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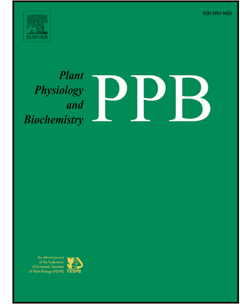

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Patrizia Rampino, Mariarosaria De Pascali, Carla Perrotta, Mariolina Gullì

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Contribution

PR planned and designed the study, performed most of the experimental part of this work, analyzed and interpreted the data, collaborated in writing the manuscript; MDP performed most of the experimental part of this work, analyzed and interpreted the data, collaborated in writing the manuscript; CP analyzed and interpreted the data, collaborated in writing the manuscript; MG planned and designed the study, interpreted the data and wrote the manuscript with the fundamental help of CP and PR. All authors contributed to the discussion and approved the final manuscript.

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New gene functions are involved in the thermotolerance of the wild wheat relative *Aegilops umbellulata*

Running title: Heat stress gene induction in *Ae. umbellulata*

Patrizia Rampino[1]*§, Mariarosaria De Pascali[1]§, Carla Perrotta[1] and Mariolina Gullì[2]

[1]Department of Biological and Environmental Sciences and Technologies, University of Salento, via Monteroni 165, 73100 Lecce, Italy

[2]Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area delle Scienze, 11/A, 43124 Parma, Italy

*Corresponding author.

E-mail address: patrizia.rampino@unisalento.it (Patrizia Rampino).

§ Both authors equally contributed to this work.

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ABSTRACT

Wheat is one of the most important food crops in the world for human consumption, like all plants it is exposed to environmental stresses including high temperatures. The deleterious effect of high temperatures negatively affects plant growth and development, leading to reduced viability and yield. These effects can be reduced by improvement of thermotolerance through innovative breeding strategies, based on the expansion of the genetic pool available, by exploring important genetic functions from wheat wild progenitors. Improving the genetic thermotolerance characteristics of wheat requires greater understanding of genetic bases of thermotolerance, through identification of high temperature stress related genes. A good source of new useful alleles is given by *Aegilops* species characterized by thermotolerant habits.

In this study we have classified as thermotolerant or thermosensitive, on the basis of physiologic tests, some accessions of wheat wild relative species belonging to *Aegilops* and *Triticum* genera. A thermotolerant accession of *Aegilops umbellulata* (AUM5) was selected, subjected to different thermal treatments and analysed at transcriptional level. By differential display reverse transcriptase polymerase chain reaction (DDRT-PCR), we investigated modulation of gene expression elicited by heat treatments. This approach allowed the identification of various transcript-derived fragments (TDFs) produced by AUM5 in response to different thermal treatments. The functions of the inducible unique genes in the molecular determination of thermotolerance process are discussed.

Keywords: *Aegilops umbellulata*, Cell membrane stability, Differential display RT-PCR, Gene expression, High temperature stress; Wheat wild relatives.

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1. Introduction

Climate changes have a deleterious impact on plant growth and development, often leading to a decrease in crop yield, due also to increasing temperatures (Lorenz et al., 2019; Janni et al., 2020). The damage extent caused by high temperature stress is influenced by plant thermotolerance that is the ability to cope with high temperatures. Although the genetic bases of thermotolerance are still not completely elucidated, it is known that thermotolerance has two components: the basal and the acquired one. Basal thermotolerance is a constitutive component corresponding to the evolutionarily achieved thermal adaptation of plants; acquired thermotolerance results in the ability of a plant to survive normally lethal temperatures after exposure to milder high temperatures. It has been postulated that these two types of thermotolerance are based on different molecular mechanisms (Song et al., 2012; Rampino et al., 2019).

Wheat is one of the five most important food crops for direct human consumption (Faostat, 2017, http://faostat3.fao.org), it is also the second most important crop providing carbohydrates and the first for vegetal protein supply in human food. Wheat, like all plants, is constantly exposed to a multitude of environmental stresses owing to its sessile nature. High temperatures have an immediate effect on plant growth and development leading to reduced viability and yield; it has been estimated that wheat production is dramatically reduced by rise in temperature (6% for each degree) (Akter and Islam, 2017; Zampieri et al., 2017). A good strategy to reduce the deleterious effects of heat stress is the improvement of wheat thermotolerance that could be achieved using new breeding strategies, such as the expansion of the genetic pool available for breeding (Feuillet et al., 2008; Tester and Langridge, 2010; Janni et al., 2020). To build upon past successes in the development of stress tolerant wheat, it will be necessary to expand the variability currently available in gene pools drawing from genetic background of wild progenitors.

Numerous studies have shown that wild progenitors have major genetic resources of plant tolerance to stressful environments (Waines, 1994; Zhang et al., 2017; Robinson et al., 2000) and that they can be an important source of new alleles useful for breeding programs. A good example is given by *Aegilops* species that were used to incorporate a number of biotic resistance genes into wheat varieties, and this genus is also contributing toward improvement of complex traits such as yield as well as drought and heat tolerance (Kishii, 2019).

Aegilops umbellulata is an important diploid donor species of several polyploid *Aegilops* that harbor the U genome. Amphidiploids between *Ae. umbellulata* and tetraploid/hexaploid wheats have been used as a bridge for transferring genes from *Ae. umbellulata* into common wheat (Song et al., 2019). *Ae. umbellulata,* a Mediterranean-western Asiatic grass, is one of the 11 diploid species in the *Aegilops* genus that possesses seven pairs of chromosomes (2n = 2x = 14, UU genome) (Edae

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et al., 2016). The standard chromosome karyotype that has been completed for *Ae. umbellulata*, indicates that six of the seven chromosomes had about the same size (Friebe et al., 1995).

The success in improving genetic characteristic of wheat for better heat stress management requires greater understanding of the genetic bases of thermotolerance through identification, cloning, and characterization of stress related genes.

