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BRIEF COMMUNICATION

Analysis of the role of mitochondrial and endoplasmic reticulum localized small heat shock proteins in tomato

P.C. NAUTIYAL[1]* and M. SHONO[2]

Directorate of Groundnut Research, Junagadh-362001, Gujarat, India[1]
Okinawa Sub-tropical Research Station, Ishigaki, 907-0002, Okinawa, Japan[2]

Abstract

This communication examines the role of small heat shock proteins (sHsps) targeted to mitochondria (Mt) and endoplasmic reticulum (ER) in tomato plants (*Lycopersicon esculentum* Mill.) under heat stress. Genetic response of transgenic and wild type plants varied under optimum, moderately elevated and elevated temperature. In optimum temperature higher biomass was recorded in wild type than the transgenic lines, whereas in moderately elevated temperature biomass increased in Mt-sHsp line. Also, net photosynthetic rate (P_N) increased in Mt-sHsp line in both the elevated temperatures, though higher in moderately elevated. Cell membrane stability (CMS) improved in all the lines after exposure to elevated temperatures, but always remained higher in transgenic lines. Transgenic lines expressed sHsps in different temperature regimes in both vegetative and reproductive parts, while wild type expressed such proteins only after 1 h of heat shock.

Additional key words: cell membrane stability, elevated temperatures, *Lycopersicon esculentum,* plant growth, photosynthesis, thermotolerance.

Global temperature is increasing, and crop plants including tomato are experiencing heat stress during one or the other stage of their life cycle (Sato *et al*. 2006, Sumesh *et al*. 2008). Heat stress in plant cells induces multiple changes that ultimately affect membrane structure and function (Wang *et al*. 2004). Plants after exposure to heat stress acquire tolerance, which involves a set of mechanisms (Thomashow 1999) including synthesis of small heat shock proteins (sHsp; Queitsch *et al*. 2000). sHsps are expressed after heat shock in mitochondria (Liu and Shono 1999), endoplasmic reticulum (Sanmiya *et al*. 2004, Mammedov *et al*. 2008), cytoplasm, nucleus and chloroplasts during vegetative, and reproductive stages and also in desiccating seeds (Wehmeyer and Vierling 2000). The sHsps are known to function as molecular chaperons and direct the proper folding of the protein as well as proper assembly of the protein complex (Vierling 1991). Some proteins promote the degradation of misfolded proteins, while others bind to different types of folding intermediates and prevent them from aggregating and still another promotes the reactivation of proteins that have already aggregated (Osteryoung and Vierling 1994). The direct evidence for the function of an individual sHsp in whole plant stress tolerance is not fully understood (Preczewiski *et al*. 2000, Nautiyal *et al*. 2005). The present study is an attempt to elucidate the basis of differential behavior of Mt- and ER-localized sHsps.

Following deletion of the β-glucuronidase gene from the binary vector pBI121 (*Clontech*, Palo Alto, CA, USA), the full-length tomato Mt- and ER-sHsp cDNA were independently sub-cloned into this vector. *Agrobacterium tumefaciens* mediated transformation and regeneration of plantlets was performed using strain LBA4404 and cotyledons from 10-d-old seedlings of tomato (*Lycopersicon esculentum* Mill.) cv. Ailsa Craig as described by Nagata *et al*. (1995). In case of Mt-sHsp four and ER-sHsp five plants were obtained from each independent transformation event. All the lines were maintained and two lines, *i.e*. Mt-sHsp T_0 (S line) and

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Abbreviations: Chl - chlorophyll; CMS - cell membrane stability; CT, MET, ET - control, moderately elevated and elevated temperatures, respectively; ER - endoplasmic reticulum; Mt - mitochondria; P_N - net photosynthetic rate.
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* Corresponding author; fax: (+91) 285 2672550, e-mail: nautiyal@nrcg.res.in

ER-sHsp T_0 (aa_1 line) were grown under optimum temperature and T_1 generation plants were developed and were confirmed to be homozygous by the germination test of their seeds on kanamycin-containing medium. After germination the seedlings were shifted in pots filled with soil, *Vermiculite* and farm-yard-manure in the ratio of 2:1:1 and grown in optimum temperature (26/20 °C). One month old seedlings of Mt-sHsp and ER-sHsp along with wild type in ten replicates were arranged in three sets. One set was retained in optimum temperature (CT), while other sets were shifted to moderately elevated (30/22 °C; MET) and elevated (32/25 °C; ET) temperature cycles. After one month, observations on photosynthetic rate (P_N) and leaf cell membrane stability (CMS) were recorded. Flowers were counted daily, after the first flower appeared. Whereas, shoot and root growth and number of mature and immature fruits were recorded at the end of the experiment. Leaf area was measured with the help of leaf area meter and plant samples were dried at 80 °C until constant mass, and specific leaf area (SLA = leaf area/leaf dry mass) was calculated.

