

EXPRESSION OF SMALL HEAT SHOCK PROTEIN23 ENHANCED HEAT STRESS TOLERANCE IN TRANSGENIC ALFALFA PLANTS

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ABSTRACT

Heat shock proteins (Hsps) are key components contribute to cellular homeostasis under stressful conditions. They function as molecular chaperone involved in protein folding, plant development, signal transduction and protecting plants against several stresses. In this study, we developed transgenic alfalfa by *Agrobacterium*-mediated genetic transformation, and integration and expression of *MsHsp23* was confirmed by polymerase chain reaction (PCR), Southern and Northern blot analyses. Transgenic (Tg1, Tg3, Tg4 and Tg7) and non-transgenic (NT) alfalfa plants were exposed to heat (42 °C) treatment for 24 h. Compared to NT control plants, Tg plants showed less wilting that maintained leaves color compared to NT plants. The expression of *MsHsp23* gene and the ascorbate peroxidase (APX) levels were higher in *MsHsp23* alfalfa lines relative to NT plants. The result indicates the chaperone activity of *MsHsp23* gene that allows for the induction of APX in transgenic alfalfa. We conclude that high level of *MsHsp23* with APX activity enhanced tolerance to heat stress in transgenic alfalfa plants.

Keywords: MsHsp23, Transgenic, Alfalfa, Heat stress, Tolerance.

INTRODUCTION

Increasing temperature is a great challenge for agricultural crops production all over the world. Field study and mathematical modelling of crop plants reveal that changing temperatures have significant effect on crop productivity (Wang *et al.*, 2015). Using light energy, plants accumulate biomass by altering inorganic carbon to carbohydrate this process is known as photosynthesis. This is one of the most heat-sensitive processes in crop plants (Sulvucci, 2008). Heat stress negatively affects on plants survival, growth and agricultural yield (Duncan and Carrow, 2001). However, series of molecular responses including genes, proteins and their expressions are found to be changed by abiotic stresses including heat, salinity, drought, and extreme temperature in plants (Li *et al.*, 2013; Rahman *et al.*, 2015; Rahman *et al.*, 2016). Long-term abiotic stresses greatly affect on plant metabolism, inhibits cellular function, reduce plant growth that leads to plants die. The effects of abiotic stresses overburden the cells, affecting the ability of the cells to function. These stresses reduce the growth of the plants and, in extreme conditions, cause the death of the plants. Therefore, plant biotechnological approach is necessary for improvement of crop plants against environmental conditions. Molecular breeding technique would be effective tool for abiotic stress tolerant crop

production (Bowler *et al.*, 1992; Noctor and Foyer, 1998).

Alfalfa (*Medicago sativa* L.) is a forage legume, cultivated as high feed value with high yield potentials globally (Kechang *et al.*, 2009). In livestock industry, alfalfa plays an important role as good source of feed nutrient for animals (Hill *et al.*, 1991). Moreover, alfalfa is excellent source of biological nitrogen fixation that increases soil fertility for crop production (Deak *et al.*, 1986). Generally, alfalfa is able to drought tolerance but suffers from extended period of heat with drought stress. Heat stress reduces plant growth, leaf water potential, photosynthesis, and carbohydrate accumulation. Moreover, heat stress induced oxidative stress subsequently generates reactive oxygen species (ROS), resulting cellular damage and lipid peroxidation in plants (Wang *et al.*, 2015). Oxidative stress leads to inhibits photosynthesis, respiration process as well as plant growth. Plants have evolved an antioxidant system where overproduced ROS are scavenged by several antioxidant enzymes including ascorbate peroxidase (APX) and superoxide dismutase (SOD). APX utilizes ascorbate as a source of electron donor to decrease H₂O₂ level. However, the function of scavenging enzyme can be interrupted by heat stress that leads to lipid peroxidation, cellular injury, and membrane damage in plants.

In this study, the objective was to develop heat tolerant transgenic alfalfa using *Agrobacterium*-mediated transgenic approach. We generated transgenic alfalfa plants over expressing small heat

shock protein 23 (*MsHsp23*) under control of CaMV35S promoter. We observed that *MsHsp23* alfalfa plant enhanced tolerance to heat stress.

