Characterization of orchardgrass p23, a flowering plant Hsp90 cohort protein

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Received: 28 July 2008 / Revised: 18 August 2008 / Accepted: 20 August 2008 / Published online: 18 September 2008
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Abstract p23 is a heat shock protein 90 (Hsp90) co-chaperone and stabilizes the Hsp90 heterocomplex in mammals and yeast. In this study, we isolated a complementary DNA (cDNA) encoding p23 from orchardgrass (*Dgp23*) and characterized its functional roles under conditions of thermal stress. *Dgp23* is a 911 bp cDNA with an open reading frame predicted to encode a 180 amino acid protein. Northern analysis showed that expression of *Dgp23* transcripts was heat inducible. *Dgp23* has a well-conserved p23 domain and interacted with an orchardgrass Hsp90 homolog in vivo, like mammalian and yeast p23 homologs. Recombinant *Dgp23* is a small acidic protein with a molecular mass of approximately 27 kDa and *pI* 4.3. *Dgp23* was also shown to function as a chaperone protein by suppression of malate dehydrogenase thermal aggregation. Differential scanning calorimetry thermograms indicated that *Dgp23* is a heat-stable protein,

capable of increasing the T_m of lysozyme. Moreover, overexpression of *Dgp23* in a yeast p23 homolog deletion strain, $\Delta sbal1$, increased cell viability. These results suggest that *Dgp23* plays a role in thermal stress-tolerance and functions as a co-chaperone of Hsp90 and as a chaperone.

Keywords Chaperone · Co-chaperone · Hsp90 · p23 · Orchardgrass · Heat stress · Thermotolerance

Introduction

Heat shock protein 90 (Hsp90) is an abundant, cytosolic molecular chaperone (Lai et al. 1984), and its expression is dramatically increased when cells are exposed to various environmental stresses (Somji et al. 2002). Hsp90 helps other proteins avoid stress-induced misfolding pathways that produce inactive or aggregated conformations and promotes folding to the native conformation (Yonehara et al. 1996; Buchner 1999). Hsp90 also functions in heterocomplexes with several co-chaperones or modulators (<http://www.picard.ch/downloads/Hsp90interactor.pdf>). The well-studied model for co-chaperones function comes from the interaction of Hsp90 with steroid receptors in mammals. Hsp90, Hsp70, Hop (an adaptor protein in Hsp90/Hsp70 chaperone machinery), Hsp40, and p23 are the minimum components necessary for assembling stable receptor–Hsp90 heterocomplex (Dittmar et al. 1997; Kosano et al. 1998).

In mammals, only Hsp90 and Hsp70 are apparently required for opening the steroid binding cleft in the hormone receptor–Hsp90 heterocomplex, and co-chaperones, Hop, Hsp40, and p23, increase the efficiency of heterocomplex (Dittmar et al. 1997; Morishima et al. 2003). In plants, the presence of Hsp90 heterocomplex has been detected using a rabbit reticulocyte folding system and wheat germ extract.

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Wheat Hsp90 formed a complex with a mouse hormone receptor in the presence of mammalian p23 (Hutchison et al. 1995). In addition, plant Hsp70 and plant Hop exhibited the same functional abilities as the homologous animal proteins to form a receptor–Hsp90 heterocomplex (Stancato et al. 1996; Zhang et al. 2003). Similarly, rape Hsp90 exhibited the same activity as rabbit Hsp90 in assembly with rabbit Hsp70/Hsp40, human Hop, and human p23 (Dittmar et al. 1997). Thus, it is believed that the Hsp90/Hsp70 heterocomplex machinery of plants is similar to that of animals.

