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Gene expression during heat-shock in embryogenic carrot cell lines

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Abstract

We have isolated an *hsp90* gene from carrot (*Daucus carota*). The deduced amino acid sequence from this cDNA revealed its similarity to the organelle-type HSP90 protein. It has high homology to other plant organelle-isoforms and shows similar homology to both cytoplasmic and prokaryotic HSP90s. To study the regulation of gene expression during heat-shock, two embryo-specific DC8 and DC59 genes, a tubulin gene and an *hsp90* gene were monitored in two embryogenic heat-stressed carrot cell lines. The expression of DC8, a LEA and DC59, an oleosin gene, decreased in both cell lines. In addition, there was a progressive degradation of the accumulated messages with time. The expression of *hsp90* gene was induced in both cell lines but with a different pattern of transcript accumulation. These results indicate that the haploid cell line responds differently from the diploid cell line and there is differential transcriptional activity in both cell lines during heat-stress.

Abbreviations: HSF – heat-shock factor; HSP – heat-shock proteins; LEA – late embryogenesis abundant proteins

Introduction

The cell's competence to sense and respond to high temperatures is a common phenomenon of adaptation to environmental stress in all organisms. Elevated temperatures trigger the expression and synthesis of a well-defined set of heat-shock proteins (HSPs) while suppress the synthesis of most other genes (Ashburner and Bonner, 1979). In eukaryotes, the HSP fall into a few major classes: HSP110, HSP90, HSP70 and a group of polypeptides ranging from 15 to 30 kDa (Lindquist and Graig, 1988). Proteins of the HSP90 class are found in the cytoplasm, nucleus (Gasc et al., 1990), chloroplasts, mitochondria, glyoxysomes (Schmitz et al., 1996; Milioni and Hatzopoulos, 1997). HSP90 proteins in animal cells are specifically associated with diverse proteins (Ziemiecki et al., 1988). The structural similarities of the HSP90s from different organisms lead to the suggestion that HSP90s modulate a variety of cellular functions (Lindquist and Graig, 1988).

The transcriptional regulation of *hsp* genes in eukaryotes is modulated by heat-shock factor (HSF), a trans-acting factor binding to HSEs (Rabindran et al., 1993; Hubel and Schoffl, 1994). The molecular mechanism transducting the heat-stress signal into the cell nucleus is unknown. Polypeptide misfolding and denaturation by heat could act as a signal. However, in prokaryotes, a single change in the RNA polymerase holoenzyme, a substitution of the sigma factor, mediates the specific capability of the holoenzyme to transcribe mostly *hsp* genes (Lewin, 1997).

In order to monitor the response of two well-defined carrot cell lines of embryogenic potential (Sung, 1976; Smith et al., 1981) under heat stress conditions, we have used two functionally different genes, DC8, a LEA (Franz et al., 1989) and DC59, an oleosin gene (Hatzopoulos et al., 1990) in addition to *hsp90*

and tubulin genes. The significance in regulating gene expression of both *hsp90* gene and embryo specific genes under heat-shock in carrot cell lines is discussed.

Materials and methods

Plant material and tissue culture conditions

Two carrot cell lines were used: HA, a haploid cell line obtained via the twin seedling method from a domesticated carrot *Daucus carota* cv. Juwarot in 1973 (Smith et al., 1981). WOO1C is a diploid cell line derived from *D. carota*, Queen Anne's Lace, grown in UK in 1970 (Sung, 1976). Tissue cultures of HA were maintained in Gamborg (B5) media (Gamborg et al., 1968) supplemented with 1 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D). The WOO1C cell line was maintained in Murashige and Skoog (1962) medium with 0.1 mg l^{-1} 2,4-D. Liquid suspension cultures were subcultured at a high cell density i.e., 8×10^5 cells ml^{-1}. Cell cultures were incubated in shakers at indicated temperatures 25, 38 or 40 °C for 1, 2 or 3 h. After the treatment, the cells were immediately collected, frozen in liquid nitrogen and kept at −80 °C.

