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ORIGINAL ARTICLE

Identification of heat shock-inducible transcripts and Hop (Hsp-organizing protein)-interacting proteins in orchardgrass (*Dactylis glomerata* L.)

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Abstract
Plants are continuously exposed to temperature changes and biotic/abiotic stresses. Current global warming dynamics cause extreme temperature fluctuations that severely affect crop productivity. Orchardgrass (*Dactylis glomerata* L.) is one of the most important perennial grasses adapted for cool temperatures. Orchardgrass displays rapid establishment, vigorous growth, and rapid recovery after cutting or grazing, but it is sensitive to heat stress. In this study, we isolated 23 heat shock-inducible partial gene fragments from orchardgrass using a differential-display RT-PCR and a reverse northern blotting. Those full-length cDNAs encode major molecular chaperones (Hsp90s, Hsp70, and small Hsp) and Hsp90 co-chaperones (Hop, p23, and Aha) were identified from a heat-treated orchardgrass cDNA library and analyzed those expressions by northern blot analysis. In addition, we further characterized Hop, which plays as an important adapter that organizes the Hsp90 and Hsp70 complex in the Hsp90-associated heteromultimeric chaperone complex. Orchardgrass *Hop* (*DgHop*) transcript levels were enhanced by heat shock stress and dramatically reduced by cold, salt, or dehydration stress. DgHop contains three conserved tetratricopeptide repeat domains, which are involved in interactions with major Hsps. Yeast two-hybrid and pull-down analysis indicated that the major DgHop client proteins were Hsp90 and Hsp70, and DgHop directly interacted with DgHsp90 and DgHsp70. Our data provide new genetic information on heat shock responses in orchardgrass. We suggest that this information contribute to generating heat shock-tolerant forage crops.

Keywords Chaperone · Co-chaperone · Differential-display RT-PCR · Heat stress · Hsp-organizing protein · Orchardgrass

Introduction

Global warming and climate change is now recognized as a serious problem with the potential to threaten all living organisms on the Earth. The Intergovernmental Panel on Climate Change (IPCC; http://www.ipcc.ch) reports that global surface temperature increased 0.74 ± 0.18 °C (1.33 ± 0.32 °F) during the past 100 years. The IPCC report concludes that temperature increase since the mid-twentieth century is due to the increase in anthropogenic greenhouse gas emissions. The increased global temperature causes abiotic stresses in plants, such as heat, drought, and salinity stresses that have reduced the productivity for most of the major crops by more than 50% (Bray et al. 2000).

Orchardgrass (*Dactylis glomerata* L.) is one of the most widely cultivated, cool season perennial forage grasses in the world. It starts to grow in early spring, develops rapidly, has high productivity, and regenerates rapidly after cutting and grazing (Van Santen and Sleper 1996). Climate change due to global warming will affect winter survival and exacerbate summer depression of perennial forage crops. Bélanger et al. (2002) studied forage crop performance using predicted future climate models based on warming, and concluded that winter injury risks to perennial crops in eastern Canada will likely increase due to reductions in cold hardening and protective snow cover during cold periods, which will expose plants to

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killing frosts, soil heaving, and ice encasement. Winter hardening of perennial crops is closely related to summer depression (Van Santen and Sleper 1996). Most perennial forage crops such as orchardgrass are well adapted to cool seasons, with optimum growth at approximately 21 °C. These forage crops are susceptible to heat stress, and plant survival and productivity dramatically declines during warm seasons (Seo et al. 1988).

Heat stress can denature proteins and disrupt cellular function and/or structure. Heat stress also elicits cell signaling pathways and cellular responses that protect against heat-induced damage, including biosynthesis of heat shock proteins (Hsps), upregulation of antioxidant pathways, and activation of kinases and transcription factors. These signaling pathways and cellular responses have been identified using proteomic analysis, DNA microarrays, and subtractive hybridization (Wang et al. 2003; Qureshi et al. 2007; Tian et al. 2008). Hsps are a unique set of proteins that accumulate to high levels in response to sudden temperature increases (Vierling 1991). Most Hsps function as molecular chaperones. They bind to unfolded or denatured proteins, prevent aggregation, induce correct refolding, and facilitate proper cell function under high-temperature conditions (Wang et al. 2004). Five major Hsp families are classified according to their molecular masses: Hsp100/ClpB (eukaryotic/*E. coli* nomenclature), Hsp90/HtpG, Hsp70/DnaK, Hsp60/GroEL, and small Hsps (Wang et al. 2004). Hsp101, Hsp70, and small Hsps were verified to confer thermotolerance in several plants (Hong and Vierling 2001; Queitsch et al. 2000; Cho and Choi 2009; Kim et al. 2012).

Many Hsp genes play in abiotic stress tolerance and those roles in response of abiotic stresses have been identified in model plants and important crop species; however, Hsp genes and those roles of perennial forage crops have not been extensively identified and characterized. The orchardgrass genome has not been completely mapped and sequenced, and very little genomic information is available. In this study, we identified 23 highly expressed genes at the transcriptional level using mRNA differential display to screen heat shock-inducible genes in orchardgrass. We further characterized a gene-encoding Hsp-organizing protein (Hop), which is an important co-chaperone that assembles the heteromultimeric Hsp complex (Pratt and Toft 2003). In addition, we confirmed its partner proteins, DgHsp90 and DgHsp70 in orchardgrass using yeast two-hybrid analysis and pull-down assay.

Materials and methods

Plant material and stress treatments

Orchardgrass (*Dactylis glomerata* L. cv. Potomac) seeds were purchased from Snow Brand Seed Co., Ltd. (Sapporo, Japan). The seedlings were grown in 250 cm^3 Erlenmeyer flasks containing 100 cm^3 of liquid B5 medium in a growth chamber at 25 °C with 16 h light/8 h dark cycles and constant shaking (3.2*g*-force). Two-week-old seedlings were exposed to a range of temperatures (25, 30, 35, 40, 45, or 50 °C) in a water bath for 1 h, and then harvested for RNA extraction. To examine the time-dependent effects of heat shock stress, seedlings were exposed to 40 °C for the indicated time intervals. Seedlings also were exposed to cold (4 °C), salt (140 mM NaCl), and dehydration stress (air-dried on filter paper).

Measurement of ion leakage and total chlorophyll content

Electrolyte leakage was measured in aerial tissues of 2-week-old seedlings, which were floated on 10 cm^3 of deionized water and exposed to various temperatures (25−50 °C with 5 °C intervals) for 1 h in a water bath. Electrolyte leakage also was measured at room temperature after 2 h of shaking (3.2*g*-force) using a conductometer (Thermo Scientific). Total electrolyte leakage from aerial tissues was measured after autoclaving (105 °C for 15 min) and subsequent shaking (3.2*g*-force) for 16 h. Chlorophyll content was measured using aerial tissues of 2-week-old seedlings that were floated in 10 cm^3 of deionized water and exposed to various temperatures and times as described for electrolyte leakage experiments. Then, the treated tissues were soaked in 80% (v/v) acetone, and total chlorophyll content was measured using spectrophotometry as described previously (Ni et al. 2009). Data represent mean ± SE with three biological repeats.

