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Acquisition of thermotolerance and *HSP* gene expression in durum wheat (*Triticum durum* Desf.) cultivars

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ABSTRACT

Plants are strongly affected by heat stress, which they overcome by modifying several physiological and biochemical mechanisms. At the cellular and molecular levels, the synthesis of heat shock proteins (HSPs) is essential in preventing or minimising the deleterious effect of high temperature. Plant responses to high temperatures are mediated by both their inherent ability to survive known as basal thermotolerance, and their ability to acquire thermotolerance after acclimation. A major aspect of the acclimation response involves the expression of *HSP* genes. Different *Triticum durum* cultivars were characterised for their response to high temperature at the physiological and molecular levels. Determination of cell membrane stability for both basal and acquired thermotolerance, and *HSP* gene expression analysis were performed. The two genotypes which contrasted most in their ability to acquire thermotolerance were exposed to different stress conditions and the expression of *HSP101C* and four small *HSP* genes was analysed. Differences in *HSP* transcripts accumulation were observed during the acclimation treatments. There is substantial evidence that induction of *HSP* gene expression has a role in the acquisition of thermotolerance; moreover, the accumulation of mitochondrial *HSP* transcripts appears to be related to the acquisition of thermotolerance.

1. Introduction

All living organisms have evolved endogenous mechanisms to cope with environmental stresses such as high temperature. Plant growth and yield are strongly affected by heat stress as it damages the functions of cells, tissues, and whole plants. The predicted levels of global warming are likely to increase the constraint to plant productivity imposed by high temperature. Differences in the sensitivity to high temperatures have been observed in crop species, due to differences at the genetic level. Several physiological and biochemical mechanisms have to be modified by plants to overcome heat stress. For instance, high temperature causes modifications of membrane fluidity, permeability and stability (Ismail and Hall, 1999; Sangwan et al., 2002), and electrolyte loss resulting from heat-induced cell membrane leakage is considered a measure of stress cellular damage (Saadalla et al., 1990; Fokar et al., 1998; Wahid et al., 2007). Another interesting, but still incompletely explained aspect of the heat shock response is the synthesis of heat shock proteins (HSPs). These proteins, through their function as chaperones and/or proteases, may play an essential role in preventing and minimising the deleterious effect of high temperature at the cellular and molecular levels (Vierling, 1991; Nover, 1991; Schöffl et al., 1999; Gulli et al., 2007). The synthesis of these proteins is known to be part of the stress tolerance strategy resulting in the ability of plants to cope with the heat stress (Iba, 2002). As a result of the HSPs production, many physiological characteristics are improved such as the membrane stability (Ahn and Zimmerman, 2006), the use of water and nutrients, and assimilate partitioning (Senthil-Kumar et al., 2007).

Plant responses to high temperatures are mediated by both their inherent ability to survive (basal tolerance), and their ability to acquire tolerance to otherwise lethal temperatures (acclimation). These two mechanisms in cereals are due to the activation of different genetic systems (Maestri et al., 2002).

Several studies have shown that an acclimated plant will survive when exposed to a temperature that would be lethal to a non-acclimated plant (Hong et al., 2003), and that this is a major aspect of the acclimation response, termed acquired thermotolerance, that involves the expression of stress-responsive genes. The

Abbreviations: CMS, cell membrane stability; HSP, heat shock protein; RT-PCR, reverse transcription-polymerase chain reaction.

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role of these genes is to maintain homeostasis at the biochemical and molecular levels during stress (Hahn and Li, 1990). Thus, the best way to study the relevance of a physiological and/or biochemical trait for thermotolerance is to pre-expose seedlings or plants to a sub-lethal acclimation temperature. In the laboratory, thermotolerance can be induced by a gradual increase in temperature leading to normally lethal temperatures, as would be experienced in natural environments (Larkindale et al., 2005; Hikosaka et al., 2006). Because thermotolerance is a multigenic trait, the genetic variability in basal and acquired tolerance needs to be assessed using different approaches. Screening techniques are required to study genetic variability in the stress response and wheat genotypes have been screened based on cell membrane thermal stability (CMS) by measuring electrolyte diffusion resulting from heat-induced cell membrane leakage (Fokar et al., 1998; Senthil-Kumar et al., 2007). Several studies have provided evidence that the genetic variability in stress responses among wheat genotypes is mainly due to differential expression of stress-responsive genes and have reported correlations between the acquisition of thermotolerance and the synthesis and accumulation of HSPs, although the mechanisms underlying the thermal tolerance are not yet completely understood (Burke, 2001; Maestri et al., 2002; Senthil-Kumar et al., 2007).

However, other studies have failed to find a relationship between relevant physiological traits and stress tolerance in known tolerant genotypes/species, mainly because expression of these traits was examined by directly subjecting the plants to severe stress (Krishnan et al., 1989; Senthil-Kumar et al., 2007). In fact, it has been shown that genetic variability in these traits is detectable only following acclimation treatment prior to severe stress (Jayaprakash et al., 1998; Kumar et al., 1999; Burke, 2001; Srikanthbabu et al., 2002).

The overall objective of the study here reported was to characterise, by physiological and molecular approaches, different *Triticum durum* cultivars (cvs) for their ability to acquire thermotolerance and to accumulate *HSP* transcripts in response to different thermal treatments.

2. Materials and methods

2.1. Plant material and evaluation of cell membrane stability (CMS)

Seeds of *T. durum* cvs Adamello, Bradano, Ciccio, Cirillo, Claudio, Gargano, Ghibli, Lesina, Messapia, Ofanto, Parsifal, Sfinge, Sorriso, Svevo, Turchese, and Vesuvio were germinated in PERLIGRAN (Deutsche Perlite, Dortmund, Germany). Seedlings were grown at 25 °C under a constant light/dark regime with 16 h light and 8 h darkness and watered with tap water. Ten-day-old seedlings with similar leaf size were selected and used for thermal treatments, physiological, and molecular analyses. Ten-day-old seedlings were collected to measure cell membrane stability (CMS).

