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Expression of five *AtHsp90* genes in *Saccharomyces cerevisiae* reveals functional differences of AtHsp90s under abiotic stresses

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ABSTRACT

The genome of *Arabidopsis thaliana* contains seven Hsp90 family genes. Three organellar and two cytosolic AtHsp90 isoforms were characterized by functionally expressing them in a temperature-sensitive *Hsp90* mutant and a conditional *Hsp90*-null mutant of *Saccharomyces cerevisiae*. The cytosolic AtHsp90-1 and AtHsp90-2 showed function similar to that of yeast in chaperoning roles; they could support the growth of yeast mutants at both permissive and non-permissive temperature. Neither the full-length nor mature forms of chloroplast-located AtHsp90-5, mitochondria-located AtHsp90-6 and endoplasmic reticulum (ER)-located AtHsp90-7 could complement the yeast Hsp90 proteins. The cytosolic AtHsp90s could stabilize the biomembrane of the temperature-sensitive *Hsp90* mutant strains under stress conditions, while the organellar AtHsp90s could not protect the biomembrane of the temperature-sensitive *Hsp90* mutant strains. Yeast two-hybrid results showed that either pre-protein or mature forms of organellar AtHsp90s could interact with cofactors cpHsp70, Hsp70, Hsp70t-2, Cyp40, p23 and a substrate protein of NOS, while cytosolic AtHsp90s could not interact with them. These results suggest that organellar and cytosolic AtHsp90s possibly work through different molecular mechanisms in forming chaperone complexes and performing their functional roles.

Introduction

Heat-shock proteins (Hsps) are key components contributing to cellular homeostasis in cells under both optimal and adverse growth conditions. They are responsible for protein folding, assembly, translocation and degradation in a broad array of normal cellular processes. They also function in the stabilization of proteins and membranes, and can assist in protein refolding under stress conditions (Wang et al., 2004). Five major families of Hsps are recognized: the Hsp60, Hsp70, Hsp90, Hsp100 and small Hsp family (Kimura et al., 1994). Different classes of Hsps appear to bind to specific non-native substrates and states.

Hsp90 is a type of essential, ubiquitous and extremely conservative protein with key roles in eukaryotic cells. Most of its known substrates are signal transduction proteins, distinguishing it from other Hsps (Pratt and Toft, 2003). In mammalian cells, Hsp90 forms complexes with the quiescent forms of a wide array of cellular signaling proteins and chaperones the conformational changes essential for their signaling-dependent activities, including ligand binding and association with partner proteins (Shiau et al., 2006). In the yeast *Saccharomyces cerevisiae*, Hsp90 is encoded by two nearly identical, functionally indistinguishable genes, *Hsc82* and *Hsp82*. Mutations in either gene are temperature sensitive for growth, and simultaneous disruption of both two genes is lethal even under normal conditions (Kimura et al., 1994; Nathan and Lindquist, 1995). Hsp90 proteins from humans, *Caenorhabditis elegans* and *Candida albicans* (Piper et al., 2003) have been shown to suppress the lethal phenotype of two Hsp90 deletion yeast strains.

In *Arabidopsis thaliana* ecotype Columbia, seven Hsp90 family members have been identified in the genome by a sequencing project. AtHsp90-1 to AtHsp90-4 proteins constitute the cytoplasmic subfamily (Milioni and Hatzopoulos, 1997; Krishna and Gloor, 2001). AtHsp90-5, AtHsp90-6 and AtHsp90-7 proteins are located in the chloroplast (Cao et al., 2003), mitochondria (Prassinos et al., 2008) and endoplasmic reticulum (ER) (Ishiguro et al., 2002), respectively. However, to date, few studies have described the physiological roles of Hsp90 chaperone complexes in plants (Queitsch et al., 2002; Samakovli et al., 2007). In this study, the functional characters of three organellar and two cytosolic AtHsp90 proteins were analyzed in the model organism yeast *S. cerevisiae*.

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Table 1
Primers used in the cloning and construction of *Hsp90* genes.

