



Overall picture of expressed Heat Shock Factors in *Glycine max*, *Lotus japonicus* and *Medicago truncatula*

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Abstract

Heat shock (HS) leads to the activation of molecular mechanisms, known as HS-response, that prevent damage and enhance survival under stress. Plants have a flexible and specialized network of Heat Shock Factors (HSFs), which are transcription factors that induce the expression of heat shock proteins. The present work aimed to identify and characterize the *Glycine max* HSF repertory in the Soybean Genome Project (GENOSOJA platform), comparing them with other legumes (*Medicago truncatula* and *Lotus japonicus*) in view of current knowledge of *Arabidopsis thaliana*. The HSF characterization in leguminous plants led to the identification of 25, 19 and 21 candidate ESTs in soybean, *Lotus* and *Medicago*, respectively. A search in the SuperSAGE libraries revealed 68 tags distributed in seven HSF gene types. From the total number of obtained tags, more than 70% were related to root tissues (water deficit stress libraries vs. controls), indicating their role in abiotic stress responses, since the root is the first tissue to sense and respond to abiotic stress. Moreover, as heat stress is related to the pressure of dryness, a higher HSF expression was expected at the water deficit libraries. On the other hand, expressive HSF candidates were obtained from the library inoculated with Asian Soybean Rust, inferring crosstalk among genes associated with abiotic and biotic stresses. Evolutionary relationships among sequences were consistent with different HSF classes and subclasses. Expression profiling indicated that regulation of specific genes is associated with the stage of plant development and also with stimuli from other abiotic stresses pointing to the maintenance of HSF expression at a basal level in soybean, favoring its activation under heat-stress conditions.

Key words: HSF, Fabaceae, bioinformatics, abiotic stress, transcription factor.

Introduction

Heat stress is one of the major factors limiting the productivity and adaptation of crops, especially when temperature extremes coincide with critical stages of plant development. The major developmental performance of plants occurs at a temperature regime between 10° and 40 °C. Temperatures below or above this range generally cause temperature-induced stresses (Treshow, 1970; Hsu *et al.*, 2010). In the case of heat stress, both the rate of temperature change and the duration and degree of high temperatures contribute to the intensity of heat stress. The degree of inherent adaptedness to heat stress of a plant is an important

determinant of its ability to survive a stress period (Efeoglu, 2009). However, the expression of HSF and HSP genes has been also observed under other abiotic and biotic stresses, as cited by Pirkkala *et al.* (2001). In response to various inducers such as elevated temperatures, salinity, drought, oxidants, heavy metals, bacterial and viral infections, most HSFs acquire DNA binding activity to the heat shock element (HSE), thereby mediating transcription of the heat shock factor genes, which results in accumulation of heat shock proteins (HSPs). Among important transcription factors, heat shock factors (HSFs) are essential for the transcription of many HSP coding genes that are active in response to sublethal heat stress leading to increased tolerance against a subsequent, otherwise lethal, heat shock (Treshow, 1970; Hsu *et al.*, 2010).

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After stress perception, intracellular changes lead to a molecular cascade of events, initiated by HSF activation and subsequent expression of HSPs limiting stress damage (Hsu *et al.*, 2010). In general, HSF proteins have a common core structure comprising a N-terminal DNA binding domain (DBD) characterized by a central helix-turn-helix (HTH) motif, an adjacent domain with a heptad hydrophobic repeat (HR-A/B) which is involved in oligomerization, a short peptide motif essential for nuclear import [nuclear localization signal (NLS)] and export [nuclear export signal (NES)], and a C-terminal AHA type activation domain (Mittal *et al.*, 2009; Hsu *et al.*, 2010).

Through the DNA binding domain, activated HSFs bind to conserved cis-acting elements called heat shock elements (HSEs). HSEs are located in the promoters of HSP genes and are defined as adjacent and inverse repeats of the motif 5-nGAAn-3, for instance 5-nGAAnnTTCnnGAAn-3 (Schöffl *et al.*, 1998).

Some HSFs have been cloned and characterized from various plant species (Nover *et al.*, 2001; Baniwal *et al.*, 2007) revealing that the network of HSF genes is highly flexible and specialized in this group. Details regarding the overall HS response network were initially not clear. However, studies in *Arabidopsis* revealed that 21 HSFs form a complex network, in which AtHSFA1a and AtHSFA1b play important roles in the induction of HSP genes in the early stage of HSR (Nover *et al.*, 2001).

An insight into the response of HSPs and HSFs to different abiotic stresses was provided through a number of genome-wide microarray datasets. *Arabidopsis* HSFs and HSPs were strongly induced by heat, cold, salinity and osmotic stresses. Furthermore, overlapping responses of HSPs and HSFs to heat and other abiotic stresses was reported, indicating that these genes are important elements in the crosstalk among different response pathways (Hu *et al.*, 2009). In rice, over-expression of OsHsp17.7 enhanced rice tolerance to heat UV-B as well as to drought (Sato and Yokoya, 2008).

Hu *et al.* (2009) identified rice *HSF* and *HSP* genes and analyzed their expression profiles under different abiotic stresses. A whole-genome microarray analysis was carried out to investigate expression changes of rice *HSFs* and *HSPs* genes in response to heat stress. By comparing their experimental data with other expression data under salt, cold, and drought conditions, Hu *et al.* (2009) found that the rice HSF and HSP families responded to different stresses in an overlapping relationship. The analysis also indicated that some *HSF* and *HSP* genes exhibited specific expression patterns in response to distinct stress types.

In *Arabidopsis*, for example, the major role of the representatives of the HsfA4/A5 group, which is generally not involved in the conventional heat stress response, may reside in cell type-specific functions connected with the control of cell death triggered by pathogen infection and/or reactive oxygen species (Baniwal *et al.*, 2007).

Although the flexible network of HSF genes has been well studied in plants, there is little information available regarding the structure and function of HSF genes in legumes. Additionally, no comparison of HSF orthologs has been carried out until now among legumes. In this study, we used well-described *Arabidopsis* HSF proteins as seed sequences in order to identify and characterize the pool of HSF genes present in the *Glycine max* genome and perform a comparative analysis against *Lotus japonicus* and *Medicago truncatula* genomes, so as to trace the panorama of the HSF genes in these leguminous plants.

Material and Methods

Based on 21 well-described *Arabidopsis* HSF genes in the AfTDB database, BLASTp searches (Altschul *et al.*, 1990) were carried out for similar sequences against the GENOSOJA database. GENOSOJA connects public and project soybean data (Nascimento *et al.*, 2012). In total, the initiative provides information on 60,747 unigenes from the NBCI, Phytozome and Soybean full-length cDNA databases (Nascimento *et al.*, 2012). Comparative searches were made in the *Medicago truncatula* and *Lotus japonicus* databases. After searching the GENOSOJA databank, only orthologs presenting the fully characteristic HSF DNA-Binding Domain (DBD) were considered for subsequent analysis. In view of the obtained soybean, *Medicago* and *Lotus* HSF candidates together with the *Arabidopsis* seed-sequences, a comparative analysis with 69 aligned proteins was performed, enabling the generation of a dendrogram, using the Neighbor-Joining (NJ) method with 2,000 bootstrap replications with program MEGA program v. 5.0 (Tamura *et al.*, 2011), to infer about HSF groups and classes within the analyzed legumes. For this purpose the sequence-coding genes from *Arabidopsis* that did not present similarity (orthology) with the studied legumes were excluded from the phenetic analysis. To prevent the influence of different sequence sizes, the alignments were trimmed aiming to exclude unequal 5' and 3' extremities.

