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Review article

The heat-shock protein/chaperone network and multiple stress resistance

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Summary

Crop yield has been greatly enhanced during the last century. However, most elite cultivars are adapted to temperate climates and are not well suited to more stressful conditions. In the context of climate change, stress resistance is a major concern. To overcome these difficulties, scientists may help breeders by providing genetic markers associated with stress resistance. However, multistress resistance cannot be obtained from the simple addition of single stress resistance traits. In the field, stresses are unpredictable and several may occur at once. Consequently, the use of single stress resistance traits is often inadequate. Although it has been historically linked with the heat stress response, the heat-shock protein (HSP)/chaperone network is a major component of multiple stress responses. Among the HSP/chaperone 'client proteins', many are primary metabolism enzymes and signal transduction components with essential roles for the proper functioning of a cell. HSPs/chaperones are controlled by the action of diverse heat-shock factors, which are recruited under stress conditions. In this review, we give an overview of the regulation of the HSP/chaperone network with a focus on *Arabidopsis thaliana*. We illustrate the role of HSPs/chaperones in regulating diverse signalling pathways and discuss several basic principles that should be considered for engineering multiple stress resistance in crops through the HSP/chaperone network.

Keywords: multistress resistance, stress acclimation, HSPs/chaperones, HSF, crop improvement.

Introduction

Stresses are defined as environmental constraints that differ from optimal conditions, ultimately impeding growth and development. As sessile organisms, plants are commonly exposed to fluctuating environments and can show a great degree of resilience to conditions that would be considered harmful to many other organisms. The process by which an organism reaches phenotypic stability despite environmental and genetic variations was termed 'canalization' (Waddington, 1961).

To improve canalization to extreme conditions, the selection of stress resistance traits has been aided by the use of associated genetic markers. Single stress resistance traits have been extensively introgressed into elite cultivars. Due to the difficulty in reproducing a specific stress of a specific strength, most genetic studies have remained limited to single stress resistance. In nature, however, stresses rarely come alone. For instance, heat stress is associated with high light, but also facilitates the spreading of pests and pathogens leading to dramatic production losses. Moreover, responses to heat will involve the opening of stomata to dampen the rise in temperature, whereas a response to drought requires the closure of stomata to avoid water loss. In this regard, it is not surprising that responses to multiple, co-occurring stresses are dramatically different than single stress responses added together. Transcriptomic analyses lead to the astonishing finding that 61% of the genes induced by dual stresses were not induced by any of the single stresses (Rasmussen *et al.*, 2013). The combination of single stress resistance traits will consequently mostly not lead to multiple

stress resistance. It is absolutely necessary to study multistress resistance pathways to understand and enhance canalization in the field (for review see Mittler and Blumwald (2010); Suzuki *et al.* (2014)).

One way to study multistress pathways would be to take advantage of the pleiotropic HSP (heat-shock protein)/chaperone network. By definition, protein denaturation is a constant direct or indirect consequence of any stress, as stresses are defined as factors impeding normal cellular functions carried out by proteins. Potentially, any stressor that induces protein misfolding would require HSP/chaperone recruitment. In this regard, chaperones are now considered as powerful buffers against environmental stress and even genetic variations (Carey *et al.*, 2006). Protein misfolding is the main feature of heat stress, so the HSPs were the first chaperones to be studied. However, since the discovery of HSPs/chaperones, it has been found that the role of these factors is not limited to heat stress management but is also involved in other stresses, such as cold, osmotic, drought, salt, UV, high light, oxidative stress and pathogen infection (Swindell *et al.*, 2007).

Multistress resistance and the HSP/chaperone pathway

HSPs and chaperones are found in most prokaryotes and eukaryotes, and even some viruses (Maaroufi and Tanguay, 2013). In a cell, more than 10 000 proteins co-exist in a limited space. Biochemists worldwide have experienced the difficulty in producing only a few of these proteins in a native conformation in concentrations comparable with *in vivo* conditions. Unfolded proteins tend to form large aggregates that severely impede normal cellular functions. The main function of HSPs/chaperones

is to act as a buffer to limit misfolding and resolve aggregates. By doing so, they minimize the impact of environmental and genetic variations on the proteome. HSP90 alone makes up for 1%–2% of the total protein content in eukaryotes (Krukenberg *et al.*, 2011). The molecular mechanisms underlying the functions of HSPs have been extensively reviewed (Al-Wahaibi, 2011; Fu, 2014; Niforou *et al.*, 2014; Wang *et al.*, 2013). Chaperone functions are not limited to folding and HSP70 and HSP90 and their cochaperones have clearly been linked to signalling, protein targeting and degradation (Huang *et al.*, 2014; Kadota and Shirasu, 2012; Kriechbaumer *et al.*, 2012; Lee *et al.*, 2009).

Transcriptional control of HSPs

The basic principles of the transcriptional control of HSPs are represented schematically in Figure 1. The main inducers of chaperones are heat-shock factors (HSF), grouped into three classes A, B and C (for review, see Guo *et al.* (2016); Nover *et al.* (2001); Scharf *et al.* (2012)). HSFs are present in all eukaryotes, but plants show a large number of HSFs (38 in soya bean, 25 in rice, 21 in *Arabidopsis*) compared with a single HSF1 in *Saccharomyces cerevisiae* or with seven members in humans (Fujimoto and Nakai, 2010). The diversity of the HSF family in plants renders their study difficult. However, sequence and expression pattern comparisons showed both distinct and overlapping functions in stress resistance and development (von Koskull-Döring *et al.*, 2007).

