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Gene expression analysis of WRKY TF in Chickpea genotypes under drought stress using semiquantitative PCR at vegetative stage

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Abstract
The chickpea (*Cicer arietinum* L.) is an important legume crop that is largely grown in arid and semiarid regions of the world. Drought is the main factor having a negative impact on chickpea growth and output. Transcription factors have been identified as the primary controllers of drought tolerance. According to reports, transcription factors are the primary controllers of drought tolerance. In this study, we examined the WRKY transcription factor's expression pattern in the leaves of susceptible (Vishal; DCP-92-3) and tolerant (ICC 4958; ICC 867) genotypes of chickpeas. To evaluate the relationship between the varying levels of this gene's expression and the associated drought tolerance of these genotypes, 40 days of after irrigation withdrawal was made. Semi-quantitative PCR analysis of gene expression revealed that drought stress greatly increased WRKY. This gene's level of expression at the most recent time point was noticeably higher than in control plants when drought conditions were present. The findings indicated that this TF might be essential for drought tolerance. It might also be a gene that responds to dryness, but further research is required before it can be applied to transgenic chickpea operations.

Keywords: *Cicer arietinum* L, drought, WRKY, semiquantitative PCR

Introduction
A significant legume crop farmed mostly in arid and semiarid regions of the world is the chickpea (*Cicer arietinum* L.). Chickpea seeds are a good source of vitamins, protein (24.6%), carbohydrates (64.6%), and other vital elements. Additionally, it has the capacity to fix nitrogen, which enables it to improve soil fertility and boost the production of other cereals (Varshney *et al*. 2013, 2013) [22]. 900 kg/ha is the global chickpea yield (Blum, 1981) [2]. This is due to the fact that it experiences numerous challenges throughout its life cycle (Jha *et al*. 2014) [7], but under ideal circumstances, chickpea yield potential has been reported to reach up to 6 t/ha. As a result, biotic and abiotic stressors are constantly being applied to chickpea. According to Jha *et al*. (2014) [7], the main abiotic stressors include salinity, cold, heat, and drought. Drought is the main factor affecting crop production and alone produces a 40–50% drop in world yield potential because 90% of chickpea crops are grown in rain-fed environments (Varshney, *et al*. 2011) [21]. Plants respond in a variety of morpho-physiological and molecular ways to drought stress. Many drought-responsive genes that code for functional proteins (like dehydrins) and regulatory proteins (like transcription factors (TFs)) are expressed to control these responses via drought signaling pathways. According to Shinozaki and Yamaguchi (2007) [19], transcription factors (TFs) that bind to DNA stimulate the expression of the target genes that control biotic and abiotic stress tolerance mechanisms. One of the main families of modulator TFs, WRKY TFs reacting to stress, are crucial in the interaction of signaling pathways (Eulgem *et al*., 2000; Chen *et al*., 2012; Agarwal *et al*., 2011) [5, 3, 1]. It has been discovered that WRKY are sensitive to chickpea injury, which is associated to biotic stress. By up-regulating the genes that code for oxidases, pathogenesis, oxidases, the phenylpropanoid pathway, and CYTP450, these genes activate defenses (Kumar *et al*., 2016; Pandey *et al*., 2017) [9, 14]. Abiotic stress tolerance was also demonstrated by several investigations to be mediated by the WRKY genes (Zou *et al*., 2004) [25]. In Arabidopsis and rice, these genes have increased drought resistance (Wu *et al*., 2009; Seki *et al*., 2002) [23, 18]. According to Wu *et al*. (2009) [23], rice OsWRKY11 enhanced transgenic plants' ability to withstand heat and drought. Under drought conditions, WRKY was differently expressed in chickpea plants (Ramalingam *et al*., 2015) [16]. As a result, these investigations have partially pinpointed the function of TFs in signaling pathways.

