

IDENTIFICATION OF COLD AND HEAT-INDUCED DIFFERENTIALLY EXPRESSED GENES IN ALFALFA (*MEDICAGO SATIVA* L.) LEAVES

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ABSTRACT

Alfalfa is an important forage legume cultivated as livestock feed crop with high yield potential. The productivity of alfalfa is reduced by multiple abiotic stresses. In this study, we identified cold and heat induced differentially expressed genes (DEGs) in alfalfa leaves using annealing controlled primer-based (ACP) approach. Three-week-old alfalfa seedlings were exposed to cold (4°C for 6 h) and heat (42°C for 6 h) treatments, respectively. Control plants were maintained at normal temperature (25°C). In this molecular technique, over 100 ACPs sets were applied for this investigation. Total 10 differentially expressed genes (DEGs) were obtained during cold or heat stresses. Out of 10 DEG, 7 (3 were cold and heat, 3 were cold, and 1 heat induced) were up-regulated response to either cold and/or heat stress. Sequencing was performed for the functional analyses of these stress induced DEGs. The identified DEGs were corresponding as ribosomal protein L20, S-adenosylmethionine decarboxylase, Cold-acclimation specific protein 30, DNAJ-like protein, and Heat shock 22 kDa protein. These genes were mostly involved in stress tolerance in plants. These stress-induced genes would be useful candidates for stress tolerant crop production.

Key words: Alfalfa, DEGs, Tolerance, Cold stress, Heat stress.

INTRODUCTION

Environmental stresses such as cold, heat, salinity, heavy metals, extreme temperature and drought are major factors which adversely affect to plant growth and productivity (Ahuja *et al.*, 2010). Plants are evolved several mechanisms for adapting to this adverse environments. Molecular programming of the plants can change the whole systems of the stressed plants (Chen *et al.*, 2006; Legnaioli *et al.*, 2009; Ramirez *et al.*, 2009; Ning *et al.*, 2010). Under stressful condition, numerous molecular changes are occurred at transcriptome, proteome and metabolome levels of plants (Li *et al.*, 2008; Shulaev *et al.*, 2008; Barkla *et al.*, 2009; Cseke *et al.*, 2009; Zeller *et al.*, 2009). Therefore, identification of new genes/enzymes with traits as well as production of stress tolerant crops are prime important. Alfalfa (*Medicago sativa* L.) is one of the important forage legumes, cultivated for the excellent nutrition quality and high yield performance (Kechang *et al.*, 2009; Rahman *et al.*, 2015). It contributes in biological nitrogen fixation and increases soil fertility (Kang *et al.*, 2011). Cold and heat are important constituents for reducing forage yield and quality (Gupta and Deswal 2012). However, it is known that several molecular and physiological changes are occurred in plants due to abiotic stresses (Chinnusamy *et al.*, 2007). Cold-stress induces reactive oxygen species (ROS) production (Xu *et al.*, 2008) and changes leaf water potential, chlorophyll level and

photosynthetic efficiency (Yu *et al.*, 2002). Electron transport chain is greatly affected due to inhibition of photosystem II (PS II) by cold stress (Jeong *et al.*, 2002). It affects to PSII by damaging of the photosynthetic repair machinery (Jeong *et al.*, 2002; Nishiyama *et al.*, 2006). Plant are evolved several mechanisms by which can adapted to cold and/or freezing condition (Thomashaw, 1999). However, heat stress is another critical environmental factor that damages growth and productivity of plants (Chauhan *et al.*, 2013; Fraser *et al.*, 2005). Cellular molecules including DNA, proteins and lipids are negatively affected by reactive oxygen species (ROS) which are produced by heat stress, leading to damage and destruction of the cell, reduces yields and quality of the crop plants (Lin *et al.* 2005; Peng *et al.*, 2004; Wanxia *et al.*, 2003). Various techniques are used for the identification of differentially expressed genes (DEGs) in plants. Numerous proteins/enzymes have been identified by proteomic approaches in alfalfa plants, and screened their biological functions response to abiotic stresses (Rahman *et al.*, 2015; Chen *et al.*, 2015; Rahman *et al.*, 2016). However, considering short time, we used annealing controlled primers (ACPs) based a convenient technique for identification of DEGs in plants. This ACP based approach was used previously by other researchers (Hwang *et al.*, 2003; Kim *et al.*, 2004). Our research group also applied this molecular technique for several plants. In barley leaves, DEGs has been identified under salt stress (Lee *et al.*, 2009), drought induced DEGs were obtained in barley leaves (Lee *et al.*, 2011). In Siberian