MATERIALS AND METHODS

Plant materials and culture explants: Alfalfa (*Medicago sativa* cv. Xinjiang Daye) seeds were collected from the National Institute of Animal Science, Rural Development Administration (RDA), Republic of Korea. Seeds were rinsed with 70% ethanol for 1-2 minutes. The seeds were then washed three times with distilled water then surface sterilized for 30 minutes using of 30% (w/v) sodium hypochlorite and 1% Tween-20. For the removal of surfactant, the sterilized seeds were rinsed properly with sterile deionized water. The sterilized seeds were germinated on hormone-free, half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 2% (w/v) sucrose and 0.3% (w/v) Gelrite. The seeds were allowed to culture in a growth chamber at 25°C with 14 h photoperiod. Hypocotyl segments of alfalfa plants were used as explants.

Vector construction: The alfalfa small heat shock proteins (*MsHsp23*) cDNA fragment was ligated into the translation ligation initiation codon within the 5 untranslated sequence of the constitutively expressed cauliflower mosaic virus promoter (35S). The resultant chimeric gene cassette was then inserted into the *KpnI/ZbaI* site of the pCambia1300 binary vector (Fig. 1). The recombinant pCAM-*MsHsp23* was introduced into *Agrobacterium tumefaciens* strain EHA105, which was used for genetic transformation.

Genetic transformation: Seven-day-old hypocotyls were used as explants for transformation. Hypocotyls were carefully excised (1–3 mm in long) from the seedlings without meristematic axillary buds. Hypocotyls were gently shaken in *Agrobacterium* suspension for 30 min then blotted on a sterile filter paper. The hypocotyls segments were transferred to the solid co-cultivation medium and maintained up to 5 days. The hypocotyls segments were transferred onto shoot-inducing medium (SIM) containing 250 mg/L hygromycin, and every three weeks, followed by sub-cultured into fresh SIM medium. The green healthy shoots were screened by MS medium containing 20 mg/L hygromycin (Hg). The seedlings were maintained in selection medium up to rooting. Putative transgenic seedlings were transferred in to pot containing nursery medium (soil: perlite; 1:1), and grown in green house.

Molecular analysis of alfalfa plants: Genomic DNA was isolated from the leaves of the wild type and putative transgenic plants using the CTAB method.

The *MsHsp23* alfalfa plants were identified by PCR screening. The amplification of *MsHsp23* and *HPT* genes were performed by PCR. The following genes primers were used for PCR analysis: 5'-TCTAGAATGGCGTCTGTTGCT-3 and 5'-TTCTCAGCTATTTAGGAGCTC-3 for *MsHsp23*, and the primers; 5'-CCTGAACTCACGACG-3 and 5'-AAGACCAAGGAGCATAT-3 were designed for the *HPT* gene, respectively. The conditions of PCR amplification was performed according to Lee *et al.* (2012a). Integration of the *MsHsp23* gene into the alfalfa plant genomes was further confirmed with Southern blot analysis. Twenty micrograms of genomic DNA was digested with *EcoRI* and separated electrophoretically on a 1.0% agarose gel. Following the each sample was transferred to a nylon membrane and hybridized with the (alpha-32P)-dCTP-labeled *MsHsp23* probe by PCR-based labeling using pCAM-*MsHsp23* plasmids as a template. Southern blot and Northern blot hybridizations were performed as described previously by Lee *et al.* (2000).

Heat tolerance assay: The *MsHsp23* transgenic and wild-type plants were kept at control environment for 24 h prior to heat treatments. Plants were placed in a heat chamber where temperatures of total system were well controlled. Three independent alfalfa lines (Tg1, Tg3, Tg4, and Tg7) were exposed to 42 °C for 24 h. Control plants were maintained at normal temperature. Following treatment leaves samples were harvested from *MsHsp23* plants and not-transgenic (NT) plants. The leaves samples were immediately frozen in liquid nitrogen then kept at -90 °C until used.