Net photosynthetic rate (P_N), stomatal conductance (g_s), internal carbon dioxide concentration (c_i), transpiration rate (E) and leaf temperature (T_{leaf}) were recorded on recently fully expanded top leaf between 09:00 and 10:00 local time concurrently on 5 plants of each line grown in CT, MET and ET, using portable infrared gas analyzer (*LCA 4*, *ACD*, Kyoto, Japan). The observations were recorded five times over a period of one month and mean values were used for statistical analysis. Leaf tissue (1 g) was sampled from second and third fully expanded leaves and analysed for chlorophyll contents following the method described by Strain and Svec (1966). For the measurement of leaf cell membrane stability (CMS) heat stress temperature was selected based on the results of our previous experiment and 48 °C for 45 min was found to be 50 % heat killing temperature and time, respectively (Nautiyal *et al.* 2005). For each observation 10 leaf discs were used in each replicate and a set of test tubes was arranged accordingly. The initial and final electrical conductivities (EC) were measured with an EC meter (*Model CM 115*, *Kyoto Electronics*, Kyoto, Japan). Relative leaf injury (RI %) was calculated following Hossain *et al*. (1995).

For heat shock treatment seedlings of wild type were grown in optimum temperature until 30 d. One week before heat shock, half of plants were exposed to water stress by decreasing daily irrigation to 50 %. Both normal and stressed plants were exposed to heat shock at 40 °C for 20, 40, 60 and 80 min in growth chambers under dark. During heat shock g_s, E and T_{leaf} were recorded on recently fully expanded top leaf using *LCA4*. After heat shock leaves were sampled and analysed for expression of Mt- and ER-sHsp.

Expression of sHsp was analysed following the SDS-PAGE and gel blot analysis. For protein extraction, 1 g fresh leaf samples were extracted in 5 cm^3 of grinding buffer (25 mM HEPES, pH 7.2, 0.35 M mannitol, 25 mM sucrose, 5 mM EDTA, 1 % (m/v) polyvinylpyrrolidone and 1 mM dithiothreitol). The pellet, in case of Mt-sHsp, was collected by mixing it with suspending buffer (20 mM HEPES, pH 7.2, 0.4 M sucrose). For the analysis of ER-sHsp supernatant was collected from different centrifugation, *i.e.* 1 000 *g* followed by 10 000 *g* for 15 min each and subsequently centrifuged at 200 000 *g* for 30 min, and mixing it with 0.1 cm^3 of suspending buffer. Samples were separated on 12 % (m/v) acrylamide gel in the presence of SDS for both Mt-sHsp and ER-sHsp by following the method of Laemmli (1970). After electrophoresis gels were either stained with Coomassie brilliant blue R-250 (CBB) or processed for Western blot analysis by electro blotting to polyvinylidene fluoride (PVDF) membrane. After electro blotting PVDF membranes were characterized for the expression of Mt-sHsp, 0.01 cm^3 of GST Mt-sHsp antibody was diluted in 10 cm^3 of blocking solution for overnight followed by second antibody, *i.e.*, anti-Rabbit IgG. Similarly, ER-sHsp were characterized by treating the PVDF membrane with ER-type antibody (first) followed by second antibody, *i.e*., anti-Rabbit IgG. Total protein in the samples was determined using the *Bio-Rad* (Hercules, USA) protein assay with bovine serum albumin as a standard. Before loading the protein samples into gel its concentration was maintained by diluting with buffer and 0.005 cm^3 of protein was loaded into each well including the standard proteins sample. Effect of heat stress was calculated on CMS and P_N by comparing the standard error at $P = 5$ %.

Vegetative and reproductive growth was influenced due to elevated temperatures. In MET number of flowers increased in all the lines including wild type, but in elevated temperature number of flowers increased only in Mt-sHsp. In contrast, number of fruits decreased in both MET and ET in all the lines (data not shown). In wild type leaf area decreased in MET and ET, but still remained higher than in the transgenic lines. In transgenic lines leaf area increased in MET and decreased in ET. On the other hand, SLA decreased in MET and ET, being lowest in Mt-sHsp line (Table 1). In transgenic lines stem and root dry masses increased in MET, while in wild type only stem mass increased in MET, while root mass decreased in ET. In CT total biomass was higher in wild type and lower in Mt-sHsp line, while in MET total biomass increased almost twice in Mt-sHsp line. Due to elevated temperatures shoot growth was affected more than the root growth.

