Journal of Animal & Plant Sciences, 32(5): 2022, Page: 1385-1393
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2022.5.0545

COMPARATIVE GENOMICS AND EXPRESSION ANALYSIS OF *KCS* GENES UNDER DROUGHT STRESS IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

P. Zaib, T. Shaheen, M. Hamyat and Mahmood-ur-Rahman*

Department of Bioinformatics and Biotechnology, GC University Faisalabad, Allama Iqbal Road, Faisalabad-38000, Pakistan
*Corresponding Author's email: mahmoodansari@gcuf.edu.pk

ABSTRACT

Water stress is considered as the main environmental factor which badly affects growth of sunflower plant. It is a water sensitive plant whose yield is greatly affected by drought stress. Several studies have been done to understand the mechanism of drought stress tolerance in plants, and exploring the role of stress tolerant genes is one of them. *KCS* genes, responsible for wax biosynthesis, have been reported to be involved in drought stress tolerance mechanism. In this study, expression profiling of *KCS* genes was done to understand their role in stress mechanism. Comparative genomics studies of *KCS* genes were carried out in sunflower and Arabidopsis by constructing phylogenetic tree. They were divided into six clades, however they were present in the same clade showing similarities between Arabidopsis and sunflower. It was further confirmed by Synteny analysis and concluded that *KCS* genes in both species share the same evolutionary origin. Further, they were amplified in sunflower by using gene specific primers. Five genes, i.e. *KCS2*, *KCS4*, *KCS5*, *KCS10* and *KCS18* were successfully amplified in sunflower variety FH-593. Then, sunflower plants were subjected to drought stress and expression profiling of amplified *KCS* genes was carried out by Real Time PCR. All the five genes were up-regulated under drought showing their role in stress conditions; however, the expression level of each gene was varied. Maximum relative expression was found for *KCS4* gene in T1, i.e. 19 fold as compared to control. The expression of other genes was found in decreasing order as indicated *KCS4>KCS2>KCS5>KCS10>KCS18* (relative expression: 19, 12, 5, 4 and 3 respectively). Biochemical analyses were also performed and significant variation was found among treatments and control. Total chlorophyll contents were decreased under drought stress while antioxidants like catalase, peroxidase, superoxide dismutase and proline were increased. Our results showed the role of *KCS* genes in drought stress which is first ever report in sunflower. This study concluded that *KCS* genes have role in drought stress tolerance and their expression is significantly up-regulated under stress conditions. The information presented here may help to further characterize *KCS* genes and their subsequent use to engineer drought stress in crop plants.

Keywords: Sunflower, wax biosynthesis, drought, gene expression profiling, *KCS* genes

Published first online April 30, 2022 Published final October 05, 2022

INTRODUCTION

Drought is a major abiotic stress which greatly reduces the crop productivity (Reddy *et al.* 2004) especially sunflower (Andrich *et al.* 1996; Reddy *et al.* 2003). There is great need to develop drought tolerant sunflower varieties which could express a greater yield potential during the severe stress conditions (Razzaq *et al.* 2017). Before development of resistant varieties, it is necessary to understand the stress tolerance mechanism and responsible genes. Extensive studies have been carried out to elucidate the drought stress mechanisms, and epicuticular wax biosynthesis is one of them. Leaf surface is covered with the lipophilic coating called cuticle to reduce the effects of dehydration in dry environments, hence reduces the effects of drought on plants. The vital roles of cuticle in protection has already been reported (Buda *et al.* 2009). During the dry conditions, cuticular waxes help the plants to prevent transpiration and saves water present in plant body. In this way, it acts as a first line of defense during dry conditions and saves the plant from non-stomatal water loss (Ahmad *et al.* 2015; Alfarhan *et al.* 2020). Moreover, it is also involved in several functions in plants including inhibition of organ fusion during organogenesis, resistance against insects, reflects UV light, decreases the retention of dust, pollen and air, increases the resistance of plants against pathogens and inhibits the insect attachment to the plant and maintenance of water potential (Nawrath, 2006; Riederer and Muller, 2006; Adamski *et al.* 2013). Cuticular waxes are long chain fatty acids and their derivatives (Shaheenuzzaman *et al.* 2019; Alfarhan *et al.* 2020). Their components include aldehydes, alkanes, fatty acids, ketones, acetones, wax esters, terpenoids and sterols (Shaheenuzzaman *et al.* 2019). Biochemical mechanism of wax biosynthesis is already known; however very less information is

available about proteins involved in the process (Ahmad *et al.* 2020).

Several genes are involved in the process of wax biosynthesis (Seo *et al.* 2011) including *KCS* genes. Expression of *KCS* genes was studied in the *Arabidopsis thaliana* and reduction in wax production was observed in *KCS1* mutant plants (Todd *et al.* 1999). *KCS1* genes appeared to be involved in production of VLCFA (very long chain fatty acids) (Blacklock and Jaworski, 2006) which are epicuticular waxes. Single mutation in *KCS1* gene resulted in complete loss of VLCFA which further led to decreased production of waxes in Arabidopsis (Go *et al.* 2014). *KCS20* and *KCS2*/*DAISY* have shown an important role in wax biosynthesis as mutation of these two genes resulted in significant reduction in wax biosynthesis on stems (Lee *et al.* 2009). *KCS* genes are well characterized in Arabidopsis however little is known about them in sunflower. So, the present study was designed to understand the role of *KCS* genes in drought stress tolerance and to carry out comparative genomics of *KCS* genes to elucidate their evolutionary origin in sunflower. Their role was characterized by transcriptional profiling of *KCS* genes in local sunflower variety.

