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Short communication

Differential expression of heat shock protein genes in sorghum (*Sorghum bicolor* L.) genotypes under heat stress

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Abstract

Various types of sorghum were subjected to thermal stress to reveal the mode of expression of genes of the heat shock protein (*hsp*) family. *In silico* sequence determination of *hsp* genes in related cereal species led to the selection of appropriate primers for PCR amplification of a segment corresponding to the *hsp90* gene from sorghum. Deduced sequence information allowed the design of gene specific primers for quantification of *hsp90* gene expression by means of real-time quantitative polymerase chain reaction (RT-qPCR). Fourteen days-old plants were exposed to a temperature of 47°C for a time period ranging from 10 to 180 min. Total RNA was extracted from stressed and control plants and subjected to reverse transcription and RT-qPCR analysis. The actin gene was used as an internal standard. Gene expression was assessed by using cDNA from all types of plant material and for all the different durations of heat stress exposure. Data from RT-qPCR analyses were analyzed using REST software. The highest level of *hsp*90 gene expression was realized upon exposure to heat for either 60 or 30 min, while expression levels differed among the genotypes studied. In addition, overall levels of *hsp90* gene expression were significantly different among varieties tested. Information of such genotypic variation in expression levels of *hsp90* gene under heat stress, coupled with related field performance data, could potentially be exploited in breeding programs.

Keywords: sorghum, heat shock proteins, *hsp90*, abiotic stress, plant breeding.
Abbreviations: HSP, heat shock protein; *hsp*, heat shock protein gene; PCR, polymerase chain reaction; RT, reverse transcription; qPCR, quantitative real-time PCR; REST, Relative expression software tool.

Introduction

Plants are generally subjected to various types of stresses, as optimal environmental conditions are only rarely prevailing (Taiz and Zaiger, 1998; Wang et al., 2003). To cope with unfavorable growth conditions, plants respond with a series of morphological, biochemical and molecular adaptations, aiming at safeguarding basic metabolic activities (Wang et al., 2001; Shao et al., 2007; Biamonti and Caceres, 2009). Proteins synthesized in response to high temperatures are called heat shock proteins (HSP) and their induction appears as a universal protective reaction occurring in all organisms ranging from prokaryotes to human beings (Lindquist, 1986; Wu, 1995; Gupta et al., 2010). In addition to heat stress, the induction of HSPs is implicated in responses to other stresses such as water deficit, salinity, radiation, heavy metal contamination as well stresses due to biotic agents (Lindquist and Craig, 1988; Blumenthal et al., 1990; Vierling, 1991; Feder and Hofman, 1999; Iba, 2002; Young and Elliott, 2002; Soransen et al., 2003; Swindell et al., 2007). HSP homologues are expressed, constitutively or under cyclic or developmental control, in every species at low levels and accumulate to very high levels in stressed cells (Waters et al., 1996; Vierling, 1997; De Maio, 1999; Young and Elliott, 2002). Members of the HSP superfamily have been classified on the basis of their approximate molecular weight into several principal classes like HSP100, HSP90, HSP70, HSP60, HSP40 as well as the low molecular mass HSPs, designated as small HSPs, (16-30 kDa) (Lindquist and Craig, 1988; Vierling, 1991; Waters et al., 1996). Higher plants possess at least 20 HSPs, whereas the corresponding number for other species exceeds the 40 HSPs (Vierling, 1991). In response to heat and other stresses, cells modulate their cellular activity and cease protein synthesis in favour of HSP synthesis (Young and Elliott, 2002). The transcription of the *hsp* genes is regulated by proteins located in the cytoplasm in an inactive state, called heat stress transcription factors (HSFs) (Hu et al., 2009). Plants are generally characterized by a large number of HSFs (at least 21) which act synergistically in regulating a range of functions and stages of response to stress such as triggering, maintenance and recovery (Tripp et al., 2009). Sorghum (*Sorghum bicolor* L. Moench) is an annual C4, grain and fodder cereal crop originating from semi-arid tropics and hence, is characterized by a considerable tolerance to drought and high temperatures. As such, sorghum provides a suitable model crop to study the molecular and biochemical mechanisms underlying resistance to high temperatures. At the same time, in view of the recent interest in exploiting sorghum's yield potential and related attributes as an important candidate energy crop, an additional upgrading of its tolerance to high temperatures is necessitated either for initial seedling establishment or for

