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# Comparative analysis of *LEA* and *Dehydrin* genes in response to drought stress in chickpea phonological different stages

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**Abstract**

The expression pattern of *LEA* and *Dehydrin* genes in two chickpea (*Cicer arietinum* L.) genotypes MCC283 and MCC80 in the various phonological stages under drought stress was investigated. For drought treatment, soil-grown 30 day-old (vegetative stage), 50 day-old (flowering stage) and 60 day-old (podding stage) plants were subjected to progressive drought by withholding water for 2, 4, and 6 day and untreated plants were used as control. RNA was extracted from leaf and then cDNA was synthesized. RT-qPCR analysis of *LEA* and *Dehydrin* expression using specific primers showed different expression patterns in different stages of both chickpea genotypes. Differential expression of *LEA* was observed in both genotypes in various phonological stage and its timing, duration and intensity of drought treatments. The expression levels of Dehydrin in both genotypes were increased significantly from 2 to 6 day of water deficit in vegetative and podding stages. Based on the obtained results, the increase in *LEA* and *Dehydrin* expression in the drought treatment for both genotypes in the Vegetative, Flowering and podding stages might be an adaptation to overcome the stress condition, supplying energy for growth and survival, thus helping the plant to survive.

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## Introduction

Chickpea (*Cicer arietinum* L.), one of the most important grain-legume crop, is grown in more than 45 countries, mostly in arid and semiarid zones (Kumar and Abbo., 2001). It's the second important legume in the world with 12.1 million ha under cultivation and with 11.1 million tonnes produced annually (FAOSTAT, 2012.). Drought, cold and salinity are the major abiotic stresses affecting chickpea in order of importance (Croser *et al.*, 2003).It has been estimated that 70% of the crop yield loss can be attributed to abiotic stresses, especially drought (Bray *et al.* 2000). Drought is a meteorological term and an environmental event, defined as a water stress due to lack or insufficient rainfall and/or inadequate water supply (Toker *et al.*, 2007).The seriousness of drought stress depends on its timing, duration and intensity (Serraj *et al.*, 2003). Worldwide, 90% of chickpea is grown under rain fed conditions (Kumar and Abbo, 2001) where the terminal drought stress during the chickpea reproductive phase results heavy yield losses (Sharma 2004-05).

Chickpea cultivars have different response to drought stress and plant densities in dry conditions (Kumar and Dhiman, 2004). Plants respond and adapt to water deficit at both the cellular and molecular levels, for instance by the accumulation of osmolytes and proteins specifically involved in stress tolerance. An assortment of genes with diverse functions are induced or repressed by these stresses (Yamaguchi-Shinozaki and Shinozaki, 2005). Most of their gene products may function in stress response and tolerance at the cellular level. Significantly, the introduction of many stress-inducible genes via gene transfer resulted in improved plant stress tolerance (Umezawa *et al.*, 2006). The expression and interaction of these genes is complex and diverse, and every gene involved forms part of a coordinated response network. The speed and coordination of expression of these genes is vital for plant survival. The identification of differentially expressed genes between 2 genotypes differing in drought tolerance is an important indicator of drought-associated genes in chickpea.

The interface between the expression of stress responsive genes and plant physiological response to drought stress is critical for translating molecular genetics into advances in crop production under stress conditions (Bruce *et al.*, 2002). A large number of genes have been described that respond to drought at the transcriptional level and the mechanisms of the molecular response to water stress in higher plants have been analyzed by studying the expression of genes responding to drought and other abiotic stresses (Seki *et al.*, 2001, Watkinson *et al.*, 2003; Oono *et al.*, 2003; Boominathan *et al.*, 2004).

Late embryogenesis abundant proteins (LEA proteins) were first found in cotton (*Gossypium hirsutum*) seeds, accumulating late in embryogenesis (Dure *et al.*, 1981). These proteins are a diverse group of stress protection proteins which are classified into six groups. *LEA* proteins comprise the vast majority of stress-responsive proteins. Many reports have described *LEA* proteins induction in vegetative tissues of several plant species under water deficit conditions imposed by the environment or accumulated as part of a developmental program in desiccation tolerant structures or stages (Garay-Arroyo *et al.*, 2000). The expression profiles strongly supported a role for *LEA* proteins as protective molecules which enable the cells to survive protoplasmic water deficit (Ingram and Bartels, 1996). An important role for at least some *LEA* proteins in cellular dehydration tolerance is indicated by their systematic expression at the onset of dehydration and the increase in stress tolerance observed upon over expression in different eukaryotic or prokaryotic hosts (Tunnacliffe & Wise, 2007). Several *LEA* genes or proteins, belonging to different groups, were induced during water-deficit stress in *Arabidopsis* (Bray, 2002) and maize (Boudet *et al.*, 2006), and played distinct roles in cells subjected to the stress. Group 2 *LEA* proteins or dehydrins are highly hydrophilic, glycine-rich and boiling stable proteins which are the most frequently described so far (Rorat, 2006).

