

Differential Transcript Accumulation of *Dehydrin* and *Beta-glucosidase* Genes to Cold-induced Oxidative Stress in Chickpea

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ABSTRACT

In this study, accumulation of H₂O₂, malondialdehyde (MDA) (as cold-induced oxidative stress indicators), the transcript levels of *dehydrin* and *beta-glucosidase* genes (involved in metabolic responses) was evaluated in chickpea cv. Jam, using qRT-PCR during control, cold acclimation (CA), cold stress (CS), recovery, and freezing phases. Results showed the existence of wide range of genetic capacity in the cultivar to increase cold tolerance when environmental conditions change. Significant increase in H₂O₂ and MDA content during CA phase indicated that seedlings perceived cold signaling that resulted in remarkable increase in the transcript levels of *dehydrin* and *beta-glucosidase* genes as part of defense responses of plants. Balancing the expression of these genes and oxidative stress indicators showed the interplay between two major defense and injury pathways. During freezing phase, the higher transcript levels of these genes in acclimated plants compared to non-acclimated plants showed a more active role for plant cells. An incapability of defense machine in non-acclimated plants was a limiting factor determining the low potential of chickpea plants to freezing phase. It was suggested that adjustment and metabolic alterations like *dehydrin* and *beta-glucosidase* genes, especially after CA phase and, thereby, decrease in oxidative stress indicators, could be a reason for relative cold tolerance in chickpea.

Keywords: Beta-glucosidase, Chickpea, Cold responses, Dehydrin gene, qRT-PCR.

INTRODUCTION

Cold tolerance is one of the major factors that can affect the extending of the growing season and geographical range of chickpea (*Cicer arietinum* L.) in dryland areas including Iran, where winter-sowing has advantages over traditional spring-sowing season, such as approximately doubling the yield, increasing water usage efficiency, and better moisture conditions (Millan *et al.*, 2006; Heidarvand *et al.*, 2011). However, the main disadvantage of chickpea winter-sowing is the risk of winter-killing due to cold stress (CS). Therefore, there is potential for expanding the range of chickpea winter-sowing by using conventional and

molecular breeding for improved cold tolerance (Habibpour *et al.*, 2012). The defense mechanisms against CS still need to be explored, but they may well be dependent on enzymatic systems that their activities under CS increase significantly. Thus, along with biochemical studies, further molecular studies are required to elucidate how chickpea responds to CS. So far, many studies showed the expression profiles of the cold-induced genes that are directly and/or indirectly involved in cold tolerance, depending on the nature of CS, its interaction with other environmental stresses, and the wide range of responses in plant species and genotypes to CS (Heidarvand *et al.*, 2010).

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Our previous results showed that a new homeostasis was established during CS in energy and primary metabolites that brought reliable balance energy-efficient growth with the ability to adapt to CS in chickpea (Heidarvand and Maali-Amiri, 2013; Kazemi Shahandashti *et al.*, 2013). These responses enhanced with cold acclimation (CA) process, are due to the reprogramming of gene expression which, results in adjusted metabolic alterations and prevent or mitigate the deleterious effects of CS (Zhu *et al.*, 2007). Of these, dehydrin proteins are highly hydrophilic that interact with cellular macromolecules, showing cryoprotective effects and scavenging activity against radicals (Hara *et al.*, 2005; Amara *et al.*, 2012). However, in plants a different dynamics of dehydrin transcripts and proteins during CS is also observed. Thus, functional links between its expression profiling and improved stress tolerance of plants need to be investigated. Beta-glucosidase enzyme is a glycoside hydrolase enzyme that breaks glycosidic bond between different components (Ketudat Cairns and Esen, 2010). This enzyme participate in various cell functions such as catalysis disaccharides and oligosaccharides bond, catalysis components of cell wall, activation of phytohormones and tolerance of abiotic stresses like CS (Kreps *et al.*, 2002; Speretto *et al.*, 2007; Baron *et al.*, 2012). Although induction of these cold-induced genes by CS have been identified by expression profiling, but in many cases, their physiological functions with respect to stress tolerance are not universal. In this study along with oxidative stress indicators we investigated the effect of CS on accumulation of dehydrin and beta-glucosidase transcripts through quantitative real-time PCR (qRT-PCR) during thermal phases in chickpea.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of chickpea cultivar Jam (Kabuli, local cultivar which is vastly cultivated in Iran) were obtained from Dryland Agricultural Research Institute (Maraghe city), sterilized (by 10% (v/v) sodium hypochlorite for 10