Analysis of ascorbate peroxidase (APX): To determine the ascorbate peroxidase (APX) activity, the total soluble proteins were extracted from the leaves of alfalfa plants using extraction buffer. The composition of the protein extraction buffer was 50 mM HEPES (pH 7.0) and 0.1 mM EDTA. The concentration of the protein was estimated with a Bio-Rad protein assay kit. The oxidation of ascorbate was initiated by H₂O₂, and the decrease to 290 nm was monitored for 1.5 minutes. The reaction mixture contained 40 mM HEPES (pH 7.0), 0.1 mM EDTA, 0.03 mM ascorbic acid (AsA), 0.1 mM H₂O₂, and enzyme samples. One unit of APX activity was defined as the amount of enzyme that caused the oxidation of 1 mole of AsA. The APX activity was determined by estimating the rate of ascorbate oxidation (extinction coefficient: 2.8 mM⁻¹cm⁻¹) as described previously by Nakano and Asada (1981).

RESULTS AND DISCUSSION

Confirmation of *MsHsp23* gene integration to plants genome: Seven independent primary transformants were obtained by plant selection marker hygromycin.

Out of these 4 lines (Tg1, Tg3, Tg4 and Tg7) have showed the presence of genes by PCR screening using gene specific primers for both *MsHsp23* and *hpt* genes (Figs. 2A,B). Southern analysis of 4 lines confirmed that genes of interest were successfully integrated into the alfalfa genome. Therefore, it was conformed that alfalfa plant contains at least one copy of transgenes and the transgenic events were truly independent (Fig. 3A). High expressions of mRNA as the genes were driven by a constitutively expressing 35S promoter. These results indicated that transgenes were successfully expressed transcriptionally and translationally in transgenic alfalfa plants. A numerous studies have been revealed that several heat shock proteins (Hsps) are most widely distributed which responded to different abiotic stresses (Sun *et al.*, 2002). These Hsps were contributed in plant development, and stresstolerance in plant under stressful conditions (Dafny-Yelin *et al.*, 2008; Chauhan *et al.*, 2012). The functional characteristics of different subcellular localized small Hsps and mitochondrial small Hsps are largely unknown. According to previous report, chaperone property of small Hsps prevents the denaturation of heat-injured proteins (Siddique *et al.*, 2008). In our study, *MsHsp23* alfalfa plants enhanced tolerance to heat stress. Previously, we discussed how alfalfa mitochondrial small heat shock proteins are overexpressed in tall fescue and tobacco plants subsequently has been proven the biological function of this gene for abiotic stresstolerance (Lee *et al.*, 2012a,b).

Over expression of *MsHsp23* transgenic alfalfa plants: We confirmed the expression of four lines (Tg1, Tg3, Tg4 and Tg7) by Northern blot analysis. The Tg4 and Tg7 were highly expressed compared to Tg1 and Tg3 (Fig. 3b). We found the *MsHsp23* gene

was highly expressed in transgenic plants during heat (42°C) stress. It has been well documented that plants respond to heat stress often with induction of HSPs (Fujimoto and Nakai, 2010; Hartlet *et al.*, 2011). The up-regulation of *MsHsp23* by heat stress suggested that this gene may play some roles related to heat stress response.

***MsHsp23* alfalfa plants enhanced tolerance to heat stress:** As a consequence of molecular results we try to find out the phenotypic difference between transgenic and NT plants at whole plant level. As shown in Fig. 4, the significant differences were not observed phenotypically before heat treatment while the phenotypic variations were found after heat treatments. The Tg7 line was less affected by heat stress compared to NT plants (Fig. 4A, B). Transgenic Tg7 line represents less number of wilted leaves while the NT plants became largely wilted and changed the leaves color. This result indicates that over expressed the *MsHsp23* may involve to protect stress-induced damages in transgenic plants. It is well known that the extreme heat treatment regulates the inactivation of diverse functional proteins and subcellular components in the cells. Moreover, changing of leaf color, reduction of photosynthesis, breakdown of the chlorophyll from the photo-oxidative damage caused by the heat stress (Feierabend and Winkelhusener, 1982; Zlatev and Yordanow, 2004; Erge *et al.*, 2008). Cytosolic and chloroplastic small Hsps have been investigated in terms of tolerance to heat and drought in plants (Sun *et al.*, 2002; Chauhan *et al.*, 2012). However, the molecular and phenotypical observations of transgenic alfalfa plants indicated that over expression of *MsHsp23* gene enhances tolerance to heat stress.