CMS test was performed according to Fokar et al. (1998); 3.5 cm long leaf segments were rinsed in distilled water and placed in a closed tube with 1 ml of distilled water. Three replicates for each cultivar were treated in a water bath at 52 °C for 1 h (T1), while the controls were kept at 10 °C (C1). 9 ml of distilled water was then added to each tube and the tubes were incubated at 10 °C for 24 h. The samples were brought to room temperature and the conductivity of the solution was measured. The tubes were autoclaved at 100 °C for 15 min (T2, C2) and the conductivity was measured again. CMS (%) was calculated as $[1 - (T1/T2)/1 - (C1/C2)] \times 100$. Means, standard deviations (SD) and comparison of the CMS values were performed using the Microsoft Excel Program. One-way ANOVA was employed for testing the significance of the resistant/sensitive genotypes.

2.2. Stress conditions and RNA extraction

Ten-day-old seedlings were heat stressed at 42 °C for 2 h (S) or acclimated by incubation at 34 °C for 1 (A_1), 6 (A_6), and 24 h (A_{24}). In some cases the acclimated seedlings were subsequently exposed at 42 °C for 2 h (A_1 + S, A_6 + S, A_{24} + S). The ability of the seedlings to recovery after the heat stress (S) was monitored by incubating the stressed seedlings at 22 °C for 2 h (R). At the end of the treatments whole seedlings were frozen in liquid nitrogen and stored at −40 °C until used for RNA extractions.

Total RNA was isolated by using the "SV Total RNA Isolation System" (Promega, Madison, WI, USA) according to the supplier's instructions, and quantified spectrophotometrically.

2.3. Database search, primer and probe design

A search of the GenBank database identified four sequences of *Triticum aestivum* encoding for HSP26 (class P), HSP23.6 (class M), HSP17.3 (class CII), and HSP16.9 (class CI) as well as a sequence coding for *T. durum* HSP101C isoform (Table 1). Primers were designed on the basis of these sequences using "Primer 3" software available at the web site http://biotools.umassmed.edu/bioapps/primer3_www.cgi and were prepared by Primm (Milan, Italy). These primers were used to obtain the corresponding *T. durum* cDNAs by RT-PCR. A fragment of the wheat *a-Tubulin* gene was also amplified as an internal control (Table 1).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Synthesis of cDNA was conducted using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions using oligo $(dT)_{15}$ as primer. PCR was performed in a reaction mixture containing 1 μl of cDNA sample in a final reaction mixture (50 μl) containing PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.1 mM dNTPs, 1 μM of each forward and reverse primer and 0.5 units of DyNAzyme DNA polymerase (Finnzymes, Espoo, Finland). In order to obtain the *T. durum HSP* specific sequences, RT-PCR was performed on RNAs extracted from heat stressed (S) seedlings, in a thermal cycler (MJ PTC-100, MJ Research, Sierra Point, CA, USA) using one step of 2 min at 94 °C, and then 30 cycles each of 30 s at 94 °C, 30 s at 57–59 °C (annealing temperature optimised for each individual gene), and 60 s at 72 °C, followed by a final step of 7 min at 72 °C. PCR products were separated in 1% (w/v) agarose, stained with ethidium bromide and observed under UV light.

2.5. Cloning, sequencing, and analysis of PCR products

PCR products were purified using the "Wizard SV gel and PCR clean-up system" (Promega) and cloned in the "PCR II TOPO Vector" included in the TA TOPO Cloning kit (Invitrogen) following the supplier's instructions. Plasmid DNA was sequenced using the "Big Dye Terminator Sequencing Kit" (Applied Biosystems, Foster City, CA, USA). Thermal cycler conditions, using MJ PTC-100, were as follows: 25 cycles each of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. Samples were analysed by an ABI Prism 3130 Genetic Analyser (Applied Biosystems).

DNA sequences obtained were compared with public databases using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

2.6. Quantitative (real-time) PCR

Primers and probes for quantitative (real-time) PCR were designed with "Primer Express 2" (Applied Biosystems) on the basis of the previously identified *T. durum* sequences. Primers

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Table 1
Primers used in RT-PCR and quantitative (real-time) PCR analysis.

Primer pairs		Sequence 5′–3′		Target and application
Forward	Reverse	Forward	Reverse	
HSP101Cf	101C3TER2	GTTGGACAGTATGAGGCCGT	CATTTCACCCCCAATTCAACAG	*TdHSP101C* RT-PCR (AF174433)
HSP26f	HSP26r	ATGGCCGCAGCGAATGCCCCCTTC	CACTGGACCTGCACGTCGATGACCTT	*TaHSP26* RT-PCR (AF097659)
HSP23.6f	HSP23.6r	ATGGCTTCCGCCGTCGATT	CTACTCGACCTTGACGTGGAA	*TaHSP23.6* RT-PCR (AF104108)
HSP17.3f	HSP17.3r	ATGGCGGGCATGGTGTTC	CAGGCGACCTGGACCTGGAT	*TaHSP17.3* RT-PCR (X58279)
HSP16.9bf	HSP16.9br	GATCGTGAGGCGGACGAA	AGCCGGAGATCTGGATGG	*TaHSP16.9b* RT-PCR (X64618)
*Ta*Tubf	*Ta*Tubr	ACCGCCAGCTCTTCCACCCT	TCACTGGGGCATAGGAGGAA	*α-tubulin* of *T. aestivum* RT-PCR (U76558)
101RTf	101RTr	CGAGAACTCCACGGTGTACATC	TGCTTGTCGACGCCATAGG	*TdHSP101C* Quantitative (real-time) PCR
HSP26.5RTf	HSP26.5RTr	AGCACAAGAAGGAGGCC	TCACTGGACCTGCACGT	*TdHSP26.5* Quantitative (real-time) PCR
HSP23.5RTf	HSP23.5RTr	AAGTACAACCGCCGCA	GAAGACGTCCTTGCGCT	*TdHSP23.5* Quantitative (real-time) PCR
HSP17.6RTf	HSP17.6RTr	CTCGGGTCCGGCGACA	CTCCGCCGTGTGCCG	*TdHSP17.6* Quantitative (real-time) PCR
HSP16.9RTf	HSP16.9RTr	CAATGCCGGATGGACTG	GTGAAGGCCGGGCTGGA	*TdHSP16.9* Quantitative (real-time) PCR
TubRTf	TubRTr	AGCCAGTTCCACTCCAACA	GAGGATGCTGCCAACAACTTC	*α-tubulin* of *T. aestivum* Quantitative (real-time) PCR
HSP101		6-FAM-TGCGCCCAGCAAG-MGB		*TdHSP101C* Taqman probe for quantitative (real-time) PCR
HSP23.5/26.5		6-FAM-AAGAACGGCGTGCT-MGB		*TdHSP23.5/26.5* Taqman probe for quantitative (real-time) PCR
HSP16.9/17.6		6-FAM-TCAGCGGCGAGCGCA-MGB		*TdHSP16.9/17.6* Taqman probe for quantitative (real-time) PCR
α-tubulin		VIC–CGTTGAAGACAAGGAA-MGB		*α-tubulin* Taqman probe for quantitative (real-time) PCR

