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Origin and Localization of Chloroplast Associated Heat Shock Proteins in *Vigna sinensis*

S KRISHNASAMY, R MANNAR MANNAN, M KRISHNAN and A GNANAM
Department of Plant Sciences, School of Biological Sciences
Madurai Kamaraj University, Madurai-625 021

The leaves of *Vigna sinensis* subjected to heat shock treatment at 40°C, showed induction of synthesis of a set of heat shock proteins (HSPs) with the molecular weight of 96, 80, 75, 22, 19, 17 and 15 kD. Four of these newly synthesized HSPs were detectable in the intact chloroplast preparations obtained from the heat shocked leaves—localized on the thylakoid membranes. These chloroplast-associated HSPs are coded by nuclear genome and the induction of their synthesis at the elevated temperatures is transcriptionally regulated.

Key Words : Heat shock proteins, Chloroplast, Thylakoid membranes, *Vigna sinensis*

Introduction

Organisms ranging from prokaryotes including photosynthetic cyanobacteria to man respond to high temperature by repressing the synthesis of most of the normally occurring proteins and inducing a new set of proteins called heat shock proteins (HSPs) (Schlesinger et al. 1982, Mannan et al. 1986). The apparent evolutionary conservation of the induction and the synthesis of HSPs at elevated temperature across the biological world is an indication for an important fundamental role for these HSPs (Tanguay 1983).

Eventhough the thermo-protective role of these HSPs are known in some organisms (Mitchell et al. 1979, Loomis & Wheeler 1982, Lin et al. 1984), the precise function of HSPs is not yet understood. Tanguay (1982) has shown that the molecular mechanism involved in the induction of thermotolerance in *Drosophila* is by the appearance of significant amount of HSPs inside the subcellular compartments. This intracellular localization of HSPs is likely to result in the non-specific lowering of the rate of irreversible loss of vital functions which were until then provided by some stress labile proteins.

The inhibition of photosynthesis in plants at high temperatures is considered to be a result of the damage within the chloroplast (Bjorkman 1975). Increase in temperature to supraoptimal levels damages the integrity of the thylakoid membranes leading to the inhibition of photosynthesis.

The present investigation attempts at understanding the site of synthesis and the intraorganellar localization of the chloroplast associated HSPs in *Vigna sinensis*.

Materials and Methods

Plant Species and Growth Condition

Vigna sinensis seedlings were grown at 25°C in a temperature-controlled growth chamber provided with illumination at a photon flux density of 45 $\mu E/m^2/sec$ with a 12 hr dark/light cycle.

In vivo *Labelling of Leaves with* ^{14}C *Amino acids*

About 1 g of leaves were cut into small bits, transferred to a 25 ml conical flask containing 10 ml of sterile water and kept in a thermostated waterbath shaker at the specified temperature. Illumination was provided at a photon flux density of 45 $\mu E/m^2/sec$. The leaf segments were preincubated for 30 min at specified temperature and then 150 μ Ci of ^{14}C-*Chlorella* protein hydrolysate (specific activity: 26 mCi/m atom of C) was added. The labelling was allowed to continue for 2 hr. In the case of studies involving site specific inhibitors, the respective inhibitors (150 μg/ml of chloramphenicol, 10 μg/ml cycloheximide and 50 μg/ml of actinomycin D) were added 30 min prior to the incubation of leaves at specified temperature. After the labelling was over the leaf segments were collected and homogenized.

Chloroplast isolation was done following the method of Fish and Jagendorf (1982) with slight modification. The leaf segments were homogenized using 20 ml of the isolation medium containing 50 mM Tris-HCl (*p*H 8.3), 350 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. The homogenate was filtered through four layers of cheese cloth and centrifuged at 3000 × g for 1 min. The chloroplast pellet was resuspended in a minimal volume of the grinding buffer and overlayered onto a 9 ml linear 25% to 92% percoll gradient containing the same ingradients as the grinding buffer. The gradient tubes were centrifuged at 5000 × g for 7 min and the lower green band of intact chloroplasts was collected and resuspended in a small volume of grinding medium.

Sub-fractionation of the Organelle

The intact chloroplast preparation obtained from the leaves labelled with ^{14}C-amino acid was fractionated into stroma, thylakoid membrane and envelope membrane following the method of Mendiola-Morgenthaler and Morgenthaler (1974).