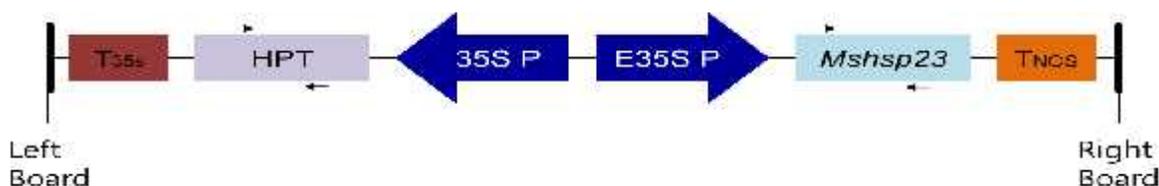


Fig. 1. Schematic representation of T-DNA region of pCAM-*MsHsp23*. The *MsHsp23* construct was used for transformation in alfalfa (*Medicago sativa* L.). *E35S*, enhanced CaMV 35S promoter; *MsHsp23*, alfalfa small heat shock protein 23; *T35S*, *HPT*, hygromycin phosphotransferase; CaMV 35S terminator; *TNOS*, nopaline synthase terminator. Arrow indicates the gene-specific primers for *HPT* and *MsHsp23*, respectively.

Ascorbate peroxidase (APX) activity increased in *MsHsp23* alfalfa plants: We analyzed the ascorbate peroxidase (APX) activity in overexpressing *MsHsp23* alfalfa plants response to heat treatment at 42°C for 48h. As shown in Fig. 5, APX activity was increased in *MsHsp23* transgenic line (Tg7) compared to NT plants.

The high accumulation APX activity in transgenic plants indicated that it regulated by heat stress. Plants have two systems for scavenging ROS: enzymatic system including APX, POD, SOD and CAT; non enzymatic including ascorbic acid (AsA). Possibly, ROS was regulated by enzymatic system including

APX. So, the high APX likely dependent on their transcription level. However, antioxidant enzymes APXs are widely distributed in different subcellular organelles including chloroplast lumen, stomata, peroxisomes, cytosol, and inner membrane space of mitochondria that effectively scavenges hydrogen peroxide (Miller *et al.*, 2010). The overexpression of small *Hsp 16.45* showed enhance tolerance to abiotic

stress in *Arabidopsis*, whereas the superoxide dismutase (SOD) and catalase (CAT) activities were highly induced (Mu *et al.*, 2013). Together these above discussion, we anticipate that over expression of *Mshsp23* gene with high level of APX activity enhanced tolerance to heat stress in transgenic alfalfa plants.

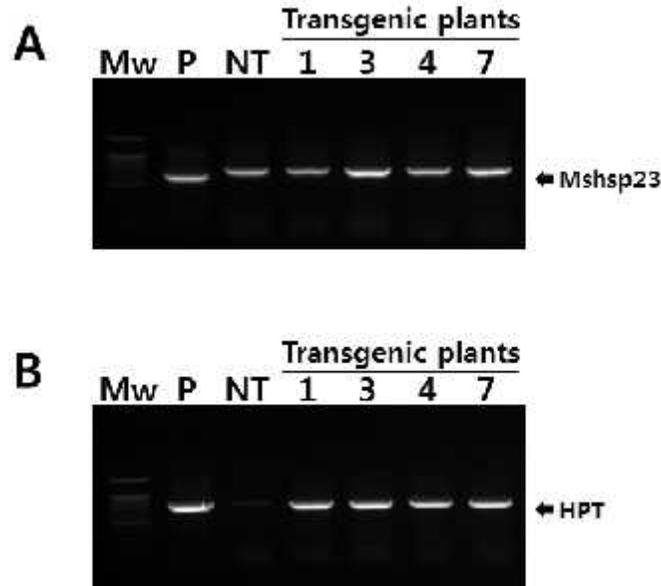


Fig. 2. Integration of transgene to the transgenic plants. PCR amplification of *Mshsp23* (A) and *HPT* (B) genes. The arrows indicate amplified gene-specific bands. *Mw*, molecular weight of marker; *P*, plasmid DNA of expression vector pCAM-MsHsp23 vector; *NT*, non-transgenic alfalfa plant; 1, 3, 4 and 7 transgenic (Tg) lines.