Target genes and their relative GeneBank accession numbers are reported.

were obtained from Primm and probes from Applied Biosystems (Table 1). Quantitative PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems), using the v. 1.1 software for data analysis. For each reaction 20 ng of first strand cDNA were used with 1× TaqMan Universal Mastermix (Applied Biosystems), 250 nM probes, 200 nM primers. Reaction conditions were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was amplified in triplicate, and each experiment was repeated at least twice with cDNAs obtained from independent experiments. *HSP* transcript levels were normalized with respect to *a-Tubulin* gene. All calculations and statistical analyses were performed as described in the ABI 7500 Sequence Detection System User Bulletin 2 (Applied Biosystems), using Microsoft Excel program. The specificities of the amplicons were determined by sequencing.

3. Results

3.1. CMS evaluation

Sixteen wheat genotypes were characterised for both basal and acquired thermotolerance by evaluation of CMS that is considered a standard method to evaluate thermotolerance (Fokar et al., 1998; Blum et al., 2001; Wahid et al., 2007). The data obtained were analysed by one-way ANOVA test to evidence variability in heat stress response. Differences were observed in their basal thermotolerance (Fig. 1) with cvs Claudio and Svevo exhibiting the highest basal thermotolerance and cvs Gargano, Ofanto, Vesuvio and Bradano exhibiting the lowest values. After acclimation, Messapia exhibited the highest CMS value which was almost twice that registered for its basal thermotolerance. In contrast, Bradano exhibited the lowest CMS value, which was even lower than the value detected for basal tolerance. Among the other genotypes, Adamello, Gargano, Cirillo, Parsifal, Sorriso, Sfinge, Lesina, Ghibli, Vesuvio, and Ofanto all exhibited increases in CMS values after acclimation, and thus appeared to be able to acquire thermotolerance. On the contrary, cvs Ciccio, Claudio, Svevo and Turchese did not appear to be able to acquire thermotolerance, with either little change in their CMS values after acclimation (Ciccio, Turchese) or decreases (Claudio, Svevo).

3.2. Cloning, sequencing, and analysis of PCR products

In order to identify possible correlations between differences in thermotolerance and the induction of *HSP* genes, we determined *HSP* gene expression by quantitative (real-time) PCR. The genes analysed were selected on the basis of their previously hypothesised correlation with thermotolerance and code for *Td*HSP101C (Gulli et al., 2007) and for small HSPs (Senthil-Kumar et al., 2007; Wahid et al., 2007), representative of four different classes: plastidial (class P), mitochondrial (class M), and cytoplasmic (classes I and II).

Primers for the *TdHSP101C* gene were designed based on a *T. durum* sequence published previously by our group (Gulli et al., 2007). Primers for the small *HSP* sequences were selected based on the corresponding *T. aestivum* genes available in GenBank (Table 1). The amplification products obtained were cloned and sequenced to verify their identity. As reported in Table 2 the sequence analysis and BLAST search comparison indicated that the cDNAs isolated were novel *T. durum* sequences belonging to the *HSP* gene family. The sequences were named according to their molecular weight as *TdHSP26.5* (class P), *TdHSP23.5* (class M), *TdHSP17.6* (class CII) and *TdHSP16.9* (class CI).

3.3. HSP gene expression

Six of the sixteen cvs were selected as representative of the various types of thermotolerance response and were used for quantitative *HSP* transcription analysis by quantitative (real-time) PCR. The cvs selected were Messapia, which had the greatest ability to acquire thermotolerance, Gargano, Vesuvio and Lesina, which were able to acquire thermotolerance although at different levels, and Claudio and Bradano that were not able to become thermotolerant. The analysis was performed on cDNAs obtained from control seedlings (C) grown at 25 °C, and seedlings subjected to the heat stress (S) or to acclimation conditions (A_{24}) followed by the heat stress (A_{24} + S). In all of these genotypes, *TdHSP101C* transcripts were already detectable, although at very low level, at 25 °C; the maximum expression level of the *TdHSP101C* gene was observed when the stress was imposed after acclimation (except for Bradano) (Fig. 2a). The plastidial *TdHSP26.5* was maximally expressed after acclimation in cvs Messapia, Gargano, Lesina, and Vesuvio (Fig. 2b). On the contrary, *TdHSP23.5* transcripts reached their maximum levels in samples deriving from non-acclimated seedlings with the exception of cv Gargano (Fig. 2c).