RNA preparations

Total RNA from cell suspensions was isolated according to Franz et al. (1989). Twenty micrograms of total RNA was loaded onto a denaturing formaldehyde-agarose gel (1.4%) and blotted without any further treatment onto nitrocellulose paper. RNA blot hybridization and washing conditions were performed as described by Church and Gilbert (1984) under stringent conditions (65 °C).

Molecular techniques

Molecular procedures were performed according to Maniatis et al. (1982). Genomic DNA was isolated as previously described (Franz et al., 1989). Digested DNA was electrophoresed on agarose gels and transferred to nitrocellulose filters (Maniatis et al., 1982). Southern blots were hybridized and washed according to Church and Gilbert (1984). Appropriate DNA fragments were subcloned for preparation of templates for sequencing as described in Franz et al. (1989). The sequencing was done by the dideoxy termination method (Sanger et al., 1977) and run on 6% polyacrylamide gels.

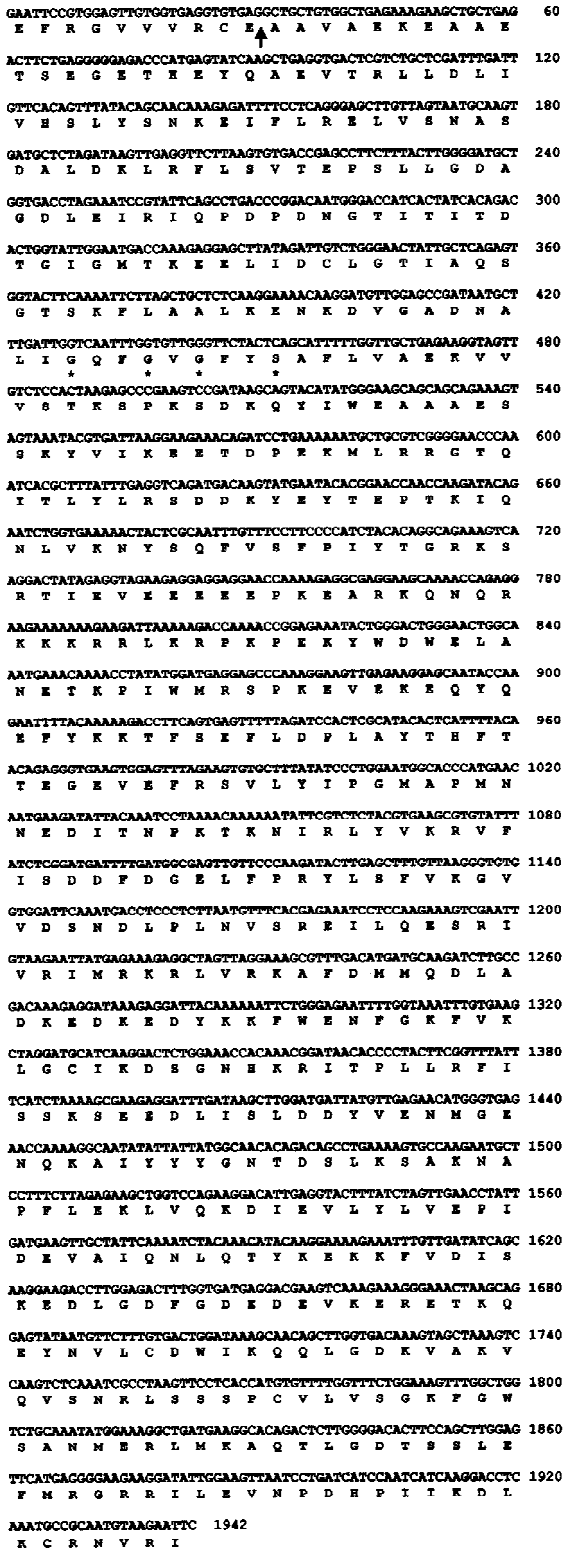

Figure 1. The cDNA sequence of DC16, an *hsp90* gene from carrots. The deduced amino acid residues are denoted below by one letter code. Arrow indicates the plausible cleavage site of the transit peptide.