p23 protein is well conserved from yeast to human as one of the Hsp90 co-chaperones. It was first identified as a component of the progesterone receptor complex and is known to be an Hsp90 binding protein (Smith et al. 1990; Johnson et al. 1994). p23 binds directly to Hsp90 in the absence of substrate, but not in the absence of ATP (Sullivan et al. 2002). p23 binding to Hsp90 is apparently involved in the conformational change, which occurs by dimerization through the nucleotide binding domains of Hsp90 with association of ATP (Chadli et al. 2000; Pratt and Toft 2003). After binding to Hsp90, p23 stabilizes the conformational state of Hsp90 by inhibition of ATP hydrolysis (McLaughlin et al. 2006). In the Hsp90–substrate complex, binding of p23 causes the dissociation of substrate from the complex in an ATP-dependent manner (Johnson and Toft 1995; Young and Hartl 2000). p23 also stabilizes the steroid receptor–Hsp90 complex and enhances the proportion of complex (Chadli et al. 2000; Pratt and Toft 2003). Human p23 has chaperone activity and binds with denatured proteins to maintain them in a folded state (Weikl et al. 1999). The C-terminal region of p23 is known to be important for this chaperone property, but is not necessary for interaction with Hsp90 (Weaver et al. 2000). Thus, p23 may have a dual role to modulate the activity of Hsp90 in the heterocomplex as a co-chaperone of Hsp90 and to prevent protein denaturation as a chaperone (Bose et al. 1996; Dittmar et al. 1997). However, the function of plant p23 has not been elucidated.

Although sequences of plant p23 homologs, including rice, corn, *Arabidopsis*, and moss have been deposited in GenBank™, no other information on these proteins is available to date. Here, we report the identification and functional characterization of an orchardgrass p23 as a co-chaperone of Hsp90 and as a chaperone.

Materials and methods

Plant material and heat treatments

Orchardgrass (*Dactylis glomerata* L. cv. Potomac) seeds were purchased from Snow Brand Seed Co. Ltd. (Sapporo,

Japan). The seedlings were grown in a growth chamber at 25°C and 16-h light/8-h dark cycles with constant shaking (120 rpm) in 250 ml Erlenmeyer flasks containing 100 ml liquid B5 medium. Two-week-old seedling were heated at various temperatures in a water bath for 1 h and then immediately frozen in liquid nitrogen. RNA was extracted from each sample and subjected to differential display RT-PCR (DD-RT-PCR). For time course analysis, same aged seedlings were exposed at 35°C for the times indicated in the “Results”. For recovery from heat stress, seedlings exposed for 1 h to 35°C were allowed to recover for 5 h at 25°C. For construction of a heat-induced complementary DNA (cDNA) library, total RNA was isolated from seedlings, which had been subjected to 35°C for 1 h.

Screening of *Dgp23* and *DgHsp90* cDNAs

Heat-induced DNA fragments were isolated from heat-treated 2-week-old orchardgrass seedlings by DD-RT-PCR using a RNAimage kit (Genhunter) and sequenced using an ABI Prism 310 genetic analyzer (Perkin-Elmer). Two fragments induced by heat treatment showed high sequence similarity with rape *p23* and barley *Hsp90*, respectively, as determined by nucleotide BLAST search (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Full-length cDNAs encoding orchardgrass *p23* (*Dgp23*) or *Hsp90* (*DgHsp90*) homologs were screened from an orchardgrass λZAPII cDNA library (Stratagene) by using each DNA fragment as a probe. The nucleotide and deduced amino acid sequences were analyzed using BLAST and BioEdit (Hall 1999).

Northern blot analysis

Total RNA from heat-treated orchardgrass seedlings was extracted according to the phenol/LiCl method described previously (Olszewski et al. 1989). Twenty micrograms of total RNA was fractionated on a 1.2% formaldehyde agarose gel and blotted onto Hybond-N⁺ nylon membrane (Amersham) with 10× SSC. Blots were hybridized with a ³²P-labeled full-length *Dgp23* using Megaprime DNA labeling system (Amersham).

Alignment of amino acid sequences and phylogenetic analysis of *Dgp23*

The deduced amino acid sequence of *Dgp23* was aligned using the multiple alignment method of ClustalX (Chenna et al. 2003) and GeneDoc (Nicholas et al. 1997) with amino acid sequences of p23 homologs from various organisms. All sequences were retrieved from the GenBank™ database after a homology search using BLAST. A phylogenetic tree was constructed using algorithms from the ClustalW in EMBL-EBI website (<http://www.ebi.ac.uk>).

