Characterization of ISSR and SCoT Markers and *TaWRKY* Gene Expression in some Egyptian Wheat Genotypes under Drought Stress

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Abstract: Ten ISSR and SCoT primers were used to estimate the genetic variability between some Egyptian wheat genotypes. A total of (141) bands across both types of markers, of which 72 ISSR bands (87.5%) and 69 SCoT bands (81.1%) were polymorphic. ISSR showed higher levels of polymorphism (P%), indicating its efficacy in separating closely related germplasm. The polymorphism information content (PIC) and resolving power (Rp) indicated no preference for any type of markers. Effective multiplex ratio (EMR), marker index (MI) indicated that ISSR revealed higher values. SCoT1 primer showed the highest P%, PIC, MI and EMR values while SCoT12 showed the highest Rp values. While, HB-11 primer showed the highest MI and EMR values, 98-A primer showed the highest P% and PIC. Across the two types of markers, a total of 54 genotype-specific markers were amplified. Most markers were showed by Shandaweel 1 genotype. Some of these markers are related to drought tolerance, and also, can be used in detecting possible relatedness among genotypes. We had profiled the expression of seven *TaWRKY* genes under PEG6000 stress. High variation in gene expression was observed between Shandaweel 1and Misr 3. All *TaWRKY* genes were expressed under different concentrations of PEG for Shandaweel 1, while Misr 3 was up regulated for all studied genes except for *TaWRKY50*. The relative *TaWRKY* genes expression showed highest and lowest levels in Shandaweel 1 and Misr 3 respectively. Moreover, *TaWRKY44* upregulated under all studied concentrations of PEG except for Shandaweel 1 at 15 % PEG, while *TaWRKY50* was downregulated for both genotypes under all studied concentrations except for Shandaweel 1 at 15 %PEG. Generally, we demonstrated high genetic variability through DNA marker, and variable gene expression studies between the studied genotypes.

Keywords: Wheat, Inter-simple sequence repeat (ISSR), Start codon targeted (SCoT), Transcription Factors, *TaWRKY*, genetic diversity

INTRODUCTION

Wheat (*Triticum aestivum* L.), one of the most important food crops, it occupies the world first rank for human stable food. There is a great interest to bridge the gap between wheat production and consumption particularly in the environments that suffer from several stresses. Egyptian wheat genotypes are characterized by withstanding of some biotic and a biotic stress, therefore they are very important economically for Egypt. The major tasks of any breeding programme, is to study the genetic diversity of the studied germplasm because it may help in selecting cultivars and lines characterized by high variability and better performance under certain conditions (Zhang *et al*., 2015). Molecular markers supply eminent sources of polymorphism which assists breeders to choose economical traits and thus increase crop production (Randhawa *et al.*, 2013). DNA markers as SCoT and ISSR are used effectively for studying genetic variation of plants (Ma *et al*., 2008; Collard and Mackill 2009; Etminan *et al*., 2016). ISSR markers could be efficiently used to evaluate genetic variations in the wheat germplasm, genetic similarity and dissimilarity among genotypes. (Sofalian *et al*., 2008, 2009). The efficiency of ISSR markers is very high and two primers were sufficient to distinguish some examined durum wheat cultivars (Pasqualone *et al*., 2000). Moreover, Abou-Deif *et al*. (2013) and Chowdhury *et al*. (2008) found that ISSR markers effective in distinguishing, fingerprint and assessment genetic diversity in a collection of wheat genotypes. However, Start Codon Targeted (SCoT) polymorphisms are reproducible markers that are based on the short-conserved region in plant genes surrounding the ATG translation start codon (Collard *et al*., 2009). SCoT markers have been used to evaluate genetic polymorphism, identify genotypes, and DNA fingerprinting in various species, including wheat, rice, check pea, sugarcane and grape (Amirmoradi *et al*., 2012; Guo *et al*., 2012; Adawy, *et al*., 2013; Hamidi *et al*., 2014; Ibrahim *et al*., 2016). Moreover, Aboulila and Mansour (2017) and Abdein *et al*. (2018) noted that SCoT marker is effective for obtaining new fingerprints for barley and tomato, respectively. One of the main cellular events occurring during stress conditions is extensive modification of gene expression (Rampino *et al.,* 200**6**). Numerous studies have shown that the expression of a vast array of genes is modulated by environmental stresses such as drought and salinity (Lata and Prasad *et al.,* 2011; Rowley *et al.,* 2011; Adams *et al.,* 2014), while drought probably has the most significant effect on growth and affects a variety of vital molecular processes in plant (Bartels and Sunkar, 2005). To date, the most studied genes are those that encode transcription factors (TFs) which have substantial role in adapting to biotic and abiotic stresses (Besseau *et al*., 2012; Chen *et al*., 2012). TFs including dehydration