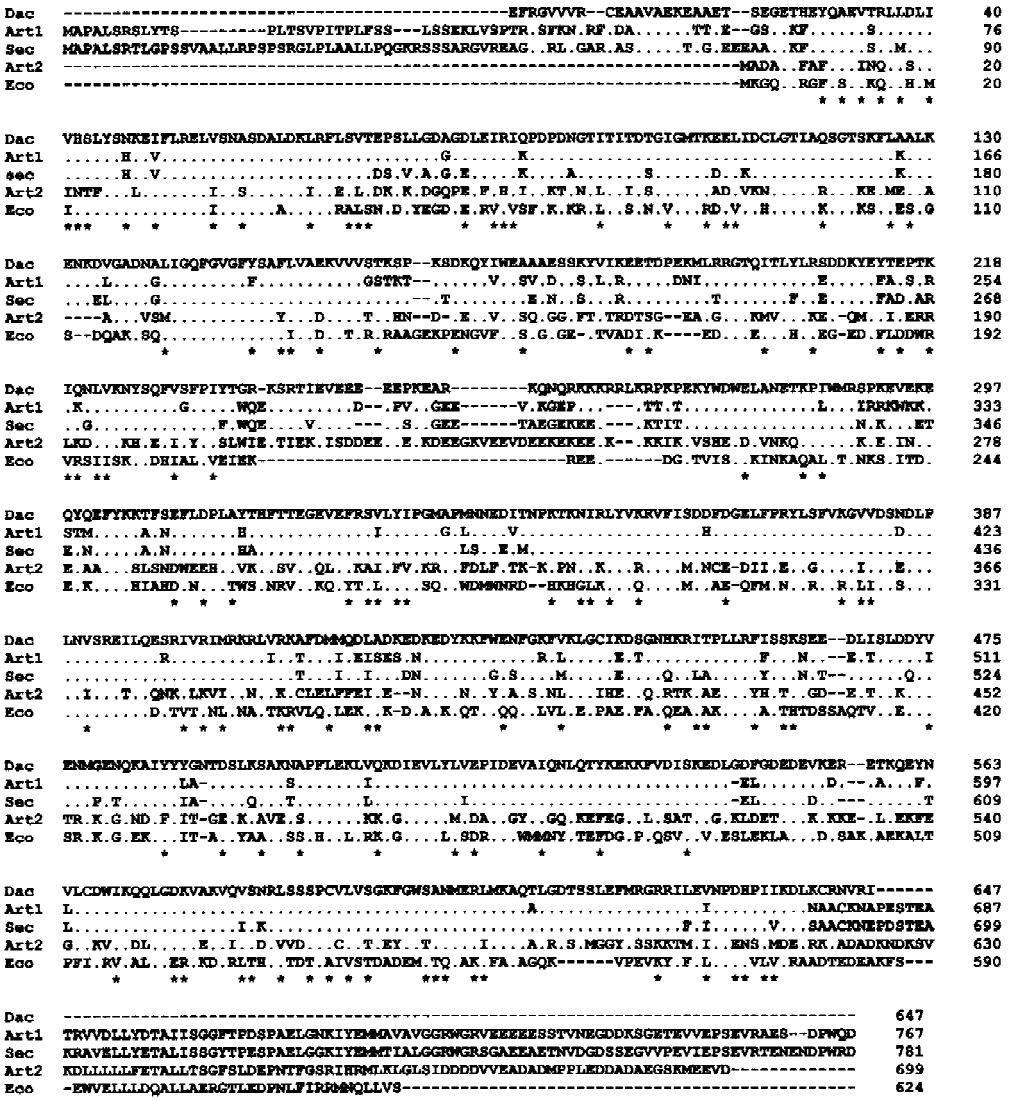

Figure 2. Alignment of the carrot amino acid sequence (Dac; *Daucus carota*) deduced from the cDNA DC16 with various protein sequences of the HSP90 family: Art1, Art2: published amino acid sequences from *Arabidopsis thaliana* (Conner et al., 1990; Milioni and Hatzopoulos, 1997); Sec, *Secale cereale* (Schmitz et al., 1996) and Eco, *Escherichia coli* (Bardwell and Craig, 1987). One letter code is used. Only amino acid differences are listed. Dots represent identical amino acids, and asterisks conserved amino acid substitutions. Dashes are inserted to maximize alignment. Numbers on the right indicate the position of the amino acids.