There has been very few *in planta* study on B and C class HSFs. Class B and C HSFs lack the activator motif AHA (aromatic hydrophobic acidic) that is necessary for the transcriptional activity of class A HSFs and are therefore considered as inhibitory HSFs. Nevertheless, HSF B class members have been demonstrated to be absolutely necessary for the proper recovery from heat stress. In *Arabidopsis*, HSF1 and HSF2b have been shown to repress the induction of HSPs during stress recovery (Ikeda *et al.*, 2011).

One unusual feature of HSF/HSP research is that many discoveries have been made on nonmodel species. It was first in tomato that the 'master regulator' *SIHSFA1* was identified. Transgenic plants overexpressing *SIHSFA1* were found to be responding better to heat stress, whereas cosuppression (CS) lines were oversensitive to heat stress (Mishra *et al.*, 2002).

In *Arabidopsis*, the transcription factors *HSFA1abd* and *e* are constitutively expressed and are responsible for triggering the HSR (heat stress response) (Yoshida *et al.*, 2011). *HSF1abde* are responsible for basal thermotolerance and also initiate the acquisition of thermotolerance. These transcription factors bind to HSEs (heat stress elements) to activate transcription of *HSPs* as well as 'transcriptional relay' *HSFs*, mainly *HSFA2*, *HSFA3* and *HSFA7a*, that will, together with *HSFA1* or separately, maintain a strong HSR during long-term, repeated stresses. In this regard, *HSFA2*, *HSFA3* and *HSFA7a* are considered as the most potent activators of *HSP* transcription during recovery. They are responsible for the heat-acclimation phenotype (Chang *et al.*, 2006; Nishizawa *et al.*, 2006; Schramm *et al.*, 2008). It is worth noting that *HSFA2* is the most highly heat-induced HSF and *hsfa2* KO mutants are the only simple mutants which are completely unable to acquire thermotolerance. This is partly explained by the fact that *HSFA2* is able to induce its own expression. However, the mechanism is not specific to *HSFA2* as other HSFs can also exert positive feedback on other HSFs. *HSFA3*, *HSFA7a* and *HSFA7b* are induced by *HSFA2* and/or *HSFA1s* after heat stress (Liu and Chang, 2013). In turn, *HSFA1e* and *HSFA7b* and *HSFB2b* are

induced by *HSFA3* overexpression (Yoshida *et al.*, 2008). Similarly, *HSFA6a* overexpression induces *HSFA6b*, *HSFA5* and *HSFA2* (Hwang *et al.*, 2014). Intriguingly, *HSFA3* overexpression in control conditions induces *HSFA1e* but not *HSFA2* expression, which is induced by *HSFA1e* after heat and/or high light stress. These complex interconnections and feedback loops demonstrate that multiple input signals can activate overlapping but different HSF/HSP responses.

This adapted HSF activity may also be a consequence of post-translational modifications (PTMs). *HSFA4a* is a target of MPK3 and MPK6 (mitogen-activated protein kinase). It was reported that phosphorylation by MPK3/6 increases the activity of *HSFA4a* (Pérez-Salamó *et al.*, 2014). It was further shown that *HSFA2* phosphorylation by MPK6 is required for its nuclear localization, but the molecular mechanism determining the subcellular localization of *HSFA2* has not been fully deciphered (Evrard *et al.*, 2013). *HSFA2* was also found to be sumoylated after heat stress (Cohen-Peer *et al.*, 2010) and an increased sumoylation was correlated with a decrease in *HSFA2* activity and diminished *HSP* induction. SUMO1-overexpressing plants showed an *hsfa2* KO phenotype with respect to heat stress tolerance. Most importantly, it is thought that homo/hetero-oligomerization is very important for the modulation of *HSP* induction. In tomato, *SIHSFB1* can positively or negatively regulate the transcription of *HSPs* by forming heterodimers with *SIHSFA1*. *SIHSFA1-SIHSFB1* can stimulate *HSP* production, whereas *SIHSFB1* alone represses the transcription of *HSPs* (Hahn *et al.*, 2011). *SIHSFA1* activity is also enhanced by *SIHSFA2* binding to such an extent that the *SIHSFA1-SIHSFA2* complex is called a 'superactivator complex' (Chan-Schaminet *et al.*, 2009). In *Arabidopsis*, despite of being an A class HSF, *HSFA5* specifically binds to and inhibits *AtHSFA4a*. In mammals, HSF1 monomers are inactive and HSF1 is known to bind DNA as a homotrimer (for the detailed structure bound to DNA, see Neudegger *et al.* (2016)). Both in mammals and plants, ROS (reactive oxygen species) directly impact HSF oligomerization. ROS are acting as second messengers in a great variety of stresses and H_2O_2 induces binding of high molecular weight protein complexes on HSEs (Volkov *et al.*, 2006) and trimerization of *HSFA1a* was shown to occur upon treatment with H_2O_2 , heat or pH variation (Liu *et al.*, 2013). ROS action on transcription factors often depends on the oxidation of one or several Cys residues, but a number of other amino acids could also be potential targets of H_2O_2 (reviewed in Driedonks *et al.* (2015)).

