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Heat Shock Factors: Regulators of Early and Late Functions in Plant Stress Response

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ABSTRACT

Heat shock factors (HSFs) among others are of great importance regarding the regulation of increased stress tolerance. Based on structural characteristics and phylogenetic comparison plant HSFs are subdivided into 3 classes and several subgroups. Recent studies showed that different HSFs play important roles during early and late stages of stress response. In this review, we focus mainly on the functional characterisation of class A HSFs of *Arabidopsis*, which are known to function as transcriptional activators of stress target genes. Recent evidence obtained from the identification of HSF-knockout mutants and microarray expression profiling indicates that different early and late HSF regulate large numbers of partially overlapping sets of target genes. Meta-analysis of microarray data generated from different experimental setup may have the potential to verify known and/or to identify novel HSF target genes. In addition, we will summarise recent work on the potential roles of oxidative stress leading to the activation of HSFs and the induction of the heat stress response.

Keywords: electrophoretic mobility shift assay, heat stress, knock out mutants, microarray, oxidative stress

CONTENTS

INTRODUCTION

The expression of heat shock proteins (HSP) is a signature of the heat shock response. It is well established that the HSP act as molecular chaperones, assisting the refolding of denatured proteins (for review see Schöffl *et al*. 1998). In all species investigated, heat stress results in the production of heat shock proteins (HSP), which have been classified into families of HSP100, HSP90, HSP70, HSP60 and small HSP (sHSP). Plants are unique with respect to the complexity of sHSP expressed upon heat stress (Schöffl *et al*. 1998). More recently a number of other "unconventional" stress genes (e.g. ascorbate peroxidase, galactinol synthase, etc.), which are co-expressed under the same conditions and showing similar kinetics have been identified (Busch *et al*. 2005).

The expression of stress genes is primarily regulated at the level of transcription. Heat stress transcription factors (HSF) are central regulators of the heat shock response. Unlike in other eukaryotes there is a high diversity of HSFs in plants. The range in the best characterised plant genomes spans from at least 18 HSFs in tomato (Baniwal *et al*. 2004), 21 in *Arabidopsis* (Nover *et al*. 2001; Xiong *et al*. 2005), 22 in maize (Fu *et al*. 2006), to 23 or more in rice (Xiong *et al*. 2005) and in soybean (Zhu *et al*. 2006). HSFs display a basic modular structure (Nover *et al*. 1996, 2001) with a DNA binding domain (DBD), an oligomerisation domain (OD), a nuclear localisation signal (NLS) and often a nuclear export signal (NES) (**Fig. 1A**). The highly conserved N-terminal DBD mediates the binding of HSFs to heat shock elements (HSEs), i.e. *cis*-acting elements in promoters of target genes (e.g. heat shock protein genes) that comprise repetitions of palindromic 'nGAAnnTTCn' motifs. Plant DBDs are encoded in two parts which are separated by an intron, whose position is identical in all HSFs (Nover *et al*. 2001). A distinguishing feature between non-plant and plant HSFs is an 11-amino acid deletion in the DBD of plant HSFs. The adjacent bipartite oligomerisation domain (OD) comprising the hydrophobic regions A and B (HR-A/B) is composed of hydrophobic heptad repeats. It is separated from the DNA-binding domain by a linker of variable length and sequence. Based on phylogeny of the DBD and the HR-A/B region, plant HSFs are assigned to three major families A, B and C with sub classes A1-A9, B1-B4 and C1/C2 (Nover *et al*. 1996, 2001; Fu *et al*. 2006). In class-A and C plant HSFs, HR-A and HR-B are separated by 21 amino acids and 7 amino acids, respectively, whereas plant HSFs of class B lack this subdomain (Nover *et al*. 2001). It is assumed that the function of the hydrophobic-repeat A/B region is responsible for trimer formation through hydro-

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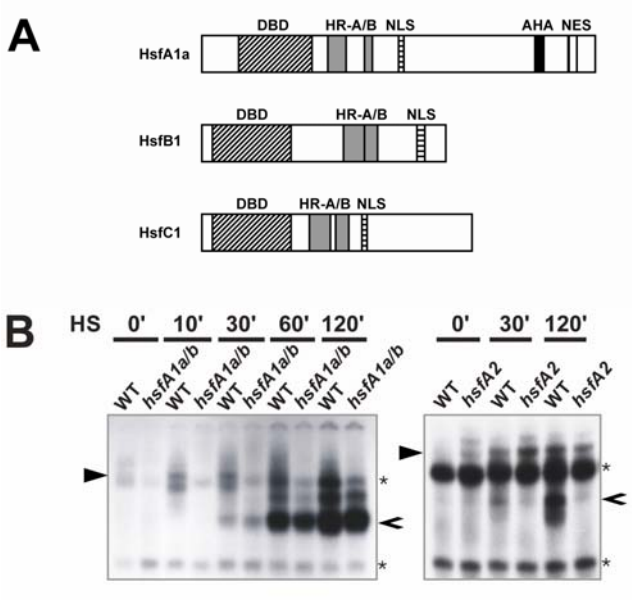