To evaluate the HSF-related tags represented in the SuperSAGE libraries generated by the GENOSOJA project, a comparative analysis using the same seed sequences and the MegaBLAST algorithm was carried out according to Altschul *et al.* (1990). For this purpose the parameters were adjusted to an e-value equal to or less than 0.1 and word size equal to 7. The low complexity filter was deactivated. Results considered only tags with identity equal to or larger than 23 bp.

The GENOSOJA databank is comprised of six SuperSAGE libraries and allowed the generation of three comparisons, including two from root tissues subjected to water deficit stress and one inoculated with Asian Soybean Rust fungus (*Phakopsora pachyrhizi*). For the water deficit libraries, seeds of a drought tolerant cultivar (Embrapa 48) and a drought susceptible cultivar (BR 16) were germinated on filter paper for four days in a growth chamber at

25 ± 1 °C and 100% relative humidity (RH). Seedlings were placed in 36 L boxes containing 50% Hoagland's solution (Hoagland and Arnon, 1950) continuously aerated and replaced on a weekly basis. These boxes were then transferred to a greenhouse under natural photoperiod of approximately 12/12 h light/dark cycle, temperature of 30 ± 5 °C and 60 ± 10% RH. The plants were allowed to grow until the V4 stage (Fehr *et al.*, 1971). The experimental plan was a randomized complete block 2x7 factorial design with three repetitions. The treatments included two cultivars (BR 16 and Embrapa 48) and seven water deficit periods (0, 25, 50, 75, 100, 125 and 150 min). Water stress was applied by removing the plants from the hydroponic solution and leaving them in boxes without nutrient solution for up to 150 min under ambient-air exposure. For each stress exposure time, roots from 10 plants were collected, pooled and frozen in liquid nitrogen before storage at -80 °C. The above mentioned exposure times were bulked together generating a library from drought tolerant genotype Embrapa 48 after stress as compared with the negative control (T0); the same procedure was also applied to the drought sensitive genotype (BR16 cultivar). The comparison regarding Asian Soybean Rust infection was generated from leaves of the resistant cultivar PI561356 collected at different times (12, 24 and 48 h) after spraying with a *P. pachyrhizi* spore suspension (6 x 10⁵ uredospores.mL⁻¹). The urediniospores were collected from *Phakopsora pachyrhizi* infected soybean fields in the state of Mato Grosso, Brazil, and maintained for over 10 generations on the susceptible cv. BRSMS-Bacuri. The suspension of spores was sprayed onto three plants per pot at the V2 to V3 growth stage (Fehr and Caviness, 1977). The same solution without the spores was used for the false inoculations (Mock). The different times were bulked together to form a single resistant library, which was compared with the false inoculated negative control collected at the same time points.

Considering the identified *G. max* EST transcripts, standard statistical methods (see Eisen *et al.*, 1998) were used to arrange the HSF genes according to their gene expression pattern, generating a graphic with colors (green, red and black) indicating their quantitative and qualitative expression (down-, up- and unregulated genes, respectively), while gray stood for absence of information. The gene expression data analyzed were collected from soybean during a variety of challenging and control conditions available at the GENOSOJA database. So as to obtain a picture of how HSFs contribute to sensing the environmental up-shifts in temperature, we applied Self-Organizing Maps followed by pairwise average-linkage cluster analysis to normalized gene expression data (Eisen *et al.*, 1998). Relationships among genes and libraries were represented by dendrograms in which branch lengths reflect the degree of gene co-expression.

An available genome browser for soybean (Phytozome database) was used to anchor identified EST candidate sequences on *G. max* virtual chromosomes, aiming to identify their distribution, relative position, and abundance. For this purpose the MegaBLAST tool was used to identify the exact location of the HSF genes in the genome, using at least 80% identity as a parameter. For the construction of a virtual karyotype representation, a CoreDRAW12 graphic application was used. The soybean chromosome information for the schematic representation was obtained from the SOYBASE site. For the design of chromosomes, considering the need for high-resolution bands (data anchored in the genome), a proportion of 1:1 (cm:Mb) was adopted for all chromosomes; thus, for the sequence positioning, each millimeter corresponded to 100,000 bp. On the representation each transversal black line corresponded to an HSF gene.

Results and Discussion

Heat and cold can have damaging consequences on both vegetative and reproductive tissues. Temperature changes can also regulate plant movements, resetting internal clocks and diurnal synchronization, flowering and germination in some species (Ruelland and Zachowski, 2010). Moreover, temperature changes can induce metabolic changes so that plants adapt and tolerate moderate cold, freezing and heat stresses (Ruelland and Zachowski, 2010). HSFs are important components of the heat shock regulatory network, with a single gene identified for yeast and drosophila, while vertebrates accounted with only four genes of this category (Swindell *et al.*, 2007). Nevertheless, unlike other organisms, plant genomes encode extraordinarily complex HSF families, both in terms of the total number of genes (usually more than 20), as well as in terms of their structural and functional diversification (Nover *et al.*, 2001). This abundance and diversity can be also seen in legumes. An extensive BLAST search of *Arabidopsis* HSF orthologs in soybean, *Lotus* and *Medicago* EST databases led to the identification of a total of 25, 19 and 21 expressed sequences, respectively (Table 1).

HSF expressed sequence tags

The characteristic HSF domains were complete in 24, 13 and 17 orthologous candidates identified among the three species, respectively (Table 1). From the 21 types of *Arabidopsis* HSF genes only 13 types were identified in soybean and *Lotus* and 14 in *Medicago* (Table 1). In our evaluation, HSFA1B, HSFA6A, HSFA7B and HSFA9 were absent in all species analyzed (Table 1). HSFA1A and HSFA1B interact as regulators responsible for immediate-early transcription of a subset of HS genes in *Arabidopsis*, and are independently important for the initial phase of HS-responsive gene expression, while their interaction enhances the expression of their target genes (Li *et al.*, 2010). The absence of HSFA1B may render soybean more sensitive to heat stress but another class A HSF may

Table 1 - *Arabidopsis thaliana* Heat Shock Factors used as seed sequences and respective matches from *Glycine max*, *Lotus japonica* and *Medicago truncatula*, with corresponding domain information. Abbreviations: HSF = Heat Shock Factor; ID, identification; Gm, *Glycine max*; Lj, *Lotus japonicus*; Mt, *Medicago truncatula*.

