# Overexpression of Organellar and Cytosolic *AtHSP90* in *Arabidopsis thaliana* Impairs Plant Tolerance to Oxidative Stress

**Hongmiao Song · Pengxiang Fan · Yinxin Li**

**Abstract** Three AtHSP90 isoforms, cytosol-localized *AtHSP90.2*, chloroplast-localized *AtHSP90.5*, and endoplasmic reticulum (ER)-localized *AtHSP90.7* genes, were constitutively overexpressed in *Arabidopsis thaliana* to study their functional mechanisms under oxidative stress. Overexpression of *AtHSP90* genes reduced germination of transgenic seeds under oxidative stress. When exposed to 10 mM $H_2O_2$, *AtHSP90* transgenic seedlings displayed lower activities of superoxide dismutase, catalase, and peroxidase; higher content of malondialdehyde; and higher levels of protein damage than detected in the wild type. This indicated that overexpression of *AtHSP90.2*, *AtHSP90.5*, and *AtHSP90.7* in *Arabidopsis* impaired plant tolerance to oxidative stress. Moreover, overexpression of chloroplast- and ER-localized *AtHSP90* resulted in lower resistance to oxidative stress than that of cytosolic *AtHSP90*. This suggested that *HSP90.2*, *HSP90.5*, and *HSP90.7* localized in different cellular compartments were involved in different functional mechanisms during oxidative stress.

**Keywords** *Arabidopsis thaliana* · Enzyme activity · Heat shock protein 90 · Oxidative stress

## Introduction

Various abiotic and biotic stresses can disrupt cellular redox homeostasis and accelerate the accumulation of reactive oxygen species (ROS) in plants (Desikan et al. 2001). Presence of hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^-$), and hydroxyl radical ($OH^-$) can result in oxidative stress, leading to the oxidization of nucleic acids, proteins, lipids, and carbohydrates that subsequently affect the integrity of cell membranes and inactivation of key cellular functions (Campo et al. 2004; Halliwell and Gutteridge 1999). To survive stress, plant cells possess specialized defense mechanisms to protect themselves by either scavenging or preventing the production of ROS and either repairing or degrading oxidatively modified molecules. Molecular chaperones belong to a large family of stress-induced proteins. They are involved in various cellular metabolic processes, by mediating folding or refolding of other polypeptides (Ellis 1990). The majority of molecular chaperones present in plant cells are heat-shock proteins (HSPs), and they play key roles in protein folding, assembly, translocation, and degradation in a wide array of normal cellular processes. These HSPs also function in stabilization of proteins and membranes and assist in protein refolding under stress conditions. Thus, they serve in defense mechanisms against various types of stress. There are five known major HSP families, including HSP60, HSP70, HSP90, HSP100, and the small HSP (Kimura et al. 1994). Different classes of HSPs appear to bind to specific non-native substrates and states.

HSP90 is an essential and ubiquitous protein with key roles in eukaryotic cells. It is one of the most abundant proteins under normal physiological conditions and accounts for 1% to 2% of cellular proteins found in eukaryotic cytosols. Unlike other chaperone systems, HSP90 tends to fold a specific set of substrates, most of which are involved in cellular

H. Song · P. Fan · Y. Li (✉)
Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
e-mail: yxli@ibcas.ac.cn

signaling pathways, including transcriptional factors, protein kinases, and key regulators (Pratt and Toft 2003). As such, HSP90 becomes the center of many cellular processes including growth, cell cycling, apoptosis, development, and evolution (Jackson et al. 2004). In plants, HSP90 is strongly induced by various abiotic stresses, such as salt, drought, low temperature, heat shock, heavy metal ions, and alkaline stress (Krishna et al. 1995; Pareek et al. 1995; Milioni and Hatzopoulos 1997; Liu et al. 2006).

In *Arabidopsis thaliana* ecotype Columbia, seven *HSP90* family members have been identified by the genome sequence project. AtHSP90.1 to AtHSP90.4 proteins correspond to the cytoplasmic subfamily (Milioni and Hatzopoulos 1997; Krishna and Gloor 2001), while AtHSP90.5 and AtHSP90.7 proteins are located in the chloroplast and endoplasmic reticulum (ER) (Cao et al. 2003; Ishiguro et al. 2002), respectively, and AtHSP90.6 is located within the mitochondria (Prassinos et al. 2008). The AtHSP90.2, AtHSP90.3, and AtHSP90.4 protein sequences are highly similar with at least 96% identity. This suggests that they may functionally overlap. However, there are few studies on the roles of HSP90 chaperone complexes in plants against oxidative stress.