Genes	Forward primers (5′ → 3′)	Reverse primers (5′ → 3′)
AtHsp90-1	TCTAGAATGGCGGATGTTCAGATGGC	CCCGGGTTAGTCGACTTCCTCCATCT
AtHsp90-2	TCTAGAATGGCGGACGCTGAAACCTT	CCCGGGGTTAGTCGACTTCCTCCATC
AtHsp90-5	TCTAGAATGGCTCCTGCTTTGAGTAGA	CCCGGGTCAATCTTGCCAAGGATCAC
AtHsp90-5M	TCTAGAGAATGGGTGAGAAGTTTGAG	CCCGGGTCAATCTTGCCAAGGATCAC
AtHsp90-6	TCTAGAATGATCAGGCTCTCTAAGCGC	CCCGGGTCATTTCTTCCCATCCACTTC
AtHsp90-6M	TCTAGAATGGCTGAGAAATTCGAGTA	CCCGGGTCATTTCTTCCCATCCACTTC
AtHsp90-7	TCTAGAATGAGGAAGAGGACGCTCGT	CCCGGGCTACAGTTCGTCCTTGGTGT
AtHsp90-7M	TCTAGAATGGCGGAGAAGTTTGAGT	CCCGGGCTACAGTTCGTCCTTGGTGT
ScHsp82	TCTAGAATGGCTAGTGAAACTTTTGA	CCCGGGCTAATCTACCTCTTCCATTT

Sequences with shadow were restriction sites.

Materials and methods

Yeast and bacterial strains and growth conditions

The haploid *Saccharomyces cerevisiae* strain iG170D (*can1-100 ade2-1 leu2-3, 12 trp-1 ura3-1 his3 hsc82::LEU2 Hsp82G170D::HIS*) was a derivate of W303 (Nathan and Lindquist, 1995). A conditional *S. cerevisiae* Hsp90 knockout strain R0005 (*can1::MFA1pr-HIS3 his3 Δ 1 leu2 Δ 0 ura3Δ0 met15 Δ 0 lys2 Δ 0 Hsp82::LEU2 hsc82::Gal1-TAP-Hsc82 TRP NAT*) was a general gift from Dr. Walid Houry's Lab (University of Toronto) and based on strain S288C. Strains were grown at 28 °C in YPD/YPG rich medium. Alternatively, specific minimal synthetic medium (SD) was used for yeast to grow under selective conditions. *Escherichia coli* DH5α was used as a host for the construction and preparation of plasmids and was grown at 37 °C in LB medium.

Plasmid constructions

The *Arabidopsis Hsp90-1*, *Hsp90-2*, *Hsp90-5*, *Hsp90-7*, *Hsp70t-2* and *cpHsp70* full-length cDNA clones were purchased from the TAIR as u22186, c105057, u13808, u12900, u22059 and u24730, respectively. *AtHsp90-6* and the Hsp90 cofactors (*Hsp70*, *Cyp40*, *p23* and *NOS*) cDNA clones were obtained by RT-PCR using mRNA prepared from *Arabidopsis* ecotype Columbia heat-shock treated calli. The *S. cerevisiae Hsp82* gene was a gift from Dr. Walid Houry's Lab (University of Toronto) in vector pProEXhta. The predicted mature form clones of AtHsp90-5 (aa57–aa780, designed as AtHsp90-5M), AtHsp90-6 (aa91–aa803, designated as AtHsp90-6M) and AtHsp90-7 (aa73–aa823, designated as AtHsp90-7M) were obtained by PCR. The *AtHsp90*, *S. cerevisiae Hsp90* or *Arabidopsis* Hsp90 cofactor genes were ligated into pGM-T vector and sequenced before plasmid construction.

To construct yeast expression plasmids, the *AtHsp90* and *ScHsp82* were moved into yeast expression vector p416GPD between *Xba* I and *Sma* I for complementation assay. These vectors were transformed into yeast strains of iG170D and R0005 by the LiAc/PEG method and selected by URA3 marker. Yeast cells with p416GPD and p*ScHsp82* acted as negative and positive controls. For yeast two-hybrid assay, the AtHsp90 and cofactor genes were ligated into $pOBD_2$ and pOAD, respectively.

The primers used are listed in Table 1.

Growth under stress on solid media

Control and transformed yeast cells were analyzed in parallel. Yeast strains were grown in liquid SD medium without supplementation of uracil to the stationary phase and then diluted to $OD_{600} = 1$. A 10-fold serial dilution was prepared and 3 μl of each dilution was spotted onto SD medium agar plates without uracil, with or without supplement of chemicals for abiotic stresses. Osmotic stress was performed on the medium supplemented with 900 mM sorbitol. 600 mM NaCl was added for salt stress, and 2 mM H_2O_2 was supplemented into the media for oxidative stress. The plates were incubated at 37 °C for 6 days as the heat-shock stress. The other plates were incubated at 30 °C for 4 days.

Growth under stress in liquid media

For comparison of growth curves, cells of the stationary phase were inoculated into the SD liquid media without uracil, which was supplemented with or without chemicals to $OD_{600} \approx 0.1–0.15$. The OD(0) values were measured before shaking, and OD(t) values were determined at 3, 6, 9, 12, 15, 18, 21, 24 h. The growth determination of yeast cultures was estimated through spectrophotometric means (Shimadzu, Japan) at 600 nm. The growth curves were obtained from three repeats for the same strain under same conditions.