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responsive element-binding factor (DREB), ABA responsive elements (ABARE), zinc-finger proteins, WRKY, and NAC TFs are upregulated by certain abiotic stresses and activate expression of abiotic stress related genes (Hu and Xiong, 2014). WRKY proteins are known to be involved in regulating diverse functional processes such as growth, development, hormone-mediated pathways, biotic and abiotic stresses (Ramamoorthy *et al.,* 2008). Many investigations reported that the expression of WRKY genes is upregulated by different environmental factors such as wounding, drought, salt, heat and cold stresses, and phytohormone treatments (Hara *et al.,* 2000; Jiang and Deyholos, 2009; Wei *et al.,* 2008). In rice, 54 WRKY genes were expressed under abiotic stress conditions (Ramamoorthy *et al*., 2008). In Arabidopsis, the transcript level of *WRKY25* and *WRKY33* increased when treated with NaCl, mannitol, ABA or cold stresses (Jiang and Deyholos, 2009). Furthermore, Niu *et al*. (2012) and Zhu *et al*. (2013) reported that *TaWRKY* Transcription Factors have a fundamental role in biotic and abiotic stress responses as well as development processes. The use of approaches integrating DNA marker and gene expression techniques has been a promising strategy in illustrating the plant stress response mechanism. We aimed to test this hypothesis in some Egyptian wheat genotypes under drought conditions. Therefore, the present study aims to: (1) evaluate the usefulness of ISSR and SCoT DNA markers in assessing and analyzing the nature and the extent of genetic diversity. (2) examine the patterns of expression levels of some *TaWRKY* transcription factor genes by qPCR under different concentrations of Polyethylene glycol (PEG6000) for some Egyptian wheat genotypes.

MATERIALS AND METHODS

Plant Material

Nine Egyptian wheat (*Triticum astivum*) genotypes (Giza 168, Misr 2, Shandaweel 1, Misr 1, Sids 12, Misr 3, Sakha 95, Bani Seuf 7 and Sohag 4) were investigated in this study.

Molecular marker analysis

Seeds were germinated in the dark and were cultivated in a greenhouse (12 h light/12 h dark cycle at 22˚C). Genomic DNA was extracted from leaves according to the CTAB method (Doyle and Doyle, 1987). ISSR and SCoT amplification was achieved as defined by Hussein *et al*. (2006) and Collard and Mackill (2009), respectively. As shown in Tables (1 and 2), ten different ISSR and SCoT primers (five per each) were selected from previous studies to be employed in this study. The similarity matrices were done using Gel works ID advanced software UVP-England Program. The relationships between the genotypes as revealed by dendrograms were done using SPSS Windows (Version 10) program. Clear and reproducible alleles were recorded as 1 for existence or 0 for absence. In order to estimate the informativeness of the markers in distinguishing the studied genotypes, PIC, EMR, MI and Rp parameters were calculated. PIC was calculated according to (Anderson *et al*., 1993), as PIC=1-Σpi^2, where pi is the frequency of the *i*th allele. Effective multiplex ratio (EMR) was calculated according to (Powell *et al*., 1996; Nagaraju *et al*., 2001): EMR = np (np/n). Where np is the number of polymorphic loci (per primer), and n is the total loci number. Meanwhile, Marker index (MI) was calculated using the formula MI = PIC × EMR according to (Powell *et al*., 1996; Nagaraju *et al*., 2001). The Rp of each primer was calculated using the formula Rp = Σ Ib, where Ib is band informativeness (the Ib can be represented on a scale of 0–1 by the following formula: where Ib = 1-(2 × ⎪0.5 - p⎪), where p is the proportion of individual containing the band (Prevost and Wilkinson, 1999).