Protein–protein interaction assay by yeast two-hybrid analysis

Vectors for yeast two-hybrid analysis were purchased from Stratagene. Open-reading frames (ORF) of *Dgp23* and *DgHsp90* were cloned into the pBD-GAL4 Cam vector and the pAD-GAL4-2.1, respectively. The bait (*Dgp23*) and the prey plasmids (*DgHsp90*, GenBank accession no. EU030446) were transformed into a pJ694A yeast strain according to the instruction manual. Yeast transformants were selected on glucose-based synthetic minimal medium (SD; 0.67% yeast nitrogen base, 2% glucose, amino acids dropout solution) deficient in tryptophan, leucine, and histidine. Interaction between *Dgp23* and *DgHsp90* was confirmed by the expression of *lacZ* reporter gene. Yeast cells harboring constructs of pBD-wt::pAD-wt and pLaminC::pAD-wt were used as positive and negative controls, respectively.

Expression and purification of Dgp23 protein

The ORF of *Dgp23* was cloned into pET41a to produce recombinant *Dgp23* with a glutathione *S*-transferase (GST) tag. The plasmid was transformed into *Escherichia coli* BL21 (DE3) for protein expression. *E. coli* cells harboring the plasmid were grown at 30°C until the OD₆₀₀ approached 0.8. Protein expression was induced by the addition of 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) for 3 h, and the cells then were harvested by centrifugation. After harvesting, the cells were resuspended in phosphate-buffered saline (PBS) and disrupted by sonication. After centrifugation, the resulting supernatant was applied onto a glutathione sepharose 4B affinity column. GST fused *Dgp23* protein was eluted using 10 mM reduced glutathione. The eluted protein was subjected to thrombin digestion (16 h/4°C) to remove the GST tag. Protein concentration was determined using a Bio-Rad protein assay. For use in a chaperone assay, *Arabidopsis* malate dehydrogenase (MDH) with a 6 $\times$ His-tag was also expressed and purified in a similar manner.

Preparation and affinity purification of antibody

A polyclonal antibody was raised against purified recombinant *Dgp23* in a New Zealand white rabbit. Affinity purification of the antibody was carried out to obtain a monospecific antibody. Purified *Dgp23* protein subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was transferred to a PVDF membrane (Immobilon-P, Millipore) and visualized by staining the membrane in a solution of 0.1% Ponceau S dye and 5% acetic acid. The area of the membrane blot corresponding to the *Dgp23* band was excised, de-stained, and blocked with 6% (w/v) powdered skimmed milk in PBS for 1 h. The membrane strip was then

incubated with the rabbit antiserum to *Dgp23* for 1 h (dilution 1/100) and then washed six times with PBS. A bound antibody was eluted with 0.2 M glycine (pH 2.5) and immediately neutralized with 2 M Tris-HCl, pH 8.0.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF)

SDS-PAGE was performed as described by Laemmili (1970). Proteins were separated in 12% acrylamide gel and then visualized by Coomassie brilliant blue staining. Isoelectric focusing (IEF) was performed in a vertical system with pH range 2–6 containing 5% polyacrylamide, 0.4% pH 3–10 ampholyte, 2% pH 4–6.5 ampholyte, and 8 M urea as described by Polenta et al. (2007). *Dgp23* protein (10 μ g) and *pI* markers were separated in the IEF gel. After electrophoresis, the gel was soaked in 10% trichloroacetic acid (TCA) for 10 min and subsequently in 1% TCA overnight and then stained with Coomassie brilliant blue.

Chaperone assay

Chaperone activity of *Dgp23* protein was assayed by measuring its capacity to suppress thermal aggregation of MDH (Basha et al. 2004). Aggregation of 0.3 μ M MDH in 40 mM HEPES, pH 7.5, was monitored in the absence or presence of *Dgp23* by measuring the absorbance at 340 nm using a Beckman DU-800 spectrophotometer attached to a thermostatic cell holder assembly at 45°C. *E. coli* thio-redoxin (EcTrx) was used as a positive control (Kern et al. 2003). Another chaperone-like activity assay was performed in SDS-PAGE as described by Kim et al. (2003), with a minor modification. An amount of 2 μ M *Dgp23*, MDH, or *Dgp23*–MDH mixture was incubated at the indicated temperature for 15 min and then cooled on ice. After centrifugation at 12,000 rpm for 15 min, the supernatant was separated in a 18% SDS-PAGE gel and transferred onto nitrocellulose membrane (Hybond-C extra, Amersham) using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). MDH and *Dgp23* were detected by the anti-His antibody (Calbiochem) and anti-*Dgp23* antibody, respectively. Peroxidase-conjugated anti-mouse IgG (Calbiochem) and anti-rabbit IgG (KPL) were used as secondary antibodies for MDH and *Dgp23*, respectively. The antigen–antibody complexes were detected using the enhanced chemiluminescence detection system (EZ-ECL, Biological Industries).

