

Minireview

Heat perception and signalling in plants: a tortuous path to thermotolerance

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Summary

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An accurate assessment of the rising ambient temperature by plant cells is crucial for the timely activation of various molecular defences before the appearance of heat damage. Recent findings have allowed a better understanding of the early cellular events that take place at the beginning of mild temperature rise, to timely express heat-shock proteins (HSPs), which will, in turn, confer thermotolerance to the plant. Here, we discuss the key components of the heat signalling pathway and suggest a model in which a primary sensory role is carried out by the plasma membrane and various secondary messengers, such as Ca²⁺ ions, nitric oxide (NO) and hydrogen peroxide (H₂O₂). We also describe the role of downstream components, such as calmodulins, mitogen-activated protein kinases and Hsp90, in the activation of heat-shock transcription factors (HSFs). The data gathered for land plants suggest that, following temperature elevation, the heat signal is probably transduced by several pathways that will, however, coalesce into the final activation of HSFs, the expression of HSPs and the onset of cellular thermotolerance.

Introduction

Temperature is a central environmental cue with a far-reaching impact on plant metabolism, development and growth. Land plants are often exposed, daily and seasonally, to wide fluctuations in temperature. Modern agriculture relies on high crop yields to meet the increasing demand for human food. Any environmental fluctuation affecting crop productivity directly concerns food security and the well-being of the human population. Considerable economic losses are caused yearly by adverse temperature extremes and the increased frequency, extent and intensity of heat waves (Long & Ort, 2010). Unlike most animals, plants are sessile and cannot flee unfavourable temperature conditions.

To survive, plants must be able to anticipate upcoming damaging conditions early enough to express genes and accumulate so-called heat-shock proteins (HSPs) involved in cellular defences against heat damage. In addition to classical plant breeding methods, several crop improvement programmes have aimed to enhance plant thermotolerance by focusing on the ectopic expression of specific genes (reviewed in Iba, 2002 and Sung *et al.*, 2003). These approaches have had some success, but have also revealed limitations because of the great complexity of the heat-induced gene network contributing to the response to high temperatures and the onset of thermotolerance (Finka *et al.*, 2010). The identification of the most upstream temperature sensor and the transduction pathway of the heat

signal is key to the development of new strategies to breed crops adapted to the warmer climates of the 21st century.

The optimal growth temperature varies among species. Yet, beyond tolerance values, all plants engage in a highly conserved response characterized by the massive up-regulation of a set of genes encoding for HSPs (Finka *et al.*, 2010). This response, involving 0.5–1% of the total genome, is of a similar amplitude in animals, yeasts and prokaryotes, and is referred to here as the heat-shock response (HSR).

The most abundantly expressed HSPs are molecular chaperones (Finka *et al.*, 2010). The timely expression of HSPs before severe heat conditions is vital for plants to acquire thermotolerance (Larkindale & Vierling, 2008; Hua, 2009). Basal and acquired thermotolerance are complex processes involving the accumulation of HSPs and other metabolites (Hua, 2009). Thermotolerance is also regulated by other processes, such as the plant hormones salicylic acid and abscisic acid (reviewed in Iba, 2002; Hua, 2009). Extensive studies in recent years have shown that the up-regulation of HSP genes is the result of a complex cascade of events, whose final steps are unquestionably the activation of heat-shock transcription factors (HSFs) and their binding to the promoters of HSP genes (Baniwal *et al.*, 2004; von Koskull-Doring *et al.*, 2007). In *Arabidopsis*, heat-activated HsfA1a, HsfA1b, HsfA2 and HsfA3 are the most downstream components of the heat signalling pathway (Baniwal *et al.*, 2004). Extensive efforts have been made to study the structure, phosphorylation, oligomeric organization, expression pattern and heat-induced post-translational modifications of plant HSFs, their association with and dissociation from chaperones before, during and after heat, their DNA-binding specificities and the type of protein partners with which they may interact (Nover *et al.*, 1996; Baniwal *et al.*, 2004; von Koskull-Doring *et al.*, 2007). Several important reviews have addressed the transcriptional regulation of HSFs (Baniwal *et al.*, 2004; von Koskull-Doring *et al.*, 2007; Hua, 2009).

In this review, we focus on the key events occurring at the earliest stages of temperature elevation to describe the upstream components of the heat-induced signalling pathway, ultimately leading to the activation of HSFs and the expression of HSPs. Table 1 describes the key components reported in land plants. They are discussed hereafter.

