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BRIEF COMMUNICATION

Overexpression of *AtHsp90.3* in *Arabidopsis thaliana* impairs plant tolerance to heavy metal stress

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Abstract

The functions of cytosolic heat shock protein AtHsp90.3 in response to heavy metal stress were characterized by using expression of *AtHsp90.3* gene in yeast and *Arabidopsis thaliana*. *AtHsp90.3* supported the *Saccharomyces cerevisiae* Hsp90 knockout strain R0005 growth and maintaining cells membrane integrity under cadmium and arsenic stresses, which was compatible with the components of ScHsc82 machinery. However, constitutive overexpression of *AtHsp90.3* in *Arabidopsis* impaired plant tolerance to Cd stress with lower germination rate and shorter root length, decreased contents of phytochelatins (PCs) and glutathione (GSH), inhibited activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), and increased content of malondialdehyde (MDA). These results suggested that proper homeostasis of Hsp90 was critical for cellular response and/or tolerance to heavy metal stress in plants.

Additional key words: antioxidants, arsenic, cadmium, heat shock proteins, malondialdehyde, phytochelatins.

The heat shock proteins (HSP) of Hsp90s family are widespread molecular chaperones found in prokaryotes and all eukaryotes. They can help other proteins avoid stress-induced misfolding pathways that produce inactive or aggregated conformations and promote folding to the native conformation (Buchner 1999). *Hsp90* genes have been isolated and cloned from many plants, and they were strongly induced by changes in temperature, salinity, heavy metals, *etc.* (Pareek *et al.* 1995, Milioni and Hatzopoulos 1997, Choi *et al.* 2006, Grigorova et al. 2011, Maksymied 2011, Wójcik and Tukiendorf 2011). Seven Hsp90 family members have been revealed in *Arabidopsis* ecotype Columbia (Krishna and Gloor 2001). AtHsp90.1 to AtHsp90.4 proteins forms the cytosolic subfamily (Milioni and Hatzopoulos 1997, Krishna and Gloor 2001). AtHsp90.5, AtHsp90.6 and AtHsp90.7 are located in the chloroplast (Cao *et al.* 2003), mitochondria (Prassinos *et al.* 2008) and endoplasmic reticulum (Ishiguro *et al.* 2002), respectively. In the previous work, we have reported that constitutive overexpression of *AtHsp90.3* gene delayed expression of heat stress transcription factors genes, *AtHsfA1d*, *AtHsfA7a* and *AtHsfB1*, and two HSP genes, *AtHsp101* and *AtHsp17*, and impaired plant tolerance to heat stress (Xu *et al.* 2010). To further understand the chaperone function of cytosolic AtHsp90.3 in plant response to abiotic stresses, the function of AtHsp90.3 under heavy metal stress was functionally analyzed by complementing the *S. cerevisiae* endogenous *Hsp90* gene and overexpressing it in *Arabidopsis*.

In present investigation, the plasmids expressed in yeast strain R0005 (*can1::MFA1 pr-HIS3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 Hsp82::LEU2 hsc82::Gal1-TAP-Hsc82 TRP NAT*) were constructed as published previously (Xu *et al.* 2010). The yeast strains were grown in liquid synthetic dextrose (SD) medium without uracil to stationary phase and diluted to absorbance A_{600} = 1. A 10-fold serial dilution was prepared and 3 mm^3 of each dilution was spotted on SD medium agar plates without or with 300 μM $CdCl_2$ or 500 μM Na_3AsO_4. Control and

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Abbreviations: CAT - catalase; GSH - glutathione; HSP - heat shock protein; MDA - malondialdehyde; PC - phytochelatins; POD - peroxidase; SOD - superoxide dismutase.
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transformed yeast cells were analyzed strictly in parallel. For membrane integrity measurement, strains were treated with 300 μM $CdCl_2$ or 500 μM Na_3AsO_4 for 9 h and stained by propidium iodide (PI). Membrane integrity was measured and calculated according to the method of Xu *et al.* (2010).

For plant heavy metal stress tolerance analysis, seeds of wild type and *AtHsp90.3* transgenic T3 generation of *Arabidopsis thaliana* lines 5, 7 and 19 (Xu *et al.* 2010) were surface sterilized and placed on ½ Murashige and Skoog (MS) agar plates supplemented with distilled water or 2 μM $CdCl_2$, and treated at 0 °C for 24 h before germination at 23 °C. To evaluate the effect of the *AtHsp90.3* on seedlings root elongation during heavy metal stress, two-day-old germinated seeds of wild type and transgenic T3 *Arabidopsis* lines were moved to ½ MS agar plates supplemented with distilled water, 50 or 100 μM $CdCl_2$. The plates were maintained vertically in the culture room for 14 d.

To evaluate the effect of the *AtHsp90.3* on antioxidants, three-week-old wild type and transgenic seedlings treated by 30 mg $CdCl_2$ kg^{-1}(soil) for 0 and 3 d, were homogenized in liquid nitrogen. Glutathione (GSH) content was determined according to the method of Ding *et al.* (2007). Phytochelatins (PCs) were determined as nonprotein thiols (NPTs) as described by He *et al.* (2005). The activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and malondialdehyde (MDA) content were assayed as described previously (Song *et al.* 2009).

There were three repetitions in each treatment and every experiment was conducted twice. All data were analyzed by one-way analysis of variance (*ANOVA*). Mean separations were performed by Duncan's multiple range test. Differences at $P \leq 0.05$ were considered as significant.