Levels of the transcripts for cytoplasmic *TdHSP17.6* and *TdHSP16.9* were much lower than those of the other transcripts analysed. The *TdHSP17.6* transcripts accumulated at the highest level after S (Fig. 2d). The levels of the *TdHSP16.9* transcripts were greatest after S in cvs Bradano and Messapia but lower in cvs Clau-

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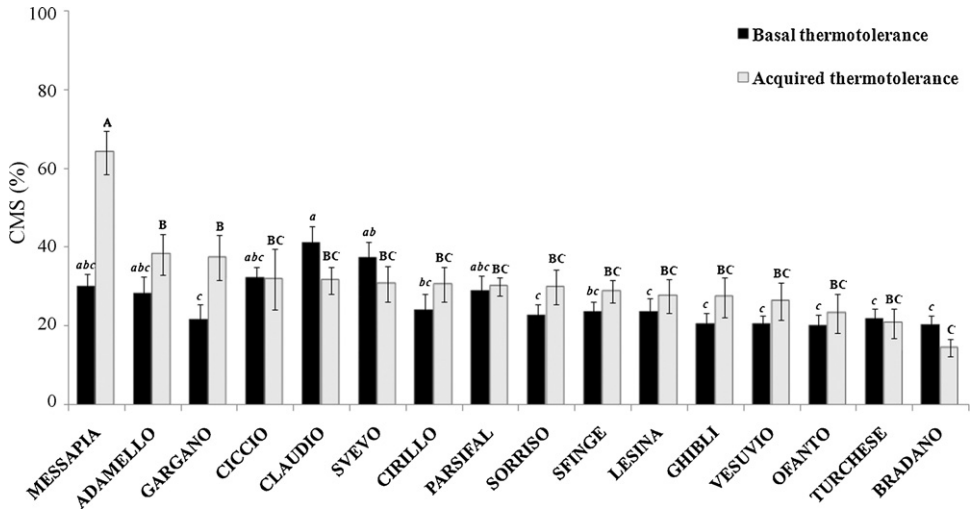

Fig. 1. Identification of the levels of basal and acquired thermotolerance in different durum wheat cultivars. Individual bars represent the CMS values determined as reported in Section 2. Ten independent measurements were performed for each test and the reported value is the mean ± S.E. The letters on the top of each column are relative to basal (a–c) and acquired (A–C) thermotolerance, and indicate statistical significant difference ($P < 0.005$, ANOVA test).

dio and Gargano. No transcripts for *TdHSP16.9* were detectable in cvs Lesina and Vesuvio under the experimental conditions used (Fig. 2e).

On the basis of the above reported results, the thermotolerant phenotypes (identified by CMS evaluation) did not correlate with the expression of the selected *HSP* genes. For this reason we decided to perform a more detailed comparison of the two most contrasting genotypes. Thus Messapia and Bradano seedlings were subjected to different types and durations of temperature stresses: 24 h at 34 °C (A_{24}), 24 h at 34 °C followed by 2 h at 42 °C (A_{24} + S), 2 h at 42 °C (S), 2 h at 42 °C followed by 2 h of recovery at 25 °C (S + R). The results reported in Fig. 3 show that acclimation (A_{24}) induced quite different levels of *TdHSP101C* transcripts in the two cultivars, with the level in Messapia being almost fivefold that observed in Bradano (Fig. 3a). When heat stress was imposed after acclimation (A_{24} + S) a dramatic increase of *TdHSP101C* transcripts was observed in Bradano, an increase, although lower, occurred also in Messapia. In the absence of acclimation (S) similar levels of *TdHSP101C* transcripts were observed in both cvs. The levels of transcripts when heat stress was followed by recovery (S + R) decreased in both cultivars although the decrease was more evident in Bradano (Fig. 3a). The production of *TdHSP26.5* transcripts was induced in the two cultivars almost at the same level by all the treatments except for A_{24} in which a difference between Bradano and Messapia was clearly evident (Fig. 3b). The *TdHSP23.5* transcript level was always higher in Messapia especially after acclimation (A_{24}) treatment (Fig. 3c). The expression of *TdHSP17.6* and *TdHSP16.9* genes was similar in Bradano and Messapia, except when acclimation was followed by heat stress (A_{24} + S), in which case the transcript levels were higher in Messapia than in Bradano (Fig. 3d and e).

The results above reported indicate that the major differences in *HSP* gene induction between the thermotolerant (Messapia) and thermosensitive (Bradano) cvs were observed when the seedlings were subjected to acclimation treatment (A_{24}). On the basis of this observation we decided to investigate how *HSP* gene expression is modulated by shorter acclimation periods, i.e. 1 h at 34 °C (A_1) and 6 h at 34 °C (A_6), followed or not by the stress (S). The results reported in Fig. 4 show that for both cvs 1 h of acclimation treatment at 34 °C (A_1) is sufficient to induce the maximum level of transcripts of all the selected genes. A prolonged acclimation period resulted in an evident reduction of the *HSP* transcripts in Bradano (sensitive cv) while in Messapia (thermotolerant cv) this reduction was much smaller (Fig. 4b). The stress treatments following the different acclimation periods did not have dramatic effects on accumulation of *HSP* transcripts in Messapia, on the contrary in Bradano a dramatic increase in the *HSP* transcripts was observed.

4. Discussion

Abiotic stresses such as drought, high salinity, cold, and heat greatly reduce the growth and yield of crops. In particular, high temperature represents a significant constraint to the cultivation of important crops, such as wheat, in large areas of the world. The ability of the plant to cope with high temperature stress varies within species and it is therefore important to identify intrinsically tolerant genotypes. So far this aspect has been limited by the lack of high throughput screening methods to screen for variability and to identify tolerant genotypes that can be used for crop genetic improvement.

Tolerance to high temperature has two components, the basal or constitutive thermotolerance, due to evolutionary thermal adaptation to the habitat, and the acquired thermotolerance, due to acclimation, i.e. the ability to survive lethal temperatures following the exposure to a mild heat stress (Burke, 2001; Klueva et al., 2001; Maestri et al., 2002).

Here we report studies at the physiological and molecular levels of a group of durum wheat genotypes differing in their basal ability to respond to heat stress and to acquire thermotolerance. The CMS

Table 2
Characteristics of cDNA clones encoding *T. durum* small HSPs.

cDNA name	Length (bp)	GeneBank accession number	HSP type	Best identity
TdHSP26.5	717	AJ971373	P	AAC96317, HSP26 (*T. aestivum*); 99%
TdHSP23.5	648	AM709764	M	AAD03605, HSP23.6 (*T. aestivum*); 99%
TdHSP17.6	483	AJ971359	CII	CAA41218, HSP17.3 (*T. aestivum*); 93%
TdHSP16.9	456	AM709754	CI	CAA45902, HSP16.9 (*T. aestivum*); 94%

cDNA length in bp, GeneBank accession number, HSP type and best identity in % of the deduced aminoacid sequences are reported.

