A highly charged region in the middle domain of plant endoplasmic reticulum (ER)-localized heat-shock protein 90 is required for resistance to tunicamycin or high calcium-induced ER stresses

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Abstract

Heat-shock protein 90 (HSP90) is a highly conserved molecular chaperone that is involved in modulating a multitude of cellular processes under both physiological and stress conditions. In Arabidopsis, there are seven HSP90 isoforms (HSP90.1–HSP90.7) that are localized in the cytoplasm/nucleus, mitochondrion, chloroplast, and endoplasmic reticulum (ER) where protein folding actively takes place. In this study, we analysed the sequence of ER-localized Arabidopsis HSP90.7 and the other ER GRP94 proteins from plants and animals, and identified a short, charged region that is specifically present in the middle domain of plant-derived GRP94 proteins. To understand the role of this charged region, we analysed transgenic plants that expressed a mutant protein, HSP90.7Δ22, which had this charged region deleted. We showed that seedlings expressing HSP90.7Δ22 had significantly enhanced sensitivity to ER stress induced by tunicamycin or a high concentration of calcium, although its general chaperone activity in preventing the model protein from heat-induced aggregation was not significantly affected. We also analysed the ATP-binding and hydrolysis activity of both wild-type and mutant HSP90.7 proteins, and found that they had slightly different ATP-binding affinities. Finally, using a yeast two-hybrid screen, we identified a small set of HSP90.7 interactors and showed that the charged region is not required for the candidate client interaction, although it may affect their binding affinity, thus providing potential targets for further investigation of HSP90.7 functions.

Keywords: Enzyme kinetics, ER stress responses, HSP90 client proteins, molecular chaperone, protein–protein interaction, transgenic plant.

Introduction

The heat-shock protein 90 (HSP90) family are well-conserved molecular chaperones in both prokaryotes and eukaryotes; they have a global role in maintaining cellular protein homeostasis and participate in a wide range of cellular processes. HSP90, in particular, is well documented in its essential role in folding a variety of protein kinases and steroid hormone receptors (Richter and Buchner, 2001; Young et al., 2001). HSP90 is also required for the assembly of multiple macromolecular structures (Makhnevych and Houry, 2012), for buffering genetic variations in Arabidopsis (Sangster et al., 2008), Drosophila (Rutherford and Lindquist, 1998), and fungi (Cowen and Lindquist, 2005), and for plant pathogen-related disease resistance (Lu et al., 2003; Takahashi et al., 2003; Boter et al., 2007).

Abbreviations: BiP, binding immunoglobulin protein; HSP90, heat-shock protein 90; ER, endoplasmic reticulum; FLAG, the 8 aa DYKDDDDK epitope; GFP, green fluorescent protein; GRP94, glucose-regulated protein 94; MS, Murashige and Skoog; CS, citrate synthase; SD, standard deviation; UPR, unfolded protein response.

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HSP90 is structurally divided into three domains, an ATP-binding N-terminal domain (Stebbins et al., 1997), a substrate-binding middle domain (Sato et al., 2000), and a C-terminal dimerization domain, which allows two HSP90 monomers to associate and form an active HSP90 protein (Minami et al., 1994). In addition, a highly charged glutamic acid-rich linker region is found between the N-terminal and the middle domains, and has been shown to facilitate client protein activation (Hainzl et al., 2009). The HSP90 family can be further divided into five subfamilies based on their subcellular localization, the cytosolic HSP90A, endoplasmic reticulum (ER)-localized HSP90B/GRP94, chloroplast-localized HSP90C, mitochondria-localized TRAP (tumor necrosis factor receptor-associated protein), and bacterial HSP90, which is also known as HtpG (high-temperature protein G) (Taipale et al., 2010). Extensive studies have revealed the evolution, structure, mechanism of action, and complex functional regulation of this protein family (Cowen and Lindquist, 2005; Pearl and Prodromou, 2006; Sangster et al., 2008; Taipale et al., 2010; Johnson, 2012; Kadota and Shirasu, 2012). However, most studies focus on cytosolic HSP90 isoforms, and the structure and regulation of the organelle-localized HSP90s in eukaryotic cells are less well-understood.

In Arabidopsis thaliana, seven HSP90 members have been identified and named as HSP90.1–HSP90.7 (Krishna and Gloor, 2001). HSP90.7 is an ER-localized HSP90 parologue and belongs to the HSP90B subfamily (Chen et al., 2006). ER-localized HSP90 is also known as glucose-regulated protein 94 (GRP94), or gp96 in mammalian cells, and facilitates the folding of many proteins such as IgG, Toll-like receptors, and subclasses of integrins (Marzec et al., 2012). ER-localized HSP90 members are also known as endoplasm (Csermely et al., 1995). Arabidopsis HSP90.7 (we refer to it as HSP90.7 rather than HSP90B or AtGRP94 in this study), has also been called SHEPHERD (SHD) in a previous study for its epistatic to the clv mutation. HSP90.7 is therefore implicated in the correct folding of CLAVATA proteins (comprising CLV1, CLV2, and CLV3), which participate in shoot apical meristem maintenance (Miwa et al., 2009; Aichinger et al., 2012; Somorjai et al., 2012). Additionally, in a study using tunicamycin to induce ER-specific stress on tobacco protoplasts, HSP90.7 was shown to have a role in supporting α-amylase secretion (Klein et al., 2006). However, whether or not HSP90.7 binds directly to α-amylase and the other candidate client proteins that require HSP90.7 function are still unknown. Moreover, analysis of the shd mutant also showed that HSP90.7 may not act as a general chaperone to bind as many newly synthesized polypeptides as the HSP70 family chaperone binding immunoglobulin protein (BiP) in the ER, and that HSP90.7 functions specifically in proliferating tissues (Klein et al., 2006).

