Complexity of the heat stress response in plants

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Plants have evolved a variety of responses to elevated temperatures that minimize damage and ensure protection of cellular homeostasis. New information about the structure and function of heat stress proteins and molecular chaperones has become available. At the same time, transcriptome analysis of *Arabidopsis* has revealed the involvement of factors other than classical heat stress responsive genes in thermotolerance. Recent reports suggest that both plant hormones and reactive oxygen species also contribute to heat stress signaling. Additionally, an increasing number of mutants that have altered thermotolerance have extended our understanding of the complexity of the heat stress response in plants.

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Introduction

Temperatures above the normal optimum are sensed as heat stress (HS) by all living organisms. HS disturbs cellular homeostasis and can lead to severe retardation in growth and development, and even death. As sessile organisms, plants are constantly exposed to changes in temperature and other abiotic factors. Worldwide, extensive agricultural losses are attributed to heat, often in combination with drought or other stresses [1•].

The accumulation of heat shock proteins (HSPs) under the control of heat stress transcription factors (HSFs) is assumed to play a central role in the heat stress response (HSR) and in acquired thermotolerance in plants and other organisms. Our current understanding of the functions of HSPs and HSFs has been summarized comprehensively in several recent reviews [2–5]. Attempts to increase thermotolerance by overexpression of a single HSF or HSP gene have had limited impact because of the genetic complexity of the HSR [6]. Data from genome-wide expression profiles of *Arabidopsis* plants that are exposed to heat stress, or of mutants in which thermotolerance is impaired, have substantially extended our knowledge of HSR in plants [7]. Comparison of expression data from different plant species experiencing HS under variable conditions, for example from different tissue types, developmental stages, growth conditions, or applications and durations of stress treatments, shows related patterns of transcript accumulation, with the expression of about 2% of the genome being affected [8–10].

This review focuses on recent publications uncovering the molecular details of HSP function and the complexity of the HSF network. In addition, we also discuss new reports defining other molecular events that are involved in sensing and surviving high temperatures, along with interactions between heat and other abiotic stresses.

HSPs as chaperones

HSPs were first identified as proteins that are strongly induced by HS. They include Hsp100/ClpB (eukaryotic/*Escherichia coli* nomenclature), Hsp90/HtpG, Hsp70/DnaK, Hsp60/GroEL and small HSP (sHSP) proteins. These HSPs are proposed to act as molecular chaperones in protein quality control. The families of HSP genes in plants are complex; because many family members are also essential for normal growth and development, it has been difficult to unravel their roles during stress ([5,7] and references therein). Other than by extrapolation from their molecular chaperone activity, there is still no specific information on how Hsp70, Hsp90 and Hsp60 contribute to survival of heat stress in plants. With regard to function during heat stress, investigations of Hsp100/ClpB and sHSPs are the most advanced.

Hsp100/ClpB proteins are members of the AAA+ family of ATPases and are involved in resolubilizing protein aggregates [11]. A cytosolic member of this family is essential for tolerance to high temperature in plants, but is not essential for normal growth [12]. Genetic analyses in *Arabidopsis* indicate that Hsp101 interacts with the sHSP chaperone system to resolubilize protein aggregates after heat stress, a process that requires complex interactions of Hsp101 protein domains [13••]. In addition to cytosolic Hsp101, plants also have nuclear-encoded, chloroplast- and mitochondrion-localized Hsp100/ClpB proteins, namely ClpB-p and ClpB-m, respectively [14•]. *Arabidopsis*

T-DNA insertion mutants in which ClpB-p and ClpB-m are disrupted do not have the same thermotolerance defects as *Hsp101* mutants, despite strong HS-dependent regulation of their transcripts [14•]. However, *Arabidopsis* ClpB-p knockouts fail to develop chloroplasts properly [14•,15]. By contrast, transgenic lines expressing an antisense construct of tomato chloroplast-localized Hsp100/ClpB showed defects in thermotolerance but no obvious developmental disorders [16]. The role of Hsp100/ClpB proteins in plant development and stress tolerance, in particular the identification of crucial substrates, requires further investigation.

sHSPs belong to a super-family of chaperones that are defined by a conserved carboxy-terminal domain of about 90 amino acids, referred to as the α-crystallin domain [5]. This is by far the most complex group of HSPs in plants. It includes members that are targeted to the nuclear-cytosolic compartment, chloroplasts, mitochondria, endoplasmic reticulum (ER) and peroxisomes [17,18], implicating these proteins in the protection of practically all cellular compartments.