Results

Molecular characterization of the hsp90 *gene from carrot*

In order to evaluate gene expression in heat-stressed embryogenic cell lines of carrot, a heat-shock gene was essential. The cDNA 16 was initially selected with embryo protein-enriched antibodies from a cDNA expression library constructed in λgt11 with polyA$^+$ RNA isolated from somatic embryos of the carrot cell line WOO1C. This cDNA contains 1942 bp and an open reading frame resulting in 647 amino acids (Figure 1). It contains most of the gene as compared to published sequences (Figure 2). The deduced amino acid sequence shows high homology to *hsp90* genes from other organisms. Sequence comparison with data bases showed that the translation product from the cDNA16 is an immature polypeptide (Figure 1) containing part of the transit peptide capable of transporting this molecule into chloroplasts. The pu-

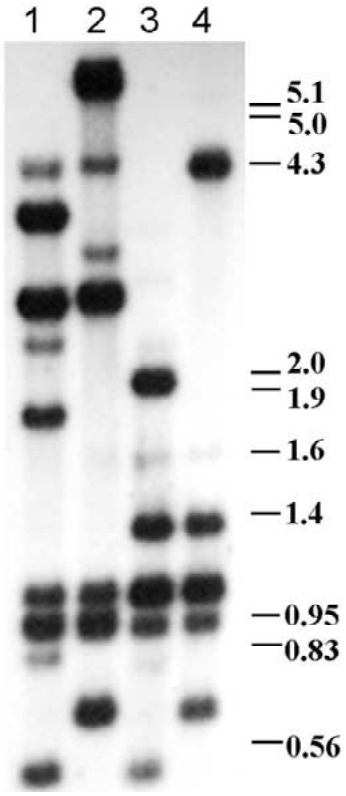

Figure 3. Southern blot hybridization analysis of *hsp90* genes in carrot cell lines. Genomic DNAs isolated from WOO1C (lanes 1 and 2) and HA (lanes 3 and 4) carrot cell lines were digested with *Eco*RI (lanes 2 and 4) and *Eco*RI/*Hin*dIII (lanes 1 and 3). The resulting Southern blot was hybridized to DC16 cDNA. All lanes have 5 μg of DNA. Numbers represent molecular mass standards in kilobases.

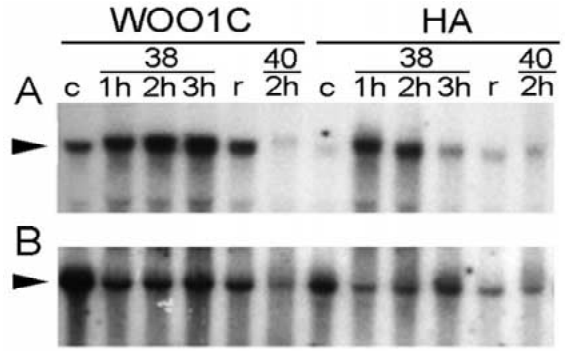

Figure 4. Induction of *hsp90* gene expression and transcript accumulation in heat-stressed carrot cell lines WOO1C lanes and HA lanes. RNAs were isolated from calli grown in liquid media. Calli were incubated at 38 °C for 1, 2 or 3 h, at 38 °C for 2 h and then returned to control temperature for 16 h (lanes r), or at 40 °C for 2 h. Lanes c, RNA from calli grown at 25 °C control temperature. Each lane contains 20 μg of total RNA. (*A*) was hybridized to cDNA16 (*hsp90* gene) and (*B*) to tubulin cDNA. Arrowheads indicate the position of the messages.