Differential display RT-PCR

Differential display RT-PCR (DD-RT-PCR) analysis was performed using the mRNA differential display system according to the manufacturer's instructions (RNAimage, GenHunter). Total RNA was extracted from each orchardgrass seedling sample and subsequently treated with DNase I. DNase-free RNA was converted into first-strand cDNA using three different one-base-anchored H-T_{11}M primers (where M may be G, A, or C) and reverse transcriptase. The RT-PCR products were subjected to secondary PCR using the same H-T_{11}M primers and an arbitrary 13-base oligonucleotide with 185 kBq of α-[^{35}S]dATP. The secondary PCR products were separated on a 6% urea-denaturing sequence gel. The gel was dried under vacuum on a gel dryer at 80 °C for 1 h and visualized on X-ray film. The dried gel corresponding to differentially expressed bands was cut and soaked in 10 mm^2 of distilled water. DNA fragments were eluted from the gel by boiling and then precipitated with 3 M sodium acetate (pH 5.2), glycogen (10 mg cm^{-3}), and 100% absolute ethanol. The DNA was

re-amplified with the same set of primers and prepared for subsequent reverse northern blot analysis.

Reverse northern blot analysis

Reverse northern blot analysis was applied to isolate strong candidates (and also for reducing false clones) that are highly expressed by heat shock treatment from large sets of DNA fragments achieved by DD-RT-PCR. The re-amplified DNA fragments were immobilized by spotting of DNA fragment onto a nylon membrane using the 96-well Bio-Dot Microfiltration Apparatus (Bio-Rad). Total RNA (20 µg) from orchardgrass seedling samples as a probe was labeled with 185 kBq of α-[^{32}P]dATP, H-T_{11}M primers, and reverse transcriptase, and subjected to the following PCR protocol: 65 °C for 5 min, 37 °C for 10 min after addition of reverse transcriptase, 37 °C for 60 min, and 75 °C for 5 min. Blots were hybridized in Denhardt's solution with SSC buffer overnight at 65 °C with radiolabeled PCR product. After hybridization, blots were washed twice with 2 × SSC containing 1% SDS for 15 min at room temperature, and twice with 0.1 × SSC containing 0.5% SDS for 10 min at 65 °C. Blots were exposed to X-ray film to visualize hybridization.

Orchardgrass cDNA library construction and full-length cDNA screening

Two-week-old orchardgrass seedlings were heat-treated at 35 °C for 1 h. Then, poly(A)$^+$ RNA from heat-treated seedlings was used to construct the orchardgrass λZAP II cDNA library (Stratagene). Full-length cDNAs of the orchardgrass cDNA library were screened using ^{32}P-labeled DNA fragments.

DNA sequencing

The selected cDNA clones were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer) and ABI 310 Sequencer (Perkin–Elmer).

Northern blot analysis

Total RNA from orchardgrass seedling samples was extracted using the phenol/LiCl method. Total RNA (20 µg) was fractionated on a 1.2% formaldehyde agarose gel and blotted onto Hybond-N$^+$ nylon membrane (Amersham) using 10 × SSC. Blots were hybridized overnight at 65 °C with a ^{32}P-labeled full-length DNA using the Megaprime DNA Labeling System (Amersham). Northern hybridization was performed using the same method described for reverse northern blot analysis. Blots were exposed and visualized using a Fuji BAS-2500 Bio-Imaging Analyzer (Fuji Photo Film). Three independent northern blot analyses were performed and the bands were quantified using ImageJ (v. 1.48).

Amino acid sequence alignment and phylogenetic analysis

The deduced amino acid sequence was aligned with homologous amino acid sequences of several species using the multiple alignment tools of ClustalX and GeneDoc. All homologous sequences were retrieved from GenBank by performing a BLAST homology search. A phylogenetic tree was constructed using NCBI-BLAST Tree View using BLAST pairwise alignment with neighbor-joining method.

Expression and purification of recombinant DgHop protein

The *DgHop* open reading frame (ORF) was cloned into *pET28a* to produce recombinant His-tagged DgHop and DgHsp90 (as a positive control for chaperone assay; Cha et al. 2009b). The plasmid was transformed into *Escherichia coli* BL21 (DE3) for protein expression. *E. coli* cells were grown at 30 °C until the OD_{600} approached 0.8. Protein expression was induced by adding 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h, and cells were harvested by centrifugation. Harvested cells were resuspended in phosphate-buffered saline (PBS), disrupted by sonication, and centrifuged. The supernatant was recovered and applied onto a nickel–nitrilotriacetic acid (Ni–NTA) affinity resin column. The column was washed with 50 mM imidazole and His-tagged DgHop was eluted with 200 mM imidazole. His-tagged DgHop was dialyzed against 40 mM HEPES (pH 7.5).

Holdase chaperone assay

The purified His-DgHop was subjected to a holdase chaperone assay as described previously (Cha et al. 2009a). Holdase chaperone activity was evaluated by measuring its capacity to suppress thermal aggregation at 45 °C of *Arabidopsis thaliana* malate dehydrogenase (MDH, EC 1.1.1.37). Activity was measured by light scattering at Abs340 using a Beckman DU-800 spectrophotometer attached to a thermostatic cell holder assembly.

Yeast two-hybrid analysis

The *DgHop* ORF was cloned into the *pBDGAL4Cam* vector containing the *GAL4* DNA-binding domain (BD). An orchardgrass cDNA library was constructed in the *pADGAL4* vector containing the *GAL4* activation domain (AD) and transformed into the yeast reporter strain pJ694A (*MATa trp1-90 leu2-3, 112 ura3-52 his3-200 gal4* Δ*gal80*

Δ*LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) harboring *pBDGAL4Cam-DgHop*. Interactions between the encoded fusion proteins were investigated by co-transforming appropriate plasmids into the yeast strain pJ694A. Transformed yeast cells carrying both plasmids were selected by plating on SD agar medium (0.67% nitrogen base without amino acids and nucleotide bases) lacking tryptophan, leucine, and histidine, and grown at 30 °C for 4 days. Histidine-positive colonies were further tested for β-galactosidase (*lacZ*) activation, according to the manufacturer's protocol (Stratagene). Positive interacting proteins were isolated from yeast cells and analyzed by sequencing. To identify protein–protein interactions between DgHop and either DgHsp90 or DgHsp70, the latter two genes were cloned into the *pBDGAL4Cam* and the *pADGAL4* vector, respectively. The bait and prey plasmids were transformed into a pJ694A yeast strain according to the manufacturer's instructions. Yeast transformants were selected and confirmed using the same steps as described previously in this section. Yeast cells harboring *pBD-wt::pAD-wt* and *pLaminC::pAD-wt* constructs were used as positive and negative controls, respectively.