The dehydrins are a class of drought-induced proteins that lack a fixed three-dimensional structure. The dehydrin sequence is highly evolved and adapted to remain disordered under conditions of severe dehydration (Mouillon *et al*., 2008).

*LEA* and *Dehydrin* genes previously validated for their significance in stress responses in various model crops and other legumes, were amplified in chickpea and sequenced after purification using gene specific primer pairs (Roorkiwal & Sharma, 2012). Earlier studies in chickpea have also reported the induction of *LEA* and *Dehydrin* under drought stress (Boominathan *et al*., 2004, Gao *et al*., 2008, Deokar *et al*., 2011). Differences in water-stress tolerance among cultivars, or within a cultivar at various developmental stages, may result from differences in the expression of genes in signal-perception and transduction mechanisms (Chinnusamy *et al*., 2004). It was important to develop a comprehensive understanding of *LEA* and *Dehydrin* expression at the RNA level in response to drought stress in phonological different stages. Using semi-quantitative method for evaluation of gene expression under stress condition has already been reported for some plants (Volkov *et al*., 2003).

The objective of this study is to evaluation of expression level of *LEA* and *Dehydrin* transcripts as two important genes induced by drought stress using RT-PCR after drought treatments of MCC283 and MCC80 chickpea cultivars in phonological different stages.

## Materials and methods

*Plant materials and stress treatments*

Two germplasme accessions, MCC283 and MCC80, with different drought-tolerance, were selected from chickpea mini-core collection based on the previous studies (Ganjeali *et al*., 2009). Both the cultivars were individually grown in 4 L pots containing a mixture of 70% soil and 30% sand. Three seeds per pot from the same cultivar were sown and a total of 15 pots per cultivar were used to have 3 replicates at each sampling. Temperature was maintained at 22± 2°C and relative humidity was about 50 ± 5%. Supplementary light giving an approximately 14 h light period and 10 h dark period was maintained during experiment. The pots were irrigated with 200 ml water every day. For drought treatment, soil-grown 30 day-old (vegetative stage), 50 day-old (flowering stage) and 60 day-old (podding stage) plants were subjected to progressive drought by withholding water for 2, 4, and 6 d respectively. In this period the soil moisture content decreased from approximately 50% to approximately 20% at the end of 6 d. As a control some plants were kept under the same condition for the same period without water stress. Drought stressed leave samples were harvested at the same time of the 2, 4, and 6 day, to avoid diurnal changes; immediately frozen in liquid nitrogen and stored at -80°C before RNA isolation.

*RNA isolation and construction of subtracted cDNA library*

Total RNA was isolated from the frozen tissues of both the drought treated and control plants using modified RNA extraction buffer containing 100 mM Tris-HCl, pH =8; 100 mM liCl; 10 mM EDTA, pH = 8; and 1% SDS, according to the hot phenol method described by Verwoevd *et al*. (1989). The RNA quality and integrity was determined by analyzing 4 ml of total RNA by agarose gel electrophoresis RNA quantity was also checked using the NanoDrop 1000 spectrophotometer (Wilmington Wilmington U.S.A). To eliminate any contaminating genomic DNA, samples of RNA were treated, prior to cDNA synthesis, with RNase-free DNase Kit (Fermentas, Hanover, MD). First-strand cDNA was synthesized from 5 mg of total RNA treated with DNase I using 200U reverse transcriptase (Fermentas). Second strand cDNA was synthesized using 10 U of DNA polymerase I and RNaseH (Fermentas) according to the manufacturer's instructions.

*Real-Time RT PCR*

Real-time quantitative RT-PCR was carried out using an ABI 7500 Real-time PCR System and 7500 System software version 1.2.3 (Applied Biosystems). The *Hsp90* gene used as reference gene (Rapacz, *et al*.,

2012) was amplified in parallel with the target gene allowing gene expression normalization and providing quantification. *LEA* and *Dehydrin* genes primers were developed. The primer sequence (designed by Primer Premier 3.0) is shown in Table 1.

RT-PCR was performed to measure the relative expression level of *LEA* (Accession Number, HO063258) and *Dehydrin* genes (Accession Number, AY170010.1). After amplification of cDNA using specific primers (Table 1), Detection of real-time RT-PCR products was done using the SYBR® Green Universal Master Mix kit (ABI) following manufacturer's recommendations. Ten micro liters of each sample were run using 2% agarose gel electrophoresis and visualized with ethidium bromide. PCR efficiencies of target and reference genes were determined by generating standard curves.

