

## Identification of *MsHsp23* gene using annealing control primer system

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**Abstract** To identify potential candidates for acquiring stress tolerance, a new annealing control primer (ACP) system was used to identify the differentially expressed genes. Alfalfa (*Medicago sativa* L.) seedlings were exposed to various abiotic stresses such as cold (4°C for 6 h), heat (42°C for 6 h), salt (300 mM for 6 h), drought (withdrawing irrigation for 48 h), copper (500 µM for 6 h), cadmium (500 µM for 6 h), and arsenic (500 µM for 6 h). Primer sets 41 and 93 were differentially expressed and identified as same sequence, which represents a mitochondrial small heat-shock protein encoding gene, *MsHsp23*. This band was markedly increased or induced in alfalfa under heat, salt, and arsenic stresses. Differential expression of *MsHsp23* was further evaluated by Northern blot analysis. Temporal expression analysis showed that mRNA pool was altered as early as 1 h of treatment. Thus, differential accumulation of *MsHsp23* under heat, salt, and arsenic stresses suggests its potential involvement in diverse abiotic stress tolerance, and thereby making a target for further molecular analysis.

**Keywords** Alfalfa · Annealing control primer · Abiotic stress · *MsHsp23* · *Medicago sativa*

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### Introduction

Plants respond to diverse abiotic stresses such as cold, heat, drought, salinity, and heavy metals either individually or in combinations. The stresses caused by abiotic factors alter plant metabolism leading to negative effects on plant growth, development and productivity (Duncan and Carrow 2001). If the stress becomes harsh and/or continues for longer period, it may lead to unbearable metabolic burden on cells, reduce growth and ultimately result in plant death in extreme cases. A key to progress toward breeding better crops under stress has been to understand the changes in cellular and molecular machinery that occur in response to abiotic stress (Bowler et al. 1992; Noctor and Foyer 1998).

Modern molecular techniques involve the identification and use of molecular markers that can enhance plant breeding programs. For instance, superoxide dismutase and ascorbate peroxidase have been considered to be marker genes of responses to oxidative stress in plants (Foyer et al. 1994; Asada 1999). Alcohol dehydrogenase is one of the key genes identified in several studies that have been significantly induced in plants under oxygen depriving conditions (Sachs et al. 1980; Nanjo et al. 2011). A recent tissue specific proteomic analysis of soybean plants revealed that small heat shock protein (sHSPs) could be used as marker protein for heat stress (Ahsan et al. 2010). Thus identification of novel genes, which determine their expression patterns in response to stress and understanding their functions in stress adaptation, will provide the basis for engineering effective strategies to improve stress tolerance (Cushman and Bohnert 2000). Therefore, the development of genetically engineered plants by the overexpression of selected genes seems to be a viable option to hasten the breeding of “improved” plants.

Intuitively, genetic engineering would be a faster way to insert beneficial genes than through conventional or molecular breeding (Wu et al. 2005; Hu et al. 2005). Also, it would be the only option when genes of interest originate from cross barrier species, distant relatives, or from non-plant sources. Indeed, there are several traits whose correlative association with resistance has been tested in transgenic plants. Following these logical steps, various transgenic technologies have been used to improve stress tolerance in plants (Allen et al. 1997).

To achieve this goal, a continuous effort for searching new ‘important’ genes is essential, which would contribute to stress tolerance in transgenic plants. Tolerance to abiotic stress may be achieved through the modification of endogenous plant pathways, often by manipulating important regulatory proteins such as transcription factors and molecular chaperons. Although genome sequencing technology has become progressively more efficient over the past decade, the sequencing of complex genomes remains expensive.

Several techniques are designed to identify differentially expressed genes (DEGs) in cells or tissues under various experimental conditions (Kim et al. 2004). It is also important in the case of abiotic stress tolerance to assess the effects and find out key gene(s) that contribute to stress tolerance. Here, we describe the annealing control primer (ACP)-based DEGs system to profile gene expression in alfalfa in responses to cold, heat, drought, salt and heavy metal stress.