SDS-Polyacrylamide Gel Electrophoresis and Fluorography

Total leaf homogenate, chloroplasts and sub-fractions of chloroplasts were precipitated with 10% trichloroacetic acid at 4°C for 30 min. The trichloroacetic acid precipitate was washed thrice with cold diethyl-ether dissolved in a small volume of 10% SDS and subsequently used for SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) with slight modifications. The gel contained a linear 7.5 to 15% acrylamide concentration gradient with 5 to 10% glycerol co-gradient. The stacking gel contained 4% acrylamide. Electrophoresis was carried out at 20°C for 10 hr at 17 mA (with initial 1 hr at 5 mA) and it was stopped after the marker dye reached the bottom of the gel. Fluorography was carried out as described by Lasky and Mills (1975) and a screen type X-ray film was exposed at −70°C for appropriate time. The gel was calibrated using the following molecular weight standards: phosphorylase A, 97000; bovine serum albumin, 66000; egg albumin, 44000; carbonic anhydrase, 29000; β lactoglobulin, 18,400; lysozyme, 14,300.

Results

Effect of Heat Shock on Protein Synthesis in Leaf Segments

Incorporation of ^{14}C-amino acids into trichloroacetic acid precipitate proteins by the leaves of *V. sinensis* incubated at different temperatures resulted in a linear increase up to 30°C (figure 1). For the fluorographic analysis, the leaves were incubated and labelled at 25°C and 40°C, the former being the control temperature and the latter the heat shock temperature.

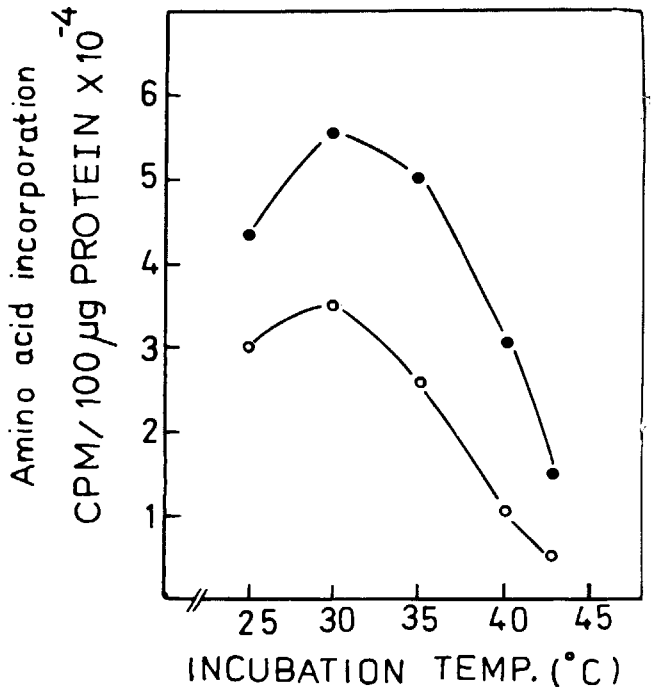

Figure 1 Effect of incubation temperature on protein synthesis by *Vigna sinensis* leaf segments.(^{14}C)-amino acid incorporation into (**a**) total leaf homogenate (closed circles) and *(b)* chloroplast fraction (open circles)

At the incubation temperature of 40°C, most of the normally occurring polypeptides were not synthesized and only a set of seven HSPs (96, 80, 75, 22, 19, 17 and 15 kD) were detectable (figure 2, A&B).

None of the higher molecular weight HSPs (96, 80 and 75 kD) could, however, be detected in the fluorographic profile of the chloroplast fraction isolated from the same sample (figure 2, F & G).

Effect of Site specific Protein Synthesis Inhibitors on the Sythesis of HSPs

Synthesis of both higher and lower molecular weights HSPs was found to be sensitive to cycloheximide, a cytoplasmic translational inhibitor (figure 2 D & I) and actinomycin D, a transcriptional inhibitor (figure 2, E & J) but not to chlorophenicol, a chloroplast specific translational inhibitor (figure 2, C & H).

