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Differential Expression of *CA7* and *NCED* Genes in Common Bean Genotypes under Drought Stress

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ABSTRACT

The response of plants to drought stress depends on several factors including the plant developmental stage and the length and severity of the stress applied. Common bean (*Phaseolus vulgaris* L.) is the most important pulse crop that is cultivated worldwide for human consumption. Understanding of the mechanisms responsible for its response to drought is, therefore, essential. An increasing number of reports show that withdrawal of water from plants growing in the controlled conditions is accompanied by changes in the expression of a number of genes. To our knowledge, regulation of gene expression in flower buds of *P. vulgaris* under stress conditions has not been reported. Our aim was to identify transcription sensitivity of *CA7* and *NCED* genes under water deficit stress at vegetative and reproductive stages of different bean genotypes. Two experiments were carried out. Within each experiment, the groups of drought-stressed plants were subjected to water withholding, while the control plants were watered every other day. Stressed plants were re-irrigated when RWC reached 66±2 percent. Our study showed that *CA7* and *NCDE* were genes differentially expressed in the studied genotypes under drought stress. The expression of these genes was strongly induced in response to drought stress in flower buds of the cultivar Jules and the line KS-21191. It seems that under stress conditions, these genes express more in the tolerant than the susceptible genotypes. Therefore, these two genes could probably be used to obtain plants relatively tolerant to water deficit stress, especially in the reproductive stage of plant growth.

Keywords: Flower buds, Gene expression, *Phaseolus vulgaris*, Water deficit**.**

INTRODUCTION

Biotic and abiotic stresses adversely affect growth and development of plants and prevent them from expressing their full genetic potentials (Sedghi *et al*., 2012). Among the different environmental stresses, drought is the constraint that induces a highly negative effect on crop production. When subjected to this constraint, plants manifest a wide range of behaviors varying from great sensitivity to high tolerance (Pasban Eslam, 2011).

Although roots are the first tissues to experience reduction in water supply, the first visible signs are evident in leaves, which appear prematurely senescent (Kavar *et al*., 2008). The response of plants to this environmental stress depends on several factors including plant developmental stage and the length and severity of the stress applied (Bray, 2002; Torres *et al*., 2006). In leaf tissues, the perception of drought stress causes stomatal closure to reduce transpiration, and, consequently, limits carbon dioxide uptake and reduces photosynthesis rates (Tu¨rkan *et al*., 2005). Plants are much more sensitive to drought at the reproductive stage, i.e. from meiosis in pollen mother cell to seed set and maturity (Saini, 1997; Cellier, 1998). Water deficit stress may result in plants synthesizing compounds that function as osmolytes to

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maintain water potential or other proteins that are proposed to protect cells from damage (O'Connell, 1995; Maggio *et al*., 2006). An increasing number of reports show that withdrawal of water from plants growing in the controlled conditions is accompanied by changes in the expression of a number of genes and, in the relatively small number of plants studied, levels of functional proteins (reviewed in Kavar *et al*. 2008). In an investigation, Seki *et al*. (2002) determined the expression profiles of 7,000 genes from the model plant *Arabidopsis thaliana* under drought stress; 277 were genes up-regulated and 79 genes down-regulated. Such changes in expression levels have been reported to be dependent on the method of water withholding employed (Bray, 2004) and on the plant species (Torres *et al*., 2006).

Some gene products are directly involved in protective mechanisms, such as the late embryogenesis abundant proteins (LEA), the synthesis of osmolytes, or ion transport functions; other gene products, e.g. transcription factors or kinases, participate in signal transduction pathways that mediate cellular responses to external stimuli (Torres *et al*., 2006; Rodriguez-Uribe and O'Connell, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007).

