HEAT SHOCK GENE EXPRESSION DURING REGENERATION IN SUNFLOWER (*Helianthus annuus* L.)

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SUMMARY

Environmental factors such as light, water and temperature, play a significant role in development of plants. Probably they interfere with the ontogenetic programme which leads to the mature multicellular organism. In fact plants, like all organisms analyzed so far, respond to temperature increase by inducing the expression of a specific set of genes, the heat shock genes, which code for the heat shock proteins (HSPs). Induction of HSPs, in response to high temperature stress, has been demonstrated in most transcriptionally active tissues and, with the exception of pollen, seems to occur at all stages of development.

The possibility that many plant species can be regenerated from cell culture in a process called somatic embryogenesis offers new tools for investigating the molecular mechanisms underlying the execution of the ontogenic programme. In particular we have investigated the interaction between gene and environment by studying the changes in the expression of hsp genes during somatic embryogenesis in sunflower.

After 3 h at 40°C many HSPs are induced, most of them are common to the three cell types analyzed: undifferentiated calli, differentiated calli, regenerated plantlets. Two groups of HSPs are synthetized, of low molecular weight (from 17 to 35 kDa) and high molecular weight (from 42 to 100 kDa). Between three stages some small differences in HSPs were detected, but they not seem indicative of any development-specific regulation.

INTRODUCTION

Plants have a very precise and orderly ontogenic programme that leads to a mature multicellular organism; the final result of these programme is also definitely influenced by other factors. Environmental factors such as light, water and temperature in particular play a significant role in plant development.

Like all the organisms analyzed so far, plants respond to heat exposure by inducing the transcription and subsequently the translation of a specific set of genes, the heat shock genes (hsp) (Key et al., 1985). The translational product of these genes, the heat shock proteins (HSPs), can be induced also in response to certain other stresses. Physiological

and genetic data indicate that the production of HSPs during stress is essential for cell survival or for recovery from the stress. There are four major classes of plant HSPs designated by their approximate molecular weight in kDa as HSP90, HSP70, HSP60 and the complex family of the low molecular weight HSPs (LMW) (15-30 kDa). These latter being the most abundant HSPs induced by heat stress (Vierling, 1991). It has been demonstrated that the induction of HSP synthesis, in response to high temperature stress, occurs in most of the tissues that are transcriptionally active. The pollen of some graminaceae could represent an exception to these generalizations. The trascription of HSP mRNAs and synthesis of HSPs during development has been studied in a number of plant species in the absence of heat stress (Nover, 1991). The results obtained clearly indicate a tissue and developmental specificity in expression of certain members of the HSP70 family and in some of the LMW-HSPs. Proteins belonging to the HSP70, HSP60 and LMW classes are localized in different cellular compartments, including mitochondria and chloroplasts.

Furthermore, evidence has been obtained, that during heat treatment, LMW-HSPs synthetized in the cytosol are imported within the chloroplasts (Vierling et al., 1988).

Plant cells are totipotent, and some species can be regenerated "in vitro" from single cells or from different tissues, to yield mature and fertile plants. The "in vitro" regeneration technique has been utilized to study the gene expression during development.

We have obtained primary callus cultures from immature embryos of sunflower, *Helianthus annuus*, L. cv Gloriasol and induced them to differentiate, to obtain plant regeneration. In these plant materials we have analyzed heat shock gene expression during the "in vitro" development of sunflower, to observe whether any variations in HSPs wich could correlate with the developmental stage.

MATHERIALS AND METHODS

Plant material : *Helianthus annuus* cv Gloriasol seeds were a kind gift of ISEA Selected seed, Falconara, Italy.

Induction of callus culture and plant regeneration: seeds were surface sterilized and placed aseptically on Murashige and Skoog medium for callus induction. The medium was adjusted to pH 6 and supplemented with sucrose (30 g/l), Fitagel (2 g/l), indole acetic acid (IAA 2 g/l), 2.4 dichlorophenoxy acetic acid (2.4-D 0.4 g/l) and kinetin (0.2 g/l). The cultures were incubated at 26°C in the light (1500 lux light intensity, 18 hours photoperiod). Calli were induced to morphogenesis placing them onto the same culture medium without 2.4-D. The somatic embryos were isolated and placed on fresh medium for further development into plantlets. Plantlets of about 1 cm were transferred to MS medium without hormons, to complete development.