The amount of free HSPs is the sensor of the cell capacity to maintain a stable proteome and feeds back on its own production. Indeed, in unstressed tissues, the commonly accepted 'chaperone titration model' specifies that HSFs are sequestered by HSP70/90 and maybe other chaperones (Guo *et al.*, 2001; Volkov *et al.*, 2006). Strong evidences obtained in tomato suggest that an increase in *SIHSFP70/90* clients leads to liberating HSFs, allowing high *HSP* and HSF production. On the contrary, excess *SIHSFP70/90* will in turn favour *SIHSFA1* inactivation and *SIHSFB* repression activity (Hahn *et al.*, 2011). However, the sequestration of TFs by HSP70/90 may not always be a simple dose-dependent effect but also a finely tuned process. In *Arabidopsis*, two FK506-binding proteins (FKBPs), named *ROF1* and *ROF2*, have been shown to regulate *HSFA2* activity. *ROF1* and *2* possess TPR (tetratricopeptide repeat) domains involved in HSP90 binding and domains involved in peptidyl-prolyl cis-trans isomerization of polypeptide bonds. It was demonstrated that *ROF1* and *2* participate in the formation of an *HSP90.1-HSFA2* complex (Meiri *et al.*, 2010). It was also observed that the *HSFA2-*

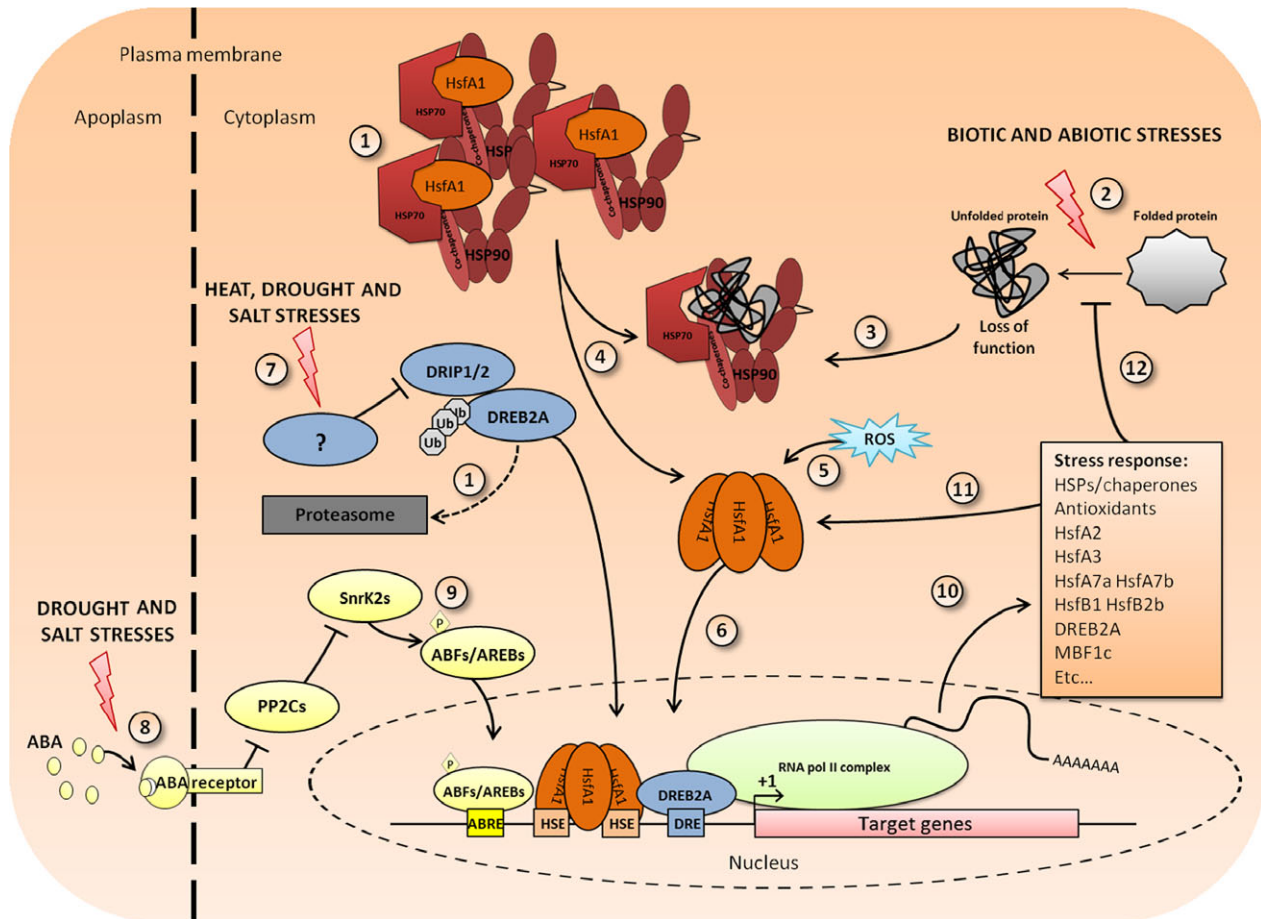


Figure 1 Schematic representation of the HSP/HSF pathway. (1) In nonstress conditions, class A1 HSFs are sequestered by HSP90/70 and their cochaperones and DREB2A is degraded through the UPS thanks to the E3 ligase DRIP1/2 (Qin *et al.*, 2008). Upon stress application (2), the high number of misfolded proteins triggers the recruitment of HSP90/70 to its client and frees the HSF1s following the chaperone titration model (4). In a high ROS context (5), the HSF1s can form oligomers and are translocated in the nucleus (6) to bind HSE on DNA and induce target genes' transcription. Trimers are represented here, in reference to mammalian HSF1 trimerization, even though the degree of oligomerization has not been established in plants except for AtHSFA1a trimerization. Other signalling pathways may interfere with the HSF/HSP pathway. Specific heat, drought and salinity stresses will lead to DRIP1/2 inhibition and DREB2A accumulation (7). Drought and salt stresses will induce ABA accumulation and binding to its receptor PYR/PYL/RCAR, leading to inactivation of PP2Cs (8). SnrK2s can then activate their target by phosphorylation (9). ABFs/AREBs and DREB2A can then enter the nucleus, cooperatively or separately bind their target DNA motif, respectively, ABRE and DRE and HSE, to activate target genes' expression (10). Induced proteins comprise stress-specific 'transcriptional relay' TFs that feedback positively on HSF and HSP transcription (11) or proteins that participate in homeostasis re-establishment (12). After the stress, the HSF/HSP content of the cell is different from the start. The quantity and the nature of the HSFs/HSP define the acclimated state.