In this study, we analysed the sequence and function of Arabidopsis HSP90.7 and showed that HSP90.7 contains a plant-specific, highly charged 22 aa fragment in the middle domain. By analysing transgenic seedlings that expressed an HSP90.7 mutant that had the charged region deleted, we showed that this region in the middle domain is essential for seedlings to resist ER stress induced by tunicamycin or a high concentration of Ca²⁺. However, the general chaperone activity in preventing model proteins from heat-induced aggregation was not affected by deletion of this charged region. Further biochemical and proteomics analyses of the mutant protein indicated that the charged region might be involved in regulating HSP90.7 ATP-hydrolysis efficiency, and not in directly binding substrate proteins.

Materials and methods

Plant materials and growth conditions

The A. thaliana ecotype Columbia (Col-0) was used as the wild-type plant. To select for transgenic plants or test plant resistance to abiotic stresses, seeds were surface sterilized and sown on 1/2-strength Murashige and Skoog (MS; Murashige & Skoog, 1962) medium containing 1% sucrose and 0.7% agar with or without supplementation by abiotic stress-inducing reagents. After stratification in the dark at 4 °C for 3–4 d, the seeds were cultured within a plant growth incubator set at 120 μmol m⁻² s⁻¹ with a 16/8 h light/dark cycle at 22 °C. Alternatively, sterilized seeds were stratified in microcentrifuge tubes and then placed over freshly prepared Pro-mix PGX® soil for growth within a plant growth chamber set at 110 μmol m⁻² s⁻¹ with a 16/8 h light/dark cycle at 22 °C.

Construction of the HSP90.7Δ22 deletion mutant

Two SpeI restriction sites (5'-ACTAGT-3') were inserted into the Arabidopsis HSP90.7 coding sequence at 1462 and 1528 bp by site-directed mutagenesis using primer 5’-CTTGGCTAGAGAT CCTTACTAGT GCATCATGATG-3’ with its reverse complement, and primer 5’-AACAGT GAGAAG AAGGGTT AAAACT GTCAATA CACAAA ATTCTGG-3’ with its reverse complement, respectively, in the Escherichia coli expression vector p11 (Savchenko et al., 2003). The HSP90.7 D488-G509 coding sequence was deleted by cleavage with SpeI and religation, and the resulting HSP90.7 mutant was designated HSP90.7Δ22. The full-length HSP90.7 and HSP90.7Δ22 coding sequences were then cloned into binary vectors pGWB402Ω and pGWB502Ω (Nakagawa et al., 2007), respectively, using Gateway cloning, generating pGWB402Ω-AhHSP90.7 and pGWB502Ω-AhHSP90.7Δ22, in which a FLAG tag coding sequence was also inserted at the C terminus before the terminal ER-retention sequence KDE.

Arabidopsis transformation and screening of transgenic plants

A. tumefaciens GV3101 carrying pGWB402Ω-AhHSP90.7 or pGWB502Ω-AhHSP90.7Δ22 plasmid was used to transform Arabidopsis Col-0 by the floral dip method (Clough and Bent, 1998). Selection of transgenic plants was performed on ½ strength MS medium with 1% sucrose supplemented with 25 μg ml⁻¹ of kanamycin for HSP90.7 transgenic plants, or 20 μg ml⁻¹ of hygromycin for HSP90.7Δ22 transgenic plants. PCR amplification and immunoblotting with anti-FLAG antibody (Sigma) were used to confirm the presence of the transgenes and expression of the FLAG-tagged proteins, respectively.
HSP90 protein expression and purification

The construct for His6-tagged canine GRP94 (73–754AA41) in pET15b (Dollins et al., 2007) was a kind gift from Daniel Gewirth (Hauptman-Woodward Medical Research Institute, Buffalo, USA). *Saccharomyces cerevisiae* cytotoxic HSP82, *Arabidopsis* cytotoxic HSP90.2, and the predicted mature forms of ER-localized HSP90.7 (R73–L823) and HSP90.7(722–754) were cloned into pBl. Constructs were introduced into *E. coli* BL21(DE3)-pRIL (Stratagene), and protein expression was induced by 1 mM isopropyl β-D-thiogalactopyranoside. His6-tagged proteins were purified using Ni-NTA resin (Qiagen), and dialysed overnight. His6-tag was cleaved with tobacco etch virus protease and removed by Ni-NTA resin. Size-exclusion chromatography with a Superdex 200 column (GE Healthcare) was used to further purify the proteins. Fractions containing the native purified protein were stored at −80 °C in buffer [25 mM Tris/HCl (pH 7.5), 150 mM KCl, 10% glycerol, 0.5 mM dithiothreitol (DTT)] until further use.