Fig. 1 Structural organisation and DNA-binding activities of HSFs. (**A**) Structural domains of representative HSFs of class A, B, and C; DBD DNA-binding domain, HR-A/B hydrophobic repeats A and B, NES nuclear export signal, NLS nuclear localisation signal, AHA aromatic, large hydrophobic and acidic amino acids. (**B**) Time course of heat stress (HS) induced DNA binding activities in protein extracts from WT and HSF knock out mutants of Arabidopsis. The double mutant *hsfA1a/b* (Lohmann *et al*. 2004) derived from WT ecotype Wassilevskija (left-hand panel), the *hsfA2* knock out mutant (Li *et al*. 2005; Nishizawa *et al*. 2006; Schramm *et al*. 2006; Charng *et al*. 2007) derived from ecotype Columbia (right-hand panel). Electrophoretic mobility shift assays were carried out essentially as described by Lohmann *et al*. (2004), using radioactively labelled synthetic HSE as a probe. Closed arrowheads mark heat-induced "early", open arrowheads heat-induced "late" HSF:HSE-binding activities; asterisks mark unspecific bands.

phobic interactions (Zuo *et al*. 1994). Most HSFs have a nuclear localisation signal neighbouring next to the OD in C-terminal direction (Lyck *et al*. 1997; Nover *et al*. 2001). Most of the class A HSFs feature a variable C-terminal activation domain whose functional motifs (for transcriptional activation of target genes) are clusters of aromatic, large hydrophobic and acidic amino acids (AHA-motif) (Czarnecka-Verner *et al*. 2000; Döring *et al*. 2000; Yuan and Gurley 2000; Kotak *et al*. 2004). None of the HSFs from classes B and C features these AHA motifs; the corresponding C-terminal region of class B HSFs is composed of neutral or positively charged amino acids. The complexity of HSFs in plants suggests diversification of functional roles in heat stress response and perhaps participation of HSFs in other cellular functions. The existence of heat inducible HSF suggests multistep mechanisms in target gene expression.

Especially plants as sessile organisms are subject to various kinds of abiotic and biotic stresses in the environment that may cause cellular damage. Therefore, effective mechanisms for prevention and repair must have evolved in plants. Environmental adaptation of plants depend on elaborate systems for stress sensing and signalling, common and stress-specific responses, and probably also on a hierarchical control of reactions. There is evidence that HSFs play important roles in stress sensing and signalling of different environmental stresses and that different stresses, including also high temperature, induce reactive oxygen species (ROS) in plants (Dat *et al*. 1998). ROS, particularly H_2O_2 are important components in abiotic stress response and signalling mechanisms. The generation of ROS, an indicator of environmental stress, may act as secondary messenger in the pathway leading to the activation of transcription factors, perhaps also HSF (Noctor *et al*. 2000; Davletova *et al*. 2005; Miller and Mittler 2006). There is increasing evidence that high light induces ROS, which causes expression of a number of common stress genes including HSP, other chaperones, and also certain HSFs (Desikan *et al*. 2001). Plants exposed to heat stress accumulate transiently enhanced levels of H_2O_2 within a very short time (15 min) and conversely H_2O_2 treated plant cells show clear indication of a heat shock response, e.g. the expression of HSP along with H_2O_2 scavenging ascorbate peroxidases (Volkov *et al*. 2006).

The present review aims to summarise recent work characterising the function and regulation of different HSFs in plants, predominantly in *Arabidopsis* as a model system. Emphasis is given to the question of communalities and differences of the so-called "early" and "late" HSFs, the use of knock out mutants and expression profiling for identification of HSF-dependent target genes and functions, and the possible role of oxidative stress and HSFs in stress sensing and signalling.

"EARLY" AND "LATE" HSFS IN HEAT STRESS RESPONSE

Besides the classification of HSF according to their structural properties into class A, B, and C, two different structure independent distinctions can be made according to their transcriptional dynamics: 1) the "early" HSFs, which are constitutively expressed at a low level and 2) the "late" HSFs, whose expression is significantly induced by heat stress (probably via the action of early HSFs). Early constitutive HSFs are believed to become immediately activated at the protein level by stress that leads to oligomerisation, DNA-binding and transcriptional stimulation of target genes. Late HSFs seem to be important for regulating later stages of the heat shock response. Both types of HSFs are members of the classes A and B (Busch *et al*. 2005). In *Arabidopsis* AtHsfA1a and AtHsfA1b are early HSFs, apparently activated by stress that leads to a monomer - trimer transition and DNA binding. To assess their functional roles single and double knock out plants of HsfA1a and HsfA1b have been investigated (Lohmann *et al*. 2004). In each of the single knock out lines the "early" DNA binding capacity (appearing immediately after onset of heat stress) was diminished and in *hsfA1a/1b* double knock out plants the "early" HSF:HSE binding complex could not be found at all (**Fig. 1B**). This demonstrates that HsfA1a and HsfA1b are the major "early" heat-activated DNA-binding factors in *Arabidopsis*. According to bandshift analysis, the action of early HSFs in WT has a maximum after 30 minutes heat stress, preceding the maximum of mRNA accumulation of HSF-target genes, which peak after 60 min heat stress (Lohmann *et al*. 2004). Interestingly, the HSF:HSE bandshift pattern changes after 30-60 min heat stress: the "early" higher molecular weight complexes become replaced by "late" lower molecular weight complexes (**Fig. 1B**). As demonstrated by Lohmann *et al*. (2004), the "late" binding complex is also formed in *hsfA1a/1b* double knock out plants, suggesting that this complex is formed by "late" HSFs. As shown by expression profiling, the major heat-induced class A HSF is AtHsfA2, others are HSFs B1, A4a, B2a, B2b, and A7a (Busch *et al*. 2005). Only the "late" HSFs B1, B2a, and A7a, but definitely not A2 are target genes of the "early" AtHsfA1/AtHsfA1b (Busch *et al*. 2005). Using *hsfA2* knock out plants we can show that the formation of the "late" complexes depends on the presence of AtHsfA2 (**Fig. 1B**).

