View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 581 (2007) 4841–4849

Four members of the *HSP101* gene family are differently regulated in *Triticum durum* Desf.

Mariolina Gullì[a], Massimiliano Corradi[a], Patrizia Rampino[b], Nelson Marmiroli[a], Carla Perrotta[b,*]

[a] *Dipartimento di Scienze Ambientali, Sez. Genetica e Biotecnologie Ambientali, Università di Parma, Italy*
[b] *Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, Lecce, Italy*

Received 6 August 2007; revised 3 September 2007; accepted 7 September 2007

Available online 14 September 2007

Edited by Ulf-Ingo Flügge

Abstract **Heat shock proteins play an essential role in preventing deleterious effects of high temperatures. In many plants, HSP101 has a central role in heat stress survival. We report the isolation and characterization of four cDNAs corresponding to different members of the durum wheat *HSP101* gene family. Expression analysis revealed differences in their induction. Accordingly, durum wheat *HSP101* genes are differently regulated, therefore having distinct roles in stress response and thermotolerance acquisition. These findings are important for further dissection of the molecular mechanisms underlying the stress response and for understanding the functions of the HSP101 family members. This information could be important for the exploitation of specific alleles in marker assisted selection for abiotic stress resistance.**
© 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Quantitative (Real-Time) RT-PCR; Heat shock response; *HSP101* gene family; *Triticum*

1. Introduction

Heat stress is one of the major constraints to plant growth and yield, as it damages cell, tissue and whole plant functioning. At the biochemical and molecular levels the synthesis of heat shock proteins (HSPs) represents the most interesting, but still not completely explained, aspect of the heat shock response. These proteins, mainly chaperones or proteases, play the essential role of preventing or minimizing the deleterious effects of heat at the cellular and molecular levels. Moreover, they help cells in recovering from the stress during the post-stress phase [1]. This role was confirmed by a study on HSP101 in *Arabidopsis*. This protein is part of a molecular complex involving also small HSPs (sHSPs), and has the role of re-solubilizing protein aggregates formed as an effect of the heat stress [2–4]. In maize, HSP101 forms complexes with HSP70, that could be part of a large multi-chaperone complex, involved in the correct protein folding [5]. Other reports have shown that HSP101 has a central role in establishing thermotolerance [6–8]. These proteins belong to a family of proteases firstly described in bacteria [9,10], but also in yeast, protozoans, and plants [11,12]. In particular they are ATPases involved in assembly/disassembly of protein complexes such as the ATP-dependent dissolution of cytosolic or nuclear protein aggregates formed during heat stress [3,13,14]. Clear evidence of the protective role of HSP101 and its involvement with thermotolerance was shown in *Saccharomyces cerevisiae* in which the survival to high temperatures and induction of thermotolerance were strictly bound to the presence of HSP104 [13,15]. Nevertheless, the essential functions which are protected or repaired by HSP101 remain to be elucidated.

In many plant species (i.e. *Arabidopsis thaliana*, soybean, rice, maize, pea, beans), many cDNAs and genomic clones, coding for different forms of HSP101, with molecular weights ranging from 100.9 to 109.4 kDa, have been isolated and characterized, suggesting that HSP101 is a member of a small gene family strongly induced by heat [16,17]. In *Triticum aestivum*, the species most similar to that of our interest i.e. *Triticum durum*, three different HSP101 coding sequences are present in GenBank [18,19].

In this paper, we report for the first time the isolation and characterization of four cDNAs coding for different forms of HSP101 in durum wheat. The relevant cDNAs were sequenced and single nucleotide polymorphisms (SNPs), specific for the different isoforms, were identified. This allowed to perform expression analysis using Quantitative (Real-Time) reverse transcription polymerase chain reaction (RT-PCR) to quantify the relative abundance of the various HSP101 isoform transcripts under different stress conditions, revealing differences in timing and level of expression.

2. Materials and methods

2.1. Plant material

Seeds of *T. durum* cultivar (cv) Ofanto, cv Creso, and of *Triticum monococcum* (ID362) were germinated on cotton pads for 3 days at 24 ± 1 °C. Seedlings were transplanted into small pots with soil and grown for 14 days at 23 °C with 16 h of light (photosynthetic photon flux density (PPFD) 80 μmol m^{-1} s^{-1}) at 65% relative humidity.

Heat treatments were performed exposing pots to 37 °C for different times (30, 60 and 90 min) or to temperatures of 29, 31, 33, 35 °C for 30 min. Acclimation was achieved by exposing plants to 34 °C for 24 h, and the temperature was then shifted to 42 °C for 2 h for stress

*Corresponding author. Fax: +39 832298858.
E-mail address: carla.perrotta@unile.it (C. Perrotta).

Abbreviations: ABA, abscisic acid; cv, cultivar; DT, di-telosomic; HSP, heat shock protein; NT, nullisomic–tetrasomic; RT-PCR, reverse transcription polymerase chain reaction; SNP, single nucleotide polymorphism

0014-5793/$32.00 © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2007.09.010

post-acclimation. Stress without acclimation was achieved by exposing plants directly to 42 °C for 2 h. After the heat stress aerial parts were collected, frozen in liquid nitrogen and kept at −80 °C until use.

2.2. Database search and primer design

A search in GenBank database showed three sequences of *T. aestivum* encoding for HSP101: *HSP101* (GenBank Accession No. AF083344), *HSP101B* and *HSP101C* (GenBank Accession Nos. AF097363, AF174433). Based on their alignment, two sets of specific primers were designed to amplify *HSP101B* (101b-forw2/101b-rev2) and *HSP101C* (101c-forw/101c-rev2) in *T. durum* (Table 1 – supplementary material). Primers were also designed to the α-*tubulin* gene sequence (αTUBF/αTUBRev2) of *T. aestivum* (GenBank Accession No. U76558) and used as an internal control for RT-PCR. Primers and probes for Quantitative (Real-Time) RT-PCR were designed with "Primer Express 2" (Applied Biosystems, Foster City, CA) for the previously identified *T. durum* sequences. Primers were obtained from MWG (Ebersberg, Germany) and probes from Applied Biosystems. Their sequences are listed in Table 1 – supplementary material.

