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ORIGINAL ARTICLE

Overexpression of *AtHsp90.2*, *AtHsp90.5* and *AtHsp90.7* in *Arabidopsis thaliana* enhances plant sensitivity to salt and drought stresses

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Abstract Three AtHsp90 isoforms, cytosolic AtHsp90.2, chloroplast-located AtHsp90.5, and endoplasmic reticulum (ER)-located AtHsp90.7, were characterized by constitutive overexpressing their genes in *Arabidopsis thaliana*. Both types of the transgenic plants overexpressing cytosolic and organellar AtHsp90s showed reduced tolerance to salt and drought stresses with lower germination rates and fresh weights, but improved tolerance to high concentration of Ca^{2+} comparing with the wild type plants. Transcriptional analysis of ABA-responsive genes, *RD29A*, *RD22* and *KIN2* under salt and drought stresses, indicated that the induction expression of these genes was delayed by constitutive overexpression of cytosolic AtHsp90.2, but was hardly affected by that of organellar AtHsp90.5 and AtHsp90.7. These results implied that *Arabidopsis* different cellular compartments-located Hsp90s in *Arabidopsis* might be involved in abiotic stresses by different functional mechanisms, probably through ABA-dependent or Ca^{2+} pathways, and proper homeostasis of Hsp90 was critical for cellular stress response and/or tolerance in plants.

Keywords *Arabidopsis* · Calcium tolerance · Heat shock protein 90 · Stress sensitivity · Signaling pathway

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Abbreviations

ABA	Abscisic acid
CaMV	Cauliflower mosaic virus
Hsc	Cognate form of Hsp
Hsp	Heat shock protein
MAPK	Mitogen-activated protein kinase
ROS	Reactive oxygen species
UPR	Unfolded protein response

Introduction

The 90 kDa heat shock protein (Hsp90) is a widespread family of molecular chaperones found in prokaryotes and all eukaryotes. It is one of the most abundant proteins under physiological conditions, about 1–2% of cellular proteins in most tissues. Hsp90 is distinct from many other well-characterized molecular chaperones and displays considerable specificity for its client proteins (Pearl and Prodromou 2000; Young et al. 2001). Its broad clients are steroid hormone receptors, protein kinases, the cell cycle control proteins and other kinds of proteins like nitric oxidase synthase and telomerase (Czar et al. 1997; Nathan et al. 1997; García-Cardeña et al. 1998; Holt et al. 1999; Pratt et al. 2001; Zhao et al. 2005). As thus, though the major function of Hsp90 is to assist proteins folding properly like other molecular chaperones, it plays a key role in signal-transduction networks, cell–cell communication, cell-cycle control, cell growth, differentiation and apoptosis. Hsp90 also acts as a buffer for phenotypic changes and is portrayed as a 'capacitor for evolution' (Rutherford and Lindquist 1998). Studies in *Drosophila* and *Arabidopsis* have revealed that Hsp90 conceals phenotypic variations under ordinary conditions, allowing them to appear only when Hsp90 is functionally impaired (Queitsch et al. 2002; Sangster et al. 2004).

Besides performing the housekeeping functions, Hsp90 is also an important stress protein. When stresses occur, it stabilizes structures of proteins and membrane systems, preventing proteins from aggregation and helping misfolded proteins refold. In *Saccharomyces cerevisiae*, Hsp90 is involved in the regulation of stress-activated high-osmolarity glycerol and mitogen-activated protein kinase (MAPK) signaling pathways (Millson et al. 2005; Hawle et al. 2007). In mammalian cells, Hsp90 is an important component of the transcriptional arm of the unfolded protein response (UPR) and plays important roles in the cellular response to cytoplasmic stresses (Marcu et al. 2002). Hsp90 could protect mammal cells from 3-hydroxykynurenine (3HK)-induced oxidative stress by reducing reactive oxygen species (ROS) levels (Lee et al. 2005), and be required for yeast cells to adapt to high osmotic stress (Yang et al. 2006).

Plant *Hsp90* genes have been identified from tomato (Koning et al. 1992), *Arabidopsis thaliana* (Takahashi et al. 1992; Milioni and Hatzopoulos 1997), maize (Marrs et al. 1993), periwinkle (Schroder et al. 1993), oil seed (Krishna et al. 1997) and rice (Liu et al. 2006), and they are strongly induced by various abiotic stresses. In *Arabidopsis* ecotype Columbia, seven Hsp90 family members have been revealed (Krishna and Gloor 2001). AtHsp90.1 to AtHsp90.4 proteins form the cytosolic subfamily (Milioni and Hatzopoulos 1997; Krishna and Gloor 2001). AtHsp90.5 and AtHsp90.7 proteins are localized in chloroplast (Cao et al. 2003) and endoplasmic reticulum (ER) (Ishiguro et al. 2002), respectively. AtHsp90.6 is localized in mitochondria (Prassinos et al. 2008). However, until recently, only a few literatures have reported the physiological roles of Hsp90 chaperone complexes in plants. Hsp90 in *Arabidopsis* is reported to be essential for the plants to provide pathogen resistance (Lu et al. 2003; Takahashi et al. 2003). Global inhibition of *Arabidopsis* Hsp90 activity reveals the buffering ability of Hsp90 for genetic variations (Queitsch et al. 2002) and increased resistance to insect herbivores (Sangster et al. 2007). Since plants do not have the advantage of motility like animals, they have to cope with various acute environmental changes, such as drought, salinity, extreme temperatures and oxidative stress. So study of Hsp90 function in plant has great potential to reveal unknown functional aspects specific to plants or shared among biological kingdoms (Sangster and Queitsch 2005).