minutes), and germinated for 3 days in the dark at 23°C on filter paper soaked with distilled water. After germination, seedlings were transferred to pots in a growth chamber at 23°C, under white light ($220 \mu\text{mol m}^{-2} \text{s}^{-1}$), a photoperiod of 16 hours, and 75% relative humidity. 20-day-old seedlings were transferred to another growth chamber with 10°C for 5 days as CA phase. Then, the temperature of growth chamber was shifted to 4°C for 2 days (LT_{50} temperature) in order to survey the effect of CS (Nayyar *et al.*, 2005). After CS, plants were transferred to control conditions (23°C) in order to survey recovery phase. Thus, our experiments have been focused on five thermal phases: control conditions (23°C), the first and the fifth days of CA, CS and recovery phase. In the other experiment, plants in control phase (23°C) and acclimated plants (after 5 days at 10°C) were placed into a climatic chamber chilled preliminary to 0°C. The temperature was lowered gradually to -10°C and plants were incubated at this temperature for 15 minutes (freezing phase). Thus, three experimental treatments in this group were plants in control phase, acclimated (cooling from 10°C to the -10°C) and non-acclimated plants (directly cooling from 23°C to the -10°C) in freezing phase. All measurements were made on the middle leaves from the apex of 3-4 plants in each treatment. Physiological experiment consisting MDA was conducted using fresh leaves while other experiments of this research were performed using samples flash frozen in liquid nitrogen and stored at -80°C.

Hydrogen Peroxide (H_2O_2) Assay

H_2O_2 content was determined according to Loreto and Velikova, (2001) method. 0.35 g fresh weight (FW) leaf fragments were ground in liquid nitrogen with a mortar and pestle and then homogenized in an ice bath with 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at $12,000 \times g$ for 15 minutes, and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1

ml of 1M potassium iodide. The absorbance of the supernatant was measured at 390 nm with a spectrophotometer (Shimadzu UV-160, Shimadzu Corporation, Kyoto, Japan). The content of H₂O₂ was expressed in $\mu\text{mol g}^{-1}$ FW.

Lipid Peroxidation Analysis

The measurement of lipid peroxidation in leaves which determines malondialdehyde (MDA), was assessed according to Heath and Packer, (1968). 250 mg FW leaflets were homogenized in 2 ml extraction buffer TCA 1% (w/v), centrifuged at 13,000×g for 15 minutes. 1 ml of the supernatant was added to 2 ml of 5% (w/v) thiobarbituric acid in 20% (w/v) TCA. The mixture was incubated in boiling water for 30 minutes and the reaction was stopped by placing the samples in an ice bath. Then the samples were centrifuged at 10,000×g for 10 minutes and the absorbance of the supernatants was measured at 532 nm with a spectrophotometer. The amount of MDA was calculated using: $C = D/E \times L$, where C is the concentration of MDA, D is the optical density, E is the molar extinction coefficient ($1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$); and L is the thickness of the layer of solution in the vessel (1 cm). Content of MDA was expressed in $\mu\text{mol g}^{-1}$ FW.

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from 0.08 g FW of leaflets, using BIOZOL (Fersion Pooyesh,

Tehran, Iran) method. The leaflets were ground in liquid nitrogen using sterile pestle and mortar before RNA extraction. The RNA samples with 260/280 ratio 1.8-2 (ratio of absorbance at 260 nm and 280 nm) were used for analysis. To confirm the RNA quality, the RNA was electrophoresed on 1.5% agarose gel containing ethidium bromide. After DNase treatment, the first strand of cDNA was constructed according to the fermentase reverse transcriptase enzyme instruction. First strand cDNA synthesis was performed using the oligo (dT)₁₈₋₂₀ primers. The reaction began by incubating 2 μg of total RNA, 0.5 μg oligo (dT) primer and DEPC treated water up to 11 μl for 5 minutes at 70°C. The tubes were placed on ice before adding the cDNA synthesis mix, which was consisted of 4 μl cDNA reaction buffer, 2 μl dNTP (10 mM dNTP mix), 20 U RNase inhibitor and DEPC-treated water to final volume of 19 μl . Then tubes were incubated for 5 minutes at 37°C. 200 U Revert Aid enzymes (MMLV-RT) were added and reactions were incubated for 60 minutes at 42°C. Finally in order to stop the reaction, the tubes were placed in hot water bath for 10 minutes at 70°C. Primers were designed using primer 3 (www.embnet.sk/cgi-bin/primer3_www.cgi) to obtain 18-21 bp length, 59-61°C melting temperature and GC content between 55 and 65% avoiding hairpins and complementarities between primers. The primers were designed based on 3'-untranslated region (3'-UTR) of genes, wherever possible (Table 1). The PCR mixture contained 5 μl of diluted cDNA, 10 μl of 2X SYBR Bio Pars (Gorgan University