What are the functions of "early" and "late" HSFs? Phenotypic analysis and expression profiling of AtHsfA1a/AtHsfA1b double or AtHsfA2 single mutants have been carried out to answer this question. Transcriptome analysis revealed that AtHsfA1a/AtHsfA1b act almost exclusively on the heat shock response since 99.95% of all differentially expressed WT genes (between WT and *hsfA1a/b* double knock out mutant) were only affected after heat stress but not at control temperature (Busch *et al*. 2005). The strongest effect of A1a/A1b was on the expression of small heat shock

proteins, i.e. Hsp26.5-P(r), Hsp15.7-CI(r), Hsp18.1-CI, Hsp22.0-ER, and Hsp25.3-P. The presence of these proteins appears to be very important throughout the heat shock response because the same subset is present among the most strongly affected target genes in *HsfA2* knock out plants (Schramm *et al*. 2006).

Surprisingly, *HsfA2*, which is not a target gene of HsfA1a/b, exhibits the highest heat-inducible expression of all *Arabidopsis* HSFs (Busch *et al*. 2005) and its heat stress-dependent accumulation of mRNA is very similar to that of conventional HSP, like the cytosolic small heat shock proteins of class CI (Schramm *et al*. 2006). *HsfA2* transcripts could be detected as early as after 15 min (Charng *et al*. 2007), whereas the first peak is reached after one hour of heat stress (Li *et al*. 2005; Schramm *et al*. 2006; Nishizawa *et al*. 2006). In the case of continued stress (Li *et al*. 2005; Nishizawa *et al*. 2006), or after subsequent recovery at control temperature (Schramm *et al*. 2006), the mRNA level rapidly declined. Following recovery at normal temperature, it was possible to increase the accumulation of A2 mRNA and protein to maximum levels during a second heat treatment (Schramm *et al*. 2006). It became evident that HSFA2 is a rather stable protein that once accumulated could have an enduring effect as a transcriptional activator. However, the rapid decline of its putative target gene transcripts in the recovery phase (Schramm *et al*. 2006) indicates that A2 is presumably inactive during recovery. At this stage A2 might be retained in the cytoplasm in complexes with heat shock proteins such as it has been shown for tomato HsfA2 (Port *et al*. 2004).

What is the phenotype of A2 mutants? Analysis of seedlings of the A2 knock out mutant uncovered a reduced basal and acquired thermotolerance and a higher electrolyte leakage during the recovery phase after stress, and the mutant is more sensitive to heat stress than wild type after a long recovery period (48 hours) while short recovery had little effect (Charng *et al*. 2007). In *HsfA2* overexpressing *Arabidopsis* lines the stress sensitive phenotype of the knock out plants was reversed and plants showed increased stress tolerance (Li *et al*. 2005; Nishizawa *et al*. 2006). Very similar phenotypes have been observed for transgenic plants overexpressing "early" HSFs (Lee *et al*. 1995; Prändl *et al*. 1998); whereas little effect on thermotolerance was observed for *hsfA1a/1b* double knock out plants (Lohmann *et al*. 2004). The relatively week phenotype of the knock out mutant may be explained by compensatory effects due to functional redundancies of HSFs and their primary target genes (heat shock proteins). In two expression profiling experiments with either A2 overexpressors versus wild type without stress (Nishizawa *et al*. 2006) or *hsfa2* compared to wild type plants after heat stress (Schramm *et al*. 2006), a set 18 genes was identical. Among the target genes not shared with HsfA1a/A1b are *Apx2* as the strongest downregulated gene in *hsfa2* mutants, *Hsp70*b and two genes of the Dreb transcription factor family, which seem to be involved in drought and cold stress responses. Besides, the small heat shock proteins described as AtHsfA1a/A1b targets are also among the highest ranked downregulated genes in *hsfa2* plants. Since AtHsfA2 expression is not regulated by AtHsfA1a/1b (Busch *et al*. 2005) the overlap in the target gene sets might be explained by cooperation between these HSFs. However, there is no indication that HSF:HSE binding undergoes a HsfA1a/b-dependent change in the transition from "early" to "late" heat shock response. Hence, it seems more likely that HSFA1a/A1b are responsible for the induction of the very early stress gene expression, and are replaced by AtHsfA2 during late expression of the same target genes.