2.3. PCR amplification and sequence analysis

HSP101B, *HSP101C* and α-*tubulin* cDNA fragments from *T. durum* cv Ofanto and *T. monococcum* (ID 362) were obtained following standard procedures. Twenty ng of cDNA were used as template for PCR reactions with the PCR Master Mix (Promega, Madison, WI) containing 200 nM of each forward and reverse primer (101b-forw2/101b-rev2, 101c-forw/101c-rev2, α-TUB-F/α-TUB-R) in the Genius Thermal Cycler (Techne, Burlington, NJ). PCR products were cloned in pGEM-T Easy vector (Promega) and amplified in *Escherichia coli* JM109 strain. Eight clones for each *E. coli* transformation were selected and sequenced, in order to verify the presence of different amplification products.

Full length cDNAs of *T. durum HSP101B* (*TdHSP101B*) and *HSP101C* (*TdHSP101C*) were amplified from cv Ofanto, exposed to 37 °C for 1 h, by RT-PCR using primer pairs 101b-5′F/101b-rev2 3′ and 101c-5′F/101c-rev3, respectively. The cDNAs were sequenced following a walking strategy. Each sequence was performed at least twice on eight independent clones. Sequence reactions were performed using CEQ™ 2000XL Sequencer (Beckman Coulter, Fullerton, CA). Nucleotide and deduced amino-acid sequences were analyzed and compared with sequences in databases using BLAST [20] and ClustalW [21] programs.

Chromosomal localization of the genes was obtained by PCR amplification of genomic DNA from Chinese Spring Nullisomic–Tetrasomic (NT) and Di-Telosomic (DT) seeds [22,23]. The primer pairs used were: 101b-5′F/101b-5′R for *TdHSP101B*, 101cA-forw/101c-revRT and 101cB-forw/101c-revRT for *TdHSP101C*. Reaction products were resolved and identified on a 15% acrylamide gel or 2% w/v agarose gel.

2.4. Southern analysis

Genomic DNA was isolated from the leaves of 14-day-old seedlings of *T. durum* cv Ofanto and *T. monococcum* ID362 as previously reported [24]. DNA was digested overnight at 37 °C with *Bam*HI, *Eco*RI, and *Xba*I restriction enzymes. Gel electrophoresis, Southern blotting, and hybridization were performed according to standard procedures [25].

2.5. Semi-quantitative RT-PCR analysis

Total RNA was extracted from 500 mg of tissue as previously described [26], and treated with DNase I (Promega) according to the manufacturer's protocol. First strand cDNA was synthesized by M-MLV Reverse Transcriptase, RNaseH Minus, Point Mutant (Promega) and random hexamers. Twenty ng of cDNA were used as template for PCR amplifications with PCR Master Mix (Promega) containing 200 nM of each forward and reverse primer (Table 1 – supplementary material). The amplicons were separated on a 2% w/v agarose gel.

2.6. Duplex Quantitative (Real-Time) RT-PCR

Quantitative (Real-Time) RT-PCR was performed by ABI PRISM 7000 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 in a total reaction volume of 25 μl with 1X TaqMan Universal Mastermix (Applied Biosystems), 250 nM of target and endogenous specific probes, 200 nM of target specific primers (101cA-forw/101c-revRT or 101cB-forw/101c-revRT; 101bA-forw/101bAR-rev or 101bB-forw/101bBR-rev), and 100/400 nM of α-*tubulin* specific primers (α-Tub-F/α-Tub-Rev2). 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 twice. The amount of target contained in each sample was determined using the relative standard curve method. *TdHSP101* transcript levels were normalized with respect to α-*tubulin*. All calculations and statistical analysis were performed as described in the ABI 7700 sequence detection system User Bulletin 2 (Applied Biosystems) using Microsoft Excel program. The specificity of the amplicons was determined by sequencing.

3. Results

3.1. Sequence isolation and characterization

To study the structure and the expression of the *HSP101* gene family members of *T. durum* we used specific primer pairs designed on the basis of *T. aestivum* sequences previously reported for two different isoforms, i.e. HSP101B and HSP101C [18,19].

Amplifications were performed on cDNAs from cv Ofanto, the amplicons obtained were cloned and sequenced. Two sequences differing in a few nucleotides between each other were obtained for both *TdHSP101B* and *TdHSP101C* genes. To verify whether the differences were due to the presence of different alleles on each genome and to distinguish between the sequences belonging to the A and B genomes, amplifications were performed on both *T. durum* (AABB genome) and *T. monococcum* (AA genome) cDNAs. As shown in Fig. 1a, in *T. durum* the amplicons for *TdHSP101B* were 188 bp long, with three SNPs that allowed us to distinguish two *TdHSP101B* forms: one named *TdHSP101B*-A, which is identical to the *T. monococcum* sequence, and the other, named *TdHSP101B*-B, which carried three SNPs. The *T. durum* amplicons for *HSP101C* (Fig. 1b) were 114 bp long: the one with two SNPs with respect to the *T. monococcum* sequence was named *TdHSP101C*-A, the other, which carried five SNPs, *TdHSP101C*-B.

Amplicons with the complete coding sequences for the four identified isoforms were obtained by RT-PCR. *TdHSP101B*-A (GenBank Accession No. AJ970533) was 2909 bp long with a 2739 bp ORF; *TdHSP101B*-B (GenBank Accession No. AJ970534) was 2950 bp long with an ORF of 2751 bp; *TdHSP101C*-A (GenBank Accession No. AJ970535) was 2878 bp long with a 2739 bp ORF; and *TdHSP101C*-B (GenBank Accession No. AJ970536) was 2933 bp long with an ORF of 2739 bp.

