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Changes in the level of chloroplast transcripts in pumpkin cotyledons during heat shock

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The levels of plastid gene transcripts are shown to be controlled by temperature in isolated pumpkin cotyledons. The temperature at which maximum transcript accumulation occurs varies between 38 and 42°C for the genes studied (*rbc*L, *psa*A, *psb*A, *psb*B, *psb*C, *psb*D, *psb*E, *atp*A). Heat shock-induced transcript accumulation is transitory with a maximum after an approximately 3 h exposure at high temperatures. On the other hand, the accumulation of *rbc*L transcript was only moderately thermosensitive. A temperature increase to 46–48°C induces a sharp decrease of transcript levels which correlates with damage to the plant. Relatively little correlation has been noted between RNA and protein patterns. However, there is a remarkable coincidence between temperature dependence of the accumulation of transcripts and the temperature dependence (28–46°C) of the synthesis of chloroplast-located heat shock proteins indicating that both processes may be related.

Plastid gene expression; Heat shock protein; Pumpkin cotyledon

1. INTRODUCTION

The temperature elevation above optimal levels induces the production of heat shock proteins (HSPs) in the cells of all organisms, plants in particular, and inhibits or decreases the protein synthesis occurring at normal habitat temperatures [1–3]. Such an alteration in the spectrum of synthesized proteins could be due to the influence of the heat shock on gene expression, processing and/or translation of mRNAs [4].

The induction and accumulation of HSP mRNAs under non-optimal temperatures has been well demonstrated in plants [2]. However, the fate of mRNAs synthesized previously under normal temperature, as well as the relationships between transcription, transcript turnover, decoding and protein stability, or the underlying regulatory control mechanisms remain unclear. Circumstantial evidence indicates specific shifts in the mRNA levels for different genes. For example, the study of mRNAs derived from nuclear genes has shown that heat shock resulted in the decrease of preexisting auxin-induced mRNA species while actin mRNA remained at the same level [4].

Different from mRNA changes influenced by light or hormones, information on RNA patterns from plastid genes under heat shock has only been reported for those of Rubisco subunits in photoautotrophic soybean cell suspensions. The mRNA content of the plastid-encoded Rubisco LS remained unchanged at temperatures optimal for HSP formation (38–40°C) although the synthesis of the polypeptide was inhibited approximately 50% indicating a translational control of the process [5]. At the same time the mRNA amounts of the nuclear-encoded Rubisco SS as well as the production of this subunit dropped by 60–75%. Contradictory results have been obtained with leaves of *Pennisetum americanum*, in which heat shock induced the degradation of Rubisco LS mRNA but did not affect the transcription of other chloroplast genes which, however, were not specified in this preliminary communication [6].

We have addressed the outlined topic including the question how sets of genes that encode complex structure are regulated. We have examined the effect of temperature elevation from 28 to 48°C on the transcript levels of genes for thylakoid proteins, including representatives of the various assemblies of this biomembrane (photosystems I and II, ATP synthase), and Rubisco LS, and compared these results with synthesis of chloroplast HSPs. Localization of HSPs in chloroplast of various plant species is well documented [7–12]. In our experiments isolated pumpkin cotyledons served as a model because their heat shock response has been previously studied in our laboratory [13].

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Abbreviations: HS, heat shock; HSP, heat shock protein; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; LS, large subunit of Rubisco; SS, small subunit of Rubisco; SDS-PAAG, SDS-polyacrylamide gel; MS, Murashige and Skoog medium.

2. MATERIALS AND METHODS

The pumpkin seeds (*Cucurbita pepo* L., Mozoleevskaya 49) were sterilized in 1% sodium hypochlorite and placed in flasks on half-strength MS medium with 1% agar [12]. The seeds were germinated for 60 h in a growth chamber at 28°C in the dark. The etiolated seedlings were then illuminated for 72 h (white fluorescent light; 1,300 lux). Detached green cotyledons were kept in distilled water in closed glasses in thermostats at discrete temperatures between 28 and 48°C.