In this study, the chaperone function of AtHsp90.2, AtHsp90.5 and AtHsp90.7 were analyzed by overexpressing them in *Arabidopsis*. Overexpression of Hsp90s in *Arabidopsis* reduced the tolerance of transgenic plants to NaCl and drought stresses but increased their tolerance to Ca^{2+}. The expressions of stress-responsive genes *RD29A*, *RD22* and *KIN2* were impaired by cytosolic AtHsp90.2 overexpression. These suggest that proper homeostasis of Hsp90 is critical for cellular stress response and/or tolerance.

Material and methods

Plasmid constructions

The *Arabidopsis Hsp90.2*, *Hsp90.5* and *Hsp90.7* full-length cDNA clones were obtained from TAIR (The Arabidopsis information resource, http://www.arabidopsis.org) with accession No. c105057, u13808 and u12900, respectively. To construct plant expression vectors, the *AtHsp90* genes were inserted into pGM-T (TianGen, Beijing) vector, sequenced and then moved into the *Xba* I-*Sma* I sites of pBI121 vector to create pBI121-*AtHsp90*.

Plant material and transformation

Arabidopsis (ecotype Columbia-0) (obtained from TAIR) was grown in a growth chamber (16 h of light and 8 h of darkness) after a 24 h vernalization period. For growth under sterile condition, seeds were surface sterilized [15 min incubation in 10% (v/v) sodium hypochlorite, and a 6 time rinse by sterile distilled water] and sown on ½ MS (Murashige and Skoog 1962) media. pBI121-*AtHsp90* vectors, in which *AtHsp90*s were driven by a cauliflower mosaic virus (CaMV) 35S promoter, were transformed into *Agrobacterium tumefaciens* C58. *Arabidopsis* plants were transformed by the floral dip method (Clough and Bent 1998). T1 seeds were collected, dried at 25°C, and sown on sterile media containing 50 μg ml^{-1} kanamycin to select the transformants. Surviving T1 plants were transferred to soil to set seeds (T2). T3 seeds were set the same way.

Northern-blot analysis

For Northern-blot analysis, total RNA was isolated from *Arabidopsis* tissues using Trizol reagent (TianGen, Beijing). RNA samples (30 μg) were electrophoresed through agarose gels in the presence of formaldehyde, followed by transfer to Hybond-N^+ membranes (Amersham International Ltd, Amersham, Bucks, UK). Northern-blots were probed with [^{32}P] dCTP-labelled cDNA probes. Prehybridization and hybridization were performed at 65°C for 18 h. Filters were washed twice for 20 min in 2 × SSC with 0.1% (w/v) SDS at 65°C, then once for 10 min in 0.1 × SSC with 0.1% (w/v) SDS at 65°C. The damp filters were autoradiographed at −80°C using two intensifying screens.

Western-blot analysis

Immunoblot analysis was used to determine the AtHsp90 proteins expression levels in the transgenic plants. Total proteins from *Arabidopsis* seedlings grown on ½ MS

media were isolated and separated by SDS-PAGE, then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using a wet trans-blot system (Bio-Rad, USA). The AtHsp90 proteins were immunoblotted using polyclonal antibody raised against yeast Hsp82 protein (Zhao et al. 2005). AtHsp90.1, AtHsp90.2 and the predicted mature form of AtHsp90.5 (G^{74}–D^{780}) were expressed as His6 versions in *E. coli* and purified by Ni-NTA before being used as positive controls.

RT-PCR analysis

Reverse transcription PCR was performed according to the manufacturer's protocol (Transgene, Beijing). For PCR amplification, 20 cycles were used for each of the samples and the control. *ACTIN* gene was used to serve as a quantifying control. The primers used for PCR amplification were shown in Table 1. Amplified DNA band were separated on 1% agarose gel.

Abiotic stress tolerance analysis

Seeds from wild type or T3 transgenic plants were placed on ½ MS agar plates supplemented with distilled water or different concentrations of 125 mM NaCl and 500 mM mannitol and treated at 0°C for 24 h before incubation at 23°C for germination. About 50 seeds of every kind of plants were placed on each Petri dish. A seed was scored as germination if the radicle completely penetrated the seed coat. The germination rates were scored daily for at least 7 days. To test the stress response of seedlings, germinated seeds were placed on ½ MS media with different concentrations of supplements (100 mM NaCl, 300 mM mannitol, 80 mM $CaCl_2$, 40 mM $MgCl_2$, 150 mM KCl) for 3 weeks at 23°C (16 h of light and 8 h of darkness). Ten seeds of every kind of plants were placed on each Petri dish. Fresh weights of seedlings were measured. All stress treated experiments were performed three times. To examine the induction of stress related genes *RD22*, *RD29A* and *KIN2*, seedlings which grown on ½ MS medium for 3 weeks at 23°C (16 h of light and 8 h of darkness) were exposed to 200 mM NaCl or 400 mM mannitol solution for different time periods at room temperature under white light before extraction of total RNA.

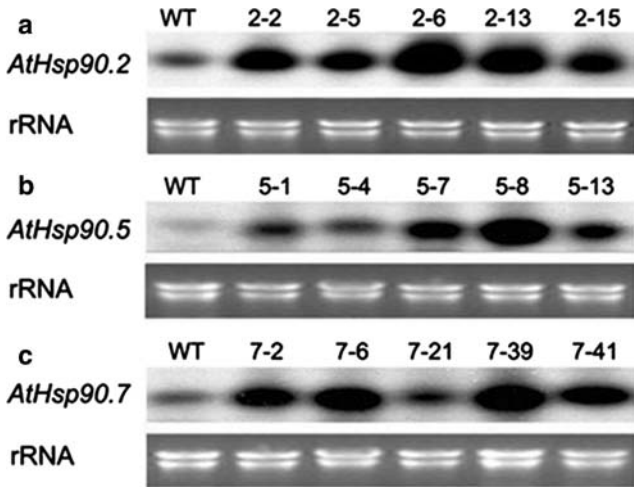

Fig. 1 Characterization of transgenic plants by Northern-blot. Hybridizations to detect transcripts of *AtHsp90.2*, *AtHsp90.5* and *AtHsp90.7* were conducted for *AtHsp90.2* (**a**), *AtHsp90.5* (**b**) and *AtHsp90.7* (**c**) transgenic plants, respectively. The independent transgenic lines were indicated on top of each lane and WT represents the untransformed wild type plant. Ethidium bromide-stained rRNA was used as RNA loading control. 2-2, 2-5, 2-6, 2-13 and 2-15 represented the *AtHsp90.2* transgenic lines. 5-1, 5-4, 5-7, 5-8 and 5-13 represented the *AtHsp90.5* transgenic lines. 7-2, 7-6, 7-21, 7-39 and 7-41 represented the *AtHsp90.7* transgenic lines

