[Plant Signaling & Behavior 3:9, 660-662; September 2008]; ©2008 Landes Bioscience

Article Addendum

Cytosolic heat shock protein 90 regulates heat shock transcription factor in *Arabidopsis thaliana*

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Key words: HSP90, heat shock transcription factor, heat shock, heat shock response element, heat acclimation, geldanamycin, radicicol

Plant survival requires the ability to acclimate to heat, which is involves the expression of heat-inducible genes. We found cytosolic heat shock protein (HSP) 90 serves as a negative regulator of heat shock transcription factor (HSF), which is responsible for the induction of heat-inducible genes in plant. Transient inhibition of HSP90 induces heat-inducible genes and heat acclimation in *Arabidopsis thaliana* seedlings. Most of upregulated genes by heat shock and HSP90 inhibitor treatments carry heat shock response element (HSE) in their promoter, which suggests that HSF participates in the response to HSP90 inhibition. *A. thaliana* HSP90.2 interacts with AtHsfA1d, which is one of the constitutively expressed HSFs in *A. thaliana*. Heat shock depleted cytosolic HSP90 activity, as shown by the activity of exogenously expressed glucocorticoid receptor (GR), which is a substrate of cytosolic HSP90. Thus, it appears that in the absence of heat shock, cytosolic HSP90 negatively regulates HsfA1. Upon heat shock, cytosolic HSP90 is transiently inactivated, and this may lead to the activation of HsfA1.

Although HSPs are responsible for refolding denatured proteins and/or for folding newly synthesized proteins, one of these HSPs, HSP90, also plays an additional role in the regulation of various cellular signaling molecules, including GR in animals and the pathogen recognition receptor, NBS-LRR-type R protein in plants. In *A. thaliana*, seven HSP90 homologues localize to the cytosol [HSP90.1 (At5g52640), HSP90.2 (At5g56030), HSP90.3 (At5g56010), and HSP90.4 (At5g56000)], mitochondria (At3g07770), chloroplast [CR88 (At2g04030)], and endoplasmic reticulum [SHD/GRP94 (At4g24190)]. The four cytosolic HSP90s are highly homologous to each other (86%-99% identity), which suggests that their activity is identical in *A. thaliana*. *HSP90.2, HSP90.3* and *HSP90.4* mRNAs accumulate constitutively, while *HSP90.1* mRNA increases extensively after heat shock in *A. thaliana* seedlings.[1] Hitherto, the function of cytosolic HSP90s has been mainly discussed within the context of the disease resistance in plant.[2-6] However, it is likely that cytosolic HSP90s play other roles in intracellular events, since depletion of HSP90 activity induces a wide variety of morphological changes.[7,8] Therefore, we investigated the effect of HSP90 inhibition to *A. thaliana* seedlings.

We used two chemicals, geldanamycin (GDA) and radicicol (RAD), to inhibit the function of HSP90. To investigate HSP90 functions on the gene regulation, we employed transgenic *A. thaliana* lines that bear β-*glucuronidase* (*GUS*) gene fused to various promoters. We found that a transgenic line that bears the heat-responsive *HSP90.1* promoter connected to *GUS* (*ProHSP90.1:GUS*)[9] gene induces the GUS after GDA or RAD treatment without heat shock. This indicates that GDA and RAD can induce heat-inducible genes in the absence of heat shock. This finding was further supported by a microarray analysis that revealed GDA or RAD treatment upregulated 157 heat-inducible genes in *A. thaliana*. Most of these genes are involved in heat shock protein/protein refolding (45 genes, 28.7%), transcription/translation (16 genes, 10.2%), and protein degradation (17 genes, 10.8%). Next, we searched the promoter regions of the upregulated 157 genes for the presence of a conserved motif. The promoters of 148 genes in the AGRIS database were analyzed by using the MEME program.[10] The identified motif is three HSE repeat (HSE3) motif, which is a specific DNA sequence (nGAAnnTTCnnGAAn) observed in the promoters of heat-inducible genes.[11] Most of 148 genes (115 genes, 77.7%) had at least one HSE3-like motif in their promoter region, which suggests that HSE3 is responsible for the induction of these genes after heat shock or GDA or RAD treatments. In a minor population of 148 genes (15 genes. 10.1%), we also identified a conserved motif (CCACGGCT) that is closely related to the unfolded protein response (UPR) element of endoplasmic reticulum (ER) stress. We speculated that GDA and RAD inhibit SHD/GRP95, an ER-localized HSP90 homologue, thereby inducing UPR.

It is known that HSF is responsible for the induction of heat-inducible genes that have HSE3 in their promoter region in various organisms, including plants. Most HSFs form a trimer to bind to HSE3. Although animals and yeast have relatively few HSFs, plants have many HSF homologues that have been classified into A, B and C subfamilies.[12] For example, *A. thaliana* has 15 class A, five class

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Submitted: 02/18/08; Accepted: 02/25/08

Previously published online as a *Plant Signaling & Behavior* E-publication: http://www.landesbioscience.com/journals/psb/article/5775

Addendum to: Yamada K, Fukao Y, Hayashi M, Fukazawa M, Suzuki I, Nishimura M. Cytosolic HSP90 regulates the heat shock response that is responsible for heat acclimation in *Arabidopsis thaliana*. J Biol Chem 2007; 282:37794–804; PMID: 17965410; DOI: 10.1074/jbc.M707168200.