Table 1. Primer sequences used in qRT-PCR amplification of known chickpea genes undergoing thermal phases.

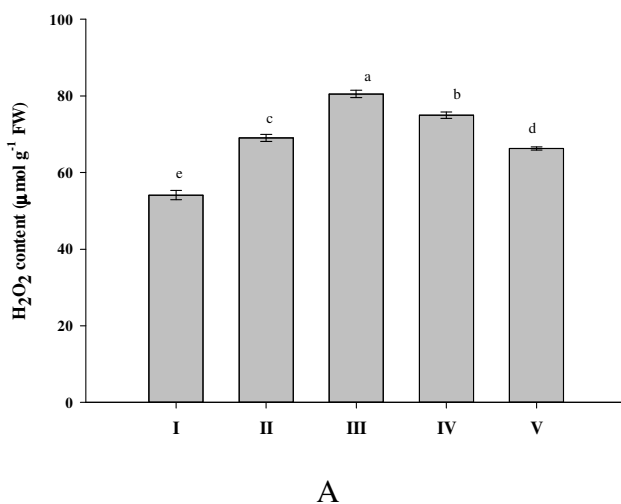
Accession number	Gene name	Primer sequence	Product length (bp)
GI/26245734	<i>Dehydrin</i>	5'-TGGTGGCACTGGAGATG-3' 5'-AACTACCTGGGTTGTGGG-3'	161
AJ630653.1	<i>Beta-glucosidase</i>	5'-GGTCAAAGGGCTGCTTCATT-3' 5'-CGGCTTCTTCTATCGGAAGTGT-3'	157
EU529707.1	<i>Actin</i>	5'-CTACGAATTGCCTGATGGAC-3' 5'-CCTCCTGAAAGGACGATGTT-3'	189



of Agricultural Sciences and Natural Resources, Gorgan, Iran) PCR Master Mix and 1 μl of each gene-specific primer (10 pmol) in a final volume 20 μl with double distilled water. PCRs were performed under following conditions: 2 minutes at 94°C and 35 cycles of 10 seconds at 95°C and 10 seconds at 60°C. The specificity of amplicons was verified by melting curve analysis (55 to 95°C) after 35 cycles. Three replicates for each sample were used for qRT-PCR analysis. Relative expression was computed using $2^{-\Delta\Delta\text{CT}}$ method. The REST software (Pfaffl *et al.*, 2002) was used to calculate ratio between the amount of target molecule and reference molecule actin within the same sample.

Statistical Analysis

Experimental data of physiological sections of this research were analyzed by SPSS 19.0 computer software on the basis of randomized complete design (RCD) with three repetitions. The treatment means were compared using Duncan's multiple range tests.



RESULTS

Change in the H₂O₂ content

The results of the H₂O₂ analysis indicated significant differences among thermal treatments. H₂O₂ content increased significantly in the first day of CA (12.7%) and in the fifth day (approximately 15%) compared to control phase (Figure 1-A). We have observed a decrease in H₂O₂ content during CS and in recovery phase. Compared to control phase, H₂O₂ content increased significantly in non-acclimated plants (approximately two-fold) and acclimated plants (by 82%) during freezing phase (Figure 1-B).