Stress conditions, protein extraction and electrophoresis: samples of plants at different developmental stages (700-800 mg fresh weight) were incubated in liquid MS medium for 24 hours at 26°C before being transferred to the control (26°C) and heat shock (40°C) temperatures for a 2 hours incorporation of labelled methionine; total protein extraction, electrophoresis and fluorography were performed as already described by Marmiroli et al. (1989).

Isolation of mitochondria: sunflower seedlings (2-3 days old) were "in vivo" labelled in 1% sucrose; 10 mM K-phosphate, pH 6.5. The samples were incubated for 1 h at the control or heat shock temperature and labelled by adding 400 μCi of ^{35}S-methionine (spec. act. 1000 Ci/mmol) and incubated for further two hours.

Isolation of plant mitochondria was performed according to Wilson and Chourey (1984). Mitochondria were dissolved in SDS buffer, proteins were separated on 10% polyacrylamide gel.

Isolation of chloroplasts: sunflower chloroplasts were isolated from 10-12 days old seedling leaves. "In vivo" labelling was done on sunflower seedlings excised above the cotyledons and incubated for 3 h with 150 μCi of ^{35}S-methionine.

Intact chloroplasts were isolated and purified through a 40% Percoll cushion according to Restivo et al. (1986). The chloroplasts were then lysed in hypotonic buffer containing 0.24 M NaCl and the protease inhibitors: 1mM phenyl-methyl sulphonyl fluoride, 1 mM benzamidine, 5 mM ε-amino-N-caproic acid. Membranes were removed, the supernatant contained soluble proteins predominantly from the chloroplast stroma. Total soluble proteins were concentrated by TCA precipitation. Soluble proteins and membrane pellets were resuspended in 60 mM Tris-HCl, pH 8.5; 60 mM dithiothreitol, 2% SDS, 15% glycerol, 5 mM ε-amino-N-caproic acid, 1 mM benzamidine (sample buffer) and heated for 1 min at 100°C before loading onto the polyacrylamide gel.

RESULTS

As schematized in Fig. 1, two hours at 40°C is sufficient to induce the synthesis of a discrete number of polypeptides of both high and low molecular weight. Some very high molecular weight proteins (250 - 120 kDa) are induced during development. In undifferentiated calli three of these proteins 250, 190, 120 kDa (Fig. 1, A) are induced, in differentiating calli the HSP of 200 kDa is induced and in plantlets the HSPs of 200 and 120 kDa are present (Fig. 1, B and C). The number of low molecular weight hsp species induced in differentiating calli and in plantlets (Fig.1, B and C) is higher then in undifferentiated calli.

Heat treatment also induces the synthesis of some HSPs associated with chloroplasts and mitochondria. Fig. 2 draws a profile of the HSPs induced in chloroplasts; the polypeptides are localized in the soluble protein fraction (70, 60, 47, 33, 31, 27, 21