ROF1-HSP90.1 complex keeps HSFA2 in a transcriptionally active state, whereas ROF2 binding inhibits HSFA2 activity. Interestingly, ROF2 is a target of HSFA2 produced 6 h after stress onset.

HSF functional diversification

The large number of HSFs and the complex modulation of their activities by hetero-oligomerization render the attribution of specific functions very difficult. In *Arabidopsis*, study of the different triple mutants *Hsfa1a, b, d*; *Hsfa1b, d, e*; *Hsfa1a, b, e* and *Hsfa1a, d, e* and also the quadruple *Hsfa1a, b, d, e* mutant revealed different specificity for stress resistance. The triple *Hsfa1a, b, d* and quadruple *Hsfa1a, b, d, e* mutants are unable to adapt to even moderately high temperatures. The *Hsfa1b, d, e* quadruple mutant was hypersensitive to salt stress. All HSF1s are involved in osmotic stress tolerance, with a preference for HSF1d and HSF1e. The presence of HSF1b and d was sufficient for normal oxidative stress tolerance. The quadruple

Hsfa1a, b, d, e mutant also showed a defect in seed development, exhibiting more than 20% abortion (Liu and Charnig, 2013).

All of these defects were partially or completely rescued by overexpressing *HSFA2*. This is consistent with the finding that *HSFA2* is a target of HSF1d and e. It has been thoroughly proven that overexpression of *HSFA2* is sufficient to enhance resistance to heat, anoxia, salt, osmotic stresses and a combination of heat, high light and oxidative stresses (Nishizawa *et al.*, 2006; Ogawa *et al.*, 2007). *HSFA2* expression can be induced by HSF1d and e, but the double KO mutant does not display full inhibition of *HSFA2* expression during combined heat and high light stresses, suggesting that another factor than HSF1d and e exists for regulating *HSFA2* expression (Nishizawa-Yokoi *et al.*, 2011). *HSFA2* is also involved in developmental processes. *HSFA2* overexpression increases callus proliferation (Ogawa *et al.*, 2007). In tomato, SlHSFA2 is up-regulated in anthers during

pollen formation (Fragkostefanakis *et al.*, 2016) and is important to mitigate pollen sensitivity to heat stress.

Apart from HSF1s, several HSFs play a role in drought and salinity stress signalling. They can be induced by ABA-dependent as well as ABA-independent signalling pathways. The latter is represented by DREB (dehydration response element binding) TFs. *HSFA3* is a target of DREB2A (Yoshida *et al.*, 2008), which is induced by multiple stresses such as drought, salt, heat and cold and to a lesser extent by oxidative stress, UV-B light and wounding (Winter *et al.*, 2007). DREB2A was first shown to be involved in drought and salt stress responses (Sakuma, 2006a). Consequently, DREB2A-dependent *HSFA3* induction may not only be important for HSP production during thermotolerance but may also lead to salt and drought stress resistance. However, resistance to the above-mentioned stresses was not investigated in *HSFA3* overexpressing plants.

ABA-dependent signalling relies on SnRK2's (sucrose nonfermenting related protein kinases 2) constitutive inhibition by PP2Cs (protein phosphatase 2 Cs; for review see (Singh and Laxmi, 2015)). PP2Cs are recruited to the ABA-bound receptors PYR/PYL/RCARs (pyrabactin resistance/pyrabactin resistance 1-like/regulatory component of ABA receptors), thereby releasing the inhibition of subclass III SnRK2s. The latter are controlling many ABA-responsive transcription factors by phosphorylation, most importantly ABF1, ABF3 (ABA response factors 1 and 3), AREB1 and AREB2 (ABA-responsive element-binding proteins 1 and 2). Consequently, *snrk2d/e/i* triple mutant seeds are highly sensitive to humidity and are not able to induce seed-specific expression of *HSFA7b* and *HSFA9* (Nakashima *et al.*, 2009) in *Arabidopsis*. *HSFA7b* is present only after heat treatment and in drying seeds. The *HSFA7b* KO did not show defects in thermotolerance. Several HSFs are transiently induced during seed imbibition, namely *HSFA2*, *HSFA9*, *HSFA7b*, *HSFA7a*, *HSFB2a* and *HSFB2b* (Chiu *et al.*, 2012). Most importantly, heat stress sustained the activation of these HSFs, leading to the accumulation of 49 HSP transcripts and is partially mediated by ABA. *HSFA9* is even more specific to seed protection processes as it is not inducible by stress. Its transcription is controlled by the seed-specific ABA-responsive transcription factor ABI3 (ABA insensitive 3) (Kotak *et al.*, 2007). Interestingly, *HSFA9* can interact with a component of auxin signalling, HalAA27 (indole acetic acid) in sunflower (*Helianthus annuus*) (Carranco *et al.*, 2010). Together with the seed abortion phenotype of the quadruple *Hsfa1a,b,d,e* mutant, these data highlight the importance of HSFs during seed development.

The promoter of *AtHSFA6a* contains two AREs (ABA-responsive elements) and is bound *in vitro* by three ABA-responsive TFs: AREB1, ABF3 and AREB3 (Hwang *et al.*, 2014). Overexpressing plants exhibited resistance to salt and drought stresses. The fact that *HSFA6a* was not found to be a target of SnRK2-dependent signalling may be due to a seed-specific modulation of ABA signalling (Nakashima *et al.*, 2009).