However, little is known about the molecular mechanisms underlying legume drought tolerance, particularly in chickpea. To create transgenic plants under drought stress, gene expression analysis can be a potential approach for studying the function of drought-responsive genes. It has been demonstrated that comparative differential gene expression analysis can show the contrasting responses of genotypes to stress and provide a better understanding of the molecular mechanisms even though these genes have been discovered in chickpea using various gene expression methods (Nguyen *et al*., 2015) [12]. One of the quickest ways to accurately assess a large number of genes for expression is semi-quantitative PCR. In this study, we used this technique to investigate the expression profile of the WRKY transcription factor in order to assess the relationship between the varied expression levels of this gene in four genotypes that are sensitive to extreme drought and were chosen from the field.

Material and Methods

Chickpea genotypes, (ICC 4958; ICC 867 and Vishal; DCP-92-3 as tolerant and sensitive genotypes respectively), were used for tissue specific expression analysis. Drought stress was applied at vegetative stage (40 DAS) grown in rainout shelter through stop watering. The leaves were collected after stress initiation, immediately frozen in liquid nitrogen and used for RNA extractions.

The extraction of total RNA from leaves was carried out using Trizol method. The cDNA synthesized using Promega cDNA Synthesis Kit, and 20 µl of reaction mixture was subsequently used for PCR by gene specific primers to validate cDNA synthesis. For semiquantitative PCR, specific primer pairs were used for WRKY TFs. The following thermal cycle conditions were used: 95 °C for 3 min, followed by 94 °C for 30 sec. 57 °C for 30 sec and 72 °C for 30s, for a total 35 cycles. For semiquantitative PCR the primer pairs were used for WRKY (Table 1) and α-Actin, respectively. qRT-PCR assays were performed in using Eppendorf thermal cycler.

Table 1: Semiquantitative PCR the primer pairs were used for WRKY and α-Actin, respectively. qRT-PCR assays were performed in using Eppendorf thermal cycler

Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
CarWRKY04	ACGTCACGTTCTAGCTTCGG	TGGAAGAACCGCGTTGTGAG
CarWRKY07	GATCTGCGGAGAAGTGGAGG	CGGAAAACCGCTGAACACTC
CarWRKY08	AACGAGCACAAGACGATCCA	ACTGAAGGCTGAAACCAACGA
CarWRKY22	GAGTCAACTTGCTGCCTTTGG	GCTGTAGCAGCAGTCCAAGT
CarWRKY51	AAAGGGCACAAGACGATCCC	TAGGCTGAGATGTGAAGCCG
α-actin	GGCATCTTTTAGCACCTTCCAGC	ACCACTTGTTGTCTAAAACCACTTT

3. Results and Discussion

3.1 RNA isolation and cDNA synthesis validation

Gel electrophoresis and nanodrop technologies have been used to verify the quantity and quality of RNA. The gel (Fig. 1) showed two distinct bands of 28s and 18s, showing the good quality of the retrieved mRNA.

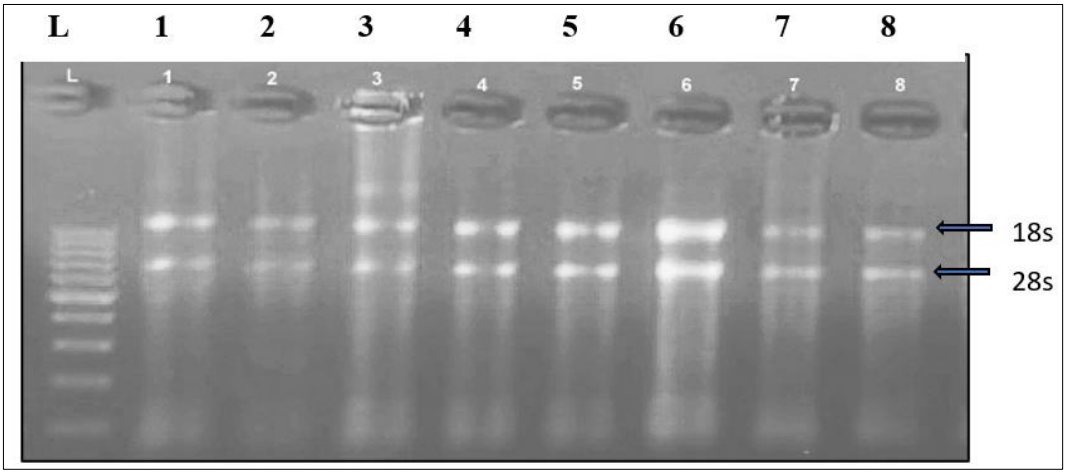

Fig 1: Agarose gel electrophoresis of isolated total RNA on 2% agarose gel, showing two clear bands