B and one class C HSFs, while tomato (*Lycopersicon esculentum*) has at least 11 class A, three class B and one class C HSFs.[12,13] Plants use these HSFs in sophisticated heat shock response systems.[12] For example, it has been shown that HsfA1 is constitutively expressed and a master regulator of heat shock response,[14] HsfB1 is a coactivator of HsfA1,[15] and HsfA2 and HsfA3 are induced by heat shock and enhance heat shock response.[16-19] Several other HSFs seem to be involved in the regulation of thermal tolerance genes during development.[12,20,21]

Like animals, plants constitutively have a pool of HSFs but do not induce heat-inducible genes without heat shock, which suggests that HSF activity is normally negatively regulated. In human cells, HSP90 interacts with HSF1 monomer and negatively regulates its function.[22] Therefore, we hypothesized that cytosolic HSP90 in *A. thaliana* normally interacts with constitutively accumulated HSF to downregulate its function. Consistent with this, the effect of GDA and RAD on *A. thaliana* was not inhibited by co-treatment with cycloheximide, an inhibitor of translation, which suggests that the constitutively accumulated HSFs were mainly responsible for the induction of heat-inducible genes. We focused on AtHsfA1d because *AtHsfA1d* mRNA is expressed constitutively and AtHsfA1d protein recognizes HSE3. When we examined the interaction between *A. thaliana* HSP90.2 and AtHsfA1d by co-immunoprecipitation and bimolecular fluorescence complementation (BiFC) analyses, we found that HSP90.2 interacts with AtHsfA1d in the cytosol and nucleus. GDA or RAD treatment abolished the interaction between HSP90.2 and AtHsfA1d. These findings suggest that in the absence of heat shock, cytosolic HSP90 negatively regulates HsfA1 activity in plant (Fig. 1).

We then analyzed about the interactions between HSP90.2 and other *A. thaliana* HSFs. While *AtHsfA4c* mRNAs are expressed constitutively in *A. thaliana* seedlings, HSP90.2 did not bind to AtHsfA4c. This suggests that cytosolic HSP90 is not involved in the regulation of HsfA4. Recent findings show that HsfA5 is a negative regulator of HsfA4.[23] The levels of *AtHsfA7a* and *AtHsfB1* mRNAs are increased by heat shock, and HSP90.2 interacts with AtHsfA7a and AtHsfB1. This suggests cytosolic HSP90 also inactivates heat-inducible HSFs during the recovery from heat shock (Fig. 1).

It has been shown that prior heat shock leads to the acquisition of tolerance to high temperatures that are normally lethal.[24,25] This so-called heat acclimation phenomenon enables plants to live without wilting in extremely hot environments. The above findings suggest that heat shock leads to the depletion of HSP90 activity, presumably because of the accumulation of denatured proteins, and that this induces HSF-mediated heat acclimation (Fig. 1). We measured HSP90 activity in vivo after heat shock by using a transgenic *A. thaliana* that exogenously expressed Gal4-VP16-GR (GVG).[26] Since the GR domain of GVG is a substrate of HSP90, the GVG activity mirrors cytosolic HSP90 activity in the presence of dexamethasone, a ligand of GR. We found that HSP90 activity was depleted by heat shock, as well as GDA and RAD treatment. To address whether transient depletion of HSP90 activity is enough for heat acclimation, we transiently treated *A. thaliana* seedlings with GDA or RAD, and then subjected them to high temperatures. The HSP90 inhibitor-treated plants acquired high temperature tolerance without heat shock, which suggests that the transient inhibition of HSP90 is sufficient for heat acclimation in plants.

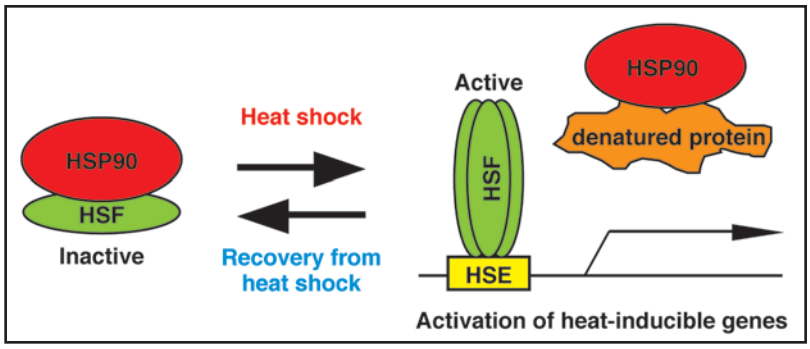

Figure 1. A hypothetical model of HSP90 function in the heat shock response. HSP90 negatively regulates HSF in the absence of heat shock. After heat shock, HSP90 releases HSF and acts to refold denatured proteins. The free HSF forms a trimer and then binds to HSE3, thereby activating heat-inducible genes. HSP90 then downregulates heat-induced HSF during recovery from heat shock.

Since plants are sessile and cannot escape to cool places, they must respond promptly to heat stress. The HSP90-HSF complex may enable plants to rapidly sense heat damage and induce thermal tolerance genes. Two recent studies also suggest that HSP90 inhibition plays an important role in plant acclimation.[3,27] Our findings complement these studies by elucidating the downstream events that occur after HSP90 inhibition. Interestingly, it has been suggested that the phosphorylation of HSF or the binding of HSP70 and small HSP to HSF may downregulate HSF activity.[11,28] It is possible that HSP90 may regulate HSF activity in partnership with these molecules, thereby fine-tuning HSF activity in vivo.

Acknowledgements

This work was supported by a Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (no. 1977040 to K.Y. and no. 16085209 to M.N.).

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