HSFB2b is a direct target of class III SnRK2s (i.e. ABA-triggered SnRK2s), but it is not known to which extent this phosphorylation event is biologically relevant (Wang *et al.*, 2013).

Similarly, *HSFA4a* was found to be induced transcriptionally during heat, salt, osmotic and cold stresses as well as during biotic stresses and *HSFA4a* overexpression leads to salt, oxidative and anoxia stress tolerance in *Arabidopsis* (Pérez-Salamó *et al.*, 2014).

Factors controlling the production of HSPs during biotic stresses are not well established. *AtHSFA2* and *AtHSFA7a* were shown to be involved in the CPR (cytoplasmic protein response), which is induced during the formation of large protein

aggregates in the cytoplasm (Sugio *et al.*, 2009). CPR is a feature of biotic stresses, as overexpression of viral proteins tends to overload the cellular machinery. In the same study, the induction of both HSFs was observed during *TuMV* (*Turnip mosaic virus*) or *TCV* (*Turnip crinkle virus*) infection, suggesting that HSFs are involved in virus resistance (Sugio *et al.*, 2009). On the contrary, *AtHSFB1* and *AtHSFB2b* negatively regulate the expression of the plant defense genes PDF1.2a and b (Kumar *et al.*, 2009). As a result, the simple mutant *Hsfb2b* and the double mutant *Hsfb1, b2* exhibited resistance to the necrotrophic fungus *Alternaria brassicicola*. Both B class HSFs are targets of HSF1s and A2 (Liu and Charng, 2013). Intriguingly, overexpression of *AtHSFA1b* induces expression of *HSFB2b* but still protects plants from biotic, as well as drought and salt stresses (Bechtold *et al.*, 2013).

HSFs were also shown massively recruited during oxidative stress. Oxidized lipids play an important role in the adaptation to oxidative stress. *HSFA1a*, *HSFA1e*, *HSFA2*, *HSFA4c*, *HSFA7a*, *HSFA8*, *HSFB1*, *HSFB3*, *HSFB4*, *HSFC1* and many HSPs are induced by OPDA (oxo phyto dienoic acid) treatment (Taki *et al.*, 2005). Another oxidized lipid derivative was recently found to specifically induce the expression of genes of the HSP/chaperone network (Mata-Pérez *et al.*, 2016), including *HSFA2* and *HSFA7b*. Gene ontology (GO) annotation revealed that 21.25% of the genes up-regulated by NO₂-Ln treatment were termed as 'chaperones', 21.25% as 'response to heat' and 6.88% as 'heat acclimation', suggesting that NO₂-Ln may play an important role in heat stress. The basis of oxylipin-mediated induction of the HSP/chaperone network is still unknown.

HSP/chaperones involvement in heat stress-independent signalling

Biotic stress signalling

Several lines of evidence indicate that the HSP/HSF pathway is involved in biotic interactions (Park and Seo, 2015). HSP90 is strongly involved in resistance proteins (R proteins) stabilization and is necessary for proper defence signal transduction (Shirasu, 2009). Most human HSP90 clients are signalling components (Taipale *et al.*, 2012) and it seems that similar principles apply to the plant kingdom (Iki *et al.*, 2010; Ishiguro *et al.*, 2002). HSP90 forms a complex with its co-chaperones SGT1 and RAR1 (salicylic acid glucosyltransferase 1 and required for *mla12* resistance 1). This complex is regulating important R proteins like RPM1 (resistance to *Pseudomonas maculicola* 1), RPS2, RPS4 (resistance to *Pseudomonas syringae* 2 and 4), RPP4 (recognition of *Peronospora parasitica* 4 (Bao *et al.* (2014) Hubert *et al.* (2003); Takahashi *et al.* (2003); Zhang *et al.* (2004)) and Rx (resistance to *Potato virus x*; Lu *et al.*, 2003). Each HSP90 isoform has its own specificity. For example, *Hsp90.2* but not *Hsp90.3* mutants are especially sensitive to *Pseudomonas syringae pv tomato* (*Pst*) carrying the avirulence factor RPM1 (*avrRPM1*).

A role of HSP70 in pathogenesis is well illustrated by the study of Jelenska *et al.* (2010) on *Pst* infection. A *Pst* effector protein, HopI1, was reported to exert its virulence functions specifically on HSP70-1 (Jelenska *et al.*, 2010). Moreover, *Arabidopsis* plants with reduced levels of HSP70-1 allowed enhanced growth of type III secretion-deficient *Pst*. Altogether, these data established a specific role of HSP70-1 in basal defence. On the contrary, heat-shock cognate 70-1 (HSC70-1), a cytosolic and nuclear chaperone, was found to down-regulate R protein-mediated resistance to pathogens (Noël *et al.*, 2007). The effect was attributed to the modulation of HSP90 functions in

immunity, as HSP70 and HSP90 often cooperate in large multichaperone complexes (Li *et al.*, 2012).

An HSP70-specific resistance pathway exists. BiP (immunoglobulin-binding protein) is an HSP70 isoform specifically located in the endoplasmic reticulum (ER) lumen. BiP has been linked with the UPR, which, like its cytoplasmic equivalent, is triggered under biotic stress conditions. The function of BiP in the UPR is reminiscent of HSP90/70-dependent regulation of SIHSA1/A2 in tomato. The accumulation of BiP clients induces the dissociation of BiP from the major UPR transducers IRE1 (inositol requiring enzyme 1), PERK [double-stranded RNA-activated protein kinase (PKR)-like ER kinase] and ATF6 (activating transcription factor 6) (Bertolotti *et al.*, 2000). However, it was found in yeast that IRE1 could induce normal UPR in the absence of BiP. BiP is now considered as a stabilizer of the UPR transducers, safeguarding the cell against unspecific UPR signalling (reviewed in Walter and Ron (2012)). Finally, *HSP101* transient overexpression in tobacco cells protects from *C. michiganensis*-induced cell death (Shafikova *et al.*, 2013), but not much is known on the involvement of other chaperones in biotic stress responses.