wild rye grass (*Elymus sibiricus* L.), this technique has been very useful for detection of heat stress-induced differentially expressed genes for first time (Lee *et al.*, 2015). The objective of this study was to identify useful genes/enzymes in alfalfa leaves response to cold and heat stresses. Further target, to clone of these new candidates and production of abiotic stress tolerant crop for sustainable agriculture in marginal crop land.

MATERIALS AND METHODS

Plant material and stress treatments: Alfalfa (*Medicago sativa* cv. Vernal) seeds were obtained from the National Institute of Animal Science, Rural Development Administration, Republic of Korea. Seeds were surface sterilized using 70% ethanol with 0.1 % of Tween 20. Then placed to plastic pots (13 cm in diameter) containing potting mix (nursery soil: perlite: 1:1). In controlled growth chamber, plants were grown for 3 weeks at 16/8 h photoperiod. Three-week-old alfalfa seedlings were exposed to cold (4°C for 6 h) and heat (42°C for 6 h) treatments, respectively. Following treatments, plant leaves were excised separately, quickly frozen in liquid nitrogen then stored at at -80°C until further analysis.

Determining of photosynthetic efficiency: The photosynthetic efficiency was determined using portable fluorometer FMS2 (Hansatech Instruments, UK) under cold and heat treatments. Alfalfa plants were exposed to cold and heat treatments as described in materials and methods sections. To determine photosynthetic efficiency, plants were adapted for 15 min prior to measurement of chlorophyll fluorescence (Fv/Fm).

Extraction of RNA: Total RNA was isolated from the leaf tissues of alfalfa plants and purified using Plant RNeasy mini kit (Qiagen, CA, USA). The isolated RNA was used for first-strand cDNA synthesis by reversed-transcriptase-catalyzed reaction. The final volume of reaction mixture was 20 µl. The conditions of reactions were maintained described previously by Lee *et al.*, (2015). After reverse transcription reactions the cDNAs were synthesized.

ACP-based GeneFishing™ reverse transcription chain reaction, gene cloning and sequencing: Annealing controlled primer (ACP)-based reverse transcription-polymerase chain reaction (DDRT-PCR) technique was used for the identification of differentially expressed genes (DEGs) according to the method of Lee *et al.*, (2014). The amplified PCR products were separated using 2% agarose gel. Nucleic acid cleaning solution (Red Safe) was used in agarose gel for the visualization of DEGs. GENCLEANÒ II Kit (Q-BIO gene, Carlsbad, CA, USA) was used for the extraction of selected DEGs from the gel. The obtained DEGs were

extracted and cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) according to the instructions of manufacturer. The ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) was used for the sequencing of cloned plasmids. The NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) tool was used for the identification and confirmation of sequenced data.

RESULTS AND DISCUSSION

Cold and heat reduced photosynthesis efficiency: The photosystem II (PSII) is a large pigment protein complex in thylakoid membrane that performs the vital reactions of photosynthesis. The photosynthesis is an essential physiological process for plants growth and development. In stressful condition, photosynthetic efficiently greatly reduced by abiotic stresses in plants (Kong *et al.*, 2014). We measured photosynthesis efficiency (Fv/Fm) of treated and not treated alfalfa plants. In this study, morphological and physiological alterations were observed between treated and normal plants. Morphological changes of alfalfa leaves were found after cold and heat treatments (Figs. 1A, B). The photosynthesis efficiency (Fv/Fm) was highly reduced by cold treatment compared to heat treatment (Figs. 1C, D). However, overall observation indicated that the normal form of alfalfa leaves changed by cold stress while heat stress has not changed significantly. This result indicated that heat stress has less effect as compared to cold stress on morphological changes as well as photosynthetic activity of alfalfa plants. The Fv/Fm efficiency reduced by heat and cold stresses compared to control plants. Possibly, photosynthetic activity decreased for suppressing electron transport in chloroplast as well as inhabiting the activity of calvin cycle (Pastenes and Horton 1996). Our results support with other researchers findings where as the chlorophyll fluorescence (Fv/Fm) reduced by cold treatment (Krivosheeva *et al.*, 1996; Roden *et al.*, 1999; Verhoeven *et al.*, 1999).