MATERIALS AND METHODS

Retrieval of protein Sequences: Protein sequences of Arabidopsis were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/). Further confirmation of KCS protein sequences was completed by using Pfam (http://pfam.sanger.ac.uk/search) and SMART (http://smart.emblheidelberg.de/) tools. Blastp tool was used to find out the similar protein sequences in sunflower.

Phylogenetic Analysis: ClustalW was used for multiple sequence alignment (MSA) of all KCS proteins of sunflower and Arabidopsis. On the basis of MSA, phylogenetic tree was constructed by using MEGA 6.0 using Neighbor Joining Method (Saitou and Nei, 1987).

Location of *KCS* genes on chromosomes: Location of *KCS* genes of sunflower and Arabidopsis was mapped on the chromosomes. Exact location of *KCS* genes on the chromosomes of sunflower was determined by using the information available on the NCBI (http://www.ncbi.nlm.nih.gov/) while chromosome mapping of Arabidopsis was done on TAIR.

Evolutionary relationship between *KCS* genes of Arabidopsis and sunflower: Synteny analysis tool, Circoletto (tools.bat.infspire.org/circoletto) was used to study the evolutionary relationship between sunflower and Arabidopsis *KCS* genes. Different colors were used to show the identity of each gene.

Plant growth and drought stress application: Seeds of sunflower varieties were collected from Oilseed Research Institute, Ayub Agriculture Research Institute, Faisalabad, Pakistan. Seeds of sunflower variety FH-593 were overnight soaked in water and subsequently sown in disposable plastic glasses containing peat moss in triplicate with five treatments. They were placed in growth chamber with a program set to 25/22 °C (day/night), 16-hours photoperiod, and relative humidity of 75%. Seedlings were emerged after one week in growth chamber which were transferred to the pots in three replications and placed in botanical garden. After one month, drought stress was applied by halting irrigation. The drought stress was applied in 2 steps for 10 days each. In first step, the samples were taken after 5 (T1) and 10 (T2) days after drought application. At day 10, the plants were irrigated once and samples were taken after 24 hours of irrigation (T3). After that, drought was again induced. In 2nd step, again samples were taken after 5 (T4) and 10 (T5) days of drought induction while "C" was control where no drought was applied. Leaf samples (one leaf per treatment per replication) were collected in Liquid Nitrogen and brought to the Plant Biotechnology Laboratory for subsequent molecular and biochemical analysis.

Total RNA isolation and PCR Amplification of *KCS* genes: Total RNA was extracted from the leaf samples with the help of Trizol reagent (ThermoFisher Scientific USA, cat#15596026). Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used for the synthesis of first strand cDNA. Oligo-dT primers and M-MuLV reverse transcriptase (ThermoFisher Scientific USA, cat#EP0352) were used for cDNA synthesis according to standard protocols. cDNA was used as a template to amplify *KCS* genes in sunflower by using sequence specific primers (**Table-1**).

Table-1: List of primers used in amplification of *KCS* genes in conventional PCR and qPCR.

S. No.	Primer Name	Primer Sequence (5′-3′)
1.	KCS2-Forward	CCGACGCCTTCGTTAT
	KCS2-Reverse	TGTACGGACGGTGTGG
2.	KCS4-Forward	TGCGGTTTTDCTATCG
	KCS4-Reverse	CTTCCACCAGCATGGA
3.	KCS5-Forward	CCAAGCCACGTACCG
	KCS5-Reverse	GAGAGACGGCGTTGGT
4.	KCS10-Forward	GCCCTCCGATGAACAC
	KCS10-Reverse	CATTGCGGACAACGAC
5.	KCS18-Forward	CGGAGACGGTCCAAGT
	KCS18-Reverse	CGATCGGCGATAGTCC
6.	Actin-Forward	TCATGAAGATCCTGACGGAG
	Actin-Reverse	AACAGCTCCTCTTGGCTTAG

Expression profiling of *KCS* genes: Gene expression levels were studied by quantitative real time PCR using

SYBER Green qPCR Master Mix (ThermoFisher Scientific, USA cat#4309155) on the CFX96 Real-Time PCR System (BIO-RAD, USA). PCR reaction was carried out in a total volume of 20 µl, containing 10 µl of SYBR Green qPCR Master Mix (ThermoFisher Scientific, USA), 0.1 µM of each specific primer, and 100ng of template cDNA. The reaction mixtures were heated to 95°C for 30s, followed by 44 cycles at 95°C for 30s, 54°C for 30s, and 72°C for 30s. The variations in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ analysis method (Ahmad *et al.* 2020). The quantification of expression was carried out by using *Actin* gene as the reference gene. The specific primers for *KCS* genes used in qPCR could be seen in **Table-1**.

Estimation of chlorophyll contents: Chlorophyll contents in sunflower were determined by taking 100mg of leaf tissue. The tissues were suspended in 10 mL of 80% acetone which were placed in dark at 4˚C for overnight. Next day, supernatant was taken by centrifugation at 5000rpm. The optical density of supernatant was measured at 645nm and 663nm. Chlorophyll contents were calculated by using the procedure described by Arnon (1949).

Determination of proline contents: Leaf tissue of 0.5g was homogenized in 3% aqueous sulphosalicylic acid. 1 mL glacial acetic acid and 1 mL of acid ninhydrin was added to the test tube and mixed well. Mixture was first heated for 10 minutes at 100˚C and then cooled in an ice bath. Extraction of mixture was performed by using the 4 mL of tuolene, vortexed for 20-30s and then finally cooled to room temperature. By using spectrophotometer, absorbance was measured at 520nm. Standard curve at 520 nm was used to calculate the amount of proline. Formula used for the measurement of proline was given by the Bates *et al.* (1973).

