understand the ability of lupin plants to tolerate changes in environmental conditions that occur

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Abbreviations: 2-DE - two-dimensional electrophoresis; EM - electron microscopy; HSG - heat-shock granule; IEF - isoelectric-focusing; IPG - immobilized pH gradient; MS - mass spectrometry; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; SG - stress granule; sHSP - small heat-shock protein.
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during the growing seasons. Many of the reported studies on heat-shock protein structure and function were investigated through *in vitro* cultured cells (Nover and Scharf 1984, Smykal *et al*. 2000*b*). In this paper we investigate the *in vivo* formation of HSGs and their component proteins in *L. albus*.

Lupinus albus L. (cv. Rio Maior) seeds were pre-germinated in distilled water for 48 h. The germinated seedlings were then transferred to sterile test tubes (one per tube) containing 15 cm^3 of full strength nutrient solution (Ferreira and Davies 1987). The seedlings were kept on sterile filter paper wicks inside the test tubes, so that only the roots were in contact with nutrient solution. The nutrient solution was replaced every day by adding fresh solution into the test tubes in a laminar flow hood. The seedlings were grown under irradiance of 340 - 400 μmol m^{-2} s^{-1} (PAR), 12-h photoperiod, night/day temperature of 20/25 °C, and relative humidity of 60 - 70 %. Heat shock treatment was given to 7-d-old seedlings by gradual increment in temperature from 25 to 42 °C over 3 h in a water bath. The seedlings received an additional heat-shock treatment for 2 h at constant temperature of 42 °C. The control set of plants did not receive the heat-shock treatment. At the end of heat-shock treatment, the seedlings were harvested and separated into leaf, shoot and root tissues for storage at -70 °C until further use. The HSGs extracted from shoot tissue are investigated in this paper.

The method of Smykal *et al*. (2000a) with some modifications was followed for the isolation and purification of HSG complexes. The shoot tissues stored at -70 °C were homogenized in twice its volume of ice-cold buffer (50 mM Tris-HCl, pH 7.8, 25 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 % (v/v) *Triton X-100*, 150 mM sucrose, 20 % (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 14 mM 2-mercaptoethanol) using a mortar and pestle. The homogenate was filtered through 4 layers of cheesecloth and nuclei and cell debris were removed by centrifugation at 12 100 *g* for 10 min. To dissociate polysomes, the supernatant was adjusted to 250 mM KCl and 30 mM EDTA, sonicated for 10 s, and centrifuged at 27 200 *g* for 10 min. Then, 15 cm^3 of the resulting supernatant was loaded on top of two cushions of (8 cm^3 each) 50 mM Tris-HCl, pH 7.8 buffer. The upper cushion was without $MgCl_2$, but containing 250 mM KCl, 0.5 % (v/v) *Triton X-100* and 250 mM sucrose, and the lower cushion without $CaCl_2$, but with 350 mM sucrose. HSGs were sedimented by 60 min of centrifugation at 61 500 *g* at 4 to 8 °C *(Beckman* ultracentrifuge with swinging bucket rotor *SW28*). The resulting pellet, containing crude high molecular-mass complexes, were dissolved in resuspension buffer (50 mM Tris-HCl, pH 7.8, 500 mM NaCl, 25 mM KCl, 30 mM EDTA, 0.5 % (v/v) *Triton X-100*, 15 % (v/v) glycerol, 5 % (v/v) sucrose), layered over 50 mM Tris-HCl, pH 7.8 buffer with 500 mM NaCl, 25 mM KCl, 1 mM EDTA, 0.5 % (v/v) *Triton X-100*, 25 % (v/v) glycerol, 15 % (v/v) sucrose and the ultracentrifugation steps repeated. For structural analysis, the HSG samples were negatively stained in 2 % (m/v) uranyl acetate on carbon support grids and viewed at 40 000× magnification (*JEM 100C*, *JEOL* electron microscope, Japan).

Protein contents in the HSG pellets were determined according to Bradford (1976), as modified by Ramagli (1999). For isoelectric focusing (IEF) electrophoresis, the *IPGphor* system was used with nonlinear immobilized (*NL-IPGs*, *Amersham Biosciences*, Sweden) pH gradient strips with a pH range of 3 - 10. Proteins (25 μg each sample) were solubilized in 8 M urea, 2 % (m/v) CHAPS, 0.04 M DTT and 0.5 % (v/v) IPG buffer. IEF was carried out at 30 V for 12 h, followed by 200 V for 1 h, 500 V for 1.5 h, 1000 V for 1.5 h, and 8000 V for 6.5 h, at 20 °C. Prior to SDS-PAGE the IPG strips were equilibrated for 2× 15 min in a buffer solution containing 0.05 M Tris-HCl pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % (m/v) SDS and a trace of bromophenol blue as tracking dye. DTT at 1 % (m/v) was added to the first equilibration step and 4 % (m/v) iodoacetamide to the second one. SDS-PAGE (15 % (m/v) acrylamide) was performed on slab gels (Laemmli 1970). 2-DE gels were stained with silver stain as described by Blum *et al*. (1987) and scanned using the *ImageQuant v3.3* densitometer (*Molecular Dynamics,* USA). After imaging, total number of heat shock proteins expressed were analyzed by spot detection and the difference in protein abundance among the different samples (control and heat-shocked) were compared. At least triplicate samples from three independent extractions were run.