## Materials and methods

### Plant materials

Alfalfa (*Medicago sativa* L. cv. Vernal) seeds were collected from Grassland and Forages Division, National Institute of Animal Science, Rural Development Administration, Cheonan, 330-801, Korea. After germination, seeds were planted in plastic pots containing Horticulture Nursery Medium (Biomedica, Korea) and grown in a growth chamber maintained at 25°C with a light intensity of 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 14-h photoperiod. Two-week-old seedlings were subjected to drought (withdrawing irrigation for 48 h), heat (42°C) cold (4°C), salt (300 mM), copper ( $\text{CuSO}_4$ , 500  $\mu\text{M}$ ), cadmium ( $\text{CdCl}_2$ , 500  $\mu\text{M}$ ), and arsenic ( $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ , 500  $\mu\text{M}$ ). For heat and cold treatments, seedlings were transferred into a growth chamber and exposed for 6 h whereas in case of salt and heavy metal treatments, seedlings were watering with mentioned concentration of the salt and chemicals and exposed for 6 h. After each treatments, leaves were

harvested separately, frozen by liquid nitrogen and were kept at  $-80^\circ\text{C}$  until use.

### RNA was isolation and ACP-based PCR

Total RNA was isolated from the leaf tissues of treated and control plants using the Plant RNeasy mini kit (Qiagen, CA, USA) and using the GeneFishing<sup>TM</sup> DEG kit (Seegene, Seoul, South Korea), DEGs were identified by an ACP-based PCR method as described by Lee et al. (2009).

### Sequence analysis

Selected DEGs were extracted from the gel using the GENCLEAN II Kit (Q-BIO gene, Carlsbad, CA, USA) and directly cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The cloned plasmids were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the M13 forward primer (5′-CGCCAGGGTTTTCCCAGTCACGA-3′) or M13 reverse primer (5′-AGCGGATAACAATTTACACAGGA-3′). Sequencing data were confirmed with the GenBank database through the Blastx program of NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>).

### Expression analysis of *MsHsp23*

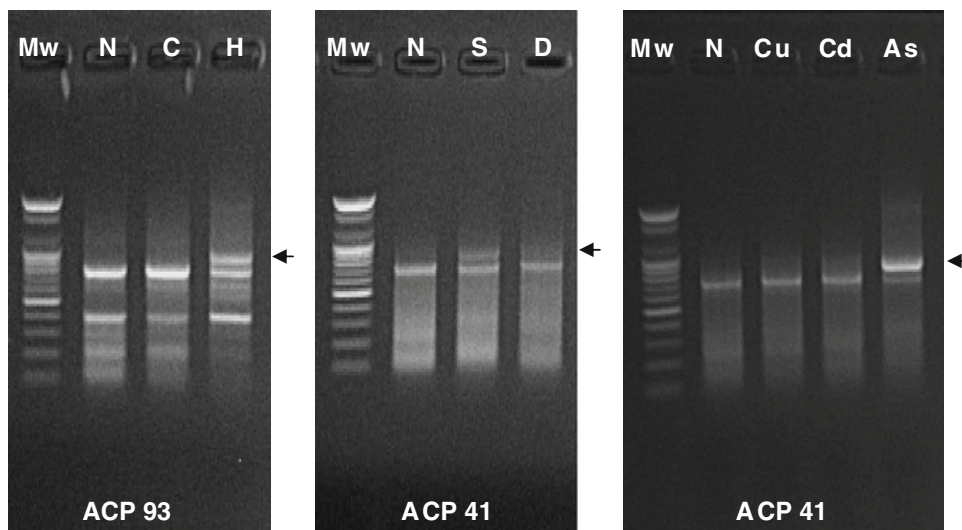
Northern blot analysis was carried out to investigate the temporal expression of *MsHsp23* gene in response to various abiotic stresses. Northern blot analysis was performed as described previously (Lee et al. 2007).