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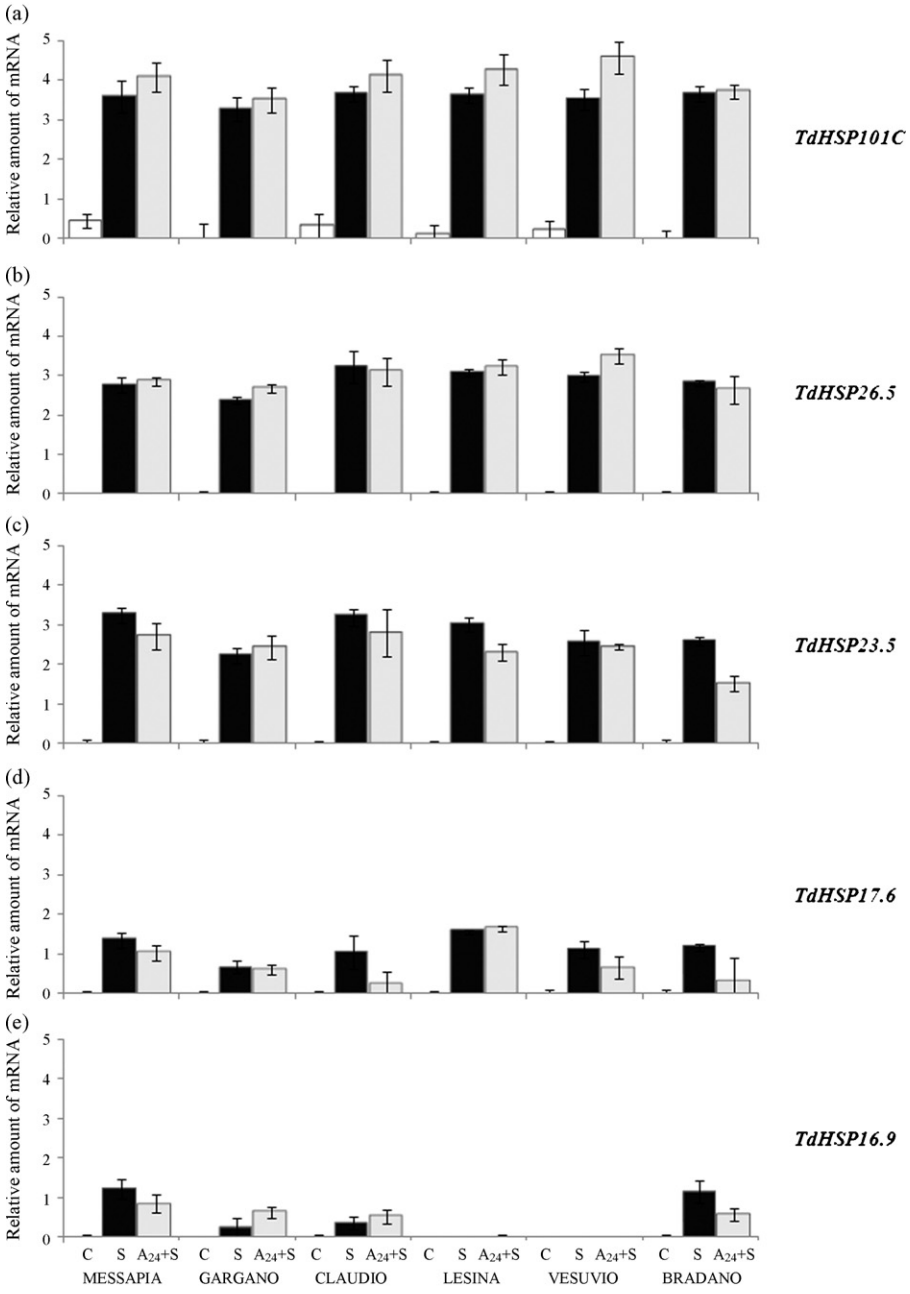

Fig. 2. Expression patterns of *TdHSP101C* (a), plastidial *TdHSP26.5* (b), mitochondrial *TdHSP23.5* (c), cytoplasmic CII *TdHSP17.6* (d), and cytoplasmic CI *TdHSP16.9* (e) genes in response to different thermal treatments obtained by quantitative (real-time) PCR in *T. durum* cvs. Error bar represents ±S.E. from three replicates for each sample. C = 25 °C; S = 2 h at 42 °C; A_{24} + S = 24 h at 34 °C + 2 h at 42 °C.

test was used to measure both types of tolerance. This test is a standard method to measure thermotolerance that has been already used to assess genetic variability in bread wheat, and is considered to be predictive of the plant behaviour under heat stress in the field (Fokar et al., 1998; Blum et al., 2001; Wahid et al., 2007). Comparison of the data obtained for basal and acquired thermotolerance showed a significant variability among the genotypes and allowed each genotype to be classified according to its capability to acquire thermotolerance. On the basis of the results of this analysis we further characterised the genotypes that exhibited contrasting behaviour. In particular, Bradano was chosen because it was one of the most sensitive to the stress, and appeared to be unable to acquire thermotolerance. Vesuvio and Lesina were among the most sensitive, but acquired good thermotolerance levels. Claudio was the most resistant but was not able to acquire thermotolerance. Gargano had low intrinsic thermotolerance, but after acclimation became quite tolerant. Messapia acquired the highest level of thermotolerance after acclimation.

It is generally accepted that a correlation exists between the development of acquired thermotolerance and the synthesis of HSPs. However, until now a strong relationship between these has not been demonstrated (Senthil-Kumar et al., 2007; Wahid et al., 2007).