<i>Arabidopsis</i> seed-sequence (Loci)	Orthologous information			BLAST results	
	Fabaceae species	Sequence ID	E-value	Score	HSF domain
ATHSFA1A (AT4G17750.1)	<i>Glycine max</i>	Gm_HSFA1A.1	1.00 e ⁻⁷⁷	166	Complete
	<i>Lotus japonicus</i>	Lj_HSFA1A.1	3.00 e ⁻³⁰	88	Incomplete
	<i>Medicago truncatula</i>	Mt_HSFA1A.1	1.00 e ⁻¹⁰⁶	381	Complete
ATHSFA1B (AT5G16820.1)	-	-	-	-	-
ATHSFA1D (AT1G32330.1)	<i>Lotus japonicus</i>	Lj_HSFA1D.1	4.00 e ⁻⁶⁷	406	Incomplete
ATHSFA1E (AT3G02990.1)	<i>Glycine max</i>	Gm_HSFA1E.1	2.00 e ⁻³²	159	Complete
		Gm_HSFA1E.2	5.00 e ⁻²³	124	Complete
	<i>Lotus japonicus</i>	Lj_HSFA1E.1	4.00 e ⁻⁹¹	487	Complete
	<i>Medicago truncatula</i>	Mt_HSFA1E.1	6.00 e ⁻⁹⁸	354	Complete
		Mt_HSFA1E.2	6.00 e ⁻⁹⁸	354	Complete
ATHSFA2 (AT2G26150.1)	<i>Glycine max</i>	Gm_HSFA2.1	6.00 e ⁻³⁰	68.9	Complete
	<i>Lotus japonicus</i>	Lj_HSFA2.1	4.00 e ⁻⁹¹	387	Complete
		Lj_HSFA2.2	1.00 e ⁻⁶⁴	373	Incomplete
	<i>Medicago truncatula</i>	Mt_HSFA2.1	4.00 e ⁻⁹²	334	Complete
ATHSFA3 (AT5G03720.1)	<i>Lotus japonicus</i>	Lj_HSFA3.1	1.00 e ⁻⁷⁴	324	Complete
ATHSFA4A (AT4G18880.1)	<i>Glycine max</i>	Gm_HSFA4A.1	1.00 e ⁻⁸²	143	Complete
		Gm_HSFA4A.2	7.00 e ⁻¹²	121	Complete
	<i>Lotus japonicus</i>	Lj_HSFA4A.1	3.00 e ⁻⁹⁰	401	Complete
		Lj_HSFA4A.2	6.00 e ⁻⁸⁹	393	Complete
	<i>Medicago truncatula</i>	Mt_HSFA4A.1	1.00 e ⁻⁸⁴	309	Complete
ATHSFA4C (AT5G45710.1)	<i>Medicago truncatula</i>	Mt_HSFA4C.1	9.00 e ⁻⁴⁷	183	Complete
ATHSFA5 (AT4G13980.1)	<i>Glycine max</i>	Gm_HSFA5.1	2.00 e ⁻⁵⁸	117	Complete
		Gm_HSFA5.2	1.00 e ⁻¹⁰⁹	392	Complete
	<i>Medicago truncatula</i>	Mt_HSFA5.1	1.00 e ⁻¹⁰⁴	374	Complete
		Mt_HSFA5.2	2.00 e ⁻⁷²	269	Complete
		Mt_HSFA5.3	2.00 e ⁻⁶¹	232	Complete
		Mt_HSFA5.4	1.00 e ⁻²¹	100	Incomplete
	Mt_HSFA5.5	2.00 e ⁻¹⁵	79.7	Incomplete	
ATHSFA6A (AT5G43840.1)	-	-	-	-	-
ATHSFA6B (AT3G22830.1)	<i>Glycine max</i>	Gm_HSFA6B.1	1.00 e ⁻⁴⁹	153	Complete
	<i>Lotus japonicus</i>	Lj_HSFA6B.1	9.00 e ⁻⁸²	291	Complete
		Lj_HSFA6B.2	7.00 e ⁻⁸¹	311	Complete
		Lj_HSFA6B.3	7.00 e ⁻⁹⁷	347	Complete
	<i>Medicago truncatula</i>	Mt_HSFA6B.1	1.00 e ⁻⁶⁶	249	Complete
ATHSFA7A (AT3G51910.1)	<i>Glycine max</i>	Gm_HSFA7B.1	5.00 e ⁻²¹	117	Incomplete
ATHSFA7B (AT3G63350.1)	-	-	-	-	-
ATHSFA8 (AT1G67970.1)	<i>Glycine max</i>	Gm_HSFA8.1	3.00 e ⁻⁶³	131	Complete
		Gm_HSFA8.2	0.0	678	Complete
	<i>Lotus japonicus</i>	Lj_HSFA8.1	9.00 e ⁻⁶⁵	318	Complete
	<i>Medicago truncatula</i>	Mt_HSFA8.1	1.00 e ⁻⁶¹	233	Complete
ATHSFA9 (AT5G54070.1)	-	-	-	-	-

Table 1 (cont.)

<i>Arabidopsis</i> seed-sequence (Loci)	Orthologous information			BLAST results	
	Fabaceae species	Sequence ID	E-value	Score	HSF domain
ATHSFB1 (AT4G36990.1)	<i>Glycine max</i>	Gm_HSFB1.1	e^{-144}	508	Complete
		Gm_HSFB1.2	e^{-127}	450	Complete
	<i>Lotus japonicus</i>	Lj_HSFB1.1	$3.00 e^{-18}$	187	Incomplete
		Lj_HSFB1.2	$1.00 e^{-26}$	68	Incomplete
	<i>Medicago truncatula</i>	Mt_HSFB1.1	$4.00 e^{-51}$	197	Complete
		Mt_HSFB1.2	$3.00 e^{-36}$	148	Complete
		Mt_HSFAB1.3	$7.00 e^{-19}$	90.9	Incomplete
ATHSFB2A (AT5G62020.1)	<i>Glycine max</i>	Gm_HSFB2A.1	$3.00 e^{-48}$	80.5	Complete
		Gm_HSFB2A.2	$5.00 e^{-73}$	270	Complete
	<i>Lotus japonicus</i>	Lj_HSFB2A.1	$9.00 e^{-30}$	197	Complete
	<i>Medicago truncatula</i>	Mt_HSFB2B.1	$3.00 e^{-06}$	48.9	Incomplete
ATHSFB2B (AT4G11660.1)	<i>Glycine max</i>	Gm_HSFB2B.1	$2.00 e^{-44}$	222	Complete
		Gm_HSFB2B.2	$5.00 e^{-81}$	296	Complete
	<i>Lotus japonicus</i>	Lj_HSFB2B.1	$8.00 e^{-68}$	280	Complete
	<i>Medicago truncatula</i>	Mt_HSFB2B.1	$6.00 e^{-67}$	251	Complete
ATHSFB3 (AT2G41690.1)	<i>Glycine max</i>	Gm_HSFB3.1	$1.00 e^{-47}$	160	Complete
		Gm_HSFB3.2	$6.00 e^{-32}$	97.4	Complete
	<i>Lotus japonicus</i>	Lj_HSFB3.1	$5.00 e^{-56}$	194	Complete
	<i>Medicago truncatula</i>	Mt_HSFB3.1	$1.00 e^{-51}$	199	Complete
ATHSFB4 (AT1G46264.1)	<i>Glycine max</i>	Gm_HSFB4.1	$3.00 e^{-61}$	96.3	Complete
		Gm_HSFB4.2	$1.00 e^{-44}$	86.3	Complete
		Gm_HSFB4.3	$1.00 e^{-126}$	448	Complete
		Gm_HSFB4.4	$1.00 e^{-120}$	427	Complete
	<i>Lotus japonicus</i>	Lj_HSFB4.1	$1.00 e^{-66}$	202	Incomplete
		Lj_HSFB4.2	$1.00 e^{-19}$	52	Complete
	<i>Medicago truncatula</i>	Mt_HSFB4.1	$2.00 e^{-89}$	325	complete
		Mt_HSFB4.2	$8.00 e^{-65}$	243	Complete
ATHSFC1 (AT3G24520.1)	<i>Glycine max</i>	Gm_HSFC1.1	$8.00 e^{-60}$	73.6	Complete

alternately play this role (Sung *et al.*, 2003; Kotak *et al.*, 2004; Li *et al.*, 2004). Whether another gene substitutes the role of HSFA1B in soybean could be tested by heterologous expression of HSFA1B; in the case of the existence of different pathways, the over-expression of HSFA1B might change the performance of soybean plants, especially under heat stress.