Measurement of membrane integrity

When strains were treated for 12 h, aliquots were removed and stained using propidium iodide (PI). Stained cells were observed and photographed using a Zeiss Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany) equipped with an individual fluorescein rhodamine filter set (Zeiss no. 15: excitation BP 546/12 nm, emission LP 590 nm), and imaged using an Axiocam MRc digital camera (Carl Zeiss, Oberkochen, Germany). Three visual fields were randomly chosen for each sample, and the number of cells in the bright field was quantified as the total number. Membrane integrity (MI) was calculated with the following formula: MI = [1 − (the number of cells in fluorescent field/the number of cells in bright field)] × 100%. The bud was counted as a yeast cell. Each treatment was replicated three times.

Yeast two-hybrid interaction assays

The *Arabidopsis Hsp90-1*, *Hsp90-2*, *Hsp90-5*, *Hsp90-6*, *Hsp90-7*, *Hsp90-5M*, *Hsp90-6M* and *Hsp90-7M* in $pOBD_2$ were transformed into yeast strain pJ69-4α. Cofactors *cpHsp70*, *Hsp70*, *Hsp70t-2*, *Cyp40*, *p23* and *NOS* genes in pOAD were transformed into yeast strain pJ69-4a and the yeast two-hybrid assays were conducted according to Uetz et al. (2000). To increase specificity, 15 mM 3-amine-1, 2, 4-triazol (3-AT) was added in the selection medium when detecting the *HIS3* reporter gene.

Yeast colonies were assayed for β-galactosidase activity using a colony-lift filter as follows: colonies were transferred to 3 MM filter paper, permeated by brief immersion in liquid nitrogen, and incubated on filter paper saturated with Z-buffer ($Na_2HPO_4{\cdot}7H_2O$ 16.1 g/L, $NaH_2PO_4{\cdot}H_2O$ 5.50 g/L, KCl 0.75 g/L, $MgSO_4{\cdot}7H_2O$ 0.246 g/L) containing 1 mg/ml X-gal at 30 °C.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by Duncan's

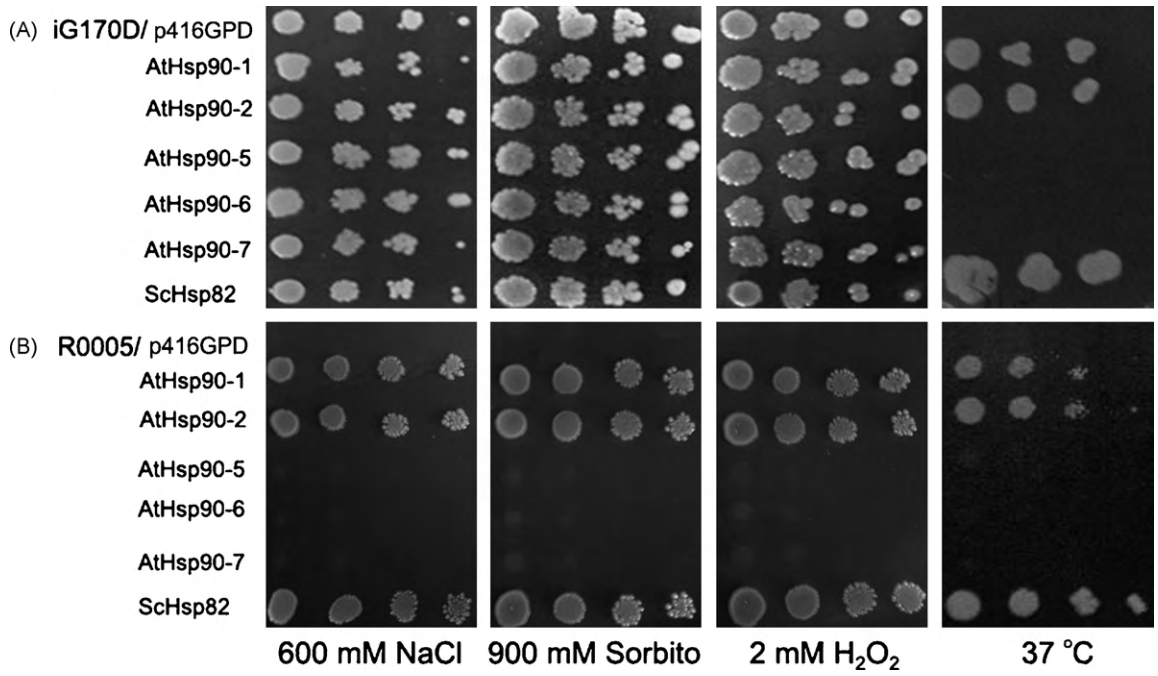

Fig. 1. Growth of iG170D (A) and R0005 (B) carrying Hsp90 orthologs expression plasmids under different stress conditions.

multiple range test. Differences at $P \leq 0.05$ were considered significant.

