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Sunflower Heat-Shock Protein Synthesis Study under Water Deficit Condition

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Abstract: To follow heat shock proteins (HSPs) expression in sunflower seeds as portion of total seed storage proteins (SSPs), we kept all agronomic factors constant except temperature. In this research, a drought-tolerant sunflower hybrid was planted under normal and drought stress conditions in order to investigate the effect of temperature on protein synthesis and accumulation during achene filling phase. The analyses of proteins were carried out by two-dimensional electrophoresis (2-DE), after total protein extraction from achene 25 days after pollination. In total, at least 542 protein spots were detected in seeds at normal growth condition versus 551 protein spots in seeds of stress condition. The presence of a polypeptide bond of 17.7 kDa as a small heat shock protein (SHSP) was only observed in seed grown in drought stress and the accuracy of identification was confirmed by micro-lab-on-chip capillary electrophoresis.

Keywords: 2D-electrophoresis, hybrid, small heat shock protein (SHSP), sunflower

Introduction

Scarcity of water is considered as one of the most severe abiotic stress on plants. Drought induces mainly genes related to heat shock through which heat shock proteins (HSPs) seem more critical. Small heat shock proteins (SHSPs) could

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either protect the plant from damage caused by the stress or help repair the damage caused by the stress (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). Stress conditions other than elevated temperatures can also lead to HSP induction (Vierling, 1991; Parsell and Lindquist, 1993).

The molecular mass of SHSPS is estimated between 15 and 42 kDa and their unusual abundance and diversity in plants is considerable (Trent, 1996).

The best-characterized example of developmental regulation is the induction of specific SHSP members during seed maturation at normal growth temperatures (Coca *et al.*, 1994; zur Nieden *et al.*, 1995). Specific SHSPs, the cytosolic class I and class II proteins, are also expressed in the absence of stress in maturing seeds of several species; the hypothetic role for these proteins is desiccation tolerance, dormancy or germination (Efeoglu, 2009). Indeed, Reddy *et al.* defined the upregulation of HSPs as a tool to combat the stress (Reddy *et al.*, 2014).

It has been observed that in other plants different HSPs express abundantly during pollen development, i.e. in maize (Magnard *et al.*, 1996; Young *et al.*, 2001). Indeed, genes for mitochondrial HSC70 and HSP22A are actively transcribed in male florets of fertile and sterile maize lines that develop unknown mitochondrial dysfunctions during microsporogenesis (Newton and Gabay-Laughnan, 1998).

It is suggested that SHSPs play a unique role to develop thermotolerant reactions that involve their binding to partially denatured proteins preventing irreversible protein inactivation and aggregation (Siddique *et al.*, 2008; Dafny-Yelin *et al.*, 2008).

In this research, we studied the protein profile of a drought-tolerant sunflower hybrid cultivated under normal and drought stress by means of two-dimensional electrophoresis (2-DE) and lab-on-chip capillary electrophoresis with the main emphasis on finding SHSPs as it presents natively or its formation as new polypeptides related to the effect of temperature.

Materials and methods

Plant materials

A drought-tolerant sunflower hybrid derived from the cross between a restorer (28 R, a drought resistant) and a female line (HA 89, Reg. no. GS-39, PI 642062). The materials have been generated in University of Udine, Italy.

Growth condition

Sunflower seeds were planted using lysimeter system (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 (0.2 m layer) for drainage and were protected from the rain by a transparent fixed canopy (Baldini *et al.*, 2002)

The soil water content in each lysimeter was measured every 3 days by Time-Domain Reflectometer (TDR) probe (model 1502 C Tektronix, Beaverton, OR, USA) using probes inserted at 20 and 40 cm depths. One lysimeter was irrigated regularly and the other one kept under drought condition with irrigation per week.

Extraction of protein

Protein extraction was performed as described by Wang *et al.* (2007). About 0.3 g of leaves was finely ground by using liquid nitrogen and then suspended in extraction buffer containing 50 mM Tris–HCl buffer (pH 7.4), 0.25 M sucrose, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM PMSF (phenylmethanesulfonyl fluoride) and 2.5% v/v β-mercaptoethanol. The samples were then centrifuged at 2,000 *g* for 5 min, the supernatant was measured for protein concentration based on Bradford assay (Bradford, 1976) and then lyophilized and kept at 4°C for further electrophoresis analysis.