In this study, one cytosolic *AtHSP90.2* and two organellar chloroplast-localized *AtHSP90.5* and ER-localized *AtHSP90.7* *AtHSP90s* were overexpressed in *Arabidopsis* to determine their function in oxidative stress and elucidate the underlying mechanism(s) of this defense response.

## Materials and Methods

### Plasmid Constructions

Full-length cDNA clones of *Arabidopsis HSP90.2*, *HSP90.5*, and *HSP90.7* and accessions c105057, u13808, and u12900, respectively, were obtained from TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org). To construct plant expression vectors, each of the *AtHSP90* genes, driven by the CaMV35S promoter, was inserted into the pGM-T vector (TianGen, Beijing, China), sequenced, and then introduced into the *Xba*I–*Sma*I sites of the pBI121 vector to create three pBI121-*AtHSP90* constructs.

### Plant Material, Transformation, and Treatment Conditions

Seeds of *A. thaliana* ecotype Columbia-0, after a 24-h vernalization period, were surface-sterilized (15 min incubation in 10% (*v/v*) sodium hypochlorite followed by six rinses in sterile distilled water) and germinated on 1/2 Murashige and Skoog (MS; Murashige and Skoog 1962) medium. These were grown in a growth chamber under 16 h of light (140±10 μmol m$^{-2}$ s$^{-1}$ provided by white fluorescent tubes) and 23±1°C.

Each of the pBI121-*AtHSP90* vectors was transformed into *Agrobacterium tumefaciens* strain C58 using the heat-shock method. *Arabidopsis* plants were transformed by the floral-dip method (Clough and Bent 1998). T1 seeds were collected, dried at 25°C, and sown on a sterile 1/2 MS medium containing 50 μg ml$^{-1}$ kanamycin to select transformants. Surviving T1 plants were then transferred to soil and grown in the greenhouse to collect T2 seeds. T2 plants were subsequently grown, and T3 seeds were set the same way.

For oxidative treatment, seeds from wild-type and T3 transgenic plants were placed on 1/2 MS agar plates supplemented with filter-sterilized 1.5 mM H$_2$O$_2$ solution. Plates were incubated in the dark at 4°C for 2 days to achieve uniform seed germination and then transferred to light condition of 16 h of photoperiod (140±10 μmol m$^{-2}$ s$^{-1}$) and 23±1°C. Stress treatments were performed three times.

For total protein extraction, seedlings were grown on 1/2 MS medium for 3 weeks at 23±1°C (16 h of light and 8 h of darkness), and then transferred to a fresh liquid medium containing 10 mM H$_2$O$_2$ and grown under 140±10 μmol m$^{-2}$ s$^{-1}$ treated for 1 and 3 h at 23±1°C.

### Preparation of Total Proteins and Immunodetection of Carbonylated Proteins

Total protein was extracted and measured using bovine serum albumin as a standard and following the protocol of Bradford (1976). Protein carbonylation was detected using the OxyBlot$^{TM}$ Protein Oxidation Detection kit (Chemicon International, Temecula, CA, USA) and according to the protocol of Xu and Tian (2008). A protein sample (30 μg) was mixed with the same volume of 12% sodium dodecyl sulfate (SDS), and a 10-mM 2,4-dinitrophenylhydrazine (DNPH; 10 μl) dissolved in 10% trifluoroacetic acid (*v/v*) was added. The reaction mixture was incubated for 15 min at 25°C and then neutralized by adding 2 M Tris base (10 μl) containing 30% glycerol. Proteins were then separated using 15% SDS-PAGE, and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) using an electroblotting apparatus (Bio-Rad, USA). Carbonylated proteins were detected using an anti-DNPH antibody and the chemiluminescence blotting substrate SuperECL plus (Applygen Technologies Inc., Beijing, China).

### The Antioxidant Enzymes and Content of Malondialdehyde Assay

Following treatment with 10 mM H$_2$O$_2$, 1 g tissue of 3-week seedlings was collected and homogenized in 5 ml ice-cold

extraction buffer and 0.1 g polyvinyl polypyrrolidone. For catalase (CAT) analysis, 50 mM sodium phosphate (pH 7.0) was used as an extraction buffer. For superoxide dismutase (SOD), peroxidase (POD), and malondialdehyde (MDA) content measurements, 100 mM sodium phosphate buffer (pH 6.4) was used. The homogenate was centrifuged at $10,000 \times g$ for 30 min at 4°C, and the supernatants were used directly for assay. For enzyme assays, three repetitions of each treatment were used, and the experiment was conducted twice.