Differential scanning calorimetry (DSC)

DSC was performed with a MicroCal VP-DSC microcalorimeter with 0.52 ml cell volume. An amount of 10 μ M lysozyme or *Dgp23*–lysozyme mixture was examined in

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B

20 mM Tris-HCl, pH 8.0, 150 mM NaCl. After degassing for 15 min at 25°C, each sample was kept under a constant pressure at 26.6 psi. Data were collected at a heating rate of 60°C/h and analyzed using the MicroCal Origin DSC software package.

Thermotolerance assay

A thermotolerance assay was modified as described by Moon et al. (2002). Yeast wild-type, BY4741 (*MATa*; *his3ΔI*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*), and yeast p23

Fig. 1 Comparison of orchardgrass p23 amino acid sequence with homologs from other organisms. **a** Alignment of orchardgrass p23 protein (accession no. DQ172836) with homologs from corn (ACF82936), rice (NP_001050241), rape (AAG41763), grape (CAO46199), *Arabidopsis* (CAC16575), japanese cedar (BAE92292), moss (XP_001768873), budding yeast (P28707), fission yeast (Q11118), human (Q15185), mouse (AAD39543), and chicken (B56211). Numbers at the right indicate amino acid residue position. Fully identical residues are shaded in black; six or seven identities in gray; and five identities in light gray. The conserved p23 domain is represented as a box; a solvent-accessible surface as a bar below the sequence; and conserved identical residues as an asterisk above the sequence. **b** Phylogenetic relationships between Dgp23 and other p23 homologs. Phylogenetic tree shows a graphical representation of evolutionary relationships and was constructed by using EBI-ClustalW algorithm

homolog deletion strain, Δ *sba1* (*MATa*; *his3 Δ 1*; *leu2 Δ 0*; *met15 Δ 0*; *ura3 Δ 0*; *YKL117w::kanMX4*), were purchased from EUROSCARF. A pYES vector and a pYES with Dgp23 ORF (pYES-Dgp23) were each transformed into Δ *sba1* strain. Wild-type and Δ *sba1* yeast cells transformed with either pYES or pYES-Dgp23 were incubated for 1 day in liquid-rich medium (2% peptone, 1% yeast extract) with 2% galactose to induce expression from the *GAL1* promoter. For the spotting assay, ten-fold serial dilutions of yeast cells from 2×10^8 cells/ml culture were spotted on rich medium with 2% galactose (YPG) and incubated at 30°C or 37°C for 3 days. In addition, the same serial dilutions of yeast cells were subjected to 25°C or 50°C for 30 min and immediately kept on ice for 5 min. These pre-heat-treated yeast cells were spotted on YPG medium and incubated at 30°C for 3 days. To measure the survival rate of Δ *sba1* cells overexpressing Dgp23, 100 μ l of cell serial dilutions were heat-treated at 50°C for 0, 10, 20, or 30 min and then plated onto YPG medium. Colonies were counted after 2 days of incubation at 30°C. The percentage viability was calculated relative to the control cells treated at 25°C.

Results

Isolation and sequence analysis of Dgp23

The transcription profiles of heat-treated 2-week-old orchardgrass were compared by DD-RT-PCR to screen heat-inducible genes. One of the partial cDNAs induced by heat treatment was identified by a NCBI-BLAST search as a rape p23 homolog. Although the sequences of p23 homologs from several plants are deposited in GenBank™, functional characterization of the plant p23 proteins has not yet been reported. With the aim of elucidating the biological roles of plant p23, we isolated a full-length cDNA from an orchardgrass cDNA library using the partial p23 fragment as a probe and named it orchardgrass p23 (*Dgp23*, GenBank accession no. DQ172836). The full-

length *Dgp23* gene encodes 911 nucleotides, with an ORF encoding 180 amino acids. The deduced amino acid sequence has a putative conserved p23 domain situated between amino acid residues 5 to 112 that contains five conserved residues, W, K, W, L, and W, at positions 9, 80, 86, 89, and 107 of Dgp23, respectively (Fig. 1a; Johnson et al. 1994; Zhao et al. 2006).