In order to determine their chromosomal location, a PCR analysis was performed for each gene on NT and DT lines of *T. aestivum* cv Chinese Spring [22,23]. Nullisomic–tetrasomic are wheat lines each lacking a different pair of homologous chromosomes, due to the replacement with its homeologous (i.e. N3AT3B means a line in which the 3A pair is missing and replaced by an additional 3B pair). Ditelosomics are euploid lines in which one arm of a given chromosome is missing, for example line DT3AS contains only the short arm of chromosome 3A and lacks its long arm. Due to their characteristics, these lines can be used in wheat to map a gene on a specific chromosome arm. As shown in Fig. 2a, using *TdHSP101C*-A specific primers, no amplification product was obtained for the N3AT3B line, while using *TdHSP101C*-

```
a
T.monococcum    TGGAGAGGAAGCGGATTCAGCTGGAGGTGGAGCTGCACGCGCTGGAGAAGGAGAAGGACA 60
TdHSP101B-A     TGGAGAGGAAGCGGATTCAGCTGGAGGTGGAGCTGCACGCGCTGGAGAAGGAGAAGGACA 60
TdHSP101B-B     TGGAGAGGAAGCGGATTCAGCTGGAGGTGGAGCTGCACGCGCTGGAGAAGGAGAAGGACA 60
                ********************************** ************************

T.monococcum    AGGCCAGCAAGGCCCGGCTGGTGGAGGTGAGGAAGGAGCTGGACGACCTGAGGGACAAGC 120
TdHSP101B-A     AGGCCAGCAAGGCCCGGCTGGTGGAGGTGAGGAAGGAGCTGGACGACCTGAGGGACAAGC 120
TdHSP101B-B     AGGCCAGCAAGGCCCGGCTGGTGGAGGTGAGGAAGGAGCTGGACGACCTGAGGGACAAGC 120
                ************************************************************

T.monococcum    TGCAGCCGCTGACGATGAAGTACAGGAAGGAGAAGGAGCGGATCGACGAGATCCGGAAGC 180
TdHSP101B-A     TGCAGCCGCTGACCATGAAGTACAGGAAGGAGAAGGAGCGGATCGACGAGATCCGGAAGC 180
TdHSP101B-B     TGCAGCCGCTGACCATGAAGTACCGCAAGGAGAAGGAGCGGATCGACGAGATCCGGAAGC 180
                ************* ********* * ********************************

T.monococcum    TGAAGCAG 188
TdHSP101B-A     TGAAGCAG 188
TdHSP101B-B     TGAAGCAG 188
                ********

b
T.monococcum    GTGAGCCGCTGGACTGGTATTCCTGTTACCAGGCTCGGACAGAATGAGAAGGCGAGGCTG 60
TdHSP101C-A     GTGAGCCGCTGGACTGGTATTCCTGTCACCAGGCTCGGGCAGAATGAGAAGGCGAGGCTG 60
TdHSP101C-B     GTGAGCCGCTGGACTGGTATTCCTGTCACTAGGCTGGGACAGAATGAGAAGGCCAGGCTG 60
                ************************** ** ***** ** ************** ******

T.monococcum    ATCGGGCTGGCTGACAGACTGCATCAGAGGGTCGTTGGACAGTATGAGGCCGTC 114
TdHSP101C-A     ATCGGGCTTGCTGACAGACTGCATCAGAGGGTGGTTGGACAGTATGAGGCCGTC 114
TdHSP101C-B     ATCGGTCTGGCTGATAGACTGCATCAGAGGGTGGTTGGACAGTATGAGGCCGTC 114
                ***** ** ***** ***************** ********************
```

Fig. 1. CLUSTAL W analysis of nucleotide sequences of the amplicons obtained using the specific primers for *HSP101B* gene (a) and *HSP101C* gene (b) on *T. durum* and *T. monococcum* cDNAs. Shadowed nucleotides indicate point substitutions.

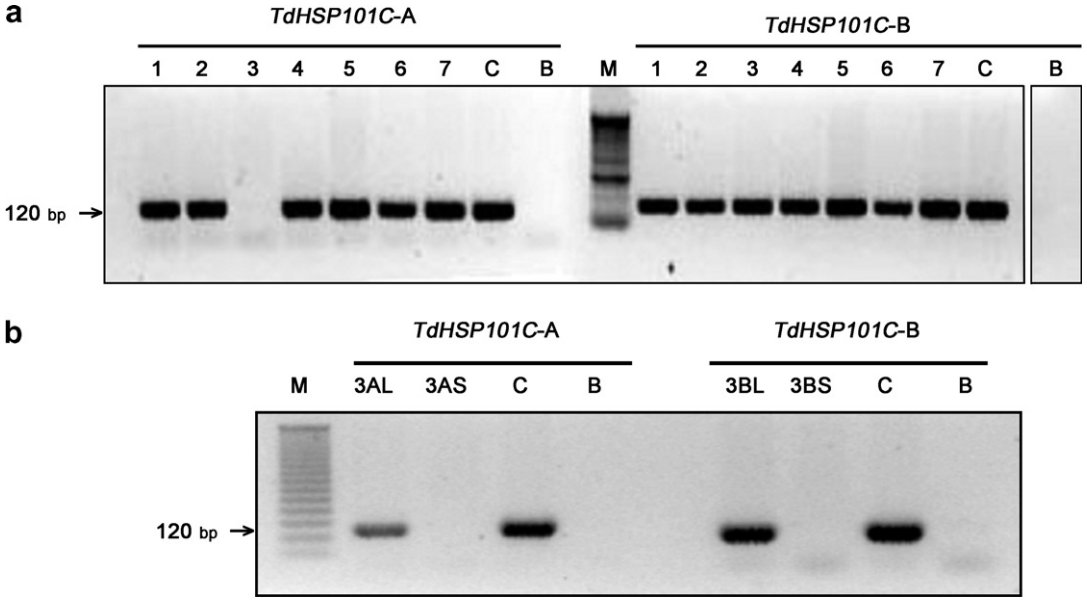

Fig. 2. Chromosome assignment of *TdHSP101C*-A and -B genes. (a) PCR products obtained from genomic DNA of the seven wheat nullisomic–tetrasomic lines (1 = N1AT1B; 2 = N2BT2A; 3 = N3AT3B; 4 = N4AT4B; 5 = N5AT5B; 6 = N66BT6A; 7 = N7AT7B) and of Chinese Spring wheat (C). In the nomenclature of the nullisomic–tetrasomic lines the "N" is followed by the number and genome of the nullisomic chromosome and the "T" is followed by the same for the tetrasomic chromosome. For the amplification *TdHSP101C*-A and *TdHSP101C*-B gene specific primers were used. M = marker, 50 bp DNA ladder; B = PCR negative control. (b) PCR products obtained from genomic DNA of four wheat ditelosomic lines (DT3AL, DT3AS, DT3BL, DT3BS) and of Chinese Spring wheat (C). In the nomenclature of the ditelosomic lines, the numeral indicates the chromosome number, the A, or B represents the specific genome, and the L or S indicates which chromosome arm is present. For the amplification *TdHSP101C*-A and *TdHSP101C*-B gene specific primers were used. M = marker, 50 bp DNA ladder; B = PCR negative control.