For the SOD assay (Wang et al. 2005), the reaction mixture (3 ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 10 μM EDTA, 2 μM riboflavin, and 0.1 ml of the enzyme extract. Mixtures were illuminated by light (60 μM $m^{-2}$ $s^{-1}$) for 10 min, and the absorbance was determined at 560 nm. Identical solutions held in the dark served as blanks. One unit of SOD was defined as the amount of enzyme resulting in a 50% decrease of the SOD-inhibitable NBT reduction. The specific activity was expressed as unit per milligram protein.

POD activity was assayed according to the method of Ippolito et al. (2000), but with slight modifications. The reaction mixture, consisting of 0.5 ml of crude extract and 2 ml of guaiacol substrate (100 mM sodium phosphate, pH 6.4, and 8 mM guaiacol), was incubated for 5 min at 30°C. The increase in absorbance at 460 nm was spectrophotometrically assayed after 1 ml $H_2O_2$ (24 mM) was added. Enzymatic activity was measured as an increase in absorbance, and a unit of activity was defined as an increase in one absorbance unit per minute under the assay conditions.

The MDA assay was performed as described by Wang et al. (2005). The reaction mixture, consisting of 1 ml of 0.5% TBA (dissolved with 15% trichloroacetic acid) and 0.6 ml enzyme preparation, was heated at 95°C for 20 min, quickly cooled in an ice-bath for 5 min, and then centrifuged at $10,000 \times g$ for 10 min to clarify the solution. Absorbance at 532 nm was measured and subtracted from the absorbance at 600 nm. MDA contents were calculated using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

CAT activity was determined by adding 0.2 ml of enzyme preparation to 2.8 ml of 40 mM $H_2O_2$, dissolved in 50 mM sodium phosphate buffer, pH 7.0, used as a substrate (Wang et al. 2005). The decomposition of $H_2O_2$ was measured by recording the decline in absorbance at 240 nm. The specific activity was expressed as unit per milligram protein, wherein 1 U of catalase converted 1 μM of $H_2O_2$ per minute.

Statistical Analysis

All data were analyzed using one-way analysis of variance. Mean separations were performed using Duncan's multiple range test. Differences at $P \leq 0.05$ were deemed significant.

## Results

### Overexpression of *AtHSP90* Reduced Oxidative Tolerance in *Arabidopsis*

To validate the functionality of the *AtHSP90* genes during oxidative stress, seeds of wild type (WT) and transgenic T3
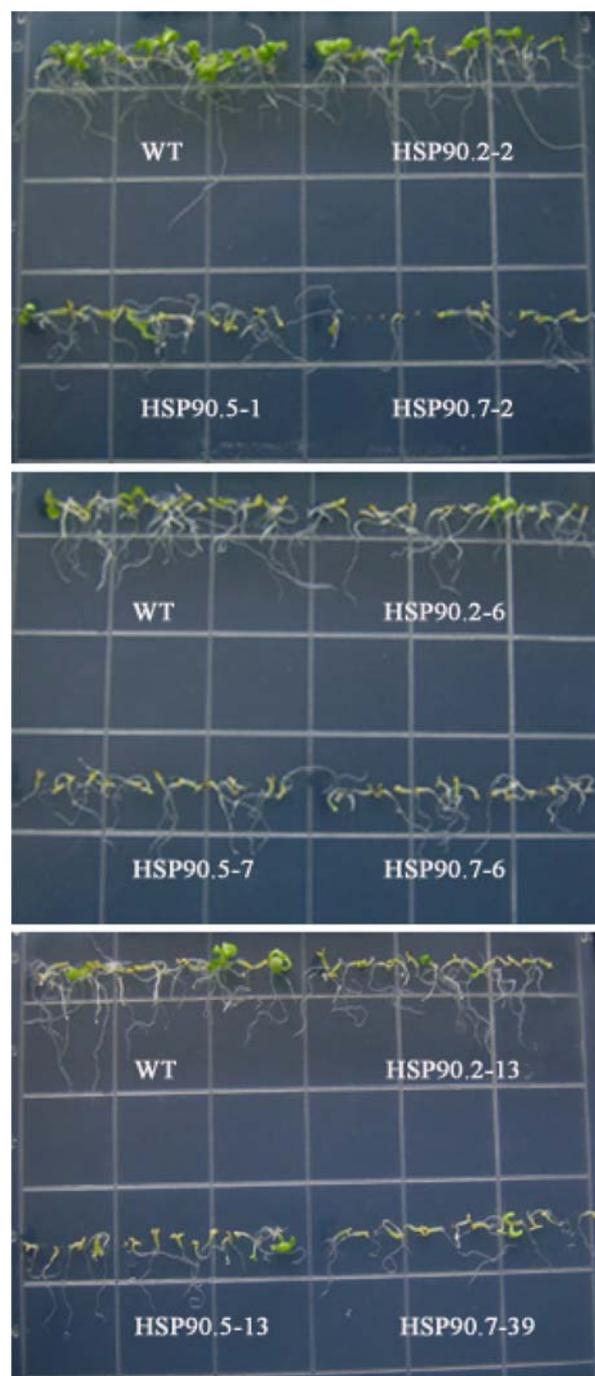


