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WRKY genes Expression profilein Egyptian tomato (Edkawy cultivar) under drought stress

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ABSTRACT
Abiotic stresses and climate changes become a serious mystery in plant breeding, particularly drought. the regulatory genes play a critical role in plants under abiotic stresses tocomplete their life cycles. WRKY transcription factors genes family are very vital in this aspect as one of stress related transcription factors. In tomato (Solanum lycopersicum), the WRKY family contains 83 members. five members of WRKY family have been selected and the study have been conducted on the two tomato cultivars; Edkawy and AC++, at three time points with two tissues (shoot and root) under drought stress conditions. Tomato plants were subjected to drought assays using water withholding treatment. The results indicated that morphological analysis of the two cultivars showed that Edkawy is highly tolerant to drought that the root length under drought stress was higher than AC++.Further the qRT-PCR expression analysis of selected five WRKY genes indicated that the expression profiling for the candidate WRKY genes showed variationin foldchange under drought conditions relative to the expression level under well-watering conditions. For instance,SLWRKY03 gene was upregulated in response to drought inEdkawy shoot and root tissues as well as in root tissue of AC++.Whereas the SLWRKY30 was shown to be up-regulate in AC++ shoots and SLWRKY58 was shown to be up-regulate in Edkawy roots while SLWRKY72 was shown to be up-regulate in Edkawy shootsand SLlWRKY75 was shown to be up-regulated in shoots of AC++.

Keywords:*Abiotic stress, Edkawy, AC++, WRKY, qRT-PCR, expression profiling and drough*t.

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I. Introduction

Tomato (Solanum lycopersicum), a member of the Solanaceae family, is one of the major economic crops. According to an estimate, tomato genome has 35,000 genes(Hoeven et al., 2002).

Edkawy is an Egyptian beefsteak tomato that has been identified as being salt-tolerant (Mahmoud et al., 1986a & b; Johnson et al., 1999) and has been selected for further research into tolerance mechanisms, particularly in relation to fruit quality.

Abiotic stresses are still the most significant constraint to crop productivity, according to (Acquaah, 2007). Abiotic stresses are considered to be responsible for over 70% of yield reduction worldwide.Drought stress is one of the abiotic stresses that has a significant negative impact on tomato plant growth and development. (Hu et al., 2021).Every year, drought reduces cereal yields by 7.0 % to 8.1 % over the world. (Lesk et al., 2016)Complicated signaling pathways may be responsible for plants' drought resistance in order to restore cellular homeostasis and promote survival. Plant adaptation to abiotic stresses requires transcriptional control, also known as transcriptome reprogramming. (Yamasaki et al., 2012). There are 2505 transcription factors present in Tomato genome and are categorized into 89 families and distributed on all 12 chromosomes(Tian et al., 2020).

WRKY transcription factors are important components family in the regulation of transcriptional reprogramming during plant stress responses, and they belong to one of the largest families of transcription factors (TFs) in higher plants (Taylor et al., 2014). These TFs also play important roles in several plant processes in response to biotic and abiotic stresses by regulating the plant hormone signal transduction pathway.WRKY proteins can bind to W-box [TGACC (A/T)] in the promoter of their target genes and activate or repress the expression of downstream genes to regulate their stress response, rendering it key TFs in plant response to biotic and abiotic stresses proteins can also interact with other transcription factors to control plant defense responses.

Because there is a lack of information on how the WRKY genes control drought resistance tolerance in tomato(Hu et al., 2021)

The current study, we investigated the impact of drought stress on the tomato plant (Edkawy and AC++cultivars) as domestic cultivar, through applicationof qRT.PCR to assess coupled with Bioinformatics

analysis to assess the types and levels of differentially expressed genes. Experiments were conducted at the seedling stage during three-time points in shoots and roots.

II. Material and Methods

The experiment was carried out at Biotechnology Department, faculty of Agriculture, Al-Azhar University.

Seeds germination and growth conditions

Seeds of the Egyptian tomato cultivars Edkawy and (Solanum 789-L. cv. Ailsa Craig; wild type) (AC++) were provided by the Tomato Genetics Resource Center (TGRC) during Cornell university. Two cultivars' seeds were planted in compressed foam trays under plastic for 7 days to increase the tray's temperature and break seed dormancy until germination at a nursery greenhouse temperature and conditions of about 25°C. The seedlings started to grow when the plastic bags were removed. When the seedlings were about 45 days old, 15 to 17 cm in height, and had formed 3 to 4 genuine leaves, a regular supply of water and nutrients was given.