The primary sensory role of the plasma membrane and Ca²⁺ ions

Several types of stimuli, such as cold, heat or osmotic stresses, can dramatically modify the physical properties of biological membranes (Falcone *et al.*, 2004; Matos *et al.*, 2007). Based on a large body of evidence from bacteria, yeasts and animals, it has been suggested that, because of their extraordinary sensitivity to environmental cues, membranes probably enclose sensory devices capable of detecting specific signals and transducing them into appropriate gene expres-

sion (Vigh *et al.*, 1998; Los & Murata, 2004). Membranes adapt their lipid composition according to the growth temperature, and can thus modulate heat-sensing components enclosed in them. Supporting this, the activation of a recombinant heat-shock promoter in the moss *Physcomitrella patens* was shown to depend on the temperature difference, rather than on the absolute temperature of induction (Saidi *et al.*, 2009). In mosses grown at different physiological temperatures, the higher the growth temperature and the more saturated the membrane lipids, the less the extent of HSR (Saidi *et al.*, 2010). In support of a central heat-sensing role for membranes, heat has also been shown to trigger a specific transient Ca²⁺ influx across the plasma membrane (Gong *et al.*, 1998; Liu *et al.*, 2006; Saidi *et al.*, 2009; Wu & Jinn, 2010). The intensity of the Ca²⁺ signature and of the following HSR was reduced in tissues with more saturated lipids and thus more rigid membranes (Saidi *et al.*, 2010). Other reports have shown that treatment with membrane fluidizers, such as benzyl alcohol, triggers a similar cascade of events: an isothermal Ca²⁺ influx within minutes, HSP expression within hours and the development of thermotolerance within days (Saidi *et al.*, 2005, 2009; Suri & Dhindsa, 2008). Conversely, exposure to rigidifying agents, such as dimethylsulfoxide, reduces the heat-mediated expression of HSPs (Suri & Dhindsa, 2008). These findings support the existence of membrane-associated thermosensors that can perceive and respond appropriately to mild changes in the fluidity of the membrane.

In mammalian cells, transient receptor potential vanilloid channels have been shown to mediate heat and pain sensations on transient entry of Ca²⁺ ions into the cytoplasm (TRPV1 and TRPV2 activated at noxious temperatures > 43°C; TRPV3 and TRPV4 activated at warm temperatures, 33–39°C and 27–34°C, respectively) (Venkatchalam & Montell, 2007). No homologues of such receptors could be identified in genomes of land plants and, so far, no genetic evidence for similar plant heat-sensitive channels exists. Yet, strong physiological and biochemical findings suggest the existence of specific calcium-permeable channels that respond to heat or membrane fluidizers. Within minutes of a temperature rise, a conserved transient calcium influx is observed in several plants (Gong *et al.*, 1998; Liu *et al.*, 2006; Saidi *et al.*, 2009; Wu & Jinn, 2010). In *Physcomitrella* protoplasts, electrophysiological analyses confirmed the presence of a heat-sensitive calcium-permeable channel in the plasma membrane that transiently opens within seconds of a sharp temperature increase and rapidly closes thereafter, despite the continuous up-holding of heat treatment (Saidi *et al.*, 2009). Moreover, when the availability of extracellular Ca²⁺ was artificially reduced, this severely, yet reversibly, inhibited the heat-induced up-regulation of HSPs, and consequently reduced the thermotolerance in *Arabidopsis*, tobacco, soybean, rice and *Physcomitrella* (Gong *et al.*, 1998; Liu *et al.*, 2005; Saidi

Table 1 Summary of the key mechanisms triggering the expression of heat-shock proteins in plants