Other members of class A, such as HSFA9, are less active or may be active only under certain conditions. The reason seems to be the presence of interesting regulators (HSFs or other transcription factors) with specialized functions. In fact, HSFA9 was found to be specific to seed development in sunflower and was exclusively detected in yellow siliques of *Arabidopsis* mRNA (Kotak *et al.*, 2004). Hence the lack of identification of some HSF classes may correlate with specialized functions other than those represented among the conditions analyzed herein.

A similar result was reported by Nover *et al.* (2001) after carrying out an analysis of HSFs in *A. thaliana*. Among the 21 described genes, HSFs A3, A6A, A6B, A7B, B2A and B3 could not be detected in any of the tissues analyzed (etiolated seedlings, roots, leaves from vegetative plants stems, flowers, siliques, and developing seeds) or conditions (heat stressed leaves and cell cultures vs. control). According to the authors it was not surprising that no matching EST was found in libraries created exclusively from RNA isolated from control tissues; a serious limitation of the data from EST libraries for these studies is the lack of samples from heat stressed tissues.

Comparing the obtained results with the data available in the Legume Transcription Factor database (Legume TFDB, Mochida *et al.*, 2009a) an increased number of *Lotus* and *Medicago* HSF representatives were observed, since the LegumeTFDB includes 18 and 16 genes, respec-

tively, and our searches identified 19 and 21, respectively, revealing that both organisms presented a similar number of HSFs as *Arabidopsis*. However, the results considering the ESTs deposited at the GENOSOJA platform revealed a surprisingly low number of HSFs (25 sequences) as compared to the LegumeTFDB information (65 sequences). This may be due to the type of databases (LegumeTFDB is sourced from large-scale shotgun sequencing whereas GENOSOJA is sourced from transcriptomic approaches), besides the fact that the LegumeTFDB bank considers both HSF and HSF-like sequences with data annotation based on different databanks (NCBI nr, *A. thaliana*, TIGR rice, *L. japonicus*, *M. truncatula*, *Populus trichocarpa* and UniProt). On the other hand, Kotak *et al.* (2004) listed 34 soybean sequences, a higher number of HSF representatives than those in GENOSOJA, but these authors did not indicate the methods and procedures used in the acquisition of these HSFs. Finally, the soybean candidates identified herein represent the active (expressed) HSFs bearing the complete DBD-domain. This set size was similar to that described for *Arabidopsis* and also for the *Lotus* and *Medicago* orthologs identified in this study; both being evolutionarily closely related species when compared to soybean (Fabaceae family, Papilionoideae subfamily).

Notwithstanding, it is important to highlight that evolutionary studies and haploid genome analysis suggested that the soybean genome experienced a tetraploidization event approximately 10-15 million years ago. Since then, the soybean genome has gone through gene rearrangements and deletions, reverting to diploid state. Therefore, soybean multigene families, including the heat shock factor family, may contain highly related but diversified genes (Mochida *et al.*, 2009b).

HSF matching to SuperSAGE tags

Regarding SuperSAGE, 68 different tags could be identified, including 26 tags unique to water deficit experiments with the tolerant comparison (water deficit stressed Embrapa 48 vs. control), 28 tags unique to water deficit experiments with the susceptible comparison (water deficit stressed BR16 vs. control) and 14 regarding Asian Soybean

Rust (PI561356 inoculated vs. control) (Table 2; Figure 1). No common tags were identified. It is important to note that among 25 HSF EST clusters, 18 had no representative in the tags database, while five clusters were represented in all libraries. The sequence Gmax_HSF3B1_SJ09-E1-R06-064-B09-UC.F was not identified in ‘Embrapa 48’ and ‘PI561356’ libraries, and Gmax_HSF3B3_Contig20961 was present in the water deficit stressed libraries only. When looked at from a different point of view, from the 14 HSF types compared, only six HSF types (HSFB1, HSFA1E, HSFB2A, HSFB3, HSFA8 and HSFA4A) were identified (Table 2; Figure 1), indicating their induction during the stress response.

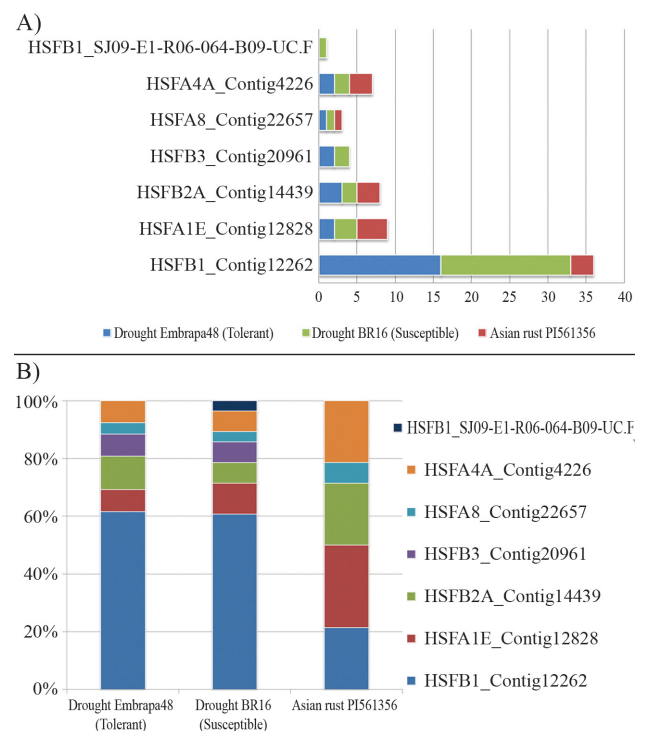


Figure 1 - Graphic representation of SuperSAGE tag distribution per cluster and compared libraries. A) Quantitative analysis (number of untags per category). B) Qualitative analysis of tag prevalence (in %).

Table 2 - Distribution of unique SuperSAGE tags in the three comparisons considered, as compared with the identified soybean EST contigs.

Soybean Contig	Drought Embrapa 48 (tolerant) vs. Control	Drought BR16 (susceptible) vs. Control	Asian Rust PI561356 vs. Control	Total
Gmax_HSF3B1_Contig12262	16	17	3	36
Gmax_HSFA1E_Contig12828	2	3	4	9
Gmax_HSF3B2A_Contig14439	3	2	3	8
Gmax_HSF3B3_Contig20961	2	2	0	4
Gmax_HSFA8_Contig22657	1	1	1	3
Gmax_HSFA4A_Contig4226	2	2	3	7
Gmax_HSF3B1_SJ09-E1-R06-064-B09-UC.F	0	1	0	1
Total	26	28	14	68

Despite the small number of identified sequences in the Asian rust 'PI561356' stress analysis, when compared to water deficit experiments, the presence of HSF representatives indicates the involvement of HS-response also during biotic stresses. The stress condition by itself can activate non-specific stress-responsive-pathways, due to the debility caused to plants by biotic stressful conditions, which can activate a crosstalk among different stress related pathways, as observed in other plants (Glombitza *et al.*, 2004; Kido *et al.*, 2011). Moreover, it is important to consider the tissue from which the library was generated, since leaves are among the first organs to present stress symptoms (especially to abiotic ones). These are necessary for the maintenance of photosynthesis and evapotranspiration processes to ensure plant survival. Moreover, the Gmax_HSF3_Contig20961 gene seems to be expressed specifically under abiotic stress, such as water deficit.