For the analysis of transcripts derived from plastid genes, the cotyledons were incubated for 2 h at constant temperatures and then fixed in liquid nitrogen. For the analysis of chloroplast HSP synthesis, 15 cotyledons used per determination were preincubated for 10 min at a constant temperature in the range of 28–48°C, and then incubated for 2 h at the same temperature with [^{35}S]methionine (1.5 MBq/mmol).

Isolation of intact chloroplasts purified by Percoll gradient centrifugation and fractionation of chloroplasts proteins by SDS-PAAG electrophoresis were carried out as described in [13]. RNA was isolated from excised pumpkin cotyledons as described by Westhoff et al. [14]. After grinding the cotyledons in liquid nitrogen, the powder was extracted with phenol and high molecular weight RNA was precipitated from the aqueous phase by LiCl. Total cellular RNA was used for all Northern and dot blot analyses.

Dot blots were prepared by denaturing total RNA, applying a two-fold dilution series of RNA to nylon membrane (Hybond N, Amersham, UK) using a Bio TM-Dot Apparatus (Bio-Rad, USA). The method was described in [15]. For Northern hybridization, RNA was electrophoresed in 1.3% agarose/formaldehyde gels and transferred to Hybond membrane by capillary blotting. RNA was fixed onto the membrane by UV irradiation. Prehybridization, hybridization and washing of the filters were made as described in Maniatis et al. [16]. Nick-translated plasmids containing segments of the spinach plastid genes *psa*A, *psb*A, *psb*B, *psb*C, *psb*D, *psb*E, *atp*A or *rbc*L were used as hybridization probes [17].

Scans of autoradiographs were made using a CD 50 densitometer (Desaga, Germany). All experiments were repeated at least three times. The data represent the means and their standard deviations of three separate experiments.

3. RESULTS AND DISCUSSION

Dot-hybridization using nick-translated *psb*A probe with total cotyledon RNA reveals a clear relationship between the signal obtained and two parameters, the RNA amount in the sample and the temperature of cotyledon exposure (Fig. 1A). Temperature elevation from 28 to 40°C generally results in transcript accumulation while incubation at 46–48°C causes a sharp decline.

Northern analysis of cotyledon RNA probed with *psb*A (Fig. 1B) demonstrates that transcripts of *psb*A gene which accumulated at 42°C had the same molecular mass as transcripts in control cotyledons (28°C) indicating the absence of both non-processed mRNA and products of mRNA degradation. These results show clearly that the steady-state level of these plastid transcripts in cotyledons are influenced by temperature. Dot-hybridization demonstrates the accumulation of transcripts from all genes, except of *rbc*L (Fig. 2) at temperature elevation to 38°C (10°C above the control level). The temperature of maximal transcript accumulation was remarkably different for various genes. For example, for *psa*A and *psb*E it occurred at 38°C, for

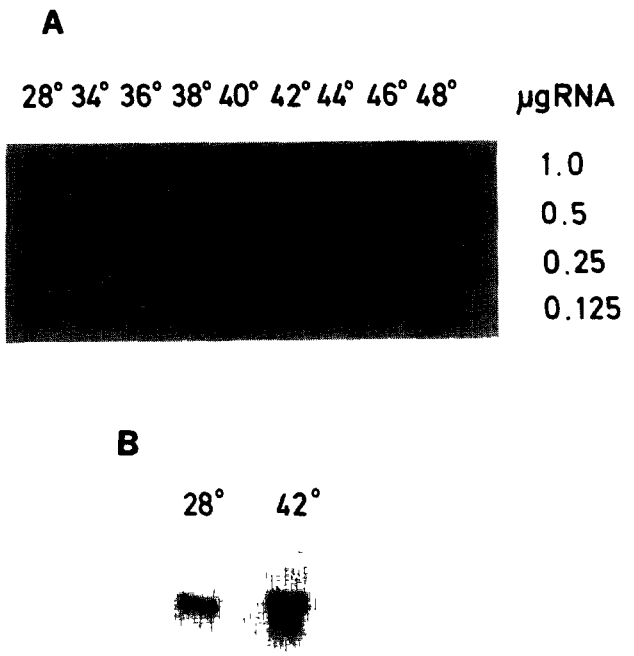

Fig. 1. Dot-blot (A) and Northern blot (B) analysis of pumpkin cotyledon RNA probed with *psb*A. RNA was isolated from cotyledons incubated for 2 h at various temperatures and used for dot-blot analysis. RNA was blotted onto nylon membrane and hybridized with nick-translated DNA containing the *psb*A gene probe.