	Plant	Treatment	Remarks	References
Membrane & Ca ²⁺	Arabidopsis (22°C)	37°C	Intracellular free calcium concentration increased	Liu <i>et al.</i> (2006)
		22°C	Induction of <i>AtHsp18.2</i> triggered by Ca ²⁺ and inhibited by calcium channel blockers and EGTA	Liu <i>et al.</i> (2005)
	Tobacco (25°C)	37°C	Hsp70 induced by membrane fluidizers and prevented by membrane rigidifiers	Suri & Dhindsa (2008)
		25°C	Triggered Ca ²⁺ influx which was blocked by EGTA and Ca ²⁺ channel blockers	Gong <i>et al.</i> (1998)
		37°C	Acquired thermotolerance diminished by EGTA and enhanced by Ca ²⁺	
		> 39°C	Ca ²⁺ is essential to MAPK induction by heat	
	Alfalfa (25°C)	37°C	DNA-binding activity of HSFs induced by Ca ²⁺	Sangwan <i>et al.</i> (2002)
	Maize (27°C)	27°C	Triggered a Ca ²⁺ influx. Acquired thermotolerance reduced by EGTA and restored by Ca ²⁺	Li <i>et al.</i> (2004)
	Rice (28°C)	> 37°C	Membrane fluidizers induced Ca ²⁺ influx, triggered HSR and enhanced thermotolerance	Wu & Jinn (2010)
	<i>Physcomitrella</i> (22°C)	22°C	Elicited Ca ²⁺ influx. Blocking Ca ²⁺ entry abolished the expression of HSPs and reduced thermotolerance	Saidi <i>et al.</i> (2009)
> 27°C		Acclimation at 30°C increased membrane rigidity and reduced HSR	Saidi <i>et al.</i> (2010)	
35°C		AtCaM3 induced by elevated temperature. DNA-binding activity of HSFs reduced in <i>cam3</i> mutant	Zhang <i>et al.</i> (2009)	
Calmodulin & kinase	Arabidopsis (22°C)	37°C	Thermotolerance significantly impaired in <i>cam3</i> mutants and enhanced in CAM3 over-expressors	Liu <i>et al.</i> (2008)
		> 45°C	AtHsFA1a specifically phosphorylated by AtCBK3 in the presence of CaM and Ca ²⁺ . Binding activity of HSFs to HSEs impaired in AtCBK3 mutants and improved in the over-expressors. Accumulation of HSPs reduced in AtCBK3 mutants and enhanced in the over-expressors	
	Tobacco (25°C)	37°C	Basal thermotolerance impaired in AtCBK3 mutants and improved in the over-expressors	Suri & Dhindsa (2008)
		37°C	Transient activation of heat-shock-activated MAP kinase activity (HAMK)	
	Alfalfa (25°C)	25°C	Hsp70 accumulation repressed by MAPKK inhibitors	Sangwan <i>et al.</i> (2002)
		37°C	Membrane fluidizers induced HAMK	
	Maize (27°C)	27°C	Elevated temperatures activated HAMK	Li <i>et al.</i> (2004)
		44°C	Antagonists of Ca ²⁺ -dependent protein kinase inhibited the activation of HAMK	
	Wheat (22°C)	37°C	Antagonists of Ca ²⁺ -dependent protein kinase inhibited the activation of HAMK	Li <i>et al.</i> (2004)
		37°C	DNA-binding activity of HSFs induced by CaM and reduced by CaM antagonist	Liu <i>et al.</i> (2003)
37°C		CaM antagonists decreased <i>hsp26</i> and <i>hsp70</i> expression		
36°C		Pretreatment with kinase inhibitor reduced HSR and negatively affected thermotolerance	Saidi <i>et al.</i> (2009)	
H ₂ O ₂ & NO	Arabidopsis (20–22°C)	22°C	Exposure to H ₂ O ₂ induced expression of <i>hsp17.6</i> and <i>hsp18.2</i>	Rentel & Knight (2004)
		20°C	H ₂ O ₂ application increased intracellular free Ca ²⁺	Volkov <i>et al.</i> (2006)
	20°C	DNA-binding activity of HSFs occurred in protein extracts from H ₂ O ₂ -treated cells	Banti <i>et al.</i> (2010)	
	23°C	HsfA2 and Hsp25 were induced by treatment with H ₂ O ₂		

Table 1 (Continued).

	Plant	Treatment	Remarks	References
		37°C	Heat increased endogenous H ₂ O ₂ levels. HSP expression is reduced by peroxide scavenger. Peroxide scavenger blocked HSF DNA-binding activity	Volkov <i>et al.</i> (2006)
		45°C	Endogenous NO levels increased during heat shock. Thermotolerance was impaired in <i>noa1</i> mutant and rescued by the addition of an NO donor	Xuan <i>et al.</i> (2010)
		37°C	DNA-binding activity of HSFs and Hsp18.2 expression were reduced in <i>noa1</i> mutant and rescued by the addition of an NO donor	
	Tobacco (24°C)	45°C	Endogenous NO levels increased during heat shock	Gould <i>et al.</i> (2003)
		28°C	Membrane fluidizer triggered H ₂ O ₂ elevation	
		> 32°C	Rapid increase in endogenous H ₂ O ₂ by heat	
		36°C	Small HSP induction reduced by inhibitor of H ₂ O ₂ generation	Konigshofer <i>et al.</i> (2008)
Cytoskeleton & protein denaturation	Arabidopsis (20°C)	20°C	Expression of HsfA2, Hsp70A and small HSPs induced by chemical generation of misfolded proteins using AZC. AZC-mediated Hsp70A expression reduced in <i>hsfA2</i> mutant	Sugio <i>et al.</i> (2009)
	Tobacco (25°C)	25°C	Microfilament and microtubule destabilizers induced HSP70 accumulations	Suri & Dhindsa (2008)
		37°C	HSP70 accumulation repressed by microfilament and microtubule stabilizers	
	Alfalfa (25°C)	37°C	Microfilament stabilizers prevented HAMK activation	Sangwan <i>et al.</i> (2002)
	Rice (28°C)	28°C	Activation of <i>Oshsp17.3</i> promoter induced by AZC	Guan <i>et al.</i> (2010)
	<i>Physcomitrella</i> (22°C)	40°C	Heat-denatured luciferase was not sufficient to induce HSPs when extracellular Ca ²⁺ was immobilized	Saidi <i>et al.</i> (2009)
HSP90 inhibition	Arabidopsis (22°C)	22°C	HSP90 inhibitors activated the transcription of HSPs and increased thermotolerance	Yamada <i>et al.</i> (2007)
		37°C	HSP90.2 binds HsfA1d in the absence of heat. Heat treatment inhibited HSP90 activity	
		37°C	ROF1 binds HSP90.1. The complex translocates to the nucleus by heat via interaction with HsfA2	Meiri & Breiman (2009)
	<i>Physcomitrella</i> (22°C)	22°C	HSP90 inhibitors induced a Ca ²⁺ -dependent HSR	Saidi <i>et al.</i> (2009)