In the present work we initially identified and characterised, in *T. durum*, four cDNAs coding for small HSPs belonging to the different classes (class P plastidial, class M mitochondrial, class I and class II cytoplasmic), and then analysed, in seedlings subjected to different heat stress conditions, the expression of these genes and of the previously isolated *TdHSP101C* (Gulli et al., 2007). The expression profiles of each gene were different, suggesting that, as already

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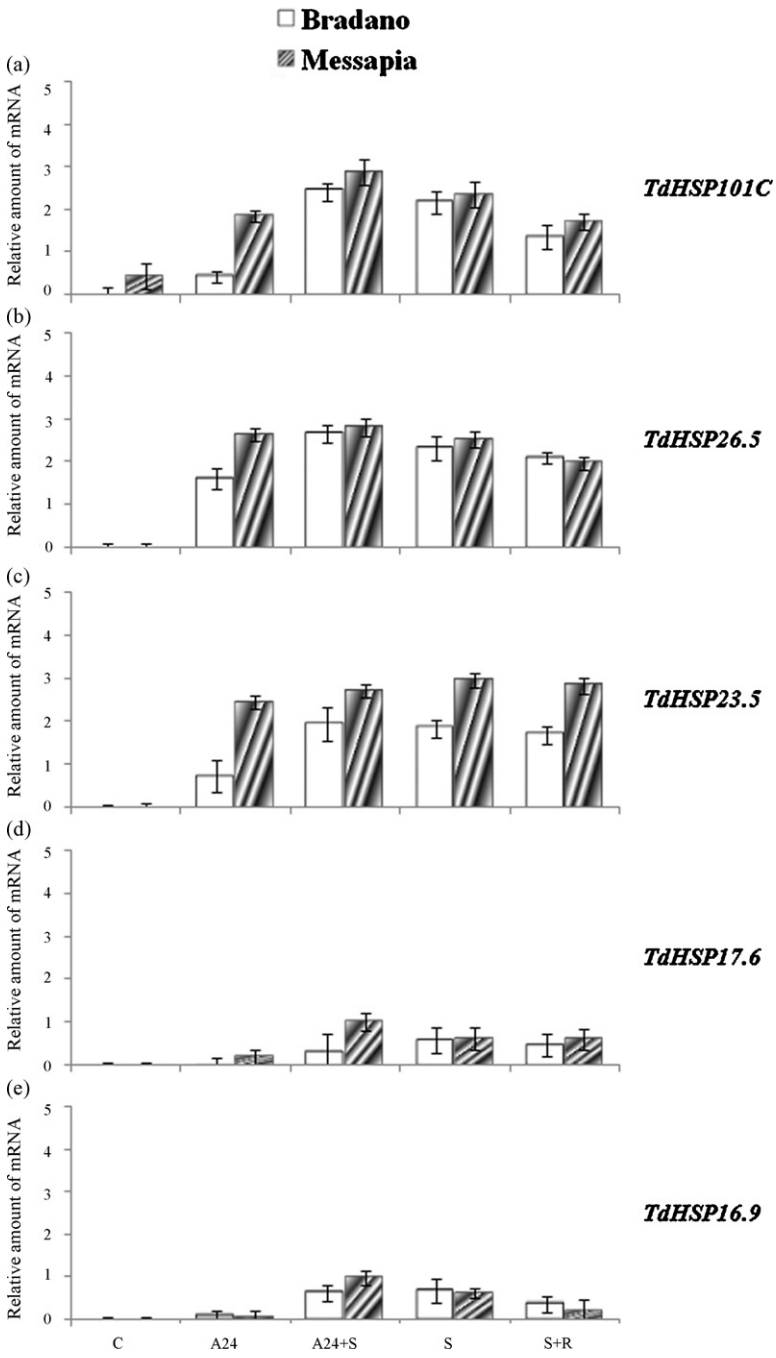

Fig. 3. Expression patterns of *TdHSP101C* (a), plastidial *TdHSP26.5* (b), mitochondrial *TdHSP23.5* (c), cytoplasmic CII *TdHSP17.6* (d), and cytoplasmic CI *TdHSP16.9* (e) genes in response to different thermal treatments obtained by quantitative (real-time) PCR in the two contrasting cvs: Bradano and Messapia. Error bar represents ±S.E. from three replicates for each sample. C = 25 °C; A_{24} = 24 h at 34 °C; A_{24} + S = 24 h at 34 °C + 2 h at 42 °C; S = 2 h at 42 °C; S + R = 2 h at 42 °C + 2 h at 25 °C.

reported, each member of the *HSP* gene family might have a distinct function in the molecular response to high temperature. However, each HSP subfamily also has a known chaperone activity mediated by a common mechanism of action. All the chaperones contributing to this network, acting in concert, are responsible for the general protective effect of HSPs (Wahid et al., 2007).

In the case of *TdHSP26.5*, transcript accumulation was higher in the genotypes that are able to acquire thermotolerance. For example, Bradano and Claudio that, on the bases of CMS values, were unable to acquire thermotolerance did not accumulate more *TdHSP26.5* transcripts when heat stress was imposed after acclimation. These results are in accordance with a number of studies showing the importance of chloroplast small HSPs in thermotolerance. In particular a genetic relationship between acquired thermotolerance and the expression of a plastid-localised HSP26 was demonstrated using recombinant inbred lines of wheat (Joshi et al., 1997). Furthermore, analyses of tomato mutants defective in one or more chloroplast small HSPs indicated that genetic variation observed in the production of chloroplast small HSPs may have a determinant role in photosynthetic system and whole plant tolerance (Heckathorn et al., 1998).

A general conclusion from the data above reported is that the diverse genotypes exhibit different levels of induction for each *HSP* gene. Nevertheless, this was not sufficient to identify strict correlations between thermotolerant phenotype and transcript accumulation for any of the genes analysed except *TdHSP26.5*.