Regulation of ABA (a crucial hormone in the adaptation of plants to different environmental stresses) biosynthesis has been mainly studied in vegetative tissues of several plant species in response to stress conditions. In water-stressed leaves, accumulation of ABA was well correlated with an increased expression of the *NCED* (9-cis-epoxycarotenoid dioxygenase) gene (Xiong and Zhu, 2003; Schwartz *et al*., 2003; Nambara and Marion-Poll, 2005; Zhang *et al*., 2008) and accumulation of the corresponding *NCED* protein (Qin and Zeevaart, 1999). Moreover, transgenic plants over expressing the *NCED* gene accumulated large amounts of ABA and were more resistant to drought stress (Thompson *et al*., 2000; Iuchi *et al*., 2001; Qin and Zeevaart, 2002; Hwang *et al*., 2010).

Common bean (*Phaseolus vulgaris* L.) is the most important food crop from the *Fabaceae* family that is cultivated worldwide for human consumption (Ramirez-Vallejo and Nelly, 1998; Kavar *et al*., 2008). Thousands of legume species exist but more common beans are eaten than any other. In some countries such as Mexico and Brazil, beans are the primary source of protein in human diets (Broughton *et al*., 2003). A major constraint to bean production is water deficit stress (Laing *et al*., 1984). Recent studies suggest that only 7% of the bean-growing area is well watered. Except for a few highland areas with abundant and well-distributed precipitation, and regions where irrigation is available, bean production is exposed to the risk of drought (Broughton *et al*., 2003). Beans are particularly susceptible to drought during reproductive stage, with significant flower and pod abortion occurring when water shortage occurs at this time (Graham and Ranalli, 1997).

Understanding of the mechanisms responsible for the response of bean cultivars to drought is therefore essential. There is genetic diversity within *P. vulgaris* for drought resistance (Lizana *et al*., 2006; Muñoz-Perea *et al*., 2006) and several genes whose expression responds to drought have already been identified (Kavar *et al*., 2008; Micheletto *et al*., 2007; Torres *et al*., 2006; Kirch *et al*., 2004). The gene for Pvlea-18, which is a member of a new late-embryogenesis-abundant (LEA) protein family that accumulates in vegetative tissues in response to water deficit, has been described by Colmenero-Flores *et al*. (1997). Some early and late dehydration responsive genes have been identified by differential display RT-PCR (DDRTPCR) in common bean roots (Torres *et al*., 2006) and leaves (Kavar *et al*., 2008). To our knowledge, regulation of gene expression in flower buds of *P. vulgaris* under stress conditions has not been reported. In the present study, our aim was to identify transcription sensitivity of

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CA7 and *NCED* genes under water deficit stress at vegetative and reproductive stages of different bean genotypes.

MATERIALS AND METHODS

Plant Material and Water Deficit Treatment

Seeds of 11 bean genotypes (Table 1) were germinated in 5.0 L pots (5 seeds each, then thinned to 3 plants) containing a mixture of soil, sand, and dung (3:1:1 by vol.) and were grown in a greenhouse at 25/15°C day/night temperatures and 14/10 hours day/night photoperiod. Two experiments (I and II) were carried out. Within each experiment, groups of drought-stressed plants (five pots per genotype) were subjected to water withholding, while the control plants were watered every other day. Leaf relative water content (RWC) of the stressed plants was measured on five different mature leaves, each from a plant per pot at a time point. Stressed plants were re-irrigated when RWC reached 66±2 percent, (8-10 days after withholding water).

The water-stress condition was induced in experiment I by withholding irrigation of plants with fully expanded first trifoliate leaves (appearance of the 3rd trifoliate). However, in experiment II the stress condition was induced by withholding irrigation of plants at the early flowering stage and only those flower buds that were undergoing meiosis during the period of stress induction were marked. Leaves in experiment I, and flower buds in experiment II, were harvested from unstressed and stressed plants in two biological repeats, and were immediately frozen in liquid nitrogen and stored at -80º C for isolation of total RNA.

RNA Isolation and cDNA Synthesis

Total RNA was isolated using the lithium chloride method proposed by Chang *et al*. (1993). For each RNA sample, leaves and flower buds from three to four plants were harvested and pooled. Isolated RNA was treated with DNaseI to remove genomic DNA. RNA concentration was measured spectrophotometrically and an RNA gel was run in order to check the quality of the RNA and the accuracy of the concentration. A sample of 500 ng of DNase-treated RNA was used for the synthesis of first-strand cDNA using the Fermentas kit (#K1620) according to the manufacturer's instructions.