With the aim to identify new genetic functions involved in thermotolerance, we have subjected to different thermal treatments some accessions of wheat wild relative species belonging to *Aegilops* and *Triticum* genera and we have classified them, on the basis of physiologic tests, as thermotolerant or thermosensitive. A thermotolerant accession of *Ae. umbellulata*, named AUM5, was selected and analyzed at transcriptional level by differential display reverse transcriptase polymerase chain reaction (DDRT-PCR), to investigate modulation of gene expression elicited by heat treatments.

DDRT-PCR is an efficient and sensitive RNA finger-printing technique that offers several advantages over other methods of gene expression analysis (Vôgeli-Lange et al., 1996; Meng et al., 2012; Huang et al., 2015). This technique has been widely utilized in plants as a simple method for identification and cloning of differentially expressed genes in absence of previous genomic information. In this study, we identified, by DDRT-PCR, a number of transcript-derived fragments (TDFs) produced by *Ae. umbellulata* in response to different thermal treatments. The corresponding transcripts were identified by an *in silico* analysis, and the function of the inducible unique genes in the basal and acquired thermotolerance process are discussed.

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2. Materials and methods

2.1. Plant material and stress treatments

Seeds of *T. turgidum subsp. dicoccoides* and subsp. *dicoccum* were kindly provided by A. Blanco (University of Bari, Italy), seeds of *Aegilops* spp. were kindly provided by C. Ceoloni (Tuscia University, Italy).

Seeds of *Triticum* spp. and *Aegilops* spp. were germinated in Petri dishes at 23°C in the dark for 3 days; seedlings were transferred in pots with soil, kept in a growth chamber at 23°C and a light/dark cycle of 16 h/8 h, 65% humidity for 2 weeks. Plants grown at 23°C were used as control (C); heat stress conditions were applied for 24 h at 34°C (Treatment 1, T1); 24 h at 34°C followed by 2 h at 42°C (Treatment 2, T2) or 2 h at 42°C (Treatment 3, T3).

For each treatment, leaves were collected, immediately frozen in liquid nitrogen and stored at -80°C

before RNA extraction.

2.2. Cell membrane stability evaluation

Evaluation of cell membrane stability (CMS) was carried out as previously described (Rampino et al., 2009) on seedlings grown for two weeks at 23°C in growth chamber (basal thermotolerance) and on seedlings that, after two weeks at 23°C, were incubated for 24 h at 34°C (T1, acquired thermotolerance). Leaf segments 3.5 cm long were collected from seedlings and rinsed in distilled water, subsequently they were placed in a tube containing distilled water (2 mL) and incubated for 1 h at 50°C to induce the heat stress; control samples were kept at 10°C. To each tube, 8 mL of distilled water were added and tubes were kept for 24 h at 10°C followed by 1 h at room temperature for stabilization. Using a Crison GLP31 conductivity meter (Crison Instruments, Barcelona, Spain), the conductivity was recorded before and after samples autoclavation for 15 min at 100°C/0.10 MPa.

Ten replicates for each genotype were analyzed and CMS (%) was calculated as [1–(T1/T2)/1–(C1/C2)] × 100, where T1=conductivity after heat shock at 50°C, T2=conductivity after autoclaving, C1=conductivity in control conditions (10°C) and C2=conductivity of control samples after autoclaving.

2.3. RNA preparation, reverse transcription and DDRT-PCR

Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI), following the manufacturer's instructions, and spectrophotometrically quantified. To remove potential residual DNA, RNA was treated with RNase-free DNase I (Takara Bio Inc., Shiga, Japan) for 30 min at 37°C. The RNA integrity was tested by electrophoresis on 1% agarose gel.

First-strand cDNA was synthesized using a M-MLV Reverse Transcriptase RNase H minus (Promega), in a total volume of 20 μL at 37°C for 60 min, with 200 ng total RNA, 4 μL 5× RT buffer, 20 μM each dNTPs, 200 nM H-T_{11}G anchor primer, RNasin 40 U. Amplification of the reverse transcribed RNA (cDNA) was performed in a total volume of 20 μL containing 2 μM dNTPs, 200 nM anchor primer, 200 nM arbitrary primer H-AP1 (AAGCTTGATTGCC) or H-AP2 (AAGCTTCGACTGT), 2 μL first strand cDNA, and 1 U Taq DNA polymerase (Takara). PCR amplifications were performed in a thermocycler programmed for one step at 95°C for 30 s and 40 cycles each of 30 s at 95°C, 2 min at 40°C, 1 min at 72°C, and a final extension for 5 min at 72°C. To identify the differentially displayed transcripts, 6% denaturing polyacrylamide DNA

electrophoresis and silver staining were used.

2.4. Amplification, cloning, and sequencing of gel-recovered fragments

Each selected TDF was excised from the gel and recovered by incubating in 100 μL of TE buffer for 15 min at 100°C; after a short centrifugation, the extracted DNA was used directly as template for PCR amplification. The PCR conditions were those used for DDRT-PCR apart from the final extension of 8 min. The re-amplified TDFs were analyzed on 1.5% agarose gel and recovered using QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany); purified TDFs were ligated into pGEM-T Easy vector (Promega) and cloned into *Escherichia coli JM83*. Plasmid DNA was purified and four clones per fragment were sequenced, using M13 universal primers (BMR Genomics, Padova, Italy).

2.5. Northern blotting and RT-qPCR analysis

For Northern blotting, 30 μg RNA were transferred to nylon membranes (Millipore, Billerica, MA, USA). Hybridization, washing and signaling detection were performed as previously described (Gullì et al., 2005). *18S RNA* was used as the reference gene.

For the RT-qPCR analysis, 500 ng of total RNA were reverse transcribed as previously reported (Iurlaro et al., 2016). Then, 5 μL of cDNA were used in a final volume of 20 μL with 2X Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and 250 nM of gene specific forward and reverse primers or 100 nM of primers specific for the housekeeping *18S rRNA* gene (Table 1). Reactions were performed with the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, USA). RT-qPCR data were analyzed by the 2-ΔΔCt method using *18S rRNA* as housekeeping gene and control samples as calibrators. The RT-qPCR data are presented as the mean values calculated from three technical replicates, and for each genotype, leaves from three independent plants were analyzed.