Abiotic stress-resistance tests

Homozygote line seeds screened from lines that contained a single transgene locus based on antibiotic selection were germinated on 1/2 strength MS medium for 6 d before being subjected to heat-shock treatment at 45 °C for 5, 10, or 30 min. The seedlings were then allowed to recover at 22 °C with a 16/8 h light/dark photoperiod. Survival rate was measured by the percentage of seedlings that had at least one green leaf 6 d after recovering from the heat shock. To test ER-specific stress resistance, seeds were germinated on 1/2 strength MS medium containing tunicamycin (0.3 or 0.4 μg ml⁻¹) for 2 or 3 d and then transferred back on to normal 1/2 strength MS medium for recovery.

In vitro chaperone activity assay

Citrate synthase (CS; Sigma) was dialysed in 20 mM HEPES/KOH (pH 7.5), 150 mM KCl, and 10 mM MgCl₂ before being used for the heat-induced aggregation assay. CS (500 nM) was prepared in a final volume of 150 μl of 20 mM HEPES/KOH (pH 7.5) and 2.8 mM β-mercaptoethanol with different amounts of HSP90 proteins. The mixtures were added to a 96-well microplate and heated at 45 °C. Light scattering at 340 nm was measured at 45 °C in a Synergy 4 spectrophotometer (BioTek). Control measurements were performed with purified HSP90 protein alone in the absence of CS.

ATP–Sepharose binding assay

An ATP–Sepharose binding assay was performed as described previously (Hernandez et al., 2002) with some modifications. Purified HSP90 protein (10 μg) was incubated with 15 μl of γ-linked ATP–Sepharose beads (Innova Biosciences) in 225 μl of binding buffer [10 mM Tris/HCl (pH 7.5), 50 mM KCl, 20 mM MgCl₂, 2 mM DTT, 20 mM Na₂MoO₄, 0.01% Nonidet P-40]. The mixture was incubated for 30 min at 30 °C with gentle shaking. The unbound fraction was collected after centrifugation, and the beads were washed four times with binding buffer. The bound protein was eluted from the beads by heating at 100 °C in SDS Laemmli buffer. Equivalent amounts of sample were separated by SDS-PAGE and visualized by silver staining.

ATP-hydrolysis assay

ATP-hydrolysis activity was measured using a coupled NADH method as described previously (Norby, 1988). The decrease in NADH absorbance at 340 nm was measured using a Synergy 4 microplate reader (BioTek). Briefly, for each assay, about 50–100 μg of purified HSP90 protein was used in the following reaction mixture: 25 mM HEPES (pH 7.5), 5 mM MgCl₂, 400 mM KCl, 0.03% Tween 20, 10% glycerol, 200 μM NADH, 3 mM phosphoenol pyruvate, 15.7 μM of pyruvate kinase (Sigma), and 24.5 μM of lactate dehydrogenase (Sigma). ATP was added in varying concentrations to determine the Kₘ values. For all assays, geldanamycin (National Institutes of Health) was added to a final concentration of 10 μM to measure geldanamycin-sensitive HSP90 activities. The turnover rate (kₐcat), binding affinity (Kₘ), and catalytic efficiencies were analysed using the Michaelis–Menten function in the statistical program Graphpad Prism.

Yeast two-hybrid screening

The HSP90.7 middle and C-terminal domains (HSP90.7732–823), designated HSP90.7MC, were cloned into the pEG202 vector for yeast two-hybrid screening. An *Arabidopsis* cDNA library constructed into prey vector pJG4-5 was a kind gift from Dr Gazzarrini (University of Toronto, Canada), and the screening of positive interaction using LEU2 and lacZ marker genes was performed in *S. cerevisiae* EGY48 cells as described previously (Tsai and Gazzarrini, 2012). Positive hits were sequenced and retransformed back to yeast cells to test interactions with wild-type HSP90.7MC, the deletion mutant HSP90.7(722–823)MC, and the bicoid-bait negative-control RFMHI (Manseau et al., 1996).

Antibodies

Polyclonal rabbit anti-HSP90.7 antibody was generated by Signalway AntiBody (College Park, USA) with purified HSP90.7 protein. The other primary antibodies used in this study were anti-FLAG monoclonal antibody (F3165; Sigma) and polyclonal anti-HA antibody (Cedarlane), anti-BiP antibody (Santa Cruz), anti-calnexin antibody (a kind gift from Dr David Williams, University of Toronto, Canada), and anti-HSP90.2 antibody, which was originally raised using purified *S. cerevisiae* HSP82 (Zhao et al., 2005) but also specifically recognizes *Arabidopsis* cytotoxic HSP90.1 and HSP90.2 but not the other organell formes of HSP90 (Song et al., 2009b).