2-3. Semi-quantitative RT-PCR Reaction

cDNA was first diluted in a 2:1 ratio and then 1.5 µL of diluted cDNA was used to perform semi-quantitative RT-PCR reaction.

Table 1. Name, source, type, and growth habit of common bean genotypes.

Genotype	Source	Type	Growth habit[a]
G-14088	CIAT	pinto	type III
G-01437	CIAT	pinto	type III
KS-21189	CIAT	pinto	type III
KS-21191	CIAT	pinto	type III
KS-21486	CIAT	pinto	type I
Tylore	CIAT	pinto	type II
Khomein	Iran	pinto	type III
Daneshkadeh	CIAT	white	type III
Kara	CIAT	white	type IV
Goynok 98	CIAT	white	type I
Jules	CIAT	white	type III

[a] I determinate: Erect; II indeterminate: Semi-spreading; III indeterminate: Spreading, IV indeterminate: Erect.

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cDNA was synthesized in a 25 µL reaction mix, using 1.25 units of Dream Taq DNA polymerase (Fermentas), 0.3 µM each of 4 primers and 300 µM dNTPs. Semi-quantitative RT-PCR was performed on the Mastercycler-gradient (Eppendorf). The PCR thermal cycle conditions were as follows: one cycle at 94°C for 3 minutes, then, 31 cycles at 94°C, for 30 seconds; 57°C, 40 seconds; 72°C, 60 seconds for NCED gene and 31 cycles at 94°C, for 30 seconds; 57°C, 35 seconds; 72°C, 45 seconds for *CA7* gene.

To determine the appropriate number of cycles, semi-quantitative RT-PCR was first performed using 4 samples for each of the two genes.

According to the earlier studies (Kavar *et al*., 2008; Torres *et al*., 2006), the following primers: PvNCED-F (CCCGAAACTCGACCCCGTCAAC), PvNCED-R (CCTCCCACGCGTTCCAGAGATG), PvCA7-F(GCAAGATTATGAAGAGGGCTTG) and PvCA7-R (CTCAAGAGCCACCAGCCTAC) were used. The Actin gene was selected as internal reference gene.

Electrophoresis was performed on PCR products using1.5% agarose gel and 0.5X TBE buffer and quantified with ImageJ software (Rasband, 2011). The gel was stained using Ethidium bromide and photography was done under UV light. Gene expression data for each sample were normalized relative to *ACT-1* expression levels and average expression levels were calculated for 2 biological repeats. Two-sample *t*-tests were used for significance testing and the *P* value calculation using Excel software.

RESULTS AND DISCUSSION

Expression of *CA7* Gene

Differential expression of *CA7* gene in leaves was observed among 11 bean genotypes exposed to drought stress at the vegetative stage. The expression of this gene increased significantly ($P< 0.05$) in lines KS-21191, KS-21486, G-14088 and cultivar Goynok98; however, its expression decreased in the cultivar Jules. For the remaining 6 genotypes (G-01437, KS-21189, Kara, Khomein, Tylore, and Daneshkadeh) differences in the expression level between control and drought stressed plants were not statistically significant (Figure 1). The results were in agreement with those obtained by Kavar *et al*. (2008). They reported that the expression of *CA7* in leaves of common bean was increased by drought stress, by about 30-fold. They also observed down-regulation of *CA7* and some other drought related genes in some genotypes, especially in the early days of stress development. Up-regulation of *ALDH*