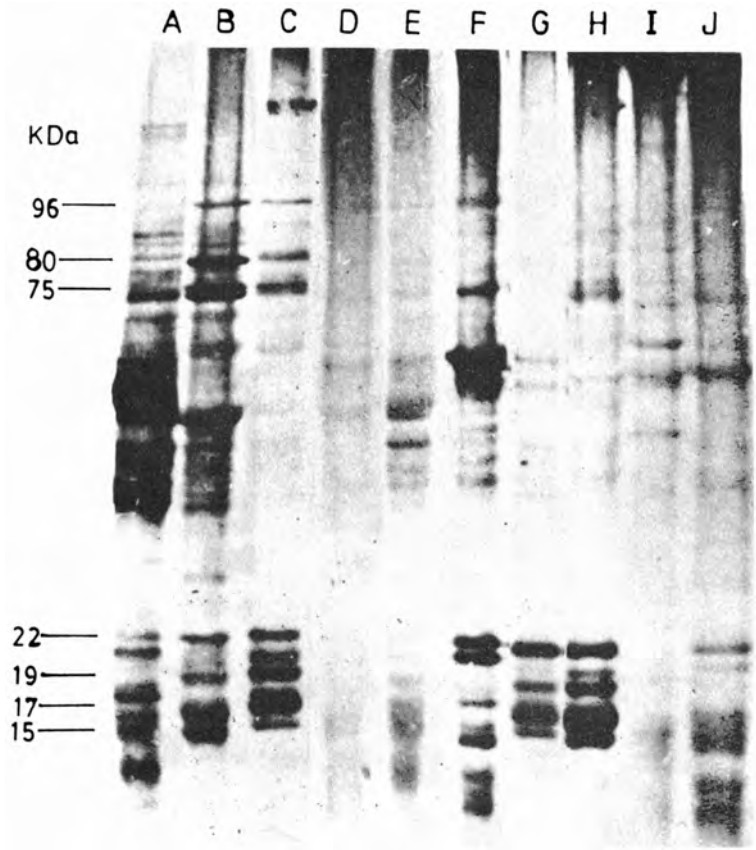

Figure 2 A–J Fluorographic profile of the total leaf homogenate and chloroplast fractions of *V. sinensis* leaf segments labelled with ^{14}C-amino acids at 25°C and 40°C in the presence and absence of various site specific protein synthesis inhibitors. A & B: Total leaf homogenate at 25°C (**A**) and 40°C (**B**); total leaf homogenate from chloramphenicol treated (**C**); cycloheximide treated (**D**) and actinomycin D treated (**E**) leaf segments **F–J** chloroplast fraction: 25°C (**F**); 40°C (**G**); 40°C–chloramphenicol treated (**H**); 40°C-cycloheximide treated (**I**); 40°C-actinomycin D treated (**J**)