Drought stress treatments

Tomato seedlings of the two types (Edkawy and AC++) were exposed to drought stress. Seedlings were transplanted into Peat Moss and Vermiculite-filled containers (3:1) and the temperature condition were optimized between 25-27 °C with 65 % humidity. Tomato seedlings were withheld water for three time points (5 days, 10 days, and 15 days) during the four-leaf stage.as a biological controlthree plants were grown in each pot. Additionally, a control sample of the two cultivars was irrigated routinely by giving the water on a regular basis. other morphological characterization was conducted such as the root length of two mentioned cultivars in each treatment and time points.

In-silico analysis of transcription factor families.

The investigation has been done in high rang of transcription factor (TF) families database for searching about transcription factors genes in tomatovarious available databases like Solgenome(https://solgenomics.net/),NCBI(https:// www.ncbi.nlm.nih.gov) investigated to determine the target family which contains the transcription factors genes that responsible for abiotic stress tolerance particularly drought ,the complete families of transcription factors in tomato listed based on the major databases in (http://planttfdb.cbi.pku.edu.cn) website, according to this families assignment rules, 1845 TFs are identified and classified into 58 families , we selected the WRKY family which contain 81 TFs genes in order to enlist the WRKY genes sequences were retrieved from PLANTTFDB (http://planttfdb.gao-lab.org/family.php?sp=Sly&fam=WRKY) The five genes from WRKY (mentioned in the table 1) have been selected as(Karkute et al., 2018)stated that these genes form nine genes showed up-regulation under drought stress mentioned in the (table 1),Meanwhile, The flat files of those five genes (CDs) coding sequence) were used to design the primers. In the same time the Tubulin gene has been selected as a housekeeping gene for comparison with the selected genes.

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Gene name	Gene ID
SlWRKY58	Solyc05g050340.2.1
SlWRKY72	Solyc02g067430.2.1,
SlWRKY30	Solyc07g056280.2.1
SlWRKY75	Solyc05g015850.2.1
SlWRKY03	Solyc02g088340.2.1

Table (1) Genes symbols and genes IDs as shown in Tomato genome database

Primer's design and specificity

The CDS sequences were extracted from tomato Solanum Lycopersicon using http://planttfdb.gaolab.org/tf.php?sp=Sly&did=Solyc05g050340.2.1 .Using the earlier retrieved sequence, the IDT (integrated DNA technologies) online software was used to design the selected genes from tomato Solanum Lycopersicon (https://www.idtdna.com/scitools/Applications/RealTimePCR/).In addition, the primer blast online program was used to evaluate the reliability and specificity of the primers, as well as to find theconsensus sequence and change the product length. .(http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK LOC =Blast Home)

The in-silico RT-PCR module and primer synthesizing

The in-silico PCR module was also used to produce all thedetails of the RT-PCR program like Tm for the forward and reverse primer to predict the PCR products Tm Calculator two web sites were used for this from NEB web site was used to determined melting temperature "tm" (http://tmcalculator.neb.com/**).** The other one

usedto give information on the aligned positions of primers for the sequences templates records was (https://www.idtdna.com/scitools/Applications/RealTimePCR) .
The primers were synthesized by Thermo scientific Invitrogen, USA. The synthesized primers were shipped as lyophilized material and kept in freezer at -20 C

RNA extraction and cDNA library preparation.

Fresh leaves sample (100 - 150 mg) were obtained and sterilized by washing with distil water and ethanol 70%, and immerged immediately in liquid nitrogen for RNA isolation.the protocol was used for RNA isolation from the previous plants using RNeasy® Plant Mini Kit(catalogue number74904) manufacturedby (Qiagen). RNA isolation protocol was carried out in accordance with to the manufacture manual. RNA concentrations were measured in ng/µl, and purity ratios (260/280 nm and 260/230 nm) were calculated using Nanodrop™ 2000 spectrophotometer (Thermo Scientific) following manufacturer's instructions. To confirm RNA concentration also run in 1% agarose gel electrophoresis to determine RNA quality using UV transilluminatorAccording to the manufacturer's instructions, first-strand cDNA was synthesized from 1.0 g of total RNA (200 ng/l concentration) in a 20-l reaction volume using a first-strand cDNA synthesis Kit.

Real time PCR reaction and amplification conditions

The intercalation dye Absolute Blue QPCR SYBR Green master mix kit (Thermo Scientific) was used as a fluorescent reporter in all qRT-PCR reactions performed in an Eppendorf Master cycler®ep real plex thermal cycler. All PCR reactions were carried out in triplicates in 25 l volumes for three biological replicates, using 1 l forward and reverse primers (25 pmol each), 12.5 l SYBR green master mix, 1 l cDNA (100 ng/l), and 9.5 l HPLC molecular biology grade water. RNA and cDNA were extracted from the two cultivars at 5, 10, and 15 days of seedling stage, and PCR products were quantified. qPCR cycling program of 1 cycle at 95 °C for 15 min, 30–40 cycles at 95 °C for 15 s, 50–60 °C for 30 s, and 72 °C for 30 s, using specific PCR primers for the gene of interest. The Tubulin gene was used as an internal reference to adjust the relative amount of mRNAs in all samples, and the 2-Ct technique was utilized to quantify PCR products (Schmittgen & Livak, 2008), The error bars show the standard errors for relative gene expression fold changes estimated from at least two biological replicates and triplicate PCR reactions for each sample.