Drought treatments

Seeds were surface sterilized in a calcium hypochlorite solution containing 5% of active chlorine, for 5 min. Seeds were rinsed with water and incubated in Petri dishes on moist sterile filter paper at 27C˚ in darkness until emergence of the radicle. Three days later germinated seeds were transferred in four 50×20 mm plastic pots/genotype (10 seeds / pot) in three replications. Plants were grown in an environmentally controlled chamber at 25 ± 2°C (day) and 21 ± 1.5°C (night), relative humidity 50% and a photoperiod of 14 h. Seedlings were watered daily with distilled water for three weeks. Subsequently drought stress treatments were imposed in the fourth week. Four pots of each wheat genotype in three replications were treated with four drought treatments. In the present study, Polyethylene glycol 6000 was used to induced water stress in plants (Emmerich and Hardegree, 1990). Drought treatments were imposed by dissolving PEG6000 at (0, 5, 15, 25%) in distilled water. After exposure to treatments for one week, the wheat plants leaves were immediately frozen in liquid nitrogen and kept at −80◦C for further analysis.

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from PEG6000 treated and untreated leaf tissues using the TRIzol® LS Reagent according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 mg of total RNA with oligo (dT) and MMLV reverse transcriptase (200U/ll, Invitrogen) according to the manufacturer's instructions. ABI A Prism 7000 sequence detection system was used for qPCR under the following cycle conditions: 10 min at 95˚C followed by 40 cycles of 15 s at 95˚C, 1 min at 60˚C. *TaActin* gene was used as internal reference gene. Genes and their corresponding primers are shown in Table (3). PCR was performed in a reaction mixture containing 2 µl of cDNA sample, 0.6 µl of each forward and reverse primer, 10 µl of SYBR Green and 6.8 µl of PCR grade water. Relative quantification was performed by applying CT method (Livak and Schmittgen, 2001).

RESULTS

ISSR polymorphism

Five ISSR primers were used to examine the genetic diversity among studied genotypes. The size of amplified bands ranged from 175 to 2160 bp. A total of 72 bands were produced Figure (1A) and Table (4). Out of which 63 were polymorphic bands and 9 bands were monomorphic. The number of polymorphic bands ranged from 9 (44-A & 44-B) to 20 (HB-11) with a mean of 12.6. The average of (P%) was 87.50 % across all the studied genotypes. The highest (P%) was (91.67%) for primer 98-A and the lowest was (75%) for primer 44-A. In addition, the PIC values varied from 0.59 (HB-15) to 0.83 (98-A) with an average of 0.70. Moreover, the highest value of MI was observed for primer HB-11 (13.26), while the lowest value related to primer 44-A (4.07). EMR values varied from 6.75 to 18.18 for primers 44-A and HB-11, respectively, whereas the mean value was 11.07. Finally, the Rp value varied from 4.00 (98A) to 13.21 (HB-15) whereas the mean value was 8.70 distinguishing the different genotypes. Generally, HB-11 and 44-A primers revealed highest and lowest values for (P%, PIC, MI and EMR%), respectively.

SCoT polymorphism

Five SCoT primers were studied to analyze the genetic differences amongst the selected genotypes as shown in Table (5) and Figure (1B). Out of 69 amplified bands, 56 and 13 bands were polymorphic and monomorphic respectively. The total bands per primer varied from 8 to 23 for (SCoT 9 and 1) respectively. The size of amplified products varied from 120 to 1820 bp. In addition, SCoT 1 and 9 primers had the highest and lowest number of polymorphic bands. The average of (P%) was 81.16% across all accessions. The highest (P%) was (95%) for primer SCoT 1and the lowest was (50%) for primer SCoT 9. PIC values varied from 0.42 (SCoT 9) to 0.77 (SCoT 1) with an average of 0.65 .On other hand, the highest value (16.17) and lowest value (0.84) of MI were observed for SCoT 1 and 9 respectively, and the average value was 6.71. In addition, EMR values ranged from 2.00 to 21.04 for primers SCoT 9 and 1 respectively, and the mean value was 9.37. The Rp values varied between 6.17 (SCoT11) to 11.46 (SCoT 12) discriminating the different genotypes. While the average Rp was 8.78. In conclusion, primers SCoT 1 and 9 revealed highest and lowest values for (%P, PIC, MI and EMR) respectively.