The lyophilized sunflower proteins were solubilized in 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.5% pH 3–10 IPG buffer v/v (GE Healthcare, USA) and 36 mM 1,4-dithio-DL-threitol (DTT) (5.6 mg/ml) via incubation at room temperature for 1 h, vortexing every 10 min, followed by centrifugation (15,000 rpm/15 min), and the supernatant was collected.

Polyacrylamide gel electrophoresis (PAGE) gels (12%) were run on an Ettan Dalt six (GE Healthcare), 0.5 h at 2.5 W per gel, then at 15 W per gel until the dye front reached the gel bottom. Upon electrophoresis, the protein spots were stained with Coomassie Brilliant Blue G-250.

Total protein extract (66 µl) was loaded onto GE Healthcare 18 cm immobilized pH gradient (IPG) gel strips (pH 3–10 L) during strip rehydration overnight, after which Isoelectric focusing (IEF) was performed for a total of 65 kV h using IPG Phor II (GE Healthcare) at 20°C.

Briefly, gels were fixed in 40% ethanol and 10% acetic acid for 30 min, and then sensitized with 30% ethanol, 0.2% sodium thiosulfate w/v and 6.8% sodium acetate w/v for 30 min 2-DE gel images acquired by GS-800 calibrated

densitometer (Bio-Rad). All images from each replicate for both treatment and control were assembled in a match set using Melanie software version 6.0.1.0.

The region containing a strongly expressed spot with MW between 14 and 21 kDa at acidic pH was cut from the 2-DE gel and placed in 400 µl extraction buffer to re-isolate protein from gel. A 4 µl freeze-dried re-extracted protein was added to 2 µl denaturing solution, this mixture and aliquot of protein 80 Ladder heated in water bath at 95°C for 5 min. After cooling down, 84 µl deionized water was added to sample and ladder. The samples were loaded on Agilent chip (Agilent 2100 Bioanalyzer Technologies) according to the manufacturer's instruction. Each chip included the standard molecular weight and a lower marker of 4.6 kDa (Bio-Rad, USA).

Statistical analysis

The experiments were carried out following experiment on the complete randomized block design with three replicates and four plants for each replication. The statistical analyses of triplicate determinations of total protein content were conducted by ANOVA (analysis of variance). Significant differences were expressed with the least significant deviation ($LSD_{0.01\%}$).

Results and discussion

There are different molecular and physiological responses to water deficit in plants. The main phase for protein synthesis and accumulation occurs during the achene maturation (Flagella, 2006).

The protein synthesis and accumulation were measured during the achene filling up phase precisely from achene formation (F4) till achene maturation (M3). Figure 1 reports protein accumulation in achene as percentage of total protein content between the flowering and achene full maturation.

The results of total relative protein content of the achene under normal and water deficit conditions (hydric stress), are provided in Figure 2. It should be taken into consideration that in the phase of maturation the biosynthesis of proteins or oil is completely affected by both temperature variations and genotype (Merrien *et al.*, 1988). In this research, significant difference in protein content of seeds at the harvest stage between two water conditions had been revealed.

2-DE was carried out and the related images of 2-DE gels of sunflower hybrid derived from the cross between HA 89 and 28 R under normal and water deficit conditions is shown in Figure 3(B) and (C). The total of 542 spots with molecular

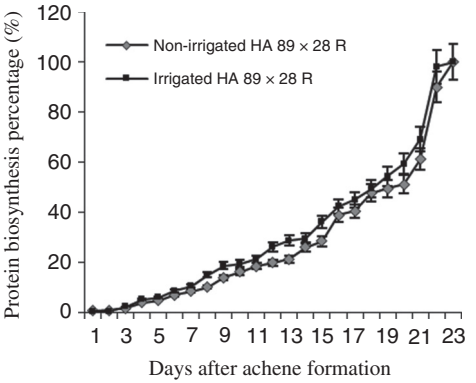

Figure 1: Achene protein accumulation (in percent) of sunflower hybrid under two irrigation regimes (non-irrigated and irrigated) in the phase between end of flowering and achene full maturation (M3).