Table 1. Primers used in RT-qPCR reactions for *hsp90* and actin genes

Primer	Sequence (5' → 3')	Product size (bp)	Primer concentration (nM)
Act-1019F	CTAGCAGCATGAAGATCAAGGTG	134	100
Act-1152R	GCCAGACTCGTCGTACTCAG		200
Hsp-118F	GGAGTTTGAGGGCAAGAAGC	138	100
Hsp-255R	CCCAGAACCTCCTTGATGAC		200

Table 2. Relative gene expression (medians) of *hsp90* and actin genes in heat stress-treated (10 min, 30 min, 60 min, 180 min) sorghum plants of the genetic material tested (Honey Graze, Dekalb, NK300 and M81). Real-time qPCR datasets were analyzed using REST v1.9.12

Treatment	Gene	qPCR efficiency	Gene expression	Std. Error	95% C.I.
Honey Graze	actin	1,0489	1	0,67-1,52	0,58-2,32
Honey Graze (10min)	*hsp90*	1,0558	58,384	46,43-74,01	42,57-81,66
Honey Graze (30min)	*hsp90*	1,0558	157,268	139,3-179,4	128,63-194,73
Honey Graze (60min)	*hsp90*	1,0558	291,232	251,1-341,4	238,13- 60,72
Honey Graze (180min)	*hsp90*	1,0558	94,621	85,32-105,67	77,17-117,51
Dekalb	actin	0,9724	1	0,71-1,49	0,68-1,50
Dekalb (10min)	*hsp90*	1,0156	8,624	7,12-11,18	6,58-12,38
Dekalb (30min)	*hsp90*	1,0156	8,746	7,07-11,62	6,91-11,98
Dekalb (60min)	*hsp90*	1,0156	15,539	11,69-22,76	11,42-23,46
Dekalb (180min)	*hsp90*	1,0156	4,77	4,17-5,71	4,06-5,92
NK300	actin	0,8648	1	0,69-1,69	0,56-1,83
NK300 (10min)	*hsp90*	1,0868	12,465	8,93-19,92	8,35-21,83
NK300 (30min)	*hsp90*	1,0868	40,002	25,46-76,91	20,85-99,54
NK300 (60min)	*hsp90*	1,0868	34,912	22,53-65,64	18,73-83,43
NK300 (180min)	*hsp90*	1,0868	7,988	6,35-11,21	5,30-14,17
M81	actin	1,2307	1	0,60-1,44	0,39-1,64
M81 (10min)	*hsp90*	1,0918	1,988	1,89-2,12	1,85-2,18
M81 (30min)	*hsp90*	1,0918	12,491	10,17-16,26	9,09-18,58
M81 (60min)	*hsp90*	1,0918	3,935	2,71-5,91	2,53-6,85
M81 (180min)	*hsp90*	1,0918	1,375	1,31-1,45	1,29-1,49

successfully coping with the increasing air temperatures due to global warming (Jaradat, 2010). In this framework, the present study aimed at investigating the response of various types of sorghum genetic material (i.e. grain, sweet, forage) to thermal stress, as far as the expression of the heat shock protein gene *hsp90* is concerned.

Materials and methods

Plant material

Hsp90 gene expression was studied in four commercial hybrid varieties of sorghum: fodder (Honey Graze), fiber (Dekalb), dual purpose (NK300) as well as a variety of sweet sorghum (M81).

Isolation and sequencing of hsp90 gene in sorghum

After an *in silico* identification of conserved sequence regions of *hsp90* genes in cereals, a pair of degenerated primers was designed for PCR amplification of the corresponding gene sequences in sorghum. DNA was extracted from young sorghum plants using the DNeasy plant mini kit (Qiagen) and PCR was performed using the degenerated primers (hsp90-F: 5'-ATCACTGGTGAGAGCAAGAAGGC-3' and hsp90-R: 5'-TTAGTCGACCTCCTCCATCTTGCT-3') and GoTaq Flexi DNA polymerase (Promega), according to the supplier's instructions. Following elution from agarose gels, amplification products were cloned into TOPO TA vector and sequenced using M13 forward and reverse primers.