Drought stress signalling

Information about the involvement of HSP/chaperones in drought stress signalling is scarce. It was found that both the overexpression of *HSC70* and the use of a dominant negative (DN) form of *HSP90* disrupted ABA-mediated stomata closure, thereby negatively affecting water loss in stress conditions. The impact of ABA treatment on *HSC70*, *HSP90*, *SGT1* and *RAR1* was investigated by Q-PCR. A decrease in *SGT1a* mRNA and an increase in *HSC70-4* mRNA was observed. *HSC70-1* and *HSC70-4* must therefore share the same function regarding the regulation of ABA signalling but under physiological conditions, only *HSC70-4* is involved in mitigating ABA signals. Surprisingly, the opposite effect was observed for ABA-mediated inhibition of germination. *HSP90* DN- and *HSC70*-overexpressing seeds were strongly hypersensitive to ABA in this assay. The targets of *HSP90* and *HSC70* are not known yet but must be downstream of SnRK2 as it was fully activated after ABA treatment, despite the use of an *HSP90* inhibitor (Clement *et al.*, 2011).

Hormone signalling and development

HSP90 and its cochaperones also regulates diverse signal transduction proteins. MAPKs are involved in many biological processes, from stress responses to cell proliferation and development (reviewed in Colcombet and Hirt (2008)). Recently in tobacco, the *SGT1*-*HSP90* complex was found to mediate the hypersensitive response (HR) induced by MEK2^{DD} (a constitutively active form of MEK2, mitogen-activated protein kinase kinase2 (Ichimura *et al.*, 2016)). MEK2 regulates HR-mediated pathogen resistance in tobacco and tomato (Oh and Martin, 2011). Interestingly, silencing of *HSP90* induced a drastic decrease in MEK2^{DD} transcripts, while *SGT1* silencing destabilized MEK2^{DD}, but not wild-type MEK2 protein levels. Several evidences point to an involvement of the same complex in the regulation of MPK4. MEK1 is necessary for the activation of MPK4 and functions in an antagonistic manner to MPK3 and MPK6. The *mekk1* mutant phenotype (dwarfism and H₂O₂ accumulation) was partially reverted at moderately high temperatures and further complemented by the *rar1* mutation at 26 °C (Ichimura *et al.*, 2006). However, it is unclear where the *HSP90*-*RAR1* module acts in the signalling pathway. Moderately high temperatures were shown

to inhibit overactivation of MPK3 and MPK6 in the *mekk1* mutant background. Chaperones may be needed to stabilize upstream positive regulators of MPK3/6 like MEK2^{DD}.

SHD (*Shepherd*) is coding for an *HSP90*-like protein residing in the ER. *shd* mutants exhibit defects similar to those induced in *clv* (*Clavata*) mutants. Like *clv* mutants, *shd* shows a disorganized shoot apical meristem (SAM), floral meristem (FM) and root apical meristem (RAM). Genetic analyses of *shd clv* and *shd wus* double mutants suggest that SHD is necessary for CLV signal transduction (Ishiguro *et al.*, 2002).

The *HSP90*-*SGT1* complex is also involved in jasmonic acid (JA), auxin and gibberellic acid (GA) signalling. It was determined that *SGT1a* and *b* were necessary for the stable expression of *CO1* (coronatine insensitive 1) and *TIR1* (transport inhibitor response 1) proteins. An effect on GA signalling was indirectly determined by observing sustained overactivation of a GA down-regulated gene (*CYP71A12*) after GA and Flg22 treatment (Zhang *et al.*, 2015). Brassinosteroid (BR) signalling is also partially dependent on *HSP90* as *BES1* [brassinosteroid insensitive1 (*BRI1*) EMS suppressor1] is an *HSP90* client (Lachowiec *et al.*, 2013). The use of the well-known *HSP90* inhibitor geldanamycin showed that *HSP90* is required for proper BR signalling, meaning that there could be more than one *HSP90* client in the BR pathway. Unfortunately, no systematic analysis of *HSP90* clients has been performed in plants.

Chaperones other than *HSP90/70* can also modulate signal transduction events. The case of the *chaos* mutant is a good example of chaperone-dependent canalization. The *CHAOS* locus is coding for a molecular chaperone named cpSRP43 (chloroplastic signal recognition particle of 43 kDa), involved in light harvesting complex proteins (LHCP) folding and chloroplast targeting. As LHCPs are intrinsic proteins, they are naturally prone to denaturation and absolutely require a chaperone for correct targeting and folding. It was shown that *cpSRP43* is down-regulated during cold and high light stress acclimation (Klenell *et al.*, 2005). The consequent decrease in LHCP levels reduced ROS production and sensitivity to oxidative stress. Moreover, *chaos* mutants were constitutively acclimated to cold and were performing better than WT under repeated stress conditions (Klenell *et al.*, 2005).