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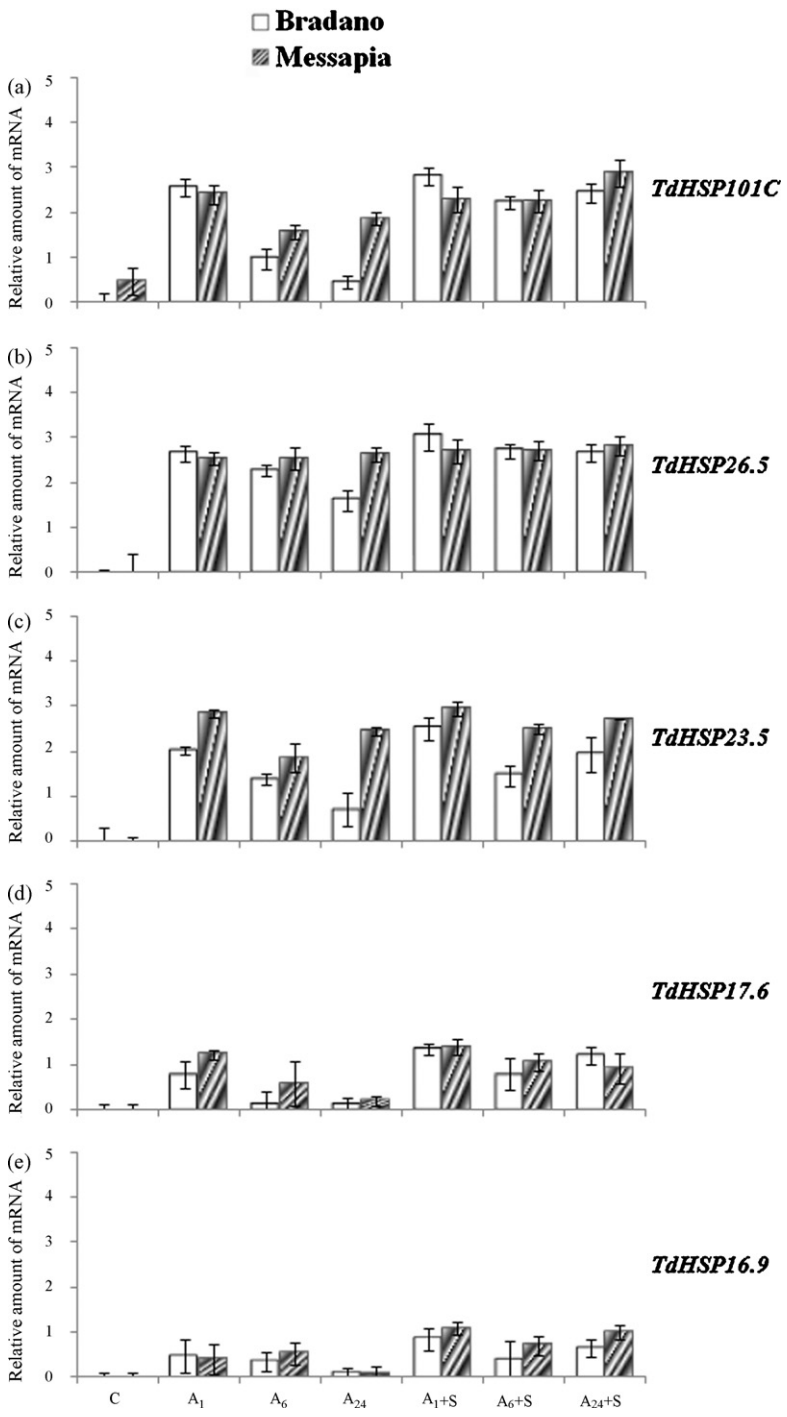

Fig. 4. Expression patterns of *TdHSP101C* (a), plastidial *TdHSP26.5* (b), mitochondrial *TdHSP23.5* (c), cytoplasmic CII *TdHSP17.6* (d), and cytoplasmic CI *TdHSP16.9* (e) genes in response to different thermal treatments obtained by quantitative (real-time) PCR in the two contrasting cvs: Bradano and Messapia. Error bar represents ±S.E. from three replicates for each sample. C = 25 °C; A_1 = 1 h at 34 °C; A_6 = 6 h at 34 °C; A_{24} = 24 h at 34 °C; A_1 + S = 1 h at 34 °C + 2 h at 42 °C; A_6 + S = 6 h at 34 °C + 2 h at 42 °C; A_{24} + S = 24 h at 34 °C + 2 h at 42 °C.

In order to establish a possible relationship between *HSP* gene induction and the acquisition of thermotolerance we have specifically compared the two genotypes exhibiting the most contrasting behaviour: Bradano (the most sensitive) and Messapia (the most thermotolerant). Different stress conditions were used in order to understand whether differences in the thermotolerant habit paralleled *HSP* transcript accumulation. Interestingly, Messapia cv is able to accumulate higher levels of transcripts for all the *HSP*s analysed and also to maintain high level of these transcripts throughout the acclimation period. In contrast, in Bradano all the transcripts accumulate quite early (A_1) but also rapidly decrease. This means that Bradano is active in the synthesis of new *HSP* transcripts when the stress is imposed, while Messapia is not, since the *HSP* specific transcripts remain at a high level throughout the acclimation period.

It should be noted that among the *HSP* genes analysed only *TdHSP23.5*, which encodes a mitochondrial small HSP, is induced in all the analysed stress conditions but at different levels in the two genotypes. In fact, the *TdHSP23.5* transcript levels are higher in Messapia, that is the thermotolerant genotype. This supports (at

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a quantitative rather than qualitative level) data reported for cereals in which the presence of multiple small mitochondrial HSPs in maize was associated with higher thermotolerance when compared to wheat and rye, in which only one mitochondrial small HSP was expressed (Wahid et al., 2007). This hypothesis is also supported by data obtained using *Arabidopsis* cell cultures (Rikhvanov et al., 2007). Although the data are still not conclusive, they suggest that in plants, as well as in animals, mitochondria are sensors of stress that initiate the cellular response to the specific stress (Jones, 2000).

Although the picture is still far from clear, there is substantial evidence that induction of *HSP* gene expression plays a role in the acquisition of thermotolerance. Furthermore, the level of mitochondrial *HSP* transcripts seems to be related to the ability to acquire thermotolerance in some cases. However, further investigations are needed to identify factors modulating plant heat stress response and to understand molecular bases of plant phenotypic flexibility which leads to thermotolerance. Direct evidence strictly linking HSP accumulation with acquired thermotolerance has been prevented by the lack of mutants needed to establish a cause and effect relationship. This shortfall may to some extent be circumvented by using the genomic tools available in *Arabidopsis*, including the large collection of T-DNA insertion lines and transgenic technologies, as well as applications of genomics, transcriptomics, and proteomics approaches in wheat. The elucidation of molecular basis of response and tolerance mechanisms is essential to obtain engineered plants that can tolerate heat stress. This will pave the way for producing crops which can give economic yield under heat stress conditions.