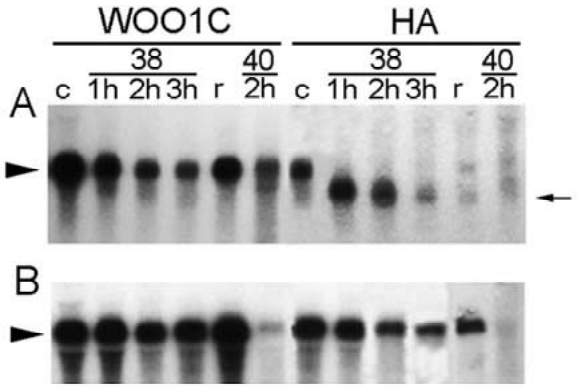

Figure 5. Comparison of oleosin and DC8 gene expression under heat-stress in carrot cell lines WOO1C and HA. RNA lanes as in Figure 4. (*A*) was hybridized to oleosin cDNA and (*B*) to DC8 cDNA from carrots. Each lane contains 20 μg of total RNA. Arrowheads indicate the position of the messages. Arrow in (*A*) indicates the truncated oleosin message.

tative cleavage site of the deduced translation product is predicted between amino acids 10 and 11. A cleavage site is frequently found and often preceded by one to several arginines within a region of 10 amino acids (von Heijne et al., 1989). The carrot HSP90 deduced amino acid sequence contains two arginines within this 10 amino acid region.

Comparison of the deduced amino acid sequence revealed that this member is an organelle directed but nuclear encoded polypeptide having high homology to other organelle-type plant HSP90s. It shows 81% identity at amino acid level to organelle-type HSP90 from rye and *Arabidopsis* (Schmitz et al., 1996; Milioni and Hatzopoulos, 1997). It is 45% identical to HSP81.1 (Conner et al., 1990), an *Arabidopsis thaliana* cytoplasmic product whereas it is 37% identical to htpG product of *E. coli* (Figure 2) (Bardwell and Craig, 1987).

To determine the number and organization of the carrot *hsp90* gene family, genomic DNA was isolated from the two different cell lines WOO1C and HA, and digested with *Eco*RI and *Eco*RI/*Hin*dIII. The digested DNA was electrophoresed, transferred to nitrocellulose filter and then hybridized to cDNA16 insert under stringent conditions (Figure 3). The hybridization pattern showed at least five strongly hybridizing bands in all cases indicating that at least five members of the *hsp90* gene family should be present within the carrot genome. Similar results were obtained with domesticated carrot cultivars (data not shown).

Heat-shock induces the hsp90 *gene in cultured carrot cells*

Figures 4 and 5 show gene expression and mRNA accumulation during heat-shock in carrot cell lines. The *hsp90* gene was constitutively expressed in these two cell lines at control temperatures. This result has been reported for other plant species (Zimmerman et al., 1989; Apuya and Zimmerman, 1992; Koning et al., 1992; Milioni and Hatzopoulos, 1997) for yeast (Borkvich et al., 1989) and for animals (Lindquist and Graig, 1988). However, the pattern of mRNA accumulation during heat-shock is different. In WOO1C, *hsp90* gene is induced at 38 °C and mRNA is accumulated from 1 to 3 h of incubation. When cells were returned to control temperature, the *hsp90* mRNA accumulation reached control level. The *hsp90* gene expression at 40 °C was almost ceased. The heat-shock protein pattern during somatic embryogenesis of carrots showed similar results (Pitto et al., 1983). In the haploid cell line HA, the *hsp90* gene induction and mRNA accumulation was higher during the first hour of incubation at 38 °C and declined during the second hour of incubation. During the third hour the message accumulation was leveled down indicating that there is concomitant mRNA degradation and gene repression. When cells were returned to control temperature the *hsp90* gene expression and mRNA accumulation reached control level. At 40 °C the expression of *hsp90* gene was ceased (Figure 4A).