The cDNA was synthesized and was validated through PCR using housekeeping gene α-actin. The result showed only a single specific band for the genes.

Expression pattern of WRKY

Semi-quantitative RT-PCR was used to estimate the transcriptional levels of WRKY in drought-treated leaves in order to study the impact of drought stress on WRKY expression.

CarWRKY04, CarWRKY08, CarWRKY22 and CarWRKY 51 gene participate in various process of plant development. To know more about the expression pattern and function of CarWRKY04, CarWRKY08, CarWRKY22 and CarWRKY 51in leaf tissue of four chickpea genotypes i.e. Vishal, DCP-93-2, ICC 867 and ICC 4958 under both control and treated condition by semiquantitative PCR were examined. The gene expression was carried out at different PCR cycles 25, 30 and 35 to campare the transcript abundance of CarWRKY04, CarWRKY08, CarWRKY22 and CarWRKY 51 gene in two tolerant and two susceptible chickpea genotypes. The cDNA was normalized using actin as a housekeeping gene. For all the samples under investigation, a target cDNA species was

amplified in semiquantitative PCR utilizing the same quantity of cycles. By evaluating the intensity of the band corresponding to the produced amplicon, after electrophoresis separation in a gel and staining with EtBr, the expression rate of the target gene is determined. The band intensity represents the quantity of target cDNA used in the PCR at the start and, consequently, the degree of target gene expression in the sample. Total cDNA concentration needs to be consistent across all examined samples in order for the analysis to produce accurate results. Each cDNA sample was originally diluted until, after a specific number of PCR cycles, the intensity of the band corresponding to the Actin gene was the same in each sample.

Characterization and expression analysis of drought related genes in chickpea leaves at vegetative stage and analysis of expression under drought stress will help identified the role of genes in drought stress. Differential level of expression was recorded for the 5 WRKY genes at vegetative stage and 4 diverse genotypes. for expression analysis total 5 genes was used (CarWRKY04, CarWRKY07, CarWRKY08 CarWRKY22 and CarWRKY51 The results show varying levels of gene expression among these five WRKY genes during the vegetative stage and across four different genotypes. In the case of CarWRKY04, it was found that this gene is upregulated in the drought-tolerant genotype ICC4958 under drought stress, while it had comparatively lower expression in the susceptible genotype Vishal under both drought stress and normal conditions. The gene showed no expression in the ICC 867 genotype. Similarly, CarWRKY08 exhibited upregulation in the drought-tolerant genotype ICC4958 under drought stress, but lower expression was observed in the ICC 867 genotype (which is also tolerant) and the susceptible genotype Vishal, under both drought stress and normal conditions. In the susceptible genotype DCP-92-3, this gene was upregulated under both drought stress and normal conditions. For CarWRKY22, upregulation was seen in the drought-tolerant genotype ICC4958 under drought stress, while lower expression occurred in the ICC 867 genotype (tolerant) and the susceptible genotype Vishal, under both drought stress and normal conditions. No expression of CarWRKY22 was detected in the DCP-92-3 genotype. In the case of CarWRKY51, upregulation was noted in the drought-tolerant genotype ICC4958 under drought stress. This gene showed comparatively lower expression in the ICC 867 genotype (tolerant) and the susceptible genotype Vishal under both drought stress and normal conditions. The susceptible genotype DCP-93-2 also showed upregulation of this gene under both drought stress and normal conditions.

Furthermore, CarWRKY51 exhibited a high level of expression across all genotypes. Overall, the study provides insights into how these specific WRKY genes respond to drought stress in different chickpea genotypes during the vegetative stage.