*psb*B, *psb*C and *atp*A at 40°C, and for *psb*A and *psb*D even at 42°C.

The various transcripts investigated also differ in the amplitude of the temperature-induced accumulation. The highest accumulation for *psa*A was 209% (at 38°C), for *psb*C 202% (40°C), *psb*D 185% (42°C), *psb*E 181% (38°C), *psb*A 172% (42°C), *psb*B 151% (40°C), *atp*A 135% (40°C), and for *rbc*L 110% (38°C).

A further temperature increase leads to reduced transcript levels which again differed in their degree of inhibition (Fig. 2). At 48°C the stationary concentrations of the *psb*A transcript decreased only slightly, that of *psb*B by 16%, of *rbc*L by 20%, *psb*D by 27%, *psb*E by 42%, *atp*A by 47%, *psa*A by 55%, and *psb*C by 58%. The level of *rbc*L transcript, in turn, varied only slightly within the temperature range of 28–48°C which is consistent with results reported for soybean suspension culture [5]. It is not known whether such a weak temperature response is common to transcripts of other stromal components as well or represents an exception among the plastid genes studied, and whether the relatively high temperature tolerance of *psb*A and *psb*D mRNA is related to the intrinsic characteristics of the photosystem II biochemistry. The polypeptides, D1 and D2, products of *psb*A and *psb*D respectively, which comprise the innermost reaction center of photosystem II that houses all redox components involved in primary photochemistry of this photosystem, are inactivated