In vitro pull-down assay

Glutathione-*S* transferase (GST), GST-DgHsp70, or GST-DgHsp90-6xHis were expressed in *E. coli* BL21 (Rosetta) as described previously (Cha et al. 2009b, 2012). Cells were lysed in buffer (1X PBS, pH 7.4, 1% Triton X-100, 0.1% 2-mercaptoethanol, 1 mg cm^{-3} lysozyme and 1 mM phenylmethylsulfonyl fluoride) with a sonicator. GST, GST-DgHsp70, GST-DgHsp90-6xHis supernatants were incubated with 30 mm^3 of pre-equilibrated Glutathione Sepharose 4B (Bioprogen) for 2 h at 4 °C with rotation. Agarose beads were precipitated by centrifugation and then washed with buffer (1× PBS, pH 7.4 and 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) for 5 min at 4 °C with rotation, three times. GST or GST-DgHsp70, GST-DgHsp90-6xHis bound beads were incubated with soluble *E. coli* lysates containing 6xHis-DgHop for 2 h at 4 °C with rotation. The beads were washed with buffer (1x PBS, pH 7.4 and 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) five times. Protein bound to the beads was separated by SDS-PAGE and analyzed by immunoblot analysis. Anti-GST and anti-His sera were used to detect GST, GST-DgHsp70, GST-DgHsp90-6xHis and 6xHis-DgHop and GST-DgHsp90-6xHis.

Statistical analysis

Statistical significances were calculated by Student's *t* test using Microsoft Excel software.

Results

Analysis of physiological responses by heat stress in orchardgrass

Plant exposure to stress conditions elicits physiological responses. This study investigated common stress-responsive parameters such as ion leakage and chlorophyll content in orchardgrass seedlings subjected to 25−50 °C (Fig. 1). Ion leakage from aerial tissues gradually increased with increasingly higher temperatures, whereas chlorophyll content declined with temperature increases. It indicates that heat shock stress causes physiological damage in orchardgrass and may reduce productivity.

Isolation and identification of heat shock-inducible genes

To isolate genes induced by heat shock, 2-week-old orchardgrass seedlings were subjected to temperatures of 25–50 °C (with 5 °C increments) for 1 h. Samples were analyzed using DD-RT-PCR, and bands from control and heat-treated seedlings were compared by analyzing sequencing gel autoradiograms. A total of 96 differentially expressed cDNA bands were excised from the dried gel and amplified by PCR using subjected arbitrary primers. These cDNA fragments were further evaluated by reverse northern blot analysis which is easy means of defining expression of large sets of genes/DNA fragments to eliminate false-positive clones. For reverse northern blot analysis, large sets of DNA fragments achieved from DD-RT-PCR were immobilized on a membrane, and then hybridized with radiolabeled RNA (as a probe) which is extracted from heat shock-treated orchardgrass seedlings (Fig. S1). The signal intensities of most PCR products increased in seedlings subjected to 35 and 40 °C compared to that at 25 °C and its transcript expression at 35 °C compared to those at 25 °C is shown and 23 cDNA fragments with > twofold increase in relative transcription levels were selected for further characterization (Fig. S2).

The 23 cDNA fragments were identified by sequencing and are listed in Table 1. These cDNA fragments had high-sequence similarity with known to heat shock protein (Hsp) genes: four *Hsp101* genes, three *Hsp90s* genes, one *Hsp70* gene, and two small *Hsps* genes. Five cDNA fragments had high sequence homology with co-chaperone genes: three Stress-induced protein (*Sti*)/Hsp-organizing protein (*Hop*) genes, one Activator of Hsp90 ATPase (*Aha*) gene, and one *p23* gene. To isolate full-length cDNA clones, we generated an orchardgrass cDNA library and used each fragment to screen for full-length clones. Eight

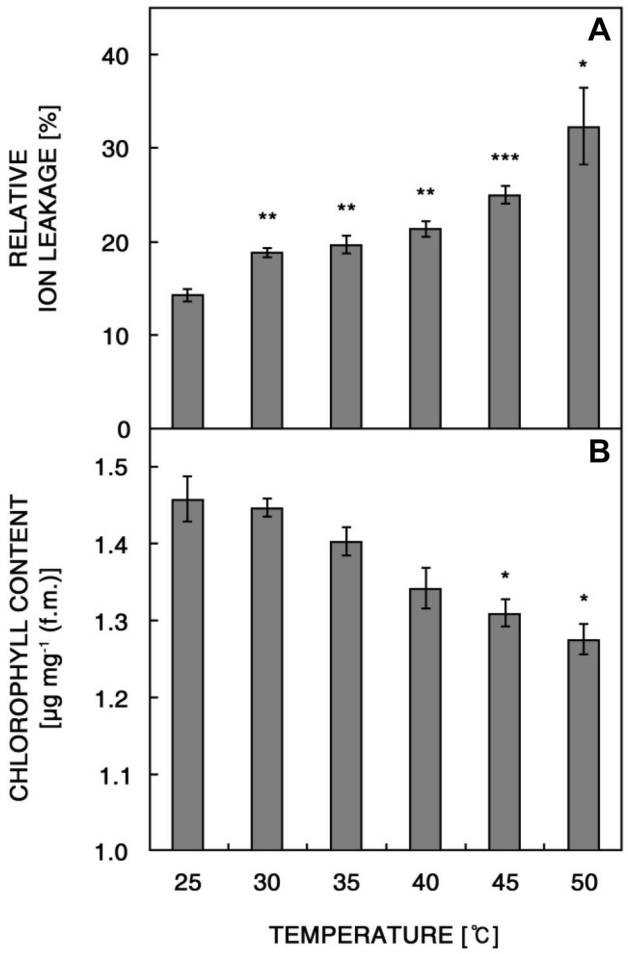


Fig. 1 Physiological responses of orchardgrass seedlings to heat shock treatments. Two-week-old seedlings were exposed to 25, 30, 35, 40, 45, or 50 °C in a water bath for 1 h. **a** Relative electrolyte leakage. Electrolyte leakage was measured 2 h after heat shock treatments, and total electrolyte leakage was measured after autoclaving plant tissue. Data represent means ± SE ($n = 3$) of relative electrolyte leakage (total leakage divided by heat shock induced leakage). **b** Chlorophyll content was measured spectrophotometrically. Data represent means ± SE ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with 25 °C treated orchardgrass seedlings; Student's t test; n refers to number of biological replicates

