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SHORT COMMUNICATIONS

Identification of Abiotic Stress Responsive Genes from Indian High Altitude *Lepidium latifolium* L.

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ABSTRACT

Abiotic stresses are major environmental factors that periodically account for significant loss in crop productivity. In order to improve the abiotic stress tolerance in vegetable crops through transgenic approaches, authors isolated and cloned six up-regulated, *LlaDREB1b* (JN214345), *LlaGPAT* (JN398166), *LlaNAC* (FJ423495), *LlaCIPK* (FJ423496), *LlaPR5* (GQ853409) and *LlaIPK* (FJ487575) and two down-regulated *LlaRan* (JN214347) and *LlaDRT* (JN214346) abiotic stress responsive genes from Indian high altitude *Lepidium latifolium* L. plant that that may be used for abiotic stress-tolerance engineering upon functional validation.

Keywords: *Lepidium latifolium*, abiotic stress responsive genes, high altitude agriculture, transgenic technology

1. INTRODUCTION

The high altitude regions owing to their unique climatic and geographic differentiation have specific agro-technological requirements, different from the ones employed elsewhere for vegetable production. The major high altitude abiotic stresses includes cold, frost, drought, salinity, low oxygen, high wind velocity and intense UV radiations etc. that negatively influence the survival, biomass production and yields of vegetable crops[1] up to 70 per cent. Since tolerance to these stresses is multigenic and quantitative in nature, a massive challenge exists to understand the key molecular mechanisms for advanced selective breeding purposes. A lot of efforts made to identify the suitable varieties/hybrids and cultivation practices, the complexity of trait of tolerance to abiotic stresses remains the major limitation in overcoming these barriers for better agricultural productivity of high altitude regions. Also, the mechanisms by which plants perceive environmental signals and further their transmission to cellular machinery to activate adaptive responses is of critical importance for the development of rational breeding and transgenic strategies to impart abiotic stress tolerance in vegetable crops[2].

Conventional breeding methods have met with limited success in improving the abiotic stress tolerance of important vegetables while biotechnology offers new and highly precise strategies that can be used to develop transgenics crop plants with improved tolerance to abiotic stress that would certainly enhance the productivity of vegetable crops at high altitude regions[3]. Although, recent advancements in genomics, transcriptomics and regulomics have enriched our understanding on stress physiology of plants, and it is hoped that this will contribute to the development of tolerant genotypes for such regions.

Timely modulation of specific sets of genes is critical for survival of plants during abiotic stress, which further dictates accumulation of mRNAs and proteins and subsequently leads to overall physiological and biochemical changes in the plants. Although a number of studies have revealed the plant genes that undergo altered expression on exposure to abiotic stress and many of these like, DREB/CBF, AP2/ERF, MYB, MYC, HD-ZIP AREB/ABF, CBL-CIPK and NAC regulons have been proposed to show their abiotic stress tolerance[4]. In addition to these genes there are several others that may play a role in abiotic stress tolerance, which are still not identified. Therefore, the identification of abiotic stress responsive genes, especially from the plants acclimatized to high altitude regions is important. Also, the current knowledge of genomics and transcriptomics emerge mainly from handful 'model plants', and often novel genes or alleles of known genes from unexplored orphan plants remain unknown to the scientific world. In order to induce abiotic stress tolerance in major vegetable crops, these resources are required to be explored. Also, the IPR and patenting issues impose the necessity for cloning of novel abiotic stress responsive genes from the indigenous high altitude plants like *Lepidium latifolium* L. (Fa. Brassicaceae). It is a herbaceous perennial weed commonly called as Pepperweed, Pepperwort or Peppergrass. This plant is native to Eurasia, and distributed from North Africa north through Europe to Norway and east to the western Himalaya[5]. In Leh-Ladakh, it grows naturally at altitudes 1200 m - 4500 m asl (4000 ft - 14,000 ft) in extreme temperatures ranging from –40 °C to + 40 °C. In these regions, most of the plants shed their leaves during winters, while Lepidium may be found growing well with green leafy appearance and surviving under snow indicates its potential as source donor of abiotic stress responsive genes.

2. MATERIALS AND METHODS

The seeds of the *Lepidium* plant were collected from

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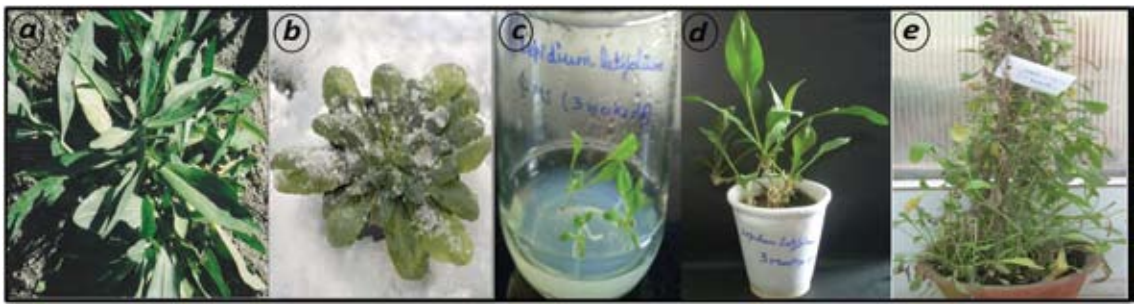

Figure 1. (a) *Lepidium latifolium*: grown at Leh in natural habitat (3600 m asl), (b) plant survived under natural abiotic stress, (c) seedling grown in ½ MS medium (3 weeks old), (d) hardened plant (3 months old), and (e) plant transferred to soil (3 years old) at DIBER, Haldwani.