Results

Plant ER-localized molecular chaperone HSP90 contains a highly charged region in the middle domain

To understand whether the ER-localized *Arabidopsis* HSP90.7 had any special structural features, we aligned it with other ER-localized HSP90 isoforms from plant and mammalian species, as well as with representative cytotoxic HSP90 members. HSP90.7 contained all conserved amino acids for ATP binding (Supplementary Fig. S1 at JXB online) in the N terminus and the γ-phosphate-binding amino acid Arg (Pearl and Prodromou, 2006) in the middle domain (Fig. 1). The mammalian GRP94 contains a ‘DGQST’ motif that is close to the ATP-binding site and forms an N-ε-carboxamidoadenosine-binding pocket in the N terminus (Soldano et al., 2003). However, HSP90.7 and the other plant ER-localized GRP94 homologues did not contain such a motif and resembled the cytotoxic HSP90 homologues (Supplementary Fig. S1). Surprisingly, HSP90.7 contained a highly charged, 22 aa sequence (D488–G509) in the middle domain, and similar charged fragments were also present in ER-localized HSP90 isoforms from barley (*Hordeum vulgare*) and periwinkle (*Catharanthus roseus*) (Fig. 1). Further analysis indicated that all 11 available ER-localized HSP90 sequences identified from the UniProtKB database for higher plants contained such a highly charged fragment,
although they were not identical (Supplementary Fig. S2A at JXB online). Additionally, the charged fragment also appeared in the ER-localized HSP90 from the unicellular green algae *Chlamydomonas reinhardtii*, but not the one from other mycota or fungal species such as *Dictyostelium discoideum*, Blastocladia leonemersonii and Coprinopsis cinerea (Supplementary Fig. S2A). This suggested that the identified charged region may be specific to plants or those species that can perform photosynthesis.

We then modelled the HSP90.7 structure using 3D-JIGSAW (Bates et al., 2001; Contreras-Moreira and Bates, 2002) and aligned the predicted structure with that of canine (*Canis familiaris*) GRP94 (CfGRP94) (Dollins et al., 2007). As shown in Supplementary Fig. S2B, the charged region in the HSP90.7 middle domain seemed to form an extra loop compared with CfGRP94. To study the function of HSP90.7 in vivo and the possible role of this charged fragment, we made two HSP90.7 constructs: FLAG-tagged HSP90.7 and FLAG-tagged HSP90.7Δ22 in which the 22 aa charged region was deleted (Fig. 2A). For both constructs, the FLAG was added before the KDEL ER-retention motif to facilitate transgene detection without impairing ER localization.

The highly charged region is required for in vivo HSP90 activity in resistance to ER stresses

To understand if the addition of a FLAG tag at the HSP90.7 C terminus affected in vivo chaperone functioning, transgenic overexpression lines were generated (Fig. 2B, C) and the homozygous progeny of overexpression lines (Supplementary Fig. S5 at JXB online) were first used to examine their tolerance to high calcium (Ca$$^{2+}$$) and heat-shock stresses. Examination of homozygous line 6-3 indicated that HSP90.7 overexpression lines conferred better resistance to 80 mM of CaCl$_2$ compared with wild-type plants, which on average had 30% less fresh weight than overexpression lines (Fig. 3A). This agreed with a previous observation that overexpression of wild-type untagged HSP90.7 helps plants resist to high concentrations of Ca$$^{2+}$$ (Song et al., 2009b). Surprisingly, seedlings expressing HSP90.7Δ22 seemed to be significantly more sensitive to a high concentration of Ca$$^{2+}$$, and resulted in much less fresh weight compared with wild-type seedlings as tested in all four independent homoygous transgenic lines (4-6-1, 11-2-5, 21-1, and 39-1) (Fig. 3A). To test if overexpression of FLAG-tagged HSP90.7 improved heat-shock resistance, transgenic seedlings grown for 6 d were heat shocked at 45 °C. It was noted that overexpression of FLAG-tagged HSP90.7 significantly improved the seedlings’ survival rate (Fig. 3B). Less than 5% of wild-type seedlings survived 30 min of heat shock, whereas approximately 45% of seedlings that overexpressed *HSP90.7* survived 30 min of heat shock. These results indicated that overexpression of HSP90.7 improves a plant’s heat-shock resistance, and that the FLAG tag does not seem to affect significantly the in vivo function of HSP90.7. However, similar assays were tested for seedlings expressing HSP90.7Δ22, and it was found that seedlings expressing HSP90.7Δ22 behaved similarly to wild-type seedlings and did not have better heat-shock resistance (Supplementary Fig. S6 at JXB online).