full-length clones were successfully isolated and entire sequences were deposited into the GenBank database with their accession numbers (Table 1). *DgHsp90* (EU030446) shows high sequence similarity with barley *Glucose-regulated protein 94* (*GRP94*), which indicates an orchardgrass Hsp90 localized to the endoplasmic reticulum (Cha et al. 2009b). *DgHsp90.1* (FJ968744) and *DgHsp90.2* (FJ968745) share 93% sequence identity with genes encoding barley and wheat *cytosolic Hsp90*, respectively. *DgHsp70* (EU541356) is identified as a *cytosolic Hsp70*. *DgHsp17.2* (DQ172835) shows high-sequence similarity with wheat *cytosolic class II small Hsp 17.2* (Cha et al. 2016). Three orchardgrass genes encoding Hsp90 co-chaperones were screened as full-length cDNAs. *DgHop* (FJ968747), *DgAha* (FJ968746), and *Dgp23* (DQ172836, Cha et al. 2009a) were identified as homologs of corn (*Zea mays*) Hop, Aha, and p23, respectively. To examine heat shock-induced transcription profiles, we performed northern blot analysis with full-length cDNA clones as probes and calculated the expression in terms of fold induction. All identified genes were strongly induced by heat shock (Table 1). This result is supported by previous reports that transcripts of *DgHsp90, DgHsp70*, and *Dgp23* were strongly enhanced by heat stress (Cha et al. 2009a, b, 2012). These genes were the most strongly induced by 35 °C, and then declined with increasing temperatures to 50 °C. Inductions of *DgHop* transcript levels were more strongly induced than those of major *Hsp* genes such as *DgHsp90.1* and *DgHsp90.2* (Fig. 2). These data suggest that heat shock stress primarily induces genes encoding Hsps and co-chaperones, and the heteromultimeric protein complex associated with Hsps and co-chaperones might be activated to repair client proteins damaged by heat shock in orchardgrass.

Identification of orchardgrass *Hop* gene

Hop protein plays an important role for assembling Hsp90 and Hsp70 in the heteromultimeric chaperone complex, and is essential for steroid receptor assembly in mammals (Pratt and Toft 2003). First, we analyzed sequence prior to evaluating whether orchardgrass Hop (DgHop) could recruit orchardgrass Hsps to associate with the multimeric chaperone complex. Full-length *DgHop* cDNA screened from the cDNA library has a length of 1937 bp and contains a 1737 bp ORF encoding 578 amino acids (Table 1, Fig. S3). The cDNA contains a 5′ untranslated region (5′-UTR) of 42 bp and a 3′-UTR of 161 bp, which includes a stop codon (TAA) and poly(A) tail (Fig. S3). PROSITE and NCBI-BLAST analysis indicates that DgHop consists of a degenerate sequence of 34 amino acids forming nine conserved putative tetratricopeptide repeat (TPR) motifs (Fig. 3a). There are three tandem-TPR motifs organizing each TPR domain at amino acid positions 2–103, 251–361, and 390–489, which has important roles in protein–protein interactions (Blatch and Lässle 1999). DgHop also contains the STI1 domain, which is known to be a heat shock chaperonin-binding motif at amino acids 527–566. Sequence alignment of DgHop with other Hop homologs (soybean, *Arabidopsis*, rice, corn, and human) shows high conservation of TPR and STI1 domains (Fig. 3a). Protein subcellular localization prediction system (LocTree3, http://rostlab.org/services/loctree2/) predicts that DgHop localizes in the nucleus with a conserved nuclear localization signal (NLS) at amino acids 246–264 (based on DgHop sequence) which is also conserved in soybean Hop

Table 1 Orchardgrass genes induced by heat shock stress

Identification	GenBank accession number	Annotation of the highest homolog (accession no.)	Length (bp)	Fold induction	*E* value[a]	References
Hsp100 family						
		Orysa sativa heat shock protein (Hsp101) mRNA (AF332981)	*P*[b]	2.76	3e−44	
		Triticum aestivum heat shock protein 101 (Hsp101b) mRNA (AF097363)	*P*[b]	2.31	5e−167	
		Triticum turgidum subsp. durum partial mRNA of heat shock protein 101 (Hsp101c-B gene) (AJ970536)	*P*[b]	2.75	2e−132	
		Triticum aestivum heat shock protein 101c (AF174433)	*P*[b]	2.46	3e−119	
Hsp90 family						
DgHsp90	EU030446[c]	*Hordeum vulgare* mRNA of GRP94 homolog (X67960)	2742	2.47	0.0	Cha et al. (2009b)
DgHsp90.1	FJ968744	*Hordeum vulgare* cytosolic heat shock protein 90 mRNA (AY325266)	2374	4.37	0.0	
DgHsp90.2	FJ968745	*Triticum aestivum* heat shock protein 90 mRNA (U55859)	2410	3.62	0.0	
Hsp70 family						
DgHsp70	EU541356[c]	*Triticum aestivum* 70 kDa heat shock protein (AF005993)	2243	2.98	0.0	Cha et al. (2012)
Small Hsp family						
		Pennisetum glaucum heat shock protein 17.9 (X94193)	*P*[b]	2.58	1e−99	
DgHsp17.2	DQ172835[c]	*Triticum aestivum* small heat shock protein 17.8 (AF350423)	882	2.93	0.0	Cha et al. (2016)
Co-chaperones						
DgSti/Hop	FJ968747	*Zea mays* clone 390399 heat shock protein STI (EU972939)	1937	3.48	0.0	
		Zea mays clone 242656 ankyrin-1 (EU962436)	*P*[b]	2.31	6e−175	
		Zea mays clone 237829 DnaJ subfamily B member 5 mRNA (EU961716)	*P*[b]	2.46	1e−108	
DgAha	FJ968746	*Zea mays* clone 277129 hypothetical protein (EU964231)	948	3.31	2e−95	
Dgp23	DQ172836[c]	*Zea mays* clone 9591 (DQ244605)	911	3.33	1e−165	Cha et al. (2009a)
Other proteins						
		Hordeum vulgare carbonic anhydrase (L36959)	*P*[b]	2.92	7e−163	
		Zea mays clone 277129 hypothetical protein mRNA (EU964231)	*P*[b]	2.35	5e−23	
		Zea mays clone 1686389 SAUR33-auxin-response SAUR family member mRNA (EU958350)	*P*[b]	2.29	9e−75	
		Zea mays clone 987937 hydrogen-transporting ATP synthase, rotational mechanism mRNA (EU976701)	*P*[b]	2.32	2e−95	
		Triticum aestivum AS2 mRNA (AY795560)	*P*[b]	2.27	2e−156	
		Zea mays clone 234675 histone H2A.2 mRNA (EU961342)	*P*[b]	2.33	5e−103	
		Hordeum vulgare subsp. vulgare cDNA clone:FLbaf12f20 mRNA (AK248944)	*P*[b]	2.24	1e−53	
		Macrocystis pyrifera mRNA of calmodulin (X85091)	*P*[b]	2.43	6e−27	

[a] *E* value was determined by BLASTn program

[b] *P* indicates that the cDNA contains a partial coding region

[c]Genes are reported by Cha et al. (EU030446, 2009b; EU541356, 2012; DQ172835, 2016; DQ172836, 2009a)