2.6. Bioinformatic analysis

The sequences of the isolated TDFs were analyzed using BLASTn and BLASTx programs within the Ensembl Plants genome browser (plants.ensembl.org/Multi/Tools/Blast). The research was directed on the available genomes of *Ae. tauschii* (Assembly Aet_v4.0), and *T. aestivum* (Assembly IWGSC), last accessed on 27/05/2020.

Primers for RT-qPCR were designed using the software Primer express 2.0 (Applied Biosystems) and their sequences are reported in Table 1.

2.7. Statistical analysis

Standard statistical analysis was performed using the statistical package SPSS (v25.0) for Windows (© Copyright IBM Corporation 1989, 2012) as appropriate. CMS data are presented as mean ± SD and analyzed by ANOVA. Expression data are presented as mean ± SD and analyzed by non-parametric Kruskal-Wallis test. A P value ≤ 0.05 was considered statistically significant.

3. RESULTS

3.1. Evaluation of CMS

Wild wheat relatives belonging to *Triticum* and *Aegilops* spp., reported in Table 2, were characterized by CMS test for basal and acquired thermotolerance. To highlight variability in response to heat stress the data obtained were analyzed by one-way ANOVA test. As shown in Table 2 and Figure 1, there is high variability for this trait, nevertheless *Triticum* spp. exhibit lower basal thermotolerance than *Aegilops* ones (p=0.00001). As far as acquired thermotolerance is concerned, both genera exhibit the same level of variability (p=0.123).

On the basis of the results obtained, the AUM5 genotype, showing the highest CMS values for both basal and acquired thermotolerance, was chosen for further molecular analysis to study modification of gene expression in response to different thermal treatments.

3.2. Identification, cloning and characterization of stress–induced cDNAs

In order to verify whether the various thermal treatments were able to induce in AUM5 the heat shock response, the expression of *HSP17.3,* a member of class II small *HSP*, was analyzed by Northern blot using the *T. aestivum TaHSP17.3* probe. The transcription level of the gene corresponding to *TaHSP17.3* is definitely induced under direct stress (T3) and, to a lesser extent, under stress imposed after acclimation (T2); on the contrary the expression of this *sHSP* is not detectable in untreated (C) as well as in the 34°C treated (T1) plants (Fig. 2). This result does not mean that a day at 34°C did not induce HSPs synthesis, but rather that, after so many hours of stress, the corresponding transcript producion declines to levels undetectable by Northern analisys.

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It is known that *HSP* genes activation is very rapid and maximum level of transcropt is reached whitin two hours from the onset of stress, as recently reported for other plant small *HSP* genes (Wang et al., 2020).

The RNAs extracted from AUM5 after the various thermal treatments were used for DDRT-PCR analysis, in order to identify genes modulated by high temperature. Figure 3 shows a representative acrylamide gel separation of fragments obtained by differential display amplification reactions.

By this analysis, many different cDNA fragments, clearly exhibiting an increased band intensity, were selected and excised from the gel. The bands recovered were named on the basis of the thermal treatment and of the position on the gel, starting from the smaller ones at the bottom. ~~Of~~ All these fragments, 19 in total, were re-amplified, and 10 positive reactions were obtained, one of which, the T2-13, produced two distinct amplicons both of which were isolated; we did not succeed in re-amplifying the two larger size amplicons T2-14 and T2-15. The re-amplification products were cloned and transformed into *E. coli*; for each transformation event, 10 putative transformed colonies were selected, to verify the presence of the inserted DNA and its size (data not shown).

We obtained clones for 11 different cDNA fragments. For each of them, four different colonies were used for DNA sequence analysis; this was necessary because the gel-isolated bands could contain more cDNA fragments of similar length, originating from different transcripts. Considering that differential display usually allows isolation of fragments corresponding to the 3' untranslated region, the sequences were extended *in silico* towards 5', in order to obtain a portion of the coding region useful to search protein databases and assign a putative function to the identified genes. DNA sequences obtained were used to search for homologies in databases, the results are reported in Table 3.

Eight clones, containing cDNAs ranging in size from 100 to 470 bp, were all homologous to the mitochondrial *atpA* gene, encoding the α subunit of ATPase; as expected, the cDNAs contained a 3' polyadenosine tract of variable length and, interestingly, their sequences differed for the position of the poly-A addition site (Fig. 4).

We found significant homologies with different wheat ESTs for all the clones except the T2-12 cl.10 and T3-4 cl.6 as shown in Table 3. In particular T1-10 cl.2 corresponds to an mRNA coding for a homologue of the EIN2 protein, part of the ethylene signal transduction pathway; T1-10 cl.3 corresponds to an mRNA coding for a predicted early nodulin-93-like; T1-10 cl.4 corresponds to an mRNA encoding for a nucleolar RNA binding protein; T2-3 cl.10 corresponds to an mRNA encoding a SMAP-domain containing protein; T2-7 cl.5 corresponds to an mRNA coding for a protein similar to a chloroplastic 50S ribosomal protein L1; T2-7 cl.8 corresponds to a

predicted cysteine-rich and transmembrane domain-containing protein 1-like; T2-12 cl.8 and T2-13 cl.1 correspond to mRNAs coding for a putative FtsH type protease; T3-6 cl.2 corresponds to an mRNA encoding a chloroplastic ferredoxin-dependent glutamate synthase (Fd-GOGAT). The remaining clones, namely T2-5 cl.6, T2-12 cl.11, T3-4 cl.6, T3-6 cl.10 are homologous to predicted transcripts of unknown function.