In tomato the situation seems to be different. Knock down of LpHsfA1 expression by an RNAi-like co-suppression resulted in a severe thermotolerance phenotype that at the molecular level was accompanied by the inability to induce expression of LpHsfA2, LpHsfB1, and HSP by heat stress (Mishra *et al*. 2002). However, it can not be excluded entirely that RNAi affected also the expression of other Hsf genes. LpHsfB1 expression, induced by LpHsfA1, seems to function as a co-activator of LpHsfA1 target genes, but the *Arabidopsis* homologue, AtHspB1, was unable to show a co-activation function in the same assay system (Barthi *et al*. 2004).

Beside a regulation of HsfA2 activity by HsfA1 through nuclear retention (Scharf *et al*. 1998; Heerklotz *et al*. 2001), it has been shown in yeast that there is a very specific interaction between HsfA2 and the cytosolic Hsp17.4-CII (Port *et al*. 2004). This interaction might be responsible for keeping HsfA2 in an inert conformation in the cytoplasm which is, according to the chaperone titration model, converted to the active form by heat stress due to the higher demand for chaperon activity by denatured proteins.

Other candidates that act as negative regulators of plant HSF-activity are the maize Emp2 and the heat-inducible Hsbp2 that are active in embryos and leaves, respectively. Both are orthologues to animal Hsbp1 that functions as an attenuator of the heat shock response by binding to Hsf1 (Satyal *et al*. 1998). Each of the maize orthologues showed specific interaction in yeast with non-overlapping subsets of maize class A HSFs (Fu *et al*. 2006). A negative function on the activity of AtHsfA4 has been reported for AtHsfA5. Both HSFs share the common features of transcription activators (Baniwal *et al*. 2007), but only the LpHsfA4 was able stimulate transcription and HsfA5 exerted a negative effect on the function of LpHsfA4.

Another interesting aspect concerning the regulation of HSFs is the finding that *AthsfA3* seems to be a downstream regulator of Dreb2A, a heat inducible transcription factor in *Arabidopsis* (Busch *et al*. 2005). Dreb2A, originally described as a drought stress responsive factor, is rapidly induced but its mRNA is also rapidly declined under heat stress (Sakuma *et al*. 2006). Conversely, *AtHsfA3* was the most strongly affected gene in plants overexpressing a constitutively active form of *Dreb2A*, and consistently was down-regulated more than 20-fold in a *dreb2a* knock-out mutant. HsfA3 expression is also regulated by heat stress but has escaped attention probably because of its very slow induction. Using RT-PCR quantification, it was shown that the A3 mRNA levels were still rising after 10 hours of heat stress. This suggests that HsfA3 is a very late regulator in *Arabidopsis* heat shock response and interconnected with drought stress. Its function is completely unknown.

Despite recent progress, our understanding of the functions and regulatory interplay of the different early and late HSF is still limited. There is a multiplicity of regulatory proteins that act on HSFs, and phylogenetically related HSFs may serve different functions. The functional roles of particular HSFs may not be readily understood by their phylogenetic relations but rather have to be explored for each HSF individually and can not be extrapolated to other species.

HEAT SHOCK FACTOR MUTANTS – IDENTIFYING TARGET GENES BY META-ANALYSIS OF EXPRESSION PROFILES

Identification of HSF target genes is crucial for understanding the functional roles of HSFs and the molecular mechanism involved in generating stress tolerance in *Arabidopsis*. Using well defined experimental conditions (short term heat stress, 1 h 37°C), transcriptome analysis of HSF knock out versus WT lines allowed a clear distinction between heat stress and HSF-dependent genes. A small set of *AtHsfA1a/1b* target genes was identified, comprising only about 4% of genes (112 out of 3056) differentially regulated during HS. It included several HSP but also a number of other stress-related genes that link the heat stress response to diverse functions, e.g. protein biosynthesis/degradation, membrane transport, oxidative stress response, and signalling (Busch *et al*. 2005). A partial overlap between the sets of putative target genes, regulated by AtHsfA1a/1b early and AtHsfA2 late in the heat stress response, was indicated by the transcriptome analysis of HSF knock out mutants

(Nishizawa *et al*. 2006; Schramm *et al*. 2006). On the other hand the *Arabidopsis* transcriptome after long term heat stress (6 h, 37°C) revealed much lower numbers of differentially expressed genes (Rizhsky *et al*. 2004). These differences probably reflect the fact that the HS-induced changes in gene expression are transient with a maximum after 1-2 h heat stress followed by a strong decline (Lohmann *et al*. 2004; Schöffl *et al*. 2006). Thus, a quantitative analysis of differences in expression levels, as a criterion for the identification of HSF target genes, has to take into account the experimental parameters of heat treatment.