Thermal stress treatments

Sorghum seedlings of all four hybrid varieties were grown in a 3:1 soil to perlite mixture in a growth chamber under controlled conditions (25˚C, 16 h light/8 h dark cycle). Fourteen days-old (14d old) sorghum plants were subjected to heat shock at four different time periods of stress exposure: 10 min, 30 min, 60 min and 180 min, at 47°C. Plants of the same age that were not exposed to thermal stress were included as controls. Samples consisted of leaf tissues collected from 3-5 plants for each of the four categories of treated and control plants. Samples were immediately frozen in liquid nitrogen and maintained at -80°C.

RNA extraction and RT-qPCR

Total RNA was extracted using the SV RNA isolation system (Promega) and quantified by spectrophotometry. RNA integrity was verified in 1 % agarose gels. First strand cDNA synthesis was performed using random primers (decamers) and the Superscript II reverse transcriptase (Invitrogen, UK), according to the manufacturer's instructions. First-strand cDNA was diluted to a final concentration of 0.5 ng/µl. Primers for RT-qPCR reactions were designed from the deduced sequence corresponding to the sorghum *hsp90* gene using Beacon Designer 5.1 software (Premier Biosoft, USA) (Table 1). Quantitative PCR was performed in 25µl reactions using gene specific primers, 1 µl of cDNA as template and the SYBRGreenER qPCR SuperMix Universal (Invitrogen, UK). Reactions were performed on the Mini Opticon Real-Time PCR system (Biorad, UK). The thermal profile for

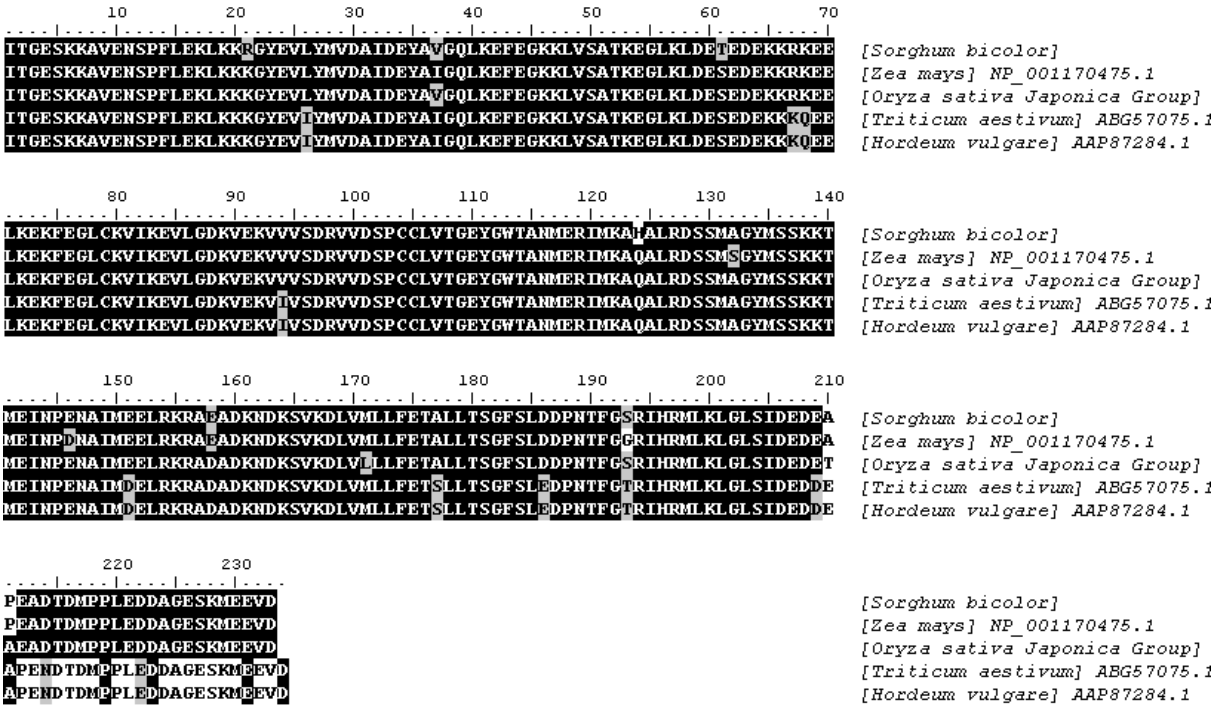

Fig 1. Multiple sequence analysis relating HSP90 of sorghum to protein homologues from *Zea mays* (Accession N. NP_001170475), *Oryza sativa* (Accession N. NP_001062159), *Triticum aestivum* (Accession N. ABG57075) and *Hordeum vulgare* (Accession N. AAP87284). The alignment was generated using BioEdit Sequence Alignment Editor v 7.0.5.3.