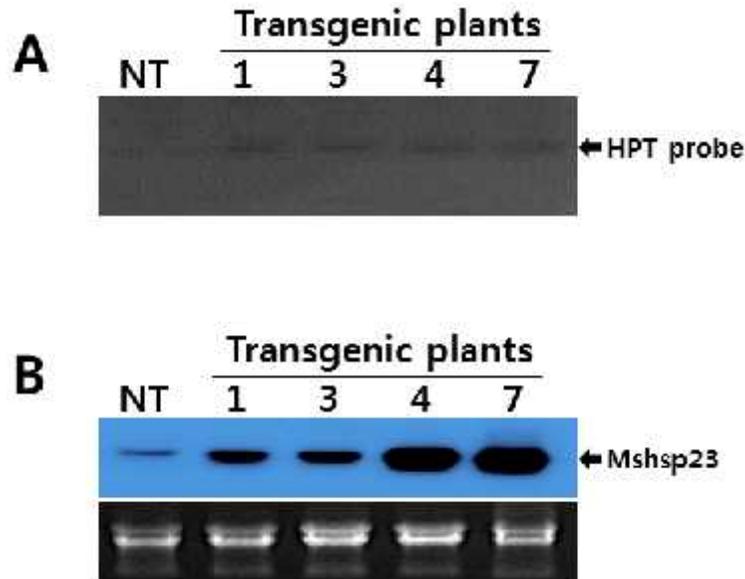


Fig. 3. Molecular characterization of *Mshsp23* gene. Southern blot analysis of *Mshsp23* gene (A), Northern blot analysis for expression of *Mshsp23* gene (B) in transgenic alfalfa plants. The ³²P-labeled DNA of HPT

was used as a probe for hybridization. The 1, 3, 4 and 7 indicate different transgenic line number of alfalfa plants.



Fig. 4. Measurement of heat tolerance of alfalfa plants. The non transgenic and transgenic lines before the heat treatment (A), and after 24 h after the heat stress treatment at 42°C (B). Twelve-week-old non-transgenic (NT), and transgenic (Tg-7) plants were exposed to 42 °C for heat treatment.

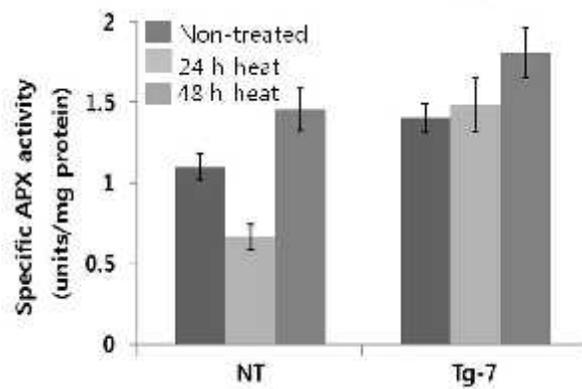


Fig. 5. Ascorbate peroxidase (APX) activity in transgenic and wild-type plants response to heat stress. The non-transgenic (NT) and transgenic (Tg7) leaves samples were harvested after 24h and 48h heat

treatments (42 °C), respectively. The assay was carried out with three independent experiments. All values present the mean of three replications.

Conclusion: We developed *MsHsp23* transgenic alfalfa plant using *Agrobacterium* mediated genetic transformation approach. The *MsHsp23* gene was driven by a constitutively over expression CaMV 35S promoter. The molecular analyses indicated *MsHsp23* genes were integrated successfully to alfalfa plants genome. In our study, we found *MsHsp23* transgenic plants exhibited tolerance in alfalfa plants to heat stress. In addition, the antioxidant enzyme APX that was highly induced in transgenic alfalfa plants. These results suggest that transgene *MsHsp23* with high level of APX enhanced heat tolerance in transgenic alfalfa

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