**Fig. 1** Seeds of transgenic plants were sensitive to oxidative stress. Seeds of WT and *AtHSP90* transgenic plants germinated on 1/2 MS medium containing 1.5 mM $H_2O_2$ for 12 days

*Arabidopsis* lines, obtained previously (Song et al. 2009), were surface-sterilized and placed on 1/2 MS medium containing 1.5 mM $H_2O_2$. Under non-stress conditions, no phenotypic differences were observed between WT and any of the transgenic plants (data not shown). However, under oxidative stress conditions, *AtHSP90.2*, *AtHSP90.5*, and *AtHSP90.7* transgenic seeds exhibited higher sensitivity to stress than WT plants (Fig. 1). This indicated that over-expression of each of these three *AtHSP90s* in *Arabidopsis* reduced oxidative tolerance in transgenic plants. Moreover, organellar *AtHSP90.5* and *AtHSP90.7* transgenic seeds showed higher sensitivity to oxidative stress than cytosolic *AtHSP90.2* transgenic seeds.

### Overexpression of *AtHSP90* Increased Protein Damage in *Arabidopsis*

Immunodetection of carbonylated proteins is a good indicator of protein damage due to oxidative stress (Ghezzi and Bonetto 2003). In this study, carbonylated proteins in WT and transgenic seedlings were determined using an immunoassay with anti-DNPH antibodies. Levels of carbonylated proteins in WT and *AtHSP90* transgenic seedlings increased with increased time and ranged from 14.4 to 94 kDa in size (Fig. 2b, d). Compared to WT seedlings, overexpression of *AtHSP90* significantly increased protein carbonylation. Following treatment with 10 mM $H_2O_2$ for 1 h, only a low level of carbonylation was observed in WT seedlings, whereas the intensity of carbonylated proteins increased significantly in *AtHSP90* transgenic seedlings, and in particular in the chloroplast-localized *AtHSP90.5* and ER-localized *AtHSP90.7* transgenic seedlings, with

molecular masses of 14.4 to 70 kDa in size (Fig. 2b). Following a 3-h treatment with 10 mM $H_2O_2$, protein carbonylation levels in all transgenic and WT seedlings were also increased. Overall, carbonylated proteins in WT seedlings were significantly lower than those in *AtHSP90* transgenic seedlings (Fig. 2d). Moreover, among all three groups of transgenic plants, *AtHSP90.5* transgenic seedlings exhibited the highest level of protein damage, and the sizes of those damaged proteins ranged from 14.4 to 94 kDa (Fig. 2d).

### Overexpression of *AtHSP90* Reduced the Antioxidant Enzyme Activities in *Arabidopsis*

As shown in Fig. 3, activities of SOD and CAT showed no significant differences between WT and cytosolic *AtHSP90.2* transgenic seedlings at 0 h. Following treatment with 10 mM $H_2O_2$, SOD and CAT activities in both WT and cytosolic *AtHSP90.2* transgenic seedlings increased at 1 h, but then dropped at 3 h, and interestingly, these activities were lower in *AtHSP90.2* transgenic seedlings than in WT (Fig. 3). Similarly, there were significant differences between WT and cytosolic *AtHSP90.2* transgenic seedlings for POD activity at 0 h, but this activity was reduced following 10 mM of $H_2O_2$ treatment for durations of both 1 and 3 h (Fig. 3). Moreover, POD activity was reduced by the overexpression of *AtHSP90.2*.