Change in the MDA Content

Comparing experimental data means of MDA have shown significant differences in thermal phases. In the first days of CA, MDA content did not change significantly but it increased 13% compared to control plants in the fifth day (Figure 2-A). Exposing to 4°C as CS phase caused a

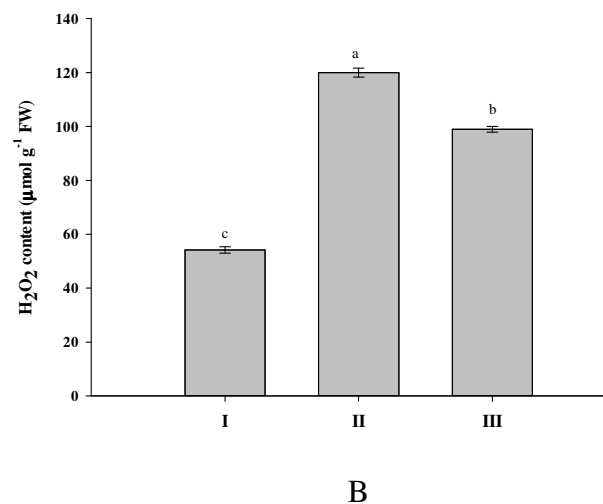


Figure 1. Effects of thermal phases on hydrogen peroxide (H₂O₂) content: (A) In the leaves of control plants (23°C) (I); acclimated plants after 1 day at 10°C (II); after 5 days at 10°C (III); cold-stressed plants (4°C) (IV), and recovery plants (V); (B) In the leaves of control plants (23°C) (I), non-acclimated plants (II) and acclimated plants after freezing phase (-10°C). The error bars represent the standard deviation (\pm SD) for replicates.

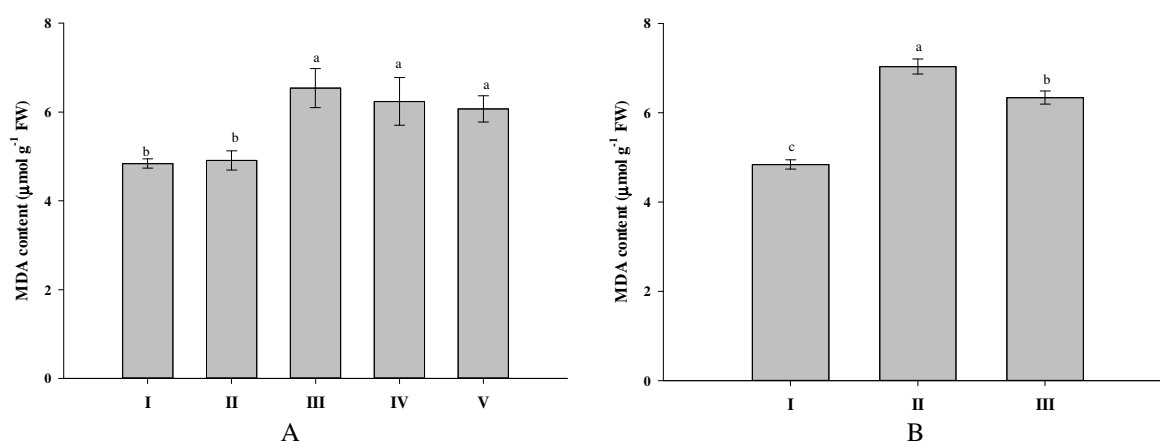


Figure 2. Effects of thermal phases on malondialdehyde (MDA) content: (A) In the leaves of control plants (at 23°C) (I); acclimated plants after 1 day at 10°C (II); after 5 days at 10°C (III); cold-stressed plants (4°C) (IV), and recovery plants (V); (B) In the leaves of control plants (23°C) (I), non-acclimated plants (II) and acclimated plants after freezing phase (-10°C). The error bars represent the standard deviation (\pm SD) for replicates.

decrease in MDA accumulation. Reducing MDA in recovery phase can be considered as plant improvement response. During freezing phase levels of MDA in non-acclimated plants and acclimated plants significantly increased (approximately 45 and 30%, respectively) (Figure 2-B).