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References

Ahn, Y.-J., Zimmerman, J.L., 2006. Introduction of the carrot HSP17. 7 into potato (*Solanum tuberosum* L.) enhances cellular membrane stability and tuberization in vitro. Plant Cell Environ. 29, 95–104.

Blum, A., Klueva, N., Nguyen, H.T., 2001. Wheat cellular thermotolerance is related to yield under heat stress. Euphytica 117, 117–123.

Burke, J.J., 2001. Identification of genetic diversity and mutations in higher plant acquired thermotolerance. Physiol. Plantarum 112, 167–170.

Fokar, M., Blum, A., Nguyen, H.T., 1998. Heat tolerance in spring wheat II. Grain filling. Euphytica 104, 9–15.

Gulli, M., Corradi, M., Rampino, P., Marmiroli, N., Perrotta, C., 2007. Four members of the *HSP101* gene family are differently regulated in *Triticum durum* Desf. FEBS Lett. 581, 4841–4849.

Hahn, G.M., Li, G.C., 1990. Thermotolerance, thermoresistance and thermosensitization. In: Morimoto, R.I., Tissieres, A., Georgopoulos, C. (Eds.), Stress Proteins in Biology and Medicine. Cold Spring Harbor Press, Berlin, Germany, pp. 79–100.

Heckathorn, S.A., Downs, T.D., Sharkey, T.D., Coleman, J.S., 1998. The small methionine-rich chloroplast heat shock protein protects photosystem II electron transport during heat stress. Plant Physiol. 116, 439–444.

Hikosaka, K., Ishikawa, K., Borjigidal, A., Muller, O., Onoda, Y., 2006. Temperature acclimation of photosynthesis: mechanisms involved in the changes in temperature dependence of photosynthetic rate. J. Exp. Bot. 57, 291–302.

Hong, S.W., Lee, U., Vierling, E., 2003. Arabidopsis *hot* mutants define multiple function required for acclimation to high temperatures. Plant Physiol. 132,757–767.

Iba, K., 2002. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annu. Rev. Plant Biol. 53, 225–245.

Ismail, A.M., Hall, A.E., 1999. Reproductive-stage heat tolerance, leaf membrane thermostability and plant morphology in cowpea. Crop Sci. 39, 1762–1768.

Jayaprakash, T.L., Ramamohan, G., Krishnaprasad, B.T., Kumar, G., Prasad, T.G., Mathew, M.K., Udayakumar, M., 1998. Genotypic variability in differential expression of *lea2* and *lea3* genes and proteins in response to salinity stress in Fingermillet (*Eleusine coracana* Gaertn) and rice (*Oryza sativa* L.) seedlings. Ann. Bot. 82, 513–522.

Jones, A., 2000. Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? Trends Plant Sci. 5, 225–230.

Joshi, C.P., Klueva, N.Y., Morrow, K.J., Nguyen, H.T., 1997. Expression of a unique plastid-localized heat-shock protein is genetically linked to acquired thermotolerance in wheat. Theor. Appl. Genet. 95, 834–841.

Klueva, N.Y., Maestri, E., Marmiroli, N., Nguyen, H.T., 2001. Mechanisms of thermotolerance in crops. In: Basra, S.S. (Ed.), Crop Responses and Adaptations to Temperature. Stress Food Products Press, Binghamton, NY, pp. 177–217.

Krishnan, M., Nguyen, H.T., Burke, J.J., 1989. Heat shock protein synthesis and thermal tolerance in wheat. Plant Physiol. 90, 140–145.

Kumar, G., Krishnaprasad, B.T., Savitha, M., Gopalakrishna, R., Mukhopadhyay, K., Ramamohan, G., Udayakumar, M., 1999. Enhanced expression of heat shock protein in thermotolerant lines of sunflower and their progenies selected on the basis of temperature-induction response (TIR). Theor. Appl. Genet. 99,359–367.

Larkindale, J., Mishkind, M., Vierling, E., 2005. Plant response to high temperature. In: Jenks, M.A., Hasegawa, P.M. (Eds.), Plant Abiotic Stress. Blackwell, Oxford, UK, pp. 100–144.

Maestri, E., Klueva, N., Perrotta, C., Gulli, M., Nguyen, H.T., Marmiroli, N., 2002. Molecular genetics of heat tolerance and heat shock protein in cereals. Plant Mol. Biol. 48, 667–681.

Nover, L., 1991. Heat Shock Response. CRC Press, Boca Raton, FL.

Rikhvanov, E.G., Gamburg, K.Z., Varakina, N.N., Rusaleva, T.M., Fedoseeva, I.V., Tauson, E.L., Stupnikova, I.V., Stepanov, A.V., Borovskii, G.B., Voinikov, V.K., 2007. Nuclear-mitochondrial cross-talk during heat shock in *Arabidopsis* cell culture. Plant J. 52, 763–778.

Saadalla, M.M., Shanahan, J.F., Quick, J.S., 1990. Heat tolerance in winter wheat: I. Hardening and genetic effects on membrane thermostability. Crop Sci. 30, 1243–1247.

Sangwan, V., Orvar, B.L., Beyerly, J., Hirt, H., Dhindsa, R.S., 2002. Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. Plant J. 31, 629–638.

Schöffl, F., Prandl, R., Reindl, A., 1999. Molecular responses to heat stress. In: Shinozaki, K., Yamaguchi-Shinozaki, K. (Eds.), Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants. R. G. Landes Co., Austin, Texas, pp. 81–98.

Senthil-Kumar, M., Kumar, G., Srikanthbabu, V., Udayakumar, M., 2007. Assessment of variability in acquired thermotolerance: potential option to study genotypic response and the relevance of stress genes. J. Plant Physiol. 164, 111–125.

Srikanthbabu, V., Kumar, G., Krishnaprasad, B.T., Gopalakrishna, R., Savitha, M., Udayakumar, M., 2002. Identification of pea genotypes with enhanced thermotolerance using temperature induction response (TIR) technique. J. Plant Physiol. 159, 535–545.

Vierling, E., 1991. The role of heat shock proteins in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 579–620.

Wahid, A., Gelani, S., Ashraf, M., Foolad, M.R., 2007. Heat tolerance in plants: an overview. Environ. Exp. Bot. 61, 199–223.

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