The reaction conditions were 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 2 min, 62°C for 30 s and 72°C for 30 s; the data were collected in the last phase (extension phase). All amplification reactions were repeated three times under identical conditions and included a negative control. For the Quantitative Real Time PCR data, the relative expression for the genes of interest was calculated using the threshold cycle (CT) method. The *CT* values were plotted against the log of the known starting concentration value and from the slope of the regression line (*y*). Quantification of the relative transcript levels was performed using the comparative *CT* method. Transcript levels of target genes were normalized against the Hsp90 gene transcript levels as described in the 7300 Real Time System (Applied Biosystems). The induction ratio (IR) was calculated as recommended by the manufacturer and corresponds to 2−ΔΔCT, where Δ*ΔCT* = (*CT*, Target gene − *CT*, Hsp90) stressed − (*CT*, Target − *CT*, Hsp90) control. Relative quantification relies on the comparison between expression of a target gene versus a reference gene and the expression of same gene in target sample versus reference sample.

## Results

*Expression of LEA*

Expression level of *LEA* transcripts in two chickpea cultivars (MCC283 and MCC80) were determined by RT-PCR. Differential expression of *LEA* was observed in both genotypes in various phonological stage and its timing, duration and intensity of drought treatments. The increase in *LEA* expression in MCC283 genotype under 6 day treatment implies a more rapid expression response, in comparison to the other periods of exposure. The expression level of *LEA* in MCC283 cultivar was increased significantly from 4 to 6 day of water deficit in vegetative and podding stages. However, the expression level of *LEA* was increased significantly from 2 to 4 day of water deficit in flowering stage. However, for the tow first drought treatment period (2 and 4 day), at the same level in vegetative stage expresses maintained. (Figure1).

**Table 1.** Primers used for RT PCR.

| Name | Sequence |
|---|---|
| FW- *LEA* | 5' GCC TTA TCT TCT ATG TTG CCA ATC 3' |
| RV- *LEA* | 5' CCA CGA CCA AAG TTA CAG AGC 3' |
| FW- *Dehydrin* | 5' AAA GTG GTG TTG GGA TGA CC 3' |
| RV- *Dehydrin* | 5' TCC TCT CTC CCG AAT TCT TG 3' |
| FW- *HSP90* | 5' CAG AGG GCT GTG TGC ATG AT 3' |
| RV- *HSP90* | 5' GAT GCG GGA GAA GAC CTC AA3' |

In the vegetative stage, there were slower and steadier increases in the expression level during the treatment periods of *LEA* in MCC80 genotype from 2 to 6 day of water deficit. In the flowering stage under 2 and 4-d drought treatments of *LEA* in MCC80 genotype, its expression increased significantly. However, the expression level of *LEA* increased significantly from 4 to 6 day of water deficit in podding stage (Figure 1).

*Expression of Dehydrin*

Expression level of *Dehydrin* transcripts in two chickpea genotypes (MCC283 and MCC80) Were determined by RT-PCR. Differential expression of *Dehydrin* was observed in both genotypes in the various phonological stage and its timing, duration and intensity of drought treatments. The expression level of *Dehydrin* in MCC283 cultivar was increased significantly from 2 to 6 day of water deficit in vegetative and podding stages. However, increases in expression level of *Dehydrin* were significant from 2 to 4 day of water deficit in flowering stage. (Fig. 1).

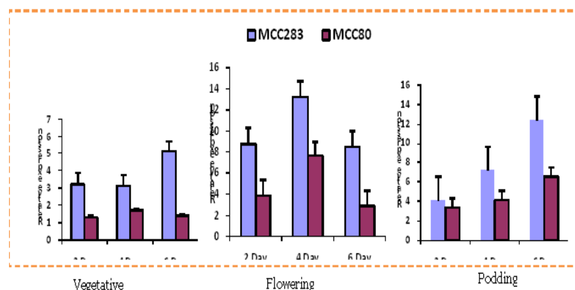


**Fig. 1**. Relative expression of *LEA* gene under drought treatment in MCC283 and MCC80 chickpea genotypes in phonological different stages. The error bars indicates the standard deviations of measurements (n = 3).

Although not statistically significant, expression increased of *Dehydrin* in MCC80 genotype when the drought increased from 2 to 6 day in flowering stage. However, the expression level of *Dehydrin* increased significantly from 2 to 6 day and from 4 to 6 day of water deficit in vegetative and podding stages respectively. In the flowering stage, there were slower and steadier increases in the expression level during the treatment periods of *Dehydrin* in MCC80 genotype from 2 to 6 day of water deficit (Figure 2).