B specific primers the amplification product was present. Considering that N3AT3B line lacks the 3A chromosome pair, that is replaced with the homeologous 3B pair, *TdHSP101C*-A was assigned to chromosome 3A and *TdHSP101C*-B to chromosome 3B. The results obtained by PCR performed on the DT lines 3AL, 3AS, 3BL, and 3BS are shown in Fig. 2b. Using *TdHSP101C*-A and -B specific primers the corresponding amplicons were obtained in the DT3AL and DT3BL lines,

but not in DT3AS and DT3BS. These results confirmed that *TdHSP101C*-A is located on chromosome 3A long arm and *TdHSP101C*-B on chromosome 3B long arm.

Using the same strategy, *TdHSP101B*-A and -B were assigned to the long arm of chromosomes 1A and 1B, respectively (data not shown).

Comparison of the deduced aminoacid sequences of these genes is shown in Fig. 3. *Td*HSP101B shares 90% identity with *Td*HSP101C, *Td*HSP101B-A and *Td*HSP101B-B share 98% identity; *Td*HSP101C-A and *Td*HSP101C-B share 98% identity. The conserved consensus sequences peculiar to plant HSP101 [17] are indicated in Fig. 3.

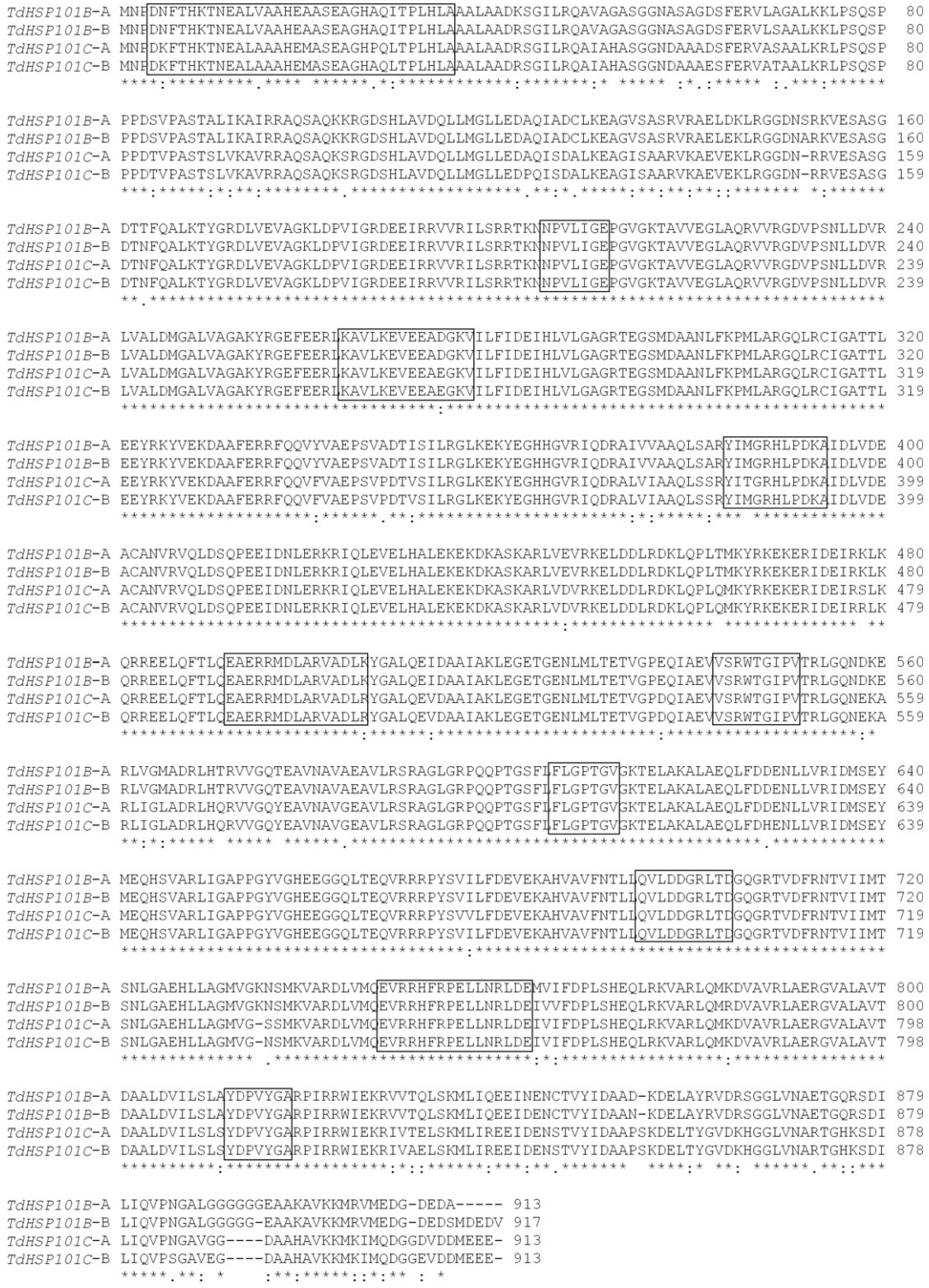

Fig. 3. Alignment of the deduced amino acid sequences of *TdHSP101B*-A, *TdHSP101B*-B, *TdHSP101C*-A, and *TdHSP101C*-B cDNAs obtained with CLUSTAL W. Gaps in the sequences are indicated by dashes. "*" indicate identical residues in all sequences; ":" indicate conserved substitutions; "." indicates semi-conserved substitutions. Boxes indicate conserved consensus sequences.

The deduced *T. durum* aminoacid sequences were compared to the known HSP101 from higher plants and a phylogenetic tree was obtained (Fig. 4).

3.2. Genomic organization of the TdHSP101 gene family

A *TdHSP101C* cDNA fragment (1169 bp) was utilized to probe *T. durum* and *T. monococcum* genomic DNA digested with *Bam*HI, *Eco*RI, and *Xba*I, the sites for which were absent in the cDNA used as a probe (Fig. 5). Hybridization patterns of the different digests showed that in *T. durum* there were two hybridization signals (ca. 9.0 and 4.8 kb) in the *Bam*HI digest, two (ca. 11 and 6.0 kb) in the *Eco*RI digest, and two (ca. 12.0 and 5.6 kb) in the *Xba*I digest. In contrast, in *T. monococcum* the hybridization bands were two (ca. 9.0 and 6.6 kb) in the *Bam*HI digest, one (ca. 11.0 kb) in the *Eco*RI digest, and one (ca. 6.1 kb) in the *Xba*I digest.

