Comparative analysis of *WRKY* gene expression in extreme chickpea genotypes under progressive water stress using real-time PCR

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Chickpea (*Cicer arietinum* L.) is an important legume crop grown mainly in the arid and semi-arid regions of the world. Drought is the major factor causes detrimental effect to chickpea growth and productivity. Transcription factors have been reported to be the key regulators of drought tolerance. In this study, we analyzed the expression profile of *WRKY* transcription factor in the leaves of tolerant (MCC537) and susceptible (MCC674) chickpea genotypes under dehydration period after 21 days of sowing at 3 time points (24, 48 and 96 hours after irrigation withdrawal) to assess the relation between the differential expression levels of this gene and related drought tolerance of these genotypes. Gene expression analysis using real-time quantitative PCR indicated that *WRKY* was significantly induced by drought stress. At the last time point, the expression level of this gene was significantly increased up to 3 fold higher than control plants under drought condition. The results demonstrated that this TF may play a critical role in drought tolerance. It also may be a potential dehydration-responsive gene but it needs further studies to be used in transgenic chickpea processes.

Keywords: drought, chickpea, transcription factors, gene expression, qPCR

1. Introduction

Chickpea (*Cicer arietinum* L.), is an important legume crop grown mainly in the arid and semiarid regions of the world such as Iran. The seeds of chickpea contain important micronutrients which are rich in protein (24.6%), carbohydrate (64.6%) and vitamins. It also has the ability of nitrogen fixation, so it can increase production of other cereal through soil fertility [1].

The yield level of chickpea is 900 kg/ha globally [2]. This is because it encounters multiple stresses during its life cycle [3], whereas under optimum conditions, chickpea yield potential was reported up to 6 t/ha. Thus, chickpea is under constant exposure to biotic and abiotic stresses. Abiotic stresses mainly include of drought, cold, heat and salinity [4]. As 90% of chickpea crops are cultivated under rain-fed conditions, drought is the major factor causing detrimental effect to crop productivity and alone causes a 40–50% reduction in global yield potential [5]. Drought stress induces a range of morphophysiological and molecular responses in plants. To regulate these responses, drought signaling pathways are involved in mediating the expression of many drought-responsive genes coding functional proteins (such as dehydrins) and regulatory proteins (such as transcription factors (TFs)). TFs binding to DNA activate expression of the target genes involved in regulating biotic and abiotic stress tolerance mechanisms [6].

WRKY TFs responding under stress conditions are one of the major modulator TFs families and play an important role in the crosstalk of signaling pathways [7, 8, 9]. *WRKY* have been found to be responsive to wounding in chickpea which is related to biotic stress. These genes activate defenses through up-regulation of genes coding oxidases, pathogenesis, oxidases, phenylpropanoid pathway and *CYTP450* [10, 11]. Several studies also showed that the *WRKY* genes involved in abiotic stress tolerance [12, 13]. These genes have increased drought tolerance in Arabidopsis and rice [14, 15]. Rice *OsWRKY11* also improved heat and drought tolerance in transgenic plants [14]. *WRKY* was differentially expressed in chickpea plants under drought condition [16]. Thus, these studies, to some extent, have identified the role of TFs in signaling pathways, however there is still little knowledge of the molecular mechanisms involved in drought tolerance in legume, especially chickpea.

Gene expression analysis can be a promising tool for understanding the function of drought-responsive genes to generate transgenic plants under drought stress. Although these genes have been identified in chickpea through different gene expression methods, it has been shown that comparative differential gene expression analysis can display contrasting response of genotypes to stress and provide a better understanding of the molecular mechanisms [17].

QRT-PCR is one of the fastest methods to test large number of genes to analyze their expression accurately. In this study, we analyzed the expression profile of *WRKY* transcription factor through this method to assess the relation between the differential expression levels of this gene in two extreme drought responsive genotypes selected in the field.

2. Materials and methods

Chickpea genotypes, (MCC537 and MCC674 as tolerant and sensitive genotypes respectively), were used for tissue specific expression analysis. Drought stress was applied progressively to 3-week-old seedlings grown in the greenhouse condition through stop watering. The leaves were collected at 24, 48 and 96 hours after stress initiation, immediately frozen in liquid nitrogen and stored at -80°c until use.