We compared the Dgp23 sequence with other p23 homologs in the GenBank™ database. While Dgp23 shows a low sequence identity with other vertebrate (26% identity) and yeast p23 proteins (28%), it shares a higher sequence identity (over 50%) with putative p23 proteins in flowering plants. p23 proteins in monocot plants (rice 78% and corn 77%) display a higher sequence identity with Dgp23 than those in dicots (grape 61%, rape 55%, and *Arabidopsis* 50%). Although the p23 domain is highly conserved, C-terminal sequences are considerably divergent among p23 proteins. From phylogenetical analysis, p23 proteins are clearly divided into three different kingdoms, plantae, animalia, and fungi. In addition, p23 proteins are classified into flowering and flowerless plants in plant kingdom. p23 proteins are well conserved and differentiated through evolution (Fig. 1b).

Expression of Dgp23 under heat-stress conditions

Expression of *Dgp23* gene under heat-stress conditions was examined by Northern blot analysis. As shown in Fig. 2a, *Dgp23* transcripts were not detected at the control temperature of 25°C, but were induced at temperatures over 30°C. The accumulation of *Dgp23* reached a maximum level at

Fig. 2 Northern blot analyses of *Dgp23*. **a** Accumulation of *Dgp23* transcripts after heat treatment at various temperatures for 1 h. **b** Time-course accumulation of *Dgp23* transcripts during heat treatment at 35°C and subsequent recovery after heat treatment. Ethidium bromide staining of the RNA gel was used to show equal loading

Fig. 3 In vivo interaction analysis of Dgp23 and DgHsp90 by yeast two-hybrid system. **+His** Transformants selected on SD medium lacking tryptophan and leucine; **−His** transformants selected on SD medium lacking tryptophan, leucine, and histidine; **X-Gal** β-galactosidase assay for the protein–protein interaction

35°C and decreased significantly at 40°C. The level of *Dgp23* transcripts changed during heat treatment at 35°C (Fig. 2b). *Dgp23* was barely detected within 30 min and was most abundantly expressed after 2 h, and its expression subsequently declined. To examine the change of expression after recovery from heat stress, seedlings exposed to 35°C for 1 h were allowed to recover at 25°C for 5 h. In samples subjected to recovery conditions, transcripts of *Dgp23* were barely detected. This temporal expression

pattern suggests that Dgp23 functions in the relatively early stages of heat stress.

Interaction between Dgp23 and DgHsp90

Deletion studies and chemical cross-linking analysis have shown that human/yeast p23 interacts with Hsp90 in vitro and that this interaction is ATP dependent (Chadli et al. 2000; Prodromou et al. 2000). An in vivo association between human Hsp90 and human p23 was also revealed by yeast two-hybrid analysis (Obermann et al. 1998). This association stabilizes the Hsp90 heterocomplex and releases the client proteins from the complex (Young and Hartl 2000). Yeast two-hybrid analysis was performed using Dgp23 and DgHsp90 to investigate the association between plant homologs of Hsp90 and p23. As shown in Fig. 3, the interaction between Dgp23 and DgHsp90 was confirmed by growth in selection medium lacking leucine, tryptophan, and histidine and expression of *lacZ* reporter gene. This result suggests that Dgp23 associates with DgHsp90 in vivo and may function as a co-chaperone of Hsp90 in the Hsp90 heterocomplex.

Purification and gel electrophoresis of Dgp23 protein

Dgp23 protein was produced using an *E. coli* expression system and purified using glutathione sepharose 4B affinity column and gel filtration chromatography. The molecular weight of purified Dgp23 was estimated to be 27 kDa by SDS-PAGE, which is similar to mammalian (Johnson et al. 1994; Weaver et al. 2000) and yeast p23 (Fang et al. 1998) proteins (Fig. 4a). Dgp23 exhibited a *pI* of 4.3 (Fig. 4b),

Fig. 4 SDS-PAGE and IEF analysis of recombinant Dgp23. **a** SDS-PAGE (12%) of protein samples from the purification steps of Dgp23. **Lane 1** Molecular weight markers; **lane 2** crude extracts after IPTG induction; **lane 3** eluate from affinity chromatography; **lane 4** purified Dgp23 after thrombin digestion. **b** IEF of purified Dgp23. Carbonic

anhydrase (*pI* 6.0), bovine serum albumin (5.6), ovalbumin (4.6), and soybean calmodulin (4.1) as IEF standards (*M*), and 10 μg of purified Dgp23 was subjected to IEF gel. All protein bands were stained with Coomassie brilliant blue

which is comparable to those of well-characterized p23 homologs (Johnson et al. 1994; Fang et al. 1998).