The analysis of SuperSAGE transcript abundance revealed a higher number of orthologous tags for the Gmax_HSF1_Contig12262 cluster (more than 50% of the identified SuperSAGE tags), followed by Gmax_HSF1E_Contig12828 (Table 2; Figure 1A). There is evidence suggesting that HSF1 plays a special role in gene activation as a cooperative partner of HSF1 and that coexpression of low levels of HSF1 with HSF1 can result in strong synergistic effects in reporter gene activation. Experiments in tomato showed that HSF1 acts as a novel type of coactivator and may be able to cooperate with HSF1a or other activators to control expression of certain housekeeping genes (Bharti *et al.*, 2004).

Evaluating the results for the comparisons among water deficit libraries (susceptible X tolerant), a similar proportion of HSF genes was observed, with the exception of the Gmax_HSF1_SJ09-E1-R06-064-B09-UC.F transcript, which was recorded exclusively in the susceptible genotype. In both libraries, Gmax_HSF1_Contig12262 (Figure 1B) was more represented, indicating that HSF genes are expressed under water stress conditions in a similar way in both susceptible and tolerant cultivars.

As expected, most SuperSAGE tags were identified from water deficit libraries. However, it is worth noting that more than 60% of the HSF gene types obtained from soybean ESTs were not identified in the SuperSAGE comparisons, suggesting that the seed EST sequences used were not complete, lacking the necessary 3' extremity for anchoring of SuperSAGE tags. This opens the possibility of identifying additional candidates upon using other annotation approaches. A role of these factors in water deficit response may exist, since their expression was reported also in association with other abiotic stresses (Kotak *et al.*, 2007). Moreover, the 68 identified tags could be potentially useful for 3' RACE (3'-rapid amplification of cDNA ends) experiments to identify the complete transcript, besides expression validation using RT-qPCR with the same mRNA samples.

Structure and evolution of HSF candidates in soybean, *Medicago*, *Lotus* and *Arabidopsis*

The functional properties of HSFs are attributed to conserved structural domains, with the highest degree of conservation being observed for the DNA-binding domain (DBD) composed of helix-turn-helix (HTH) structures, and an adjacent domain with a heptad hydrophobic repeat (HR-A/B) which is involved in oligomerization. In addition, there are two further characteristic components: (i) the short peptide motif essential for nuclear import (NLS: nuclear localization signal) and export (NES: nuclear export signal), and (ii) a C-terminal AHA type activation domain (Li *et al.*, 2010). Primarily based on the structural features of the oligomerization domain, plant HSFs are classified into three evolutionarily-conserved classes, namely A, B and C, bearing 14 sub-classes (Nover *et al.*, 2001). The high degree of conservation within the HSF family is corroborated by our *in silico* analysis, as in the generated dendrogram it was possible to observe the differentiation of sequences according to their classes, and within each class there was a grouping of sequences according to their subclasses (Figure 2). A clear differentiation among the HSF classes A and B classes from a basal ancestral sequence has been established, as expected, since class B- and non-plant-HSFs differ from class A- and C-HSFs by an additional 21 or 7 amino acids, respectively, which separate the two subdomains HR-A and HR-B located in the hydrophobic regions (Nover *et al.*, 2001). Furthermore, the AHA type acidic activation domain is exclusively represented by class A members (Mittal *et al.*, 2009).

With respect to class A, two main groups emerged in the present evaluation: one (I) with HSF4 and HSF5 representatives and the other (II) with the remaining HSF4 and HSF5 members (Figure 2). This is a predictable result, since HSFs A4 and A5 form a group distinct from the remaining HSFs by structural features of their oligomerization domains and by a number of conserved signatures. This is also consistent with their role, since A4 HSFs are potent activators of heat stress-related gene expression, whereas A5 HSFs act as a specific repressor of HSF4 activity, while other members of class A are not affected due to the high specificity of their oligomerization domains (Baniwal *et al.*, 2007).

The second group included three branches, with a basal one including HSF8 and HSF1 (Figure 2). Although class C is more similar to class A than to B, it was expected that this class would behave as a separate group. Nevertheless, the high diversity in the response of different HSF genes to different stresses suggests that there is a high degree of specialization regarding the response of specific HSFs to a particular stress condition. This is consistent with the fact that both HSF8 and HSF1 presented increased expression under cold stress (Miller and Mittler, 2006), indicating that this adaptive response to tolerate cold conditions may be responsible for characteristics shared by

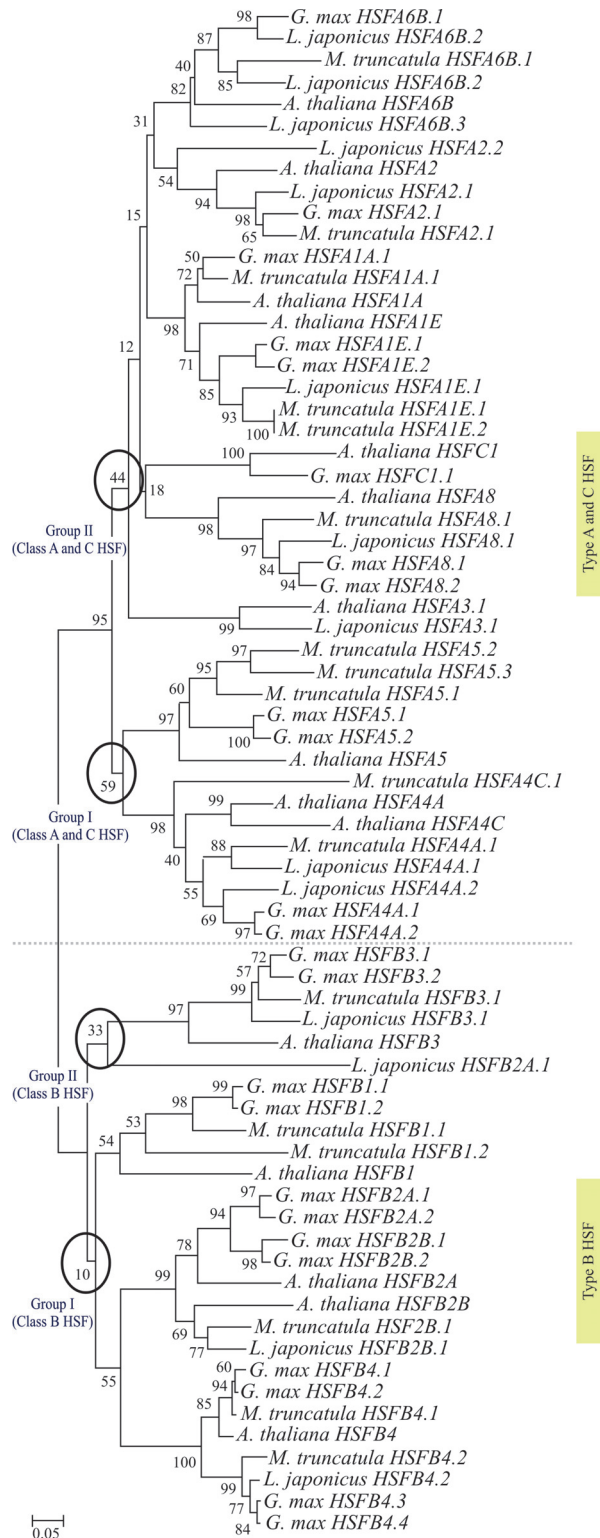


Figure 2 - Dendrogram showing relationships among HSF proteins of *A. thaliana*, *G. max*, *L. japonicus* and *M. truncatula*. Numbers on the base of the nodes represent bootstrap values. Dotted line divides Type A and C from type B. Gray circles indicate each node shared by HSF groups: 'Group I (Class A and C HSFs)' = HSF4 + HSF5, 'Group II (Class A and C HSFs)' = HSF1 + HSF3 + HSF6 + HSF8 + HSF8 + HSF8, 'Group I (Class B HSF)' = HSF1 + HSF2 + HSF4 and 'Group II (Class B HSF)' = HSF3 + *L. japonicus* HSF2A.1. Bar represents similarity coefficient.