Results

Generation and characterization of *AtHsp90* overexpressing transgenic lines

To investigate the specific function of individual AtHsp90 in plants, *AtHsp90.2*, *AtHsp90.5* and *AtHsp90.7* genes driven by CaMV 35S promoter were introduced into *Arabidopsis* plants, respectively, via *Agrobacterium* mediated transformation. By kanamycin resistant seeds screening and PCR confirmation, 15, 18 and 41 independent T1 transgenic lines were obtained for *AtHsp90.2*, *AtHsp90.5* and *AtHsp90.7*, respectively. Five independent transgenic lines of each *AtHsp90* gene were randomly chosen and tested for *Hsp90* mRNA expression levels. As shown in Fig. 1, all transgenic *Hsp90* genes were highly expressed at transcription level.

To test if the transgenes were overexpressed at the protein level, we chose the *AtHsp90.2* transgenic lines as an exam-

Table 1 Primers used in PCR amplification of stress-responsive genes

Genes	Forward primers (5′→3′)	Reverse primers (5′→3′)
RD29A	CACACACCAGCAGCACCCAGA	CCAGAAAGCAGAGAGACCGGA
RD22	CATGGTAGTGGCGAT TGCGGC	GCCGCGTTAGGATCGTCGTGG
KIN2	TCAGAGACCAACAAGAATGCC	CTACTTGTTCAGGCCGGTCTT
ACTIN	GGAAAGGATCTGTACGGTAAC	TGTGAACGATTCCTGGAC

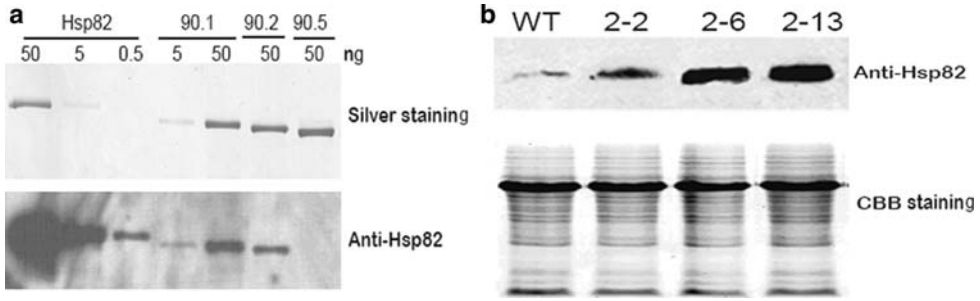

Fig. 2 Characterization of transgenic plants by Western-blot. Immunobloting of *AtHsp90.2* transgenic plants showing the overexpression of *AtHsp90.2*. **a** Purifed Hsp82 (50, 5 or 0.5 ng), AtHsp90.1 (indicated as 90.1, 5 and 50 ng), AtHsp90.2 (indicated as 90.2, 50 ng) and AtHsp90.5 (indicated as 90.5, 50 ng) were resolved by SDS-PAGE and silver stained (*top panel*) or immunoblotted using antibody raised against yeast Hsp82 (*bottom panel*). **b** Crude protein samples from wild type (WT) or *AtHsp90.2* transgenic lines 2-2, 2-6 and 2-13 were resolved by SDS-PAGE and stained by commassie brilliant blue (*bottom panel*) or immunoblotted with anti-Hsp82 antibody (*top panel*)

ple. An antibody raised against yeast Hsp82 was originally shown to have very high titration in detection of yeast Hsp82 proteins (Zhao et al. 2005). To test if this antibody is able to detect the plant Hsp90 proteins, we did an immunoblotting using purified AtHsp90.1, AtHsp90.2 and the predicted mature form of AtHsp90.5 (G^{74}-D^{780}) which were expressed as His6-tagged versions and purified by Ni-NTA (Qiagen). As shown in Fig. 2a, this antibody cross-reacted with both AtHsp90.1 and AtHsp90.2 but not with AtHsp90.5. This antibody did not cross-react well with AtHsp90.7 either (data not shown). This antibody was therefore used to analyze the cytosolic Hsp90 protein expression level in wild type and *AtHsp90.2* transgenic lines. As shown in Fig. 2b, with equivalent loading of crude protein samples from transgenic seedlings, comparing with wild type, transgenic lines 2-2, 2-6 and 2-13 showed pronounced higher level of Hsp90 proteins. Although this immunoblotting could not distinguish well the expression level of AtHsp90.1 and AtHsp90.2, it was likely that the higher level of Hsp90 was conferred by high expression of the *AtHsp90.2* transgenes. The protein expression levels of *AtHsp90.5* and *AtHsp90.7* in transgenic lines were also likely high since their RNA levels were higher as shown in Fig. 1, though their expression level had to be confirmed by further immunoblotting when suitable antibodies were available.

To examine the possible phenotypes of transgenic lines, T3 progeny of the *AtHsp90* overexpressing lines and the wild type plants were grown in the greenhouse under normal conditions. No obvious different phenotypes were observed between transgenic plants and the wild type (data not shown).