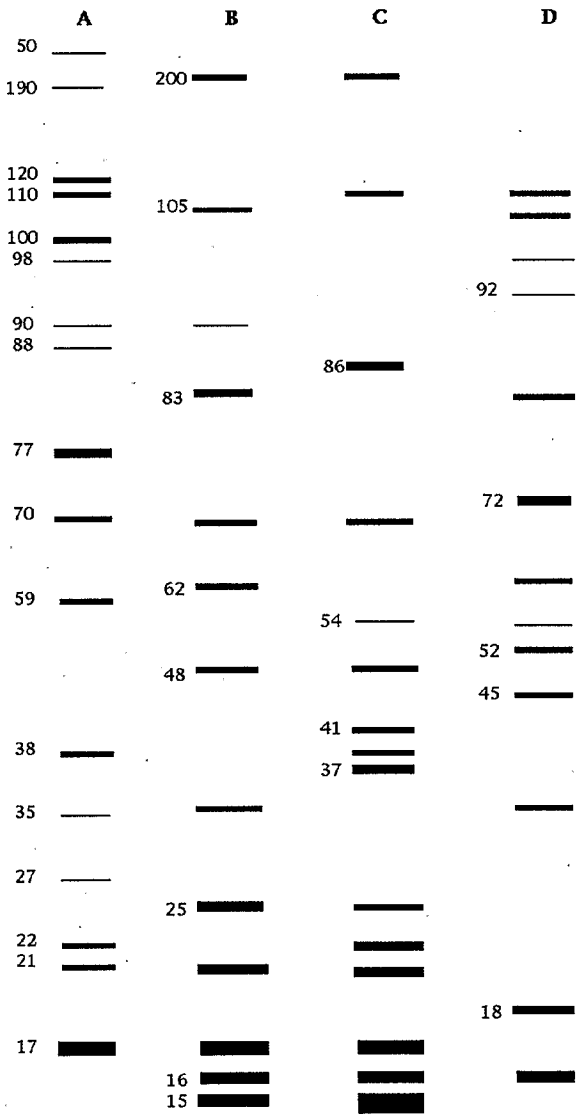

FIG. 1 Outline of the heat shock proteins induced in (A) undifferentiated calli, (B) differentiating calli, (C) regenerated plantlets and (D) seedlings, after 2 hours at 40°C. The apparent molecular weight of each stress protein is indicated.

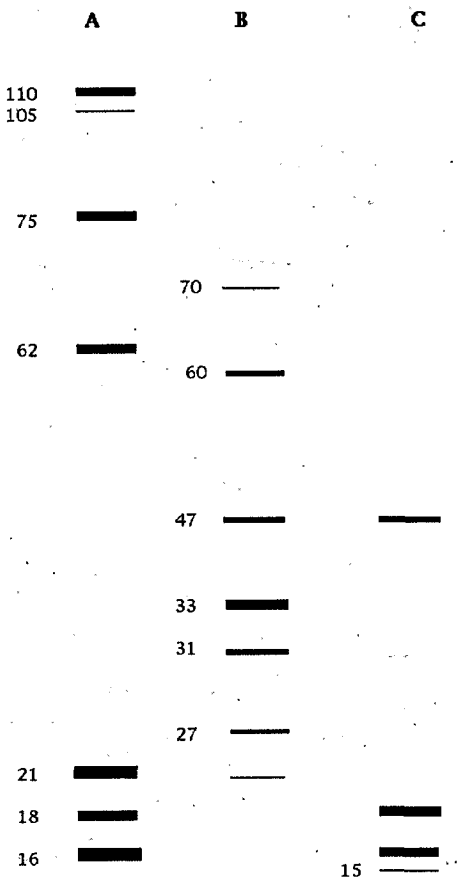

FIG. 2 Outline of the heat shock proteins localized in mitochondria (A), chloroplast stroma (B) and membranes (C).

kDa) or are associated with the thylakoid membranes (62, 47, 19, 16, 15 kDa) as shown in Fig. 2, B and C.

In mitochondria heat shock induces the synthesis of high molecular weight (110, 105 kDa) HSPs, as well as "chaperonins" (75 and 62 kDa) and low molecular weight HSPs (21, 18, 16 kDa (Fig. 2A).

DISCUSSION

The expression of genes during development has been studied in a number of plants (Key et al., 1985). As already mentioned there is clearly tissue and developmental specificity in the expression of certain HSP genes in plants. The results in this study clearly show that in sunflower the most prominent classes of HSPs are induced at all the three developmental stages analyzed. The polypeptides induced belong to the class of high molecular weight HSPs (from 42 to 100 kDa and above) as well as to the class of low molecular weight HSPs (from 17 to 35 kDa). Although none of these polypeptides can be associated with a specific developmental stage, the very high molecular weight proteins (200, 190, 120 kDa) induced at high temperature, seem to be present only in the "in vitro" system.

The electrophoretic analysis indicates that the major classes of HSPs synthetized by young seedlings of sunflower are fairly homologous to HSPs of other plant species (Nover L. et al., 1991). Some of these HSPs are associated with chloroplasts and mitochondria. Most of the HSPs found in chloroplasts are nucleus-encoded proteins imported into the organelles (Key et al., 1985). The heat stress inhibits the chloroplasts' function and the synthesis of the two subunits of chloroplast ribulose biphosphate carboxylase-oxygenase (Vierling and Key, 1985). This physiological alteration may affect the expression of the genetic potential of the plants by negatively influencing the chloroplasts photosynthetic function. The existence of HSP localized within the chloroplasts may therefore answer to the physiological need for thermoprotecting the organelles.

The HSPs associated with mitochondria are also imported into the organelles, but one of the HSP60 present in sunflower mitochondria seems to be encoded by the mitochondrial genome (data not shown), as also reported in maize and Brassica (Sinibaldi and Turpen, 1985; Prasad and Hallberg, 1989).

The study of HSPs regulation in developmental mutants could be a useful approach to understanding the effect of mutation on plant stress response, as well as in elucidating the regulation of the plant genes and the role of HSPs during morphogenesis.

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