3.3. Analysis of the expression of cDNAs isolated by differential display

In order to confirm that the various cDNAs really correspond to genes induced by heat stress, a preliminary Northern analysis was performed on AUM5 RNAs using as probes the isolated TDFs. By this analysis, we detected hybridization signals only for T2-13a cl.6, corresponding to the *atpA* gene. All other probes did not detect any signal, probably due to the low level of expression of the corresponding genes. The *atpA* gene showed a very high level of transcription (Fig. 5) and its induction was confirmed in all the different heat treatments. Hybridization signals, quantified using Instant Imager, indicate that in AUM5 the *atpA* gene expression in T1 condition is 4-fold, while in T2 and T3 it is 3-fold, in comparison to the control. *atpA* gene expression was also analysed in a wild *Triticum* genotype, namely MG5473 (*T. turgidum* subsp. *dicoccum*), already identified as heat sensitive (Table 2), in which we observed constitutive expression of *atpA* at almost the same level after all treatments.

The expression level of the genes, whose expression was undetectable by Northern blot, was analysed by RT-qPCR in RNA samples obtained from AUM5 plants subjected to the same temperature conditions as those used for differential display. In general, all the genes were expressed under the stress treatments in accordance to the previous differential display analysis and some of the obtained results are shown in Figure 6. The RT-qPCR analysis allowed to confirm the differential expression in response to thermal treatments for most of the cDNAs; for example, T1-10 cl.2 showed the highest expression under T1 (FC 12) ; T2-7 cl.8 and T3-4 cl.6 showed the highest expression under T2 (FC 16 and 7,5 respectively) and a moderate induction in T3 (FC 6,4 and 4, 5 respectively); T2-3 cl.10 was induced both in T1 and T2, but at a lower extent (FC 1, 7 and 2,4).

4. Discussion

4.1. Thermotolerance in crops

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Plants adaptation to challenging environments requires different strategies to maintain all the molecular activities assuring cell metabolic balancing, normal plant growth and development. In particular, we have evaluated the variability of both basal and acquired thermotolerance in two groups of genotypes, namely *Aegilops* and *Triticum* spp. In the case of basal thermotolerance, *Aegilops* spp. showed a higher level of thermotolerance in comparison with *Triticum* spp. In the case of acquired thermotolerance, both groups of genotypes showed a wide variability with overlapping distribution of the trait. This may be explained considering that basal and acquired thermotolerance have different genetic basis as previously reported (Maestri et al., 2002; Rampino et al., 2009). In fact, basal or constitutive thermotolerance is the thermal adaptation to the habitat, while the acquired thermotolerance is due to acclimation. This preliminary evaluation allowed us to select the most thermotolerant genotype, AUM5, for the following molecular analyses.

Stress response activates molecular networks able to sense the stress and to trigger a re-programming of cellular activities requiring major changes in gene expression; this activation was investigated by many research groups for different kinds of abiotic stresses in many plant species (see Janni et al., 2020 for a review), however a huge work has still to be done to better understand the molecular mechanisms underlying these processes. One of the most important aspects of these studies is the identification of novel gene functions, activated under stress that could account for genetic variability of stress response and, at the same time, could be the basis for improving thermotolerance in agronomic important species, such as wheat. Due to plants extreme genetic variability in heat sensitivity, generating thermotolerant crops appears to be a challenging task. Although important advances have been made by traditional breeding, a time consuming and quite expensive technology, screening of wheat germplasm to identify thermotolerant traits seems, at the moment, the most promising approach (Awlachew et al., 2016; Singh et al., 2018). To obtain new thermotolerant varieties, the enrichment of the genetic pool, useful for marker-assisted selection, could be achieved by highlighting the genetic background of thermotolerance in wheat wild relatives and landraces.

4.2. Analysis of gene expression in a heat tolerant Ae. umbellulata

With the aim to elucidate molecular basis of thermotolerance in wheat wild relative species, we performed the analysis of gene expression on an accession of *Ae. umbellulata*, named AUM5, never subjected to this kind of analysis and classified, through physiologic tests, as heat stress tolerant (for basal as well as for acquired thermotolerance). A number of sequences differentially induced by various temperature treatments were identified and characterized. Among these, the

most peculiar is a group of sequences, differing in length and characterized at 3' end by 9 different positions of the polyA addition site, all homologous to the *atpA* mitochondrial gene. Few data are available on occurrence and function of polyadenylation into organelles, nevertheless according to some authors (Adamo et al., 2008), polyadenylated mitochondrial transcripts are more stable and their level appear significantly enhanced following heat stress. In durum wheat the protein coded by *atpA* is translated from the polycistronic mRNA *atpA-atp9* (Laser et al., 1995). It is well known that the main mitochondrial function is to produce ATP through oxidative phosphorylation by the F_0F_1-ATP synthase complex, located on the internal mitochondrial membrane, responsible for coupling the dissipation of electrochemical protonic gradient to ATP production (Rühle and Leister, 2015). This function needs to be regulated in accordance to the extremely variable requirements of the different plant developmental stages or in relation to environmental condition changes. Modulation of mitochondrial gene expression are involved in these adaptive processes through transcription regulation, variation in copy number of mtDNA or amplification of sub-genomic molecules (Shedge et al., 2010). The induction of *atpA* gene observed in our study may be explained by inhibition of photosynthesis and oxidative stress caused by high temperature. Cells exposed to stress, in fact, reveal lower ATP pool content due to reduction of chloroplast synthetic activity coupled to an enhanced ATP consumption; thus, promoting respiratory activity could be an adaptive reaction aimed at restoration of the normal cell ATP amount.