Chaperone activity of Dgp23

It has been reported that p23 protein in yeast and mammals mainly functions as a co-chaperone in Hsp90 heterocomplex. Furthermore, human p23 has an additional function as a chaperone (Bose et al. 1996). However, Hop, another human co-chaperone of the Hsp90 heterocomplex, does not have this function (Bose et al. 1996). To examine the chaperone function of Dgp23, we measured the aggregation rate of MDH at 45°C in the absence or presence of Dgp23. Dgp23 suppressed the heat-induced aggregation of MDH in a dose-dependant manner (Fig. 5a). The maximum aggregation protection (over 90%) of MDH under heat stress was achieved in a molar ratio of one subunit of MDH to five subunits of Dgp23. Thioredoxin, which functions as a chaperone (Kern et al. 2003), showed similar activity to that of Dgp23. In the absence of MDH, the light scattering value of Dgp23 did not change at 45°C, indicating that Dgp23 was acting as a heat-stable chaperone protein.

Chaperone proteins facilitate protein folding and maintain substrates in soluble form (Huang et al. 2001). We performed Western blot analysis using anti-Dgp23 antibody for Dgp23 or anti-His antibody for MDH to examine whether Dgp23 increases the solubility of MDH under heat treatment conditions. Dgp23, MDH, or the same molar ratio of Dgp23 and MDH mixture was exposed to various temperatures for 15 min. Heat-denatured proteins were removed by centrifugation, and the supernatants were subjected to SDS-PAGE. While Dgp23 remained soluble up to 50°C, MDH was stable only up to 35°C. However, in the MDH and Dgp23 mixture, MDH stability was increased up to 40°C (Fig. 5b). These data indicate that Dgp23 not only protects MDH from heat-induced aggregation but increases its solubility at high temperature.

Heat stability by differential scanning calorimetry (DSC) analysis

Proteins are often denatured and aggregated by heat stress. Thermal damage of proteins has been proposed as a trigger for Hsps activation in the cell (Li et al. 1991). Hsps effectively prevent the aggregation of thermally denatured proteins (Pivovarova et al. 2005). In order to examine the effect of Dgp23 on thermal denaturation of substrate protein, we performed DSC analysis using egg-white lysozyme as a substrate (Babu and Bhakuni 1997; Fig. 6). The heat sorption peak (T_m) for lysozyme was measured as 73.39°C. When a mixture of lysozyme and Dgp23 was subjected to DSC, the peak was observed at 97.23°C, which was much higher than that for lysozyme alone. This

indicates that Dgp23 is a heat-stable protein that can prevent the aggregation of lysozyme.

Complementation of Dgp23 in a Sba1 deleted yeast mutant

Sba1 is known as a yeast homolog of human p23 (Fang et al. 1998) and is expressed constitutively during glucose deficiency (Fairhead and Dujon 1994). Therefore, we examined whether Dgp23 could increase the thermotolerance of yeast in vivo using a Sba1 deletion yeast strain ($\Delta sba1$). Although the growth rate of $\Delta sba1$ harboring Dgp23 ($\Delta sba1$ +Dgp23) was the same as that of wild-type or $\Delta sba1$ at 30°C, $\Delta sba1$ +Dgp23 cells grew better than other cells at 37°C (Fig. 7a). When heat-treated at 50°C for 30 min prior to spotting onto plates, $\Delta sba1$ +Dgp23 cells showed better cell viability than wild-type or $\Delta sba1$ cells after a 3-day incubation at 30°C (Fig. 7b). The number of

Fig. 5 Chaperone assay of recombinant Dgp23. **a** Chaperone activity of Dgp23. MDH (0.3 μM) was incubated in the absence (open circle) or presence of 0.3 μM (closed square), 0.6 μM (closed diamond), 1.5 μM (closed triangle) Dgp23 or 0.3 μM EcTrx (open diamond). Dgp23 (0.3 μM) was incubated without MDH (open square). **b** p23 protection against MDH thermal aggregation. Two micromolar MDH, p23 or MDH-p23 mixture, was heat-treated at the indicated temperatures for 15 min. After centrifugation, supernatants were analyzed by 18% SDS-PAGE. The blots of each gel were hybridized with anti-His antibody for MDH and anti-Dgp23 antibody for Dgp23