Although growth size of R0005 clone with *AtHsp90.3* was smaller than that with *ScHsp82*, the cytosolic AtHsp90.3 supported the yeast under heavy metal stresses, which indicated that *Arabidopsis* AtHsp90.3 was compatible with the components of yeast Hsp90 machinery under heavy metal stresses (data not shown). To measure the viability of R0005 cells with *AtHsp90.3* and *ScHsp82* under heavy metal stresses, the R0005 cells grown in presence of 300 μM $CdCl_2$ or 500 μM Na_3AsO_4 were stained with PI. Viable cells with an intact plasma membrane excluded PI, otherwise, the unhealthy cells accumulated it. R0005 cells expressing *AtHsp90.3* and *ScHsp82* had the similar percentage of membrane integrity under Cd and As stresses as in control conditions, indicating that *AtHsp90.3* could help the R0005 cells to exclude the toxic PI.

Table 1. Membrane integrity of R0005 cells under Cd and As stress based on PI staining. Means ± SE, $n = 3$. Values followed by different letter were significantly different according to Duncan's multiple range test at $P < 0.05$.

	ScHsp82	AtHsp90.3
Control	98.93 ± 1.17a	97.76 ± 0.88a
Cd	87.81 ± 1.76b	83.71 ± 3.51b
As	90.73 ± 1.29b	88.39 ± 1.52b

The germination rates of wild type and *AtHsp90.3* overexpressing lines did not show any obvious difference (data not shown). However, under Cd stress, the germination of *AtHsp90.3* overexpressing seeds was greatly impaired comparing with wild type (Table 2). Moreover, under Cd stress condition, *AtHsp90.3* transgenic seedlings showed more sensitivity than that of wild type, and their root growth were significantly inhibited (Table 2), suggesting that overexpression of AtHsp90.3 in *Arabidopsis* reduced plants tolerance to heavy metal stress.

To detect the effect of overexpression of cytosolic *AtHsp90.3* on oxidative stress induced by heavy metals, GSH, PC and MDA contents and the activities of SOD, CAT and POD were measured in leaves collected from transgenic and wild type plants after exposure to Cd. Results showed that no significant difference was found between wild type and *AtHsp90.3* transgenic seedlings before stress. However, after being treated by Cd for 3 d, compared to wild type, the overexpression of *AtHsp90.3* significantly decreased the GSH and PC production, increased the MDA content, and reduced the activities of SOD, CAT and POD in transgenic *Arabidopsis* (Table 3), suggesting that constitutive overexpression of *AtHsp90.3* decreased the antioxidant defense response of plant cells under heavy metal stress.

Table 2. Seed germination and root length of wild type and *AtHsp90.3* transgenic plants under Cd stress. Means ± SE, $n = 3$. Values followed by different letter were significantly different according to Duncan's multiple range test at $P < 0.05$.

	Cd [μM]	WT	AtHsp90.3-5	AtHsp90.3-7	AtHsp90.3-19
Germination rate [%]	2	70.00 ± 1.25a	45.00 ± 2.08b	44.58 ± 0.83b	45.42 ± 1.08b
Root length [cm]	0	7.83 ± 0.20a	7.79 ± 0.10a	7.75 ± 0.10a	7.83 ± 0.10a
	50	1.64 ± 0.18a	1.21 ± 0.19b	1.33 ± 0.14b	1.35 ± 0.06b
	100	1.35 ± 0.04a	0.88 ± 0.02b	0.87 ± 0.02b	0.90 ± 0.02b

Table 3. Effect of *AtHsp90.3* overexpression on the content of GSH, PC and MDA [μmol g^{-1}(protein)] and the activities of antioxidant enzymes [U mg^{-1}(protein)] in transgenic plants before (0 d) and after (3 d) Cd treatment [30 mg($CdCl_2$) kg^{-1}(soil)]. Means ± SE, $n = 3$. Values followed by different letter were significantly different according to Duncan's multiple range test at $P < 0.05$.

	WT		AtHsp90.3-5		AtHsp90.3-7		AtHsp90.3-19	
	0 d	3 d	0 d	3 d	0 d	3 d	0 d	3 d
GSH	0.14±0.02a	0.53±0.02a	0.14±0.01a	0.47±0.01b	0.14±0.01a	0.51±0.01b	0.14±0.02a	0.51±0.01b
PC	0.12±0.00a	0.49±0.01a	0.12±0.01a	0.39±0.00b	0.11±0.00a	0.39±0.00b	0.12±0.01a	0.40±0.01b
MDA	19.67±0.98a	34.62±4.39b	20.41±1.23a	49.62±4.50a	20.36±1.18a	46.15±2.88a	20.39±1.28a	45.58±3.46a
SOD	132.83±2.91a	180.58±9.71a	131.59±1.94a	161.17±7.77b	132.15±3.88a	162.14±14.6b	133.71±3.30a	163.11±12.4b
CAT	5.92±0.17a	17.08±1.15a	5.96±0.38a	11.31±1.29b	5.67±0.13a	10.92±1.54b	5.77±0.15a	10.37±1.35b
POD	1.17±0.11a	1.66±1.12a	1.18±0.13a	1.40±0.06b	1.04±0.10a	1.38±0.19b	1.08±0.08a	1.39±0.13b

Generally, an appropriate intracellular balance between reactive oxygen species (ROS) generation and scavenging mechanisms exists in all cells. In addition, the redox homeostasis requires the efficient coordination of reactions in different cell compartments and is governed by complex signal transduction pathways. The constitutively overexpressed cytosolic *AtHsp90.3* in *Arabidopsis* may shift the equilibrium of Hsp90 with its substrates, suggesting that proper homeostasis of cytosolic Hsp90 is critical for cellular stress response.

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