Functional Characterization of Siberian Wild Rye Grass *EsHSP 16.9* Gene Conferring Diverse Stress Tolerance in Prokaryotic Cells

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Functional characterization of Siberian wild rye grass EsHSP 16.9 gene conferring diverse stress tolerance in prokaryotic cells

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Introduction

Siberian wild rye (*Elymus sibiricus* L.) is a perennial, caespitose, and self-pollinating grass indigenous to Northern Asia and also is widely distributed from Northern Europe to Japan. The plant shows strong environmental adaptability with tolerance to drought and cold; thus, it is often used as forage resources (Yan *et al*., 2007). Environmental stresses caused by global warming are acknowledged to be as a serious issue in agriculture due to reductions of crop productivity (Ahuja *et al*., 2010). Genetic natural breeding of Siberian wild rye would potentially increase the productivity of forage crops; however, genetic studies on this grass have yet to be conducted. Heat shock proteins (Hsps) are the well characterized stress inducible proteins playing as molecular chaperones in prokaryotes and eukaryotes. We have also identified two differently localized small Hsps: rice chloroplastic and alfalfa mitochondrial Hsps confer tolerance to oxidative and heat stresses in tall fescue and to salinity and arsenic stresses in *E. coli*, tobacco, and tall fescue, respectively (Lee *et al*., 2012a; Lee *et al*., 2012b). Here, we cloned the small *Hsp16.9* gene from various heat stress-induced fragments in Siberian wild rye using differentially expressed gene (DEG) analysis. We examined the mRNA expression of *EsHsp16.9*, *in vitro* molecular chaperone activity and *in vivo* stress tolerance by using a prokaryotic system against diverse environmental stresses.

Materials and Methods

Plant materials and growth conditions: Seeds of Siberian wild rye were collected from Ulaanbaatar, Mongolia. Seeds were planted in pots containing Horticulture Nursery medium and grown in a growth chamber maintained at 25°C with a light intensity of 400 μmol m^{-2} s^{-1} and a 14-h photoperiod.

Isolation of EsHsp16.9: Four-week-old seedlings were subjected to 42°C heat treatments or 25°C non-treatment (control) for 6 h. After treatment, leaves were harvested and frozen with liquid nitrogen. Total RNA was isolated from the leaf tissues of treated and control plants using TRIzol reagent (Qiagen, CA, USA). Using the GeneFishing™ DEG kit (Seegene, South Korea), *EsHsp16.9* was isolated using an annealing control priming-based polymerase chain reaction method, as described by Lee *et al*., 2012c.

Sequence analyses: The nucleotide and deduced amino acid sequences and the conserved domain were analyzed using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Bioedit (version 7.2.3) software. The isoelectric point (pI) and molecular weight (Mw) of EsHsp16.9 were calculated using the Compute pI/Mw tool in ExPASy (http://web.expasy.org/compute_pi/). Amino acid sequences of small Hsps in *Arabidopsis* and homologs of EsHsp16.9 from different plant species were retrieved from the GenBank™ database after a homology search using blastp in NCBI-BLAST. Multiple sequence alignment was performed using ClustalX2 and GeneDoc (version 2.7) software. A phylogenetic tree was constructed by the neighbor joining method with 1000 bootstrap replicates by software MEGA6.0 (http://www.megasoftware.net).

Abiotic stress treatments to Siberian wild rye plants: Four-week-old Siberian wild rye seedlings were exposed to conditions of drought (withdrawing irrigation), heat (42°C), cold (4°C), salt (NaCl, 300 mM), copper ($CuSO_4$, 500 μM), cadmium ($CdCl_2$, 500 μM), arsenate ($Na_2HAsO_4 \cdot 7H_2O$, 500 μM), methyl viologen (MV, 1 mM), and hydrogen peroxide (H_2O_2, 10 mM). For heat and cold treatments, seedlings were transferred into a temperature-controlled growth chamber (42 or 4°C). For salt and heavy metal treatments, seedlings were irrigated with water containing indicated concentrations of salt and heavy metals. For MV and H_2O_2 treatments, leaf segments were floated on water containing indicated concentrations of MV and H_2O_2. Stress-treated plants were harvested, frozen in liquid nitrogen and maintained at -80°C until use.

Northern blot analysis: To investigate transcriptional expression of the *EsHsp16.9* gene in response to various abiotic stressors, total RNA was isolated from stress-treated leaves using TRIzol reagent (Qiagen, CA, USA). The extracted total RNA (10 μg) was fractionated by electrophoresis on a 1.2% agarose gel containing formaldehyde and then blotted onto a Hybond-N^+ nylon membrane (Amersham, Little Chalfont, UK). The blots were hybridized overnight at 65°C with a ^{32}P-labeled probe, which is PCR-amplified *EsHsp16.9* gene.