The present research on stress response increasingly takes advantage of microarray expression data, which are available to the scientific community. It would be highly beneficial if such data, obtained from different experimental designs, conditions, mutants and transgenic lines could be exploited for addressing specific questions about the differences in the functions of key regulators of the stress response, e.g. HSFs. For example, common approaches for determining gene function are loss of function (gene knock out or knock down) and gain of function (transgenic, transient or ectopic overexpression) approaches. Such mutants are already available for a small number of *Arabidopsis* HSF genes, including *AtHsfA1a* (Lee *et al*. 1995; Lohmann *et al*. 2004), *-1b* (Prändl *et al*. 1998; Lohmann *et al*. 2004) and *AtHsfA2* (Nishizawa *et al*. 2006; Schramm *et al*. 2006). It has been shown, that gain of function mutants, resulting from transgenic overexpression of a given HSF results in a constitutive expression of HSP under non-HS conditions and enhanced basal thermotolerance (Lee *et al*. 1995; Prändl *et al*. 1998). On the other hand T-DNA knock out mutants of the same HSF genes exhibit a weak phenotype of compromised acquired thermotolerance that correlates with an impaired expression of several HSP genes following heat stress (Lohmann *et al*. 2004).

Based on the rational, that gain of function and loss of function mutants would show a differential expression of the same set of target genes, just in opposite ways, the question arose, whether it is possible to exploit microarray profiling data to get conclusive results on the array of high confidence target genes for a given HSF. Using the closely related, functionally redundant AtHsfA1a/1b as a model we compared the available microarray data of knock out mutant plants (Busch *et al*. 2005) with microarry data (unpublished results) obtained for transgenic AtHsfA1b-overexpressing plants (Prändl *et al*. 1998). It was expected that a true AtHsfA1b target gene should be characterised by the following properties:

i) heat-induced mRNA level in WT plants
ii) increased expression at normal temperature in *AtHsfA1b* overexpressing lines
iii) no significant induction by heat stress in hsfA1a/1b knock out plants compared to room temperature.

Direct comparison between two independent experiments in two different *Arabidopsis* accessions with different genotypes is inappropriate. This is illustrated by the fact that based on a fold change cut off of $\geq$2, a significant number of genes is differentially expressed between Wassilewskija and C24 ecotypes at normal temperature (~18%) and after heat stress (~33%, unpublished results).

In statistics, meta-analysis combines the statistical information from several independent experiments and therefore enhances the use of information of each experiment. It has been shown to increase the statistical power of detecting small changes in gene expression in microarray experiments (Choi *et al*. 2003), and has been successfully applied to studies combining loss of function and gain of function experiments (Levesque *et al*. 2006). The meta-analysis of HSF-dependent target gene expression was carried out by applying a z-score transformation of the fold changes of each mutant/control pair and then setting in both experiments z-score cut offs of $\geq$1.88 or 1.25, that conform the top 3% or respectively 10.65% of the normal curve of distribution.

The z-score analysis with a cut off at 1.88 yielded a total of 16 genes (**Table 1**) including 6 HSP, 2 co-chaperones, 2 HSFs, and galactinol synthase genes, which represent cano-

Table 1 Meta analysis of microarray data - identification of HSF-dependent target genes.

Array	*HsfA1b*-TP vs Wt at RT[1)]		*hspA1a/b* vs Wt after HS[2)]		Annotation
identifier	Fold change↑	z-score	Fold change↓	z-score	
254059_at	58.97	29.52	0.34	2.53	mitochondrial HSP23.6-M
249575_at	30.55	15.02	0.03	43.71	class I-related HSP15.7-CI
264402_at	20.11	9.69	0.33	2.7	HSP100, putative
246450_at	19.35	9.3	0.09	13.83	heat shock factor HsfA1b (Hsf3)
259913_at	19.31	9.28	0.27	3.69	development. regulat. GTP-binding protein
253884_at	17.7	8.47	0.04	29.41	chloroplast precursor HSP25.3-P
250304_at	13.7	6.42	0.29	3.21	elongation factor eEF1Bα1
247691_at	13.33	6.24	0.03	40.41	cytosolic HSP18.1-CI
262148_at	13.29	6.21	0.02	80.11	class I-HSP26.5-P
263320_at	12.02	5.56	0.13	8.74	galactinol synthase 3 (GolS3)
252081_at	10.82	4.95	0.46	1.53	heat shock factor HsfA7a
262307_at	10.13	4.6	0.48	1.43	DNAJ N-terminal domain-containing protein
254263_at	9.51	4.28	0.08	16.2	expressed protein
247780_at	9.27	4.16	0.16	7.15	dehydrodolichyl diphosphate synthase
255891_at	9.25	4.16	0.3	3.17	expressed protein
253949_at	8.82	3.93	0.27	3.69	co-chaperone grpE family protein
247851_at	7.57	3.3	0.43	1.77	lipocalin, putative
250013_at	6.34	2.67	0.44	1.66	expressed protein
250074_at	5.67	2.33	0.47	1.45	UTP-glucose-1-phosphate uridylyltransferase
254414_at	5.6	2.29	0.14	8.54	(2R)-phospho-3-sulfolactate synthase-related
255787_at	4.82	1.89	0.22	4.83	cinnamoyl-CoA reductase family
246554_at	4.5	1.73	0.24	4.26	HSP100, putative
264814_at	**4.42**	**1.69**	**0.43**	**1.76**	**zinc finger (MYND type) family protein**
250899_at	4.02	1.48	0.23	4.5	cell division cycle protein 48, putative
262582_at	3.83	1.39	0.12	10.03	aspartate-glutamate racemase family
245243_at	3.73	1.34	0.06	19.93	hypothetical protein
253689_at	3.69	1.32	0.17	6.66	expressed protein
254076_at	3.64	1.29	0.28	3.47	immunophilin-related
258984_at	3.58	1.26	0.45	1.62	DNAJ N-terminal domain-containing protein
264968_at	3.57	1.25	0.22	4.82	rubber elongation factor (REF) family protein