Results

The cytosolic AtHsp90s can complement yeast Hsp90 function

To determine the general function of AtHsp90 proteins, five *AtHsp90* genes were investigated for complementation of the yeast endogenous *Hsp90* genes. As shown in Fig. 1A, all transformed iG170D cells showed similar growth sizes on solid media under salt, osmotic and oxidative stresses. However, at a non-permissive temperature of 37 °C, iG170D cells harboring *AtHsp90-5*, *AtHsp90-6* and *AtHsp90-7* could not survive and showed the temperature-sensitive phenotype. In the yeast strain R0005, our results showed that AtHsp90-5, AtHsp90-6 and AtHsp90-7 could not rescue the yeast growth on solid media containing glucose as a carbon source. This suggested that *Arabidopsis* organellar AtHsp90s did not fit the components of yeast Hsp90 machinery. The cytosolic AtHsp90-1, AtHsp90-2 could support the yeast under salt, osmotic, oxidative and heat stresses, although growth sizes of cells with *AtHsp90-1* and *AtHsp90-2* were smaller than with *ScHsp82* (Fig. 1B). This indicates that *Arabidopsis* Hsp90-1 and Hsp90-2 are compatible with the components of yeast Hsp90 machinery under these testing conditions. To verify the observed phenotype, iG170D and R0005 cells were grown in liquid stress media. The growth rate measured in liquid media was fully consistent with the phenotypes observed on plates (Fig. 2).

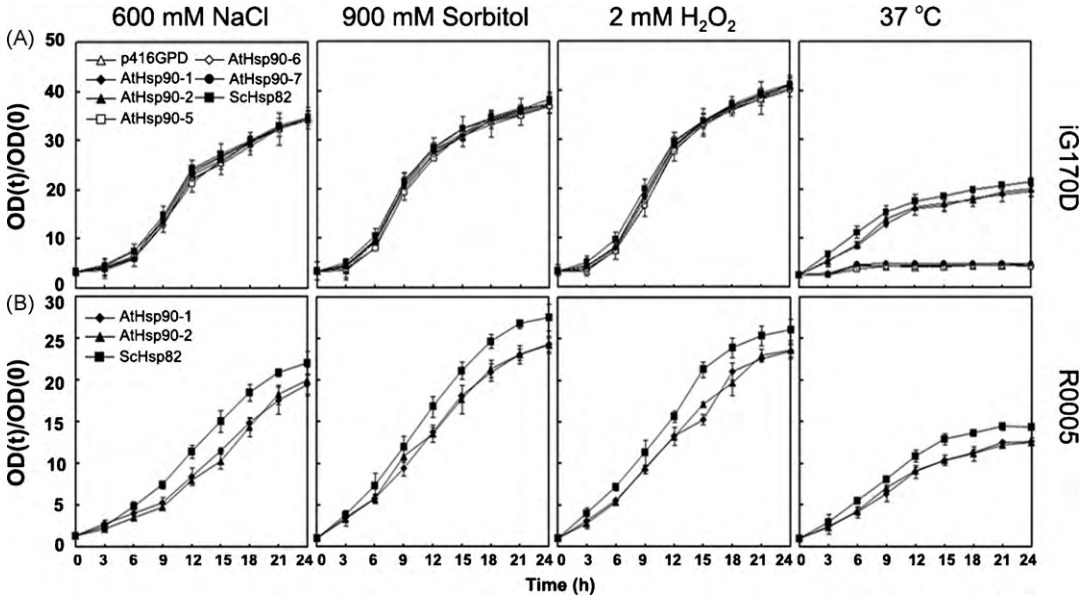

Fig. 2. Growth curves of iG170D (A) and R0005 (B) cells carrying Hsp90 orthologs expression plasmids in SD liquid medium under different stress conditions. Each value is the mean ± SD of three independent replicates.

It has been reported that, when an additional sequence like GST is attached to the N-terminus of cytosolic Hsp90, the Hsp90 activity will be affected (Nathan et al., 1997). However, a TAP tag fused to the yeast Hsp82 N-terminus could support yeast growth but cause somewhat temperature-sensitive effects (Zhao et al., 2005). The organellar targeting sequences for AtHsp90-5, AtHsp90-6 and AtHsp90-7 might affect activities of the Hsp90 in the mature forms in yeast cells, so we tested whether their mature forms could support the growth of yeast mutant R0005. The results showed that mature forms of AtHsp90-5, AtHsp90-6 and AtHsp90-7 still could not complement the yeast Hsp90 (Fig. 3).