Identification of cold and heat-induced genes in alfalfa: In this current research, we obtained cold and heat-induced genes in alfalfa leaves using annealing control primer (ACP)-based GeneFishing approach. We have identified several genes including ribosomal protein L20 (RpL20; DEG9), S-adenosylmethionine decarboxylase (AdoMetDC; DEG7), Cold-acclimation specific protein 30 (CAPs; DEG 32), DNAJ-like protein (DNAJ-LP; DEG41), and Heat shock 22 kDa protein (HSP 22; DEG54) using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) tool. Total 10 differentially expressed genes (DEGs) were induced under cold or heat stresses. We identified 7 (3 were cold and heat, 3 were cold, and 1 heat induced) DEG, were exhibited up-regulation response to either cold or heat

stress (Table 1). These candidates were mostly involved in stress tolerance in plants. In the following sections we were discussed with the putative biological role response to abiotic stresses.

Ribosomal protein L20 (RpL20; DEG9), was up regulated response to cold and heat stresses (Fig.1, Table 1). The Rpl20 is essential for cell survival in plants under stress condition. Rpl33, a nonessential plastid-encoded ribosomal protein that is required under cold stress conditions in tobacco plants (Rogalski *et al.*, 2008). However, in our study, the up-regulation of RpL20 indicated that it is stress inducible protein that have potential role in alfalfa plants under cold and heat stresses.

We identified cold-induced S-adenosylmethionine decarboxylase (AdoMetDC; DEG7), cold acclimation specific protein 30 (DEG32) and DnaJ-like protein (DEG41). S-adenosylmethionine decarboxylase (AdoMetDC) plays an essential regulatory role in the polyamine biosynthetic pathway by generating the n-propylamine residue required for the synthesis of spermidine and spermine from putrescein (Pegg *et al.*, 1998). AdoMetDC also known to influence the rate of ethylene biosynthesis. AdoMetDC regulates plant growth and development as well as plant stress responses.

Proteins including AdoMetDC, and AdoMet synthase were identified in alfalfa and their role were mentioned under drought and salt stresses (Bagga *et al.*, 1997; Rahman *et al.*, 2015). S-adenosylmethionine (AdoMet) has a pivotal role by involving the processes of nucleotide and amino acid metabolism. AdoMet acts as methyl donor to DNA, RNA, proteins and small molecules, as a propylamine donor in the synthesis of the polyamines (PAs). These PSs play pivotal role in cell growth, and development and respond to stress tolerance to various environmental factors.

Cold-acclimation specific protein 30 (CAPs; DEG 32) that was indentified in alfalfa leaves under cold stress. CAPs are belongs to Group III protein found in several organism including plants (Berger *et al.*, 1996; Monroy *et al.*, 1993). Caps were reported previously in several plants (Monroy *et al.*, 1993; Wolfrim *et al.*, 1993; Mohapatra *et al.*, 1989). The above authors mentioned that expression of cold acclimation-specific (cas) genes were significantly correlated with cold induced freezing tolerance in plants. Therefore, we predicted that the up regulation of CAP would be effective candidate for low-temperature tolerance in plants.