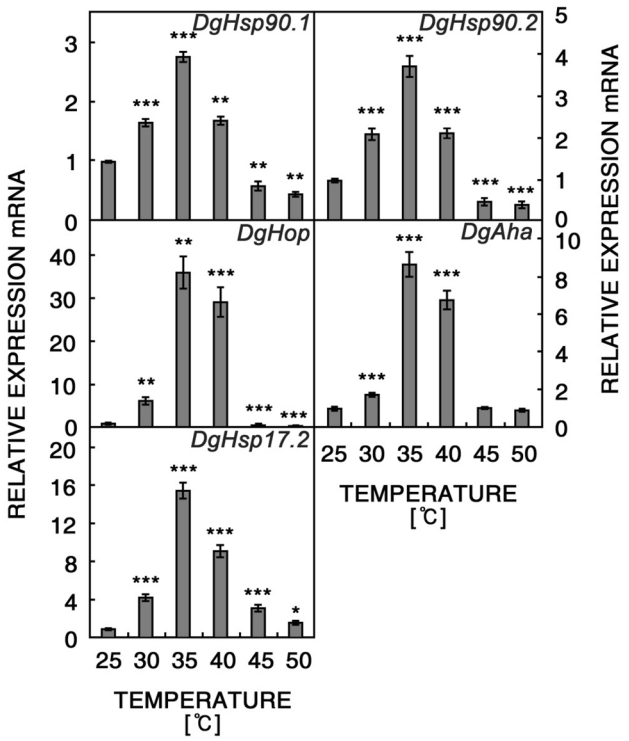


Fig. 2 Expression levels of heat stress-induced genes in orchardgrass. Orchardgrass seedlings were subjected to heat treatment at the indicated temperatures for 1 h, and then total RNA was isolated. Relative expression of *DgHsp90.1, DgHsp90.2, DgHop, DgAha*, or *DgHsp17.2* was calculated with total RNA (20 μg) verifying by ethidium bromide staining. Three independent northern blot analyses and intensity was analyzed using ImageJ. Data are mean ± SE ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with 25 °C treated orchardgrass seedlings; Student's *t* test; *n* refers to number of biological replicates

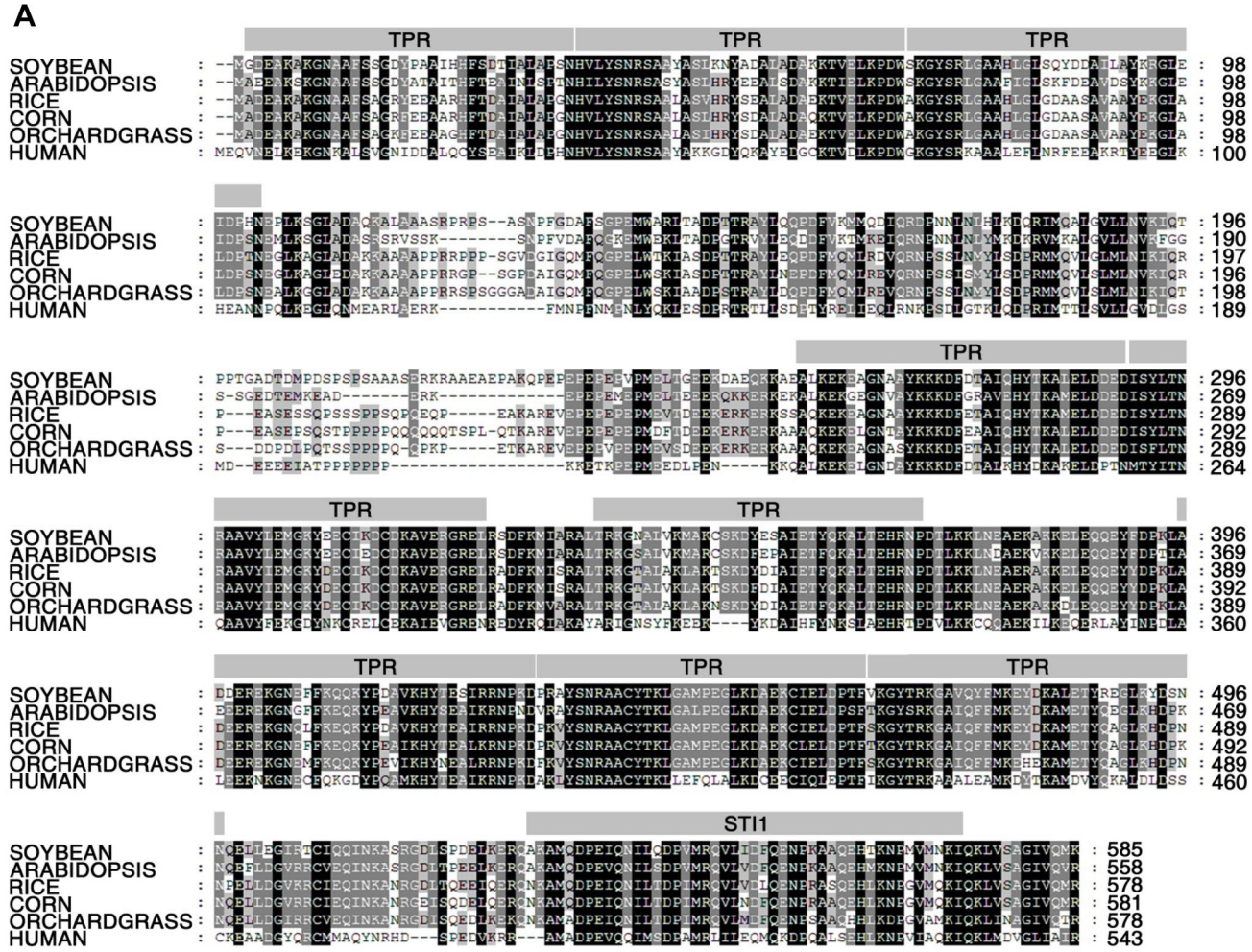
(Zhang et al. 2003). DgHop has higher sequence identity with Hop proteins in monocots (over 82% sequence identity) than with those in dicots (71–74% identity) (Fig. 3b). However, it does still remain elusive how nuclear Hop protein interacts with different subcellular localized Hsp90 and Hsp70.

We performed northern blot analyses to examine *DgHop* transcription levels in orchardgrass seedlings subjected to various abiotic stresses. *DgHop* transcripts were expressed at basal levels until 30 min after exposure to heat shock stress at 40 °C, but these levels were dramatically enhanced at 1 and 2 h since heat treatment, and then declined to basal levels after subsequent exposure to 25 °C for 5 h as a recovery (Fig. 4). Seedlings also were exposed to cold (4 °C), salt (140 mM NaCl), or dehydration (air-dried on filter paper) stress for 0, 1, 6, and 12 h (Fig. 5). *DgHop* transcripts were slightly induced in response to cold stress. In contrast, *DgHop* transcripts were dramatically reduced in response to salt and dehydration stress (Fig. 5). These results indicate that *DgHop* expression was induced primarily by extreme temperature stress. We measured the chaperone activity of recombinant DgHop in vitro using the model substrate malate dehydrogenase (MDH). MDH was fully denatured under heat denaturing conditions at 45 °C and DgHop did not effectively suppress MDH aggregation (Fig. S4). Neither in 1:1 or 1:10 molar ratio of MDH to DgHop could protect heat-induced aggregation of MDH, while almost 90% of MDH aggregation was suppressed by DgHsp90 in a 1:1 molar ratio of MDH to DgHsp90 (Fig. S4). It suggests that DgHop functions as an adaptor protein to assemble the chaperone complex, rather than acting as a chaperone protein.