Expression Pattern of Dehydrin

In the first day of CA phase, transcript

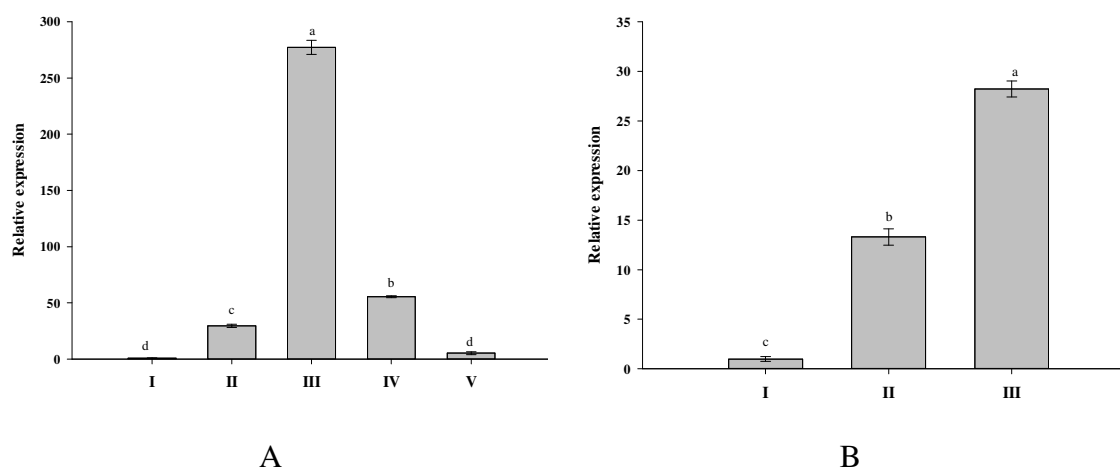


Figure 3. The gene expression ratio of dehydrin: (A) In the leaves of control plants (at 23°C) (I); acclimated plants after 1 day at 10°C (II); after 5 days at 10°C (III); cold-stressed plants (4°C) (IV); recovery plants (V); (B) In the leaves of control plants (at 23°C) (I); non-acclimated plants (II), and acclimated plants (III) after freezing phase (-10°C). The error bars represent the standard deviation (\pm SD) for replicates.

levels of dehydrin increased with a low slope compared to control phase, but highly expressed in the fifth day CA phase (Figure 3-A). The expression of dehydrin was the strongest at CA phase (5 days after the initial acclimation) of chickpea seedlings. Assessing the effect of CS, the transcript level of this gene has shown decreasing trends compared to CA phase so that this decrease trend continued in recovery phase. The exposure of seedlings to freezing phase caused a significant increase in the dehydrin



transcripts in acclimated plants and non-acclimated plants. However, the accumulation of transcripts in freezing phase in acclimated plants was more than that of non-acclimated ones (Figure 3-B).

Expression Pattern of Beta-glucosidase

In CA phase, beta-glucosidase transcripts increased in the first day, but it decreased significantly in the fifth day. However, the level of transcripts was highly more observed compared to control phase. Results have shown an increase in the transcript level of this gene during CS and a downturn of that in recovery phase. The exposure of seedlings to freezing phase caused a significant increase in the beta-glucosidase transcripts in acclimated as well as non-acclimated plants. However, in freezing phase its level in acclimated plants was approximately two-fold than that of non-acclimated plants [Figure 4 (A and B)].

DISCUSSION

As known, the physiological and molecular changes have a crucial role in cell activities upon exposure to CS. The present

results showed that, thermal phases changed H_2O_2 content that probably was one of the reasons for changing in the MDA level (Maali-Amiri *et al.*, 2007; Maali Amiri *et al.*, 2010, Deryabin *et al.*, 2005). It was supposed that the changes in H_2O_2 and MDA contents in CA phase probably were a sign that indicate this level of H_2O_2 could not be considered as an intense damage. Several studies showed that H_2O_2 level was recognized as the signal molecules to activate transcription factors and gene expression involved in tolerance to stress (Vanacker *et al.*, 2000). The increased H_2O_2 content followed by MDA accumulation probably activates defense mechanisms involved in cold tolerance when exposed to less temperature. Non-significant, and then significant increase in MDA content during CA phase indicated that seedlings perceived cold signaling and then increased transcript levels of *dehydrin* gene as a part of the defense responses of plants. It seemed that CA phase due to reprogramming of gene expression may led to adjusted metabolic alterations (Heidarvand and Maali, 2010). In the fifth day of CA phase, an increase of *dehydrin* transcripts may reflect a potential acclimation in chickpea leaves. This reprogramming of *dehydrin* gene expression along with other defense mechanisms