III. Results And Discussion

Drought stress analysis

As show in (figure 1) the seedlings were grown in a nursery for 45 days before exposed to water-deficit stress for 5, 10, and 15 days. After 5 days of drought stress, just a few leaves on AC++ plants rolled; however, Edkawy plants were still good without rolled leaves. After a ten-day of drought stress, the most of the AC++ leaves were rolled and showed chlorosis (Figure 1). Only a few Edkawy leaves, were rolled. The most of AC++ leaves died after 15 days of drought stress (Figure 1) Moreover, only a few Edkawy leaves were rolled and started to showed chlorosis symptoms. Which agree with (El-din et al., 2017) So these results showed that Edkawy tomato cultivar plants were more drought tolerant than AC++ plants .while AC++ is a moderate to susceptible cultivar to drought stress,(Bian et al., 2019) while (Shamim et al., 2016)stated that accessions 'Ailsa Craig',(AC++) was ranked as susceptible to drought. While (Abdelmageed & Gruda, 2009; Amjad et al., 2013; Wahb-Allah, Alsadon, & Ibrahim, 2011; Mahmoud. A et al., 2011 ; Alsadon, Sadder, & Wahb-Allah, 2013) reported Edkawy was tolerant to abiotic stress .

Root length measurement

the results showed (figure 2) that the root length in AC++ started with 9.2 cm after 5 days in control, while it was in the treatment 8.2 cm and then increased to be 10.8 cm after 15 days, moreover the root length in Edkawy started from 9.5 cm in control then increased to be 12 cm after 15 days. in contest the root length in Edkawy is higher than the root length in AC++ in different time points of treatment.
(Shamim et al., 2016)reported that the root length in AC++ shorter than Edkawy while the mean of shoot and root length was closely, in our study the root length have been conducted in three replicates for each cultivar in every time point and the results agreed with (Shamim et al., 2016).

Figure 1. Drought tolerance in Edkawy tomato plants was compared to AC++ tomato plants. Water was withheld from five-week-old tomato plants for five days, ten days, and fifteen days. For each cultivar, about thirty seedlings were used.

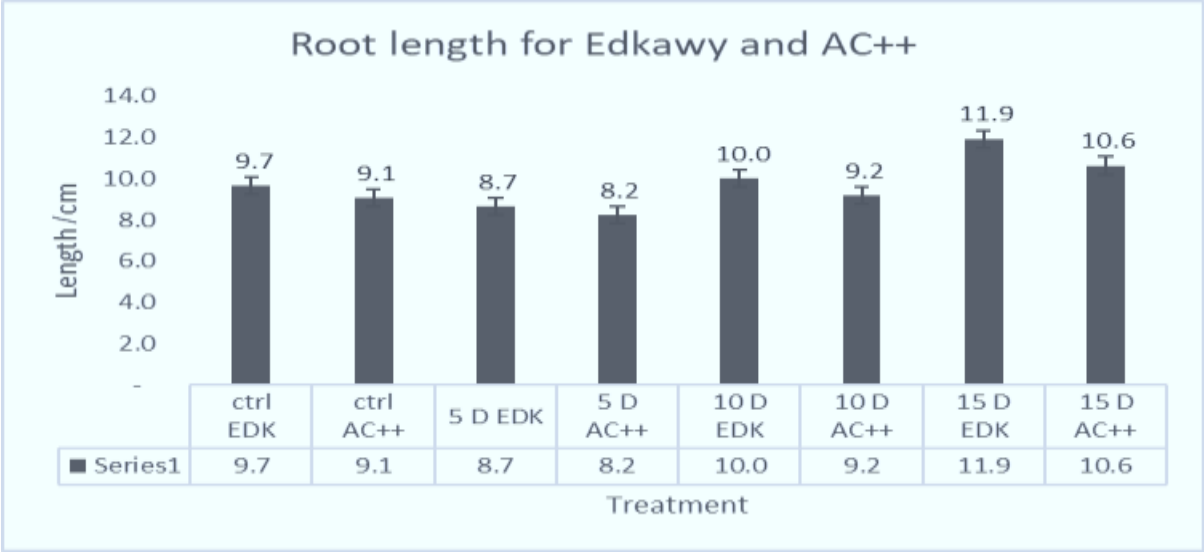

Figure 2. Root length in Edkawy tomato plants was compared to AC++. Three replicates of seedlings for five days, ten days, and fifteen days. For each cultivar, three seedlings were used in each evaluation.