The data reported in this paper show the induction of other sequences at variable level; among these we identified a homologous of *ein2* (Alonso et al., 1999), a gene isolated from ethylene insensitive *Arabidopsis* mutants, coding a transmembrane protein that plays a role in the ethylene signal transduction pathway (Zheng and Zhu, 2016). Ethylene is involved in the regulation of many development-related processes (seed germination, cell elongation, fruit maturation, leaf abscission, senescence) and in the regulation of biotic and abiotic stress response (Khan et al., 2017; Dubois et al., 2018). Ethylene increased synthesis was reported in response to abrupt water stress and mild heat stress (up to 35°C), being on the contrary inhibited by gradual water stress or temperatures exceeding 35°C (Morgan and Drew, 1997). Larkindale and Knight (2002) reported ethylene involvement in defense from oxidative damage induced by heat stress in *Arabidopsis*; ethylene ability to increase antioxidant defense was also demonstrated in *Agrostis stolonifera* var. *palustris* (Larkindale and Huang, 2004). Another report (De Paepe et al., 2004) on modification of gene expression in *Arabidopsis* after ethylene application indicates that there is a large number of induced genes involved in recovery of cell functions and in defense mechanisms; among these, one of the most abundantly and early induced is *hsp17.4-CI*, a thermotolerance related gene. Taken together, these data indicate a protective role of ethylene in thermotolerance acquisition; the

increase in transcription level of the *ein2* homologous gene, observed in AUM5 after acclimation (24 h at 34°C), is in agreement with this hypothesis. To our knowledge, at the present no data have been reported about temperature regulation of this gene.

Another gene identified in this study as related to heat stress is the one coding for a putative FtsH-like protease. This kind of proteases, firstly described in *E. coli* (Wagner et al., 2012), are involved in gene expression modulation through the degradation of heat shock transcription factor σ^{32} (Lopes et al., 2018), they are also present in plant chloroplasts and mitochondria. In *Arabidopsis*, seventeen *FtsH* sequences were identified, thirteen are chloroplastic and three are located in mitochondria; the only one (FtsH 11) targeted to both organelles (Wagner et al., 2012) is involved in thermotolerance (Chen et al., 2006). Most of these proteases were shown to play a major role in assembly and maintenance of the plastidial membrane system (Wagner et al., 2012), moreover their expression is enhanced by high light intensity and, to a lesser extent, by heat stress; these genes are characterized by extreme variability in their expression level (Sinvany-Villalobo et al., 2004). The observed induction of the gene coding for a FtsH-like protease only in extreme stress conditions might indicate that its activation is a consequence of the response to stress damage (rather than an acclimation mechanism), aimed at increasing degradation of damaged proteins produced under stress. Furthermore, considering that the removal of damaged components is a key point in PSII repair, it is plausible that heat stress resistant plants are particularly efficient in this repair process.

Another identified TDF corresponds to a sequence coding for a ferredoxine-dependent glutamate synthase (Fd-GOGAT), a plastidial enzyme involved in recycling photorespiration-produced NH_3. As temperature increases, C3 plants increase their photorespiration rate in order to cope with high temperature adverse effects, like accumulation of cell toxic molecules (i.e. NH_3) and loss of volatile nitrogen. In general, abiotic stresses (drought, salinity, extreme temperatures) affect N uptake and assimilation pathways through down-regulation of key genes (Popova et al., 2002; Goel and Singh, 2015) and this could account for reduction of plant growth and development under stress. Nevertheless, our data indicate a slightly increased expression of *Fd-GOGAT* gene and this could be due to the intrinsic thermotolerant habit of AUM5.

As for the other clones identified by differential display, homology analyses did not provide useful information to hypothesize their possible function, due to the fact that they are homologous to transcribed sequences of unknown function. In particular, the gene corresponding to T2-3 cl.10 cDNA codes for a protein that exhibits homology with a putative small rice ribonucleoprotein, an *Arabidopsis* protein of unknown function, and a human protein similar to a splicing factor. Expression data reported in this study suggest that this gene is not promptly induced

or that its expression at high temperatures requires a previous acclimation treatment. Searching in the "Genevestigator" (https://www.genevestigator.ethz.ch) database, we found that the expression of the *Arabidopsis* homologous gene is induced under high temperature conditions; this may suggest that this gene has a conserved function in both species in relation to thermal stress. Moreover, if really this gene encodes for a component of the splicing complex, an increase in its transcription could be related to the ability of AUM5 to acquire thermotolerance following acclimation.

5. Conclusion

In this study we report the physiological evaluation of basal and acquired thermotolerance of several wheat wild relative genotypes and the identification of the most thermotolerant among them, namely the *Ae. umbellulata* accession AUM5. The gene expression analysis carried out for the first time in the thermotolerant AUM5 under various high temperature stress treatments, revealed novel genetic functions, related to stress response. In particular, we identified some transcripts that had never been directly related to thermotolerance. We also observed some 'new' sequences that are not yet properly annotated and which could enrich the genetic pool of sequences, specifically induced by heat stress, not belonging to already known and well-studied gene classes such as the ones coding for HSPs.

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Contribution

PR planned and designed the study, performed most of the experimental part of this work, analyzed and interpreted the data, collaborated in writing the manuscript; MDP performed most of the experimental part of this work, analyzed and interpreted the data, collaborated in writing the manuscript; CP analyzed and interpreted the data, collaborated in writing the manuscript; MG planned and designed the study, interpreted the data and wrote the manuscript with the fundamental help of CP and PR. All authors contributed to the discussion and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure Legends

Figure 1. Evaluation of thermotolerance. Box plots represent mean CMS values distribution for acquired and basal thermotolerance in *Triticum* species (white box) and *Aegilops* species (gray box). P-values obtained by ANOVA are shown.

Figure 2 Expression of *HSP17.3* in AUM5. Northern blot hybridization showing the transcript corresponding to *TaHSP17.3* in the *Ae. umbellulata* AUM5 genotype in response to different thermal treatments: C=23°C, T1=24 h at 34°C, T2=24 h at 34°C followed by 2 h at 42°C and T3=2 h at 42°C. rRNA bands are shown to confirm equal amount of RNA loaded on the gel.

Figure 3. DDRT-PCR analysis in AUM5. Portion of an acrylamide gel showing some of the fragments selected (white arrows) as differentially expressed in AUM5 in response to the different thermal treatments (T1, T2 and T3) respect to the control condition (C). C=23°C, T1=24 h at 34°C, T2=24 h at 34°C followed by 2 h at 42°C and T3=2 h at 42°C.