AtHsp90 overexpressing plants are more sensitive to salt and drought stresses

In *Arabidopsis*, stress sensitivity is most evident at the early developmental stage. To investigate the effect of change in *Hsp90* expression on abiotic stress sensitivity, the germinations of *AtHsp90* overexpressing lines grown under salt and drought stress conditions were first scored. On ½ MS medium, the germination rates of wild type and *AtHsp90* overexpressing lines did not show any obvious difference (data not shown). Most seeds (about 80%) started to germinate on the second day. On medium supplementing with 125 mM NaCl or 500 mM mannitol, however, the germination of tested seeds was greatly impaired. No seeds germinated in the first two days. On the third day, seeds began to germinate for all tested lines. The germination of *AtHsp90* overexpressing seeds was monitored for at least 7 days under stress conditions. For *AtHsp90.2* overexpressing seeds, the salt and drought (Fig. 3) stresses did not significantly change their final germination rates, although their germination was slightly delayed comparing with wild type. However, the germination of *AtHsp90.5* and *AtHsp90.7* overexpressing seeds was dramatically delayed on stress medium containing NaCl or mannitol (Fig. 3). At the time when the germination rates of wild type and *AtHsp90.2* overexpressing seeds approached 100%, only up to 70% *AtHsp90.5* and *AtHsp90.7* overexpressing seeds germinated.

To determine whether modification of *Hsp90* expression affects early plant development, germinated seeds placed on different stress media were continuously grown for 3 weeks. It seemed that salt and drought stresses inhibited the growth of *AtHsp90.5* and *AtHsp90.7* overexpressing seedlings. The fresh weights of *AtHsp90.5* and *AtHsp90.7* overexpressing seedlings were significantly less than wild type and *AtHsp90.2* overexpressing seedlings under salt and drought stresses (Fig. 4).

AtHsp90.2 overexpression impairs the expression of stress-responsive genes

To elucidate the molecular mechanism of AtHsp90 during the stress response, we monitored the transcript levels of some typical abiotic stress marker genes including *RD29A*,

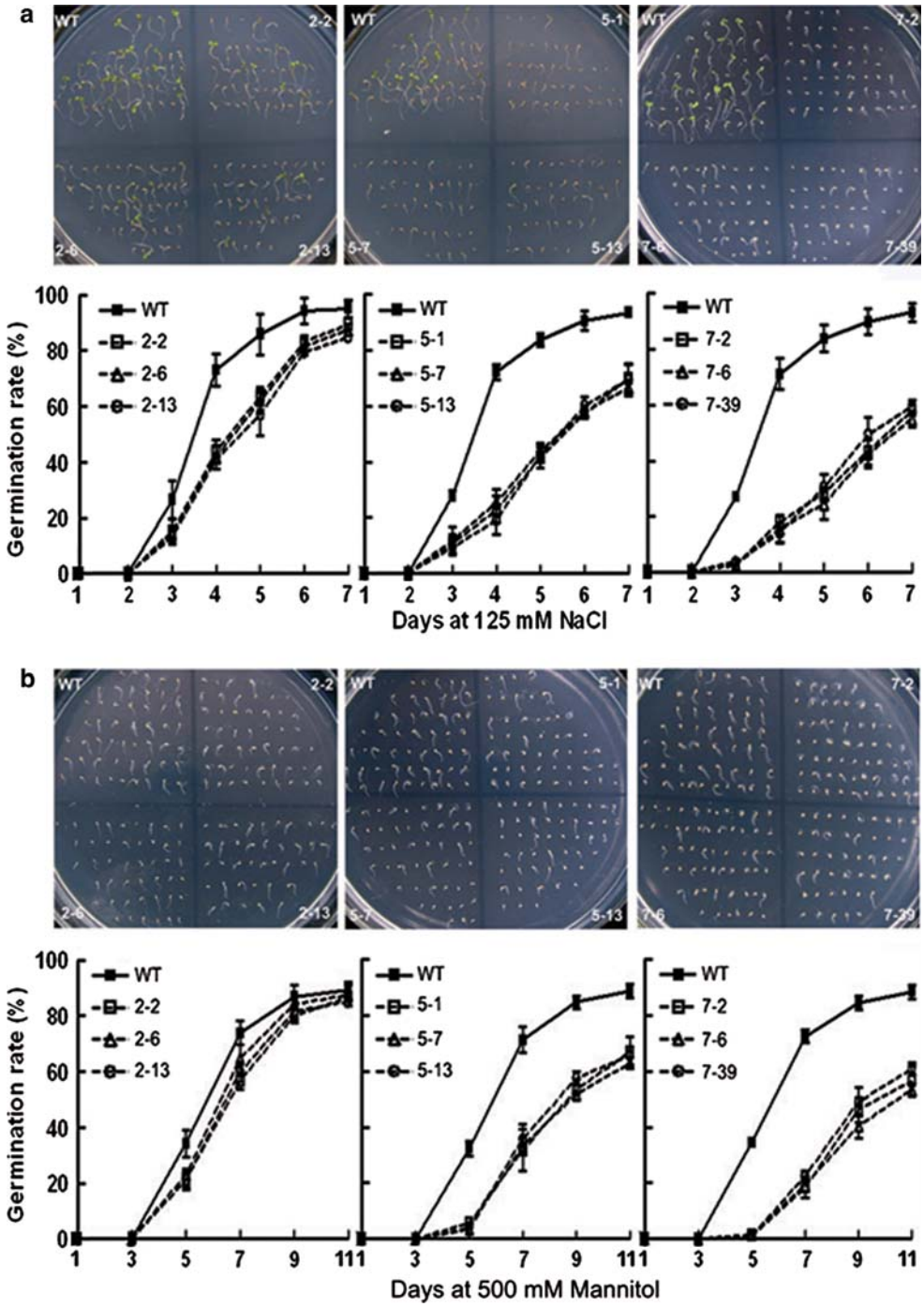

Fig. 3 Responses of seed germination to salt and drought stresses. **a** Seed germination and germination rates of wild type and *AtHsp90* transgenic plants on ½ MS medium containing 125 mM NaCl for 7 days. **b** Seed germination and germination rates of wild type and *AtHsp90* transgenic plants on ½ MS medium containing 500 mM mannitol for 11 days. *Error bars* indicate SD; $n = 3$. 2-2, 2-6 and 2-13 represented the *AtHsp90.2* transgenic lines. 5-1, 5-7 and 5-13 represented the *AtHsp90.5* transgenic lines. 7-2, 7-6 and 7-39 represented the *AtHsp90.7* transgenic lines