Leh and have been successfully germinated and maintained in culture room under white light at 25 °C with 16 h light and 8 h dark photoperiods. Fully grown mature plants were subsequently transferred to soil in glass house (Fig. 1) at Defence Institute of Bio-Energy Research (DIBER), Haldwani, India. In Leh-Ladakh, it grows naturally in extreme temperatures ranging from - 40 °C to + 40 °C (Fig. 1(a)). In these regions, most of the plants shed their leaves during winters, while *Lepidium* may be found growing well with green leafy appearance and surviving under snow (Fig.1(b)) indicates its potential for the isolation of abiotic stress responsive genes. The seeds of the plant collected from in and around the city of Leh and have been successfully germinated and maintained in culture room under white light at 25 °C with 16 h light and 8 h dark photoperiods (Figs. 1(c) and 1(d)). Fully grown mature plants are subsequently transferred to soil in glass house conditions (Fig. 1(e)) in the laboratory at Haldwani.

Total RNA was isolated from young leaves of three months old *Lepidium* seedling by using standard methods of HiPurA^{TM} Plant RNA isolation Kit (HiMedia laboratories Pvt. Ltd, India). Total RNA was given DNAase treatment (RNAase free) for removing the DNA contamination[6]. The cDNA was synthesized for Rapid Amplifications of cDNA Ends (RACE) amplifications by following manufacturer's instructions of FirstChoice^{R} RLM-RACE kit (Ambion Inc. Texas, USA). RACE fragments of selected abiotic stress responsive genes were amplified by using their respective gene specific primers and following standard methods of commercial RACE kit (Ambion Inc. Texas, USA). These RACE fragments were cloned in pBluescriptII SK + vector (Stratagene, USA) and sequenced commercially from Vimta Labs Ltd, Hyderabad, India. After that, extreme forward and reverse gene-specific primers were designed (primer sequences are shown in Table 1) for all selected genes and used to amplify their full-length cDNA by following standard method of commercial reverse transcription-PCR (RT-PCR) kit (Clontech, USA). The RT-PCR product was subsequently sequenced for further confirmation. Sequence analysis and protein predictions were made by using the NCBI ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

3. RESULTS AND DISCUSSION

Despite its agronomic potential, economic and ecological importance, *Lepidium* has escaped the focus of scientific community and till date there are fewer reports on this important species, that too mostly relating to its medicinal and edible uses[7-8]. Therefore, authors found it appropriate to introduce *Lepidium* as source donor for many abiotic stress responsive genes as shown in Fig.1.

Earlier efforts from this laboratory have led to the identification and isolation of 232 abiotic stress responsive genes from Lepidium using suppressive subtraction hybridization (SSH)[9]. Out of these, 175 and 75 genes were up and down-regulated in response to cold stress, respectively. Among them, based on the expression patterns and vitality of the function, some of the important genes were selected for further detailed studies and subsequently their full-length cDNA sequences were isolated by using RACE and RT-PCR methods. So far, six up-regulated, *LlaDREB1b* (JN214345), *LlaGPAT* (JN398166), *LlaNAC* (FJ423495), *LlaCIPK* (FJ423496), *LlaPR5*

Table 1. List of RT-PCR primers used for amplification of full-length cDNA sequence of isolated abiotic stress responsive genes from *Lepidium latifolium* L.

Gene	Extreme forward primer	Extreme reverse primer
LlaDREB1b	5′-CGGGATCCATCAATGGACTCTTTTTCGTCTT-3′	5′-GAGAGCTCTTTTAATAATTCAAAGCGACAGG-3′
LlaGPAT	5′-CGGGATCCAGCGATGTCTGAGCTTGTTCGG-3′	5′-GAGAGCTCCGCGGAGTTTAAAATCGTGTATTG-3′
LlaNAC	5′-ATGGAGAGCACCGATTCTTC-3′	5′-TTAAGAAGGGTACCAGTTTA-3′
LlaCIPK	5′-ATGGAGAAGAAAGGGTCTGT-3′	5′-TCAGTGCCAAGCCAATACAA-3′
LlaPR	5′-ATGGCTCTTCCGTTGCCATTGA-3′	5′-TCAAAAGAGCCGCCACATGCGA-3′
LlaIPK	5′-ATGCTAAAGGTCCCTAAACACCAAG-3′	5′-CTAGGGCCCATTGTCAAGCTGGGA-3′
LlaRan	5′- ATGGCTCTACCTAACCAGCAAA -3′	5′- TTACTCAAAGACGTCGTCATCAT -3′
LlaDRT	5′-ATGGCCTCAGTAACCTCAGCCG -3′	5′-TTAGTTAACGGTGACTTTACCG-3′

Table 2. Isolated and cloned abiotic stress responsive genes of *Lepidium latifolium* L.