3.3. Expression analysis

The level of expression of the different *HSP101* genes in *T. durum* was investigated by Quantitative (Real-Time) RT-PCR. To establish any variation in response of the durum wheat *HSP101* genes to heat stress, their expression was compared in different thermal conditions. Preliminary expression analysis was performed by semi-quantitative RT-PCR to verify primers specificities and amplicons identities (data not shown). Further, to discriminate the expression of the individual genes belonging to this family, a duplex Quantitative (Real-Time) RT-PCR system was set up. The expression of the A and B forms of *TdHSP101B* and *TdHSP101C* was evaluated in two durum wheat cvs (Ofanto and Creso) exposed to the different thermal regimes. The results obtained by this analysis are reported in Figs. 6 and 7; in the cv Ofanto the transcripts of *TdHSP101B*-A and -B forms were already detectable at 23 °C, although at very low level, and abruptly increased after 30 min at 29 °C. The expression of both *TdHSP101B* forms reached almost the maximum level at 33 °C and remained very high along all the thermal treatments tested (Fig. 6a). On the contrary *TdHSP101C*-A and -B forms in the cv Ofanto were induced starting from 29 °C. During the stress, *TdHSP101C* transcripts gradually accumulated reaching a maximum level after 60 min at 37 °C but decreased by 90 min at 37 °C. Compared with *TdHSP101C* forms, the level of expression of *TdHSP101B* forms was considerably higher under all the thermal treatments (Fig. 6a).

In the cv Creso (Fig. 6b) the *TdHSP101B*-B transcripts were already detectable at 23 °C. *TdHSP101B*-A and -B forms were strongly induced after 30 min at 29 °C, and reached their maximum level at 35 °C. The level of expression remained very high at all the 37 °C treatments. Transcripts of the *TdHSP101C*-A and -B forms in the cv Creso were induced at 29 °C, reached their maximum level after 60 min at 37 °C with comparable values, and remained high also after 90 min at 37 °C. In this cv also, the levels of transcripts of the *TdHSP101B* forms were considerably higher than that of *TdHSP101C* forms under all the thermal treatments (Fig. 6b).

The possible different roles of the two *Td*HSP101B and *Td*HSP101C isoforms in conferring thermotolerance, were analyzed in seedlings after a treatment at very high temperature (42 °C) performed with and without the pre-treatment at 34 °C.

As shown in Fig. 7a and b, in both cvs *TdHSP101B* (A and B forms) are transcribed under all the thermal treatments tested although the highest level of expression was induced when heat shock (2 h at 42 °C) was imposed after a long term pre-treatment (24 h at 34 °C). On the contrary in both cvs heat shock

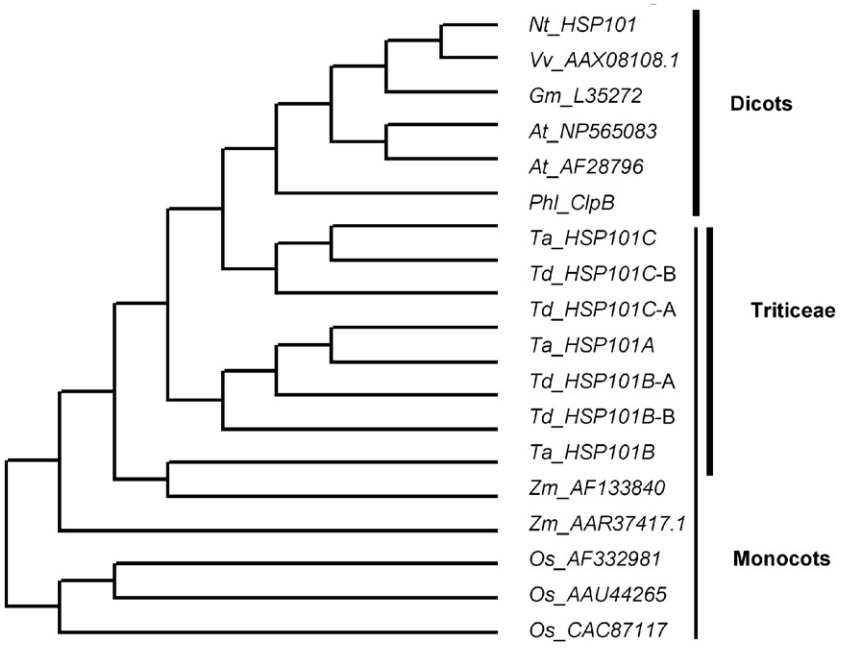

Fig. 4. Phylogenetic tree based on amino acid sequences showing the relationship of *Td*HSP101B and *Td*HSP101C with other HSP101 from higher plants: *Arabidopsis thaliana* (GenBank Accession Nos. NP565083, AF218796), *Triticum aestivum* (GenBank Accession Nos. AAC83689.2, AF174433, AF097363), *Zea mays* (GenBank Accession Nos. AF133840, AAR37417.1), *Oryza sativa* (GenBank Accession Nos. Q6F2Y7, CAC87117, AF332981, AAU44265), *Vitis vinifera* (GenBank Accession No. AAX08108.1), *Nicotiana tabacum* (GenBank Accession No. AAC83688.2), *Glycine max* (GenBank Accession No. L35272), *Phaseolus lunatus* (GenBank Accession No. AAF91178).