Fig. 6 DSC. Thermal unfolding of lysozyme (closed square) and lysozyme–Dgp23 mixture (closed circle) were measured in DSC. The inset shows the melting temperature

Δ *sba1*+Dgp23 colonies surviving after heat treatment was more than that of wild-type or Δ *sba1* (Fig. 7c). Therefore, considering that Δ *sba1* exhibited mild growth defects at high temperatures (Fang et al. 1998), these data show that Dgp23 increased the heat tolerance of yeast cells.

Discussion

Hsp90 is one of the most thoroughly characterized chaperones and is known to play a role in numerous cellular

processes, including protein folding, cell cycle, apoptosis, and signal transduction, by cooperating with various client proteins such as kinases, transcription factors, and regulating enzymes. During these processes, Hsp90 is differentially modulated by many co-chaperones, TPR proteins (Hop, Hip, FKBP, PP5, and others), Hsp40, Cdc37, Aha1, and p23 (Caplan et al. 2003). Hsp90 also plays important roles in development, innate immunity, and buffering of genetic variation in plants (Queitsch et al. 2002; Sangster and Queitsch 2005). Plant Hsp90 acts in cooperation with other co-chaperones such as FKBP42 (Kamphausen et al. 2002), SGT1 (Muskett and Parker 2003), RAR1 (Takahashi et al. 2003), RPM1 (Hubert et al. 2003), Hop (Zhang et al. 2003), PP5 (de la Fuente van Bentem et al. 2005), FKBP62, and FKBP65 (Aviezer-Hagai et al. 2007). Nonetheless, p23, a well-conserved co-chaperone of Hsp90 heterocomplex chaperone machinery in mammals, is not yet characterized in plants.

Our study provides the first characterization of plant p23, which is involved in heat stress. Dgp23 has hydrophilic residues in the C-terminal region as seen in human p23, although the residues are not identical (Weikl et al. 1999). The unstructured C-terminal region of human p23 is important for chaperone activity but not for binding to Hsp90 (Weikl et al. 1999; Weaver et al. 2000). The conserved domain (CD) structure and phylogenetic features of Dgp23 suggest that Dgp23 encodes a p23 homolog in orchardgrass. Recently, SGT1 was characterized as a co-chaperone of Hsp90. *Arabidopsis* SGT1 has a CS (CHORD-SGT1) domain, similar to human p23 protein, and plays an important role in R-gene mediated disease resistance (Takahashi et al. 2003). Although *Arabidopsis*

Fig. 7 Thermotolerance of yeast cells overexpressing Dgp23. Yeast spot assays (a and b) and survival rate (c) were carried out to determine the thermotolerance of yeast transformants. **a** Wild-type (BY4741, WT), Δ *sba1* (pYES alone), and Δ *sba1*+Dgp23 were incubated at 30°C or 37°C for 3 days. **b** Serially diluted yeast cells were exposed to 25°C or

50°C for 30 min before spotting and incubated at 30°C for 3 days. **c** Survival rate after heat treatment at 50°C for the indicated times. After 2 days incubation at 30°C, visualized colonies were counted to calculate the percentage survival relative to yeast cells treated at 25°C

SGT1 contains a p23-like domain, it exhibits low sequence identity (15% with human p23), molecular weight, pI, and CD structure, which are characteristics that differ from those commonly observed in other p23 proteins. Thus, the existence and function of p23 homolog in plants has been questionable to date. We investigated the expression level of Dgp23 in response to abiotic stresses. Levels of *Dgp23* transcripts were highly increased by heat stress. However, the transcripts were not induced by cold, dehydration, or salt (data not shown). Some plant co-chaperones of Hsp90 such as FKBP and Hop were also induced by heat stress (Zhang et al. 2003; Aviezer-Hagai et al. 2007), and their expression is likely linked to Hsp90 function under stress conditions.