Performance of Mt-sHsp in MET and ET was better than of ER-sHsp and wild type (Table 1). P_N increased in MET and ET in Mt-sHsp and decreased in rest of the lines. Also, P_N was highest in Mt-sHsp (11.15 μmol m^{-2} s^{-1}) in MET; this resulted in higher biomass assimilation. Correspondingly, c_i was higher in wild type and lower in Mt-sHsp lines. Total chlorophyll (Chl) content increased in MET and decreased in ET in all the lines, however, increase in Chl content in MET was highest in wild type (Table 1). Change in Chl *a*/Chl *b* ratio in different temperature regimes was more pronounced in Mt-sHsp than in rest of the lines. This might be an adaptation to

Table 1. Leaf area [cm^2 $plant^{-1}$], SLA [cm^2 g^{-1}], stem dry mass [g $plant^{-1}$], root dry mass [g $plant^{-1}$], total biomass [g $plant^{-1}$], stomatal conductance, g_s [mmol m^{-2} s^{-1}], photosynthetic rate, P_N [µmol m^{-2} s^{-1}], Chl contents [mg g^{-1}(f.m.)] and relative leaf injury, RI [%] of Mt-sHsp and ER-sHsp lines along with wild type in optimum (26/20 °C, CT), moderately elevated (30/22 °C, MET) and elevated (32/25 °C, ET) temperatures.

Lines		Leaf area	SLA	Stem dry mass	Root dry mass	Total biomass	g_s	P_N	Chl	RI
Mt-sHsp	CT	324	205	10.3	2.3	16.6	16.1	9.4	7.1	53.2
	MET	555	165	19.2	3.1	38.8	15.0	11.1	10.5	44.3
	ET	298	135	8.8	2.0	15.8	12.3	9.8	6.0	46.5
ER-sHsp	CT	255	210	9.7	2.5	22.4	14.4	10.6	8.6	58.1
	MET	603	183	17.9	4.6	32.7	12.3	9.7	10.1	50.0
	ET	200	157	8.5	2.2	20.0	12.0	9.0	5.8	48.2
Wild type	CT	670	228	11.0	3.5	27.1	15.5	9.3	7.5	65.2
	MET	662	194	19.0	3.2	38.0	12.2	8.7	10.8	56.2
	ET	300	177	10.0	3.0	20.5	11.0	8.5	5.6	55.3
$LSD_{0.05}$		168	28	4.9	1.0	7.6	0.01	0.23	0.27	2.5

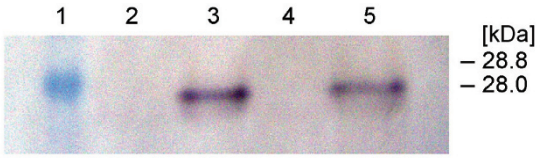

Fig. 1. Expression of Mt-sHsp (28.0 kDa) in wild type after heat shock treatment at 40 °C for 1 h. Plants were exposed to 100 % (control) or 50 % soil moisture (water deficit) one week before heat shock treatment. 1 - protein standard, 2 - leaf from control plant (no heat shock treatment), 3 - leaf from control plant (after heat shock treatment), 4 - leaf from water deficit plant (no heat shock treatment), 5 - leaf sample from water deficit plants (after heat shock treatment).

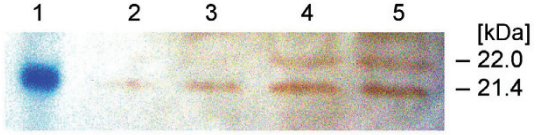

Fig. 2. Expression of ER-sHsp (22.0 kDa) in wild type plant after heat shock treatment at 40 °C for 1 h. Plants were exposed to 100 % (control) or 50 % soil moisture (water deficit) one week before heat shock treatment. 1 - protein standard (21.4 kDa), 2 - leaf from control plant (no heat shock treatment), 3 - leaf from water deficit plant (no heat shock treatment), 4 - leaf from water deficit plant (after heat shock treatment), 5 - leaf from control plant (after heat shock treatment).

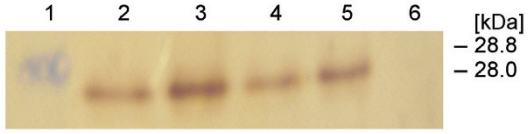

Fig. 3. Expression of Mt-sHSP (28.0 kDa) in vegetative and reproductive parts in T_1 plant. 1 - protein standard (28.8 kDa); 2 - stem, 3 - leaf, 4 - flower, 5 - immature fruit, 6 - leaf of wild type (control plant).