Genotype-specific markers

On other hand, the number of genotype-specific markers (positive and negative) scored across studied genotypes was as high as 54 in which 27 of them were generated from ISSR analysis, while 27 from SCoT analysis Tables (6). However, in ISSR marker primer 98-A reveled highest number of genotype-specific markers (nine markers) while in SCoT marker primers SCoT 10 & 11 showed highest number of genotype-specific markers (six markers). The highest number of genotype specific markers across both types of markers was scored for genotype Shandaweel 1 (18 amplicons) 11 markers of them revealed by ISSR and 7 markers revealed by SCoT, while the lowest was scored for genotype Misr 3 (2 amplicons). Interestingly, we noticed that genotype Giza 168 generated genotype-specific markers via just ISSR marker.

Genetic similarity (GS) and cluster analysis using SCoT and ISSR data

Genetic similarity values showed clearly substantial differences among the studied wheat genotypes. Tables (7 - 9) revealed that the lowest genetic similarity was (0) between (Bani Seuf 7 vs. Sohag 4), (Misr 3 vs. Sids 12) and (Misr 1 vs. Shandaweel 1) for ISSR, SCoT and combined date respectively. Meanwhile, the highest genetic similarity was 1 between (Misr 2 vs. Shandaweel 1), (Misr 2 vs. Sohag 4) and (Misr 2 vs. Shandaweel 1) for ISSR, SCoT and combined date respectively. In the same context, the two markers were used to construct dendrogram based on UPGMA cluster analysis. The dendrograms in Figure (2A-C) indicated that the ISSR and SCoT markers succeeded in distinguishing the studied genotypes in relation to their genetic background and geographical origin. According to ISSR analysis all the studied genotypes divided into five different clusters, Figure (2A). First cluster include (Sids 12), second cluster include (Bani Seuf 7 & Sohag 4), third cluster include (Sakha 95 & Giza 168), fourth cluster include (Shandaweel 1 & Misr 1), fifth cluster include (Misr 2 & Misr 3). On other hand, according to SCoT analysis all the studied genotypes divided into five different clusters, Figure (2B). First cluster include (Misr 2), second cluster include (Shandaweel 1 & Sohag 4 & Sakha 95), third cluster include (Misr 1 & Giza 168), fourth cluster include (Bani Seuf 7), fifth cluster include (Sids 12 & Misr 3). Finally, according to the combined data analysis for all the studied genotypes, five different clusters were obtained. First cluster include (Misr 2), second cluster include (Misr 3 & Sids 12), third cluster include (Bani Seuf 7 & Sohag 4), fourth cluster include (Sakha 95 & Giza 168), fifth cluster include (Shandaweel 1 & Misr 1).

Gene expression studies

It appears from the aforementioned investigations that high number of new genotype-specific markers were revealed by both types of markers. Expression of some *TaWRKY* transcription factors under drought stress conditions was investigated in order to determine the association between genotype-specific markers and gene(s) for drought tolerance in the studied wheat genotypes. Shandaweel 1 (had highest number of genotype-specific markers) and Misr 3 (had lowest number of genotype-specific markers) were selected to study their degree of drought tolerance. Figure (3A) showed that the expression of all studied genes i.e. *TaWRKY2, TaWRKY4, TaWRKY8, TaWRKY20, TaWRKY31, TaWRKY44 and TaWRKY50* was induced

