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Heat-shock Protein Synthesis Study by Micro-chip

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ABSTRACT

Keeping constant all agronomic factors except temperature is a way to follow Heat Shock Proteins (HSPs) expression. Three different sunflower hybrids derived from the cross between an inbred line, Cms HA 89, and three different restorer inbred lines, with resistance, susceptible, and normal reactions to drought, were produced. In order to investigate the impact of temperature on protein accumulation during achene filling phase, they were cultivated in two different geographical areas: Karaj in Iran and Udine in Italy. Total protein content and structural polypeptide fractions of Seed Storage Proteins (SSPs) were determined. The analysis of HSPs was carried out by means of lab-on-chip capillary electrophoresis. It was revealed that protein accumulation in achene occurs at a greater rate during the achene filling phase, i.e. approximately 9-25 days after pollination, in all examined hybrids. Besides, the presence of a polypeptide band of 17.7 kDa supposed to be of small Heat Shock Proteins (sHSPs) family was recorded in all three hybrids grown up in hot and arid environment, Karaj, which implied independent sHSPs expression from the paternal restorer line.

Keywords: Hybrid sunflower, Lab-on-chip capillary electrophoresis, Paternal restorer line, Small Heat Shock Protein (sHSPs).

INTRODUCTION

Small Heat Shock Proteins (sHSPs) are produced ubiquitously in prokaryotic and eukaryotic cells upon heat (Sun *et al*., 2002). However, over heat stress, other kinds of environmental stress conditions, such as infection, inflammation, exercise, exposure of the cell to toxins (ethanol, arsenic, trace metals and ultraviolet light), starvation, hypoxia (oxygen deprivation), nitrogen deficiency, or water stress that would lead to the production of great amounts of HSPs (Santoro, 2000). sHSPs could either protect the plant from the damage, or help repair the damage, caused by the stress. Moreover, there are some overlaps in the function of different stress proteins, for example, one stress (and its HSPs, subsequently) could induce protection against another (Ya-Lin *et al*., 2010; Chao *et al.*, 2009).

In general, the sHSPs are not found in normal vegetative tissues, but accumulate to high levels in response to heat stress (Puigderrajols *et al*., 2002). Specific sHSPs are also expressed during various phases of plant development as part of the endogenous developmental program. Thus, although sHSPs are apparently not essential for basal cell functions (Such as high molecular weight HSPs: HSP90, HSP70 and HSP60), their functions are likely to be critical for survival and recovery from heat stress as well as for specific developmental processes (Wang *et al*., 2004).

The molecular mass of sHSPs is estimated between 15 to 42 kDa and their unusual abundance and diversity in plants is considerable. Up to now, six classes of sHSPs have been identified in plants based on their intracellular localization and sequence relatedness (Sun *et al*., 2002). Specific sHSPs, the cytosolic class I and

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class II proteins are also expressed in the absence of stress in maturing seeds of several species and hypothetic role for these proteins is desiccation tolerance, dormancy, or germination (Efeoglu, 2009). Further, the upregulation of HSPs has been sometimes described as part of the stress response (Puigderrajols *et al*., 2002) and this overexpression is transcriptionally regulated (Tomanek and Somero, 2002). Obviously, this upregulation is the principal part of the heat shock response which is induced mainly by heat shock factors. The mechanism by which heat-shock (or other environmental stressors) activates the heat shock factor has not been determined. However, some studies suggest that an increase in damaged or abnormal proteins brings sHSPs into action.

sHSPs located in the chloroplast, the endoplasmic reticulum, and the mitochondria are characterized by a conserved sequence at their C terminus (Sun *et al*., 2002). Biochemical analysis indicates that sHSPs are found in high molecular weight complexes between 200–400 kDa that are most likely composed solely of multiple sHSPs subunits (Taiz and Zeiger, 2006). However, evolutionary analyses suggest that *sHSPs* gene families arose by gene duplication and divergence prior to the radiation of angiosperms (Waters *et al*., 1996, Wu *et al*., 2007). It is suggested that sHSPs act *in vivo* as a type of molecular chaperone to bind partially denatured proteins preventing irreversible protein inactivation and aggregation, and that sHSP chaperone activity contributes to the development of thermo tolerance (Siddique *et al*., 2008, Dafny-Yelin *et al*., 2008).

In this research, we studied the impact of temperature on protein accumulation (total protein and individual HSP synthesis) during achene filling phase. We aimed to use lab-on-chip capillary electrophoresis to evaluate the differences between naturally occurring polypeptides of the hybrids Seed Storage Proteins (SSPs) and the formation of new polypeptides related to the effect of temperature fluctuation.