qPCR was: 10 min at 95°C, followed by 40 cycles each consisting of 95°C for 15 sec, 60°C for 30 sec and 72°C for 15 sec. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 3 % agarose gel. The expression levels of sorghum actin gene were used as internal standards for normalization of cDNA template quantity using actin-specific primers (Accession N. AF282624) (Table 1). For each stress condition as well as for controls, expression measurements were performed using duplicate biological replications and three technical replications. Controls with no cDNA template were also included in qPCR analysis. Three point reference curves were created from all treated and control plants and each point was represented with three replicates. Data analysis was performed using REST (Relative Expression Software Tool) software v1.9.12 (Corbett, Germany). The corresponding real-time qPCR efficiency of one cycle for each gene was determined with the slope of a linear regression model, according to the equation: $E = 10[-1/\text{slope}]$. Relative expression was calculated based on qPCR efficiency (E) and the crossing point difference (ΔCP) of a treated *vs* control for both target and reference genes according to the formula: Ratio= (Etarget)ΔCPtarget(control – sample)/(Eref)ΔCPref(control – sample) (Pfaffl et al., 2002). Relative expression ratios are calculated on the basis of group means for target gene versus reference gene and group ratio results are tested for significance. Here, the *hsp90* gene expression was normalized by actin expression, which is a non-regulated reference gene, and then the normalized value was used to calculate differential gene expression in samples. Expression ratios were calculated in the 95 % confidence interval.

Results and discussion

Sequence determination of the hsp90 gene in sorghum

The sequence of the 698 bp-amplicon corresponding to the *hsp90* gene of sorghum was determined and subsequently, its nucleotide and amino acid sequence was compared with previously published sequences of gene homologues from other cereal species such as maize, rice, wheat and barley. BLAST search revealed that the percentages of sequence identities among the HSP90 protein of sorghum and other members of this protein family amount to more than 90 %. More specifically, the HSP90 protein of sorghum scored the highest sequence identity with the HSP90 from *Zea mays* (97 %) (Accession N. NP_001170475) and *Oryza sativa* (97 %) (Accession N. NP_001062159). In addition, the HSP90 protein of sorghum presented an amino acid sequence identity level of 94 % with the corresponding proteins from *Triticum aestivum* (Accession N. ABG57075) and *Hordeum vulgare* (Accession N. AAP87284) (Fig. 1).

Assessment of relative gene expression of hsp90 in sorghum under heat stress

The expression response patterns of the *hsp90* gene in sorghum were analyzed with respect to four different time periods of thermal stress. The relative gene expression of