Notes: temperature values in parentheses against the name of the model plants indicate the growth temperature used. AtCBK3, Arabidopsis calmodulin-binding protein kinase3; AZC, L-azetidine-2-carboxylic acid; CaM, calmodulin; CAM3, calmodulin 3 gene; CBK3, calmodulin-binding protein kinase3; EGTA, ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacetic acid; HAMK, heat-shock-activated MAP kinase; HS, heat shock; HSE, heat-shock element; HSF, heat-shock factor; HSPs, heat-shock proteins; HSR, heat-shock response; MAPK, mitogen-activated protein kinase; NO, nitric oxide; *noa1*, nitric oxide associated1.

et al., 2009; Wu & Jinn, 2010). This conserved effect shows the importance of the transient entry of extracellular Ca²⁺, possibly regulated by the plasma membrane, for the optimal initiation of HSR in land plants. Blockers of calcium channels reduced the amplitude of the heat-induced Ca²⁺ influx, as well as HSP synthesis (Liu *et al.*, 2005; Saidi *et al.*, 2009).

The role of Ca²⁺ ions in HSF activation has also been shown. In the cell, the heat-activated HSFs recognize

and bind conserved DNA sequences, called heat-shock elements (HSEs), in HSP promoters (von Koskull-Doring *et al.*, 2007). When maize cell extracts were treated with ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacetic acid (EGTA), the ability of HSFs to bind HSEs was reduced (Li *et al.*, 2004), an effect that was restored on addition of Ca²⁺. This result shows that the final step in HSF-mediated HSP expression depends on a Ca²⁺ signal. Electrophysiology has demonstrated that the origin of the

Ca²⁺ involved in heat signalling is extracellular. Moreover, in *Physcomitrella*, the artificial release of Ca²⁺ ions from internal stores, using ionomycin or thapsicargin, during heat treatment, was unable to mediate HSR, as long as extracellular Ca²⁺ was maintained low by EGTA (Saidi *et al.*, 2009).

The timing of heat-induced Ca²⁺ entry has also been proven to be critical. The delayed availability of extracellular Ca²⁺ during the first minutes of a temperature rise (22–38°C) reduced significantly the activation level of a small HSP promoter and negatively affected the acquired thermotolerance (Saidi *et al.*, 2009). Remarkably, heat did not trigger the osmotic-responsive promoters, nor did osmotic stress trigger the specific heat-inducible promoters (Saidi *et al.*, 2009). This demonstrates the great specificity of the heat sensors and osmosensors, although they are both dependent on an influx of external calcium. To maintain such high specificity in the cytoplasm, whilst using the same secondary messengers as other receptors, heat-sensitive channels probably rely on specific Ca²⁺-dependent effectors, such as calmodulins (CaMs).

Calmodulins and kinases

In plants, CaMs have been shown to carry out signalling functions in response to several environmental cues (Snedden & Fromm, 2001). CaMs have been reported to be involved in heat signalling and HSP expression. The expression of *Arabidopsis* CaM3 and CaM7 is increased during heat shock (Liu *et al.*, 2005; Zhang *et al.*, 2009), and the addition of CaM antagonists clearly decreases the expression of *hsp26* and *hsp70* genes at high temperature (Liu *et al.*, 2003). Interestingly, the addition of CaM proteins to maize protein extract increases the DNA-binding activity of HSFs *in vitro* under non-heat-shock conditions (Li *et al.*, 2004). When CaM antagonists were added at elevated temperatures, this DNA-binding ability was decreased (Li *et al.*, 2004), accounting for reduced HSP expression under similar conditions. This argues in favour of CaMs acting downstream to the heat-sensitive Ca²⁺ channels. Recent genetic evidence supports this in *Arabidopsis*, where CaM3 has been shown to play a key role in HSR (Liu *et al.*, 2008; Zhang *et al.*, 2009). The accumulation of HSP transcripts is down-regulated in *cam3* mutants and up-regulated in CAM3 over-expressing plants (Zhang *et al.*, 2009). In line with the above findings, the DNA-binding activity of HSFs is reduced in *cam3* mutants, even at high temperature. Consequently, *cam3* mutants become heat sensitive, whereas CAM3 over-expressing plants exhibit an improved thermotolerance (Zhang *et al.*, 2009). Therefore, the modulation of HSF DNA-binding activity, leading to HSP expression and the establishment of thermotolerance, appears to be under the control of Ca²⁺-dependent activation of specific CaMs in plants.