The expression and message accumulation of a house keeping gene, carrot tubulin gene was also monitored (Figure 4B). The tubulin mRNA levels in most manipulations remained about the same except for the 40 °C heat-shock treatment. The increase of tubulin mRNA in HA haploid cell line during heat-shock could also result from differences in loadings, even though the amount of total RNA in gels was almost the same and the profile did not show any detectable degradation pattern (data not shown).

Heat-shock represses the expression of embryo-specific genes

In order to monitor expression and mRNA accumulation of embryo-specific genes in these two heat-stressed cell lines, DC8 and DC59 genes were used as probes. Both genes are also expressed in embryogenic calli. The kinetic analysis showed that in the diploid cell line WOO1C, the level of both DC59 and DC8 gene expression and mRNA accumulation declined when cells are heat-shocked at 38 °C (Figure 5). This is obvious during the second or the third hour of incubation at 38 °C. However, the rate of reduction of DC59 mRNA levels was more profound when compared to that of DC8. DC59 and DC8 gene expression almost ceased when cells were incubated at 40 °C for 2 h. At 40 °C, there was an obvious degradation of the DC59 mRNA resulting in a band at around 550 nucleotides long hybridizing to oleosin probe. When cells are heat-shocked for 2 h at 38 °C and then returned to control temperature, both DC59 and DC8 gene expression and mRNA accumulation was relieved and almost reached control levels (Figure 5).

The reduction level of both gene expression and mRNA accumulation was more rapid in the haploid cell line, HA. The profile of DC59 gene expression and mRNA accumulation was unique (Figure 5A). During the first hour of incubation at 38 °C, the mRNA levels declined concomitantly with the size of the message at a discrete 550 nucleotide long band. Further incubation at 38 °C showed that the size of the message remained the same, 550 nucleotides long, but the mRNA levels declined even further. When heat-shocked cells were returned to control temperature, gene expression was restored as it was obvious from the regular size of oleosin message 750 nucleotides. At 40 °C the expression of the oleosin gene was completely abolished and degradation of the message was rapid to a level that was undetectable (Figure 5A). In the diploid cell line the profile of DC59 gene expression and mRNA degradation was gradual with time. However, at 40 °C the degradation pattern was started to appear (Figure 5A).

DC8 mRNA accumulation declined during the first hour of incubation at 38 °C (Figure 5B). This decline was even greater during the second or third hour of incubation. DC8 gene expression was almost abolished at 40 °C at least for the haploid cell line (Figure 5B). These results indicate that for either of the genes tested, the gene expression and mRNA accumulation during heat-shock in carrot follows a unique pattern.

Discussion

The deduced polypeptide from the cDNA 16 shows that it is an organelle-type HSP90. The HSP90 from carrot has high homology to other organelle-type HSP90s (Schmitz et al., 1996; Milioni and Hatzopoulos, 1997) and shows low similar homologies to the cytosolic or bacterial counterparts. Sequence analysis showed that the transit peptide could be in-

volved in transporting and establishing the mature polypeptide into chloroplasts. The *hsp90* gene family in carrot is composed of several members as has been found in other plants (Milioni and Hatzopoulos, 1997).

It is known that HSP90 is expressed in developing seeds of *Brassica napus* during all stages of seed development (Reddy et al., 1998). In maize, *hsp90* genes are expressed in embryos as well as in heat-shock embryos (Marrs et al., 1993). Other *hsp* genes are also expressed during embryo development (Almoguera and Jordano, 1992; Coca et al., 1994; Carranco et al., 1997; Almoguera et al., 1998; Rojas et al., 1999; Wehmeyer and Vierling, 2000). A small heat-shock gene is activated during induction of tobacco pollen embryogenesis by starvation (Zarsky et al., 1995). Embryogenic developmental competence of microspores is correlated to HSP protein synthesis in response to 32 °C treatment (Cordewener et al., 1995). Binarova et al. (1997) showed that a short severe heat-shock is required to induce somatic embryogenesis in late bicellular pollen of *B. napus*. In white spruce *hsp* genes are developmentally regulated during somatic embryogenesis (Dong and Dunstan, 1996). Therefore, there is a relationship between *hsp* gene expression and embryogenesis.