RD22 and *KIN2*. *RD29A*, *RD22* and *KIN2* genes are barely expressed under normal growth conditions. With salt or drought treatments, the expressions of *RD29A/RD22/KIN2* genes were strongly induced in wild type *Arabidopsis* plants (Kurkela and Borg-Franck 1992; Yamaguchi-Shinozaki and Shinozaki 1993a, b). As shown in Fig. 5a, when wild type and, *AtHsp90.5* or *AtHsp90.7* overexpressing lines were treated with salt or drought stresses, *RD29A*/*RD22*/*KIN2* genes were strongly induced. However, the induction of these three genes was impaired in the *AtHsp90.2* overexpressing lines.

Because three independent transgenic lines of *AtHsp90.2*, *AtHsp90.5* and *AtHsp90.7* behaved the same way under stress conditions (Figs. 3, 4), we used *AtHsp90.2* transgenic line 2-13, *AtHsp90.5* transgenic line 5-7 and *AtHsp90.7* transgenic line 7-6 for further analysis of the stress-responsive gene expression patterns by RT-PCR. Under both salt and drought stresses conditions, the expression

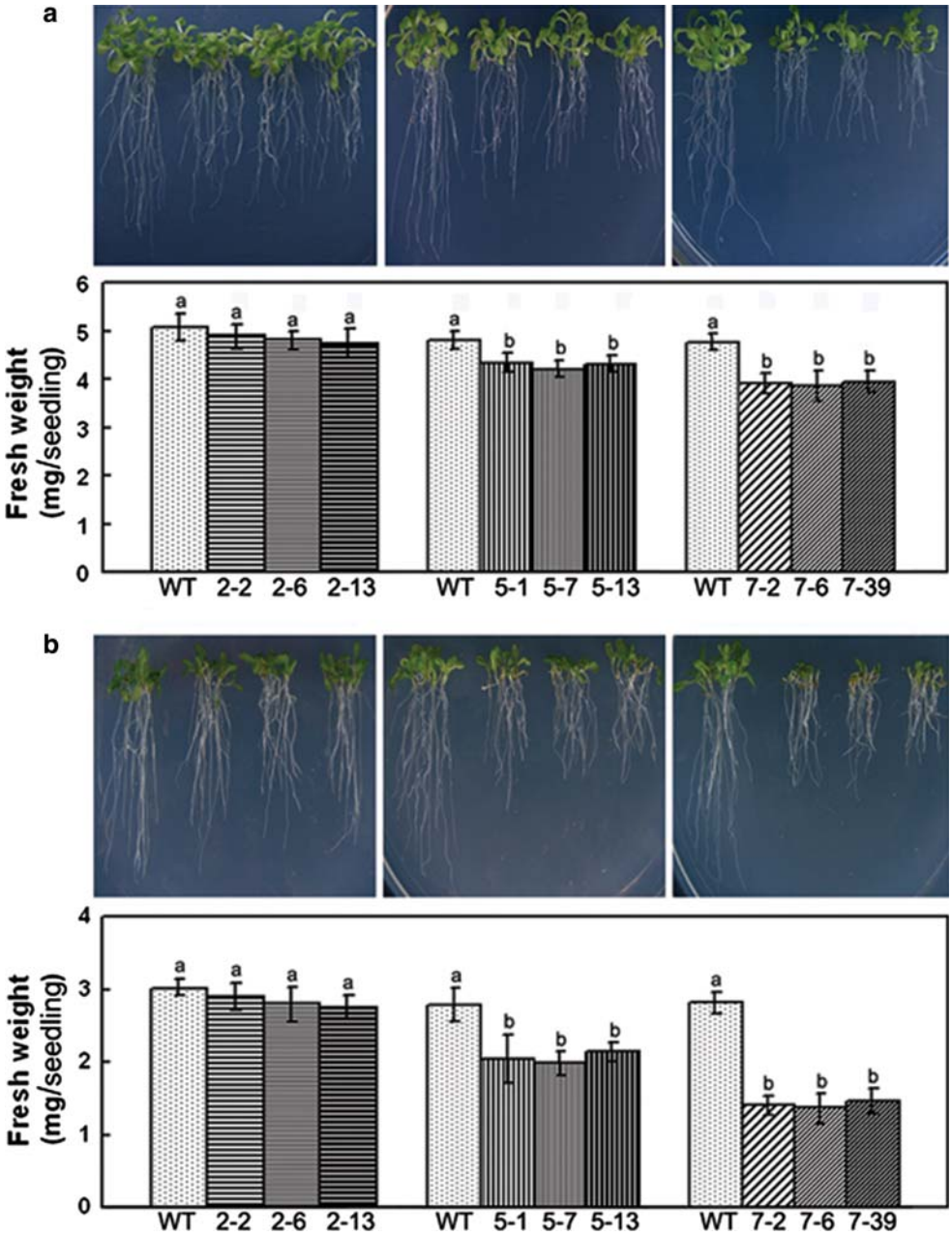

Fig. 4 Effect of *AtHsp90* overexpression on salt and drought tolerance in transgenic plants. Seedlings of wild type and transgenic plants grew on ½ MS medium supplementing with 100 mM NaCl and 300 mM mannitol for 3 weeks. Fresh weights of wild type and transgenic plants on ½ MS medium supplementing with **a** 100 mM NaCl or **b** 300 mM mannitol. *Error bars* indicate SD; $n = 30$. *Different letters* indicate means are significantly different at the $P < 0.05$ level. 2-2, 2-6 and 2-13 represented the *AtHsp90.2* transgenic lines. 5-1, 5-7 and 5-13 represented the *AtHsp90.5* transgenic lines. 7-2, 7-6 and 7-39 represented the *AtHsp90.7* transgenic lines

patterns of *RD29A*/*RD22*/*KIN2* genes showed no significant differences between the wild type and the *AtHsp90.5* and *AtHsp90.7* overexpressing plants, however, the transcriptional accumulation peak of three genes appeared to be delayed in the *AtHsp90.2* overexpressing plants (Fig. 5b).