these two genes. In fact, in the multiple alignment analysis, two regions comprising 15 residues each (amino acid positions 125 to 139 and 154 to 168) were shared by both HSF8 and HSFC protein sequences, though absent in other class A HSF members. Furthermore, peculiarities shared by HSFs, such as deletions of six amino acids at position 106-111 and probable mutations in two segments (intervals: 161-168 and 195-220) may justify the differentiation of class C proteins from class A ones, as evidenced in the dendrogram.

Regarding the specific function of class C, remarkable little information is currently available. According to Nover *et al.* (2001), HSFs were well represented in expressed sequence tags (ESTs) from libraries of tomato, soybean, potato, barley and *Arabidopsis*. The HSF type is clearly separated from all others by sequence details of the DBDs and by the characteristics of the HR-A/B region. However the significance of these extended oligomerization domains in class A and C HSFs for the coiled-coil structure and oligomerization behavior is not yet clear (Nover *et al.*, 2001).

We denoted a conservation in the position and function of AHA motifs and NES in the C-terminal regions of class A. These regions, in addition to the flanking amino acid residues, were sufficient to identify the HSFs without prior knowledge about the respective DBDs or HR-A/B regions (Kotak *et al.*, 2004). Furthermore, the results were positive for ESTs encoding representatives of HSF groups A1, A2 and A6 (Kotak *et al.*, 2004). Thus, it can be inferred that the observed grouping formed by HSF1, HSF2 and HSF6B in the dendrogram (Figure 2) was based on the similarity of AHA motifs and NES in the C-terminal regions.

It is noteworthy that the C-terminal domains (CTDs) of class B HSFs are completely different (Nover *et al.*, 2001), justifying their isolation in a separate branch, composed of two main groups. The first one includes the B3 sub-class members together with a single member of the B2 sub-class from *L. japonicus*. This unexpected grouping of the *Lotus* B2 sub-class member seems to result from a deletion in a region rich in alanine, valine, isoleucine and methionine. Apparently, this deletion was responsible for the exclusion of this sequence from the branch including the remaining class B members. The second group includes B1, B2 and B4 sub-classes, these being separated in different branches according to their sub-classes (Figure 2). This grouping may be explained by differences observed in a cluster containing arginine and lysine residues close to the C-terminus of HSF1, probably responsible for permanent nuclear localization (Heerklotz *et al.*, 2001) and also by the fact that similar motifs were found in other representatives of this group and also in groups B2 and B4 (with the exception of the HSF3 sub-class) which is the smallest of all HSFs identified so far.

Although our knowledge is still limited, functional diversification seems to be the main reason for the coexis-

tence of more than 20 HSF types in plants (Baniwal *et al.*, 2007). A systems analysis of tomato HSFs revealed two interesting peculiarities: (i) there are at least four different HSF groups (Scharf *et al.*, 1990, 1993; Treuter *et al.*, 1993; Bharti *et al.*, 2000) belonging to two classes (*i.e.*, class A with HSFs A1, A2, and A3 and class B with HSF B1), and (ii) two of the four HSFs (HSFA2 and B1) are heat stress-inducible proteins (Nover *et al.*, 2001; Kotak *et al.*, 2004). In most cases, all identified gene classes and sub-classes were expressed and identified in the four evaluated legumes, suggesting that the family members diverged before the species differentiated. Alternatively, such gene classes and sub-classes may have already functioned as independent genes in the common ancestor, thus favoring divergent evolution.

HSF expression in soybean

Plant cells constitutively express a pool of HSF proteins that are maintained in an inactive state. Certain results suggest that heat-induced protein denaturation participates in the activation of these HSFs (Yamada *et al.*, 2007). This molecular device is normally based on changes in protein conformation and can respond very quickly, playing therefore a central role in transcriptomic remodeling induced upon heat exposure. Accordingly, all HSFs expressed in soybean identified in this study were derived from experiments in the absence of heat stress.

Moreover, it is well known that heat often occurs in combination with drought or other stresses that cause extensive agricultural losses worldwide. HSFs serve as the terminal components of signal transduction, mediating the expression of HSPs and other HS-induced transcripts, but their diverse temporal and spatial expression has also been demonstrated under the influence of other abiotic stresses (Kotak *et al.*, 2007).

HSFs are involved in stress sensing and signaling but can also be part in the regulation of other cellular processes, including development, where a role is strongly suggested by expression profiles in libraries of tissues from young stages. The only exceptions seen herein were mature adult and drought-stressed leaves where the expression of HSF B1 and HSF B2A1 was diametrically and remarkably down- and up-regulated, respectively (Figure 3).

Plant HSFs may also function as H₂O₂ sensors, as is also the case in humans and *Drosophila*, where HSFs directly sense H₂O₂ and assemble into homotrimers in a redox-regulated manner. HSFA2 controls expression under prolonged HS and recovery conditions. Interestingly, its expression is induced by high luminosity and exposition to H₂O₂, emphasizing its importance under various stress conditions (Miller and Mittler, 2006). HSFA4A and HSFA8 are likely to act as sensors of reactive oxygen species (ROS), with HSFA5 acting as a repressor of HSFA4. Indeed, in soybean the profiles of HSFA4A and HSFA8 were quite similar, considering the number of libraries where

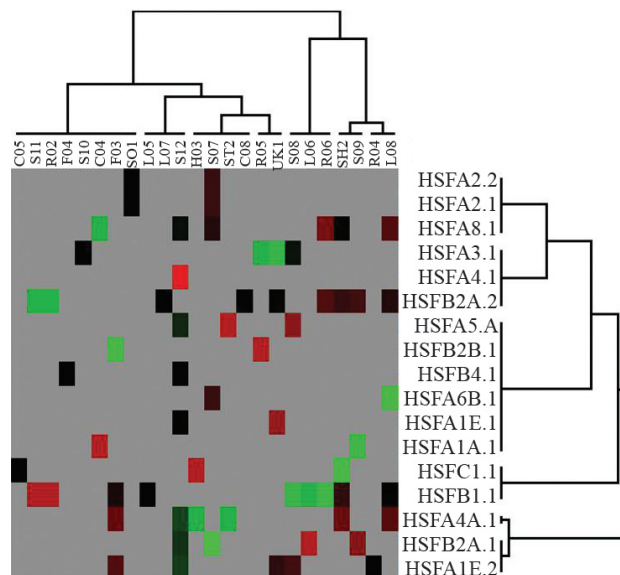


Figure 3 - Hierarchical clustering (Cluster3.0) of up-regulated (red), down-regulated (green) and non-regulated (black) soybean EST clusters ($p < 0.05$) related to HS response; gray stands for absence of information. Dendrograms above and to the left of the graph show the relationships among libraries and expressed genes, respectively. Library codes: C04, immature cotyledons of greenhouse grown plants; C05, 8-day-old cotyledons; C08, 3- and 7-day-old cotyledons; F03, mature flowers of field grown plants; F04, floral meristem; H03, hypocotyl and plumule, germinating seeds; L05, unexpanded leaves and shoot tips of 2-week-old seedlings; L06, drought stressed leaf tissue; L07, leaf, 3-week-old, greenhouse grown; L08, leaf; R02, roots of 7-day-old plants; R04, roots of bulked times; R05, roots of 8-day-old seedlings; R06, root; S07, seed coats of greenhouse grown plants; S08, 11-day-old seedlings; S09, whole seedlings of greenhouse grown plants; S10, seedlings; S11, seedlings, minus the cotyledons; S12, Seeds containing globular-stage embryos; SH2, germinating shoots; SO1, *in vitro* cultivated somatic embryos; ST2, stem tissue of greenhouse grown plants.