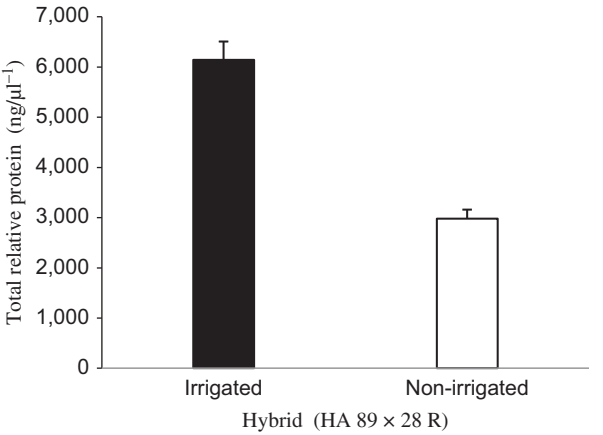

Figure 2: The comparison of total relative protein concentration of sunflower hybrid under two irrigation regimes (non-irrigated and irrigated).

weight varying from 10 to 110 kDa was reproducibly detected across three replicate gels from hybrid under normal irrigation (Figure 3(B)). However, more nine spots were presented in hybrid grown under hydric stress in which their presence was also statistically significant by Student's t-test at $p \leq 5\%$ (Figure 3(C)).

Based on the 2-DE profile, the region with differential spots with molecular weight between 14 and 21 kDa was suspect to contribute SHSPs.

The micro-lab-on-chip protein analysis confirmed two modifications in the picks as demonstrated in Figure 3(A) and (D). One of them that do not exist in sample grown under normal irrigation appeared as a sharp and highly intense

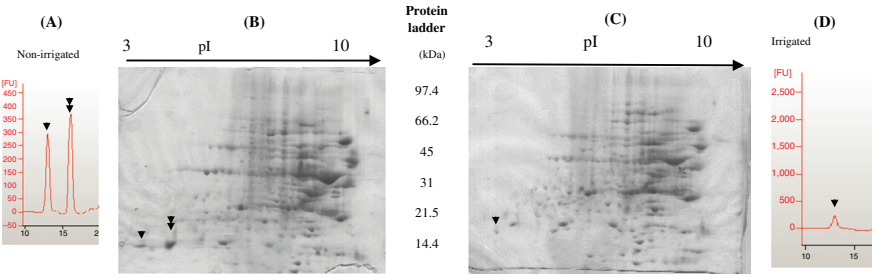

Figure 3: Comparative proteome analyses of sunflower achene proteins (25 DAF). (B) and (C) 2-DE gel for non-irrigated and irrigated lysimeter, respectively. (A) and (D) Micro-lab-on-chip analysis of candidate differential SHSPs for non-irrigated and irrigated lysimeter, respectively.

pick representing 17.7 kDa polypeptide from SHSPs family (marked by double triangle in Figure 3(B)). The other pick that existed in sample grown under normal irrigation was over-expressed (Figure 3(C) and (D)).

Conclusion

As high temperature causes a serious threat to cellular viability, several heat-induced responses have developed to cope better with the stress conditions. Of them, SHSPs with molecular mass of 15–30 kDa plays an important role against stressful conditions and recently have been reported to have molecular chaperone activity in vitro.

It has been reported that the biosynthesis of a new polypeptide SHSPs depends on adverse environmental conditions such as temperature (Dafny-Yelin *et al.*, 2008). In this research, the protein synthesis/accumulation during the achene filling has followed up to investigate up/downregulation of genes affecting the improvement of defense mechanisms against hydric/heat stresses. It has been proved that temperature variations have a direct effect on SHSPs synthesis/expression, which directly refers to genetic status behind the plant.

The appearance of specific newly synthesized protein (17.7 kDa) from SHSPs family in plants grown under hydric stress identified through micro-lab-on-chip confirms its synthesis induction as a consequence of heat stress. Furthermore, we used lysimeter trials by which all parameters were kept unchanged but irrigation rate between two experiments. It was sufficient to make sure that the newly synthesized protein or over-expressed protein in the plant grown under hydric stress are from HSP family.

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