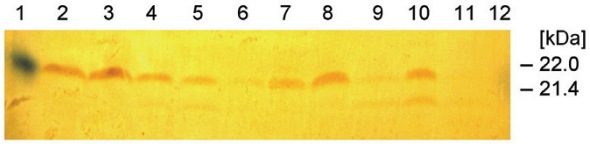

Fig. 4. Expression of ER-sHsp (22.0 kDa) in leaf of T_1 plant. 1 - protein standard (22 kDa), 2 to 10 - leaf samples of individual lines expressing ER-sHsp, 11 - leaf sample from wild type (control plant) and 12 - protein standard (21.4 kDa).

heat stress (data not shown). Overall, relative injury (RI) was lower in transgenic lines than the wild type (Table 1). The lower leaf injury in transgenic lines indicated the function of sHsp as molecular chaperons in maintenance of CMS. However after exposure to ET, RI decreased in all the lines.

After heat shock treatment, both control and water-stressed plants of wild type expressed Mt-sHsp and ER-sHsp in 60 min, and also significant differences were recorded in g_s, E and T_{leaf} (data not shown). During heat shock g_s in water-stressed plants was much lower than in controls. In water-stressed plants during initial heat shock (20 min) E was much lower than in the control plants and increased thereafter, while remained steady in control plants. These results clearly showed that control plants under heat shock maintained their T_{leaf} due to higher E, while T_{leaf} was higher in water-stressed plants. After 60 min of heat shock recovery in g_s and E was recorded and this coincided with the expression of Mt-sHsp and ER-sHsp. These proteins were expressed when T_{leaf} was 3 °C higher than in the control leaves (data not shown). Protein gel blot analysis of leaf samples showed distinct protein bands of 28 and 22 kDa of Mt-sHsp and ER-sHsp, respectively (Figs. 1,2).

Mt-sHsp line was tested for the expression of protein in T_1 both vegetative and reproductive parts (Fig. 3), and ER-sHsp in vegetative parts in T_1 (Fig. 4). However, such protein bands could not be detected in wild type grown in

CT. Rapid growth in vegetative stage and early development of reproductive organs in ER-sHsp (T_1) line (seed collected from T_0 plants grown in ET) was observed (data not shown).

Present investigation has defined *in vivo* role of Mt- and ER-sHsp in thermotolerance of P_N and CMS. Thus in MT-sHsp line, the change in expression of the respective protein caused modest change in P_N and electrolyte leakage, whereas the protein change in ER-sHsp line caused only modest change in electrolyte leakage. The overall performance of Mt-sHsp line was superior to the ER-sHsp line and wild type, indicating that Mt-sHsp could play an important role in thermotolerance in tomato plants. Mitochondria appear to be the key component for thermotolerance because they function during both photorespiration and dark respiration (Heckathorn *et al*. 1998). Also increase in P_N in Mt-sHsp line in elevated temperatures could be explained on the basis of electron transport activity caused in part by change in membrane fluidity (Salvucci and Crafts-Brandner 2004). In addition, the coincidence between the time of recovery in g_s and expression of sHsp, in this study, indicates the function of sHsp as molecular chaperons. This supports the fact that under heat stress membrane-bound complexes are disrupted and induction of various Hsp genes takes place (Liu and Shono 1999). A correlation between photosynthetic thermotolerance and production of sHsp has also been demonstrated in tomato plants (Preczewiski *et al*. 2000) and yield in crop plants has been positively correlated with the P_N (Nautiyal *et al*. 1995, 1999).

In this study, the Mt-sHsp line showed lower biomass in CT and higher biomass in MET, this could be due to additional requirement of photosynthates for the expression of sHsp. It is presumed that sHsps perform their function in ET and additional requirement of photosynthates is compensated in terms of acquired thermotolerance. This makes the transgenic lines, especially the Mt-sHsp, superior over the others. Thus incorporation of heat tolerance through Hsp in crop plants will have an energetic cost, and would require additional carbon assimilates (Mitra and Bhatia 2008). In addition to the molecular and physiological attributes, Mt-sHsp line showed better adaptation in morphological features in relation to lower SLA (thicker leaf), leaf rolling and leaf trichomes (data not shown). The negative association between SLA and expression of sHsp was reported by Knight and Ackerly (2001). In addition, a negative relationship between SLA and water use efficiency (Nautiyal *et al*. 2002) in groundnut cultivars, and SLA and thermotolerance (Nautiyal *et al*. 2008) in wild *Arachis* spp. was reported.

These findings indicate that Mt-sHsp would be more beneficial than the ER-sHsp, because the previous one was able to provide thermotolerance to both P_N and CMS, which are vital for plant growth. More studies however are required to investigate the role of sHsps in reproductive organs in different crops to manipulate long termotolerance.

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