they were detected. On the other hand, and considering the same libraries, HSFA5 was absent, except in immature seeds containing globular embryo stages where none of the three genes were detectably expressed (Figure 3). It is also interesting to note that HSF B1.1 was up-regulated in seven-day-old root libraries (R02) and in seedlings (without cotyledons) (S11), situations in which HSF B2A.2 was down-regulated, indicating that these genes may act as antagonists during the initial phases of plant development. This assumption is corroborated by the fact that HSF B1.1 was down-regulated, while HSF B2A.2 was up-regulated in the mature root library (L08).

The similarity in expression patterns of HSF genes in specific libraries (in specific developmental stages or conditions) indicates that the activation of these genes might be evoked by the same *cis*-regulatory elements in their promoters. Such co-expression was observed for HSFA2.1, HSFA2.2, HSFA6B.1 and HSFA4A.1 in the library S07 from 'seed coats of greenhouse grown plants'. Co-expression could indicate that these genes play the same role or are co-participants in the same pathway.

The induction of transcriptomic remodeling through the HSF network is very important but complex, as it in-

volves several HSFs. This network is only a part of the orchestration that contributes to survival under high temperature stress. The panel exposed by our work suggests that HSFs also mediate cross-talk between signaling cascades in soybean for HS and other abiotic stresses, with possible roles in soybean development. Nevertheless, the questions raised here may have to be addressed in subsequent experiments in which the tissues and conditions should be pooled for different and sequential time points.

Distribution of HSF genes in the soybean genome

The comparative analysis of *G. max* EST sequences (25 in total) and genomic sequences enabled the identification of 62 loci bearing HSF genes (Table 3; Figure 4) from 65 HSFs previously described for soybean (Mochida *et al.*, 2009a), a crop with a supposed polyploid recent past (McClellan *et al.*, 2010). From the 25 obtained candidates, two did not align significantly with the characterized heat

Table 3 - Correspondence among identified GENOSOJA expressed sequence tags (ESTs) and characterized genes of *Glycine max*. Abbreviations: ID = identification; HSF, Heat Shock Factor; Gm, *Glycine max*.

Genosoja ID	Gene ID	Genosoja ID	Gene ID
Gm_HSFA1A.1	Glyma01g01990	Gm_HSFA1A.4	Glyma10g07620
Gm_HSF1.1	Glyma01g22910	Gm_HSFA2.3	Glyma10g09460
Gm_HSF3.1	Glyma01g34490	Gm_HSFA5.2	Glyma10g36910
Gm_HSF4.1	Glyma01g34490	Gm_HSF2B.1	Glyma10g38240
Gm_HSF1.2	Glyma01g39260	Gm_HSFA1E.2	Glyma10g38930
Gm_HSF2A.1	Glyma01g42640	Gm_HSFA1E.3	Glyma11g01190
Gm_HSFA1E.1	Glyma01g44330	Gm_HSF2A.2	Glyma11g02800
Gm_HSF4.2	Glyma02g44670	Gm_HSF1.5	Glyma11g06010
Gm_HSF3.2	Glyma03g29190	Gm_HSFA2.4	Glyma11g33630
Gm_HSFA1A.2	Glyma03g31380	Gm_HSFA1A.5	Glyma13g16510
Gm_HSFA6B.1	Glyma03g31380	Gm_HSFA1A.6	Glyma13g21490
Gm_HSFA7A.1	Glyma03g31380	Gm_HSF1.6	Glyma13g24860
Gm_HSFA1A.3	Glyma03g34900	Gm_HSFA4A.3	Glyma13g29760
Gm_HSF4.3	Glyma04g04200	Gm_HSF4.7	Glyma14g04070
Gm_HSF4.4	Glyma04g04200	Gm_HSF4.8	Glyma14g09190
Gm_HSFA2.1	Glyma04g05500	Gm_HSFA1A.7	Glyma14g11030
Gm_HSFA2.2	Glyma04g05500	Gm_HSFA4A.4	Glyma15g09280
Gm_HSF1.3	Glyma05g20460	Gm_HSFA1A.8	Glyma16g13400
Gm_HSF1.4	Glyma05g20460	Gm_HSFA1E.4	Glyma16g19500
Gm_HSFA5.1	Glyma05g28460	Gm_HSF3.3	Glyma16g29750
Gm_HSFA4A.1	Glyma05g28460	Gm_HSF2A.3	Glyma16g32070
Gm_HSFA4A.2	Glyma05g29470	Gm_HSFA1A.9	Glyma17g06160
Gm_HSFA8.1	Glyma05g34450	Gm_HSF1.7	Glyma17g20070
Gm_HSF4.5	Glyma06g04390	Gm_HSFA1A.10	Glyma17g34540
Gm_HSFC1.1	Glyma07g09510	Gm_HSF4.9	Glyma17g35980
Gm_HSFC1.2	Glyma07g09520	Gm_HSF2A.4	Glyma18g14700
Gm_HSF4.6	Glyma07g36370	Gm_HSFA1E.5	Glyma19g26460
Gm_HSFA8.2	Glyma08g05220	Gm_HSFA5.4	Glyma19g26750
Gm_HSFA5.3	Glyma08g11460	Gm_HSF3.4	Glyma19g31940
Gm_HSFA4A.5	Glyma08g12630	Gm_HSF3.5	Glyma19g31940
Gm_HSF2A.5	Glyma09g26510	Gm_HSFA6B.3	Glyma19g34210
Gm_HSF2A.6	Glyma09g26510	Gm_HSFA8.3	Glyma19g37580
Gm_HSFC1.3	Glyma09g32300	Gm_HSF4.10	Glyma20g08250
Gm_HSFA1A.11	Glyma09g33920	Gm_HSFA1A.13	Glyma20g28870
Gm_HSFA1A.12	Glyma10g00560	Gm_HSF2B.2	Glyma20g29610
Gm_HSFA6B.2	Glyma10g03530	-	-