Bioinformatics and data mining approach to identify and characterize of WRKY genes

In current study five genes (SlWRKY58 SlWRKY72,SlWRKY30,SlWRKY75,SlWRKY03 and SlWRKY77) from WRKY family have been evaluated for expression analysis by quantitative real time PCR in three different time points (after 5 days ,10 day and 15 day) with two varieties Edkawy and AC++ under drought stress on two tissues shoot and root , so , we have twelve level for each gene Based on the PLANTTFDB is a database of plants. (S. Huang et al., 2012) stated that the number of WRKY genes in tomato (Solanum lycopersicum) is 81 genes in different crops,, also (S. Huang et al., 2012) reported that the number of WRKY genes in tomato (Solanum lycopersicum) is 14549 genes. According to. (Karkute et al., 2018) the total number of WRKY transcription factors in tomato is 83. In white pear, soybean, and capsicum annuum. genome wide analysis of WRKY transcription factors has been done in white pear , soybean and *capsicum annuum* L(X. Huang et al., 2015),(Xu & Hu.,2016),(Diao et al., 2016).As shown in (table 2) we selected the genes based on their function as reported from bioinformatic data mining research and indicate that the protein name, the molecular functionto perform the binding in the specific integration of gene for regulation and the biological process with the localization of each gene from the five mentioned genes.

As shown in figure (9) Solyc02g088340 (sol WRKY 03) and Solyc02g067430(sol WRKY 72) are located in chromosome no. 2 while Solyc05g015850 (sol WRKY 75) and Solyc05g050340 (sol WRKY 58) are located in chromosome no. 5. meanwhile, the Solyc07g056280 (sol WRKY 30) are located in chromosome no. 7.

The SLWRKY03 gene description usingin-silico approach and qRT-PCR.

As shown in (Figure 7) ,the qRT-PCR results for SlWRKY03 gene in AC++ under drought stress in shoot after 5 days was down regulate but in root was up-regulate and continue up- regulating after 10 days in root with highly up-regulated (13.4 fold)while in shoot was down regulate also after 15 days in root and shot turned back todown-regulating , meanwhile SlWRKY03 gene in Edkawy after 5 days in shoot was up-regulated but in root was down regulate also after 10 days in shoot was highly up-regulated (92 fold) also in root was up-regulated, after 15 days was down-regulate in shoot and root. So most of this results agreed with (Karkute & Gujjar, 2017) which reported that SLWRKY03 classified in group 1 and the Cis-acting regulatory elements in the promoter region involved in the abscisic acid responsiveness was 5UTR Py-rich stretch; ABRE; Box 4; Box I; CAAT-box; CGTCA-motif; Circadian; ERE; G-Box; HSE; Skn-1_motif; TATA-box;TCA-element; TC-rich repeats; TGACG-motif; W box .however this results not matched with the database plant efp which stated that The expression level of sol WRKY 03 was highly expressed in fruits and moderate expressed in leaves and low expressed in roots. Figure ..

In addition to the vital role of SLWRKY03 as a positive regulator of induced resistance in response to nematode invasion and infection, mostly during the early stages of nematode infection(Chinnapandi & Bucki, 2019) The functional characteristics of SLWRKY03 have been investigated, and the results have been discovered: SLWRKY03 (Solyc02g088340.2.1; accession no. KU674829).SlWRKY03 has two WRKY domains (WRKYGQK/ WRKYGQK), as well as a zinc-finger-like motif ligand, C-X6-C-X27-H-X1-H, according to in-silico research the function as a TF is supported by the presence of a nuclear-localization signal sequence (KKKVER) at position 250, PTKRRK at position 275, RKYGQK at location 223 and RKYGQK at position 393.(S. Huang et al., 2012) by Pfm tool http://pfam.xfam.org/protein/A0A3Q7F961 we determined the strat and end of the SLWRKY03 ad it was start 16 and end 400 with a description Protein kinase domain .

this results not matched with the database *plant efp*http://bar.utoronto.ca/eplant_tomato/ which stated that The expression level of sol WRKY03 was highly expressed in fruits and moderate expressed in leaves and low expressed in roots. Figure (8)

The SLWRKY58 and SLWRKY72genes description using in-silico approach and qRT-PCR analysis.