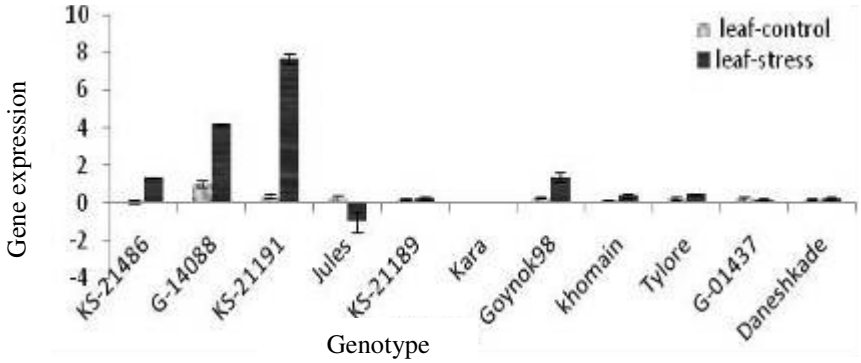

Figure 1. Relative expression of *CA7* gene in leaves of common bean cultivars in response to drought stress at vegetative stage by semi-quantitative RT PCR.

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genes under saline or drought conditions has been reported in many plant species, indicating that the corresponding enzymes may play an essential role during osmotic stress adaptation (Kotchoni and Bartels, 2003). Of the five selected *ALDHD* genes analyzed in *Arabidopsis thaliana*, the strongest dehydration stress response was observed in *ALDH7B4* (Kirch *et al*., 2004), whose sequence is closely similar to that of CA7.

The gene *CA7* was expressed in flower buds of all studied genotypes in response to drought stress at reproductive stage, but the rate of expression was different among genotypes (Figure 2). Expression of this gene was highly significant ($P< 0.01$) in genotypes G-14088, KS-21191, Jules, KS-21189 and Goynok98. According to Op Den Camp and Kuhlemeier (1997), two tobacco mitochondrial *ALDH* genes, *TobALDH2a* and *TobALDH2b* were expressed in reproductive tissues and exhibited a high acetaldehyde oxidizing activity *in vitro*. However, expression of this gene in flower buds of *P. vulgaris* under stress conditions has not yet been reported.

Kirch *et al*. (2001) discussed that expression data for *Craterostigma plantagineum* aldehyde dehydrogenase (Cp-ALDH) suggest its involvement in the dehydration stress response; and expression profile of *Ath-ALDH3* in response to dehydration essentially corresponded to the *Cp-ALDH* transcript accumulation. It seems that under stress conditions, *CA7* gene expresses more in tolerant than susceptible genotypes. Therefore, we suggest that CA7 could be used to obtain plants with tolerance to water deficit stress in both vegetative and reproductive stages of plant growth. Sunkar *et al*. (2003) also suggested use of *Ath-ALDH$_3$* to obtain plants with tolerance to diverse environmental stresses. They studied the expression of *Ath-ALDH$_3$* gene in *Arabidopsis* transgenic lines and indicated that transgenic plants improved tolerance to dehydration. Increased activity of *Ath-ALDH$_3$* appears to constitute a detoxification mechanism that limits aldehyde accumulation and oxidative stress, which is primarily because of excessive accumulation of relative oxygen species (ROS).

Expression of *NCED* Gene

Relative expression of *NCED* gene under water deficit conditions was studied in 11 bean cultivars /lines (Figure 3). In KS-21486, KS-21191 and Goynok98, expression of *NCED* in leaf tissues of stressed plants increased by 12, 8 and 17 fold in comparison to the control plants, respectively; however, increased expression of this gene in stressed plants relative to their control was not statistically significant ($P> 0.05$) in some genotypes (Figure 4).