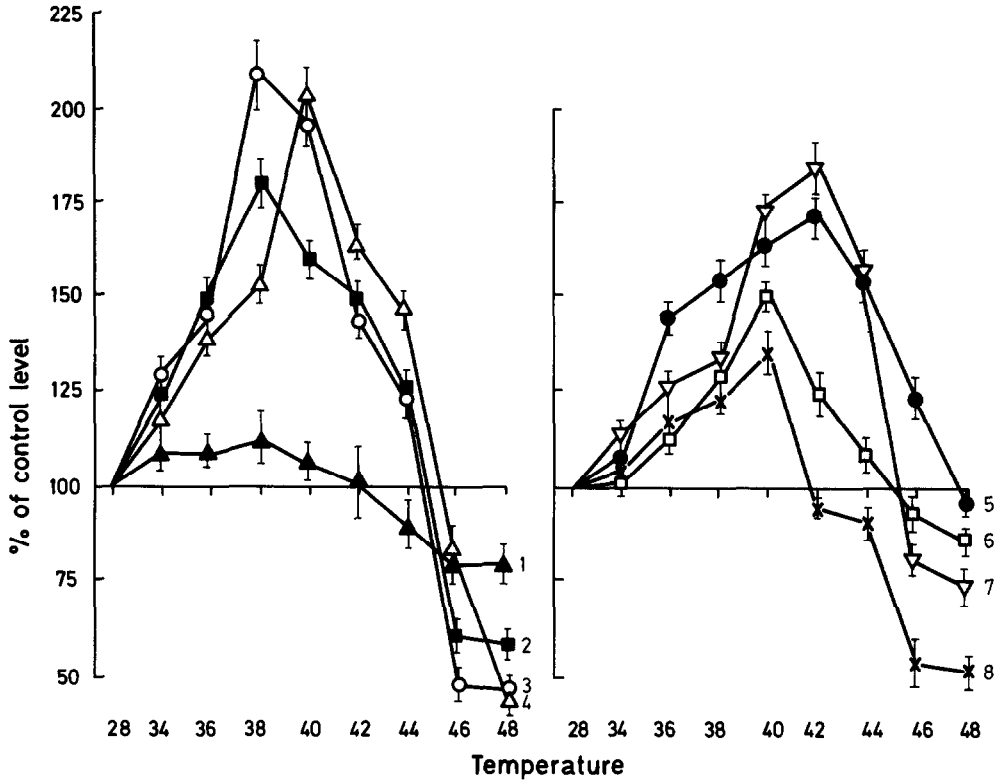

Fig. 2. Effect of temperature on transcript levels of plastid genes. Details of the experiment are shown in the legend to Fig. 1 and in section 2. RNA was hybridized with nick-translated DNA containing the following plastid genes: (1) *rbc*L, (2) *psb*E, (3) *psa*A, (4) *psb*C, (5) *psb*A, (6) *psb*B, (7) *psb*D, (8) *atp*A. The results of densitometric scanning of fluorographs for transcripts at different temperatures are given as % of transcript amounts at control temperatures (28°C). Levels in cotyledons after HS are expressed relative to the level in control cotyledons (100% at 28°C). The data represent the means and their standard deviations of three separate experiments.

and turn over rapidly in response to light and various stress. Their rapid degradation is balanced by an induction or maintenance of gene expression that compensates for the loss of these proteins [18,20].

Fig. 3 demonstrates the changes in steady-state transcript levels of *psa*A and *psb*D during continuous heating of cotyledons at 40°C. An increased transcript content was observed only during the first hours of heating, the maximum value being at 3 h. Then the transcript levels drop sharply and remain almost at control levels during the next 5–9 h. This implies that transcript accumulation during heat shock is transitory, at least for these genes.

Fig. 4 represents a fluorograph of PAAG-electrophoresis of polypeptides isolated from the intact chloroplasts after the incubation of cotyledons with [^{35}S]methionine at various temperatures. It is evident from the corresponding scans that the radioactivity in the band of Rubisco LS does not decrease dramatically up to 42°C, but comprises only 30% of the control level at 44°C and 5% at 46°C. On the other hand, Fig. 2 illustrates that the amount of Rubisco LS transcripts changes only slightly within the 28–46°C temperature range. Consequently, the inhibitory influence of temperatures between 44 and 46°C on the synthesis of LS

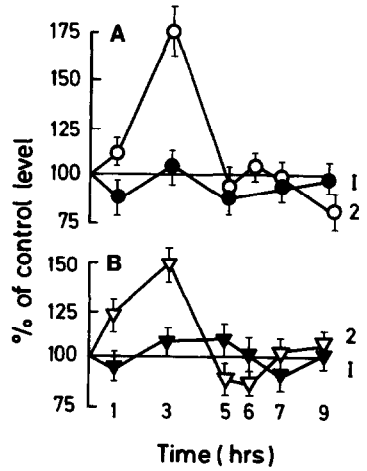

Fig. 3. Chloroplast gene transcript levels during heat shock as a function of time. Total RNA was isolated from cotyledons incubated at 28°C (1) and 40°C (2). Total RNA of cotyledons used for dot blot analysis was probed with radio-labelled *psa*A (A) and *psb*D (B). The results of densitometric scanning of fluorographs are given relative to that of control cotyledons (cf. Fig. 2).