Many aspects of the current model of sHSP function have been derived from studies of plant and cyanobacterial sHSPs [5]. Although plant sHSP monomers are around 16–30 kDa, most sHSPs form large oligomers (of 8 or more monomers) in the native state. The only available X-ray structure of a eukaryotic oligomeric sHSP is that of the dodecameric cytosolic Hsp16.9 from wheat [19]. *In vitro*, sHSPs bind to partially unfolded proteins in an ATP-independent manner, preventing their irreversible aggregation. Substrates that are denatured in the presence of sHSPs can be refolded and reactivated by Hsp70/DnaK with the participation, in some cases, of Hsp100/ClpB and GroEL [20,21]. In the current model of sHSP function, sHSP oligomers dissociate to dimers and bind substrates through both their non-conserved amino-terminal domain and the conserved α-crystallin domain [22,23]. During HS, *in vivo* cyanobacterial sHSPs are associated with diverse proteins, which might represent protected substrates [24]. In addition to chaperone function, sHSPs are also proposed to modulate membrane fluidity and composition [25].

Defining plant sHSP function *in vivo* remains challenging. Transcripts of all major sHSPs accumulate dramatically during HS, and specific sHSPs are also expressed during development and some other stresses [7,26]. Unfortunately, limited T-DNA insertion lines are available in *Arabidopsis* to allow studies of sHSP-deficient plants. Nevertheless, overexpression of the chloroplast-localized sHSP in tomato and tobacco provides evidence that this sHSP protects photosystem II under some stress conditions [27•,28]. Likewise, overexpression of a mitochondrial sHSP enhanced thermotolerance in tobacco [29]. Immunomodulation of cytosolic sHSPs led to plant heat sensitivity [30]. Whether the chaperone model adequately explains the function of all sHSPs, and if and how these proteins protect specific critical targets are major open questions.

Heat stress transcription factors

HSFs serve as the terminal components of signal transduction, mediating the expression of HSPs and other HS-induced transcripts. Plants possess multiple HSF-encoding genes, with 21 members defined in *Arabidopsis*. Plant HSFs comprise three conserved evolutionary classes, A, B and C, which are mainly distinguished by the structural features of their oligomerization domains [31]. Our knowledge of the molecular mechanisms of plant HSF function is predominantly based on analyses of HSFs in tomato and *Arabidopsis*. In tomato, HsfA1a, HsfA2 and HsfB1 seem to form a regulatory network that is responsible for the expression of HS-responsive genes ([3] and references therein). HsfA1a is constitutively expressed and regulates the HS-induced expression of HsfA2 and HsfB1. HsfA1a was therefore defined as a master regulator of HSR in tomato [32], whereas HsfA2 is the major HSF in thermotolerant cells. HsfA1a also functions as a nuclear retention factor and co-activator of HsfA2 by forming a HsfA1a–HsfA2 hetero-oligomeric complex. Because of the absence of AHA-type activation motifs, which are typically found in the carboxy-terminal domain of class A HSFs [33], class B HSFs seem to act as attenuators of class A HSFs [34]. Tomato HsfB1 has, however, been identified as a novel type of co-activator of class A HSFs and other transcription factors [35].

Genome-wide transcriptome analysis of wild type and several HSF mutant lines of *Arabidopsis* has extended our knowledge of the temporal and spatial expression of HSFs under the influence of heat and other abiotic stresses. Analysis of *HsfA1a*, *HsfA1b* and *HsfA2* knockout mutants suggests that HsfA1a and HsfA1b are important for the initial phase of HS-responsive gene expression, and that HsfA2 controls expression under prolonged HS and recovery conditions [36,37•,38•,39]. The latter finding is supported by results showing that thermotolerance under repeated HS treatments declines more rapidly in an *HsfA2* knockout mutant [40•].