after expose to PEG6000 for Shandaweel 1. In the same trend, Misr 3 was upregulated for all studied genes except for *TaWRKY50*. Moreover, the expression of Shandaweel 1 was higher than Misr 3 for all studied genes except the expression of *TaWRKY4* was the opposite. However, *TaWRKY44* represented highest mRNA transcript levels (11.64) for Shandaweel 1. Interestingly, the same gene had the lowest mRNA transcript levels (1.11) for Misr 3. It worthy to mention, that the studied concentrations of PEG revealed clear variability in the expression of both genotypes. As it is evident, in Figure (3A) the highest expressions folds at 5% PEG were (2.6 for *TaWRKY31*) & (1.88 for *TaWRKY4*), at 15 % (2.52 for *TaWRKY20*) & (1.78 for *TaWRKY4*) and at 25 % (11.64 for *TaWRKY44*) & (3.41 for *TaWRKY20*) for Shandaweel 1 and Misr 3 respectively, comparing with the untreated control. Moreover, it was noted that *TaWRKY44* upregulated under all studied concentrations except for Shandaweel 1 at 15 % PEG. Meanwhile, *TaWRKY50* was downregulated for both genotypes under all studied concentrations except for Shandaweel 1 at 15 % PEG. To distinctly indicate the contrasting performance of studied genotypes, a summary was generated based on the performance of differential expression genes grouped in clusters. In Figure (3B) it was noted that there are five expression clusters for the studied genes /genotypes. First cluster involve all the genotypes that its expression was upregulated for all the studied genes at 5, 15, 25% of PEG. Expression of *TaWRKY 2, 20* for Shandaweel 1 and *TaWRKY 2,31* for Misr 3 belongs to this pattern. Second cluster involve all the genotypes that its expression decreased after exposed to 5% PEG and then increased after exposed to15% PEG and finally its expression decreased after treated with 25% PEG. Expression of Shandaweel 1for *TaWRKY50* and Misr 3 for *TaWRKY44* belongs to this pattern of expression. Third cluster involve all the genotypes that its expression was increased after exposed to 5% PEG and then decreased after exposed to 15% PEG and finally its expression increased after exposed to 25% PEG. In this context, expression of Shandaweel 1 for *TaWRKY4,8,31,44* and Misr 3 for *TaWRKY20* were belongs for this pattern. The fourth cluster include the genotypes that upregulated at 5 and 15 % PEG and expression of Misr 3 for *TaWRKY4,8* belong to this pattern. Finally, fifth cluster involve all the genotypes that its expression was downregulated for studied genes at 5,15,25% of PEG. The expression of Misr 3 for *TaWRKY50* is the example for this pattern. To sum up, gene expression results showed molecular genetic diversity for the studied wheat genotypes and *TaWRKY* genes.

DISCUSSION

The main aspect of this study is to evaluate the molecular genetic diversity of nine Egyptian wheat genotypes by using DNA based markers and gene expression profiling. ISSR and SCoT markers have been demonstrated to be helpful in genetic variability assessment because of their high reproducibility and great power for the detection of polymorphism in wheat (Pasqualone *et al*., 2000; Chowdhury *et al*., 2008; El-Assal and Gaber, 2012; Abou-Deif *et al*., 2013; Pakseresht *et al*., 2013; Etminan *et al*., 2016). Ten ISSR and SCoT primers were investigated for their efficiency to reveal polymorphic patterns in the studied genotypes. Our results showed that the average level of polymorphism revealed by ISSR primers was higher than the average level of polymorphism revealed by SCoT primers across all the genotypes. But in the same time, the highest level of polymorphism was revealed by SCoT 1 primer (95%) followed by primer ISSR 98-A (91.67%). The detected degree of polymorphism between the two techniques indicated that ISSR markers were more efficient in detecting genetic variability between the tested genotypes. Our finding were in accordance with several studies establishing the roles of RAPD, ISSR and SSR markers and reported that the ISSR markers revealed recurrence, polymorphism and promising in discrimination between different cultivars (El-Assal and Gaber, 2012). Moreover, Carvalho *et al*. (2008) found a higher %P than that reported here (98.5%) using 18 ISSR primers in 99 wheat accessions. On the contrary, many investigations documented lower %P than our results among wheat genotypes (54.66%, 69.77%, 80.2%) reported by (Najaphy *et al.,* 2012; Khaled *et al.,* 2015; Haiba *et al.,* 2016) respectively. Compared to other molecular markers used for genetic diversity in wheat, our % P values were higher than those of SSR (50.3%) (Maric *et al.,* 2004), TRAP (40%) (Al-Doss *et al*., 2010), RAPD (52.6%) (Wynne *et al.,* 1970), AFLP (50.2%) (Eivazi *et al.,* 2007), and SRAP (54.81%) (Farshadfar *et al.,* 2003).The effectiveness of ISSR and SCoT markers is assessed by calculating four marker parameters such as PIC, MI, EMR and Rp. Based on our results, values of PIC, MI, EMR in ISSR was higher than SCoT marker. PIC value of markers indicated the usefulness of DNA markers for gene mapping, molecular breeding and germplasm evaluation (Peng *et al.,* 2005). Many investigations have been extensively studied the PIC index (Tatikonda *et al.,* 2009; Thudi *et al.,* 2010). In this study ISSR 98-A primer had the highest PIC value than all studied primers. The moderate values of PIC for both marker primers could be attributed to evaluation of genetic diversity. In the same time, in this study, the average of PIC values was higher than the values that found by (Zamanianfard *et al*., 2015) using ISSR, and by (Hamidi *et al.*, 2014) using SCoT markers in wheat germplasm. EMR is the product of the fraction of polymorphic bands and the number of polymorphic bands. In addition, MI is the product of PIC and EMR, thus the higher polymorphism provides higher EMR and MI values. In this regard, SCoT 1 primer had the highest values of MI and EMR than all studied primers. Our average values of