[1)] *HsfA1b* transgenic plants versus wild type, in C24 background, at room temperature (RT), data from one experiment (this paper)
[2)] *hsfA1a/b* double knock out plants versus wild type, in Wassilewskija background, after heat stress (HS), analysis of original data from Busch *et al*. (2005)

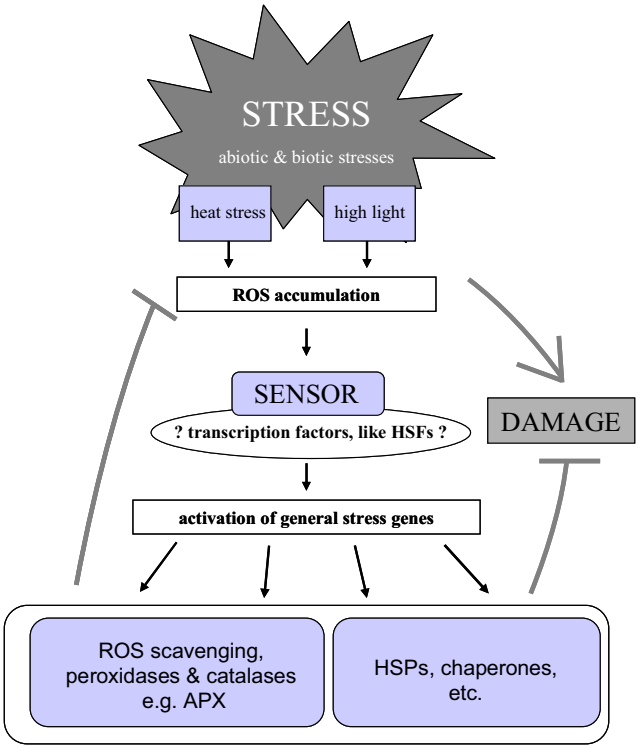

Fig. 2 Model of putative oxidative stress signalling pathway. Abiotic and biotic stresses, like high light or heat stress, cause the production of ROS in plants. ROS accumulation may cause damage to cells, but it might also act as signal for the activation of stress response and defence reactions. Transcription factors like the HSFs may act as potential stress sensors but also as regulators for activating several different defence pathways that in turn may counteract ROS signalling and cellular damage.

nical heat stress and HS-induced HSF-dependent targets. This whole gene set is a subset of previously identified *AtHsfA1a/1b* dependent genes (Busch *et al*. 2005). Using a z-score of 1.25, a total of 30 genes were selected. Among these genes was only one, which was thus far not identified as an *AtHsfA1a/1b* target. It is a member of the zinc finger family protein MYND (myeloid *trans* location protein 8 type, Nervy and DEAF-1) that exists in mammals, yeast and plants. It has been shown that members of this family are involved in the development of cancer (Spadaccini *et al*. 2006). In *Caenorhabditis elegans* it was shown that proteins containing a MYND domain are involved in chromatin and gene regulation (Ansieau and Leutz 2002). Not unexpectedly, this may suggest that chromatin effects are involved in HSF-dependent regulation of gene expression.

In summary, this analysis confirms a number of genes as HSF-targets activated in the early phase of the heat stress response, which have previously been identified in single microarray experiments. The potential of meta-analysis is demonstrated by the identification of a novel HSF target gene, which escaped attention when analysing single experiments. With the availability of increasing numbers of *Arabidopsis* HSF knock out and transgenic mutants it will become a useful tool for determining the individual and overlapping functions in the complex regulatory network in plants with high confidence.

ARABIDOPSIS HSFS: INVOLVEMENT IN OXIDATIVE AND HEAT STRESS SIGNALLING AND RESPONSES

There is ample evidence that heat stress and oxidative stress are connected in the abiotic stress responses in plants. In this review, we would like to summarise the current state of knowledge about the roles of HSFs as sensors and regulators integrating stress sensing and signalling (**Fig. 2**). A breakthrough has been the work on *Drosophila* and mammalian HSFs showing that HSFs may play an important role as direct sensors of heat and oxidative stress (Zhong *et al*. 1998; Ahn and Thiele 2003). It was demonstrated that both, heat stress and H_2O_2 treatment had the capacity to convert recombinant mammalian HSF1 monomers into DNA-binding trimers in a redox-dependent fashion (Ahn and Thiele 2003). Much less is known about the involvement of plant HSF in oxidative stress sensing and signalling, however, recent publications shed some light on the possible roles of certain *Arabidopsis* HSFs in these processes.