Estimation of antioxidant enzymes: Leaf samples were grinded and standardized in a 0.2M phosphate buffer at pH 7.8. Samples were filtered by using the four layers of muslin cloth. Extracts were obtained after centrifugation at 15000rpm for 10 minutes at 4˚C temperature. Catalase (CAT) contents were calculated according to Aebi (1984) while peroxidase (POD) and superoxidase dismutase (SOD) were estimated according to Zhang (1992).

Statistical Analysis: The experiment was designed according to Completely Randomized Design (CRD) in triplicate with five treatments. *Microsoft Excel 2003*® was used to calculate standard errors (SEs) of replicates and treatments for chlorophyll contents, proline contents and antioxidant enzymes. The variation among treatments was determined by one-way analysis of variance (ANOVA) by using *Statistix 8.1*® (Analytical Software, Tallahassee, USA) followed by least significant difference (LSD) test at $P < 0.05$. The % change in the unit values of chlorophyll contents, proline contents and antioxidant enzymes was calculated by the formula given below:

$$\% \text{ Change in Unit Values} = \frac{\text{Unit value of Treatment} - \text{Unit value of Control}}{\text{Unit value of Control}} \times 100$$

RESULTS

Phylogenetic analysis: KCS proteins of both species were divided into six distinct clades, however clade 1 and 3 have fewer members as compared to others. *KCS11* and *KCS20* genes of both species were present in clade 1 while *KCS10* was present in clade 2. *KCS4* and *KCS9* genes of sunflower and Arabidopsis were also present in the same clade 4. In the same way, *HanKCS5* and *AtKCS5* were present in same clade 5 and *KCS7, KCS9* and *KCS12* were clustered in clade 6. However, *HaKCS2* gene was available in clade 2 while it was classified in clade 1 in Arabidopsis. This pattern of distribution clearly showed that *KCS* genes are widely scattered in the genomes of both species. However, most of the *KCS* genes were classified in the same clade which clearly showed the homology between *A. thaliana* and sunflower (**Figure-1**). It is also a clear indication that *KCS* genes in both species have same evolutionary origin.

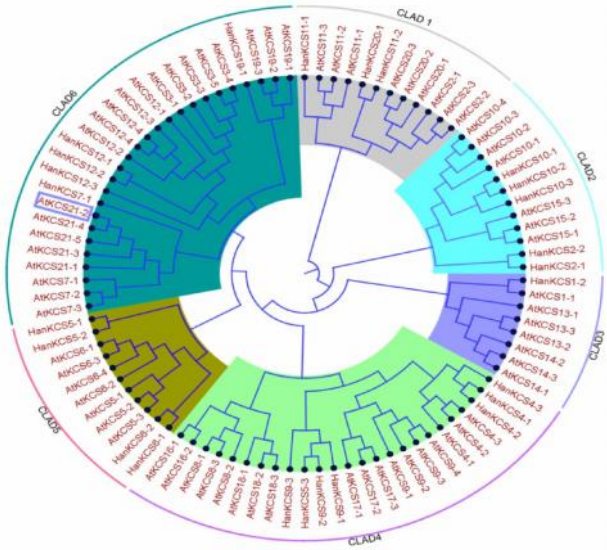

Figure-1: Phylogenetic analysis of KCS proteins from sunflower and *A. thaliana*. The phylogenetic tree classified the KCS proteins into 6 distinct clades indicating their diverse nature.

Chromosome mapping of *KCS* genes of sunflower and Arabidopsis: *KCS* genes were distributed on all 5 chromosomes at diverse locations in *A. thaliana.* On chromosome number 1, 7 *KCS* genes were present while on chromosome 2, 6 *KCS* genes were studied. Two *KCS* genes were present on the chromosome 3 while 3 were

available on the chromosome 4 and 5 each (**Figure-2A**). In the same way, in sunflower *KCS* genes were mapped on 17 chromosomes while some chromosomes were observed to have no KCS genes. Chromosome 1 was observed to have *KCS20* gene while *KCS4* was present on chromosome 2. Chromosome 15 contained *KCS13* gene while chromosome 7 had *KCS12* gene. *KCS2* and *KCS10* were present on chromosome 10. *KCS6* gene was present on chromosome 12 and *KCS19* gene was on chromosome 14 while *KCS11* and *KCS5* genes were mapped on chromosome 16 and 17 respectively (**Figure-2B**).

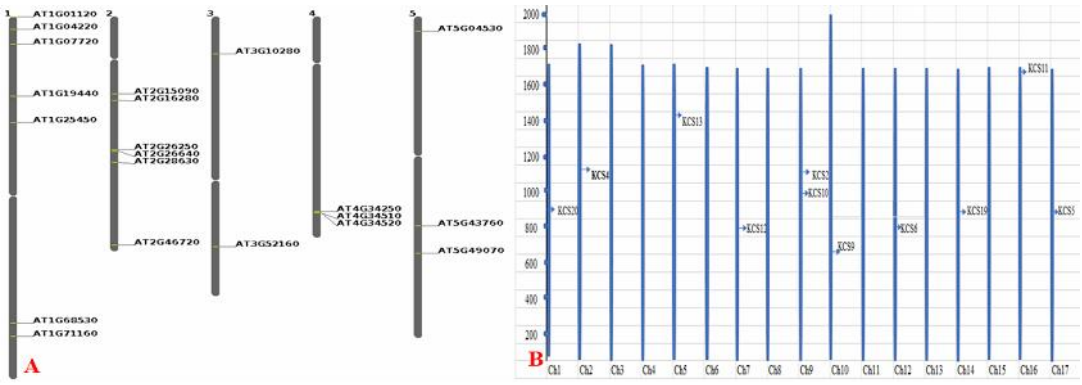

Figure-2: Distribution of *KCS* genes on A) Arabidopsis and B) sunflower chromosomes.