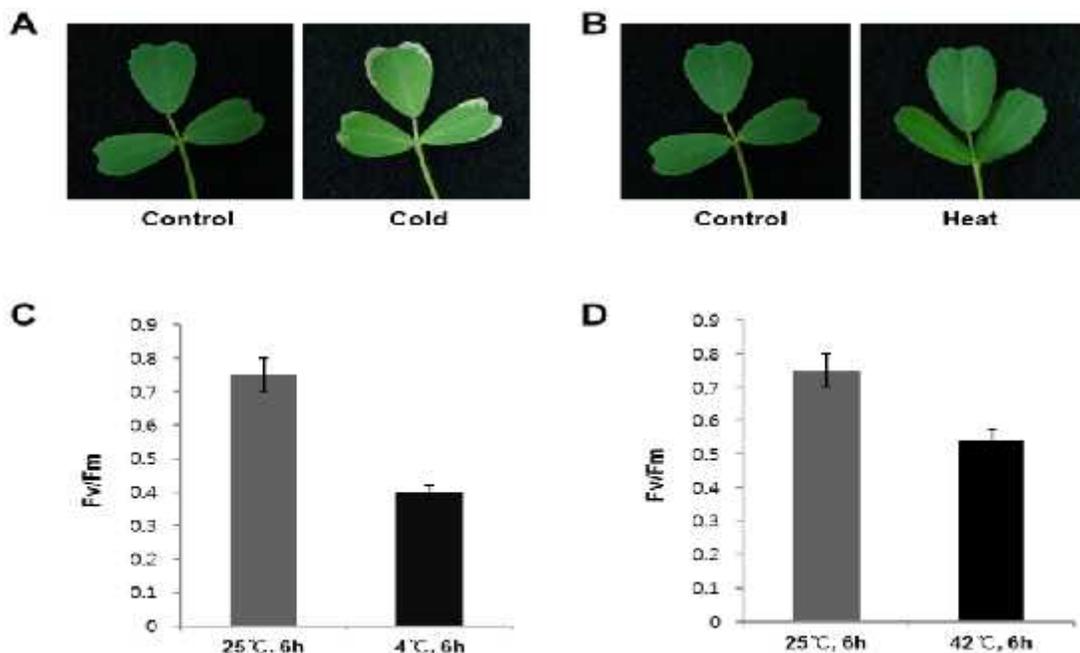


Fig 1. Effect of cold and heat stresses on the leaf and photosynthetic efficiency. Morphological changes of alfalfa leaves after cold (A), and heat stress treatments (B). Reduction of chlorophyll fluorescence (Fv/Fm) in alfalfa leaves due to cold (C) and heat (D) stresses.

Differentially expressed gene 41 (DEG41), identified as DnaJ-like protein (DnaJ-LP) under cold stress treatment (Table 1). This protein was newly reported in this study that observed in alfalfa leaves. Now, it has been cloned from alfalfa for development of stress tolerant crop using transgenic approach in our progress

research. DnaJ-LP a type of molecular chaperone involved in proper folding of protein as well as showed abiotic stress response in plants. Therefore, we proposed that our cloned homolog would be useful for freezing or low temperature tolerant crop production. We obtained, heat-induced protein corresponding as heat shock 22 kDa

(HSP 22; DEG54) in alfalfa leaves. HSPs are belongs to type IV (molecular mass between 15~ 30kDa) chaperone group. In pea plants, HSP22 was induced by heat stress, observed the expression of this protein was detected within 1 h (Wood *et al.*, 1998). Moreover, the author

suggested that HSP22 had pivotal contribution in thermotolerance in pea plants. In our study, as a consequence of legume groups, our identified HSP22 (DEG 54; Table 1) would be recommended as potential candidate for development of thermo-tolerant crops.