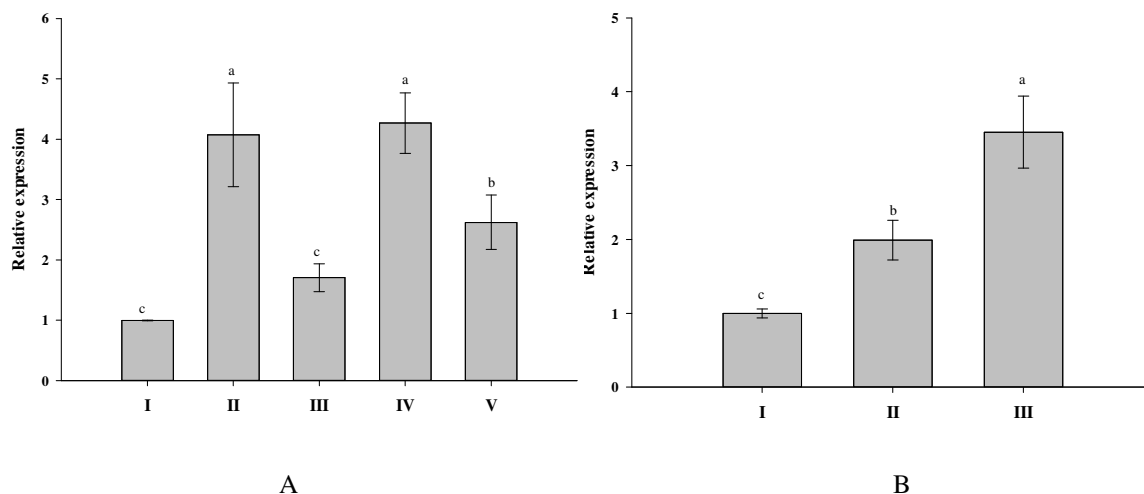


Figure 4. The gene expression ratio of beta-glucosidase: (A) In the leaves of control plants (at 23°C) (I); acclimated plants after 1 day at 10°C (II); after 5 days at 10°C (III); cold-stressed plants (4°C) (IV), recovery plants (V); (B) In the leaves of control plants (at 23°C) (I); non-acclimated plants (II), and acclimated plants (III) after freezing phase (-10°C). The error bars represent the standard deviation (\pm SD) for replicates.

resulted in decrease of the oxidative indicators (H_2O_2 and MDA) during CS. Previously, we have shown that the decreased MDA content during CS was related to the enhanced cold tolerance and plants with higher levels of unsaturated fatty acids showed decreased MDA (Maali Amiri *et al.*, 2007). In our study, the decline in MDA content could possibly be associated with the composition, properties of membrane and more activity of defense systems like antioxidants (Kazemi Shahandashti *et al.*, 2013). Because of the complexity of CS response network, we assumed that the cold responses of chickpea might be partly due to the stabilization of their membranes composition and physical properties. The decreasing of dehydrin transcripts during CS probably could be from a potential damage of the translation process or from low damage of CS because of CA phase. Data in MDA content confirmed the later. Transcript levels of *dehydrin* gene were gradually decreased under recovery phase and this trend also observed in H_2O_2 and MDA content. The freezing phase stimulated increasing of H_2O_2 and MDA contents especially in non-acclimated compared to acclimated plants that probably triggered oxidative stress in plants. It was possible that the leaf system was incapable of developing tolerance to freezing phase in non-acclimated plants and therefore, oxidative indicators increased significantly. The higher level of dehydrin transcripts in acclimated plants compared to non-acclimated plants particularly showed a more active role for plant cells protection. The contribution of dehydrins to freezing tolerance in plants could be partly due to their protective effect on membranes (Puhakainen *et al.*, 2004). But in non-acclimated plants low increase of dehydrin transcripts probably along with weak activity of other defense factors could not prevent cellular injuries. Thus, our results indicated that dehydrins probably involve in the increase of tolerance level in chickpea.