HSP90.7 is an ER-localized molecular chaperone, and ER stress usually results from improper protein homeostasis within the ER (Gardner et al., 2013). To examine if overexpression of FLAG-tagged HSP90.7 conferred specific resistance to ER stress, we tested seedling growth on medium supplemented with tunicamycin, a commonly used reagent that specifically inhibits the first step of the N-glycosylation pathway within the ER (Duksin and Mahoney, 1982). It was noted, however, that seedlings overexpressing FLAG-tagged wild-type HSP90.7 had very similar average fresh weights to wild type after 11 d of recovery, indicating that overexpression of HSP90.7 did not significantly improve ER-specific stress resistance, at least under the tested conditions (Fig. 4A, B). Interestingly, seedlings expressing FLAG-tagged HSP90.7Δ22 had increased sensitivity to tunicamycin. None of the four tested homoygous expression lines recovered well under the tested conditions (Fig. 4A, B). Combined with the stress test with a higher Ca$$^{2+}$$ concentration (Fig. 3A), this suggested...
that the 22 aa fragment in the middle domain is essential for proper HSP90.7 function in vivo and that deletion of this charged fragment caused a dominant-negative effect in both tunicamycin and high Ca\(^{2+}\) resistance. Nevertheless, a close look at the transgenic lines that expressed HSP90.7\(^{\Delta 22}\) did not reveal any significant growth defect at early or late development stages under normal growth conditions (16 h photoperiod, 22 °C, 110 μmol m\(^{-2}\) s\(^{-1}\)), by examining their leaf initiation, rosette radius, flowering, and flower organ development (Supplementary Fig. S3 at JXB online).

Deletion of the highly charged sequence in the HSP90.7 middle domain does not affect its general molecular chaperone activity in vitro

To understand why deletion of the charged region in the middle domain of HSP90.7 exerted a dominant-negative effect under ER-specific stress conditions, we examined the chaperone activity of HSP90.7 and HSP90.7\(^{\Delta 22}\) in preventing CS from heat-induced aggregation. The predicted mature forms of HSP90.7 and HSP90.7\(^{\Delta 22}\) were purified and mixed with CS. CS is an unstable protein that is denatured when heated at 45 °C and gradually aggregates; the amount of aggregate particles can be monitored by its light-scattering ability (CS+buffer in Fig. 5). When purified HSP90.7 or HSP90.7\(^{\Delta 22}\) was added to the CS mixture, both proteins were shown to inhibit heat-induced CS aggregation and the effect was concentration dependent (Fig. 5A, B). When equimolar amounts of HSP90.7 or HSP90.7\(^{\Delta 22}\) were added, heat-induced aggregation of CS was almost completely inhibited. There was no significant difference between HSP90.7 and HSP90.7\(^{\Delta 22}\) in preventing CS aggregation. As positive and negative controls, we also tested the effects of canine GRP94, and green fluorescent protein (GFP) on the inhibition of heat-induced aggregation of CS. Canine GRP94 was able to inhibit the aggregation of CS, while GFP was unable to inhibit the heat-induced aggregation of CS as expected (Supplementary Fig. S4 at JXB online). Interestingly, when geldanamycin, which

Fig. 2. HSP90.7 constructs and screening of transgenic plants that express HSP90.7 or HSP90.7\(^{\Delta 22}\). (A) Schematic diagrams of HSP90.7 and HSP90.7\(^{\Delta 22}\) coding sequence constructs in binary vectors used for plant transformation. The charged fragment D488–G509 in HSP90.7 is deleted in HSP90.7\(^{\Delta 22}\). The C-terminal FLAG tag and ER-retention sequences are shown above. (B, C) Immunoblotting of transgenic plants expressing FLAG-tagged wild-type HSP90.7 (B) and HSP90.7\(^{\Delta 22}\) (C). Total proteins were prepared from 10-d-old T2 seedlings, resolved by 8% SDS-PAGE, and immunoblotted with anti-FLAG and anti-HSP90.7 antibodies.
tightly binds to the ATP-binding pocket of HSP90, was added, both HSP90.7 and HSP90.7Δ22 lost their ability to inhibit CS aggregation (Fig. 5C). These data indicated that deletion of the highly charged region in the HSP90.7 middle domain did not affect its in vitro chaperone activity and that the chaperone activity is probably conferred by a specific HSP90.7 conformation that can be blocked by the HSP90 inhibitor geldanamycin.

Deletion of the highly charged fragment enhances the catalytic efficiency of HSP90.7 on ATP hydrolysis

Since the functional cycle of HSP90 in vivo is a complex process that requires ATP binding and hydrolysis for both cytosolic HSP90s (Pearl and Prodomou, 2006; Taipale et al., 2010) and mammalian GRP94 (Ostrovsky et al., 2009), we then analysed the ATP-binding affinity of HSP90.7 together with the other representative HSP90 isoforms using an ATP-Sepharose binding assay. As shown in Fig. 6A, CfGRP94 and ScHSP82 had much higher ATP-binding affinity than Arabidopsis HSP90.2, HSP90.7 and HSP90.7Δ22 (top two panels of Fig. 6A). Close examination of the band intensity and quantitative analysis of the bands revealed that cytosolic

Fig. 3. FLAG tag does not significantly affect HSP90.7 function in vivo. (A) High calcium resistance test for transgenic plants overexpressing FLAG-tagged HSP90.7 and HSP90.7Δ22. Seeds were germinated on ½ strength MS medium containing 80 mM CaCl2 and grown for 3 weeks. The average fresh weight of seedlings was measured for wild-type Col-0 (WT), homozygous HSP90.7 overexpression line 6-3, and four homozygous HSP90.7Δ22 expression lines (4-6-1, 11-2-5, 21-1, and 39-1). Error bars represent standard deviation (SD) from at least 40 seedlings, and bars with different letters on top are significantly different as examined by t-test (P<0.05). (B) Heat-shock resistance test for transgenic plants overexpressing FLAG-tagged HSP90.7. Seeds were germinated on ½ strength MS medium for 6 d before heat-shock treatment for 5, 10, or 30 min at 45 °C. Survival rates were calculated as the percentage of seedlings that had at least one green leaf after 6 d of recovery from 30 min heat-shock treatment. The survival rates were averaged from three independent transgenic lines. Error bars represent SD.