Evolutionary relationship of sunflower and Arabidopsis *KCS* genes: In Synteny analysis, genes dissecting the circle at several locations showed out that *KCS* genes have similar evolutionary origin but separated during the evolutionary process. They were divided in to the 3 groups, i.e. red, orange and green. *HanKCS9* and *AtKCS9* genes were observed to have the same evolutionary origin. *HanKCS4* gene and *AtKCS4* also shared the same evolutionary origin. Evolutionary relationship between the *HanKCS5* gene and *AtKCS5* gene was also found the same way. *HanKCS10* gene was observed to be originated from the *AtKCS10* gene. *HanKCS7* and *AtKCS7* genes of both species shared the same evolutionary origin and in the same way *HanKC12* and *AtKCS12* genes also exhibited the same results. Results of synteny analysis confirmed that the *KCS* genes of sunflower and Arabidopsis were closely related to each other and shared the same evolutionary origin (**Figure-3**).

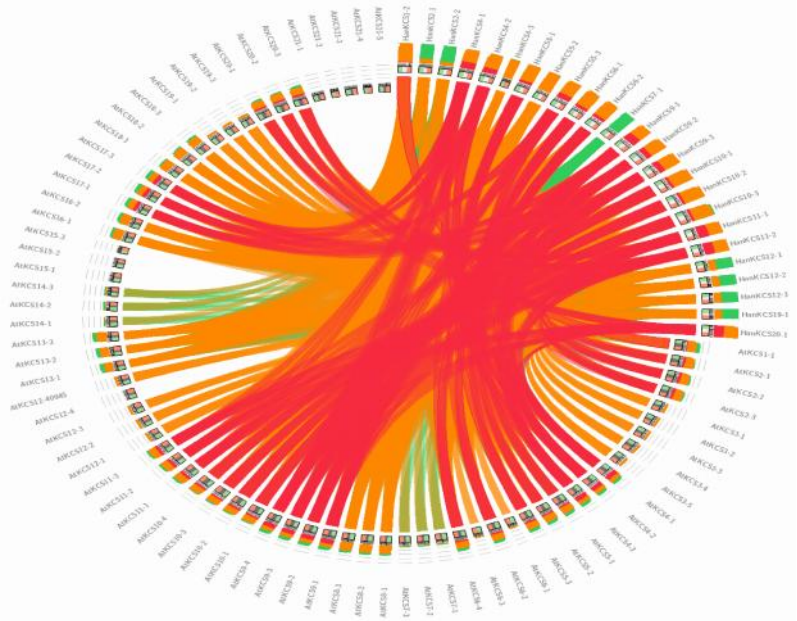

Figure-3: Evolutionary relationship between *KCS* genes of Arabidopsis and sunflower through Synteny analysis. Genes having same evolutionary origin were indicated with the same color.

Amplification of *KCS* genes in sunflower: Various *KCS* genes were amplified in sunflower using cDNA as template in PCR reaction with the help of gene specific primers. Five genes (*KCS2, KCS4, KCS5, KCS10* and *KCS18*) out of 13 KCS genes were amplified in sunflower (**Figure-4**). *KCS2* gene with required band size of 321bp, *KCS4* gene with 351bp, *KCS5* gene 353bp, *KCS10* gene 319bp and *KCS18* with band size 370bp was obtained in sunflower.

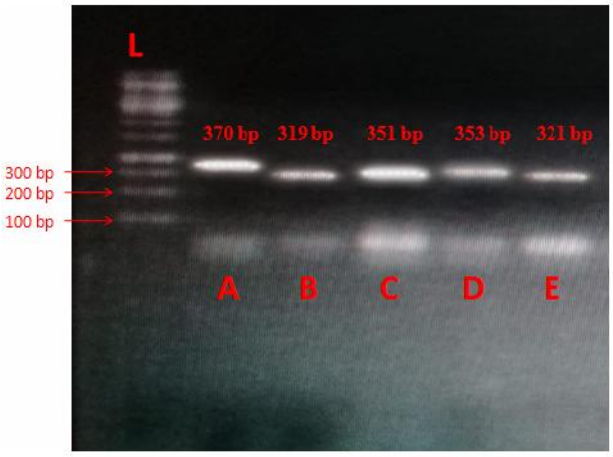

Figure-4: Amplification of *KCS* genes in sunflower using conventional PCR. Lane L: 100bp ladder, Lane-A *KCS18* gene Lane-B *KCS10* gene Lane-C *KCS4* gene Lane-D *KCS5* gene and Lane-E *KCS2* gene amplification.

Expression analysis of *KCS* genes by using real time PCR: During different drought stress treatments, expression of *KCS* genes was found to be up-regulated when compared with control. Expression of *KCS2* gene was observed to be 9 fold more in T1, 12.25 fold in T2, 3 fold in T3 (after re-hydration), 5 fold in T4 and 11 fold in T5 as compared to the control (**Figure-5A**). *KCS4* gene was also observed to be up-regulated during the drought stress conditions. Expression of *KCS4* in T1 was incresed by 19 fold, T2 13 fold, T3 4 fold (after re-hydration), T4 11 fold and T5 17 fold as compared to the control (**Figure-5B**). Five fold increase in expression of *KCS5* gene in T1, 7 fold in T2, 3 fold in T3 (after re-hydration), 13 fold in T4 while 9 fold in T5 was observed in a drought stressed variety as compared to the control (**Figure-5C**). Gradual increase in expression of *KCS10* gene was also noted. Expression of *KCS10* during drought stress was observed to be 3 fold increased in T1, 5 fold in T2, 3 fold in T3 (after re-hydration), 14 fold in T4 and 15 fold in T5 as compared to the control (**Figure-5D**). *KCS18* expression was investigated to be enhanced during the severe water stress conditions as its expression was greatly enhanced at 20 days water stress. Drought stress resulted in 2-fold more in *KCS18* expression in T1, 3-fold in T2, 2-fold in T3 (after re-hydration), 12-fold in T4 and 19-fold in T5 as compared to the control (**Figure-5E**).