How HSFs become phosphorylated and activated during heat shock, and how this is connected to the preceding upstream events, is still ambiguous. A CaM protein phosphatase, PP7, has been reported to interact with both AtCaM3 and AtHsfA1a, suggesting a possible role in the activation of HSFs (Liu *et al.*, 2007). In addition, Ca²⁺ entry has been shown to be essential to the specific heat activation of a mitogen-activated protein (MAP) kinase (HAMK) (Sangwan *et al.*, 2002), suggesting that the activation of specific kinases is a downstream element of the Ca²⁺-dependent heat signalling pathway. Indeed, recently, genetic evidence has demonstrated a key role played by the *Arabidopsis* CaM-binding protein kinase 3 (AtCBK3) (Liu *et al.*, 2008). In *cbk3* mutants, submitted to a temperature increase, both the HSF DNA-binding activity and the accumulation of HSPs were reduced, but were enhanced in plants over-expressing AtCBK3. Consequently, the basal thermotolerance was shown to be affected by AtCBK3. Liu *et al.* (2008) have also demonstrated that AtCBK3 interacts with AtHsfA1a *in vivo* and promotes the latter's phosphorylation *in vitro*, which, remarkably, requires the presence of both Ca²⁺ and CaM. AtCBK3 can also bind CaM3 in a Ca²⁺-dependent manner (Wang *et al.*, 2004). Therefore, it is reasonable to speculate that a heat-induced Ca²⁺ transient acts as a primary mediator activating AtCaM3 and promoting its interaction with AtCBK3, thus leading to the activation, by phosphorylation, of HsfA1a. In line with this, other reports have shown that high temperatures induce a transient increase in HAMK activity in alfalfa and tobacco (Sangwan *et al.*, 2002; Suri & Dhindsa, 2008). Antagonists of Ca²⁺-dependent protein kinase inhibit HAMK activity (Sangwan *et al.*, 2002) and inhibitors of MAPKK repress the accumulation of Hsp70 at 37°C (Suri & Dhindsa, 2008). In *Physcomitrella*, pretreatment with a kinase inhibitor (dicoumarol) strongly reduced HSR and negatively affected thermotolerance (Saidi *et al.*, 2009). As the heat-induced Ca²⁺ influx is highly conserved among land plants, the identification and characterization of CBK3 homologues in other plants are central to the confirmation that activation/phosphorylation of HSFs occurs via a Ca²⁺–CaM–kinase pathway. It is also essential to investigate whether the subcellular localization of HSFs is regulated by these upstream components.

Hydrogen peroxide (H₂O₂) and nitric oxide (NO)

H₂O₂ and NO have been shown to play a role as signalling molecules in plants (Neill *et al.*, 2002). Although damaging at high concentrations, H₂O₂ is involved in several protective pathways and regulates the expression level of defence genes under various stresses (Neill *et al.*, 2002). It is well known that exposure to elevated temperatures produces H₂O₂ and induces oxidative stress in plant cells (Larkindale & Knight, 2002). H₂O₂ increases following heat shock have

been reported to take place within 5 and 15 min in tobacco and *Arabidopsis* suspension cells, respectively (Konigshofer *et al.*, 2008; Banti *et al.*, 2010). It has been established recently that the production of H₂O₂ contributes to the transduction of the heat signal into the expression of HSPs (Volkov *et al.*, 2006; Konigshofer *et al.*, 2008). At non-heat-shock temperatures, treatment of *Arabidopsis* suspension cells and seedlings with exogenous H₂O₂ induces HsfA2 and small HSP transcription (Volkov *et al.*, 2006; Banti *et al.*, 2010). Conversely, when peroxide scavengers or inhibitors of H₂O₂ generation are added during high-temperature treatment, HSP expression is reduced in both *Arabidopsis* and tobacco (Volkov *et al.*, 2006; Konigshofer *et al.*, 2008). The DNA-binding activity of HSFs has been demonstrated to occur in protein extracts treated with H₂O₂ at 20°C (Volkov *et al.*, 2006), indicating that H₂O₂-mediated HSP expression occurs by way of post-translational HSF activation. Substantiating this observation, the addition of peroxide scavengers blocks the DNA-binding ability of HSFs even at high temperatures (Volkov *et al.*, 2006). It is thus apparent that H₂O₂ *per se* can induce an HSF-dependent HSR. To confirm that H₂O₂-mediated up-regulation of HSP genes modulates the physiology of the plant, it would be interesting to investigate the effect of peroxide scavengers or inhibitors of H₂O₂ generation on the plant-acquired thermotolerance following initial priming at mild temperatures. This could also help to distinguish necessity from sufficiency of H₂O₂ in HSR. Interestingly, exposure to membrane fluidizers triggered a rapid and transient elevation of cellular H₂O₂ (Konigshofer *et al.*, 2008). Moreover, H₂O₂ application has been shown to trigger an increase in intracellular free Ca²⁺ (Rentel & Knight, 2004). It is thus likely that H₂O₂ stimulates directly MAP protein kinases leading to effective gene expression (Fig. 1).