Overexpression of *AtHsp90* confers *Arabidopsis* higher tolerance to calcium

AtHsp90.2 transgenic line 2-13, *AtHsp90.5* transgenic line 5-7 and *AtHsp90.7* transgenic line 7-6 were chosen for the following further phenotypic analysis. It was previously reported that *Hsc82* overexpressing yeast cells were sensitive to various stresses especially to high concentrations of NaCl, but were tolerant to high concentration of calcium (Imai and Yahara 2000). In order to investigate if *Hsp90* overexpressing *Arabidopsis* had the similar response, seeds of wild type and transgenic lines were tested on ½ MS medium containing 80 mM $CaCl_2$. The wild type seedlings displayed more seriously inhibited growth than the transgenic seedlings under high concentration of calcium treatment (Fig. 6a). The transgenic plants, especially the *AtHsp90.5* and *AtHsp90.7* overexpressing plants, developed more fresh weight under high concentration of $CaCl_2$ (Fig. 6a). To test if the growth of *AtHsp90* overexpressing plants was affected by other metal ions, transgenic plants were also treated with 40 mM $MgCl_2$ and 150 mM KCl. However, only *AtHsp90.5* transgenic seedlings showed

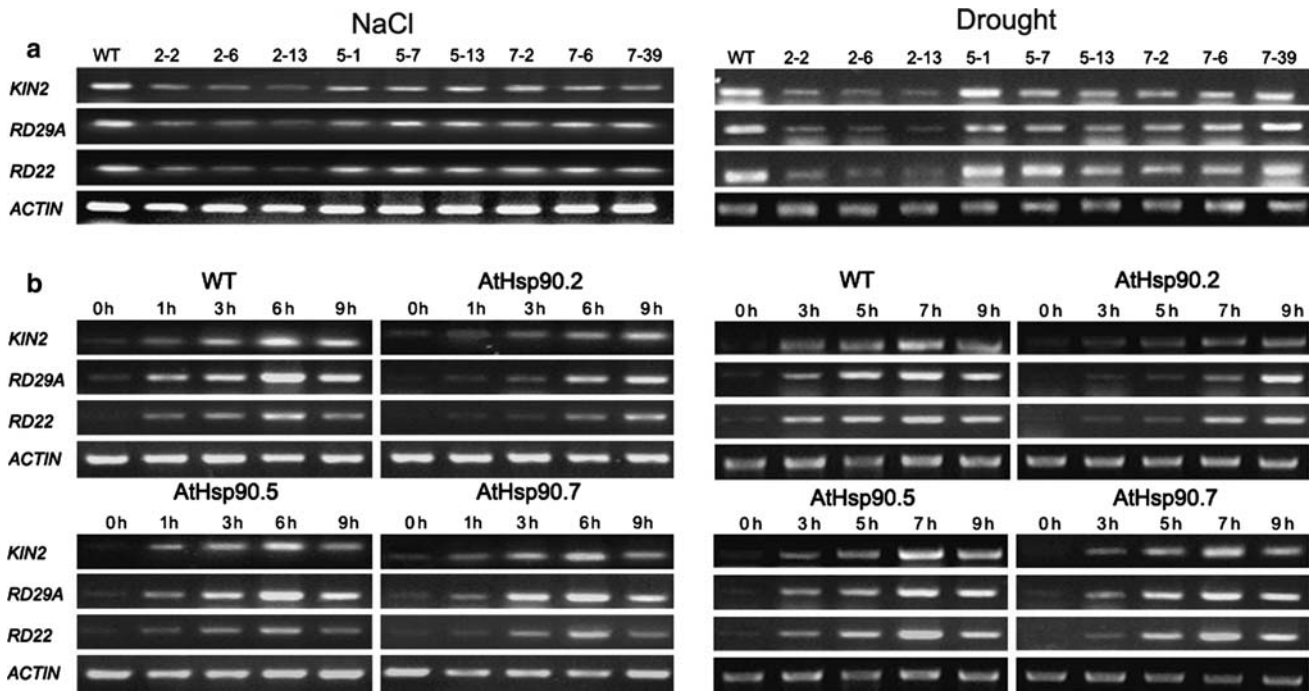

Fig. 5 RT-PCR analysis of stress-responsive genes under stress conditions. **a** Expression of *RD29A*, *RD22* and *KIN2* in wild type and transgenic plants after treatment with 200 mM NaCl for 3 h and 400 mM mannitol for 5 h. **b** Expression patterns of stress-responsive genes in wild type and transgenic plants (*AtHsp90.2*-13, *AtHsp90.5*-7 and *AtHsp90.7*-6) induced by salt (200 mM NaCl) and drought (400 mM mannitol) at different time points. *ACTIN* served as a quantifying control. 2-2, 2-6 and 2-13 represented the *AtHsp90.2* transgenic lines. 5-1, 5-7 and 5-13 represented the *AtHsp90.5* transgenic lines. 7-2, 7-6 and 7-39 represented the *AtHsp90.7* transgenic lines