As shown in (figure 3 A) SlWRKY58 gene in Edkawy after 5 days in shoot was up-regulated but in root was down regulate also after 10 days in shoot was down regulate but in root was up-regulated, after 15 days in shoot was down-regulate while in root was highly upregulate (25.9 fold) . , SlWRKY58 gene in AC++ under drought stress in shoot and root after 5 days was down regulate and continue down regulated after 10 days in shoot and rootalso after 15 days in root was down-regulated while in shoot after 15 days was high up-regulate (24 fold), this results not matched with the database *plant efp*http://bar.utoronto.ca/eplant_tomato/ which stated that The expression level of sol WRKY 58 was highly expressed in fruits and moderate expressed in leaves and low expressed in roots. (Figure 5 A)

Meanwhile, as shown in (figure 3 B) , SlWRKY72 gene in Edkawy after 5 days was high up-regulated in shoot (22.5 fold) but in root was down regulate also after 10 days in shoot and root was up-regulate but after 15 days in shoot and root was down-regulate while in root was upregulate. While in AC++ under drought stress in shoot and root after 5 days was down regulate and continue down regulated after 10 days in shoot and root also after 15 days in root was down-regulated while in shoot after 15 days was up-regulate, (Karkute & Gujjar, 2017) stated that SlWRKY58 and SlWRKY72 are possibly responsible for

providing drought resistance to tomato plants, according to his work and previous reports of paralogous genes.so, the SlWRKY58 and SlWRKY72 genes can be used to develop drought-tolerant transgenic crops. In the same time this results not matched with the database plant efp which stated that The expression level of sol WRKY 72 was highly expressed in roots and low expressed in leaves and roots. (Figure 5 B)

The SLWRKY75 and SLWRKY 30genes description using in-silico approach and qRT-PCRanalysis.

As shown in (figure 4 A) , SlWRKY75 gene in AC++ under drought stress after 5 days in root was up- regulate and shoot was down regulateand continuedown regulated after 10 days in shoot and root however in shoot after 15 days washigh up-regulate (34.5 fold) , meanwhile SlWRKY75 gene in Edkawy after 5 days in shoot was up-regulated but in root was down regulate also after 10 days in shoot and root was down regulate , after 15 days in shoot was down-regulate while in root was upregulate . In the same time this results not matched with the database *plant efp* which stated that The expression level of sol WRKY 75 was highly expressed in roots and fruits with low expressed in leaves (Figure 6 A)

Meanwhile, as shown in (figure 4,B) , SlWRKY30 gene in Edkawy after 5 days was high up-regulated in shoot (16.9 fold) but in root was down regulate also after 10 days in shoot and root was down regulate also after 15 days in shoot and root was down-regulate. While in AC++ in shoot and root after 5 days was down regulate and continue down regulated after 10 days in shoot however rootwas high up- regulate (35 fold) but after 15 days in shoot and root was down-regulated, Meanwhile this results not matched with the database *plant efp* which stated that The expression level of sol WRKY 30 was highly expressed leaves in roots with low expressed in fruits (Figure 6 B)

The results of qRT-PCR analysis demonstrated the different expression patterns reported by each time point individually SlWRKY03, was up-regulate in shoot of Edkawy after 10 days and root of AC++. while SlWRKY58 was up-regulate in root of Edkawyafter 15 days and shoot of AC++.in the same time SlWRKY72, was up-regulate in shoot of Edkawy after 5 days in the other side it considers down regulate in AC++ at all time points. The SlWRKY75 was up- regulate in Edkawyafter 5 days and shoot of AC++ after 15 days. also, The SlWRKY30 was up- regulate in Edkawyafter 5 days in shoot also in AC++ after 10 days inshoot andconsider down regulate in other time points in two cultivars. Gene expression level data under different abiotic stress level is lacking so our study will support the data base for gene expression by adding more information about the scanning genes.

Gene name	Gene ID	Protein name	Go Term			Data base link
			Molecular function	Biological process	Localization	
SlWRKY58	Solyc05g050340.2.1	Uncharacterized protein	DNA binding	Regulation of transcription, DNA-templated	Nucleus	https://www.ebi.ac.uk/QuickGO/annotations?geneProductId=A0A3Q7GLM1
SlWRKY72	Solyc02g067430.2.1	WRKY domain-containing protein	DNA binding	regulation of transcription, DNA-templated	Nucleus	https://www.ebi.ac.uk/QuickGO/annotations?geneProductId=A0A3Q7F087
SlWRKY30	Solyc07g056280.2.1	WRKY16 protein	sequence-specific DNA binding	regulation of transcription, DNA-templated	Nucleus	https://www.ebi.ac.uk/QuickGO/annotations?geneProductId=K4CGG1
SlWRKY75	Solyc05g015850.2.1	WRKY domain-containing protein	DNA-binding transcription factor activity - sequence-specific DNA binding	regulation of transcription, DNA-templated	Nucleus	https://www.ebi.ac.uk/QuickGO/annotations?geneProductId=A0A3Q7GE34
SlWRKY03	Solyc02g088340.2.1	Protein kinase domain-containing protein	RNA polymerase II CTD heptapeptide repeat kinase activity	positive regulation of transcription elongation from RNA polymerase II promoter	Nucleus	https://www.ebi.ac.uk/QuickGO/annotations?geneProductId=A0A3Q7F961