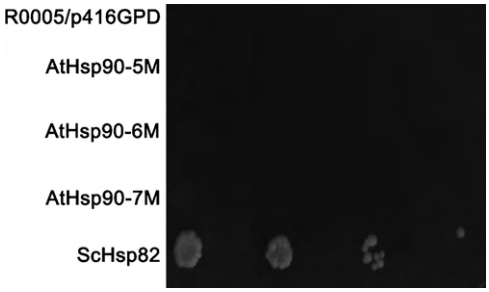

Fig. 3. Growth of R0005 with the mature forms of organellar AtHsp90 expression plasmids on the plate with glucose as carbon source instead of galactose.

AtHsp90s confer differential abiotic resistance to yeast cells

AtHsp90-5, AtHsp90-6 and AtHsp90-7 cannot support R0005 growth, as shown in Fig. 1B, so we examined whether *Arabidopsis*

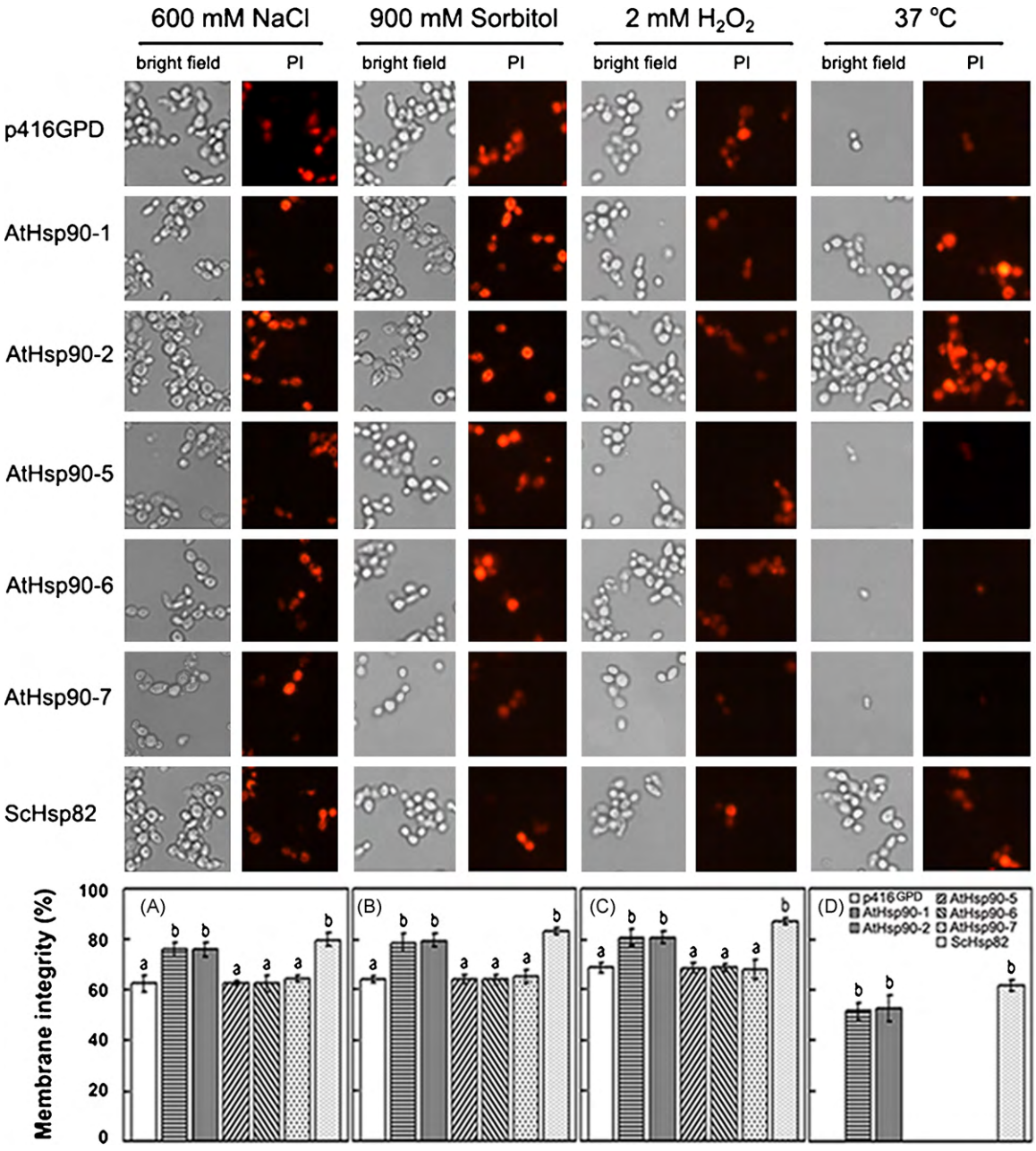

Fig. 4. Microscopy images and membrane integrity of iG170D cells under four stress conditions based on PI staining. (A) 600 mM NaCl; (B) 900 mM sorbitol; (C) 2 mM H_2O_2 and (D) 37 °C. Each value is the mean ± SD of three independent replicates. Different letters indicate statistically different means as at $P \leq 0.05$.