As known, a large proportion of genes corresponded to well characterized enzymes

that were required for energy or metabolic processes, such as photosynthesis, respiration, and intermediary metabolism (Byun *et al.*, 2009) which are up-regulated by physiological needs or environmental stresses. The early highly induction of this gene in the first day of CA phase seemed that enables plants to accomplish rapid increases in reprogramming of genes in response to CA when yet chickpea plants were not acclimated (Brautigam *et al.*, 2009). But under longer periods of CA the transcript level was decreased although the level of transcripts yet was approximately two-fold of as compared to control plants. This decrease in the beta-glucosidase transcripts probably could show acclimation status in plants. Previous reports indicated that beta-glucosidase contributed to abscisic acid homeostasis in plant cells under environmental stresses (Dietz *et al.*, 2000; Lee *et al.*, 2006). The increasing of beta-glucosidase expression during CS, probably established a new homeostasis against CS so that in CS phase, H_2O_2 and MDA contents compared to CA phase, decreased. Thus, it seems that beta-glucosidase induction as a primary metabolite is from early chickpea responses for encountering against CS along with the induction of dehydrin. The transcript levels of beta-glucosidase were gradually decreased under recovery phase. It indicated that chickpea plants perceived thermal changes and intended to reach to its level in control plants whereas the damages caused by CS had compensated. In freezing phase the higher level of beta-glucosidase transcripts in acclimated plants compared to non-acclimated plants showed a more active role in plant responses but these changes could not alleviate cellular injuries in non-acclimated plants. Thus, it seems that probably changes in beta-glucosidase transcripts along with other gene functions, involve in the increase of cold tolerance in chickpea.

In conclusion, under thermal phases the chickpea plants showed different physio-molecular responses that confirmed the existence of wide range of genetic capacity



in a cultivar to enhance cold tolerance when environmental conditions change. These responses effectively may be triggered in CA phases to encounter CS in chickpea. This could be inferred from low H_2O_2 and lipid peroxidation contents and accumulation of transcript levels in *dehydrin* and *beta-glucosidase* genes. Freezing phase could be useful in the assessment of relative capability of chickpea to CS injuries and helped better understanding of the relationships observed in cellular responses. An incapability of defense machine was a limiting factor, determining the low potential of non-acclimated chickpea plants to freezing temperature. Detailed studies of chickpea plants may be interesting in elucidation of survival mechanisms of cold-sensitive plant tolerance.

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تغییر رونوشت ژن‌های دهیدرین و بتاگلوکوزیداز تحت تنش اکسیداتیو القا شده توسط سرما در نخود

م. خزائی، ر. معالی امیری، ع. ر. طالعی، و س. رمضانپور

چکیده

در این پژوهش محتوی پراکسید هیدروژن (H_2O_2) و مالون دی آلدئید (MDA) (به عنوان شاخص-های تنش اکسیداتیو) و سطوح رونوشت دهیدرین و بتاگلوکوزیداز (ژن‌های درگیر در پاسخ‌های متابولیکی) به کمک qRT-PCR، در دوره‌های کنترل، سازگاری، سرما، بهبودی بعد از سرما و زیر صفر درجه سانتی‌گراد در رقم نخود جم ارزیابی شد. نتایج بیانگر وجود ظرفیت ژنتیکی افزایش تحمل به سرما در این رقم در حین تغییر شرایط محیطی می‌باشد. افزایش معنی‌دار در میزان H_2O_2 و MDA در دوره سازگاری نشان داد که گیاهچه‌ها سیگنال سرما را درک کرده و سلول افزایش قابل توجهی در رونوشت ژن‌های دهیدرین و بتاگلوکوزیداز (به عنوان بخشی از پاسخ‌های دفاعی) ایجاد کرد به طوری که توازن بیان این ژن‌ها و شاخص‌های تنش اکسیداتیو به اثرات متقابل مسیرهای خسارت و دفاع اشاره دارد. در دمای زیر صفر درجه سانتی‌گراد، سطوح رونوشت بیشتر ژن‌ها در گیاهان سازگار شده در مقایسه با گیاهان سازگار نشده نقش فعال‌تر سلول‌های گیاهی را نشان داد. ناتوانی ماشین دفاعی در گیاهان سازگار نشده عامل محدود کننده در تعیین پتانسیل کم گیاه نخود به دمای زیر صفر درجه بود. تنظیم تغییرات متابولیکی شامل ژن‌های دهیدرین و بتاگلوکوزیداز مخصوصاً بعد از سازگاری و همچنین کاهش در شاخص‌های تنش اکسیداتیو علت تحمل نسبی به سرما در نخود می‌باشد.