Chaperones of the *HSP100* family play a crucial role in development. *HSP100* chaperones are essential components of the protein quality control (PQC) process. They act in concert with *HSP70* chaperones to thread and degrade toxic protein aggregates (Mogk *et al.*, 2015). Major metabolic pathways require these *HSP100*s to reactivate or degrade misfolded enzymes following environmental stresses (Pulido *et al.*, 2016). They are also involved in protein targeting as they process the signal peptide of specific precursor proteins once they have reached their destination. For instance, *ClpC1* was shown to be involved specifically in photosystem biogenesis. In the *clpc1* mutant, chlorophyll, PSI and PSII levels were down-regulated and growth was consequently strongly impaired (Sjögren *et al.*, 2004). *HSP100* chaperone's role is not limited to proteins. *HSP101* was shown to bind a nucleotide sequence in the 5'UTR (untranslated region) of some mRNAs. It was demonstrated that *HSP101* was able to stabilize target mRNAs and enhance their translation (Ling *et al.*, 2000; Wells *et al.*, 1998; Wu *et al.*, 2013).

Engineering the HSFs for multistress resistance

The *HSP*/chaperone pathway is exerting pleiotropic regulation of gene expression on both the translational and post-translational

levels. In addition to protect a broad spectrum of proteins, chaperones regulate a great number of signal transduction components. Four major features define the HSP/chaperone pathway: it is *ubiquitous*, able to *memorize* stress, it can respond to a variety of stresses thanks to a great degree of *modularity* and shows complex *feedback loops*, both positive and negative. The HSP/chaperone pathway provides stability both in control and stress conditions. As a consequence, it should be considered as a key actor of canalization. The impact of HSF overexpression is varied. It was demonstrated that the overexpression of *AtHSFA1b*, *AtHSFA2*, *AtHSFA3*, *AtHsfA4a* and *AtHSFA6a* induces chaperone production and improves stress resistance in *Arabidopsis*. Nonetheless, it also modulated developmental programs (reviewed in Fragkostefanakis *et al.* (2014)).

DREB2A modulates *HSFA3* expression, and deletion of the regulatory domain of *DREB2A* leads to the production of a CA (constitutively active) form of the protein. The resulting plants constitutively express *HSFA3* and exhibit resistance to drought, salt and heat stresses. Nonetheless, transgenic plants displayed growth retardation (Sakuma *et al.*, 2006b). *AtHSFA3* overexpression has been shown to elevate thermotolerance but also produced moderate to severe dwarfism (Yoshida *et al.*, 2008). The impact of *AtHSFA3* overexpression on other stresses was not investigated. Tomato *SIHSFA3* overexpression in *Arabidopsis* gave a different phenotype. Plants were resistant to heat but more sensitive to salt stress. Flowering was also delayed, but they were not dwarf as 35S::*AtHSFA3* plants (Li *et al.*, 2013). It would be interesting to compare *SIHSFA3* and *AtHSFA3* targets to identify genes responsible for the dwarf phenotype.

Constitutive induction of *HSFA4a* was detrimental to plants as *HSFA4a* overexpression showed 20%–30% growth reduction in control conditions. However, when exposed to salt, anoxia or oxidative stress, *HSFA4a*-overexpressing plants performed better than control. Expression of *HSFA4a* was induced by numerous other stresses like UV-B, cold, drought ozone or pathogens (Pérez-Salamó *et al.*, 2014). The impact of *HSFA4a* overexpression on resistance to those stresses was not investigated but could be very interesting.

AtHSFA6a and *AtHSFA6b* are not induced by heat stress treatment but are specifically induced by ABA, salt, drought and osmotic stress (Huang *et al.*, 2014, 2016; Hwang *et al.*, 2014). Consistently, the promoter of *AtHSFA6a* contains two ARE and was bound *in vitro* by three ABA-responsive TFs: AREB1, ABF3 and AREB3. Similarly, *AtHSFA6b* promoter contains several ABREs bound *in vitro* by AREB1. Overexpressing plants exhibited resistance to salt and drought stress. A genetic screen aiming at discovering mutations inducing constitutive expression of *HSFA6a* and *AtHSFA6b* would be promising.

HSFA1b is particularly promising with respect to its multistress resistance potential. *HSFA1b* overexpression confers resistance to drought, salt and biotic stress and enhances seed yield (Bechtold *et al.*, 2013). Analysis of an HSF1b-specific heat-shock element in the promoter of *HSFA1b*-differentially expressed genes (DEGs) allowed the identification of 55 direct targets among the DEGs. Among those 55 genes were several transcription factors related to multiple stresses (in particular *HSFA7A*, *HSFB2b*, *HSFB2a* and *MBF1c*) that should be responsible for the resistance traits (Bechtold *et al.*, 2013).

The particular role of *HSFA2* as a response amplifier and in stress acclimation makes it a strong candidate for resistance engineering. A relatively small overexpression of *AtHSFA2* did not negatively impact biomass but led to a weak resistance to heat

stress, oxidative stress and a combination of heat, high light and oxidative stresses (Li *et al.*, 2005; Nishizawa *et al.*, 2006). High-level overexpression of *HSFA2* was achieved with the *El2Q* promoter, resulting in a 400-fold increase in *HSFA2* expression. *El2Q::AtHSFA2* plants displayed dwarfism as well as resistance to heat, osmotic and salt stresses (Ogawa *et al.*, 2007). Nevertheless, this difficulty might be overcome using inducible instead of constitutive promoters. Interestingly, overexpression of *SIHSFA1* in tomato (which constitutively activates *SIHSFA2*) led to an increase in biomass as well as heat stress resistance (Mishra *et al.*, 2002). This observation suggests that it should be possible to uncouple HSF-mediated resistance and growth inhibition. A genetic screen aiming at reverting the dwarf phenotype of *El2Q::AtHSFA2* would be as difficult as it would be interesting.