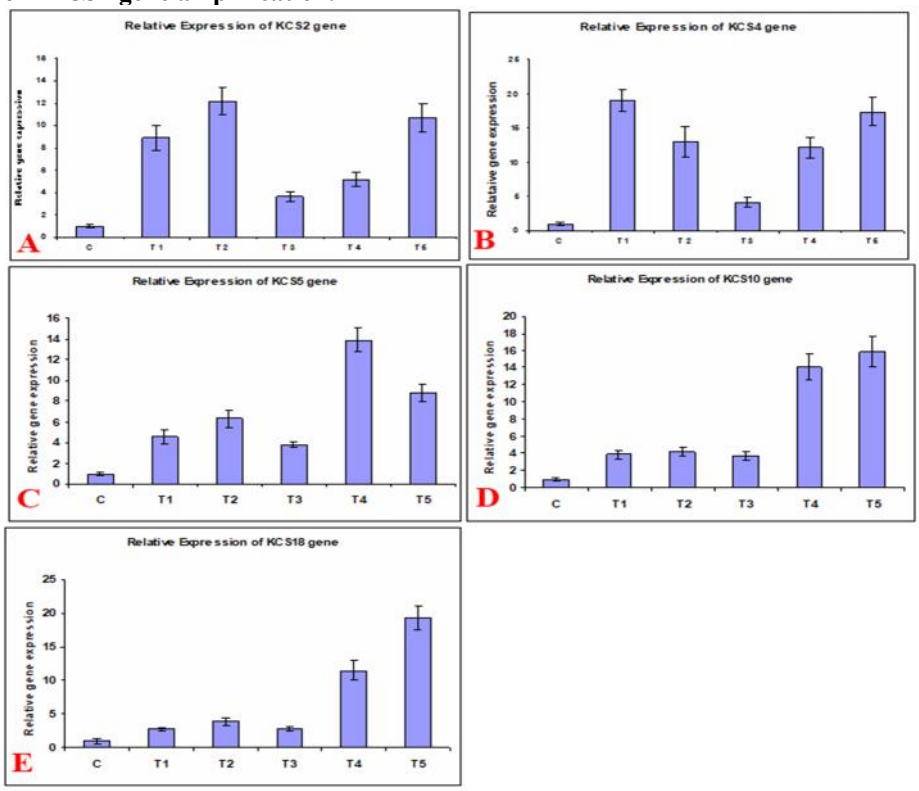

Figure-5: Relative expression of *KCS* genes using real time PCR; A) *KCS2*, B) *KCS4*, C) *KCS5*, D) *KCS10* and E) *KCS18* where treatments were C = no drought, T1 = 5 days of drought, T2 = 10 days of drought, T3 = 24 after re-hydration, T4 = 5 days drought after re-hydration and T5 = 10 days drought after re-hydration.

Chlorophyll, proline and antioxidant enzymes under drought stress: Level of chlorophyll was observed to be 12% decrease in T1, 14% in T2, 14% in T3, 22% in T4 and 23% decrease in T5 as compared to control (**Figure-6A**). It is well documented phenomenon that chlorophyll contents are decreased under stress conditions. Higher amounts of CAT were observed during the water stress conditions, i.e., 38% increase in T1, 31% in T2, 29% in T3, 27% in T4 and 31% in T5 was observed (**Figure-6B**). While 20% increase in proline level in T1, 9% in T2, 12% in T3, 3% in T4 and 7% in T5 was observed in comparison with control (**Figure-6C**). In comparison with control, 6% increase in SOD level in T1, 10% in T2, 6% in T3, 4% in T4 and 5% in T5 was observed under stress conditions (**Figure-6D**). Level of POD was also increased under drought stress. About 8% increase in T1, 6% in T2, 8% in T3, 9% in T4 and 9% in T5 was observed as compare to control (**Figure-6E**).