Total RNA extractions from leaves were performed using a column RNA Isolation Kit (DENA Zist, Asia). The cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas), and 1 ml of the reaction mixture was subsequently used for PCR by gene specific primers to validate cDNA synthesis. For qRT-PCR, specific primer pairs were used for *WRKY* and IF4α. The following thermal cycle conditions were used: 95°C for 3 min, followed by 94°C for 15s, 60°C for 30s and 72°C for 30s, for a total 40 cycles. Relative expression levels of gene in each sample were normalized to the expression level of *IF4α* as a reference gene. For real time quantitative PCR the primer pairs (GCGAGGAAACAAGTGGAGAG, forward), (TTCCCCCATTGGT AAAAACA, reverse) and (GTCTCAGCAACTCATGGAGACA, forward), (CACGTCAATACCACGAGCTA GA, reverse) were used for *WRKY* and *IF4α,* respectively. qRT-PCR assays were performed in triplicate using BIORAD CFX96 (Fig. 3). Following PCR, a melting curve analysis was performed (Fig. 4). Relative expression level was calculated as follows:

$$2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct\ \text{treated} - \Delta Ct\ \text{control})}$$

3. Results and discussion

3-1 RNA isolation and cDNA synthesis validation

The quality and quantity of RNA have been confirmed through gel electrophoresis and nanodrop Two separate bands of 28s and 18s were appeared on the gel (Fig. 1), indicating the high quality of extracted mRNA.

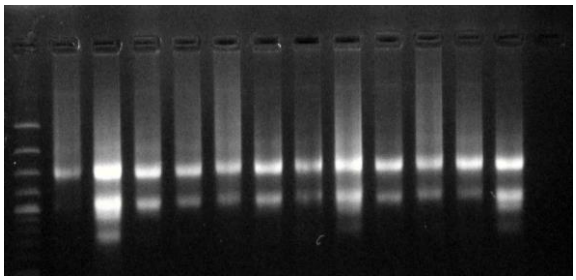

Figure 1: Gel electrophoresis of extracted RNA on 1.5% agarose gel, showing two clear bands.

Nano-drop also was used to determine the concentration and purity of isolated RNAs. The presence of protein and other contamination can generally inhibit cDNA synthesis and PCR process [18]. The ratio of 280/260 and 230/260 was around 2, validating purity of RNAs against phenolic, and carbohydrate contamination (Fig. 2).

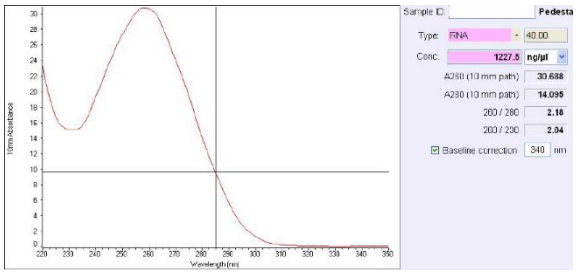

Figure 2: Nano-drop plot and its related data.

The cDNA was synthesized and was validated through PCR using gene specific primers of *WRKY* and *IF4α*. The result showed only a single specific band for the genes. Wells from 1-4 and 5-8 are PCR products of *IF4α* and *WRKY* (figure 3).

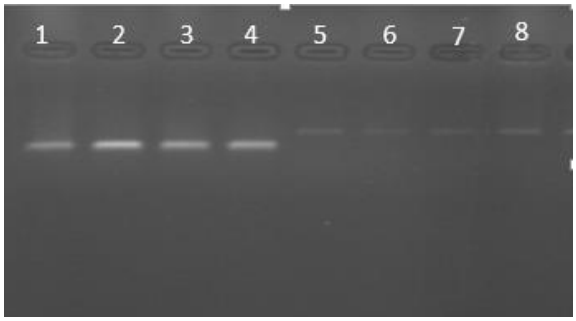

Figure 3: cDNA validation by PCR. Wells from 1-4 and 5-8 are PCR products of *IF4α* and *WRKY*

3-2 Expression pattern of *WRKY*

To examine the effect of drought stress on *WRKY* expression, the transcriptional levels of *WRKY* in leaves under drought treatment were determined by quantitative RT-PCR. Melting curve analysis also was performed and confirmed the specific amplification of *WRKY* gene and the absence of primer dimer (Fig. 4).