The production of transgenic plants with altered HSF/HSP expression has also been employed to produce stress resistance in crops. Overexpression of endogenous HSF has been performed in tomato (Mishra *et al.*, 2002) and soybean (Zhu *et al.*, 2006), successfully increasing plant tolerance to stress. However, the HSF families in crop species are also diverse and crop transformation is time-consuming. Overexpression of one, randomly chosen, HSF could be inadequate to increase resistance to a specific set of stresses. To overcome these difficulties, several HSFs from wheat or rice were first characterized in transgenic *Arabidopsis*. For instance, a role of *OsHSF7*, *OsHSFA2a* and *TaHSFA2d* in stress responses has been confirmed in *Arabidopsis* (Chauhan *et al.*, 2013; Liu *et al.*, 2009; Yokotani *et al.*, 2008). These genes could now be used for stable *in planta* overexpression in crop plants.

Future perspectives of technology transfer to crops

Generally speaking, it seems that the most promising targets in terms of enhancing stress resistance are the most upstream components of a given signalling pathway, as exemplified by *AtHSFA1b* and *SIHSFA1*. It may be that activating a whole branch of a signalling pathway allows a better tuning of the response than activating just one downstream element. Another promising way to increase resistance would require understanding the principles of growth arrest in stress-resistant plants. It may be possible to uncouple the functions of growth inhibition from those of stress protection in a given signalling pathway. A genetic screen aiming at finding a reversion of the dwarfism of *HSFA2* or *HSFA3* overexpressors could lead to mutants that disconnect resilience and growth.

Still, will these results be applicable to crop resistance engineering? Several lines of evidence point towards a broad conservation of the HSP/chaperone network as a multiple stress protection mechanism among all land plants. Indeed, plant land adaptation was shown to be strongly dependent on HSPs as exemplified by the diversification of *HSP70*. The green algae *Chlamydomonas reinhardtii* possesses only six *HSP70* isoforms, whereas *Physcomitrella patens*, *Oryza sativa* and *Populus trichocarpa* have, respectively, 21, 24 and 20 *HSP70* isoforms (Tang *et al.*, 2016). Gene expression analyses revealed moss *HSP70* isoforms were responsive to ABA, drought and salt stresses, in addition to heat stress (Tang *et al.*, 2016). Gene overexpression and knockout studies showed *HSP70s* from tobacco, soya bean and citrus play a protective role during dehydration stress (Yu *et al.*, 2015). In rice, *OsAHL1* was shown to directly induce *HSP101* and *HSP90* expression leading to drought resistance (Zhou *et al.*, 2016). *OsHSP90-2* and *OsHSP90-4* were also found up-regulated after drought, cold, heat and salt stresses (Zhang *et al.*, 2016). When introduced in *E. coli*, *OsHSP90-2* was

sufficient to induce resistance to heat, high salinity and drought (Zhang *et al.*, 2016). A similar modulation of *HSFs* and *HSPs* was found in tomato in response to heat, drought and salinity (Fragkostefanakis *et al.*, 2015). In wheat, overexpression of *Triticum aestivum HSA6f* was demonstrated to direct the expression of several *HSPs*, leading to thermotolerance (Xue *et al.*, 2015). Most strikingly, the expression of a maize *HSF*, *ZmHSF06* (*Zea mays HSF06*), is sufficient to confer heat and drought stress resistance to *Arabidopsis* (Li *et al.*, 2015). The conservation of HSF signalling from monocots to dicots definitely provides evidence for a strong conservation of HSP-based multiple stress responses in crops.

From the above-mentioned facts, it is clear that many good targets to enhance multistress resistance are defined. Nonetheless, the technology is not readily transposable to crop species. Up to now, most attempts to enhance resistance have used transgenic 35S promoter-driven overexpression of class A HSFs, sometimes from stress-resistant species. However, two major obstacles restrict the use of this technique. Firstly, GMOs are 'associated with unnaturalness and trigger disgust' (Blancke *et al.*, 2015) in the general population and this feeling is now translated into the official European regulation. Even though the ban on GMOs is now limited to Europe, it is a rising concern worldwide and must be considered carefully. Secondly and most importantly, 35S-driven overexpression is unreliable. It does not produce a normally distributed high-level expression of genes and may be subjected to gene silencing (Butaye *et al.*, 2004). Performing a genetic screen to identify *Arabidopsis* mutants with enhanced *HSF* expression (using a reporter gene to monitor HSFs' activity) would be a more adequate approach. The TILLING (targeted induced local lesion in genomes) technique can then be used to apply knowledge gained from *Arabidopsis* directly into many cultivated species. Numerous mutant populations of crop species already exist and can be screened for a specific variant. Mutant *loci* can be identified in a matter of weeks thanks to the massive expansion of NGS (next generation sequencing) techniques (Kurowska *et al.*, 2011). The recently discovered CRISPR-CAS9 (clustered regularly interspaced short palindromic repeat-CRISPR associated 9) system would also allow a rapid technology transfer in crops. The advantage of CRISPR-CAS9 compared with TILLING would be that the latter requires a significant number of crosses to introgress one mutation in a competitive cultivar. Also, mutagenic agents used in TILLING introduce many unwanted mutations in the genetic background that need to be 'cleaned'. In this regard, genome editing by CRISPR-CAS9 would be cleaner and faster (Bortesi and Fischer, 2015). Nonetheless, CRISPR-CAS9 still relies on transgenic DNA insertion and may be considered as a GMO and subjected to the same regulation, even though the end product does not contain any transgene. Regardless, mutations enhancing HSF expression or activity undoubtedly are valuable targets to engineer multistress-resistant crops.

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Conflict of interest

The authors declare no conflict of interest, in accordance with the policy described in the Instructions for Author (<http://onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291467-7652/homepage/ForAuthors.html>).

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