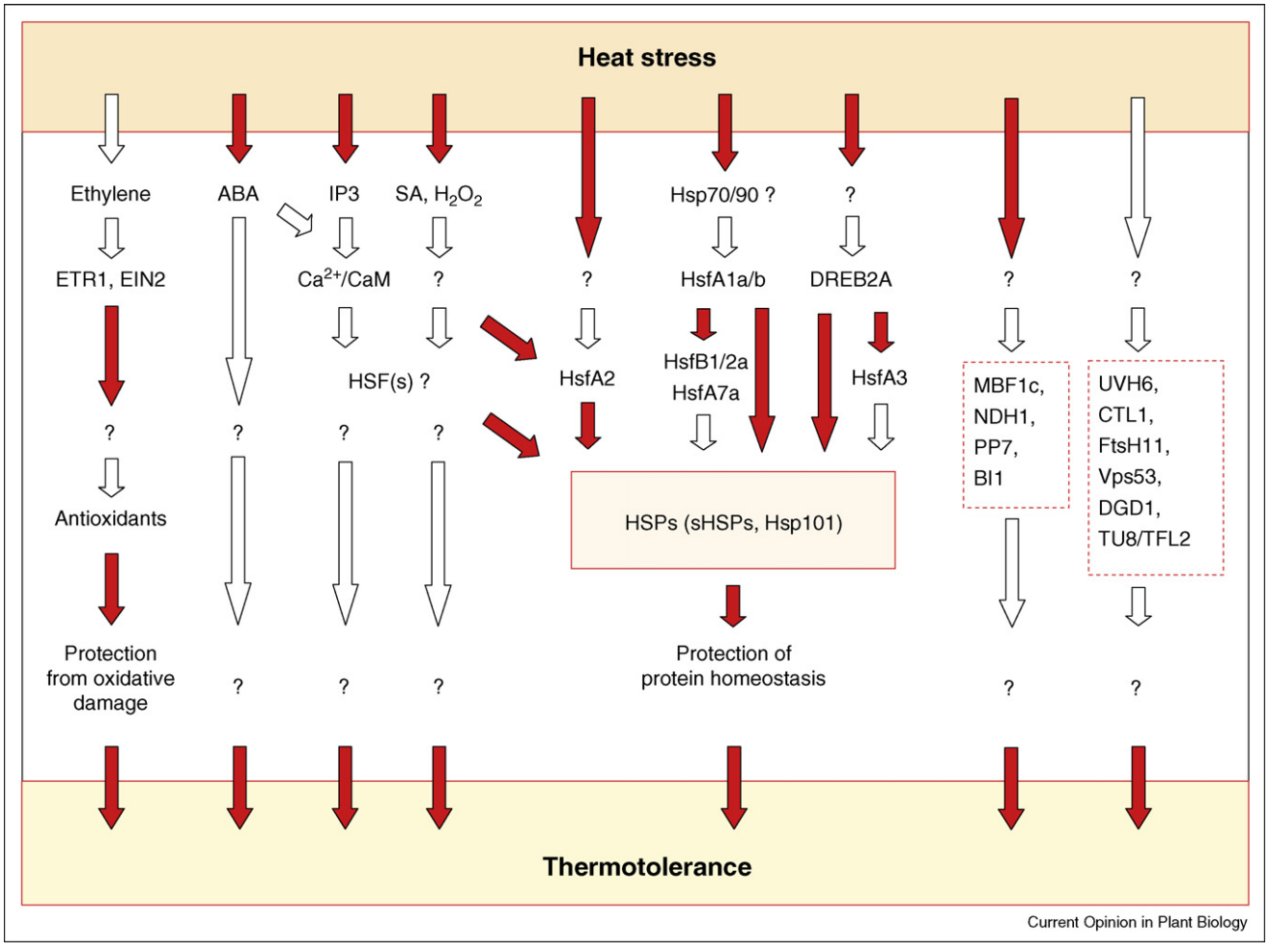
Remarkably, the HS-induced expression of HsfA2 in *Arabidopsis*, in contrast to tomato, is not regulated by HsfA1a or HsfA1b [37•]. Further studies based on knockouts of other members of the HSF family might be required to evaluate the HS-induced regulation of HsfA2 in *Arabidopsis*. Interestingly, the expression of HsfA2 was also shown to be induced by high light and H_2O_2 [39]. Its stringent involvement in the regulation of ASCORBATE PEROXIDASE 2 (APX2), a key enzyme of oxidative stress, further emphasizes the importance of HsfA2 under various abiotic stress conditions.