Fig. 4. Expression of AtHSP90.7Δ22 shows a dominant-negative effect on ER stress resistance. (A) Left: seeds were germinated on ½ strength MS medium and grown for 11 d. Right: seeds were germinated on ½ strength MS medium containing 0.4 μg ml−1 tunicamycin for 3 d and then transferred to ½ strength MS medium for 11 d. WT represents wild-type Col-0 seedlings, 6-3 is a homozygous FLAG-tagged HSP90.7 expression line, and 4-6-1 and 11-2-5 are two examples of homozygous FLAG-tagged HSP90.7Δ22 expression lines. (B) Average fresh shoot weight per seedling. The fresh shoot weights were measured after 11 d of recovery as shown in (A). Four different homozygous FLAG-tagged HSP90.7Δ22 lines (4-6-1, 11-2-5, 21-1, and 39-1) and one FLAG-tagged HSP90.7 line (6-3) are shown. Three replicates were performed and error bars represent SD. Bars with different letters on top are significantly different as examined by t-test (P<0.05).
Arabidopsis HSP90.2 had a slightly higher ATP-binding affinity than HSP90.7Δ22, while HSP90.7Δ22 had a higher ATP-binding affinity than the wild-type HSP90.7 (bottom panel of Fig. 6A).

The ATP-binding and hydrolysis activities for HSP90.7 and HSP90.7Δ22 were further analysed by NADH-coupled enzymatic assays and their kinetics were determined to be slightly different (Fig. 6B). Fitting the kinetics data to the Michaelis–Menten equation indicated that HSP90.7Δ22 had a similar turnover rate to wild-type HSP90.7 (Table 1), while the $K_m$ value of HSP90.7Δ22 was only about 58% of that of the wild-type HSP90.7. This suggested that the deletion mutant HSP90.7Δ22 had a higher ATP-binding affinity, in agreement with the ATP-Sepharose binding assay (Fig. 6A), and as a result, the catalytic efficiency of HSP90.7Δ22 was almost twice as high as that of wild-type HSP90.7. Assays performed on ScHSP82 and CfGRP94 showed that they both had much lower $K_m$ values compared with HSP90.7, albeit having much lower activity.
Expression of HSP90.7 \(^{\text{Δ}22}\) does not induce an unfolded protein response (UPR) under normal growth conditions

To further understand whether the enhanced sensitivity to tunicamycin and a high concentration of Ca\(^{2+}\) for HSP90.7 \(^{\text{Δ}22}\) expression seedlings was due to excessive ER stress, we analysed the protein level of BiP, whose overexpression is a hallmark for ER stress (Gardner et al., 2013). Interestingly, the expression level of BiP was not upregulated in FLAG-tagged HSP90.7 \(^{\text{Δ}22}\) transgenic seedlings compared with wild type (Fig. 7A, top panel). The expression of cytosolic HSP90 or ER-chaperone calnexin was also not affected in transgenic HSP90.7 \(^{\text{Δ}22}\) seedlings (Fig. 7A). This suggested that the general protein homeostasis in both the cytosol and the ER lumen might not be significantly affected under normal growth conditions. We also tested whether expression of HSP90.7 \(^{\text{Δ}22}\) affected the UPR if treated with tunicamycin or DTT, the two commonly used UPR-inducing reagents. We noticed that both tunicamycin and DTT induced the expression of BiP well; however, there is no difference between wild type and HSP90.7 \(^{\text{Δ}22}\) expression lines (Fig. 7A bottom panel), suggesting that expression of HSP90.7 \(^{\text{Δ}22}\) might not significantly affect the normal UPR, at least not for the induction of BiP under ER stress. In an attempt to understand whether deletion of the charged region interfered with the association of any specific HSP90.7 clients, we purified both FLAG-tagged HSP90.7 and HSP90.7 \(^{\text{Δ}22}\) complexes by affinity purification. Although both FLAG-tagged HSP90.7 and HSP90.7 \(^{\text{Δ}22}\) were purified well, no other protein was significantly co-purified as shown by SDS-PAGE and silver staining (Fig. 7B). We tried to analyse the HSP90.7FLAG and HSP90.7 \(^{\text{Δ}22}\)-FLAG protein complexes by liquid chromatography/tandem mass spectrometry; however, no robust HSP90.7 binding partners were identified (data not shown). This is presumably because client proteins bind HSP90.7 so weakly that the HSP90.7 protein complex was not well preserved in the affinity purification.