## Results and discussion

### Identification of *MsHsp23* gene

To identify differentially expressed genes under high or low temperatures, salinity, drought and heavy metals, ACP-based GeneFishing PCR technology was used with a combination of 120 arbitrary primers. Out of 120 ACP primers, ACP 93 and ACP 41 showed differentially expressed DNA bands under heat, salt and As. The bands size was about 1 kb (Fig. 1). The DNA bands were identified as same sequence, which represents a mitochondrial small heat-shock protein coding gene, *MsHsp23* (Fig. 1). This band was increased in alfalfa under heat, salt and As stresses and it suggests that *MsHsp23* may involved in those stresses. It has been reported that small heat-shock proteins are usually undetectable in vegetative tissue under normal growth conditions, but can be induced by

**Fig. 1** Agarose gel electrophoresis shows results of annealing control primer system for the identification of DEGs in response to various abiotic stresses indicated. *N* control; *C* cold; *H* heat; *S* salt; *D* drought; *Cu* copper; *Cd* cadmium and *As* arsenic. Arrows indicating the induction of the gene under the following stresses. *ACP* indicates the set of primers used for each PCR reaction. The expression of small HSP was increased under heat (H), salt (S) and As stress



developmental stimuli or by environmental stresses including heat (Sun et al. 2002; Lee et al. 2007).

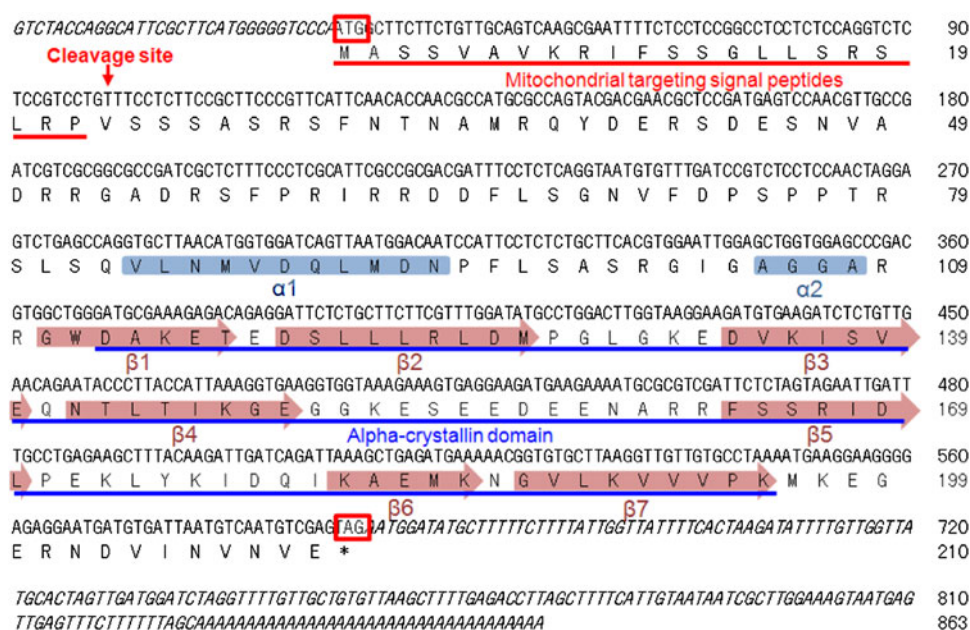
Sequencing of the *MsHsp23*

The full-length cDNA of the *MsHsp23* gene had a size of 863 bp with 5'- and 3'-untranslated regions (UTRs) and a polyA tail. The open reading frame (ORF) was flanked by a 198-bp 5'-UTR and a 32-bp 3'-UTR. An ORF search showed that *MsHsp23* contained a 633-bp ORF encoding a protein with 213 amino acids (Fig. 2). The calculated molecular weight of the encoded protein was approximately 23.36 kDa, with a theoretical isoelectric point of 5.246.