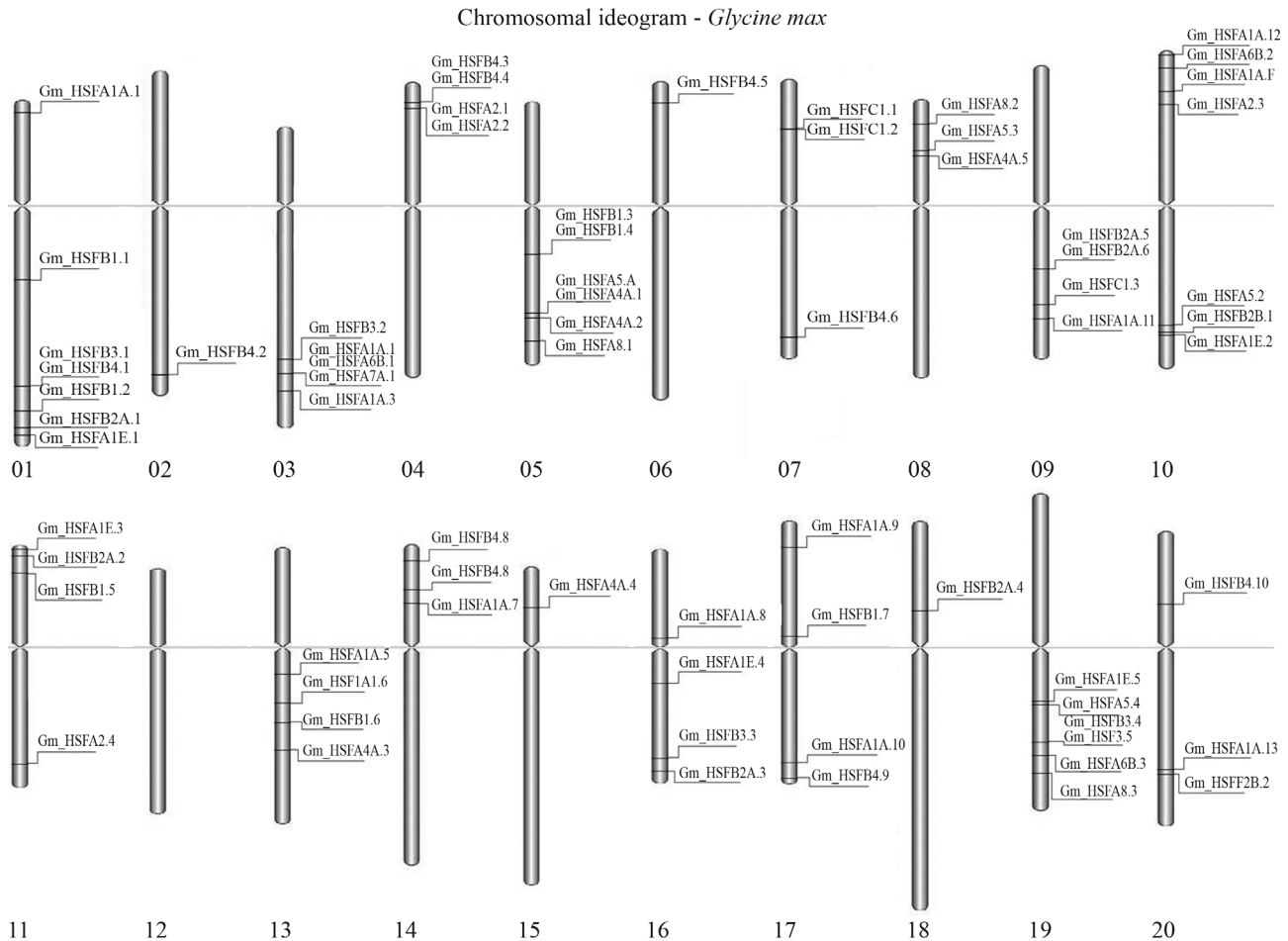


Figure 4 - *In silico* hybridization of HSF sequences against the SOYBASE database. Schematic representation of clusters that were anchored in soybean based on BLAST similarity results (see Table 3 for correspondence between EST cluster identification and HSF described genes).

shock factor genes, which can be justified by differences in the cultivars used in genomic and expression sequencing projects. In addition, three described genes for soybean were not identified among the EST sequences, indicating a lack of expression of these genes in the libraries of the GENOSOJA database. Differences among the analyzed cultivars may also explain this lack of similarity.

With respect to the genomic distribution of the HSF family, nine gene clusters could be identified in chromosomes 01, 03, 04, 05, 10, 11 and 19 (Figure 4). According to Mochida *et al.* (2009a) these clusters may consist of paralogous genes. In soybean, the relative physical distribution of transcription factor genes is of interest, and two types of clusters can be distinguished based on their evolutionary history. The first type consists of a series of genes that arose through repeated tandem duplications (originated from a founding locus). The second type, which is not considered as consisting of paralogous genes, probably arose independently and then relocated to form these duplications and clusters (Mochida *et al.*, 2009b). Pairs of duplicated genes on different chromosomes are common and gene clusters of three or more highly related genes are also widely found

(Mochida *et al.*, 2009a). Considering the distance of their occurrence, a few of the duplicated genes could be classified arbitrarily as either genes that were not duplicated in tandem on the same chromosome, or genes that were so (Mochida *et al.*, 2009a).

Moreover, none of the EST clusters aligned on chromosome 12. This was expected, since in this chromosome there is no description of HSF family members (Mochida *et al.*, 2009b), while other chromosomes (02, 06, 15 and 18) presented a single representative of the group.

Concluding Remarks

Results from the present investigation indicate that gene duplication and diversification occurred during plant evolution, whilst differences in their expression patterns caused species-specific variability in the composition of the HSF family members, which can be divided into three different classes and several sub-classes according to their particular motifs and residue-specific rich regions. Although not all of the previously described genes could be found for the three species studied when using a transcriptomic approach, we expect that experiments directed at

heat-stress conditions may provide additional sequences related to the HS response, including other HSF genes. Furthermore, the absence of soybean ESTs for some HSF members did not impair the evaluation of the distribution of the HSF family in the soybean genome. The family is present in 19 of the 20 chromosomes, including clustered distribution in some.

To understand the complexity of a plant's HSF family and stress response systems in general, it is important to consider that when plants became adapted to terrestrial habitats they evidently had to face and become specialized to rapidly changing and extreme environmental conditions. The present approach represents the first evaluation considering only expressed HSF genes, revealing 25 expressed ESTs and 68 SuperSAGE tags, with emphasis on root tissue (water deficit) libraries. Some HSF candidates present in *Arabidopsis*, that are apparently missing in the transcriptome of the evaluated legumes (for example HsFA1B), may be important candidates for biotechnological approaches in soybean and other legumes directed towards increasing their performance under temperature stress conditions. Moreover, some genes found to be induced under water deficit may constitute interesting target genes for inferences regarding the association of heat and cold stresses, especially considering current climate change scenarios.

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Internet Resources

- AtTFDB - Arabidopsis transcription factor database at Agris, <http://arabidopsis.med.ohio-state.edu/AtTFDB/> (Jun/2011).
- GENOSOJA database, Brazilian Soybean Genome Consortium, <http://bioinfo03.ibi.unicamp.br/soja/> (Jun/2011).
- Lotus japonicas* database, <http://www.kazusa.or.jp/lotus> (Jun/2011).
- Medicago truncatula* database, <http://www.medicago.org/> (Jun/2011).
- Phytosome Soybean genome browser, <http://www.phytozome.net/cgi-bin/gbrowse/soybean/> (Jun/2011).
- SOYBASE site, <http://soybase.org/gbrowse/cgi-bin/gbrowse/gmax1.01/#search> (Jun/2011).
- Legume Transcription Factor Database, LegumeTFDB, <http://legumetfdb.psc.riken.jp/> (Jun/2011).

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