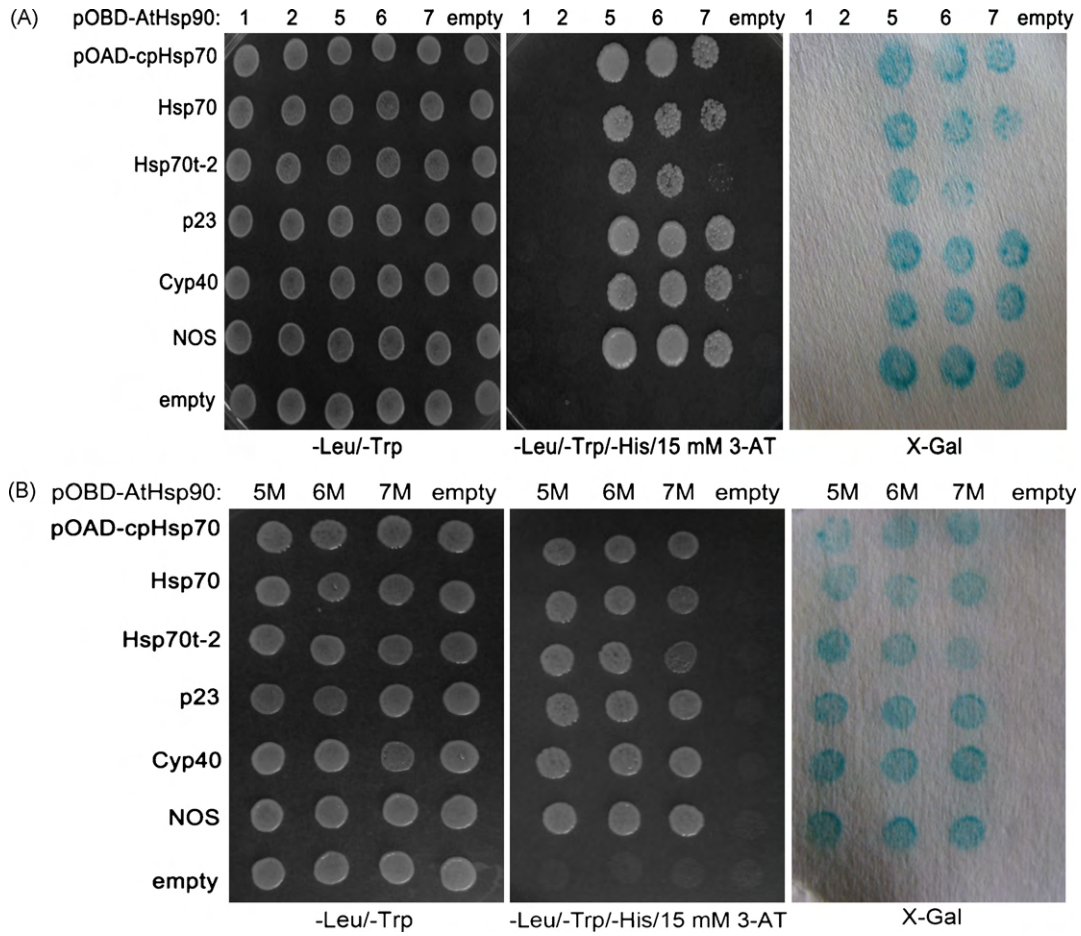

Fig. 5. Interactions of (A) AtHsp90 proteins and (B) mature forms of organellar AtHsp90s with cofactors. Diploid cells mated by pJ69-4a and pJ69-4α transformed cells on non-selective medium (-Leu, -Trp) and selective medium (-Leu, -Trp, -His) in presence of 15 mM 3-AT was observed. cpHsp70 is located in chloroplast. Hsp70 is located in cytoplasm. The location of Hsp70t-2 is unknown.