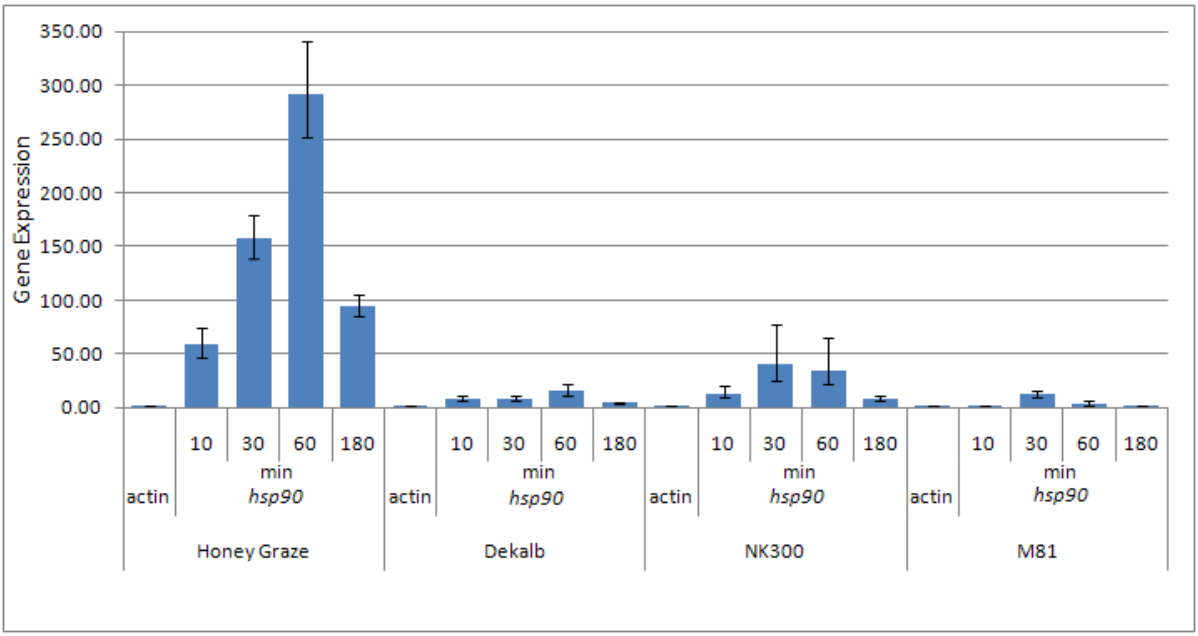

Fig 2. Accumulation of *hsp90* gene transcripts in four sorghum varieties subjected to thermal stress for four different time periods. Total RNA was isolated, reverse transcribed to cDNA, and subjected to real-time quantitative PCR using gene specific primers. Relative *hsp90* gene expression was calculated with respect to the expression level in control plants after normalization with the levels of the actin transcripts. Data was analyzed using REST software v1.9.12. Bars show medians with standard error.

hsp90 of heat stressed plants versus the control plants was calculated using the REST software which enables a correction of the qPCR efficiencies and the mean crossing point deviation between treated and control plants (Nolan et al., 2006, Pfaffl et al., 2002). The expression profiles of the *hsp90* revealed that this gene is highly expressed in response to heat stress in sorghum, a crop known for its ability to thrive in warm regions. However, different expression levels were obtained for the four time periods of exposure to thermal stress when quantitatively assessed. In the varieties Honey Graze and Dekalb, induction of the *hsp90* gene was recorded at 10 min of heat stress whereas the highest level of gene expression was determined in plants that were subjected to high temperature for 60 min. In contrast, NK300 and M81 presented the highest levels of *hsp90*-transcript after 30 min of heat stress. In all varieties tested, the relative mRNA amount of *hsp90* declined after having reached its highest level. Overall levels of *hsp90* gene expression were highest for Honey Graze, followed by NK300, while Dekalb and M81 presented significantly lower expression levels (Table 2) (Fig. 2). The pattern of initial gene induction followed by a gradual *hsp* transcript accumulation, the reach of a peak level and the subsequent transcript decline is a common feature of similar studies with other crops. Different *hsp90* expression and related protein production patterns observed as far as duration of exposure to heat and plant genotypes are concerned, has also been reported in these studies (Milioni et al., 2001; Ahn et al., 2004). The decline after having reached the highest transcript accumulation most probably occurs due to mRNA degradation and possibly gene repression. Given that differential gene expression is the result of a cell's response to altered external and internal signals, varying exposure durations at which the peak level is attained, as well as overall transcript level accumulation at peak among genotypes, might well reflect their different genetic background (Milioni et al., 2001). In sorghum, the production of early heat shock proteins allows for a rapid recovery of cells and continuation of growth. In contrast, late HSP production may result in growth inhibition and eventually, cell death. However, it is still not well elucidated whether the early elicitation of *hsp* transcripts acts protectively against thermal stress. To this respect, it is challenging to determine the correlation of *hsp90* gene induction with the phenotype of sorghum plants subjected to heat stress under field or greenhouse conditions. If a positive correlation of *hsp90* induction with plant's thermotolerance is well established, the expression level of *hsp90*-trancsripts could be practically exploited as a functional molecular marker for screening tolerance to heat stress.

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