The expression level of *NCED* gene in flower buds of both control and stressed

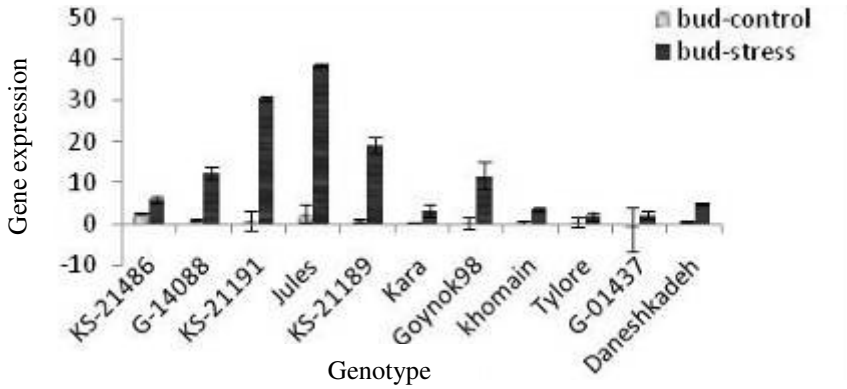

Figure 2. Relative expression of *CA7* gene in flower buds of common bean cultivars in response to drought stress at reproductive stage by semi-quantitative RT PCR.

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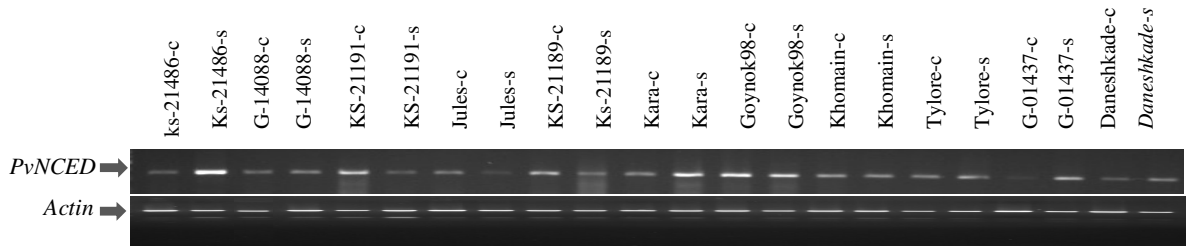

Figure 3. Detection of *PvNCED* expressions in leaves of common bean genotypes and the effect of drought on *PvNCED* expression by semi quantitative RT-PCR (C: Control and S: Stress, 28 PCR cycles

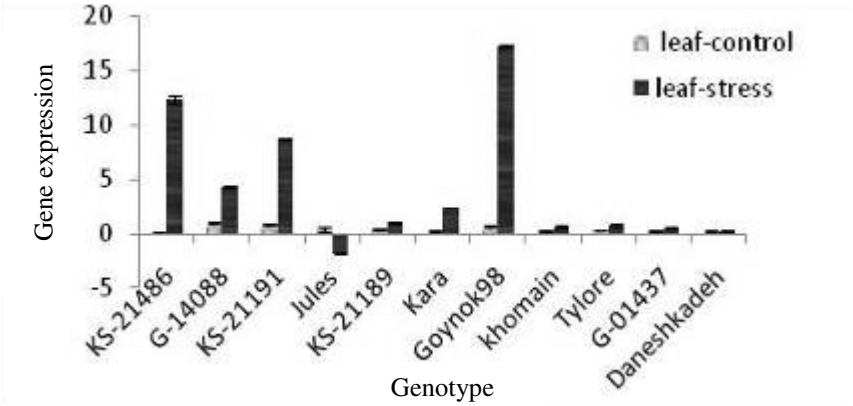

Figure 4. Relative expression of *NCED* gene in leaves of common bean cultivars in response to drought stress at vegetative stage by semi-quantitative RT PCR.

plants was higher than that in leaf tissues. Water stress at reproductive stage caused an increase in the expression of *NCED* gene in flower buds of all, except G-01437 and Daneshkadeh genotypes. In response to water stress, relative expression of this gene was 137 and 152 fold of that in the control plants in KS-21191 and Jules, respectively (Figure 5).