NO is a secondary messenger that is also implicated in a variety of biotic and abiotic stresses (Gould *et al.*, 2003). Endogenous NO levels have been shown to increase in tobacco and *Arabidopsis* within 30 min of heat shock (Gould *et al.*, 2003; Xuan *et al.*, 2010). The isolation of mutants impaired in NO synthesis provides a valuable tool to reveal a direct link between thermotolerance and NO levels. Compared with the wild-type (WT), the *Arabidopsis noa1* mutant displays reduced NO levels and an impaired basal thermotolerance (Xuan *et al.*, 2010). Corroborating this observation, the accumulation of AtHsp18.2 following temperature elevation is less pronounced in mutants impaired in NO synthesis. Pretreatment of the *noa1* mutant with an NO donor, sodium nitroprusside (SNP), has been

shown to restore temperature-induced AtHsp18.2 expression and to rescue an optimal thermotolerance. The connection between NO and thermotolerance has also been demonstrated to be mediated by HSFs. The heat-induced DNA binding of HSFs is weaker in the *noa1* mutant and, interestingly, the supplementation of SNP has been proven to be sufficient to rescue the HSF–DNA complexes (Xuan *et al.*, 2010). Internal NO levels have also been shown to regulate AtCaM3 transcript levels. AtCaM3 levels increased as a function of NO levels and the heat-induced up-regulation of AtCaM3 failed in the *noa1* mutant background and increased after SNP pretreatment (Xuan *et al.*, 2010). The application of SNP alone failed to enhance the thermo-

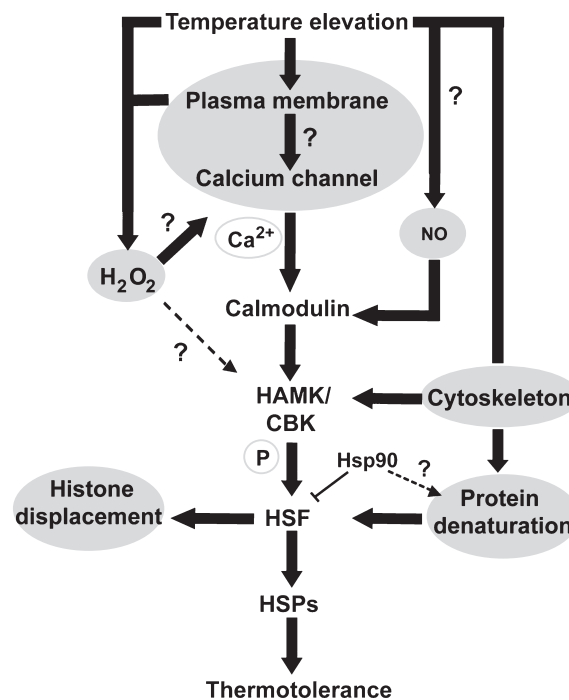


Fig. 1 Schematic diagram illustrating the key components of the plant temperature signalling pathway leading to optimal thermotolerance via heat-shock transcription factors (HSFs) and heat-shock proteins (HSPs). Temperature elevation induces an increase in membrane fluidity, activating unknown Ca²⁺ channels and triggering a transient Ca²⁺ influx. Ca²⁺ activates calmodulin (CaM) and promotes the phosphorylation (P) of HSFs via interaction with CaM-binding protein kinase (CBK) (or heat-activated MAP kinase, HAMK). Activated HSFs bind to HSP promoters and probably cause histone displacement. HSP expression is thus induced and contributes to enhance thermotolerance. The temperature increase (as well as changes in the membrane state) also increases hydrogen peroxide (H₂O₂) levels that might activate a similar pathway. Heat-induced nitric oxide (NO), cytoskeleton rearrangements and protein denaturation act at different levels to trigger HSF-dependent HSP expression. Black arrows indicate pathways supported by evidence in the literature and dotted arrows show hypothetical processes. Question marks indicate the unknown players. Note: Ca²⁺, H₂O₂ and NO affect other pathways that are not shown in this model for the sake of clarity.

tolerance of the *cam3* mutant. However, CAM3 over-expression, although having no effect on NO levels, improved the thermotolerance of the *noa1* mutant (Xuan *et al.*, 2010). This strongly indicates that AtCaM3 is an active component of the NO-mediated heat signalling pathway acting downstream of NO production.

Cytoskeleton and protein denaturation

Being sessile organisms, plants are prone to wide temperature variations that may affect protein homeostasis, cause protein misfolding or induce reversible dissociation of labile protein complexes. When exposed to various stresses, the dynamics of the cytoskeleton (microtubules, actin and their associated proteins) play an essential role in regulating cell architecture and intracellular signalling (Nick, 2007). A transient and reversible depolymerization of cytoskeletal components is observed in intact *Arabidopsis* and tobacco cells at nonlethal yet higher temperatures (Muller *et al.*, 2007; Malerba *et al.*, 2010). The targets of cytoskeletal rearrangement following a temperature increase are not well known. Nevertheless, the isothermal modulation of cytoskeleton organization has allowed the identification of HAMK as a downstream target. Not only do destabilizing microfilaments trigger HAMK activity at 25°C in alfalfa cells, but, in addition, HAMK activation, normally induced by heat or membrane fluidizers, has been shown to be repressed by microfilament stabilizers (Sangwan *et al.*, 2002). Further, Hsp70 accumulation is prevented at 37°C in the presence of microfilament and microtubule stabilizers, and, conversely, is induced after the application of destabilizing agents at 25°C (Suri & Dhindsa, 2008). These data indicate that the cytoskeleton is involved in the response to an increase in temperature and contributes to the regulation of HSP expression (Fig. 1).