Figure 4. Sequence of the T2-13a cl.6 fragment, homologous to *atpA* gene. The H-AP2 primer is underlined and the different poly-A addition sites are highlighted in grey.

Figure 5 Northern blot hybridization showing the level of *atpA* transcripts detected by the probe T2-13a cl.6 in *Ae. umbellulata* AUM5 genotype and *T. turgidum* subsp. *dicoccum* MG5473 genotype in response to different thermal treatments: C=23°C, T1=24 h at 34°C, T2=24 h at 34°C followed by 2 h at 42°C and T3=2 h at 42°C. rRNA bands are shown to confirm equal amount of RNA loaded on the gel.

Figure 6 Expression analysis of TDFs obtained by differential display in *Ae. umbellulata* AUM5 genotype. The relative expression (Fold Change) of each gene was measured by RT-qPCR in leaves samples of plants subjected to different temperature treatments: T1=24 h at 34°C, T2=24 h at 34°C followed by 2 h at 42°C and T3=2 h at 42°C. All values are normalized using *18SrRNA* as housekeeping gene and control sample as calibrator. Average mean values (±SD) were derived from three biological replicates.

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Table 1. Primers used for the RT-qPCR-analysis

Target sequence	Primer sequences (5' to 3')	
T1-10 cl. 2	For-GCAATTCACTCTGGTTTTTCCC	Rev-AAGCAGCTACAGTGACCGTTCA
T1-10 cl. 3	For-CGGCGATTTTCAGTTTCAGG	Rev-CCTAGCAGGCACCAACTCTCTT
T1-10 cl. 4	For-CCATTTCTGAGAGTTGTTGCAG	Rev-TTTTCAGTGTGTCCCAATCCTG
T2-3 cl. 10	For-ATCATCGTGTGTCTCATAAACAGC	Rev-CCGGGTGAGAAGATACAAGTGTC
T2-7 cl. 5	For-GAACACTGGGAGGTGCTTAGGT	Rev-GCACTGTTTAACTGCCGATGTC
T2-7 cl. 8	For CCACGTTGTTGCCACGCTAT	Rev-GGATTCGAGTTACACAGGCAGG
T2-12 cl. 10	For-AACACGGTGAGCAACTGAGCA	Rev- CAGGCAAAAACAGAACCCGA
T2-12 cl. 11	For-TTTCGCAATCGCAAGAGGTG	Rev-TCCACAACAGAAAGCAGCAGC
T2-13a cl. 6	For- CTAAGTAAACAGGCGGTGGC	Rev-CGTCTCCAGCTTGTGTTTCA
T2-13b cl.1	For-TCCGAAGTGGGGTAAACTTG	Rev-GGGCTTCATGTTGGGTAAAA
T3-4 cl. 6	For-TAAGCGTTCCAGCCAAAGTCC	Rev-GTCAAACAACTCGTAGCAGCGG
T3-6 cl. 2	For-TGCGAGTTGCATGTGCTTTAGT	Rev-CCACCGATTATCGACACCTGA
18S rRNA	For-TGCCCTATCAACTTTCCATGG	Rev-TGGATGTGGTAGCCGTTTCTC

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Table 2. List of the *Triticum* and *Aegilops* wild accessions utilized and their CMS values for basal and acquired thermotolerance

Species	Genotypes	CMS % ± SD	
		basal thermotolerance	**acquired thermotolerance**
Triticum turgidum subsp. *monococcum*	ID 362	29.53±1.12	81.89±3.02
	ID 529	23.33±1.27	36.06±4.33
	ID 1331	22.97±2.09	66.60±4.37
Triticum turgidum subsp. *dicoccoides*	MG 29881	24.84±0.81	67.55±2.98
	MG 29896	23.99±0.91	87.63±5.33
	MG 3533	23.73±1.33	54.05±3.28
	MG 4316	20.89±2.21	24.98±3.45
	MG 4317	25.30±2.34	76.90±2.98
	MG 4327	20.68±1.89	76.01±4.88
	MG 4328	26.25±1.98	69.90±3.86
	MG 4330	25.43±2.09	81.35±5.43
	MG 4337	27.29±1.54	46.76±3.91
	MG 4343	29.23±2.70	38.09±1.58
	MG 5444	17.45±0-97	73.42±3.23
Triticum turgidum subsp. *dicoccum*	MG 3521	21.01±1.45	37.88±1.88
	MG 4365	21.36±1.79	32.91±3.45
	MG 4375	21.09±2.03	52.68±1.11
	MG 5323	20.41±1.97	67.37±6.08
	MG 5473	17.25±2.56	21.29±0.11
	MG 5510	19.80±2.02	69.90±3.21
Aegilops kotschyi	AKO 6	33.07±2.05	66.18±2.44
	AKO 11	29.71±2.01	57.43±2.90
	AKO 14	33.99±2.51	32.87±2.93
	AKO 15	34.07±1.97	69.52±2.80
Aegilops longissima	ALO 2	66.80±2.98	67.72±2.71
	ALO 8	43.31±2.01	56.33±2.77
	ALO 12	30.03±1.93	54.17±3.24
Aegylops umbellulata	AUM 1	29.91±3.01	53.73±3.88
	AUM 3	46.30±1.77	80.35±4.51
	AUM 4	33.61±2.98	84.90±3.85
	AUM 5	67.70±2.02	88.35±1.05
Aegilops variabilis	AVA 1	30.27±3,04	48.50±1.89
	AVA 4	55.13±1.98	78.08±2.76
	AVA 5	30.57±3.02	47.16±o.76
	AVA 8	30.48±2.99	79.57±1.01
	AVA 17	30.11±2.87	54.10±1.04

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Table 3. Identification of genomic sequences homologous to transcript derived fragments (TDFs) isolated in the *Ae. umbellulata* AUM5 genotype by DDRT-PCR. For each TDF, the name, length and accession number are indicated; the genomic location of the overlapping gene identified in *Ae. tauschii*, the gene name, gene function and Gene Ontology (GO) categories are reported when available