p23 associates with Hsp90 at the last stage of the Hsp90 heterocomplex assembly and stabilizes the glucocorticoid receptor–Hsp90 complex (Dittmar et al. 1997). Dgp23 has a conserved peptide sequence of WPRLXKX (residues 86–92), which is a signature of p23 proteins. Weaver et al. (2000) defined this region as the solvent-accessible surface of a cavity having polar side chain walls and an apolar floor, which may accommodate a side chain from Hsp90 or substrates. This observation suggests that Dgp23 can interact with Hsp90, but physical interaction assays are required to confirm this. Thus, we examined the interaction between Dgp23 and DgHsp90, which were screened from an orchardgrass cDNA library by yeast two-hybrid analysis. The interaction between Dgp23 and DgHsp90 was confirmed (Fig. 3), indicating that Dgp23 binds to Hsp90 in vivo and may function as a co-chaperone of Hsp90 in plants in a manner similar to human p23.

Hsp100, Hsp90, Hsp70, and small Hsps are well-known molecular chaperones in eukaryotes (Miernyk 1999; Kim et al. 2007). Some co-chaperones such as FKBP and p23 in human also function as molecular chaperones, but Hop does not exhibit this activity (Bose et al. 1996; Buchner et al. 1998). Hop is believed to function only as an adaptor protein to organize Hsp90 and Hsp70 in both mammals and plants (Chen and Smith 1998; Zhang et al. 2003). To characterize the function of plant p23 as a molecular chaperone, we measured the chaperone activity of Dgp23. Dgp23 suppressed over 90% of MDH heat-dependent aggregation at a ratio of 1:5 subunits of MDH to Dgp23, and the activity was increased in a dose-dependant manner similar to that of human p23. Human p23 suppressed the aggregation of citrate synthase by 50% and 90% when mixed in a molar ratio of 1:8 (citrate synthase/human p23; Bose et al. 1996) and 1:5, respectively (Weaver et al. 2000). In the absence of MDH, Dgp23 was stable under thermal conditions. Molecular chaperones are heat-stable proteins and protect substrates against thermal aggregation (Fu and Liang 2003; Kim et al. 2004). The chaperone function of Dgp23 was further examined by DSC analysis (Fig. 6). The

stability of heat-exposed lysozyme was increased by the presence of Dgp23. These results support the idea that Dgp23 functions as a chaperone.

When cells are exposed to heat stress, several classes of Hsps accumulate and function as molecular chaperones. In the absence of Hsps, thermotolerance was reduced (Solomon et al. 1991; Schirmer et al. 1994). Although p23 is not an Hsp, Dgp23 exhibits chaperone activity in vitro. We tested whether Dgp23 could confer thermotolerance in vivo by examining in vivo thermotolerance in a *Sba1* deletion yeast strain, $\Delta sba1$, overexpressing Dgp23. Fang et al. (1998) reported that $\Delta sba1$ cells grew slowly at both low and high growth temperatures compared to wild-type cells, while the cells did not change in growth phenotype. However, $\Delta sba1$ cells overexpressing Dgp23 showed better cell growth and viability against heat treatments. Major molecular chaperones such as Hsp101 (Queitsch et al. 2000), Hsp70 (Lee and Schöffl 1996), and small Hsp (Kim et al. 1997) have been reported to confer thermotolerance, but co-chaperones have not been well characterized. Although transcription levels of Hsp90 co-chaperones Hop and FKBP are induced by heat stress (Zhang et al. 2003; Aviezer-Hagai et al. 2007), only FKBP has a chaperone property (Bose et al. 1996), and FKBP20 overexpression in yeast showed thermotolerance (Nigam et al. 2008). Therefore, co-chaperones that exhibit chaperone activity may confer thermotolerance to cells. However, further investigation is required to determine whether in vitro chaperone activity is indicative of in vivo thermotolerance activity.

In conclusion, our results show that heat-inducible Dgp23, a p23 protein in plants, is a co-chaperone of Hsp90 and suppresses the thermal aggregation of substrates with its intrinsic chaperone activity. The chaperone activity of Dgp23 may confer thermotolerance in vivo.

Acknowledgements This research was supported by a grant from the KRF (2008-531-F00006), the EB-NCRC (R15-2003-012-01001-0 to CDH and DS, and R15-2003-002-01001-0 to KHL) funded by MEST, and the TDPAF (201056-03-3-SB010) funded by MOAF, Korea. JYC, NE, MHJ, and MS were supported by scholarships from the BK21 program, MEST, Korea.

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