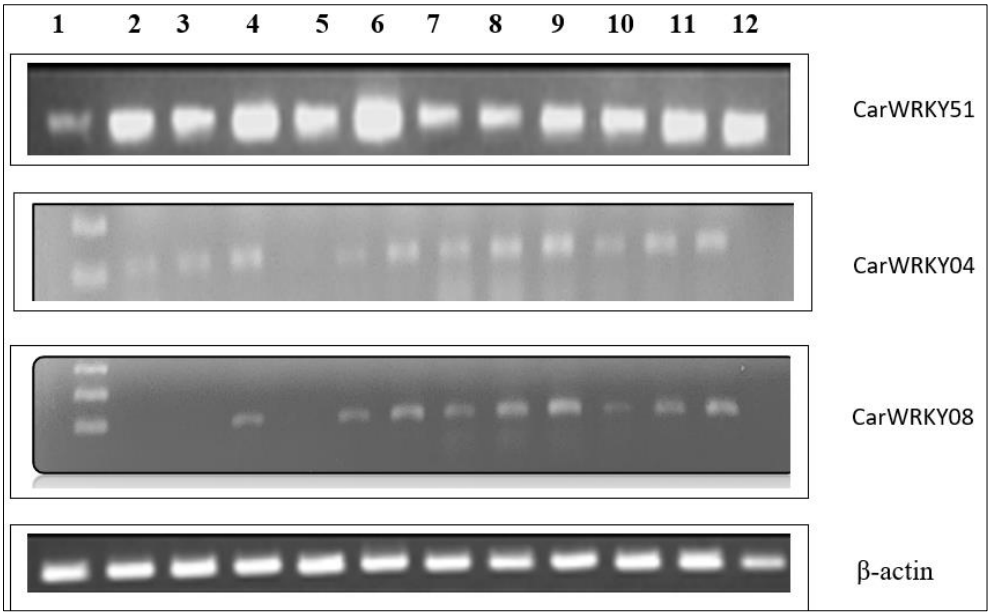

Fig 2: Semi-Quantitative RT-PCR analysis of CarWRKY genes in chickpea under drought stress

Previous investigations have demonstrated that WRKY is activated by dehydration and is essential for controlling plant drought tolerance (Ren *et al*., 2010, Van Eck *et al*., 2014) [17, 20]. According to Konda *et al*. (2017) [8], the WRKY TF was variably up-regulated in conditions of drought, salt, and coldness. It was discovered that WRKY from Arabidopsis has dramatically increased levels of ABA and dehydration response genes. Genes that are activated by heat shock or dehydration were induced in rice as a result of the WRKY. Senna was the subject of a pioneering study on WRKY TF, one of six genes related to drought stress (Mehta *et al*., 2017) [11]. Similar research revealed that wheat WRKYs (*Triticum aestivum* L.) served as stress-responsive genes involved in stress tolerance. Their overexpression has also improved Arabidopsis' resistance to salt, dehydration, and frost (Niu *et al*., 2012) [13]. Drought, heat in transgenic Arabidopsis, and enhanced stress tolerance were also associated with the expression of TaWRKY33 genes in Arabidopsis (He *et al*., 2016; Qin *et al*., 2015; Chen *et al*., 2015) [4, 6, 15]. Similar findings were made previously; showing that over-expression of the soybean gene WRKY in transgenic Arabidopsis could increase the plant's tolerance to salinity, cold, and drought stress (Zhou *et al*., 2008) [24]. These findings show that the genes CarWRKY04, CarWRKY08, CarWRKY22, and CarWRKY51 are expressed greater in drought-stressed conditions in chickpeas than in control conditions.

Conclusion

The findings showed that WRKY TF may play a significant part in enhancing drought resistance. Our findings also imply that WRKY may be helpful in defining candidate genotypes that are drought-tolerant, and it appears that choosing prospective dehydration-responsive genes may make it easier to create transgenic chickpea varieties.

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