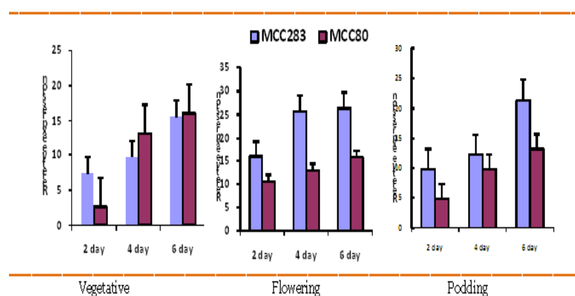


**Fig. 2**. Relative expression of *Dehydrin* gene under drought treatment in MCC283 and MCC80 chickpea genotypes in phonological different stages. The error bars indicates the standard deviations of measurements (n = 3).

**Discussion**

Abiotic stresses, such as drought, can limit the geographical distribution of plants and limit the growth and yield of economically important species. Substantial efforts have been devoted to determine the nature of the injury caused by these stresses and the plant-protection mechanisms involved in tolerance responses (Bray, 2004). Tolerance of drought is a complex phenomenon, because it changes according to drought intensity and duration, and the plant developmental stage during which drought occurs. Differences in water-stress tolerance among cultivars, or within a cultivar at various developmental stages, may result from differences in the expression of genes in signal-perception and transduction mechanisms (Chinnusamy *et al.*, 2004). Understanding the mechanisms involved in the response of plants to adverse environmental conditions is, without a doubt, the first step in the generation of crops with higher tolerance to stress. Molecular responses to water deficiency have mainly been investigated in terms of survival of stress. (Iuchi *et al.*, 2000). Gene products involved in water-deficit responses can be includes proteins, osmolytes, and other compounds that probably confer direct tolerance to abiotic stresses, including chaperones, late embryogenesis abundant (LEA) proteins, such as LEA14 (Singh *et al.*, 2002), mRNA-binding proteins, such as glycine-rich protein (Bocca *et al.*, 2005), key enzymes for osmolyte biosynthesis, such as galactinol synthase and delta-1-pyrroline-5-carboxylate synthetase, involved in synthesis of raffinose-family oligosaccharides (Hannah *et al.*,2006) and proline (Schafleitner *et al.*, 2007).

This study provides a comparative of genotype-specific expression patterns of two unigenes in chickpea phonological different stages in response to drought. The regulation of this gene was confirmed by real-time qPCR.

To determine whether drought stress is involved in the expression level of *LEA* and *Dehydrin* genes quantification of transcripts of these genes under performed by RT-PCR analysis to find the changes in transcript level of these genes under phonological different stages.

In the present study, *LEA* and *Dehydrin* genes in both chickpea genotypes showed different expression patterns in different stages (Figure1, 2). Data from a number of previous studies suggested accumulation of *LEA* and *Dehydrin* was correlated with stress tolerance in chickpea (Gao *et al.*, 2008, Deokar *et al.*, 2011), oat (Maqbool *et al.*, 2002), rice (Moons *et al.*,1997), wheat (Ried and Walker-Simmons, 1993), and tobacco (Kim *et al.* 2005).

The increase in *LEA* and *Dehydrin* expression in the drought treatment for both genotypes in the Vegetative, Flowering and podding stages might be an adaptation to overcome the stress condition, supplying energy for growth and survival, thus helping the plant to survive (Figure1, 2). But the drought-tolerant genotype, MCC283 that we studied seemed to increase synthesis of osmotic regulators for protection against water-deficit damage, more, the drought-sensitive genotype MCC80. In other hand, drought-tolerant MCC283 showed higher molecular responses to drought at the all stages; it probably perceives water stress more quickly than the sensitive genotype MCC80, allowing it to adapt and ensure reproduction because of the role of *LEA* and *Dehydrin* proteins as protective molecules that enable cells to survive protoplasmic water depletion. In contrast, MCC80 presented later perception of, and defense to, stress, conferring survival ability. However, our molecular data, gene-expression values, suggest that the same molecular mechanisms operate in both genotypes at different stages in response to drought.

More in depth expression studies involving the use of additional genotypes and more time-points supplemented with physiological observations during stress imposition may possibly provide a better insight into the role of the proposed genes in drought responses in chickpea. The identification of genes involved in tolerance to these stresses and their required timing of expression shall greatly aid development of elite chickpea cultivars through molecular breeding or genetic manipulation.

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