This work demonstrates the *in vivo* formation of cytoplasmic granular aggregates or complexes, termed as HSGs, with several distinct proteins as major structural components in *L. albus,* under high temperature stress. The EM studies on isolated granules showed that they are globular to irregular in shape and ranged between 20 to 60 nm in size (Fig. 1). This is in good agreement with other published data on HSGs and can be attributed to different bound proteins and the oligomeric nature of sHSPs (Nover *et al*. 1989, Groenen *et al*. 1994, Jinn *et al*. 1995, Lavoie *et al*. 1995, Smykal *et al*. 2000*a*). The purity of isolated HSG complexes relied on the stability of the HSG complex in high salt buffers (500 mM NaCl and 30 to 50 mM EDTA). The presence of high salt concentrations coupled with repeated sucrose cushion ultracentrifugation steps in the isolation of HSG complexes limited the possibility of ribosomal/polysomal contamination. The use of high salt buffers and repeated ultracentrifugation steps were reported in other studies (Mansfield and Key 1988, Nover *et al*. 1989).

The 2-DE analysis of HSG proteins showed different profiles under heat shock or control (non-stress) conditions (Fig. 2). The heat shock treatment induced *de novo* synthesis of several proteins that were either absent or present in much lower amounts in control HSG fraction (Fig. 2). Three to four hundred protein spots were detected for heat shock conditions. A unique set of newly expressed protein spots with molecular masses

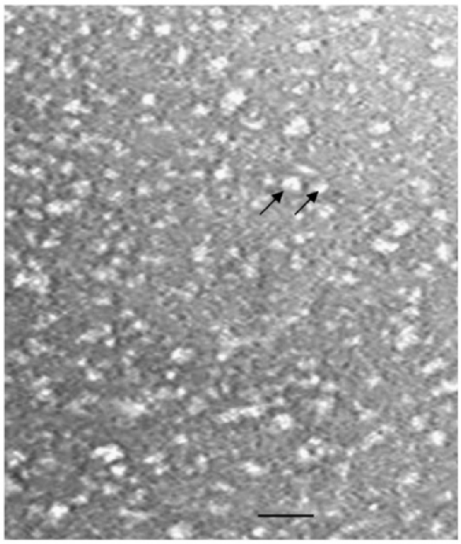

Fig. 1. Electron microscopic view of heat-shock granules from *Lupinus albus*. *Bar scale* 40 nm. *Arrows* indicate individual heat-shock granules.

15 to 20 kDa were detected in heat shock when compared to control (Fig. 2). Several protein spots of high molecular masses were also part of the HSGs under heat shock treatment. Some protein spots in the acidic range present in the control gel were absent under heat shock conditions (Fig. 2). This could be a part of the house keeping proteins which undergo rapid translation under heat shock conditions. However, in both gels (control and stressed) the protein abundance was higher in the pH range 4 to 8 and lower at extreme pH values of 3 and 10.

Our results, along with other reported studies, suggest that HSPs are also in control cells but their synthesis increases under heat-shock conditions (Nover and Scharf 1984, Smykal *et al*. 2000*a*). In plants, heat shock response is a ubiquitous response resulting in altered gene expression and protein translation (Sule *et al*. 2004). It is possible that some of the new protein spots detected under heat shock conditions in *L. albus* HSG may result from modification or phosphorylation of previously existing proteins, particularly HSP of 70, 66 and 40 kDa. Under stress conditions, the amount of inducible HSP70 increases in the cytosol and it could bind to