For those transgenic seedlings expressing organellar *AtHSP90.5* or *AtHSP90.7*, SOD, CAT, and POD activities were lower than those of WT in the absence of $H_2O_2$ treatment (0 h; Figs. 4 and 5). However, following 10 mM of $H_2O_2$ treatment for 1 h, SOD and CAT activities

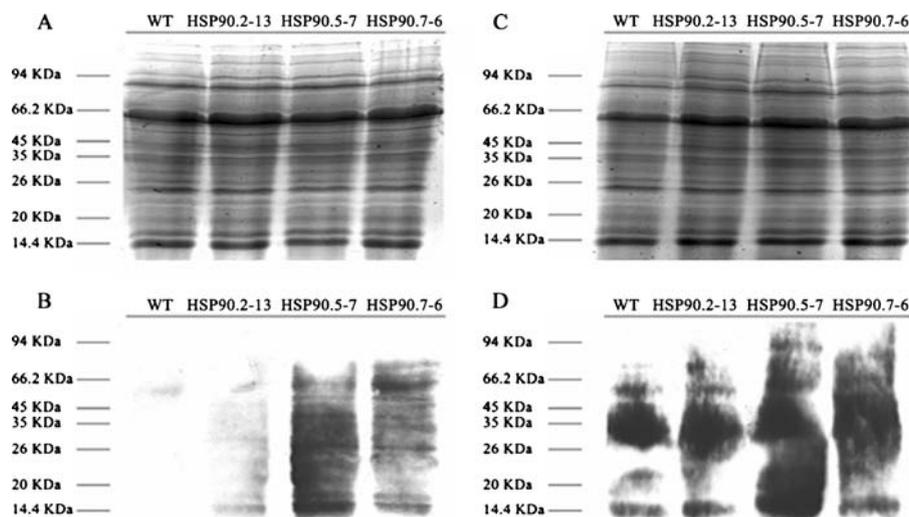**Fig. 2** Overexpression of AtHSP90s increased proteins damage in *Arabidopsis*. **a** proteins of WT and transgenic plants treated by 10 mM $H_2O_2$ for 1 h; **b** immunodetection of carbonylated proteins of WT and transgenic plants treated by 10 mM $H_2O_2$ for 1 h; **c** proteins of WT and transgenic plants treated by 10 mM $H_2O_2$ for 3 h; **d** immunodetection of carbonylated proteins of WT and transgenic plants treated by 10 mM $H_2O_2$ for 3 h. The proteins were stained with Coomassie Brilliant Blue R-250
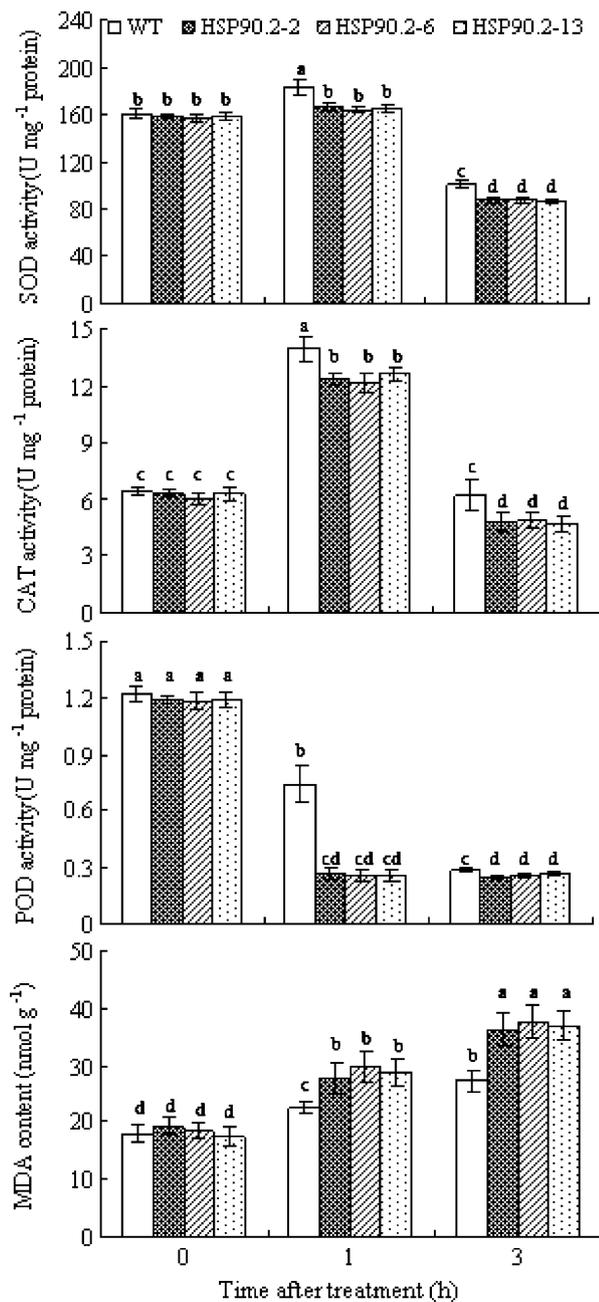
**Fig. 3** Effect of *AtHsp90.2* overexpression on the activities of antioxidant enzymes and the content of MDA in transgenic plants under oxidative stress. *Error bars* indicate SD; *n*=6. *Different letters* indicate statistical difference at $P \le 0.05$