Since a number of hsp genes are expressed during zygotic and somatic embryogenesis it was important to analyze the expression of an *hsp90* gene in both embryogenic cell lines under heat-shock. The message of the *hsp90* gene is prominent in both unstressed cell lines indicating that the embryogenic competence of both cell lines may also be supported by the active expression of *hsp* genes and their mRNA accumulation. However, the message of the *hsp90* gene is accumulated in diploid cell line in higher levels.

The mRNA pattern showed that during heat-shock, although there was a differential induction in *hsp90* gene expression depending on the cell line, there was a reduction in gene expression and transcript accumulation for both embryo-specific genes. The expression of *hsp90* gene and mRNA accumulation during heat-shock it was more prominent and persistent in the diploid cell line. The mRNA was detected in higher levels 3 h after induction. In the haploid cell line, the mRNA levels of *hsp90* decreased 2 h after induction. Moreover, the mRNA accumulation profile of DC59 is different from that of DC8 during heat-shock. The degradation of the mRNAs is profound at least in the case of DC59. The gene induction/suppression of expression and mRNA accumulation was relieved when the heat-shock stress was removed.

The results suggested that the haploid cell line HA was more sensitive to heat-stress when compared to the diploid cell line WOO1C or the diploid cell line can tolerate more the heat-stress. Alternatively, the effect of the heat-shock to the carrot haploid cell line was more drastic when compared to that of the diploid cell line. This response may also be attributed to different genetic background of the two cell lines.

Differential gene expression is the result of the cell's response to altered signals, internal or external. The heat-shock signal transduction pathway is believed to mediated through polypeptide misfolding and protein denaturation. The expression of HSPs is primarily regulated by a heat-dependent activation of the heat-shock transcription factor (HSF) that recognizes the conserved DNA-binding sites, the heat-shock element (HSE) localized in the promoter region of most *hsp* genes (Rabindran et al., 1993; Hubel and Schoffl, 1994; Zuo et al., 1994).

The heat-shock response of the cell involves a two-fold reaction: the induction of the HSPs and the suppression of synthesis of most other proteins. The selective transcription profile under normal or heat-shock conditions could be attributed to induction/suppression of different genes under either condition. However, it will be necessary for the cell to establish or express a suppressor gene able to repress diverse genes as LEA and oleosins. This suppressor should be induced by heat-shock. Alternatively, changes in the RNA polymerase constitute(s), a eukaryotic analogue of the sigma-70 factor, could account for such changes. It is known that nuclear encoded RNA polymerases could initiate plastid sigma-70-type promoters (Hajdukiewicz et al., 1997). Recently, nuclear encoded sigma-70 factor genes have been isolated from *Arabidopsis* (Isono et al., 1997). These results suggested that changes in expression pattern of plastid genes could be attributed to alteration of nuclear encoded sigma factors (Hajdukiewicz et al., 1997; Isono et al., 1997). In prokaryotes, a single change in the holoenzyme composition of the RNA polymerase, a substitution of the sigma factor mediates the specific capability of the holoenzyme to transcribe mostly *hsp* genes (Lewin, 1997). A change in the RNA polymerase holoenzyme could halt most gene expression as in the case of DC8 and DC59 genes, and cellular activities, making the cell competent to overcome this stress by mostly expressing *hsp* genes. It is conceivable to expect that sigma-70-like factor in plants could bind to RNA polymerase apoenzyme releasing the normal sigma-like factor and

changing the expression pattern of the cell. Since HSPs are chaperones and generally associated with diverse polypeptides, the function of such polypeptide that binds to RNA polymerase apoenzyme during heat-shock could be attributed to one of the many HSPs.

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