Extreme heat stress may cause protein denaturation *in vivo*. Indeed, the perception of high temperature and the expression of HSPs have long been thought to be carried out at the cellular level by some unknown heat-sensitive proteins, which, on denaturation in the cell, presumably activate HSFs by causing the dissociation of HSF and Hsp70 and/or Hsp90 complexes (Morimoto, 1998). This model was mainly adapted from animal studies, but very little indirect evidence supports it in plants. Interestingly, the chemical generation of misfolded proteins, using L-azetidine-2-carboxylic acid (AZC), induces the transcriptional activation of HsfA1, Hsp70A and small HSP genes in *Arabidopsis* (Sugio *et al.*, 2009) and activates the *Oshsp17.3* promoter in rice (Guan *et al.*, 2010). Similar results have been observed using virus infection and ectopic protein expression (Jockusch *et al.*, 2001; Sugio *et al.*, 2009). The AZC-mediated expression of HSPs appears to occur via HSFs. AtHsp70A expression measured after the generation of misfolded proteins was significantly reduced in the *hsfA2*

mutant background when compared with WT (Sugio *et al.*, 2009). These recent results suggest that the accumulation of misfolded proteins is a genuine trigger leading to the activation of HSFs in plants. Several reports have suggested that HSPs themselves play a regulatory role and act as negative regulators that bind a fraction of HSFs at optimal growth temperatures, thus maintaining them inactive. Following temperature elevation, Hsp70 and Hsp90 are presumably recruited to repair protein damage, hence releasing HSFs and allowing their activation. *Arabidopsis* Hsp70 and Hsp90.2 have been shown to interact with HsfA1a and HsfA1d, respectively (Kim & Schoffl, 2002; Yamada *et al.*, 2007). Nevertheless, the identity of the heat-sensitive proteins titrating this interaction during heat shock is unknown. In *Physcomitrella*, heat denaturation of recombinant thermolabile luciferase is not sufficient to induce HSR when extracellular Ca²⁺ is prevented from entering the cell (Saidi *et al.*, 2009). Whether this Ca²⁺ dependence and protein unfolding independence is specific to bryophytes, or whether it is a general property of land plants, remains to be elucidated. It is reasonable to speculate that, to establish optimal thermotolerance, plants need to sense a temperature rise before protein damage has occurred. They would favour rapid responses in anticipation of damage, and not as a result of it. Protein unfolding/misfolding could activate some HSFs, but this would take place only at very high temperatures, and would be unlikely to contribute to the onset of thermotolerance.

The role of Hsp90 in HSR

Inhibitors of Hsp90 activity (geldanamycin and radicicol) have been shown to induce a heat-shock-like response and to enhance thermotolerance in *Arabidopsis* and *Physcomitrella* (Yamada *et al.*, 2007; Saidi *et al.*, 2009). The overlap between heat- and Hsp90 inhibitor-induced genes is significant (Yamada *et al.*, 2007; Finka *et al.*, 2010). Most of the up-regulated genes contain specific HSF-binding sequences in their promoters, suggesting that HSP activation by Hsp90 inhibitors may occur via HSFs (Yamada *et al.*, 2007). Yamada *et al.* (2007) have also shown that *Arabidopsis* cytosolic Hsp90 activity is reduced during heat shock. This suggests that Hsp90 may be an inhibitor of HSFs in the absence of heat. It has therefore been hypothesized that, under heat shock, the inactivation of Hsp90 would allow the release of HSFs and trigger HSP transcription. However, other findings in *Physcomitrella* have shown that the induction of HSPs via the isothermal inhibition of Hsp90 is also dependent on the entry of extracellular Ca²⁺ (Saidi *et al.*, 2009). In the presence of EGTA, neither geldanamycin nor radicicol was able to activate HSP promoters, suggesting that calcium must be a necessary component of the pathway. It is still unclear to what extent and how exactly Hsp90 may regulate or mediate HSF

activity, and what is the nature of the signal controlling the interactions between Hsp90 and HSFs. Given the above observation, it is essential to re-assess the fraction of chaperone-free and chaperone-bound HSFs at non-heat-shock and warm temperatures. Thus far, it seems that only a minor fraction of HSFs, reflected by the extent of HSR, can be activated by the presumed chemical dissociation of Hsp90 and HSFs (Saidi *et al.*, 2009).

Recently, *Arabidopsis* Hsp90.1 has been found to bind ROF1 (a peptidyl prolyl *cis/trans* isomerase), and the so-formed complex localizes to the cytoplasm under optimal non-heat-shock conditions (Meiri & Breiman, 2009). The *in vivo* interaction between AtHsp90.1 and AtHsfA2 has also been reported (Meiri & Breiman, 2009). Interestingly, heat-shock induced the nuclear localization of the Hsp90–ROF1 complex but only in the presence of HsfA2 (Meiri & Breiman, 2009). The expression levels of small HSPs were found to be reduced significantly in *rofl* mutants that exhibited a low survival rate when exposed to high temperatures (Meiri & Breiman, 2009). The interaction of Hsp90 and ROF1 has therefore been suggested to play an important role in the prolongation of thermotolerance. The upstream signal that regulates the subcellular localization of Hsp90–ROF1 was not addressed in the above study and remains unknown.