**Fig. 4** Effect of *AtHsp90.5* overexpression on the activities of antioxidant enzymes and the content of MDA in transgenic plants under oxidative stress. *Error bars* indicate SD; *n*=6. *Different letters* indicate statistical difference at $P \le 0.05$

significantly dropped in organellar *AtHSP90.5* and *AtHSP90.7* transgenic seedlings, while these increased in WT seedlings (Figs. 4 and 5). Though POD activity in WT seedlings decreased at 1 and 3 h following 10 mM of $H_2O_2$ treatment, it remained higher than that in either chloroplast-localized *AtHSP90.5* or ER-localized *AtHSP90.7* transgenic seedlings (Figs. 4 and 5).

Under oxidative stress as well as normal physiological conditions, chloroplast-localized *AtHSP90.5* and ER-localized
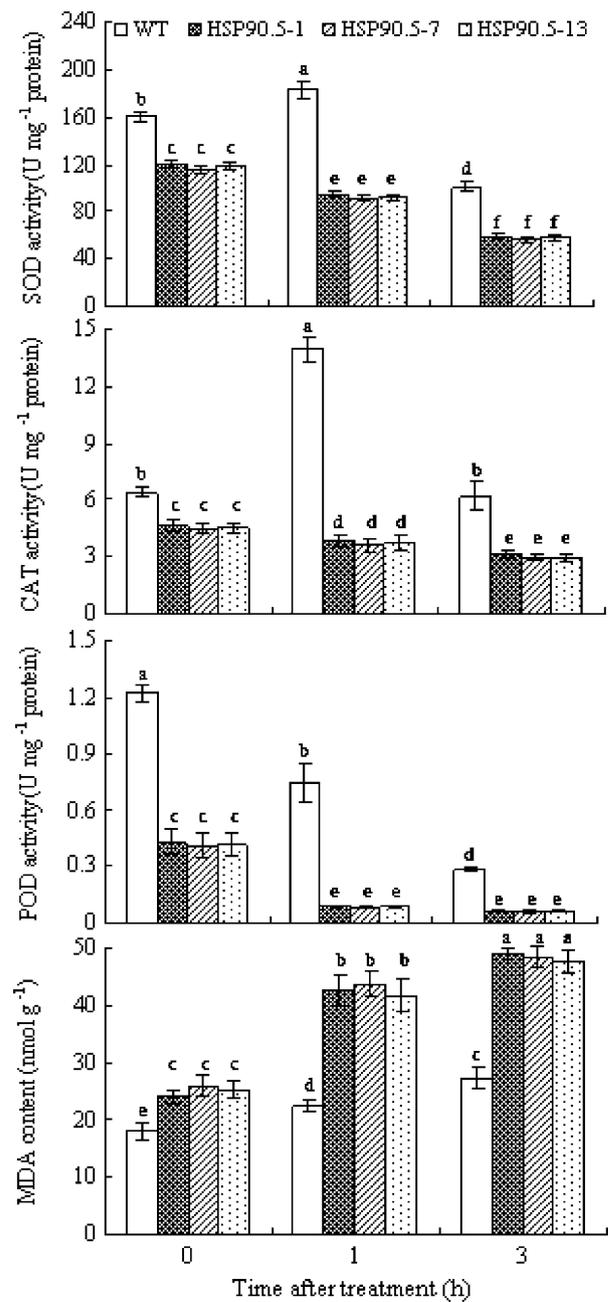
*AtHSP90.7* transgenic seedlings showed significantly lower levels of antioxidant enzyme activities than those in cytosolic *AtHSP90.2* transgenic seedlings (Figs. 3, 4, and 5).