Identification of Hop-interacting proteins

TPR motif-containing proteins including Hop/Sti, Hip, Cyp40, FKBP, CDC, and PP5 have been identified in organisms ranging from bacteria to humans (Blatch and Lässle 1999). Hop participates in Hsp90 heterocomplex assembly through TPR motifs by recruiting Hsp90 to preexisting Hsp70–client complexes (Chen and Smith 1998; Smith 2004). However, plant Hop and Hop-interacting proteins are still largely unknown. To screen DgHop client proteins, we performed a yeast two-hybrid analysis with an orchardgrass cDNA library in the *pADGAL4* vector. After excluding the false clones, we identified 15 proteins as DgHop-interacting proteins. Hsp90 proteins had the strongest reproducible interactions with DgHop (Table S1). Hsp70 and class I small Hsp also were identified as DgHop-interacting proteins. Cinnamyl-alcohol dehydrogenase, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS), glycine decarboxylase subunit, adenosine kinase, and the 10 kDa polypeptide of PSII also were screened as interacting proteins. These results suggest that DgHop may function as an organizing protein that interacts with Hsp90 and Hsp70 in orchardgrass.

Furthermore, we examined direct interactions between DgHop and DgHsp90 or DgHsp70. The yeast two-hybrid proteins were constructed as follows: *DgHsp90* or *DgHsp70* ORFs were inserted into the activation domain (AD) vector to generate prey fusion proteins, and the *DgHop* ORF was inserted into the binding domain (BD) vector to generate a bait protein. Both DgHsp90 and DgHsp70 strongly interacted with DgHop (Fig. 6a). To further confirm and independently examine the physical interaction, we used GST pull-down assays. Recombinant GST as a negative control, GST-DgHsp70 and GST-DgHsp90-6xHis were expressed in *E. coli* and then purified by affinity chromatography using glutathione agarose beads. 6xHis-DgHop was also expressed in *E. coli* and then the soluble protein extracts were incubated with the glutathione agarose beads immobilized GST, GST-DgHsp70 or GST-DgHsp90-6xHis. 6xHis-DgHop was co-purified with GST-DgHsp70 or GST-DgHsp90-6xHis, but not with GST alone (Fig. 6b). This result provides an additional evidence that DgHop interacts with DgHsp70 or


A
TPR
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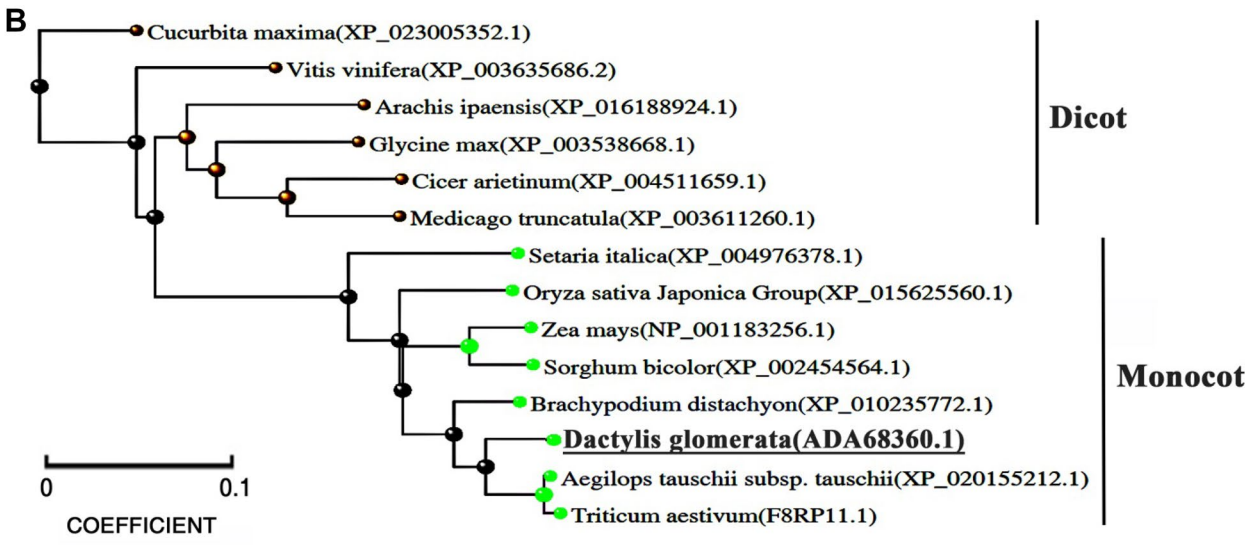
B
Cucurbita maxima(XP_023005352.1)
Vitis vinifera(XP_003635686.2)
Arachis ipaensis(XP_016188924.1)
Glycine max(XP_003538668.1)
Cicer arietinum(XP_004511659.1)
Medicago truncatula(XP_003611260.1)
Setaria italica(XP_004976378.1)
Oryza sativa Japonica Group(XP_015625560.1)
Zea mays(NP_001183256.1)
Sorghum bicolor(XP_002454564.1)
Brachypodium distachyon(XP_010235772.1)
Dactylis glomerata(ADA68360.1)
Aegilops tauschii subsp. tauschii(XP_020155212.1)
Triticum aestivum(F8RP11.1)
Dicot
Monocot
0
0.1
COEFFICIENT

◂**Fig. 3** Sequence analysis of orchardgrass Hop (*DgHop*). **a** DgHop alignment with homologs from soybean (Hop-1) (Zhang et al. 2003), *Arabidopsis* (Sti1, CAB45987), rice (Sti1, BAD25789), corn (Sti, ACG45057), and human (Hop, NP_006810). Numbers at the right indicate amino acid residue position. Fully identical residues are shaded in black, five identities are shaded in gray, and four identities are shaded in light gray. The conserved TPR and STI1 domains are represented as gray boxes above the residues. **b** Phylogenetic relationships between DgHop and other Hop homologs. The phylogenetic tree was produced in NCBI-BLAST Tree View using BLAST pairwise alignment with neighbor-joining method

DgHsp90 physically. Therefore, DgHop has a similar function to that of mammalian Hop proteins and acts as an adaptor protein that assembles the Hsp90 heterocomplex.