(Table 2)Gene and the protein name, the molecular function and the biological process with the localization of each gene from the five mentioned genes based on the database

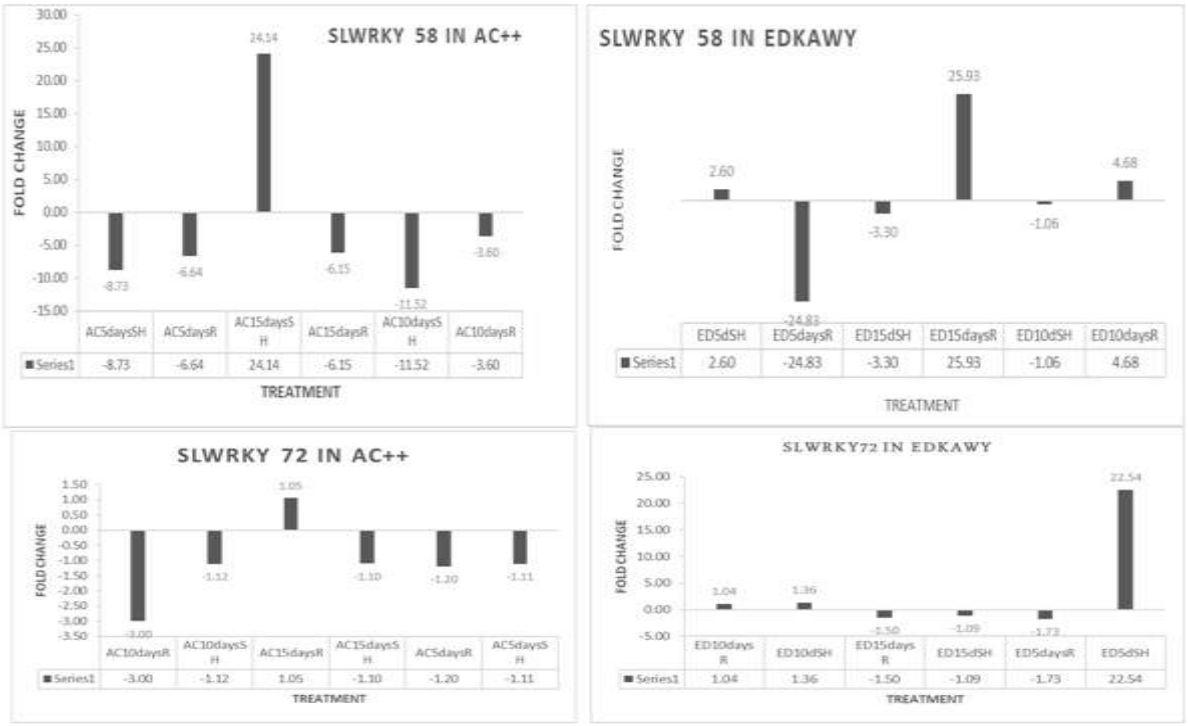

Figure 3.(A) Quantitative expression of SLWRKY58 and (B) SLWRKY72 gene in shoot and root of Edkawy and Ac++ tomato under drought stress. On the axis, the positive values represent upregulation and negative values represent downregulation.