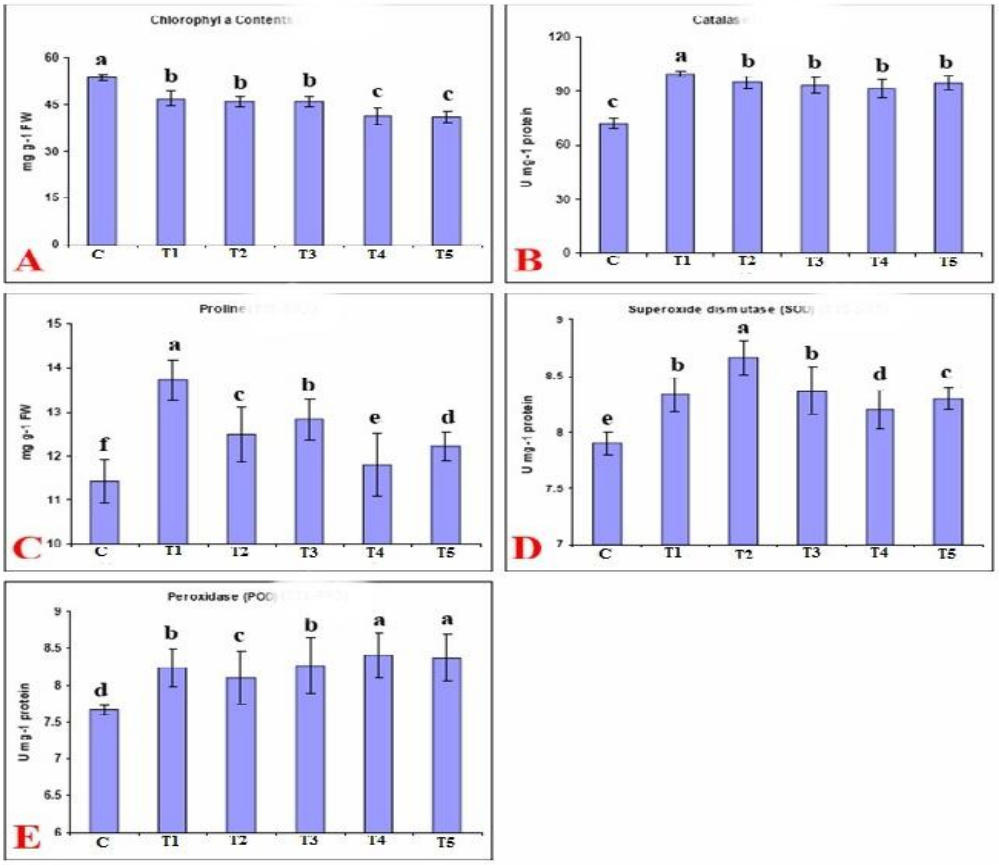

Figure-6: Biochemical analysis of drought stressed sunflower plants; A) chlorophyll contents, B) Catalase, C) Proline, D) Superoxide dismutase and E) Peroxidase where treatments were C = no drought, T1 = 5 days of drought, T2 = 10 days of drought, T3 = 24 after re-hydration, T4 = 5 days drought after re-hydration and T5 = 10 days drought after re-hydration.

DISCUSSION

Abiotic stresses badly effect the growth and development of crop plants causing huge yield losses (Zhou *et al.* 2020). Cuticular waxes play important role to protect the plants from various abiotic stresses such as, drought, salinity, cold and ultraviolet radiation (Ahmad *et al.* 2015; Shaheenuzzaman *et al.* 2019). Wax biosynthesis gene families, i.e. *KCS, LACS*, *CER*, *KCR* and *FAR* play important role in abiotic stress tolerance (Ahmad *et al.* 2015; Shaheenuzzaman *et al.* 2019). *KCS* genes are extensively characterized in Arabidopsis and are known as to play an important role in wax biosynthesis, however, little is known in sunflower. *KCS* genes encode 3-ketoacyl-coA synthase enzyme which is involved in synthesis of very long chain fatty acids (VLCFA) that further resulted in production of epicuticular wax in

plants. They were observed to be over expressed during osmotic and drought stresses (Todd *et al.* 1999).

In view of the importance of wax biosynthesis genes, it is important to identify sunflower varieties having wax biosynthesis genes. In this study, locally hybrid sunflower varieties were selected and wax biosynthesis genes were amplified and their expression analysis was carried out under drought stress conditions. It is significant to mention that it is first ever report to study expression profiling of *KCS* genes in sunflower. Previously, phylogenetic analysis, structure prediction and expression analysis of *KCS* genes in Arabidopsis was conducted (Joubès *et al.* 2008). However, in current study, *KCS* genes of Arabidopsis and sunflower were compared and their evolutionary studies were carried out. Further, chromosomal locations of *KCS* genes in Arabidopsis and sunflower were also determined. Results of our study revealed the close relationship of Arabidopsis and sunflower *KCS* genes. It was further determined that both the species share the same evolutionary origin. Our results showed the same pattern as of the distribution of Trithorax homolog genes in chickpea (Qasim *et al.* 2018), WRKY TFs in Arabidopsis (Sultan *et al.* 2016), Potassium transporter genes in chickpea (Azeem *et al.* 2018) and WRKY TFs in chickpea (Waqas *et al.* 2019).

There are 21 *KCS* genes reported in Arabidopsis (Ouvrard *et al.* 1996; Cellier *et al.* 1998; Gopalakrishna *et al.* 2001; Liu and Baired, 2003; Dezar *et al.* 2005; Herrera Rodriguez *et al.* 2007) however, we were able to amplify five genes (*KCS2, KCS4, KCS5, KCS10* and *KCS18*) in sunflower in this study. All the five genes were up-regulated under drought stress. Similar results were found earlier when after exposing to the drought stress, *KCS1, KCS3* and *KCS20* genes expression level was observed to be increased 10 times more than the control plants in Arabidopsis (Joubès *et al.* 2008). Level of *KCS1, KCS6, KCS9, KCS10* and *KCS16* gene expression was also observed to be increased in drought-stressed plants (Joubès *et al.* 2008). Down-regulation of *KCS1, KCS3* and *KCS6* was reported under low temperature and darkness stress however, up-regulation of these genes was observed under drought stress conditions (Joubès *et al.* 2008). In another experiment, drought stress was applied to Arabidopsis plants by using the Polyethylene glycol. The expression of *KCS6* gene was observed to be increased (Hooker *et al.* 2002). In this study, *KCS10* and *KCS18* were highly up-regulated in plants that remained under drought stress for longer period of time.

It is well documented that drought stress resulted in enhanced level of SOD (Abedi and Pakniyat, 2010) and POD in plant species like wheat (Csiszar *et al.* 2005), brassica (Das and Uprety, 2006) and poplar (Xiao *et al.* 2008). The current data also indicated the higher levels of POD and SOD in sunflower after drought stress which is in agreement with the reported results. It was also reported earlier that sunflower varieties showed lower level of chlorophylls (Manivannan *et al.* 2008) under drought stress while higher proline contents (Baloğlu *et al.* 2012). Our results showed the same pattern and it was found that chlorophyll contents were decreased under drought stress while proline contents were found to be increased. Drought tolerant variety of safflower and *Brassica napus* showed the higher level of CAT contents than the control variety (Sajedi *et al.* 2012). In this study, the same pattern was observed and the current data suggested that drought stress greatly enhanced the level of CAT in sunflower.