Qin and Zeevaart (1999) studied ABA biosynthesis and expression of PvNCED1 in common bean in response to water stress and found a close correlation between the abundance of PvNCED1 mRNA and

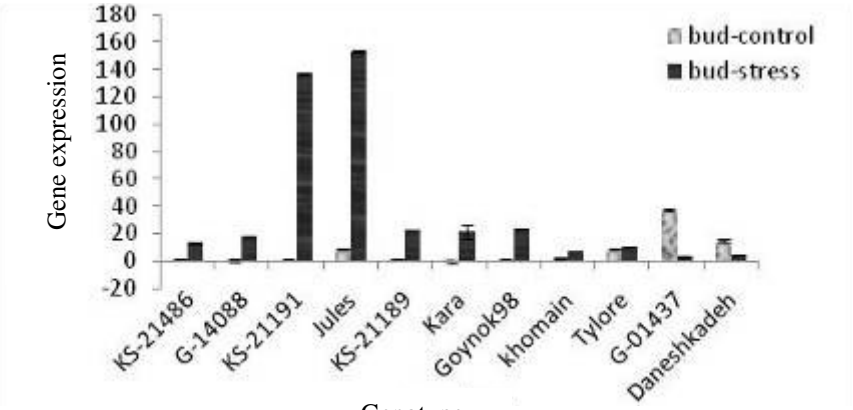

Figure 5. Relative expression of *NCED* gene in flower buds of common bean cultivars in response to drought stress at reproductive stage by semi-quantitative RT PCR.

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protein, and increase in ABA levels in leaves and roots. In other plant species also, *NCED* expression is induced in response to water-deficit and regulates ABA biosynthesis (Nambara and Marion-Poll, 2005; Yang and Guo, 2007). Overexpression of *NCED* gene in transgenic plants results in the increase of ABA accumulation and drought tolerance (Iuchi *et al*., 2001; Qin and Zeevaart, 2002; Lefebvre *et al*, 2006; Wan and Li, 2006; Thompson *et al*, 2007; Zhang *et. al*., 2008; Hwang *et al*., 2010). Our study showed that the expression of *CA7* and *NCDE* genes was strongly induced in response to drought stress both in leaves and flower buds of the cultivar Jules and the line KS-21191. Therefore, one of the conclusions to emerge from the present study is that, within the evaluated cultivars/lines, KS-21191 and Jules seem to be relatively drought tolerant. However, these results must be confirmed experimentally under field conditions.

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بیان متفاوت ژنهای *CA7* و *NCED* در ژنوتیپ‌های لوبیا در شرایط تنش خشکی

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چکیده

واکنش گیاهان به تنش خشکی به چندین عامل از جمله مرحله رشد و نمو گیاه و مدت و شدت تنش اعمال شده بستگی دارد. لوبیا (*Phaseolus vulgaris* L.) از مهمترین حبوبات است که در کل جهان برای مصرف انسان کاشته می شود. بنابراین آگاهی از ساز وکار های عکس‌العمل این گیاه به تنش خشکی حائز اهمیت است. گزارشات مختلف نشان داده است که عدم آبیاری گیاهانی که در شرایط کنترل شده کشت شده اند با تغییراتی در میزان بیان ژن های متعدد در آنها همراه بوده است. تا کنون گزارشی مبنی بر چگونگی تنظیم بیان ژن در جوانه های گل لوبیا در دسترس نیست. هدف از این مطالعه تعیین میزان حساسیت بیان ژن های *CA7* و *NCED* تحت تنش خشکی در مراحل رشد رویشی و زایشی ژنوتیپ های مختلف لوبیا بود. بدین منظور دو آزمایش انجام شد. در هر آزمایش گیاهان شاهد هر دو روز یکبار آبیاری شدند حال آنکه گیاهان تحت تنش از زمان اعمال تنش تا رسیدن محتوای نسبی آب برگ (RWC) به ۲±۶۶ درصد آبیاری نگردیدند. این ژن ها در پاسخ به تنش خشکی در جوانه های گل رقم Jules و لاین KS-21191 به شدت بیان شدند. با توجه به اینکه در شرایط تنش خشکی این ژن ها در ارقام متحمل بیشتر از ارقام حساس بیان می شوند، به نظر می رسد بتوان از این دو ژن برای تولید ارقام نسبتا مقاوم به خشکی، خصوصا در مرحله زایشی گیاه استفاده نمود.

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