TDF	Length	Accession number	Genomic location (plants.ensembl.org)	Overlapping gene	Gene name	Uniprot	Function	Other species	GO categories
T2-3 cl.10	116 bp	AJ627877.1	4D:396055602-396055702	AET4Gv20575500	/	M8BH50	SMAP domain-containing protein	/	/
T2-5 cl. 6	162 bp	AJ627878.1	7D: 615,230,247-615,230,489	AET7Gv21262800.1 LOC109757586	/	/	PREDICTED: *Aegilops tauschii* subsp. *tauschii* uncharacterized LOC109757586 (LOC109757586), mRNA	/	/
T3-6 cl. 10	185 bp	AJ627880.1	7D: 615,230,247-615,230,489	AET7Gv21262800.1 LOC109757586	/	/	PREDICTED: *Aegilops tauschii* subsp. *tauschii* uncharacterized LOC109757586 (LOC109757586), mRNA	/	/
T3-6 cl.2	192 bp	AJ627879.1	2D: 79,034,073-79,051,075	AET2Gv20260800 XM_020292432.1	/	/	PREDICTED: *Aegilops tauschii* subsp. *tauschii* ferredoxin-dependent glutamate synthase, chloroplastic (LOC109733233), mRNA	*Arabidopsis thaliana*, GLU2	GO:0006537: glutamate biosynthetic process; GO:0019676: ammonia assimilation cycle; GO:0016041:glutamate synthase (ferredoxin) activity; GO:0009570: chloroplast stroma
T2-7 cl.8	199 bp	AJ627882.1	5D: 514,896,049-514,897,207	AET5Gv21038300.2 XM_020322300.1	/	/	PREDICTED: *Aegilops tauschii* subsp. *tauschii* cysteine-rich and transmembrane domain-containing protein 1-like (LOC109763452), mRNA	/	/
T2-7 cl.5	199 bp	AJ627881.1	1D: 338,254,580-338,258,576	AET1Gv20591100 XM_020340715.1	/	R7WD93	PREDICTED: *Aegilops tauschii* subsp. *tauschii* 50S ribosomal protein L1, chloroplastic (LOC109782125), mRNA	/	/

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T1-10 cl. 4	258 bp	AJ627885.1	3A: 53,388,996-53,399,238, 3D: 604,960,237-604,961,330	TRITD_3Av1G026360AE T3Gv21163500	*NOP5-2*	A0A446N5D7 R7WED2	Nucleolar RNA binding protein G	NOP5B_ARATH - Probable nucleolar protein 5-2	GO:0042254:ribosome biogenesis; GO:0030515: snoRNA binding; GO:0016021: integral component of membrane
T1-10 cl.2	259 bp	AJ627883.1	4D: 17,829,564-17,836,477	AET4Gv20071900	*EIN2*	M8BSP5	Ethylene-insensitive protein 2	/	GO:0016021: integral component of membrane
T1-10 cl.3	260 bp	AJ627884.1	6D: 196,446,592-196,466,214	AET6Gv20475200 LOC109733116	*ENOD93*	/	PREDICTED: *Aegilops tauschii* subsp. *tauschii* early nodulin-93-like (LOC109733116), transcript variant X1, mRNA	HORVU6Hr1G040900	/
T3-4 cl.6	261 bp	AJ627214.1	/	/	/	/	/	/	/
T2-12 cl. 8	326 bp	AJ627886.1	3D: 317,067,416-317,074,391	AET3Gv20548300 XM_020309562 LOC109750604	*hflB*	/	PREDICTED: *Aegilops tauschii* subsp. *tauschii* ATP-dependent zinc metalloprotease FTSH 9, chloroplastic/mitochondrial (LOC109750604), mRNA	/	GO:0006508: proteolysis; GO :0051301: cell division; GO:0004222 metallo-endopeptidase activity; GO:0016887: ATPase activity
T2-12 cl. 10	340 bp	AJ627887.1	/	/	/	/	/	/	/
T2-12 cl. 11	341 bp	AJ627888.1	7D: 615,230,247-615,230,489	AET7Gv21262800.1 LOC109757586	/	/	PREDICTED: *Aegilops tauschii* subsp. *tauschii* uncharacterized LOC109757586 (LOC109757586), mRNA	/	/
T2-13b cl1	413 bp	AJ627890.1	3D: 317,067,416-317,074,391	AET3Gv20548300 XM_020309562 LOC109750604	*hflB*	/	PREDICTED: *Aegilops tauschii* subsp. *tauschii* ATP-dependent zinc metalloprotease FTSH 9, chloroplastic/mitochondrial (LOC109750604), mRNA	//	GO:0006508: proteolysis; GO :0051301: cell division; GO:0004222 metallo-endopeptidase activity; GO:0016887: ATPase activity

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T2-13a cl.6	474 bp	*AJ627889.1*	Mithocondrial genome *Ae.squarrosa* and *T. aestivum*	//	*atpA*	/	ATP synthase, F1 complex, alpha subunit	//	GO:0015986:ATP synthesis coupled proton transport; GO:0046933 Proton-transporting ATP synthase activity, rotational mechanism; GO:0045261: Proton-transporting ATP synthase complex, catalytic core F(1)

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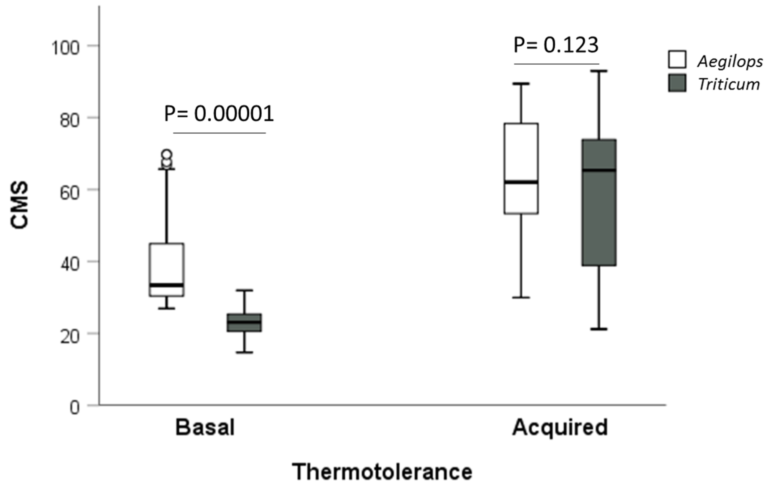