Results and Discussion

Small heat shock proteins (Hsps) are one of most conserved molecular chaperones that protect stress-inducible denaturation of substrates in living organisms. Small Hsps consist of a large subfamily categorized by subcellular localization ranging in size from 12 to 40 kDa. Here, we identified and characterized a small Hsp 16.9 gene (*EsHsp16.9*) from Siberian wild rye (*Elymus sibiricus* L.). *EsHsp16.9* is a 456-bp cDNA with an open reading frame predicted to encode a 151-amino acid protein. It possesses a conserved α-crystallin domain, which is a unique domain for small Hsps; shares high sequence similarity with cytosolic class I small Hsps among the small Hsp subfamily in *Arabidopsis*; and is close (96% similarity) to small Hsp in wheat. Northern blot analysis showed that *EsHsp16.9* transcripts were enhanced by heat, drought, arsenate, methyl viologen, and H_2O_2 treatments. Moreover, we expressed and purified recombinant EsHsp16.9 proteins in *Escherichia coli* to confirm its activity as a molecular chaperone. We found that recombinant EsHsp16.9 exhibits effective molecular chaperone activity, as determined by inhibition of thermal aggregation of malate dehydrogenase (MDH), which is broadly used as a model substrate. In addition, *in vivo* stress tolerance was examined using the *E. coli* system, which showed that cells overexpressing *EsHsp16.9* grew better in the presence of NaCl, arsenate, and polyethylene glycol whereas cells harboring an empty vector retarded growth. These data suggest that EsHsp16.9 belongs to cytosolic class I small Hsps and acts as a molecular chaperone that could enhance stress tolerance in living organisms.

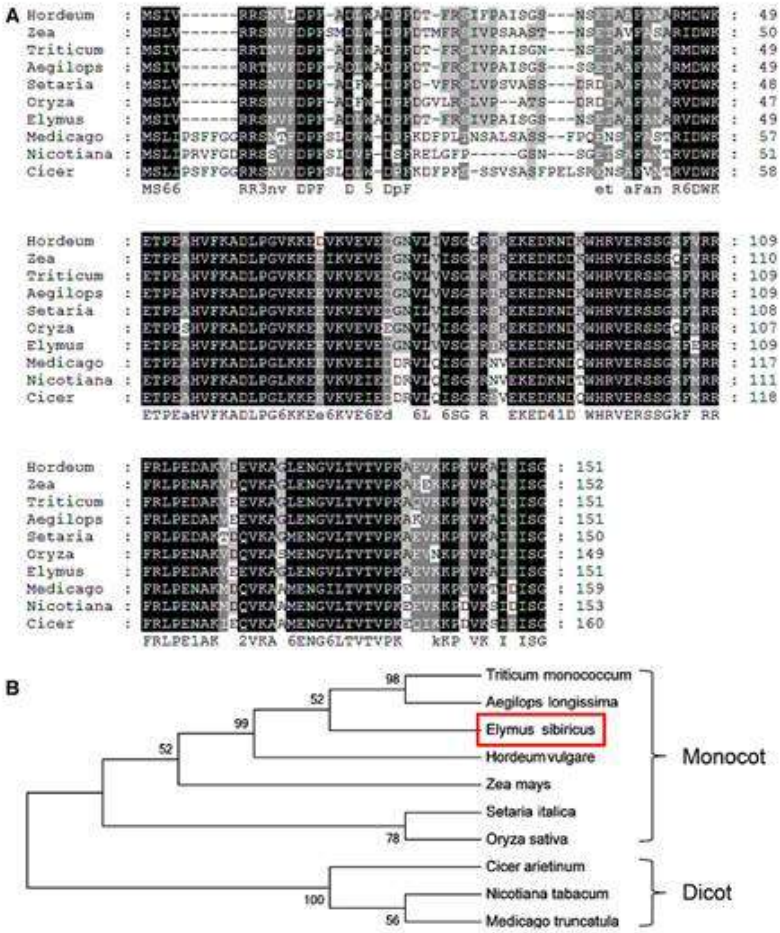

Fig. 1. Amino acid sequence comparison and phylogenetic analysis of EsHsp16.9 with small Hsps from different plant species. (A) Alignment of *Elymus sibiricus* Hsp16.9 (EsHsp16.9) protein with homologs from *Hordeum vulgare* (Hsp17; ADW78607), *Zea mays* (Hsp16.9 class I; NP_001150783), *Triticum monococcum* (Hsp17; CAM96547), *Aegilops longissima* (Hsp16.9; CAM96537), *Setaria italica* (Hsp16.9 class I; XP_004968026), *Oryza sativa* (Hsp;

AAB39856), *Medicago truncatula* (Hsp18.2 class I; XP_003619703), *Nicotiana tabacum* (Hsp 3B class I; ADK36668), and *Cicer arietinum* (Hsp8.5 class I; XP_004505085). **(B)** Phylogenetic analysis of EsHsp16.9 with homologs from different plants. The *scale bar* represents the number of amino acid substitutions per site.