Discussion

To cope with heat stress, plants activate a large set of specific genes that results in the accumulation of specific heat stress-associated proteins, which is the heat shock response (HSR) (Vierling 1991). HSR is regulated at the transcriptional level by heat shock transcription factors (HSFs). HSFs subsequently induce expression of *Hsp* genes by binding to heat shock elements (HSEs) (Schöffl et al. 1998). Hsps prevent protein aggregation, and promote proper folding of unfolded proteins and confer enhanced thermotolerance in plants (Vierling 1991). Although most of these proteins are expressed in response to heat stress, expression of their mRNA transcripts has not been fully elucidated. Microarray analysis revealed that most of heat-induced transcripts as well as Hsp coding genes in heat-exposed *Arabidopsis* suspension cells showed a peak at 1 h after heat treatment, and then gradually declined that is consistent with our results (Fig. 2; Lim et al. 2006). Prolonged heat stress and lethal temperature decrease transcription of heat-shock (HS) related genes, accumulate unfolded proteins in cell, and consequently result plant death, but short-term exposure to sub-lethal temperature (such as heat-acclimation) increases thermotolerance through prolonged transcription of HS genes (Schöffl et al. 1998; Amano et al. 2012). Thus, exposure of heat stresses over 45 °C for 1 h could be lethal to orchardgrass and Arabidopsis caused by reducing transcription of HS genes (Fig. 2; Lim et al. 2006). In addition, several plant Hsp90 co-chaperones, including soybean Hop and bentgrass p23, were reported that those transcription is enhanced by heat stress (Zhang et al. 2003; Tian et al. 2008). However, a comprehensive molecular approach to isolate heat-responsive genes is still restricted in model plants such as *Arabidopsis* (Lim et al. 2006).

In this study, heat-inducible genes were isolated and identified from orchardgrass, one of the most important pasture crops in the world, using mRNA differential display and cDNA library screening. Table 1 shows full-length genes encoding Hsp family member (*Hsp90s, Hsp70*, and *small Hsps*) and Hsp90 co-chaperones (*Hop, Aha*, and *p23*) with enhanced transcripts in heat-stressed orchardgrass seedlings (Fig. 2).

The Hsp90 heterocomplex plays as an important role for regulating several cellular responses (Pratt and Toft 2003). A minimal Hsp90 heterocomplex containing Hsp90, Hsp70, Hop, Hsp40, and p23 has been demonstrated in animals based on the presence of a hormone receptor. In the molecular leves, Hop/Sti1 enables to form Hsp90 heterocomplexes with Hsp90 and Hsp70 for recognition of the client proteins of Hsp90 (Pratt and Toft 2003; Fellerer et al. 2011). Co-chaperones such as Hop, Hsp40, and p23 enhance the efficiency of Hsp90 heterocomplex assembly (Dittmar et al. 1997). However, all components in this complex have not been fully identified in plants. Recently, although biological function of Hop/Sti1 is still elucidative, Hop is involved in a trafficking system of rice chitin receptor OsCERK1 and contributes to OsRac1 immune complex (Chen et al. 2010). Interestingly, the plant Hsp90 heterocomplex has been detected using a rabbit reticulocyte folding system and wheat germ extracts. Plant Hsp90 and Hsp70 cross-react not only with plant co-chaperones but also with animals' to form a heterocomplex assembly (Hutchison et al. 1995; Stancato et al. 1996; Dittmar et al. 1997; Zhang et al. 2003). Thus, it is believed that the Hsp90/Hsp70 heterocomplex machinery in plants may be functionally identical to that in animals. Hsp90 function also is coupled to ATP binding and hydrolysis through association with ATPase-regulating stimulator and suppressor co-chaperones Aha and Hop, respectively (Scheibel et al. 1998; Caplan et al. 2003). Although *E. coli* contains a single Hsp90 homolog (HtpG), eukaryotic Hsp90 proteins are distributed in distinct organelles, including the cytoplasm, chloroplast, mitochondria, and endoplasmic reticulum (ER) (Krishna and Gloor 2001).

In orchardgrass seedlings, we identified three heat-inducible *DgHsp90* genes, one ER-localized *DgHsp90*, and two cytosolic *Hsp90*s (*DgHsp90.1* and *DgHsp90.2*) (Table 1). We previously reported that transcriptional expression of *DgHsp90* was enhanced by heat and oxidative stresses, and was reduced by cold and dehydration (Cha et al. 2009b). DgHsp90 also displayed strong ATP-dependent chaperone activity, and overexpression in yeast cells conferred thermotolerance. However, two cytosolic DgHsp90.1 and DgHsp90.2 have not yet been identified. Another major chaperone protein Hsp70 is also abundant and localizes to several subcellular regions. Cytosolic DgHsp70 was reported to have ATP-dependent chaperone activity and confer thermotolerance (Cha et al. 2012). We also characterized the orchardgrass Hsp90 co-chaperone protein Dgp23 previously (Cha et al. 2009a). Dgp23 was the first identified p23 homolog in plants; it exhibited chaperone activity and

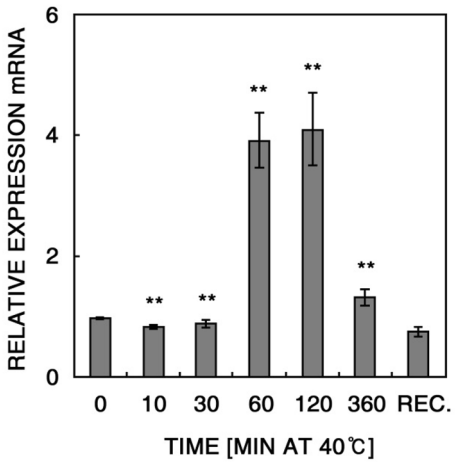

Fig. 4 Time-course accumulation of *DgHop* transcripts during heat stress. Orchardgrass seedlings were exposed to heat (40 °C) for indicated time. REC represents the seedlings subsequently recovered from heat (at 40 °C for 60 min and then at 25 °C for 300 min). Total RNA was isolated and analyzed by northern blot analysis. Equal loading was verified by ethidium bromide staining. The band intensity of northern blot was normalized using rRNA and analyzed using ImageJ. Data are mean ± SE ($n = 3$). $^{**}P < 0.01$ compared with non-treated (zero time point) orchardgrass seedlings; Student's *t* test; *n* refers to number of biological replicates

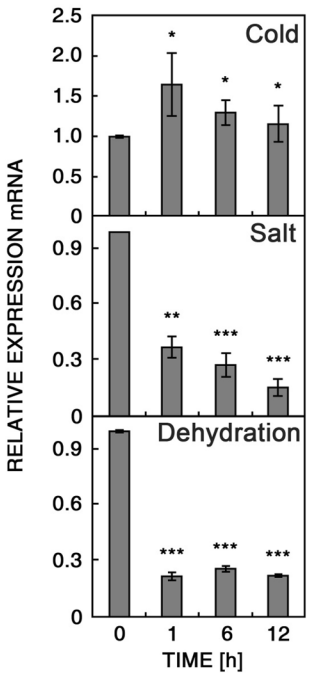

Fig. 5 *DgHop* mRNA expression levels in response to different abiotic stresses. *DgHop* transcript levels were examined in response to cold (4 °C), salt (140 mM NaCl), or dehydration (air-dried) treatment. Stress-treated seedlings were harvested at the indicated time points, and analyzed by northern blotting after total RNA extraction. Relative expression was calculated with total RNA (20 µg) verifying by ethidium bromide staining. Three independent experiments were analyzed using ImageJ. Data are mean ± SE ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with non-treated (zero time point) seedlings; Student's *t* test; *n* refers to number of biological replicates

interacted with DgHsp90. Although p23 can bind directly to Hsp90 in the absence of substrate, it cannot bind to Hsp90 in the absence of ATP (Sullivan et al. 2002). And, p23 stabilizes Hsp90 conformation by inhibiting ATP hydrolysis (McLaughlin et al. 2006).