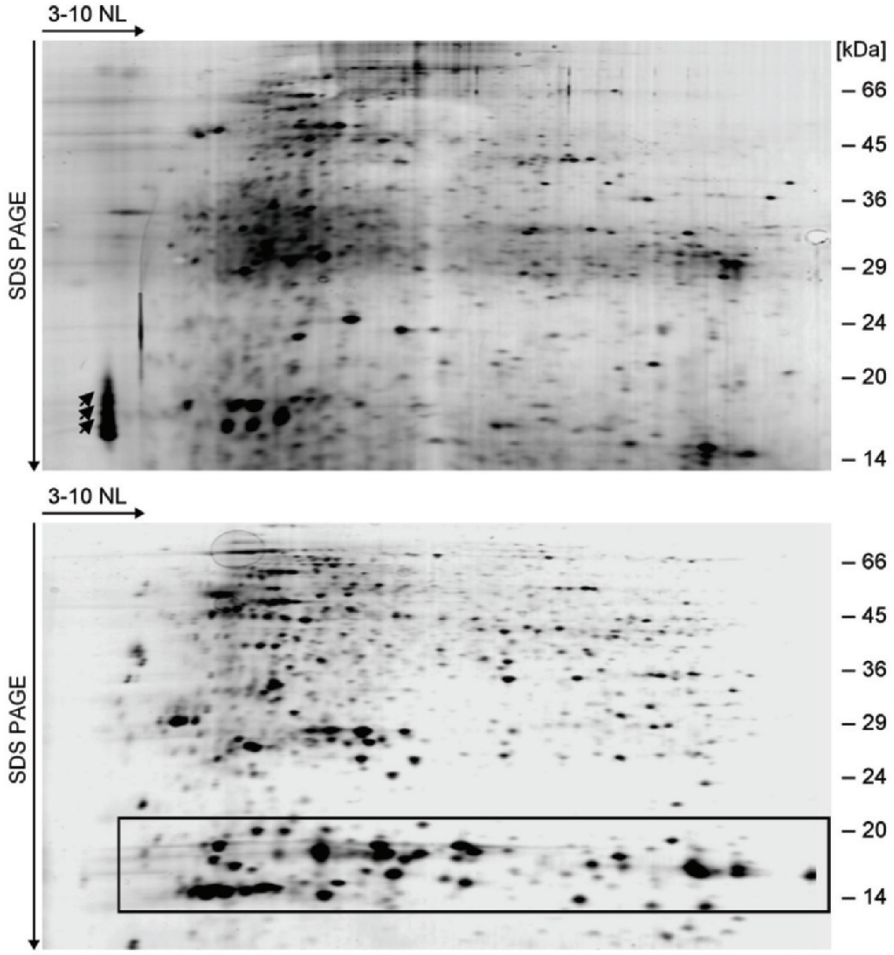

Fig. 2. Silver stained 2-DE maps of heat-shock granule proteins from the shoot tissue of *Lupinus albus* under control (*upper*) and heat-shock (*lower*) conditions. *Arrows* represent the acidic protein spots present only in control tissue. The *boxed area* represents the unique set of protein spots expressed under heat shock condition.

unfolded proteins or protect others from denaturation. It may be noted that even under normal growth conditions, the binding of HSP70 occurs mediating the folding of nascent polypeptides emerging from polysomes (Frydman *et al*. 1994). Smykal *et al*. (2000*a*) assumed that some of the HSG proteins (HSP70, HSP40) in association with HSP20 may act as molecular chaperones and help to prevent thermal aggregation and may enhance and facilitate the refolding of partially denatured proteins during heat shock. *In vitro* experiments suggest that sHSPs prevent thermal denaturation and aggregation of bound proteins (Buchner 1996, Collada *et al*. 1997). Nover and Scharf (1984) suggested that under heat shock the synthesis of proteins either continued or discontinued and started again once the stress is removed. They concluded that reversible segregation of distinct mRNA species from the translation apparatus contributed to the heat-shock specific pattern of protein synthesis in plants. In another study, Nover *et al*. (1989) proposed a model for explaining the putative role of HSGs in plant systems. Their model explains the storage of a specific subset of total cellular mRNAs into the HSG complex. They represent the untranslated and transiently stored mRNAs coding for the bulk of control proteins, where the heat-shock mRNAs are segregated in the polysomal fraction. However, recent studies have revealed that the corresponding stress granules (SGs) in mammalian cells contain several RNA binding proteins that rapidly shuttle in and out of SGs and regulate mRNA translation and decay (Anderson and Kedersha 2006, Hoyle *et al*. 2007). Therefore, it would be interesting to test whether plant HSGs could complement these results. However, at present it seems that HSGs may function in the storage of control mRNAs during high temperature conditions, which will be translated later during recovery period and in this way plants cope with frequent temperature alterations experienced especially during hot summer days. Further proteomic analysis involving 2-DE coupled with mass spectrometry (MS) could reveal the distinct isoforms of the HSG proteins in lupin plants and such an in-depth analysis will be necessary, in order to understand their role in heat tolerance.

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