Hsp90 proteins could help yeast cells to cope with abiotic stresses in the Hsp90 temperature-sensitive mutant strain iG170D. To quantitatively measure the viability of iG170D cells under different stress conditions, the iG170D cells grown under different conditions were stained with propidium iodide. Viable cells with an intact plasma membrane will exclude the chemicals outside of the cells. Otherwise, the unhealthy cells will accumulate fluorescent propidium within the cells. As shown in Fig. 4, iG170D cells expressing *AtHsp90-1* and *AtHsp90-2* excluded PI better than the cells containing an empty vector alone when they were exposed to 600 mM NaCl (Fig. 4A), 900 mM sorbitol (Fig. 4B), 2 mM H_2O_2 (Fig. 4C) or 37 °C (Fig. 4D). *AtHsp90-5*, *AtHsp90-6* and AtHsp90-7 expressing cells showed similar percentages of integrity cells under these stress conditions, indicating that AtHsp90-5, AtHsp90-6 and AtHsp90-7 do not help the iG170D cells at all in excluding the toxic PI. This implies that *Arabidopsis* organellar Hsp90s were unable to perform their function in the yeast system.

Interactions between AtHsp90s and possible AtHsp90 cofactors

Hsp90 machinery requires cochaperones or cofactors that either bind non-native substrates and transfer them to Hsp90, or regulate the Hsp90 activity in folding the substrates at the final step. Hsp90 cochaperones often directly interact with Hsp90 and act as components of the Hsp90 multiple-protein complex (Pratt and Toft, 2003). To better understand the different functions among AtHsp90s, we analyzed the interactions between AtHsp90 isoforms and some of the potential AtHsp90 cofactors using a yeast two-hybrid assay. As shown in Fig. 5A, Cyp40, p23, chloroplast Hsp70 (cpHsp70) and NOS had strong interactions with AtHsp90-5 and AtHsp90-6, while these cofactors had weak interaction with AtHsp90-7. The Hsp70t-2 hardly interacted with AtHsp90-7, and had a weak interaction with AtHsp90-6, but it strongly interacted with AtHsp90-5. Cytosolic Hsp70 had the strongest interaction with AtHsp90-5. However, the cytosolic AtHsp90-1 and AtHsp90-2 did not interact with any of the tested cochaperones. We also used the X-Gal β- galactosidase assay to confirm the protein-protein interactions, and the two results were consistent.

To further investigate the role of the signature motifs in the interactions between organellar Hsp90s and the cofactors, the mature forms of the organellar Hsp90s were tested in the yeast two-hybrid assay. As shown in Fig. 5B, a similar pattern was observed in the interactions between the mature organellar Hsp90s with tested proteins of cpHsp70, Hsp70, Hsp70t-2, Cyp40, p23 and NOS, except that AtHsp90-7M had slightly stronger interactions with these proteins. The counterpart X-Gal β- galactosidase assay also confirmed the results.

Discussion

Seven Hsp90 family members from the *Arabidopsis* genome were revealed, with four localized in the cytoplasm, one in ER, one in chloroplast and one in mitochondria (Milioni and Hatzopoulos, 1997; Krishna and Gloor, 2001). In this study, we amplified the *AtHsp90-6* gene through RT-PCR with the mRNA extracted from a heat-shock treated *Arabidopsis* callus as template. Together with

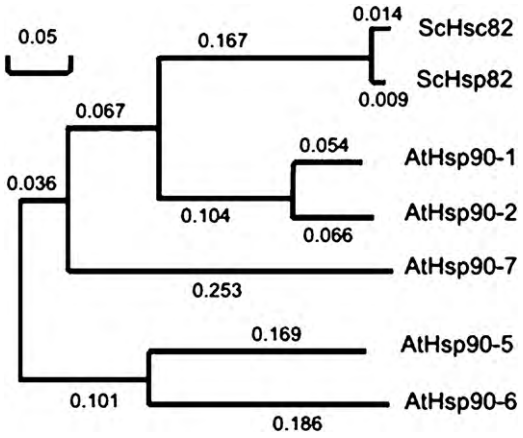

Fig. 6. The amino acid sequences homology analysis of *A. thaliana* and *S. cerevisiae* Hsp90 proteins.

another four *AtHsp90* genes, *AtHsp90-1*, *AtHsp90-2*, *AtHsp90-5* and *AtHsp90-7*, we analyzed the functions of the five *AtHsp90* genes by expressing them in yeast.