HsfA4a and HsfA8 are hypothesized to act as sensors of reactive oxygen species (ROS) [41], and HsfA5 was identified as a specific repressor of HsfA4 isoforms in tomato and *Arabidopsis* [42]. Recently, *Arabidopsis* HsfA3 was shown to be regulated by DREB2A, a transcription factor involved in the regulation of dehydration-responsive genes [43•]. These findings suggest HSF-mediated cross talk between HS and other abiotic stress signaling cascades. Moreover, the unique role of HsfA9 during seed development, and not during environmental stresses [26,44], further emphasizes the specific functions of individual HSFs under different conditions.

Signaling

Multiple signaling pathways are implicated in the HSR, some of which control HSPs whereas others control the production or activation of diverse effector components (Figure 1). There is emerging evidence that HS is accompanied by some degree of oxidative stress, and that there is cross-talk between heat and oxidative stress signaling. A burst of H_2O_2 was reported to occur after very short periods at high temperature, apparently as a result of NADPH oxidase activity [45]. This burst has been correlated with the induction of HS-responsive genes, a process assumed to be mediated through direct sensing of H_2O_2 by HSFs [4,46]. Pretreatments with H_2O_2 or menadione also lead to increased tolerance of HS [47], and mutants of two NADPH oxidases (*atrbohB* and *atrbohD*) show defects in thermotolerance [48•]. Moreover, specific HSPs are upregulated in maize mitochondrial mutants that have respiratory deficiencies [49]. These data indicate that the generation of ROS can

Figure 1



Overview of signaling pathways and factors involved in thermotolerance. The figure shows signaling components involved in the heat stress response and protective factors leading to thermotolerance that are described in the text. The most-characterized part of the network contains heat stress transcription factors (HSFs) that regulate genes encoding heat stress proteins (HSPs), which act as molecular chaperones. Red arrows indicate connections with experimental evidence, open arrows mark hypothesized connections. Question marks represent as-yet-unidentified factors in the corresponding signal transduction pathways. Boxes that have red dotted lines represent a collection of gene products that are known to affect thermotolerance, but whose particular functions in the network are still unknown.

induce HSP synthesis, but the involvement of ROS in regulating HS-induced HSP expression is less clear.

A series of papers address the possible role of Ca^{2+}-dependent signaling in HSR. HS-induced cytosolic Ca^{2+} transients were measured in *Arabidopsis* cell cultures and in wheat, and a link through calmodulin (CaM) was proposed [50]. However, Ca^{2+} transients were detected only during recovery from HS in *Arabidopsis* seedlings (see references in [7]). Pretreatment of maize cell extracts with Ca^{2+} increased *in vitro* DNA-binding of HSFs at room temperature, whereas Ca^{2+} chelators abolished HSF-binding activated by heating [51]. Other data implicate the activity of inositol 1,4,5-trisphosphate (IP3) upstream of Ca^{2+} in the HSR [52]. Interestingly, a HS-inducible *Arabidopsis* phosphatase, AtPP7, was shown to be involved in thermotolerance. The protein interacts with both CaM and HSF and is required for induction of Hsp70 and Hsp101 [53]. However, additional work is necessary to verify the functional linkage of these observations to the HSR, and to explain the discrepancies between detection of Ca^{2+} transients in different studies.

Phytohormones, such as abscisic acid (ABA), salicylic acid (SA) and ethylene, have also been linked to HS signaling in different plant species [7,47,48•]. A transient peak in ABA levels was reported in response to HS in pea plants [54] and during recovery from HS treatments in creeping bentgrass [47]. The *Arabidopsis* ABA signaling mutants *abscisic acid insensitive 1* (*abi1*) and *abi2* showed reduced survival after HS, but the accumulation of HSPs was not affected in these mutants [48•].