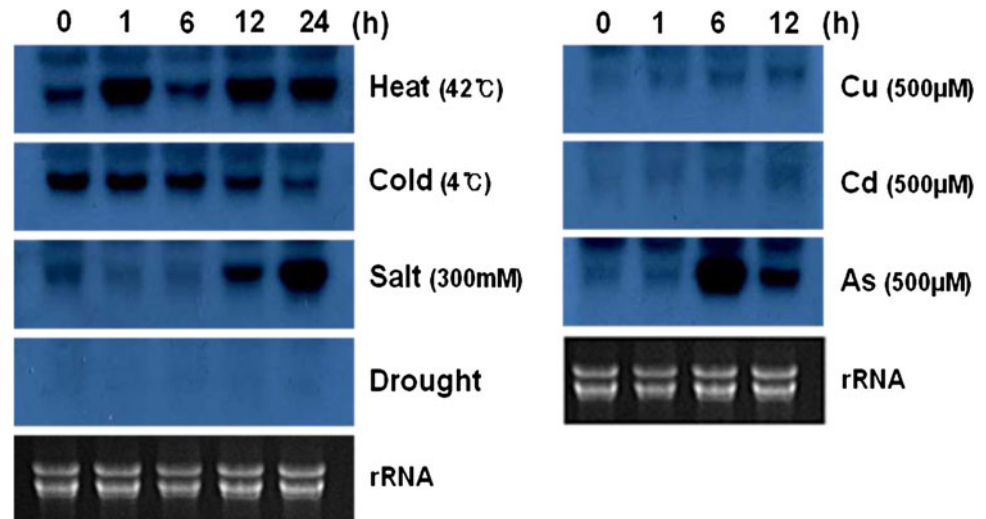
Expression of the *MsHsp23* gene under various abiotic stresses

To investigate the temporal mRNA expression patterns of *MsHsp23*, northern blot analysis was carried out. As shown in Fig. 3, results of a northern blot analysis were virtually consistent with that of the ACP-based RT-PCR with minor discrepancies. To confirm more clearly, heat, salt or As stresses cause high accumulation of *MsHsp23* transcripts, while it was reduced under cold stress. In case of drought stress, the expression difference is not detectable. Above all, the ACP technique allowed the isolation of differentially expressed genes under the conditions tested. Our results clearly indicate that the differential accumulation

**Fig. 2** Nucleotide and deduced amino acid sequences of alfalfa small Hsp23. Numbers at the right indicate nucleotide and amino acid position. The untranslated regions of 5' and 3' of *MsHsp23* are denoted by *italic*. The mitochondrial targeting signal peptides and their cleavage site are shown as red line and arrow, respectively. The alpha-crystallin domain is underlined (blue)



**Fig. 3** Expression levels of the *MsHsp23* gene under various abiotic stresses. The treated alfalfa seedlings were harvested at the indicated times



patterns of *MsHsp33* transcripts were observed by the different stress treatment, and it shows the possibility of which *MsHsp23* may be involved in diverse stress conditions and confer the stress tolerance. It has been reported in several studies that in addition to heat stress, plant sHSPs are also accumulate in response to a large number of other stresses such as heavy metals, oxidative stress and salt conditions (Wang et al. 2003; Ahsan et al. 2007; Sarkar et al. 2009). Moreover, Yeh et al. (2002) also reported that the overexpression of sHSPs in *E. coli* showed enhance thermotolerance, as well as changes in the protein aggregation patterns.

The molecular responses to various abiotic stresses were investigated by the approaches with transcriptomic analysis based on an ACP system. Here we identified differentially expressed genes under abiotic stresses in alfalfa seedlings and they were mostly unknown genes and a few common stress-related genes (data not shown). Among them, mitochondrial small Hsp23, *MsHsp23*, was responded by the diverse stress treatment such as heat, salt, As stresses and thus it could be a strong candidate that may confer the abiotic stress tolerance to plants.

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