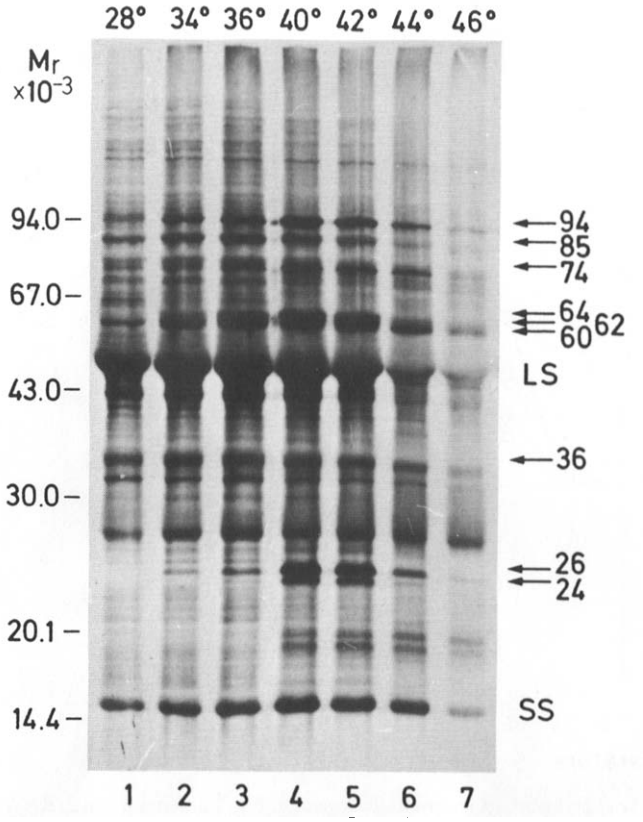

Fig. 4. Temperature dependence of heat shock protein synthesis in pumpkin cotyledons. Excised green cotyledons were preincubated at various temperatures for 10 min; 100 mCi of [^{35}S]methionine was then added during an additional 120 min of incubation. The chloroplast proteins were fractionated on SDS-PAA gels (7.5–15%) and visualized by fluorography. Equal amounts of protein were loaded on each lane. Arrows denote major heat shock bands. The positions of large (LS) and small (SS) Rubisco subunits are indicated on the right, those of M_r markers on the left: 94,000, 67,000, 43,000, 30,000, 20,100, 14,400. The set of HSPs was the same in protease-treated and untreated chloroplasts [12].

seems to operate primarily at a posttranscriptional, probably translational level. This conclusion is consistent with the results of Vierling and Key [5]. Comparably, Western blot analysis demonstrates that there is no increase in the amount of 32 kDa polypeptide (gene *psb*A), P700/1 chlorophyll a apoprotein (*psa*A) or α subunit of ATP synthase (*atp*A; data not shown), and therefore there is no obvious correlation between transcript and polypeptide accumulation under heat shock conditions for these proteins.

The patterns of high and low molecular weight chloroplast HSPs are presented in Fig. 4. The synthesis of the former class (94, 85, 74 and 64–60 kDa) can be observed at 28°C, is strongly promoted with temperature increase (34–36°C) and reaches the maximum at 40°C, slightly decreases at 42°C, and essentially ceases at 44°C. The synthesis of the 94, 85 and 74 kDa HSPs decreases by approximately 60% at 44°C while that of the 64–60 kDa class is more thermostable. It should be emphasized that 60 kDa HSPs resolve into three components in two-dimensional gel electrophoresis and belong to thylakoid membrane proteins. Circumstantial evidence indicates that these proteins are all encoded in plastid chromosomes [12]. At 46°C only traces of high molecular HSPs were found.

Low molecular chloroplast HSPs are not synthesized at normal temperatures (28°C). Their appearance is induced under heat shock. Among low-molecular HSPs those of 26 and 24 kDa are synthesized most actively. Their synthesis differed in the response to temperature elevation. For example, 26 kDa HSPs are formed at 34°C while 24 kDa HSPs appeared at 36°C. The fluorograph scanning has revealed the following kinetics of 26 kDa HSP synthesis (in arbitrary units): 34°C 1; 36°C 2.4; 40°C 10.4; 42°C 9.2; 44°C 2.8; 46°C traces. For the 24 kDa component the following kinetics were recorded: 34°C below detection; 36°C 1; 40°C 8; 42°C 6.2; 44°C 1.2; 46°C not detectable.

Thus, there is some difference in the temperature dependence of individual chloroplast HSP synthesis. As discussed above, level of chloroplast mRNAs also did not change uniformly when the temperature increased. Nevertheless, the comparison of the outlined temperature dependence in the accumulation of transcripts for plastome-encoded genes with the synthesis of chloroplast HSPs has unraveled a remarkable coincidence between transcript levels and the production of chloroplast-located HSPs. Thus, the temperature range of 34–40°C induced an increase in the level of examined chloroplast transcripts (except *rbc*L) and simultaneously activated HSP synthesis. Whereas the higher temperature (44–46°C) decreased dramatically the mRNA levels and suppressed HSP synthesis. Although a causal relationship between the latter processes remains to be verified, the correlation may not reflect mere coincidence. This not only raises questions as to the underlying mechanisms of regulation (transcription, processing, turnover or stability of corresponding mRNAs and their interplay), but alerts to the intriguing and testable possibility that HSPs may participate in the stabilization of plastid mRNA under heat-shock conditions.

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