Gene	GenBank Acc. No.	Complete cds (bp)	Putative function
LlaDREB1b	JN214345	998	Cold and drought stress-induced dehydration responsive element binding transcription factor.
LlaGPAT	JN398166	1615	Cold responsive chloroplast glycerol-3-phosphate acyltransferase gene involved in biosynthesis of phosphatidylglycerol.
LlaNAC	FJ423495	1388	NAC domain proteins are involved in developmental processes and responses to cold stress.
LlaCIPK	FJ423496	1870	CIPK signaling system is a newly emerging plant-specific and Ca^{2+}-dependent network mediating cold tolerance.
LlaPR5	GQ853409	1422	Pathogenesis related proteins are induced under various abiotic stresses.
LlaIPK	FJ487575	1149	Cold-induced IPK2 BETA-like protein involved in stress signaling pathway.
LlaRan	JN214347	1326	Ran (Ras-related GTP binding protein) gene gets down-regulated on cold stress.
LlaDRT	JN214346	757	DNA damage repair protein/transcription factor gets down-regulated on cold exposure.

(GQ853409) and *LlaIPK* (FJ487575) and two down-regulated *LlaRan* (JN214347) and *LlaDRT* (JN214346) genes have been successfully isolated and cloned from Lepidium (Table 2).

Among the up-regulated genes, dehydration responsive element binding (LlaDREB1b) transcription factor gene belongs to AP2/EREBP family, which binds to CRT/DRE element (TACCGACAT), in the promoter region of the many cold-regulated (COR) genes and regulates their expression in response to both low temperature and water deficit via ABA-independent pathways10-11. Glycerol-3-phosphate acyltransferase (LlaGPAT) gene encodes a protein that increases the unsaturation of fatty acids present in the plasma membrane that gives resistance during cold-induced membrane injury. It has been proved that, chilling sensitivity of plants can be manipulated by modulating levels of unsaturation of fatty acids of membrane lipids by the actions of acyl-lipid desaturase and GPAT12-13. While, NAC (No apical meristem, ATAF and cup shaped cotyledon) gene family belongs to a novel class of transcription factors unique to plants, which is reported to be involved in developmental processes, including formation of the shoot apical meristem, floral organs and lateral shoots, as well as being implicated in responses to various environmental stresses14-15. Another up-regulated gene identified was calcineurin B-like protein (CBL) interacting protein kinase (LlaCIPK) gene known to function in stress and ABA responses16. PR5-like gene (LlaPR5) showed possible role in the regulation of cold/low temperature response in addition to its role in various stresses induced by pathogens in the plants17 thereby suggesting of crosstalk between signal transduction pathways leading to stress tolerance against various types of stresses (Table 2). Inositol 1,4,5-trisphosphate kinase (LlaIPK) plays an important role in signal transduction in cells by phosphorylating inositol 1,4,5-trisphosphate (IP3) to inositol 1,3,4,5 tetrakisphosphate (IP4). Both IP3 and IP4 are critical second messengers which regulate calcium (Ca2+) homeostasis. Over-expression studies involving AtIpk2β gene in model plants suggests a role for AtIpk2β as a transcriptional control mediator and possible involvement in plant stress responses18. On the other hand, among down-regulated genes, Ras related GTP binding (LlaRan) gene encodes Ran protein, which is remarkably conserved among plants, animals and fungi and plays a fundamental role in cell division and nuclear function in all eukaryotes19. This line of evidence falls in direct agreement to the well established fact that during stress, the molecular and biochemical machinery of a cell is directed to enhance the chances of survival and not towards growth by cell division. Over-expression of OsRAN2 affects the sensitivity to salt stress in rice20. Likewise, DNA damage repair protein/transcription factor (LlaDRT) gene also gets down-regulated under abiotic stresses.

The full-length cDNA sequence of these genes were cloned separately between *Bam*HI and *Sac*I restriction endonuclease sites in pCAMBIA0390 plant expression vector under the control of stress inducible rd29 promoter. The cloning was further confirmed by restriction and PCR analysis. After that, these gene constructs were transformed into *Agrobacterium* strain LBA4404 by electroporation and transformed colonies were screened by PCR for confirming gene integrity. These *Agrobacterium* strains harbouring the gene constructs would be used for genetic transformation of vegetable crops for improving abiotic stress tolerance. These aspects are presently under study.

Vegetable crop transformed with isolated abiotic stress responsive genes will involve one time investment, but their cultivation will ensure availability of nutrient rich fresh food even during snow bound periods. Additionally, transformed vegetable crops would not only increase productivity and production but also bring additional areas under cultivation.

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