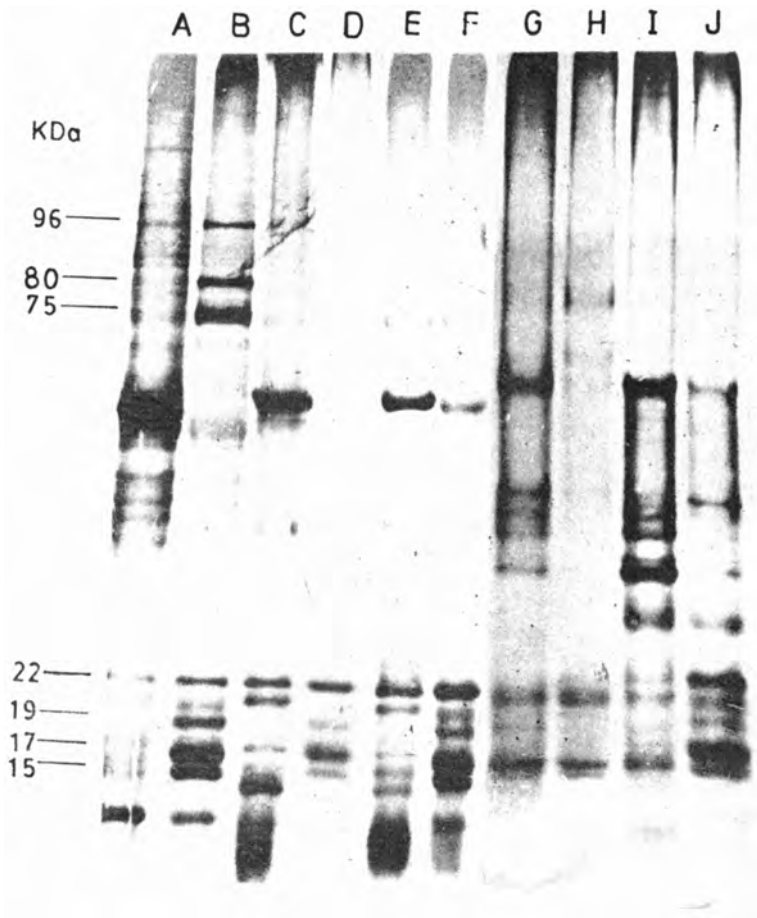

Figure 3 **A–J** Fluorographic profile of the total leaf homogenate, chloroplast and subchloroplast fractions of the *V. sinensis* leaf segments labelled with ^{14}C-amino acids at 25°C and 40°C. **A & B** total leaf homogenate at 25°C (**A**) and 40°C (**B**). **C & D**, chloroplast fraction at 25°C(**C**) and 40°C (**D**). **E & F** thylakoid membranes at 25°C (**E**) and 40°C (**F**); **G & H**, stromal fraction at 25°C (**G**) and 40°C (**H**). **I & J**, envelope membranes at 25°C (**I**) and 40°C (**J**)

Intra-organelle localization of HSPs

In order to find out the intra-organelle localization of the lower molecular weight HSPs detectable in the chloroplast fractions isolated from heat shocked leaves, the chloroplasts were further fractionated into thylakoid, stroma and envelope fractions and their fluorographic profiles analyzed (figure 3). The lower molecular weight HSPs detectable in the total chloroplast fraction were found associated mainly with the thylakoid membranes and to some extent with the envelope fractions and were totally absent in the stromal fraction.

Discussion

The results clearly demonstrate that the leaves of *V. sinensis* have got a definite heat shock response with the induction of synthesis of a set of HSPs by an abrupt increase in the incubation temperature.

The constitutive synthesis of 75 kD HSP is highly significant as the HSPs are reported to be responsible for the induction of thermotolerance (Lin et al. 1984). Under the field conditions, the leaves are most likely to experience brief periods of temperature increase during the course of the day and therefore, the maintenance of threshold levels of HSPs might provide sufficient protection with an increase in temperature for a brief period.

The fact that the synthesis of HSPs was inhibited by cycloheximide and not by chloramphenicol suggests that the HSPs are likely to be coded by the nuclear genome and synthesized on the 80S cytoplasmic ribosomes. The observation that the treatment with actinomycin D, a transcriptional inhibitor inhibits the synthesis of HSPs indicates that the induction of synthesis of HSPs in *V. sinensis* at the elevated temperatures is regulated at the level of transcription.

In a photosynthetic tissue, chloroplast is the most important organelle and its functioning is very much affected at high temperatures due to the damage to membrane components (Berry & Bjorkman 1980). The ability to repair such a damage is of obvious importance to the survival of the plant at the elevated temperatures and the molecular mechanism for this adoptation to high temperature stress is yet to be elucidated.

The present observation on the localization of the HSPs within the chloroplast is of great value to propose that these HSPs could play a role in the induction of thermotolerance as suggested by Lin et al. (1984). The observation that the HSPs detectable inside the photosynthetic apparatus are getting localized on the thylakoid membranes is an additional evidence to suggest a functional role for the HSPs since of all the components of the photosynthetic apparatus, the thylakoid membranes are most susceptible to heat stress.

Kloppstech et al. (1985) have reported that in the heat shocked pea leaves and *Chlamydomonas* cells only one HSP (22 kD) is getting localized into the chloroplast. It is not clear at this time whether the four lower molecular weight HSPs associated with the chloroplast fraction of the heat shocked *V. sinensis* leaf segments are four different polypeptides or all of them are derived from a single polypeptide by a mild proteolysis. It could be resolved by doing cell free translation using Poly A RNA from the heat shocked leaves and *in vitro* uptake of the products by the isolated chloroplast as reported by Vierling et al. (1986) in the case of pea, soybean and corn.

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