MATERIALS AND METHODS

Plant Materials

Three sunflower hybrids derived from the crosses between three different restorers (13S as drought susceptible, 28R as drought resistant, and AC4122 as normal) and one female line (HA89, Reg. no. GS-39, PI642062). Inbred line 28R was derived from the cross between

H. argophyllus×*H. annuus* (De Romano and Vázquez, 2003). The materials have been generated in National Institute of Genetics and Biotechnology (NIGEB), Iran, and University of Udine, Italy.

Growth Condition

Trials were done in 2010 (and for further confirmation in 2014 the cultivation was repeated) at the Experimental Farm of Udine University (46° 02'N, 13° 13' E and 110 m asl) and NIGEB of Iran, Karaj (37º 23' N, 46º 16' E, 1450 m asl using lysimeter systems (length 1.1 m, width 0.8 m and depth 0.70 m). The lysimeters were filled with loam soil (20%, 42%, and 38% of clay, silt, and sand, respectively) (0.5 m layer) and with sand, gravel and fine pebbles at the bottom (0.2 m layer) for drainage, and were protected from the rain by a transparent fixed canopy.

The soil water content in each lysimeter was measured every 3 days by TDR (Tektronics 1502C) using probes inserted at 20 and 40 cm depths. Therefore, a programmed irrigation was executed to keep the same moisture content of the soil for both localities.

The main climatic characteristics, divided into the pre-and post –flowering stages of the crop, were recorded at an automatic weather station close to the experimental site (Table1).

Protein Analysis

Total sunflower seed proteins were extracted from 50 mg of sunflower seeds in

Table 1. Average values of minimum temperature (Min T), maximum temperature (Max T), relative humidity (RH), and solar radiation (Radiation), during the time between end of flowering and end of physiological maturity periods.

Localities	Min T (°C)	Max T (°C)	RU (%)	Radiation (MJ m^{-2} day^{-1})
Karaj (Iran): End flowering-physiol. maturity (Growth period)	18.7	37	40	25.8
Udine (Italy): End flowering-physiol. maturity (Growth period)	12.72	23	70	21.6

0.4 mL of 2M urea 15% glycerol, 0.1M DDT and 0.1M Tris/HCl, pH 8.8, using an ultra-sonic water bath for 15 minutes. Extracts were centrifuged at 11,000×g for 5 minutes (Wang *et al*., 2007).

Size, purity, and concentration of the protein samples had been identified daily, after flowering till the end of achene maturation by lab-on-chip. A final extraction volume of 400 µL of extracted protein was selected as optimal, based on the resolution and intensities of peaks reported by the Agilent software. A 4 µL protein sample was added to 2 µL denaturing solution, this mixture and aliquot of protein 80 Ladder heated in water bath at 95°C for 5 minutes. After cooling down, 84 µL deionized water was added to the sample and ladder.The samples were loaded on Agilent chip (Agilent 2100 Bioanalyzer Technologies) according the manufacturer's instruction. Each chip included the standard molecular weight and a lower marker of 4.6 kDa (Biorad, USA).

Statistical Analysis

The experiments were carried out following a factorial arrangement in the complete randomized block design with three replicates and four plants for each replication. The first factor, genotype, constituted the three hybrids, and the second factor i.e. locality, with two levels (Karaj and Udine). We conducted statistical analyses of triplicate determinations of total protein content of the considered genotypes by ANOVA. Significant differences were expressed with the least significant deviation ($LSD_{0.01\%}$).

RESULTS AND DISCUSSION

Sunflower seeds are a significant source of proteins and a number of authors have studied correlations between the protein content and other seed characteristics (Joksimović *et al.*, 1999).

The protein synthesis and accumulation were measured during the achene maturation phase precisely from achene formation (F4) till achene maturation (M3). The protein accumulation has been expressed as a percentage of total protein content at the end of achene maturation phase (Figure 1). The ongoing increase of protein concentration during the achene maturation establishes the fact in which the biosynthesis of proteins in sunflower achene is a phenomenon that happens in the maturation phase of achene (Flagella, 2006).

The results of the total relative protein content of the achene in different hybrids cultivated in the two localities, Karaj and Udine, are provided in Figure 2. Higher amount of protein synthesis accumulation was registered in the hybrids cultivated in Karaj. It should be taken into consideration that, in the phase of maturation, the biosynthesis of proteins or oil is completely affected by temperature variations besides genotypes Merrien *et al*. (1998). Environmental factors affect certain physiological processes within the seed during seed formation. In this research, significant difference in protein content of

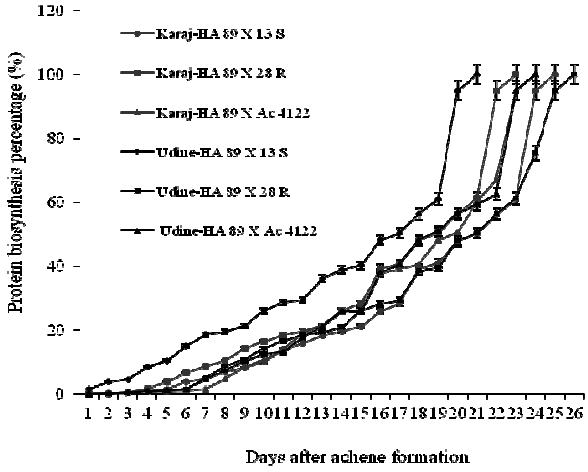

Figure 1. Achene protein accumulation (in percent) of different sunflower hybrids in the phase between end of flowering and achene full maturation (M3).