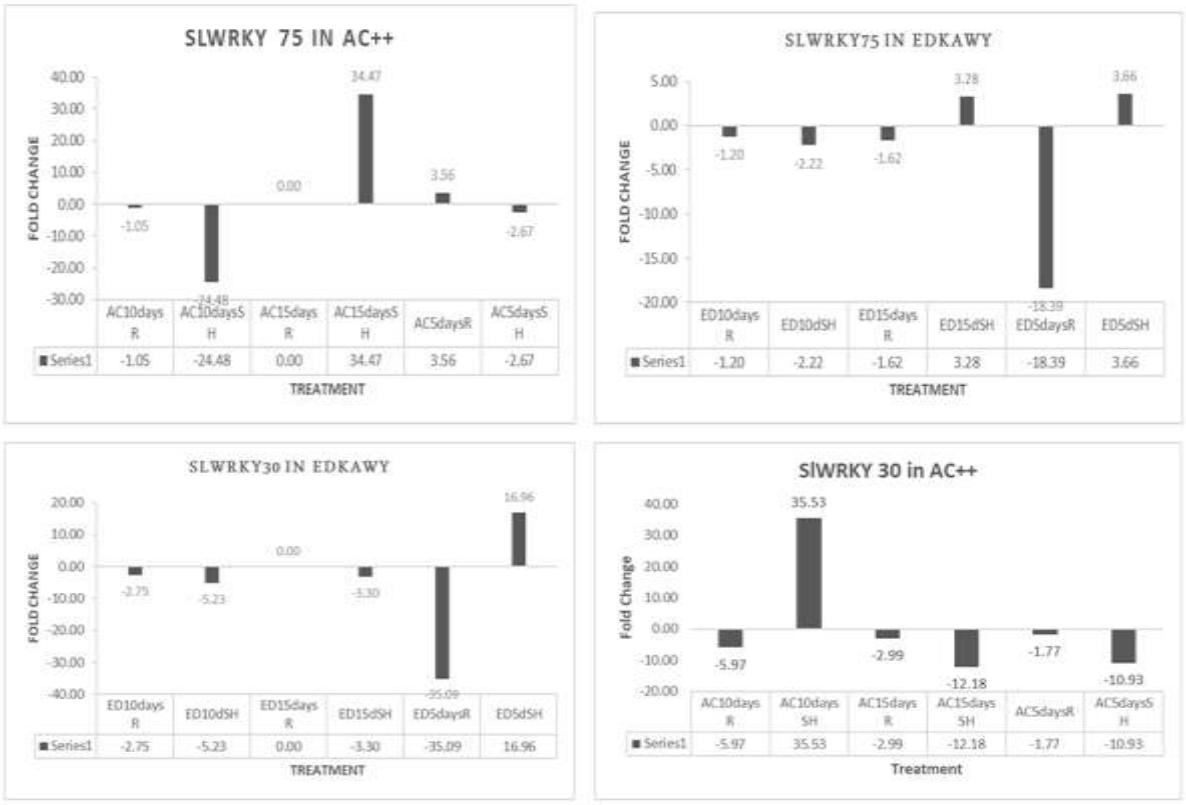

Figure 4. (A)Quantitative expression of SLWRKY75 and (B) SLWRKY30 gene in shoot and root of Edkawy and Ac++ tomato under drought stress. On the axis, the positive values represent upregulation and negative values represent downregulation.

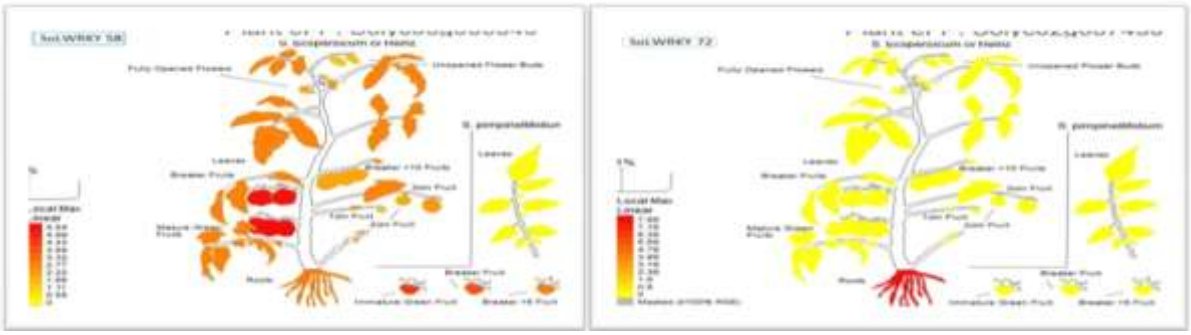

Figure 5(A) The expression level in sol WRKY 58 was highly expressed in fruits particularly in clusters 1 &2 and moderate expressed in leaves and roots(B) while solWRKY 30 is highly expressed in leaves and roots with a moderate expression level in fruit

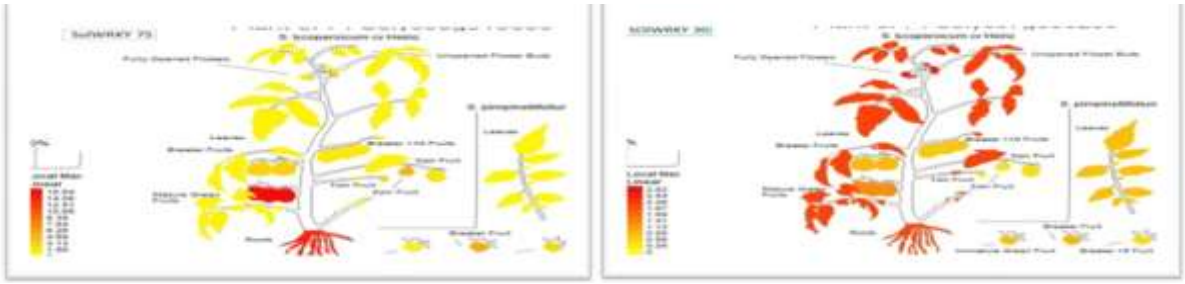

Figure 6(A) The expression level of sol WRKY 03 was highly expressed in fruits and moderate expressed in leaves and low expressed in roots(B) while gene solWRKY 75 is highly expressed in roots and fruits with low expression level in leaves