Overexpression of *AtHSP90* Enhanced MDA in *Arabidopsis*

Lipid peroxides were determined by measuring MDA in levels in plants. As shown in Fig. 3, there were no
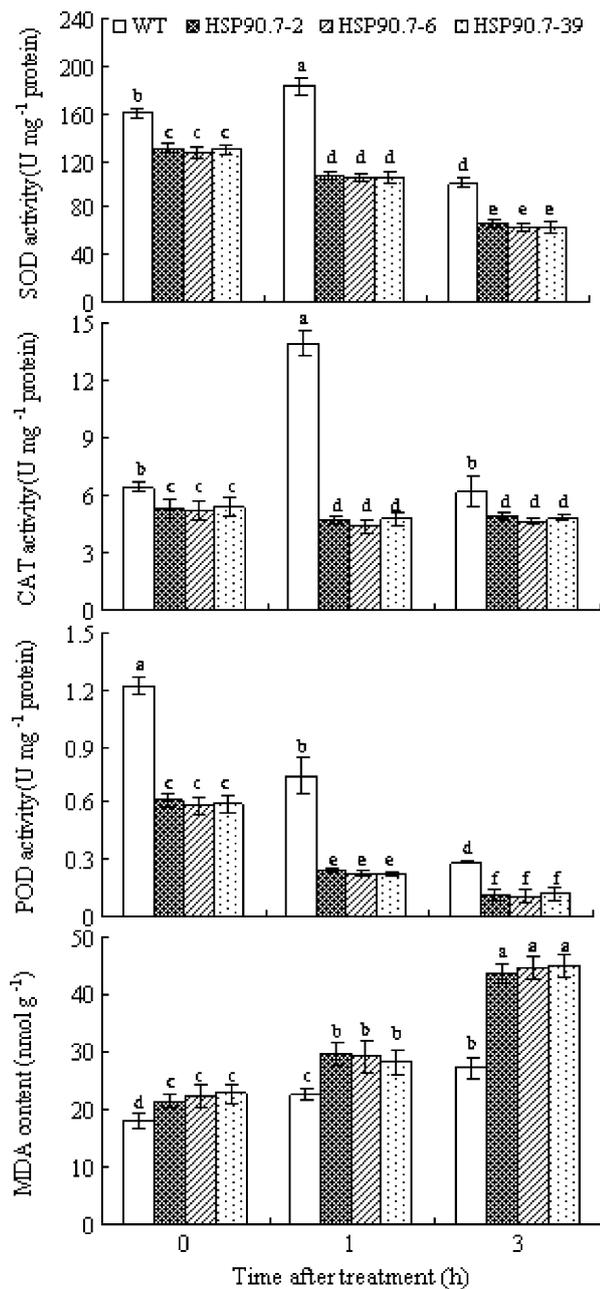
Fig. 5 Effect of *AtHsp90.7* overexpression on the activities of antioxidant enzymes and the content of MDA in transgenic plants under oxidative stress. *Error bars* indicate SD; $n=6$. *Different letters* indicate statistical difference at $P \leq 0.05$

significant differences in MDA levels between WT and cytosolic *AtHSP90.2* transgenic seedlings prior to $H_2O_2$ treatment. However, following 10 mM of $H_2O_2$ stress treatment, higher levels of MDA were observed in cytosolic *AtHSP90.2* transgenic seedlings than in WT seedlings. For both chloroplast-localized *AtHSP90.5* and ER-localized *AtHSP90.7* transgenic seedlings, MDA levels were higher than those in the WT seedlings at 0, 1, and 3 h (Figs. 4 and 5). These indicated that over-

expression of AtHSP90s aggravated oxidative damage of plant cells.

## Discussion

To develop a better understanding of the functional mechanisms of *Arabidopsis* HSP90 proteins in plants grown under oxidative stress, cytosol-localized AtHSP90.2, chloroplast-localized AtHSP90.5, and ER-localized AtHSP90.7 proteins were investigated in transgenic *Arabidopsis* plants.

In general, overexpression of *AtHSP90s* reduced germination of transgenic seeds under oxidative stress compared to the WT seeds (Fig. 1). Furthermore, carbonylated protein content was higher in transgenic seedlings than in WT seedlings, particularly at 1 and 3 h following oxidative stress treatment (Fig. 2), thus indicating that overexpression of *AtHSP90.2*, *AtHSP90.5*, and *AtHSP90.7* impaired plants tolerance to oxidative stress in transgenic *Arabidopsis*.