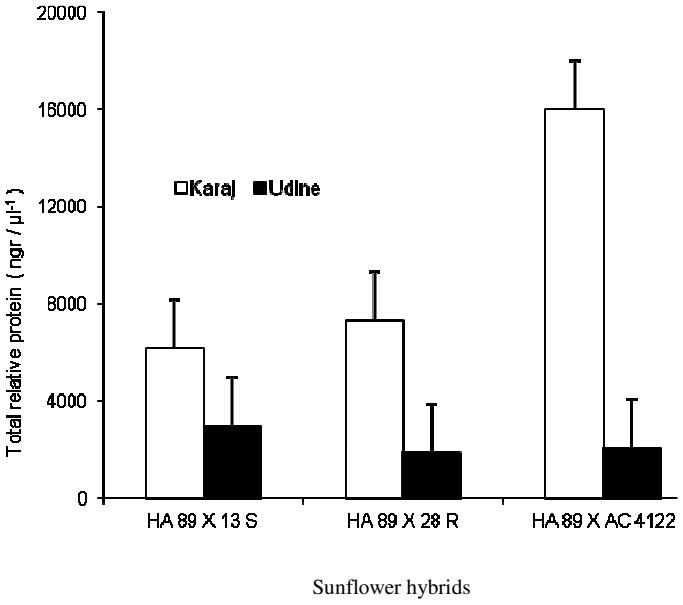

Figure 2. The comparison of total relative protein concentration of three sunflower hybrids in two localities, Karaj, Iran, and Udine, Italy.

seeds at the harvest stage between the studied hybrids and the two localities were revealed. The protein contents of the hybrid derived from restorer AC4122, drought-susceptible hybrid derived from 13S, and the pure line 28R resistant to drought, were 12,995.98, 7,107.04, and 5,971.62 ng μL^{-1}, respectively.

The interaction between temperature variations and hybrids was highly significant, which confirmed the key role of restorer in hybrid making, based on the fact that all those hybrids had the same CMS, female line (Alba *et al*., 2010).

Lab-on-chip protein analyzer diagnoses the proteins of 3-80 kDa. Based on the chromatogram profile, the appearance of a

sharp and highly intense peak (about 400 FU) 17 s after gel run represents a 17.7 kDa polypeptide from sHSPs family, which is marked by a cycle in Figure 3A. This peak has been detected in the samples grown in the hot-arid climate. This implies that the expression of this small polypeptide is the consequence of higher temperatures, which triggers the synthesis of small heat shock protein. However, this signal has not been detected for the hybrids grown in Udine.

Tishkov and Popov (2004) reported four proteins as heat protein shock expressed in soya at critical environmental conditions. Among them, only one, a small molecular mass HSP, was reported to be induced by high temperature stress.

Figure 3 demonstrates a Lab-on-a-Chip chromatogram of proteins extracted from the achenes after 9-25 DAP of different sunflower hybrids cultivated in two localities. Chromatogram provides banding pattern of the sample's polypeptides.

CONCLUSIONS

As plants cannot move away from heat, they have evolved a battery of specialized "*stress genes*", the sHSPs. These are expressed in response to heat in all sub-cellular compartments and allow plants to cope better with the stress conditions on site.

Dafny-Yelin *et al*. (2008) and Dahlia *et al*. (2009) stated that the biosynthesis of a new polypeptide sHSPs depends on the environmental factors such as temperature. In the present study, the importance of the up/down regulation of the related genes and, consequently, the amount of specific synthesized protein (17.7 kDa), has been emphasized. However, the temperature variations have a direct effect on sHSPs expression, which is directly linked to germplasm resources used for breeding program in the restorer inbred line construction. The presence of a polypeptide band of 17.7 kDa (presumed to be from