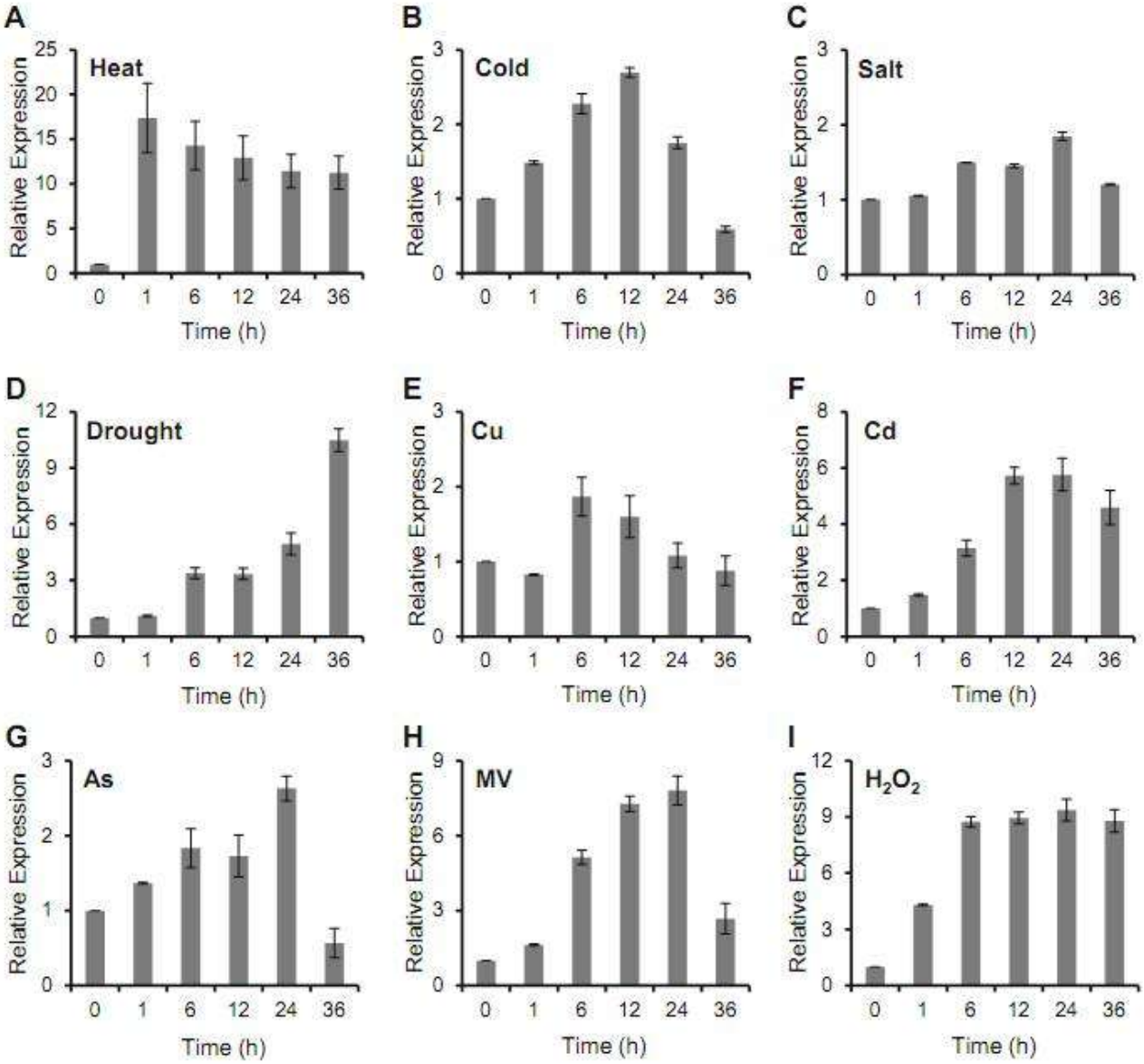

Fig. 2. Expression of EsHsp16.9 transcripts in response to abiotic stressors. Expression of EsHsp16.9 in seedlings treated with heat (a), cold (b), salt (c), drought (d), copper (e), cadmium (f), arsenate (g), methyl viologen (h) and hydrogen peroxide (i) for 0, 1, 6, 12, 24 and 36 h. Three independent northern blot analyses were performed and the band intensity was analyzed using ImageJ. Data are mean ± SE (n = 3)

Conclusion

Siberian wild rye grass is an important forage crop in northern Asia and has strong adaptability to unfavorable environments. Here, we screened for useful genetic materials from Siberian wild rye by DEG analysis using the gene-fishing technique. Using this approach, we identified a small Hsp 16.9 (EsHsp16.9) which we isolated, successfully cloned, and categorized as falling into the cytosolic class I subfamily. Enhanced expression of *EsHsp16.9* transcripts were also confirmed under various environmental stressors. Small Hsp genes are found in various organisms including plants, act as molecular chaperones to protect cellular proteins against damage from diverse stressors, and have been known to induce stress tolerance. Using a recombinant protein expression system in *E. coli*, we expressed and purified recombinant EsHsp16.9 protein and found that it effectively exhibited molecular chaperone activity to protect thermal aggregation of malate dehydrogenase. We also revealed that *E. coli* cells expressing EsHsp16.9 exhibited better growth under salt,

arsenate, PEG, and heat stressors. Thus, *via* genetic manipulation the *EsHsp16.9* gene has the potential to confer tolerance into crops to multiple environmental stressors.

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