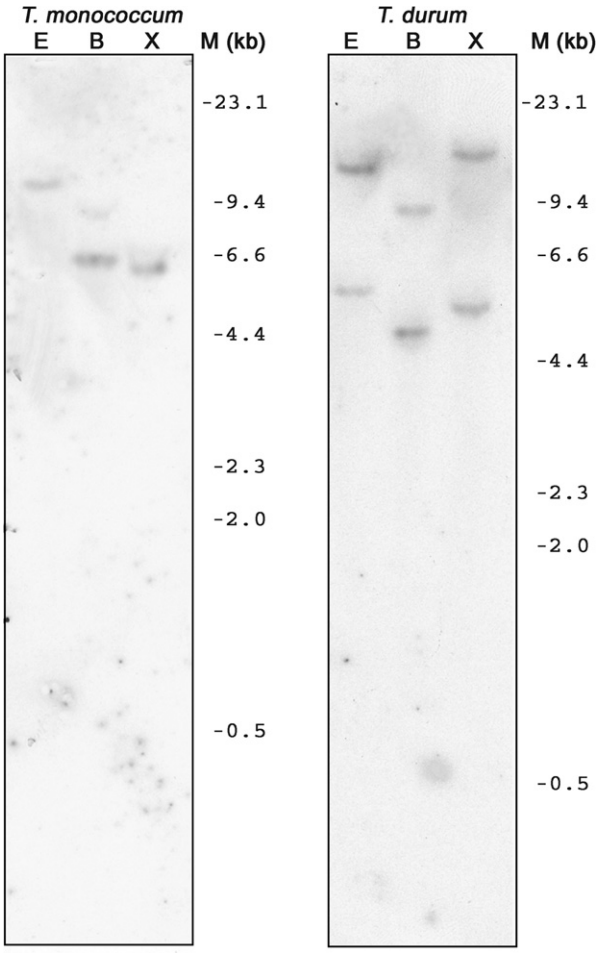

Fig. 5. Southern hybridization of genomic DNA from *T. durum* wheat and *T. monococcum* with the *TdHSP101(B-C)* cDNA probe. Total DNA (30 μg each sample for durum wheat and 15 μg each sample for *T. monococcum*) was digested with either *Bam*HI (B), *Eco*RI (E), or *Xba*I (X), and separated on 0.7% agarose gel. M = molecular masses of markers (λ-*Hind*III) in kb.

treatment at 42 °C for 2 h did not induce a high level of expression of *TdHSP101C* (A and B forms), unless a long term pre-treatment (24 h at 34 °C) was performed to induce thermotolerance. Moreover, after the pre-treatment at 34 °C all isoforms reached their maximum level of expression, though the induction of *TdHSP101B*-A and -B was higher than induction of *TdHSP101C*-A and -B in both cultivars; nevertheless, some differences can be observed between the two cvs, in fact the level of expression of the two isoforms was higher in the cv Creso.

4. Discussion

HSP101 is a key stress protein induced by abscisic acid (ABA), heat, drought, and cold stresses and its synthesis is increased to promote stress tolerance under such abiotic stress conditions [27]. In the last few years significant progress was obtained about the characterization of *HSP101* gene family in many plants, however so far no information exists about genetic organization of this gene family in durum wheat. We have identified in *T. durum* four stress-induced cDNAs, that were not reported before. The molecular characterization of these cDNAs indicated that they code for two different HSP101 isoforms (i.e. *Td*HSP101B and *Td*HSP101C) that, due to the presence of SNPs, can be distinguished in A and B forms. Previous investigations of the *HSP101* genes in other cereals showed that the maize *HSP101* gene is located on chromosome 6 bin 6.06 [28], while, the rice gene for the only HSP101 cytoplasmic isoform (Os05g44340), so far identified, is located on chromosome 5 position 25,720,401-25,723,954 (TIGR Rice Genome Annotation – Release 4). The genes for *T. durum* HSP101 isoforms here reported mapped on wheat chromosomes 1 long arm (*Td*HSP101B) and 3 long arm (*Td*HSP101C). These data are in accordance with the comparative analysis of maize, rice and wheat available maps indicating that maize chromosome 6, rice chromosome 5, and wheat chromosome 1 share an overall synteny. Nevertheless, since the wheat *HSP101* have not been mapped so far, we can not have a confirmation about the position of these genes on the wheat genome.

Phylogenetic analysis indicated that *T. durum Td*HSP101B-A and -B forms cluster with the other monocot sequences and that they are separated from *Td*HSP101C. Moreover the two *T. durum* HSP101C are more related to the dicots, and form with the *T. aestivum* HSP101C a cluster that seems to be peculiar to the Triticeae, suggesting that this isoform might have originated in these species, and that the *HSP101* genes in *T. durum* belong to a small gene family. This was further confirmed by Southern analysis on *T. durum* and *T. monococcum* indicating that different members of the *HSP101* gene family are present per haploid genome in *T. durum*. Other authors [19] reported the same kind of evaluation concerning the genomic organization of the *T. aestivum HSP101* gene family. In fact, in their studies they suggested that in the hexaploid genome of *T. aestivum* the *HSP101* gene family consists of a small number of genes in each wheat genomes.

The capacity of genotypes to cope with stress appears to be correlated with qualitative and/or quantitative variations of HSP synthesis. In particular, the expression of *HSP101* genes has been linked to increased thermotolerance in many organisms [6–8]. In fact, as already reported for *T. aestivum* and other species like *E. coli*, yeast, *Arabidopsis,* and maize [9–12], the different *HSP101* gene family members are involved in the response to different stresses, and exhibit a particular role in the heat stress response. Nevertheless, specific roles for the different isoforms have not been attributed. This task is particularly difficult to achieve in the presence of gene family in polyploid genomes like the *T. durum* one. In order to investigate whether the four identified genes are different not only in structure but also in function, we have studied their expression profiles in different conditions, with the aid of quantitative transcriptomic tools like Quantitative (Real-Time) RT-PCR. This is a useful approach when the aim of the work is to identify and quantify transcripts belonging to a gene family, as this technique allows to distinguish exactly among the contribution to the transcriptome of each of the different members. This would be difficult in allotetraploid species such as durum wheat, because of the presence of transcripts from each genome. In our case, this analysis was made possible because the complete sequence of the different cDNAs, corresponding to the different isoforms, essential for this kind of evaluation, had been determined. The presence of regions characterized by SNPs allowed the design of specific primers and probes for each specific gene.