Conclusion: Cuticular waxes are the first line of defense against various biotic and abiotic stresses in plants. There are several genes which are responsible for cuticular wax biosynthesis. They are well documented in Arabidopsis but little is known in sunflower. In this study, the five wax biosynthesis *KCS* genes (*KCS2, KCS4, KCS5, KCS10* and *KCS18*) were amplified in the sunflower for the first time. Further, they were characterized for their role in drought stress tolerance in local sunflower varieties. It was reported that the amplified *KCS* genes were up-regulated under stress condition however; the expression of *KCS10* and *KCS18* was highly significant. This study may help to understand the role of KCS genes in drought stress in sunflower. The information obtained may be useful to engineer drought tolerant sunflower plants.

Acknowledgements: Funding from Higher Education Commission, Islamabad, Pakistan (NRPU Project No. 5579) to corresponding author and Principal Investigator of the project is highly acknowledged.

REFERENCES

Abedi, T., and H. Pakniyat (2010). Antioxidant enzymes changes in response to drought stress in ten cultivars of oilseed rape (Brassica napus L.). Czech J. Genet. Plant Breed. 46(1): 27-34.

Adamski, N. M., M.S. Bush, J. Simmonds, A.S. Turner, S.G. Mugford, A. Jones, K. Findlay, N. Pedentchouk, P. Wettstein-Knowles and C. Uauy (2013). The I nhibitor of wax 1 locus (I w1) prevents formation of β-and OH-β-diketones in wheat cuticular waxes and maps to a sub-cM interval on chromosome arm 2 BS. Plant J. 74(6):989-1002.

Aebi, H. (1984). Catalase in vitro. *In*: Methods in Enzymology 105:121-126.

Ahmad, H.M., Mahmood-ur-Rahman, F. Azeem, T. Shaheen, M.A. Irshad (2020). Genome-wide analysis of long chain acyl Co-A synthetase (LACS) genes in sunflower (Helianthus annuus)

suggests their role in drought stress. Intl. J. Agric. Biol. 24:863–870.

Ahmad, H.M., Mahmood-ur-Rahman, Q. Ali and S.I. Awan (2015). Plant cuticular waxes: a review on functions, composition, biosyntheses mechanism and transportation. Life Sci. J. 12:60-67.

Alfarhan, A.H., R. Rajakrishnan, A. Mohamed, Al-Shehri, A.S.M. Al-Tamimi, S. Al-Obaid and S. Khalaf (2020). Analysis of the cuticular wax composition and ecophysiological studies in an arid plant *Z. nummularia* Wight & Arn. Saudi J. Biol. Sci. 27:318-323.

Andrich, G., A. Zinnai, S. Balzini, S. Silvestri and C. Galoppini (1996). The effect of drought stress on some characteristics of sunflower seeds. Agricoltura Mediterranea, 126:285-291.

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant physiol. 24(1):1.

Azeem, F., B. Ahmad, R. M. Atif, M.A. Ali, H. Nadeem, S. Hussain, H. Manzoor, M. Azeem and M. Afzal (2018). Genome-Wide Analysis of Potassium Transport-Related Genes in Chickpea (Cicer arietinum L.) and Their Role in Abiotic Stress Responses. Plant Mol. Biol. Rep. 36:451-468.

Baloğlu, M. C., M. Kavas, G. Aydin, H.A. Öktem and A.M. Yücel (2012). Antioxidative and physiological responses of two sunflower (Helianthus annuus) cultivars under PEG-mediated drought stress. Turk. J. Bot. 36(6):707-714.

Bates, L.S., R.P. Waldren and I. Teare (1973). Rapid determination of free proline for water-stress studies. Plant Soil 39(1):205-207.

Blacklock, B.J. and J.G. Jaworski (2006). Substrate specificity of Arabidopsis 3-ketoacyl-CoA synthases. Biochem. Bioph. Res. Com. 346(2):583-590.

Buda, G.J., T. Isaacson, A.J. Matas, D.J. Paolillo and J.K. Rose (2009). Three-dimensional imaging of plant cuticle architecture using confocal scanning laser microscopy. Plant J. 60(2):378-385.

Cellier, F., G. Conéjéro, J.C. Breitler and F. Casse (1998). Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower: Accumulation of dehydrin transcripts correlates with tolerance. Plant Physiol. 116(1):319-328.

Csiszar J., E. Feher-Juhasz, E. Kotai, K.O. Ivankovits, G.V. Horvath, A. Mai, A. Galle, I. Tari, J. Pauk, D. Dudits and L. Erdei (2005): Effect of osmotic stress on antioxidant enzyme activities in transgenic wheat calli bearing MsALR gene. Acta Biol. Szeged. 49:49–50.

Das R. and D.C. Uprety (2006): Interactive effect of moisture stress and elevated CO2 on the oxidative stress in Brassica species. J. Food Agric. Environ. 4:298–305.

Dezar, C.A., G.M. Gago, D.H. González and R.L. Chan (2005). Hahb-4, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to Arabidopsis thaliana plants. Transgenic Res. 14(4):429-440.

Go, Y.S., H. Kim, H.J. Kim and M.C. Suh (2014). Arabidopsis cuticular wax biosynthesis is negatively regulated by the DEWAX gene encoding an AP2/ERF-type transcription factor. Plant Cell 26(4):1666-1680.

Gopalakrishna, R., K. Ganesh, B.T. Krishnaprasad, M.K. Mathew, M.U. Kumar and G. Kumar (2001). A stress-responsive gene from groundnut, Gdi-15, is homologous to flavonol 3O-glucosyltransferase involved in anthocyanin biosynthesis. Biochem. Bioph. Res. Com. 284(3):574–579.