We further applied a yeast two-hybrid screening using the HSP90.7 middle and C-terminal domains as bait to identify potential HSP90.7 interactors with an Arabidopsis cDNA library (Tsai and Gazzarrini, 2012). The middle and C-terminal domains were chosen as bait because the middle domain is responsible for the client binding, and screening using these domains has proven successful to identify both co-chaperones and client proteins for cytosolic HSP90 isoforms (Zhao et al., 2005). As a result, from over 100 colonies that grew on the selection medium, we identified seven potential HSP90.7 interactors, each of which were identified at least twice (Supplementary Table S1 at JXB online). Interestingly, analysis of three of the interactors, PRXR1 (At4G21960.1), the peroxidase 42, SMT2 (At1G20330.1), a sterol-C24-methyltransferase, and a calcineurin-like metallophosphoesterase (At4G24730.2), indicated that they all interacted with both wild-type and mutant HSP90.7MC but not with the negative-control protein (Fig. 7C). As a control, immunoblotting indicated that the three prey proteins, which all contained a signal peptide and are targeted to the ER during their in vivo synthesis, were expressed well in the test yeast cells (Fig. 7D). These data suggested that the charged region might not be required for client protein binding, at least not for the three tested candidates.

### Discussion

In this study, we analysed HSP90 family proteins that are located in different subcellular compartments of the cell. We particularly identified a short, charged region in the middle domain of plant ER-localized HSP90s, and this charged sequence was absent from any cytosolic, plastidic, mitochondrial, or metazoan-derived ER-localized HSP90 homologues (Fig. 1). Phylogenetic analyses have shown that GRP94 and cytosolic HSP90 family members evolved separately from a common ancestor, distinct from the ancestor of Hsp70 and TRAP (Chen et al., 2006; Marzec et al., 2012). The charged region in the middle domain is only observed in plant ER-localized GRP94 species, suggesting that this region may have evolved concurrently with the early symbiosis event from which cyanobacteria were harboured and had evolved into the chloroplast (Goksoyr, 1967; Osteryoung and Nunnari, 2003; Glynn et al., 2007).

By analysing the ATP-hydrolysis activity, we found that deletion of the charged region in HSP90.7 middle domain resulted in a significantly higher ATP-hydrolysis efficiency due to increased ATP-binding affinity. The \(k_{\text{cat}}\) of HSP90.7 was comparable to that of HSP82 (0.566 ± 0.080 min\(^{-1}\)) (Table 1), bacterial HptG (0.47 min\(^{-1}\)) (Panaretou et al., 1998), and mitochondrial TRAP (0.1 min\(^{-1}\)) (Owen et al., 2002), but much higher than that of human cytosolic HSP90 (0.04 min\(^{-1}\)) (McLaughlin et al., 2002) and mammalian GRP94 (Table 1) (Dollins et al., 2007). This suggests that plant ER-localized HSP90 species might better inherit the ATP-hydrolysis activity of its ancestor, the HptG group A protein, very early on in the formation of the eukaryotic cell (Chen et al., 2006). Additionally, in terms of the mechanism of ATP hydrolysis, the higher catalytic activity of HSP90.7 suggests that it may generally adopt a catalytically active and closed conformation among apo, open and closed states (Southworth and Agard, 2008). It has been shown that the N terminus of HSP82 dimerizes and shifts to the closed conformation upon ATP binding, while the N terminus of GRP94 moves to a

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**Table 1. ATP hydrolysis activity of HSP90.7 and its homologues**

<table>
<thead>
<tr>
<th>HSP90 species</th>
<th>(k_{\text{cat}}) (min(^{-1}))</th>
<th>(K_m) (mM)</th>
<th>Catalytic efficiency (mM (^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP90.7</td>
<td>2.26 ± 0.17</td>
<td>0.77 ± 0.18</td>
<td>2.94 ± 0.91</td>
</tr>
<tr>
<td>HSP90.7(^{\text{Δ}22})</td>
<td>2.34 ± 0.15</td>
<td>0.44 ± 0.11</td>
<td>5.34 ± 1.67</td>
</tr>
<tr>
<td>ClGP94</td>
<td>0.072 ± 0.004</td>
<td>0.065 ± 0.013</td>
<td>1.11 ± 0.27</td>
</tr>
<tr>
<td>ScHSP82</td>
<td>0.66 ± 0.08</td>
<td>0.38 ± 0.19</td>
<td>1.72 ± 0.58</td>
</tr>
</tbody>
</table>

\(^a\) CIGP94 represents GRP94 from Canis familiaris and ScHSP82 represents HSP82 from Saccharomyces cerevisiae.
more open conformation, to which the nucleotide is more accessible (Dollins et al., 2007). The open conformation does not allow efficient ATP hydrolysis, thus explaining the lower turnover rate of GRP94. Therefore, the ATP-hydrolysis mechanism of HSP90.7 may resemble the functional cycle of HSP82 and HptG, rather than GRP94 in mammals.