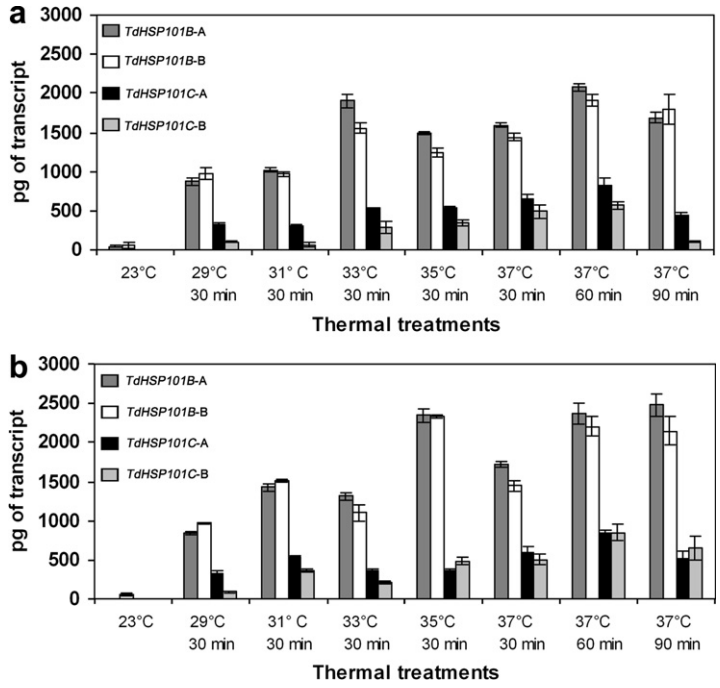

Fig. 6. Expression patterns of *TdHSP101B* (A and B form) and *TdHSP101C* (A and B form) genes in response to temperature increase (from 23 °C to 29, 31, 33, 35 °C for 30 min, or 37 °C for 30, 60, 90 min) by duplex Quantitative (Real-Time) RT-PCR in *T. durum* cvs Ofanto (a) and Creso (b). Error bar represents ±S.E. from three replicates for each sample.

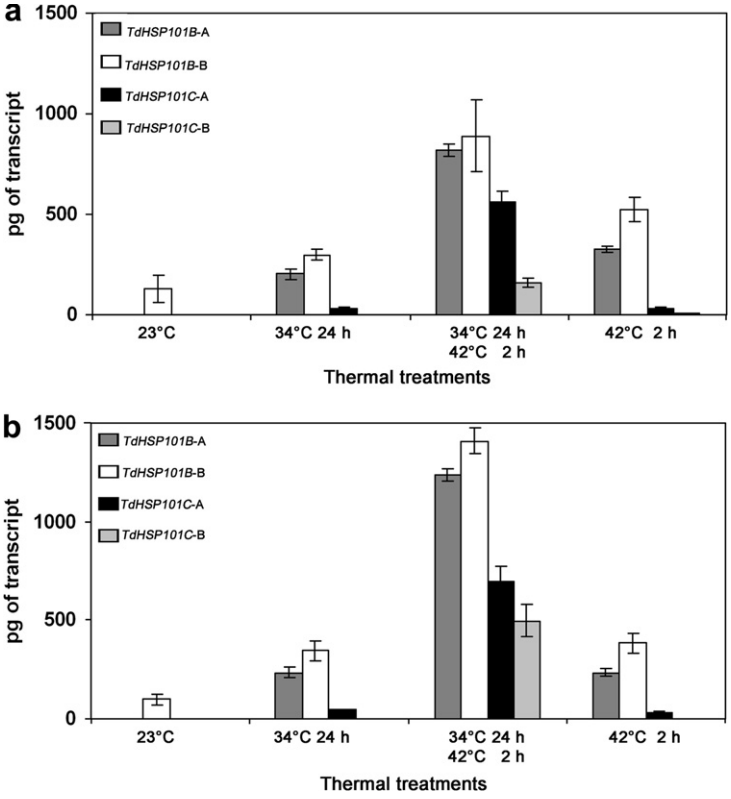

Fig. 7. Expression patterns of *TdHSP101B* (A and B form) and *TdHSP101C* (A and B form) genes in response to different thermal treatments (23 °C as control, 34 °C for 24 h; 42 °C for 2, after a pre-treatment at 34 °C for 24 h; 42 °C for 2 h without pre-treatment) by duplex Quantitative (Real-Time) RT-PCR in *T. durum* cvs Ofanto (a) and Creso (b). Error bar represents ±S.E. from three replicates for each sample.

According to the data here reported, *TdHSP101B* and *TdHSP101C* are characterized by an extremely different level of expression; thus suggesting that the two isoforms might have different roles/functions in the heat shock response. In particular, they might have different roles during acquisition of thermotolerance. So far the role of HSP101 in the acquisition of thermotolerance has been demonstrated in many organisms [8,15], for instance in some *Arabidopsis* mutants it has been shown a clear link between *HSP101* expression and thermotolerance [7,29], although the specific role for each isoform has not yet been demonstrated.

High temperature tolerance in plants has two main components: an inherent thermotolerance, i.e. the constitutive component resulting from evolutionary thermal adaptation of the species to their habitat, and an acquired thermotolerance, i.e. the ability of a plant to survive lethal temperatures, following the exposure to a mild heat stress (acclimation) [30,31].

In order to establish whether *Td*HSP101B and *Td*HSP101C isoforms have different roles in conferring thermotolerance, the heat shock was performed with and without the thermotolerance inducing pre-treatment. Through this analysis, differences in timing and level of expression for the two isoforms were observed in both cvs analyzed, thus confirming the different role for *TdHSP101C* gene with respect to *TdHSP101B*. In particular, *TdHSP101C* (A and B forms) being massively expressed only after the long term treatment at 34 °C might be the leading actor in the acquisition of thermotolerance.

According to our findings, in *T. durum* there are different members of *HSP101* gene family, located on A and B genomes, actively transcribed in response to thermal stress, and differently expressed under various thermal treatments. However, to dissect their regulatory mechanisms further analysis is needed. A better elucidation of the relationships between molecular diversity within the *HSP101* gene family, and the comprehension of the role of the different members will help us to understand the functions of these genes in stress responses and induction of tolerance in durum wheat; moreover, this knowledge will be useful to improve the tolerance of durum wheat to abiotic stresses.

Acknowledgements: We thank Prof. Steve Quarrie for his helpful comments and discussion. This work was supported by Grants from Italian MiPAF (AMIFRUGAM) and MIUR (PRIN 2003) to Carla Perrotta.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.09.010.

References

[1] Larkindale, J., Mishkind, M. and Vierling, E. (2005) Plant responses to high temperature in: Plant Abiotic Stress (Jenks, M.A. and Hasegawa, P.M., Eds.), pp. 151–180, Blackwell Scientific Publications, Oxford.

[2] Agarwal, M., Sahi, C., Katiyar-Agarwal, S., Agarwal, S., Young, T., Gallie, T.R., Sharma, V.M., Ganesan, K. and Grover, A. (2003) Molecular characterization of rice *hsp101*: complementation of yeast *hsp104* mutation by disaggregation of protein granules and differential expression in indica and japonica rice type. Plant Mol. Biol. 51, 543–553.