Elevated SA levels in response to HS have been measured in different plant species [47,54,55]. One study suggested that SA acts downstream of ABA and upstream of a phosphatidyl-inositol-4,5-bisphosphate (PtdIns[4,5]P_2)-specific phospholipase C (PLC) [54]. But like ABA, SA does not appear to be required for HSP synthesis during HS [48•,55].

Ethylene signaling mutants, such as *ethylene resistant 1* (*etr1*) and *ethylene insensitive 2* (*ein2*), are sensitive to HS, particularly under high-light conditions, but acquire thermotolerance normally. There is no direct evidence for the involvement of ethylene signaling in HSP induction [48•]. One highly HS-inducible gene encoding the MULTIPROTEIN BRIDGING FACTOR 1c (MBF1c) is, however, involved in thermotolerance and assumed to be linked with ethylene signaling [56•].

Novel components in thermotolerance

It has become increasingly obvious that HSPs alone cannot support optimal thermotolerance. Amelioration of oxidative stress is one likely role for other factors. The production of glycinebetaine in tobacco reduced HS-induced photo-oxidation by enhancing the protection of photosystem II. This correlated with elevated levels of antioxidants and increased thermotolerance [57]. Protection against photo-oxidation was assumed to occur through cyclic phosphorylation via NAD(P)H dehydrogenase (NDH), which is highly HS-inducible [58]. Other pathways include HS-induced production of specific sugars, such as raffinose and galactinol, by galactinol synthases [37•]. However, mutation of GALACTINOL SYNTHASE 1 (GolS1) caused no defect in thermotolerance [59].

Several mutant screens have identified other genes that are involved in thermotolerance. In a screen for the HS-acclimation of dark grown hypocotyl elongation, seven loci (*hot* loci) were identified [60]. The first mutant from this screen (*hot1*) was defective in Hsp101, one of the chaperones essential for thermotolerance. However, *HOT2* was recently reported to encode CTL1, a chitinase-like protein, which is not only required for acquired thermotolerance but also involved in salt stress and required for proper development [61]. These mutations are not simply temperature-sensitive alleles, as null mutations at these loci cause HSR defects that are the same as or more severe than those caused by missense mutations. The identities of the other *hot* mutants remain to be reported.

Another screen for genes that are involved in thermotolerance was based on chlorophyll accumulation after HS in dark-grown seedlings [62,63]. This screen identified a mutation in DIGALACTOSYLDIACYLGLYCEROL SYNTHASE 1 (DGD1) [62] and a mutation affecting the chloroplastic FtsH11 protease, which is associated with strongly reduced photosynthetic capability after HS [63]. Mutants that have reduced thermotolerance after longer recovery periods have also been isolated [40•,64]. Such screens identified HsfA2 and one highly HS-upregulated gene of unknown function (Hsa32).

Mutants identified through other screens are also HS-sensitive or unable to acquire thermotolerance. One, *hit1-1*, encodes a homologue of yeast *Vps53p*, which is involved in vesicle trafficking, a role not yet confirmed in plants [65]. The *tu8* mutant had a defect in thermotolerance, among other pleiotropic phenotypes, and showed reduced accumulation of Hsp90, sinapine, glucosinolate, and anthocyanin after HS [66]. The gene that is mutated in *tu8* encodes HETEROCHROMATIN PROTEIN 1 (also known as *TERMINAL FLOWER 2* [*TFL2*]). Mutations in *Arabidopsis* BAX-INHIBITOR 1 (AtBI1) are normal under non-stress conditions, but cause increased sensitivity to HS-induced programmed cell death [67].

Conclusions

Response to and survival of heat stress is a complex phenomenon in plants (Figure 1). The induction of

classical HSPs (chaperones) through the HSF network is clearly important, but even this response is not simple and involves several HSFs. Other transcription factors and multiple signaling pathways also orchestrate the HSR, regulating a range of effector components, all of which contribute to survival under high temperature stress.

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