Herrera Rodriguez, M.B., R. Perez Vicente and J.M. Maldonado (2007). Expression of asparagines synthetase genes in sunflower (Helianthus annuus) under various environmental stresses. Plant Physiol. Biochem. 45(1):33–38.

Hooker, T.S, A.A. Millar and L. Kunst (2002). Significance of the expression of the CER6 condensing enzyme for cuticular wax production in Arabidopsis. Plant Physiol. 129:1568–1580.

Joubès, J., S. Raffaele, B. Bourdenx, C. Garcia, J. Laroche-Traineau, J., P. Moreau, F. Domergue and R. Lessire (2008). The VLCFA elongase gene family in Arabidopsis thaliana: phylogenetic analysis, 3D modelling and expression profiling. Plant Mol Biol. 67(5):547.

Lee, S.B., S.J. Jung, Y.S. Go, H.U. Kim, J.K. Kim, H.J. Cho, O.K. Park and M.C. Suh (2009). Two Arabidopsis 3-ketoacyl CoA synthase genes, KCS20 and KCS2/DAISY, are functionally redundant in cuticular wax and root suberin biosynthesis, but differentially controlled by osmotic stress. Plant J. 60(3):462-475.

Liu, X. and W.V. Baird (2003). The ribosomal small-subunit protein S28 gene from Helianthus annuus (Asteraceae) is down-regulated in response to drought, high salinity, and abscisic acid. Am. J. Bot. 90(4):526–531.

Manivannan, P., C.A. Jaleel, Z. Chang-Xing, R. Somasundaram, M. Azooz and R. Panneerselvam (2008). Variations in growth and pigment compositon of sunflower varieties under early season drought stress. Global J. Mol. Sci. 3(2):50-56.

Nawrath, C. (2006). Unraveling the complex network of cuticular structure and function. Curr. Opin Plant Biol. 9(3):281-287.

Ouvrard, O., F. Cellier, K. Ferrare, D. Tousch, T. Lamaze, J.M. Dupuis and F. Casse-Delbart (1996). Identification and expression of water stress-and abscisic acid-regulated genes in a drought-tolerant sunflower genotype. Plant Mol. Biol. 31(4):819-829.

Qasim, M., M. Azeem, M. Zubair, U. Qaiser, A.A. Shah, H. Nadeem, H. Manzoor, S. Hussain, B. Ahmad and F. Azeem (2018). Phylogenetic Analyses of the Trithorax Homologs and Related Proteins in *Cicer arietinum*. Int. J. Agric. Biol. 20:575-582.

Razzaq, H., M.H.N. Tahir, H.A. Sadaqat and B. Sadia. (2017). Screening of sunflower (*Helianthus* annus L.) accessions under drought stress conditions, an experimental assay. J. Soil Sci. Plant Nutr. 17(3):662-667.

Reddy, A.R., K.V. Chaitanya and M. Vivekanandan (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J. Plant Physiol. 161(11):1189-1202.

Reddy, G., K.S. Dangi, S. Kumar and A. Reddy (2003). Effect of moisture stress on seed yield and quality in sunflower. J. Oilseeds Res. 20:282-283.

Riederer, M. and C. Muller (2006). Annual Plant Reviews, Biology of the Plant Cuticle (23): Blackwell Publishing Ltd., Oxford, UK.

Saitou, N. and M. Nei (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4):406-425.

Sajedi, N., M. Ferasat, M. Mirzakhani and M.M.A. Boojar (2012). Impact of water deficit stress on biochemical characteristics of safflower cultivars. Physiol. Mol. Biol. Plants 18(4):323-329.

Seo, P.J., S.B. Lee, M.C. Suh, M.J. Park, Y.S. Go and C.M. Park (2011). The MYB96 Transcription Factor Regulates Cuticular Wax Biosynthesis under Drought Conditions in Arabidopsis. Plant Cell 23:1138–1152.

Shaheenuzzaman, M., T. Liu, S. Shi, H. Wu and Z. Wang (2019). Research advances on cuticular waxes biosynthesis in crops: A review. Intl. J. Agric. Biol. 21:911–921.

Sultan, S., M.A. Ali, R.M. Atif, F. Azeem, H. Nadeem, M.H. Siddique, E. Filiz, K. Hussain and A. Abbas (2016). Genome wide analysis of stress responsive WRKY transcription factors in *Arabidopsis thaliana*. Turk. J. Agric-Food Sci. Technol. 4:279-290.

Todd, J., D. Post-Beittenmiller and J.G. Jaworski (1999). KCS1 encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in Arabidopsis thaliana. Plant J. 17(2):119-130.

Waqas, M., M. T. Azhar, I.A. Rana, F. Azeem, M.A. Ali, M.A. Nawaz, G. Chung and R.M. Atif (2019). Genome-wide identification and expression analyses of WRKY transcription factor family members from chickpea (*Cicer arietinum* L.) reveal their role in abiotic stress-responses. Genes & genomics, 41:467-481.

Xiao X., X. Xu and F. Yang (2008). Adaptive responses to progressive drought stress in two Populus cathayana populations. Silva Fenn 42:705–719.

Zhang, X.Z. (1992). The measurements and echanisms of lipid peroxidation and SOD POD and CAT activities in biological system. In: Zhang, X.Z. (ed) Research Methodology of Crop Physiology, Beijing Agriculture Press, pp 208-211.

Zhou, T., D. Yu, H. Dong, Z. Sun, Y. Tan, X. Sun, X. Sheng, M. Duan and D.Y. Yuan (2020). Genome-wide identification and expression profile of *NINJA* and *AFP* genes in rice. Intl. J. Agric. Biol. 23:171–182.