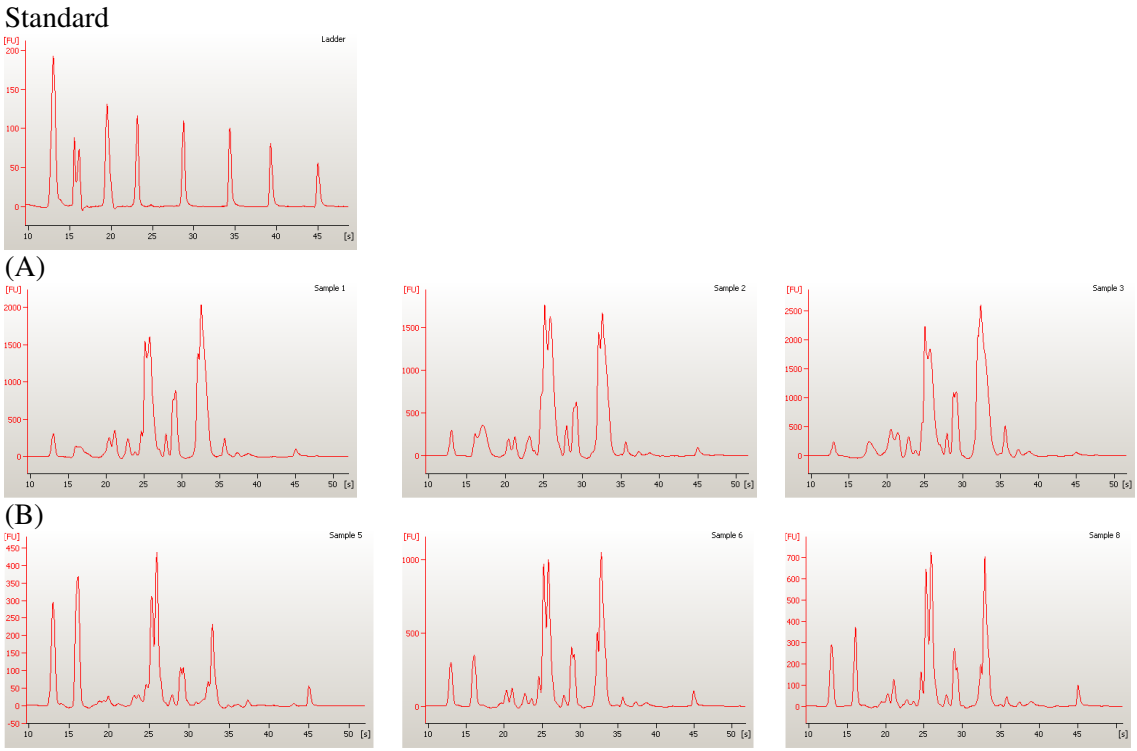

Figure 3. Lab-on-a-Chip chromatogram of total proteins extracted from achene 25 DAPS of three sunflower hybrids, cultivated in two localities: (A) Udine (samples 1HA 89×13S, 2HA89×28R, 3HA89×AC4122), and (B) Karaj (5HA89×13S, 6HA89×28R, 8HA89×AC4122). The chromatogram of standard protein is placed as ladder.

sHSPs family) was observed in all three hybrids grown in hot and arid environment. This implies the independent sHSPs expression and synthesis from the paternal restorer line.

In the present research, water availability was limited by using lysimeter trials to investigate the effect of one parameter, i.e. temperature, on protein synthesis. Consequently, the synthesis of a 17.7 kDa protein demonstrated the correlation between higher temperature and sHSPs synthesis induction.

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مطالعه سنتز پروتئین های شوک گرمایی با استفاده از میکروچیپ

س. طهماسبی انفرادی

چکیده

یک راه مناسب جهت تعقیب بیان پروتئین های شوک گرمایی ثابت نگه داشتن تمام فاکتورهای زراعی به جز فاکتور دما میباشد. در این تحقیق سه هیبرید مختلف آفتابگردان که از لقاح بین یک اینبردلاین، Cms HA89، و سه رستورر مختلف (که نسبت به استرس خشکی مقاوم، حساس و نرمال هستند) به دست آمده و مورد استفاده قرار گرفتند. هیبریدهای مورد نظر را در دو ناحیه جغرافیایی مختلف، کرج و اودینه ایتالیا به منظور بررسی تاثیر دما بر تجمع پروتئین ها در فاز پرشدگی آکنه، کشت دادیم. مقدار تام پروتئین ها و فراکسیونهایی از پلی پپتیدهای ساختاری از پروتئین های ذخیره ای دانه (SSPs) تعیین شدند. آنالیز HSPs با استفاده از سیستم الکتروفورزی موئینه Lab-on-Chip شناسایی شدند. مشخص شد که تجمع پروتئین ها در آکنه با نرخ بالایی در فاز پرشدگی آکنه، یعنی تقریبا بین ۹ تا ۲۵ روز پس از گرده افشانی اتفاق می افتد. مضافا، حضور یک باند پلی پپتیدی ۱۷.۷ کیلودالتون به عنوان یکی از پروتئین های خانواده پروتئین های کوچک شوک گرمایی (SHSPs) در هر سه هیبریدی که در محیط گرم و خشک (کرج) پرورش یافتند مشاهده شد که بیانگر بیان مستقل SHSPs مستقل از رستورلاین پدری است.