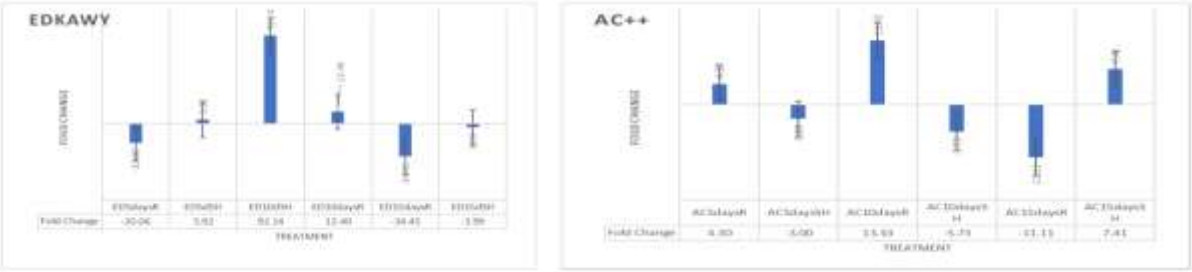

Figure 7 . Quantitative expression of SLWRKY03 gene in shoot and root of Edkawy and Ac++ tomato under drought stress. On the axis, the positive values represent upregulation and negative values represent downregulation.

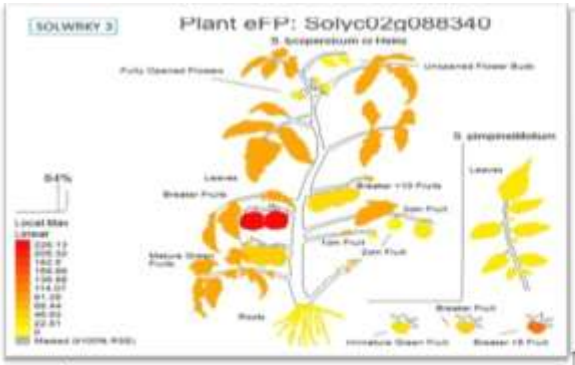

Figure 8 The expression level of sol WRKY 72 was highly expressed in roots and low expressed in leaves and fruits

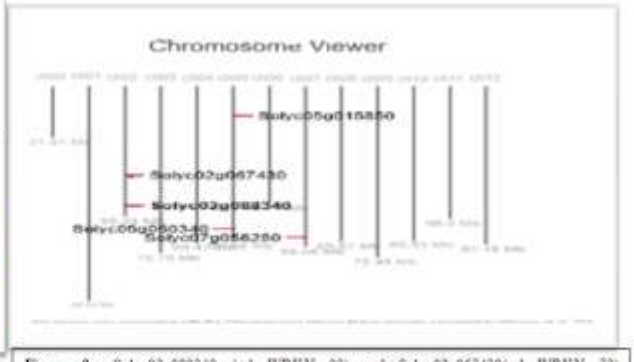

Figure 9:- Solyc02g088340 (sol WRKY 03) and Solyc02g067430(sol WRKY 72) are located in chromosome no. 2 while Solyc05g015850 (sol WRKY 75) and Solyc05g050340 (sol WRKY 58) are located in chromosome no. 5. meanwhile, the Solyc07g056280 (sol WRKY 30) are located in chromosome no. 7

IV. Conclusion

In the current study in order better understanding of the drought tolerance mechanisms in tomato, expression profiling was performed, therefor the comparison between Edkawy and Ailsa Craig' (AC++) were conducted under drought stress.The morphological observationsindicated that Edkawy is more tolerant to drought than Ailsa Craig, also the root length measurements showed that Edkawy root was higher than the root of AC++. Our study offers an investigation in WRKY family and by five genes of this family (slWRKY58, slWRKY30, slWRKY03, slWRKY72, slWRKY75) the relative expression was observed the variation of up-regulation and down-regulation in root and shoot , some results matched and confirmed the previous literature such as slWRKY03 the expression of this gene in shoot of Edkawy after ten days of stress was up-regulate so this led us to suggest that this gene play a role of the drought tolerance or induce other genes to response to stress .and other genes showed different results such as slWRKY58 up-regulate in root of Edkawy after 15 days also up-regulate in Ac ++ in shoot .The current investigation may be start in decode gene regulatory networks in Edkawy as a domestic cultivar and Opens up a new horizons for the use of the Edkawy to overcome soil problems as it has a strong root system .our results showed that the Edkawy variety is high tolerance to drought stress conditions. so, we recommended that from now on the evaluation and selection of varieties should be based on a key component when dealing with drought stress and used that in guide decisions. particularly, that the scarcity of water became It poses real danger all over the world.

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