There is a strong link between oxidative stress and the expression of HSP genes. The application of H_2O_2 and other oxidative compounds are capable of inducing heat shock gene expression, like sHSP and ascorbate peroxidase (Apx) genes, which are not expressed in unstressed *Arabidopsis* cells (Volkov *et al*. 2006). Some genes are induced to comparable levels by moderate heat stress as well as by application of H_2O_2 (Panchuk *et al*. 2002; Volkov *et al*. 2006). *Apx2* has been identified as a prime target of HSF; possible candidates for its regulation are AtHsfA1a, AtHsfA1b and AtHsfA2 (Panchuk *et al*. 2002; Schramm *et al*. 2006; Volkov *et al*. 2006). Its role as an important antioxidant enzyme in plants with a high affinity for H_2O_2 constitutes Apx as the most important H_2O_2-scavenging enzyme. Genes of the cytosolic Apx of *Arabidopsis*, *AtApx1* and *AtApx2*, are AtHsfA1b dependently expressed as indicated by the analysis of HsfA1b-overexpressing transgenic *Arabidopsis* plants (Panchuk *et al*. 2002). The heat stress induction of Apx expression suggests that its function is required during or after heat stress. In fact, it has been demonstrated recently that heat stress causes transiently H_2O_2/ROS production in *Arabidopsis* tissue culture cells, which implicates that the heat-induced Apx-action might be involved in controlling oxidative damage or signalling in plants (Volkov *et al*. 2006).

Searching for common features, promoter analysis revealed that not only conventional heat shock genes (HSP) contain HSF-binding motifs, such elements are also present in many promoters of defence and transcription factor genes, which are involved in oxidative stress signalling (Storozhenko *et al*. 1998; Davletova *et al*. 2005), including also the Apx genes (Panchuk *et al*. 2002; Schramm *et al*. 2006). HSF-HSE binding seems to be involved in the heat induction of *Apx1* and *Apx2* expression (Storozhenko *et al*. 1998; Schramm *et al*. 2006). However, HSFs appear not only to be regulators of genes related to oxidative stress, but also the expression of some HSFs is enhanced by oxidative stress.

Based on the digital northern tool from Genevestigator (Zimmermann *et al*. 2004), Miller and Mittler (2006) identified higher transcript levels (>2-fold) of *AtHsf*s *A2*, *A4a*, *A8* and *B1*, under oxidative stress conditions and also a temporary peak during high light stress as well as in Apx1 knock-out mutants. Except for *AtHsfA8*, which is only weakly induced by oxidative stress but not by heat stress, the transcript levels of all other four HSFs are also enhanced after heat shock (Busch *et al*. 2005).

AtHsfA2 seems to be one of the most important HSFs in the heat shock response because it shows the strongest induction of mRNA levels of all *Arabidopsis* HSFs after heat stress (Busch *et al*. 2005), a strongly increased expression (approximately 50-fold) under oxidative stress (Miller and Mittler 2006) and by combined high light and heat stress (Li *et al*. 2005; Nishizawa *et al*. 2006; Schramm *et al*. 2006). The analysis of knock out mutants demonstrated an involvement of AtHsfA2 in basal and acquired thermotolerance and oxidative stress tolerance (Li *et al*. 2005; Charng *et al*. 2007). The phenotype of the knock out mutant is relatively weak but the role of this HSF in stress tolerance was confirmed by the analysis of transgenic overexpression of *AtHsfA2* (Li *et al*. 2005; Nishizawa *et al*. 2006). It is speculated that the function of AtHsfA2 in generating heat and oxidative stress tolerance is carried out through its putative target genes including some HSP genes and *Apx1*, whose expressions in *AtHsfA2* knock out plants are reduced under heat stress, but not completely shut down (Li *et al*. 2005). It

is not surprising that AtHsfA2 is not the sole regulator of these genes, other factors like AtHsfA1a and A1b, which are immediately active upon onset of heat stress, seem to serve the same functions through activating a similar set of target genes.

In the case of AtHsfA4a, a functional role at a relatively early stage of the oxidative stress acclimation response was suggested. *AtHsfA4a* is constitutively expressed, but rapidly increasing mRNA levels are detected after H_2O_2 stress (Davletova *et al*. 2005). This HSF appears to be involved in the regulation (activation) of *Apx1* and *Zat12* expression during high light stress, because their expression was negatively affected in transgenic *Arabidopsis* overexpressing a dominant negative *AtHsfA4a* construct devoid of the activation domain (Davletova *et al*. 2005). Zat12, a zinc-finger protein, is required for the expression of *Apx1* during oxidative stress. The presence of HSEs in the promoter regions of *Zat12* and *Apx1* genes (Rizhsky *et al*. 2004) suggests that AtHsfA4a may be involved in the regulation of both genes. Furthermore, Apx1 may be involved in a feedback regulatory circuit, since in an *Apx1* knock out line the mRNA levels of *AtHsfA4a* and *AtHsfA8* are increased more than 2-fold during the early response to high light (Davletova *et al*. 2005). It seems conceivable that without the scavenger activity of Apx1 oxidative stress should increase, which causes activation of constitutively expressed HSF and eventually enhanced expression of inducible HSFs.