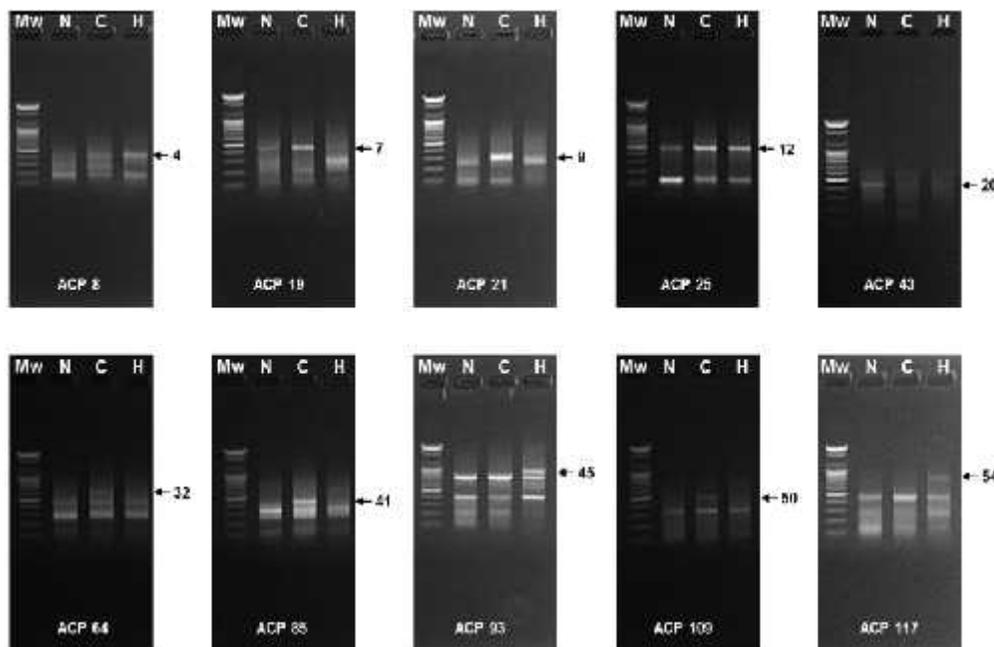


Fig 2. Agarose gel image of differentially expressed genes (DEGs) in alfalfa leaves. Arrows indicate differentially expressed cold and heat-induced genes compared to the control. N, non-treated; C, cold treated; H, heat treated samples of alfalfa plants

Table 1. Cold and heat-induced differentially expressed genes (DEGs) in alfalfa leaves obtained by BLAST- (<http://www.ncbi.nlm.nih.gov/BLAST/>) tool.

Serial no.	Accession no.	Gene name (species)	Product size (bp)	Score	E Value
<i>Cold and heat induced</i>					
DEG 4 ↑	ABK96239.1	Unknown (hybrid poplar: <i>Populus trichocarpa</i> x <i>Populus deltoides</i>)	428	418	e-116
DEG 9 ↑	YP_001381705.1	Ribosomal protein L20 (RpL20) (<i>Medicago truncatula</i>)	491	188	7.00E-47
DEG 12 ↑	ACJ85344.1	Unknown(<i>Medicago truncatula</i>)	515	688	0.0
<i>Cold induced</i>					
DEG 7 ↑	ABO77440.1	S-adenosyl methionine decarboxylase (<i>Medicago sativa</i> subsp. <i>falcata</i>)	449	371	e-102
DEG 32 ↑	ABX80061.1	Cold-acclimation specific protein30 (<i>Medicago sativa</i> subsp. <i>falcata</i>)	627	256	4.00E-67
DEG 41 ↑	AAB36543.1	DnaJ-likeprotein (<i>Phaseolus vulgaris</i>)	511	255	e-142
<i>Heat induced</i>					
DEG 54 ↑	CAA60120.1	Heat shock 22 kDa protein, mitochondrial (<i>Pisum sativum</i>)	519	627	e-179

Conclusion: In this study, we used an ACP-based RT-PCR approach for the identification of differentially expressed cold and heat induced genes in alfalfa leaves.

These identified genes were mostly involved in abiotic stress tolerance in plants. In addition, we obtained newly identified genes including cold-acclimation specific

protein 30 (CAPs; DEG32), DNAJ-like protein (DEG41) and Heat shock 22 kDa protein (DEG54). We expect, as a new report this study discloses the latest insight of molecular mechanisms in alfalfa plants under stress conditions. However, identification of these genes with some new candidates would be useful for abiotic stress tolerant crop production. Further efforts are going to characterize of these promising candidates for development of abiotic stress tolerant crop using biotechnological approach.

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