Reduced expression of HSP90.7 has been shown to repress CLV signalling activity (Ishiguro et al., 2002). However, we did not observe any significant phenotype in HSP90.7 or HSP90.7Δ22 expression transgenic plants under normal growth conditions (Supplementary Fig. S3). One reason could be that either the enhancement or inhibition of the CLV signalling pathway by HSP90.7Δ22 expression did not...
reach a threshold that is necessary to trigger an observable phenotype. In agreement with this hypothesis, a previous study showed that Arabidopsis plants can tolerate up to a 10-fold high inducible CLV3 expression without displaying any phenotype (Muller et al., 2006). Secondly, the previously observed phenotype associated with the shd mutant is from Arabidopsis ecotype Wassilewskija (Ishiguro et al., 2002), and a similar shd mutant phenotype may not be observed in the Col-0 or Landsberg erecta (Ler-0) background due to polymorphism of CLV genes.

Nevertheless, HSP90.7Δ2 transgenic plants in the Col-0 background showed a dominant-negative effect in resistance to tunicamycin and developed much less fresh weight during the recovery stage (Fig. 4), while overexpression of FLAG-tagged wild-type HSP90.7 did not induce such an effect. Because the expression of BiP or calnexin was not significantly induced (Fig. 7A), it is evident that the expression of HSP90.7Δ2 did not induce UPR in the ER. Additionally, we showed that HSP90.7Δ2 has a similar general chaperone activity in preventing CS from heat-induced aggregation (Fig. 5). Therefore, it is plausible to assume that the dominant-negative effect is not associated with the general chaperone activity of HSP90.7. We propose that there is a currently unknown mechanism that uniquely and sensitively monitors the altered ATPase activity of HSP90.7 and subsequently induces an HSP90.7-specific response in Arabidopsis. In agreement with this hypothesis, both ATP binding and hydrolysis for mammalian GRP94 have been shown to be essential for in vivo chaperone activity (Ostrovsky et al., 2009). A recent study also showed that knockdown of GRP94 only selectively induces a subset of UPR-related chaperones, and such a response cannot be rescued by an ATPase-deficient GRP94 form (Eletto et al., 2012), therefore leading the authors to propose that cells monitor the activity state of GRP94. We hypothesize that the enhanced ATP-binding and hydrolysis activity of HSP90.7Δ2, albeit by only 2-fold, may already signal that there is no need for the upregulation of certain specific ER-localized chaperones or foldases (Gupta and Tuteja, 2011) upon ER stress, thus leading to hypersensitivity, although the hallmark protein BiP is still upregulated (Fig. 7A).

We also observed that overexpression of HSP90.7 helped seedlings to resist a high concentration of Ca2+ and heat-shock stress. This agrees with previous studies on non-FLAG-tagged AtHSP90.7 (Song et al., 2009a) and on an orchard grass ER-localized HSP90, which helps yeast resist heat-shock stresses (Cha et al., 2009). Since the ER-localized HSP90 parologue is one of the few abundant Ca2+-binding and storage proteins (Macer and Koch, 1988), overexpression of wild-type HSP90.7 may help Ca2+ storage and alleviate high Ca2+ toxicity. Surprisingly, expression of HSP90.7Δ2 did not help resistance against high Ca2+ stress and instead also exerted a dominant-negative effect, similar to that in tunicamycin resistance. The aforementioned mechanism that monitors the cellular HSP90.7 ATPase activity may also work in the Ca2+ stress and signalling pathway. It should be noted that, although the charged linker region between the N-terminal and middle domains binds Ca2+ and regulates cellular Ca2+ storage as shown for mammalian GRP94 (Biswas et al., 2007), the role of the charged region in the HSP90.7 middle domain in directly modulating cellular Ca2+ binding and storage capacity requires further investigation. Finally, we screened a small set of HSP90.7 interactors (Supplementary Table S1) and demonstrated that the charged region in the HSP90.7 middle domain was not required for binding three of the candidate client proteins by a yeast two-hybrid assay (Fig. 7C). Our study therefore provides potential targets to further investigate the role of HSP90.7 in modulating cellular Ca2+ homeostasis and/or the tunicamycin resistance.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** N-terminal domain sequence alignment of ER-localized AtHSP90.7 with HSP90 homologues.

**Supplementary Fig. S2.** Amino acid sequence alignment and modelled 3D structure of HSP90.7 highlighting the highly charged region in the middle domain.

**Supplementary Fig. S3.** Transgenic plants expressing HSP90.7Δ2 do not show significant growth defects under normal growth conditions.

**Supplementary Fig. S4.** CIGRP94 prevents heat-induced aggregation of citrate synthase while purified GFP protein does not.

**Supplementary Fig. S5.** Immunoblotting of HSP90.7 proteins from homozygous transgenic lines.

**Supplementary Fig. S6.** Transgenic seedlings expressing HSP90.7Δ2 do not show better heat-shock resistance than wild type.

**Supplementary Table S1.** HSP90.7 interactors identified by yeast two-hybrid assay using the middle and C-terminal domains as bait.

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**References**