QRT-PCR showed that *WRKY* was significantly up-regulated by drought stress. As shown in Fig. 5, the expression level of *WRKY* in MCC537 was not changed at two first time points after stress, whereas in the sensitive genotype (MCC674), *WRKY* expression trend was down-regulated at two first time points (24h and 48h after stress) compared with its control. Moreover, in both sensitive and tolerant genotypes (MCC674 and MCC537), *WRKY* was expressed at higher level in the last time point (96h after stress) with severe water stress. At this time point (96 h), the expression level of this gene was approximately 3 fold higher than control genotype under drought condition. This study suggests that *WRKY* may play an important role as transcriptional activator; this more likely is involved in the response to drought stress especially at later times after stress.

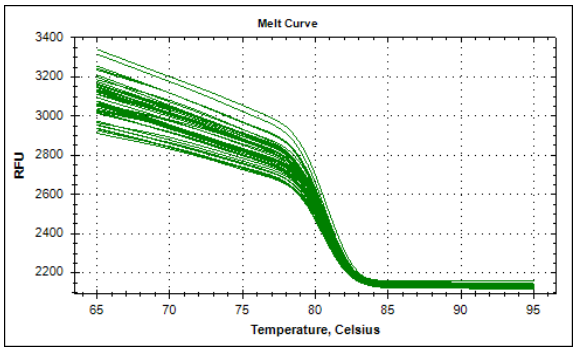

Figure 4: Melting curve analysis showing specific amplification of *WRKY* gene and the absence of primer dimer.

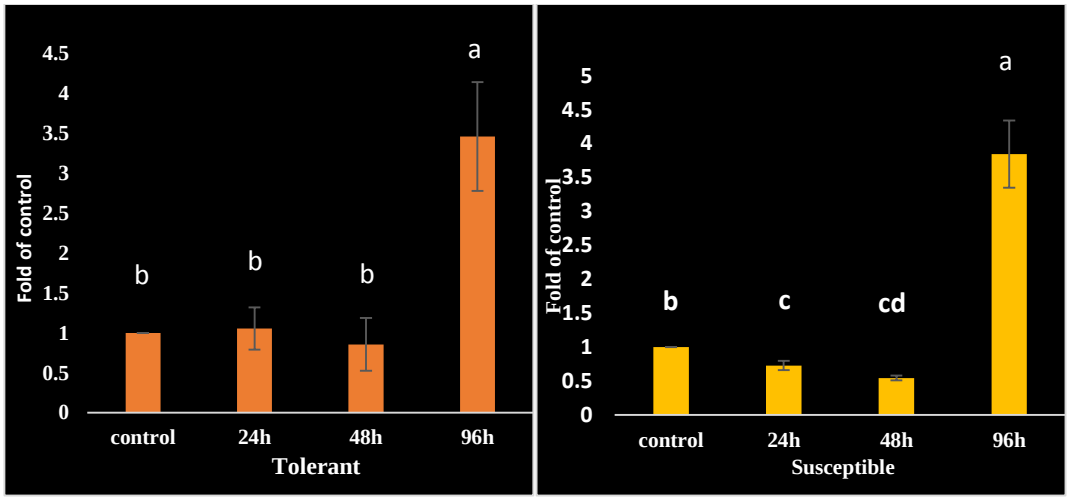

Figure 5: Differential expression of *WRKY* gene in two tolerant and susceptible genotypes at various times points (24, 48 and 96hours) after stress.

It has shown in the previous studies that *WRKY* is induced by dehydration and play critical role in regulating drought tolerance in plants [19, 20]. The *WRKY* TF was differentially up-regulated in drought, salinity, cold condition [21]. *WRKY* from Arabidopsis was found to be significantly enhanced level of ABA and dehydration responsive genes. The *WRKY* in rice resulted in the induction of dehydration or heat shock-inducible

genes. *WRKY* TF, one of six genes involved in drought stress, was studied for the first time in senna [22]. In a similar study, *WRKYs* from wheat (*Triticum aestivum* L.) functioned as stress-responsive genes in stress tolerance. Their over-expression have also increased salt, drought and freezing tolerance in Arabidopsis [23]. The expression of *TaWRKY33* genes in Arabidopsis was also expressed by drought, heat in transgenic Arabidopsis, and improved stress tolerance [24, 25, 8]. Similar observation was previously reported in which over-expression of *WRKY* from soybean in transgenic Arabidopsis could improve resistance to drought, cold and salinity stress [26]

In conclusion, the results demonstrated that *WRKY* TF may play a critical role to improve drought tolerance. Our results also suggest that *WRKY* may be useful to define candidate drought-tolerant genotypes and it seems that the selection of potential dehydration-responsive genes could facilitate the development of transgenic chickpea varieties.

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