Hsp70 function is coupled to an ATP hydrolysis cycle by associating with various proteins, which commonly possess TPR motif-containing co-chaperones such as Hip, Hop, and CHIP. These co-chaperones interact with both Hsp90 and Hsp70 through TPR motifs. The TPR motif is a degenerate 34 amino acid repeat that is often arranged in tandem arrays, although it is not unusual to find one or more repeats separated from such an array (Blatch and Lässle 1999). The binding of co-chaperones at TPR motifs differentially regulates Hsp90 and Hsp70 functional activity, translocation, and subcellular localization (Chen and Smith 1998; Pratt et al. 1999; Prodromou and Pearl 2003). Human Hop and its TPR domains function as an adaptor protein to assemble Hsp90 and Hsp70, and disrupt mature receptor complex assembly (Chen and Smith 1998). The transcription levels of mouse and soybean Hop transcription levels increased by heat stress (Lässle et al. 1997; Zhang et al. 2003). Hop homologs are defined by presenting of nine TPR motifs clustered into domains (Fig. 3). The Hsp90–Hop–Hsp70 complex is formed via Hop TPR2a and TPR1 binding each C terminus of Hsp90 and Hsp70, respectively (Carrigan et al. 2004). The Hsp90 central domain is also necessary to interact with Hop (Onuoha et al. 2008).

We also identified that DgHop transcript levels are dramatically induced by heat stress and are reduced by cold, salt, and dehydration stress (Figs. 2c, 4, 5). The results demonstrate that DgHop acts positively during heat-specific stress to modulate heteromultimeric complex assembly with Hsp90 and Hsp70. However, DgHop could not play as a chaperone protein itself, suggesting that DgHop may be important to organize mature form of Hsp90 heteromultimeric complex. We confirmed that Hsp90 and Hsp70 are major interacting partner proteins of DgHop in orchardgrass through yeast two-hybrid and pull-down assay (Table S1, Fig. 6). Interestingly, several novel DgHop-interacting proteins, such as cinnamyl-alcohol dehydrogenase, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, glycine decarboxylase subunit, adenosine kinase, and the 10 kDa polypeptide of PSII, are identified in this study (Table S1). Loss of function of *Medicago truncatula cinnamyl-alcohol dehydrogenase 1* which is involved in the last step of monolignol biosynthesis displays temperature-sensitive growth defects (Zhao et al. 2013). DAHPS catalyzes the first step in the shikimate pathway triggering a synthesis of diverse

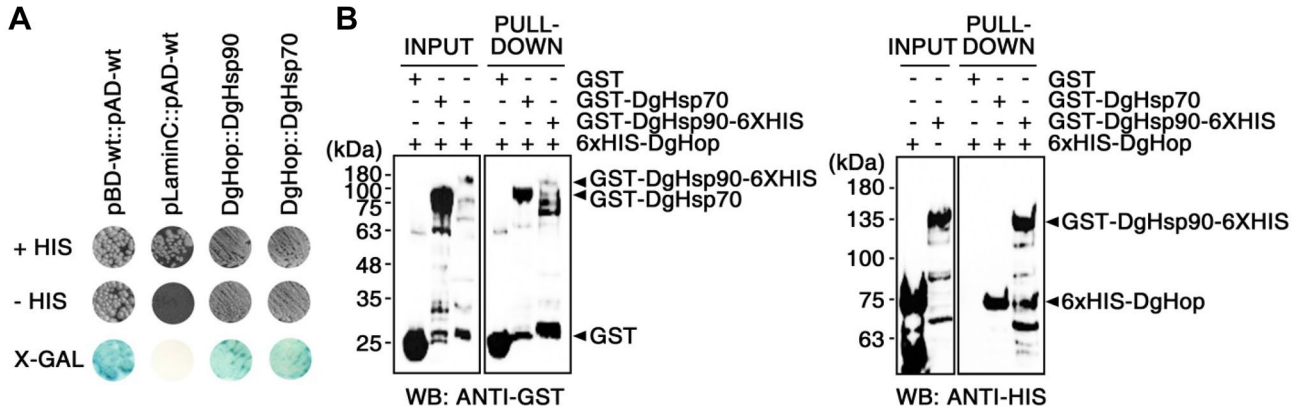

Fig. 6 Protein–protein interaction between DgHop and DgHsp90 or DgHsp70. **a** Yeast two-hybrid interaction analysis. + His, transformants isolated on SD medium lacking tryptophan and leucine; – His, transformants selected on SD medium lacking tryptophan, leucine, and histidine; X-Gal, β-galactosidase assay for protein–protein interaction. **b** GST pull-down assay. Bacterially expressed GST, GST-DgHsp70 and GST-DgHsp90-6xHis were independently immobilized onto glutathione agarose beads and incubated with soluble extracts of 6xHis-DgHop expressed in *E. coli*. Co-purified 6xHis-DgHop with GST-DgHsp70 or GST-DgHsp90-6xHis was examined with anti-His antibody

aromatic compounds (Derrer et al. 2013). Proteomic analysis revealed that heat stress causes wide range of proteomic changes, especially in photosynthesis, carbon metabolism and signaling identified in leaves (Wang et al. 2017). Glycine decarboxylase subunit and component of PSII are involved in photorespiration and photoreaction, respectively. Gene-encoding *adenosine kinase 1* in mustard hill coral is decreased by heat stress (Kenkel et al. 2013). Based on the characteristic of these DgHop interactors, it could be possible to be responsive or client proteins of Hsp90 heteromultimeric complex. Thus, DgHop may plays as a role not only in organizing protein for Hsp90–Hsp70 association, but also in bridging between Hsp90–Hop–Hsp70 complex and clients. However, it still remains elusive.

In conclusion, we screened 23 unique fragments of heat shock-inducible genes in orchardgrass seedlings using DD-RT-PCR. Eight full-length genes were isolated from the orchardgrass cDNA library, which encoding major molecular chaperones (Hsp90, Hsp70, and small Hsp) and Hsp90 co-chaperones (Hop, Aha, and p23). The expression of these genes was induced during heat stress and this result supports DD-RT-PCR screening. We isolated and characterized the first monocot Hop gene (*DgHop*) in orchardgrass, and showed that *DgHop* expression is enhanced by heat shock but not by other abiotic stresses such as cold, salt, and dehydration. Yeast two-hybrid analysis and in vitro pull-down assay using DgHop indicated that DgHop primarily functions as an adaptor protein involved in the formation of the Hsp90–Hop–Hsp70 chaperone complex. Our data provide new approaches to utilize Hsp genes to enhance thermotolerance in heat-sensitive crops.

Author contribution statement JYC and DS initiated the project, and JYC, DB, MSC and SH performed the experiments. JYC, SHL and DS analyzed the data, and JYC and DS wrote the paper. All authors discussed the results and approved the manuscript.

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