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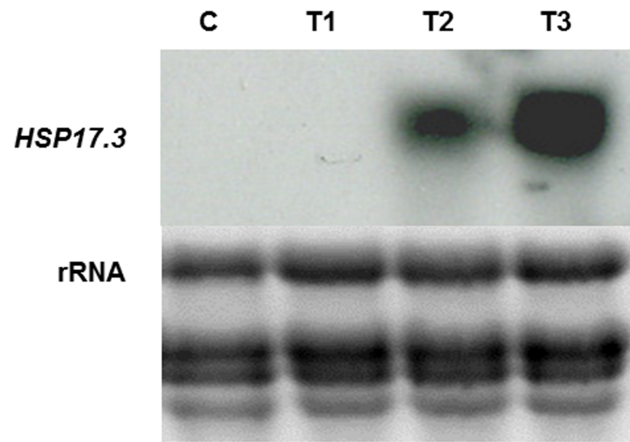

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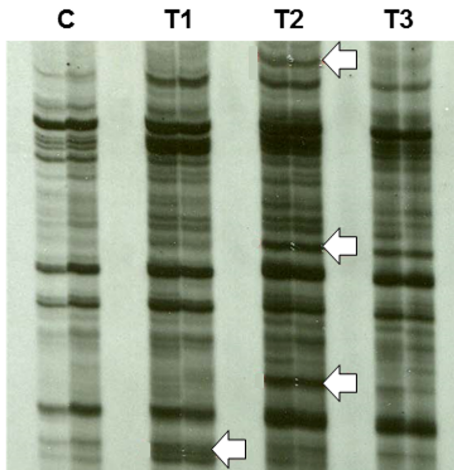

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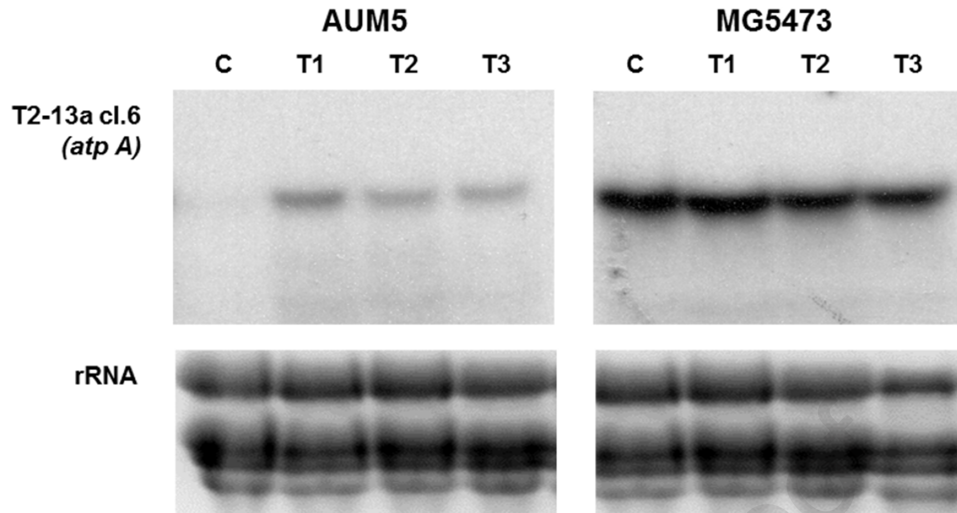

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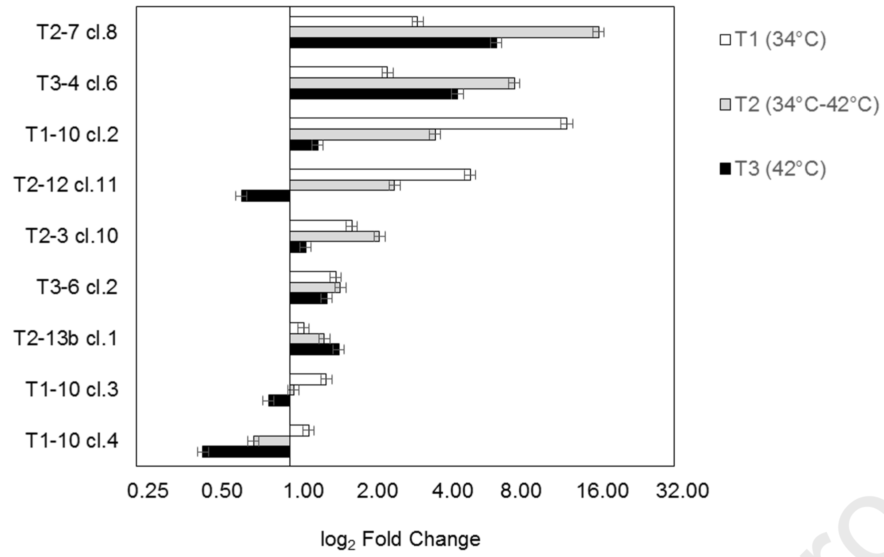

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Highlights

Aegilops umbellulata: a source of new genes to increase thermotolerance in wheat.

First example of heat stress response analysis at molecular level of a wheat wild relative, namely the *Ae. umbellulata* accession AUM5.

Novel genetic functions, not belonging to already known gene classes such as the ones coding for HSPs, are induced by heat stress in *Ae. Umbellulata*.

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Declaration of interests

X☐ **The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.**

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