The role of histones and nucleosome occupancy

Heat can cause histone acetylation, methylation, ubiquitination, phosphorylation, glycosylation, ADP-ribosylation and sumoylation (Clapier & Cairns, 2009). These modifications of amino-terminal histone tails protruding from nucleosomes can regulate the active or repressed state of the associated DNA sequence in a code-like manner (Jenuwein & Allis, 2001; Liu *et al.*, 2010). In plants, histone acetylation seems to be concomitant with DNA demethylation during transcriptional gene activation (Zilberman *et al.*, 2008). Reversible histone acetylation homeostasis is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Although plant HATs and HDACs are involved in abiotic stresses, there are no data indicating their involvement in the modulation of HSR, but it has been shown that the Rpd3L HDAC complex is essential for the yeast HSR (Ruiz-Roig *et al.*, 2010).

Recently, chromatin immunoprecipitation has shown that *Arabidopsis* H2A histone variant, H2A.Z, is present in smaller amounts at promoter regions of Hsp70 and other temperature up-regulated genes following warming from 17 to 27°C (Kumar & Wigge, 2010). A mutant, *Arp6*, failing to incorporate H2A.Z into nucleosomes, showed constitutive up-regulation of Hsp70 and exhibited a gene expression pattern of plants grown at warmer temperatures (Kumar & Wigge, 2010). In addition, Kumar & Wigge (2010) have reported a decrease in local DNA wrapping

when using nucleosomes purified from 17°C-grown plants and exposed to 27°C. However, these temperature-mediated changes in DNA wrapping, observed *in vitro*, were very subtle and do not reproduce the significant drop in nucleosome occupancy of chromatin from plants grown at 27°C. It has been suggested that H2A.Z might act as a direct temperature sensor and mediate changes in gene expression associated with temperature response through nucleosome occupancy. Yet, the *Arp6* mutant deregulated > 2000 non-HSP genes (Kumar & Wigge, 2010), and H2A.Z is also associated with other genes responsible for phosphate starvation and plant immunity (March-Diaz *et al.*, 2008; Smith *et al.*, 2010). It is thus reasonable to assume that the specificity of HSR must take place upstream of nucleosomes. Moreover, a presumed initial temperature sensing at the nucleosome level would have to be compatible with the dependence of the plant HSR on Ca²⁺ entry, CaMs and HSF activation.

Since the initial observation of chromosome puffs in heat-treated cells of *Drosophila* salivary glands, it is generally accepted that nucleosomes, which are stably bound to the heat-inducible genes at rest, need to become dynamic and specifically displaced during heat shock for optimal transcription (Dyson *et al.*, 2005). This displacement of nucleosomes correlates with a specific increase in histone H3 phosphorylation at HSP genes. Work with mice *hsf1*–/– mutants has confirmed that it is Hsf1 that dictates the specificity of histone H3 and H4 dynamic displacement from HSP genes, whereas the same histones remain stably bound to non-HSP genes (Thomson *et al.*, 2004). Similarly, in yeast, the dynamic displacement of histones at heat-shock genes during warming has been shown to correlate with increased levels of histone H3 acetylation. Remarkably, both H3 acetylation and dynamic displacement are reduced in strains expressing truncated Hsf1, confirming that, as in animals, heat-activated Hsf1 provides a specific signal to render histones transiently more dynamic, thereby allowing an optimal transcription of HSP genes (Erkina & Erkin, 2006). Together, this indicates that, in yeast and animals, Hsf1 is the central component of the heat signalling pathway that, by virtue of its ability to recognize and bind exclusively to HSP promoter elements, confers high specificity of gene expression during HSR. Unlike other eukaryotes, plant genomes enclose a large number of HSF genes. It would be exciting to test whether a plant HSF can accomplish a similar function.

Conclusions

Genetic screens, mutant analyses, genomic and pharmacological approaches have recently allowed significant progress in the identification of several components of the heat signalling pathway in plants. Fig. 1 summarizes the current knowledge and shows an overview of the different possible

mechanisms reported in plants so far. Many questions remain open and the future avenues of research should turn towards the identification of the heat-activated Ca^{2+} channels and the connection between Ca^{2+} , HSF activation and nucleosome occupancy during and following mild temperature rise. Although the precise identity of the most upstream temperature sensor in land plants remains unknown, it is doubtful that cytosolic protein misfolding acts as a primary thermosensor, especially during a mild temperature increase where protein damage is unlikely. However, recent physiological and biochemical evidence points to fluidity-sensitive Ca^{2+} channels in the plasma membrane as plausible candidates. Specific CaMs and MAP kinases are probably involved in the downstream events transducing the signal from the membrane to transcription factors. In turn, the activated HSFs would provide the specificity of HSR, via displacement of histones from HSP genes, whose optimal up-regulation enhances plant thermotolerance (Fig. 1).

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