Plants possess an array of antioxidants including ascorbate, glutathione, hydrophobic molecules, and detoxifying enzymes that operate in different cellular organelles to scavenge ROS and protect cells from oxidative damage (Noctor and Foyer 1998). SOD, CAT, and POD are the most important detoxifying enzymes, and these act together with other enzymes of the ascorbate–glutathione cycle to promote scavenging of ROS (Hernandez et al. 2001). In this study, those observed lower levels of SOD, POD, and CAT in transgenic *HSP90.2*, *HSP90.5*, or *HSP90.7* seedlings, compared to WT, following oxidative stress, suggested that overexpression of *AtHSP90* restrained the activities of antioxidant enzymes in these transgenic plants. This further demonstrated that overexpression of *AtHSP90.2*, *AtHSP90.5*, or *AtHSP90.7* impaired plant tolerance to oxidative stress in transgenic *Arabidopsis*. Moreover, the observed differences in sensitivity to oxidative stress between either chloroplast-localized *AtHSP90.5* or ER-localized *AtHSP90.7* and cytosolic *HSP90* transgenic plants indicated that these compartmentalized HSP proteins were involved in different mechanisms of stress response.

HSP90 proteins maintain substrates in signaling competent states, thus enabling them to receive upstream signals. These substrates typically undergo conformational changes, thereby propagating signal transduction to downstream effectors (Sangster et al. 2007). Both eukaryotic and prokaryotic HSP90s have been reported to transiently bind to unfolding intermediates of substrates, thereby apparently stabilizing enzymes and suppressing aggregation. Since the HSP90 proteins interaction with its substrates is a dynamic process, based on relative binding affinities, its levels may already be optimized (Sangster et al. 2004). Constitutive overexpression of HSP90 proteins may disrupt the

association between HSP90 and its substrates. For instance, in *Hsc82*-overexpressed yeast cells, calcineurin is trapped by excess HSC82 resulting in cell sensitivity to abiotic stress (Imai and Yahara 2000). Furthermore, an HSP70/HSP90 co-chaperone, the carboxyl-terminus of HSC70-interacting protein (CHIP), contains a U-box domain homologous to those of the E4 ubiquitination factors and may present chaperone-bound misfolded proteins for both ubiquitination and degradation. In terms of the kinetic model of protein folding and degradation, the CHIP activity suggests that substrates, residing longer on HSP90, are more likely to be ubiquitinated and degraded (Murata et al. 2001; Ree et al. 2006). Thus, some HSP90 client proteins that are involved in plant stress response may be held back by those overexpressed HSP90 proteins and then targeted for degradation by CHIP. In this study, overexpression of *AtHSP90.2*, *AtHSP90.5*, and *AtHSP90.7* in *Arabidopsis* may shift the equilibrium of HSP90 with the client-bound state, disrupt plant stress signal transduction, and reduce activities of antioxidant enzymes, thereby impairing plant tolerance to oxidative stress. This may partly explain the observed impaired plant tolerance to oxidative stress in transgenic *Arabidopsis*.

In plants, reaction centers of photosystem I and photosystem II in chloroplast thylakoids are major sites for ROS generation (Asada 2006). In this study, overexpression of *AtHSP90.5* in *Arabidopsis* may disrupt plant stress signal transduction, thereby inhibiting antioxidant enzymes activity and increasing accumulation of ROS in chloroplast, as AtHSP90.5 is located in the chloroplast. Overexpression of chloroplast-localized *AtHSP90.5* results in higher sensitivity to oxidative stress than cytosolic *HSP90*. The ER is critical for the formation of disulfide bonds and newly synthesized secretion and proper folding and transport of membrane-bound proteins. Various disturbances can cause accumulation of unfolded proteins in the ER (Ma and Hendershot 2004). In this study, overexpression of ER-localized *AtHSP90.7* has resulted in higher sensitivity to oxidative stress than that of cytosolic *HSP90*. It is likely that overexpression of ER-localized AtHSP90.7 may disrupt the association of HSP90 and its substrates, thereby destroying the normal function of ER. Excessive and prolonged ER stress can trigger cell suicide, usually in the form of apoptosis deemed as a last resort of multicellular organisms to dispense dysfunctional cells (Xu et al. 2005).

In conclusion, overexpression of *AtHSP90.2*, *AtHSP90.5*, and *AtHSP90.7* in *Arabidopsis* impairs plant tolerance to oxidative stress, thus suggesting that proper homeostasis of HSP90 is critical for cellular stress response and/or tolerance in plants. Considering the important roles of AtHSP90 in coordinating cellular redox homeostasis,

further research should be carried out to provide further insights into the mechanism of function of HSP90 proteins in plant defense response against various environmental stresses.

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