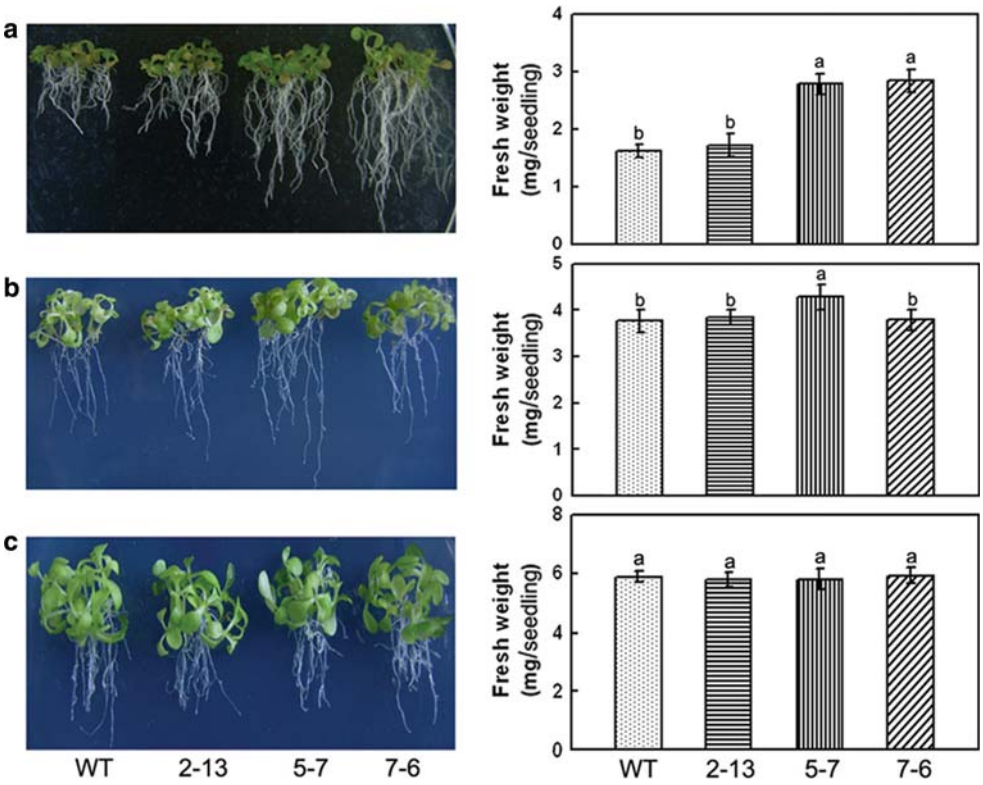

Fig. 6 Increased tolerance of *AtHsp90* overexpressing transgenic plants to high concentration of $CaCl_2$. **a** Seedlings and fresh weights of wild type and transgenic plants grew on ½ MS medium supplementing with 80 mM $CaCl_2$ for 3 weeks. **b** Seedlings and fresh weights of wild type and transgenic plants grew on ½ MS medium supplementing with 40 mM $MgCl_2$ for 3 weeks. **c** Seedlings and fresh weights of wild type and transgenic plants grew on ½ MS medium supplementing with 150 mM KCl for 3 weeks. *Error bars* indicate SD; $n = 30$. *Different letters* indicate means are significantly different at the $P < 0.05$ level. 2-13 represented the *AtHsp90.2* transgenic lines. 5-7 represented the *AtHsp90.5* transgenic lines. 7-6 represented the *AtHsp90.7* transgenic lines

slightly increased tolerance to high concentration of Mg^{2+}, and no obvious difference was observed among seedlings on KCl supplemented media (Fig. 6b, c).

Discussion

Hsp90 provides an essential function in all eukaryotic cells and plays key roles in a variety of stresses and developmental responses. It is believed that chaperone mechanism of cytosolic Hsp90 is conservative, but there is rare report on the organellar Hsp90s. *Arabidopsis* Hsp90 family contains seven members. AtHsp90.1 to AtHsp90.4 belong to cytosolic subfamily. The AtHsp90.2, AtHsp90.3, and AtHsp90.4 protein sequences are at least 96% identical to each other. This suggests that they may have very similar biochemical function. AtHsp90.5 and AtHsp90.6 are prokaryote originated Hsp90 paralogs (Krishna and Gloor 2001). In this study, the function of AtHsp90.2, AtHsp90.5 and AtHsp90.7 were investigated by overexpressing them in *Arabidopsis*. Our results showed that *AtHsp90.2*, *AtHsp90.5* and *AtHsp90.7* transgenic plants were more sensitive to salt and drought stresses (Figs. 3, 4), but more tolerant to high calcium than wild type plants (Fig. 6). Moreover, transgenic seeds and seedlings of organellar Hsp90s of *AtHsp90.5* and *AtHsp90.7* overexpressing *Arabidopsis* were more sensitive to NaCl and drought stresses comparing with *AtHsp90.2* transgenic plants (Figs. 3, 4).