Only one of the class B HSF, *AtHsfB1*, showed an increased transcript level induced by oxidative stress (Miller and Mittler 2006), but at present its functional role in the oxidative stress response is unclear. Of all class B HSFs, *AtHsfB1* shows a strong induction by many environmental stimuli including: pathogens, wounding, salt, ozone, light, H_2O_2, and heat (Genevestigator: Zimmermann *et al*. 2004). Recently the homologue in tomato, LpHsfB1, was shown to function as a co-regulator of other HSFs (LpHsfA1 and LpHsfA2), enhancing their transcriptional activity on target genes (Barthi *et al*. 2004). The co-activation function is dependent on a histone-fold-like motif in its C-terminal domain LpHsfB1. Interestingly, the *Arabidopsis* AtHsfB1 lacks a crucial lysine residue in this motif and can not function as a co-activator (Barthi *et al*. 2004). AtHsfB1 may rather be a negative regulator of gene expression as indicated by its effects on transient expression in promoter-reporter gene expression analyses, where the crucial role of this inhibitory effect on class A HSFs was mapped to the C-terminal region of B1 (Czarnecka-Verner *et al*. 2004). Nevertheless, no direct interaction was detected between AtHsfB1 and LpHsfA1 or other *Arabidopsis* HSFs (Barthi *et al*. 2004). Thus the function of the *Arabidopsis* AtHsfB1 is still obscure. Its induction by a number of environmental stimuli indicates that it might have a very general but central function in stress response regulation and thus may be linked to oxidative stress response.

There are indications that further HSFs are involved in oxidative stress signalling, even if their transcription level is not induced after heat or oxidative stress. The analysis of the double knock out *Arabidopsis* plant *hsfA1a/hsfA1b* provided evidence that these HSFs are early response regulators for the heat stress response (Lohmann *et al*. 2004). These HSFs are constitutively expressed, but at very low levels and are not enhanced by heat stress, oxidative stress or any other treatment. Investigations of the *hsfA1a/hsfA1b* knock out lines revealed a participation of AtHsfA1a and AtHsfA1b in the formation of immediate early high molecular weight HSF-HSE binding complexes (Lohmann *et al*. 2004). Such HSFs should be suitable candidates for the activation by oxidative stress. By the criterion of high molecular weight HSF-HSE binding complexes, H_2O_2 has been identified as an inducer of AtHsfA1a/AtHsfA1b activity *in vivo* (Volkov *et al*. 2006) The involvement of H_2O_2 in HSF activation by heat stress was indicated by the negative effects of ascorbate, a non-enzymatic scavenger of H_2O_2, on both the formation of high molecular weight HSF-HSE binding complexes and the expression of heat shock genes (Volkov *et al*. 2006). The definite roles of class A factors AtHsfA1a and AtHsfA1b in oxidative stress sensing and signalling have still to be determined. However, from its array of target genes, which overlaps a great deal with that of the strongly heat and oxidative stress induced AtHsfA2, it becomes apparent that HSFs have redundant functions in regulating the expression of stress genes and possibly also in stress signalling.

CONCLUSIONS

Recent analyses of HSF in *Arabidopsis* led to a better understanding of some aspects of the complex HSF network in plants. There is clear evidence that "early" and "late" class A HSF share common sets of target genes (HSP and others), indicating that the adaptive response to long-term heat stress requires a sustained expression of a number of stress genes. It becomes also clear that other as yet unidentified class A HSF are required for regulating the expression of other "early" and "late" stress genes, since the "early" AtHsfA1a/b and the "late" AtHsfA2 do not cover the complete array of heat stress-regulated genes in plants. In particular it is still unknown which HSF regulates the heat-induced expression of A2. The overlap in the sets of HSF target genes between e.g. heat stress and oxidative stress may be due to the participation of ROS (e.g. H_2O_2) and HSF in both signalling pathways. Future research will have to clearly identify the relevant HSFs (and other transcription factors) as well as the molecular mechanisms of activation and regulation of common stress gene expression.

The functions of class B HSFs are less well understood. There is evidence that some B factors may act as co-activators, like LpHsfB1, or negative regulators as suggested for its *Arabidopsis* homologue AtHsfB1. Although, the exact functional divergence between these closely related HSF are not understood, it became clear that the sequence relationship between HSFs in different species do not allow predictions of common functions. The current progress in understanding the functions of HSF is largely dependent on the identifications of putative target genes using expression profiling of HSF knock out mutations and well defined experimental conditions. The exploitation of microarray data by meta-analysis will shed more light on the functional roles of HSFs in the complex regulatory network in plants.

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