To determine the function of cytosolic *AtHsp90*s, *AtHsp90-1* and *AtHsp90-2* were expressed in the temperature-sensitive *Hsp90* mutant iG170D and conditional *Hsp90*-null mutant R0005 of *S. cerevisiae*. Since the sequences of cytoplasmic AtHsp90-1 and AtHsp90-2 share the highest similarity with yeast ScHsc82 and are closely related to yeast ScHsp82, as shown in Fig. 6, it is not surprising that both can support the growth of yeast. AtHsp90-2 also shares high similarity with the other two *Arabidopsis* cytosolic Hsp90 homologues AtHsp90-3 and AtHsp90-4 (at least 96% identical) that belong to the same functional group (Milioni and Hatzopoulos, 1997). AtHsp90-1 and AtHsp90-2 are both strongly heat-inducible and AtHsp90-2 is also constitutively expressed (Milioni and Hatzopoulos, 1997); they seem to mimic the two inducible and constitutive expression members of Hsp90 homologues, Hsp82 and Hsc82 in *S. cerevisiae* (Borkovich et al., 1989). Recent research on cytosolic *AtHsp90-1* to *AtHsp90-4* mutants showed that the stochastic mechanisms that potentially produce changes in phenotypic traits within the signals and pathways dynamic network of the developmental processes were normally restricted by *Hsp90* (Samakovli et al., 2007). Sangster et al. (2007) also indicated that the expression inhibition of cytosolic *AtHsp90-1* to *AtHsp90-4* by siRNA enhanced the expression of genes generally responsible for stress responses such as those to ABA stimuli, water deprivation and the jasmonic acid biosynthetic process.

Neither pre-protein forms of AtHsp90-5, AtHsp90-6 and AtHsp90-7 nor their mature forms can alone support the growth of yeast mutant R0005 (Figs. 1, 3). The reason may be partly related to their large differences in amino acid compared to cytosolic Hsp90s. Previous studies of hsp90 complexes in mammal and yeast cells have revealed that Hsp90 cooperates with a number of cochaperones for its functions (Pratt and Toft, 2003). The hsp90-based chaperone complex contains hsp90, Hop, hsp70, hsp40, p23 and immunophilin. In this complex, Hsp70 and Hsp90 do not directly interact with each other, but are connected by a cofactor of Hop. Cyp40 is an immunophilin that binds to the domain of the Hsp90 C-terminal MEEVD sequence (Taylor et al., 2001). Cytoplasmic Hsp90 proteins from both plants and animals include the C-terminal pentapeptide MEEVD, which specifically binds the TPR domain that exists in many of Hsp90 cochaperones, including Hop/Sti1 and immunophilins (Scheufler et al., 2000). However, organellar AtHsp90-5, AtHsp90-6 and AtHsp90-7 do not contain the MEEVD motif at the C-terminus as does cytosolic Hsp90s (Krishna and Gloor, 2001). Client proteins are implicated in binding to the middle segment of Hsp90 (Meyer et al., 2003). Deletion studies suggest that human and yeast p23 interacts with both the N-terminal and middle domains of Hsp90β *in vitro* (McLaughlin et al., 2006), whereas plant p23 is not like human and yeast p23, interacting with cytosolic Hsp90 (Krishna and Zhang, 2003). However, in a recent study, it was shown that p23 from orchardgrass could interact with DgHsp90, which is a Hsp90 homolog in orchardgrass located in the ER (Cha et al., 2009). In this study, organellar and cytosolic AtHsp90s showed different interactions with the tested cofactors, cpHsp70, Hsp70, Hsp70t-2, Cyp40, p23 and a substrate protein of NOS, organellar AtHsp90s could interact with most of the tested cofactors, while cytosolic AtHsp90s could not interact with these cofactors (Fig. 5A). Moreover, the interactions between the mature organellar Hsp90s and the tested proteins showed a similar pattern, even though the AtHsp90-7M seemed to have slightly stronger interactions with these proteins (Fig. 5B), implying that the signature motifs were either not engaged or had very little effect on the interactions between AtHsp90s and the cofactors. These results suggest that organellar AtHsp90s, combined with their cochaperones and substrates, and integrated into the heterogenous protein complexes through a specific molecular mechanism. The molecular differences between the cytosolic and organellar Hsp90s in forming chaperone complexes and performing functions might be related to the failure to complement the yeast Hsp90 mutant with *Arabidopsis* organellar Hsp90s (AtHsp90-5, AtHsp90-6 and AtHsp90-7). Assays using a second method such as pulldown or co-immunoprecipitation is desirable to further confirm the interaction between AtHsp90s and cofactors.

In conclusion, yeast complementary results showed that the functions of cytosolic Hsp90 are conserved in plants and yeast. Chloroplast-localized AtHsp90-5, mitochondrial-localized AtHsp90-6 and ER-localized Hsp90-7 could not complement the yeast Hsp90 mutant having analogous functional properties, possibly due to the large differences in amino acid sequences compared to cytosolic Hsp90s, to different molecular mechanisms in forming complexes, or due to different compartmentalization. This preliminary study provides important information about the function of AtHsp90 family members against abiotic stress. Considering the important roles of AtHsp90 in sustaining cellular homeostasis under adverse conditions, further research should be carried out to study their detailed functional mechanisms in the plant defense response against environmental stresses.

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