In the previous studies, *Arabidopsis* chloroplastic AtHsp90.5 was found to participate in photomorphgenesis by mutant analysis. The point mutant of *AtHsp90.5*, *cr88*, displayed long hypocotyls in red light and the delayed greening process. In cotyledons and young leaves, plastids were less developed than those of the wild type. In addition, mutation decreased the expression of some light-induced genes (Cao et al. 2003). An *Arabidopsis* mutant *shepherd* was previously identified with a T-DNA insertion in the *AtHsp90.7* gene 5′ leading sequence which disrupted the *AtHsp90.7* gene expression. Characteristics of the *shepherd* mutant suggested that AtHsp90.7 is required for apical meristem expansion, activation of the CLV1/CLV2 receptor complex (Ishiguro et al. 2002), and protein secretion (Klein et al. 2006). In this study, overexpressing *AtHsp90.5* and *AtHsp90.7* in *Arabidopsis* resulted in higher sensitivity of transgenic seeds and seedlings to NaCl and drought stresses comparing with wild type, even comparing with *AtHsp90.2* transgenic plants (Figs. 3, 4). This may represent a different scenario that Hsp90 is involved in signal transduction networks and stress responses. Excessive Hsp90 may bind and hold those proteins responsible for export of toxic ions like Na^+ or transcription factors which are responsible for induction of osmolytes required for osmotic balance. For instance, excessive Hsp90 in ER may slower the secretion and targeting of plasma membrane or vacuole membrane ion transporters to exclude excessive cytosolic Na^+ (Klein et al. 2006). In addition, the interaction between Hsp90 and its substrates is dynamic based on relative binding affinities, and its levels may already be optimized. Increased availability of Hsp90 may delay client protein maturation or transport (Sangster et al. 2004). Nevertheless, we can not rule out the possibility that overexpression of Hsp90 stimulates the maturation of certain protein kinases or transcription factors involved in stress response pathways. However, constitutive overexpression of Hsp90 shifts its general functions from protective to disruptive ones under salt and osmotic stresses. *Arabidopsis* has four cytosolic Hsp90 genes, in which the nucleotide divergence between *AtHsp90.2* and *AtHsp90.3* is less than 6%. Such duplications might allow functional diversification of the Hsp90s and eliminate possible biochemical drawbacks to overexpression (Sangster et al. 2004). Additionally, excessive Hsp90, especially in chloroplast or ER, might introduce a general stress itself in those cellular compartments, either by competing for the organellar import/export system or disturbing the native protein homeostasis necessary for normal salt and osmotic stress resistances. Interestingly, in a report that constitutively reduced cytosolic Hsp90 by using siRNA enhances the expression of genes generally responsible for stress responses such as those to ABA stimulus, water deprivation and jasmonic acid biosynthetic process (Sangster et al. 2007). These suggest that Hsp90 level in plant is critical for homeostasis of stress response and/or resistance proteins.

To better understand the molecular basis of the impaired stress tolerance of the *AtHsp90* transgenic plants, the expression pattern of well characterized stress-related genes, such as *RD29A*, *RD22* and *KIN2*, were investigated. These genes have been used as convenient markers for monitoring the ABA and stress response pathways in plants (Pandey et al. 2004), primarily because their promoter regions contain ABA-responsive element and therefore strongly induced by ABA, a key player in drought response. Our data showed that *AtHsp90.2* transgenic plants delayed the expressions of *RD29A*, *RD22* and *KIN2* transcripts comparing with wild type plants under salt and drought stresses conditions (Fig. 5b), suggesting that cytosolic Hsp90 is involved in plant stress response probably in an ABA-dependent manner. *AtHsp90.5* or *AtHsp90.7* overexpression did not significantly affect the expression of *RD29A*, *RD22* and *KIN2* under salt and drought stresses conditions (Fig. 5), suggesting that these two Hsp90 isoforms are not directly involved in the expressions of these genes, probably because these stress-related proteins are localized in cytosol (Gilmour et al. 1996) or nucleus and not directly linked with the chloroplast or ER-localized Hsp90.

Imai and Yahara (2000) reported overexpression of cytosolic *Hsc82* in *S. cerevisiae* conferred yeast cells more sensitivity to NaCl and sorbitol but higher tolerance to Ca^{2+} and that, this phenotype was similar to calcineurin-defective cells and suppressible by overexpression of calcineurin catalytic subunit Cna2. It was proposed that excessive Hsc82 present in cells trapped Cna2 and negatively regulated the calcineurin function (Imai and Yahara 2000). Calcineurin is present in all eukaryotes investigated except higher plants (Hogan and Li 2005). However, two EF-hand Ca^{2+}-binding proteins homologous to calcineurin regulatory subunit CNB encoded by *AtCBL* gene which is capable of interacting with rat CNA in vivo (Kudla et al. 1996) and *SOS3* (Liu and Zhu 1998) have been found in *Arabidopsis*. It is likely that one or more calcium binding proteins are regulated by Hsp90 in *Arabidopsis*. Overexpression of *AtHsp90s* may change the regular homeostasis of those calcium binding proteins and interrupt the normal Ca^{2+} signaling pathway, thus making the *Hsp90* overexpressing transgenic plants higher tolerance to high calcium concentration (Fig. 6a), and resulting in more sensitivity to salt and drought stresses (Figs. 3, 4).

In the previous studies, *Hsp90* transcripts were found to be accumulated to high levels during high temperature and pathogen infection in plants (Milioni and Hatzopoulos 1997; Takahashi et al. 2003; Liu et al. 2006). Inhibition of Hsp90 function resulted in the attenuation of several R-gene signaling pathways, leading to increased sensitivity to biotrophic pathogens, including viruses, microbial pathogens, and certain metazoan parasites (Kanzaki et al. 2003; Lu et al. 2003; Takahashi et al. 2003; Bhattarai et al. 2007). Moreover, Sangster et al. (2007) demonstrated that the *Arabidopsis* cytosolic Hsp90-reduced lines consistently showed greater resistance to insect herbivores, and showed later flowering than wild type. Given the above literatures, in our next studies, it will be interesting to see how these *AtHsp90* overexpression lines perform in response to temperature stress, pathogen and herbivore attack as well as flowering time, and to further comprehensively understand the mechanism of Hsp90 in plant stress responses.

In conclusion, overexpression of *AtHsp90.2*, *AtHsp90.5* and *AtHsp90.7* in *Arabidopsis* may shift the equilibrium of Hsp90s with their client-bound states, disrupt ABA-dependent or Ca^{2+} pathways, and by which impairs plant tolerance to abiotic stresses, suggesting that proper homeostasis of Hsp90 is critical for cellular stress response and/or tolerance in plants. Although the detailed mechanism of *AtHsp90* involvement in stress is not yet clear, the characterization of *AtHsp90* function will provide new insights into stress-responsive pathways.

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