[3] Lee, S., Sowa, M.E., Chai, J.M. and Tsai, F.T.F. (2004) The ClpB/Hsp104 molecular chaperone – a protein disaggregating machine. J. Struct. Biol. 146, 99–105.

[4] Lee, U., Vie, C., Escobar, M., Williams, B., Hong, S.W. and Vierling, E. (2005) Genetic analysis reveals domain interactions of *Arabidopsis* HSP100/Clp and cooperation with the small heat shock protein chaperone system. Plant Cell 17, 559–571.

[5] Zhang, C. and Guy, C.L. (2005) Co-immunoprecipitation of Hsp101 with cytosolic Hsc70. Plant Physiol. Biochem. 43, 13–18.

[6] Queitsch, C., Hong, S.W., Vierling, E. and Lindquist, S. (2000) Heat shock protein 101 plays crucial role in thermotolerance in *Arabidopsis*. Plant Cell 12, 479–492.

[7] Hong, S.W. and Vierling, E. (2000) Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress. Proc. Natl. Acad. Sci. USA 97, 4392–4397.

[8] Hong, S.W. and Vierling, E. (2001) Hsp101 is necessary for heat tolerance but dispensable for development and germination in the absence of stress. Plant J. 27, 25–35.

[9] Gottesman, S., Clark, W.P. and Maurizi, M.R. (1990) The ATP-dependent Clp protein *Escherichia coli*: sequence of *ClpA* and identification of Clp-specific substrates. J. Biol. Chem. 265, 7886–7893.

[10] Parankiewicz, J., Wang, J. and Clarke, A.K. (1999) New insights into the ATP-dependent Clp protease: *Escherichia coli* and beyond. Mol. Microbiol. 32, 449–458.

[11] Schirmer, E.C., Glover, J.R., Singer, M.A. and Lindquist, S. (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem. Sci. 21, 289–296.

[12] Vierling, E. (1997) Plant HSP100/ClpB in: Handbook of Molecular Chaperones and Protein Folding Catalysts (Gething, M.J., Ed.), pp. 253–255, Oxford University Press, Oxford, UK.

[13] Parsell, D.A., Kowal, A.S., Singer, M.A. and Lindquist, S. (1994) Protein disaggregation mediated by heat shock protein Hsp104. Nature 372, 475–478.

[14] Katiyar-Agarwal, S., Agarwal, M., Gallie, D.R. and Grover, A. (2001) Search for cellular functions of plant Hsp100/Clp family proteins. Crit. Rev. Plant Sci. 20, 277–295.

[15] Sanchez, Y. and Lindquist, S. (1990) HSP104 required for induced thermotolerance. Science 248, 1112–1115.

[16] Agarwal, M., Katiyar-Agarwal, S., Sahi, C., Gallie, D.R. and Grover, A. (2001) *Arabidopsis thaliana* Hsp100 proteins: kith and kin. Cell Stress Chaperon. 6, 219–224.

[17] Agarwal, M., Katiyar-Agarwal, S. and Grover, A. (2002) Plant Hsp100 proteins: structure, function and regulation. Plant Sci. 163, 397–405.

[18] Wells, D.R., Tanguay, R.L., Le, H. and Gallie, D.R. (1998) HSP101 functions as a specific translational regulatory protein whose activity is regulated by nutrient status. Genes Dev. 12, 3236–3251.

[19] Campbell, J.L., Klueva, N.Y., Zheng, H.G., Nieto-Sotelo, J., Ho, T.H.D. and Nguyen, H.T. (2001) Cloning of new members of heat shock protein *HSP101* gene family in wheat (*Triticum aestivum* L. *Moench*) inducible by heat, dehydration, and ABA. Biochim. Biophys. Acta 1517, 270–277.

[20] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

[21] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

[22] Sears, E.R. (1966) Nullisomic–tetrasomic combinations in hexaploid wheat in: (Riley, R. and Lewis, K.R., Eds.), Chromosome Manipulations and Plant Genetics: The Contributions to a Symposium held during the Tenth International Botanical Congress, Edinburgh, 1964, p. vii, Plenum Press, New York.

[23] Sears, E.R. and Sears, M.S. (1978) The telocentric chromosomes of common wheat. In: Proceedings of the 5th International Wheat Genetics Symposium of the Indian Society of Genetics and Plant Breeding (Ramanujam, S., Ed.), pp. 389–407, New Delhi, India.

[24] Marmiroli, N., Pavesi, A., Di Cola, G., Hartings, H., Raho, G., Conte, M.R. and Perrotta, C. (1993) Identification, characterization, and analysis of cDNA and genomic sequences encoding two

different small heat shock proteins in *Hordeum vulgare*. Genome 36, 1111–1118.
[25] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[26] Rampino, P., Spano, G., Pataleo, S., Mita, G., Napier, J.A., Di Fonzo, N., Shewry, P.R. and Perrotta, C. (2006) Molecular analysis of a durum wheat "stay green" mutant: Expression pattern of photosynthesis-related genes. J. Cer. Sci. 43, 160–168.
[27] Batra, G., Singh Chauhan, V., Singh, A., Sarkar, N.K. and Grover, A. (2007) Complexity of rice Hsp100 gene family: lessons from rice genome sequence data. J. Biosci. 32, 611–619.
[28] Nieto-Sotelo, J., Martinez, L.M., Ponce, G., Cassab, G.I., Alagón, A., Meeley, R.B., Ribaut, J.-M. and Yang, R. (2002) Maize HSP101 plays important roles in both induced and basal thermotolerance and primary root growth. Plant Cell 14, 1621–1633.
[29] Hong, S.W., Lee, U. and Vierling, E. (2003) *Arabidopsis hot* mutants define multiple functions required for acclimation to high temperatures. Plant Physiol. 132, 757–767.
[30] Klueva, N.Y., Maestri, E., Marmiroli, N. and Nguyen, H.T. (2001) Mechanisms of thermotolerance in crop in: Crop Responses and Adaptation to Temperature Stress (Basra, A.S., Ed.), pp. 177–217, Food Production Press, Binghamton, NY.
[31] Burke, J.J. (2001) Identification of genetic diversity and mutations in higher plant acquired thermotolerance. Physiol. Plant. 112, 167–170.