MI and EMR were higher than values reported by (Najaphy *et al.,* 2012) (1.34 and 5.69) and (Tonk *et al*., 2014) (1.58 and 4.79) using ISSR marker analysis respectively. The resolving power (Rp) is another method used to measure the ability of primers or techniques to distinguish between genotypes (Prevost and Wilkinson, 1999). The average of Rp values of SCoT marker was higher than ISSR marker but the differences were not significant. Meanwhile, SCoT 12, ISSR-HB-11 and ISSR-HB-15 primers possessed highest Rp values than all other studied primers. Tonk *et al.* (2014) showed that the Rp values varied from 0.13 for ISSR 851 primer to 6.00 for ISSR 824 primer with average 2.34 which is lower than our average value. In the same context, Najaphy *et al.* (2011) found that the highest Rp was revealed by primer UBC-845 (16.5) which is higher than our values. All studied genotypes/ markers were collectively characterized by high number of cultivar-specific markers (54 amplicons) in which 27 were generated across both types of markers. Shandaweel 1 exhibited higher number of specific markers (18 amplicons) while Misr 3 exhibited lower specific markers (2 amplicons) Tables (6). Meanwhile ISSR 98-A and SCoT 10 & 11 primers revealed highest number of genotype-specific markers 9 & 6 amplicons respectively. These specific markers can be considered as a useful marker for screening for drought tolerance in studied wheat genotypes. In this context, Moghaieb *et al.* (2010) and (Haiba *et al*., 2016) determined 13 ISSR positive and negative specific markers. However, Abd El-Hadi (2012) showed four genotype–specific ISSR markers. The similarity matrix was carried out to produce right relationships based on large and diverse genome regions as shown in Tables (7 - 9). The results of genetic similarity indicate that ISSR and SCoT succeeded to detect high genetic distances which showed a high diversity among the genotypes. Using combined data of ISSR and SCoT the highest similarity indices resulted between (Misr 2 vs. Shandaweel 1) while the lowest was between (Misr 1 vs. Shandaweel 1). In this regard, the genetic similarity was studied among wheat varieties by different investigations and it was rated (71%, 77% and 83%) by (Abou-Deif *et al.,* 2013; Zamanianfard *et al.,*2015; Shirnasabian *et al.,* 2014). While it was ranged between (0.34 to 0.68) and (0.933 and 0.080) by (Baraka *et al.,* 2010; Aida *et al*., 2012). To understand the level of genetic divergence between studied genotypes, cluster analysis was calculated. The dendrogram based on ISSR and SCoT markers divided the wheat genotypes into five main groups with some variation. Moreover, the results of the dendrograms did not separate the genotypes with possible similar ancestor. Similar results found by (Abdel-Lateif *et al.,* 2018) whereas the generated dendrogram based on SCoT markers classified Sakha-93 and Sakha-94 cultivars into different groups. In contrast, Giza-168 and Giza-171 genotypes were classified together in the same cluster. Also studying the polymorphism using SCoT marker by (Xiong *et al.*, 2011) showed that not all peanut genotypes related to the same variety were classified in the same group. These results confirm the capability of SCoT as an excellent marker to research the genetic relationships between various cultivars and obtaining new specific clustering (Xiong *et al.,* 2011; Etminan *et al.,* 2016). On other hand, Carvalho *et al.,* (2009) showed that most of studied wheat cultivars belong to the same botanical variety and were clustered in the same main group. Meanwhile, Malik *et al.* (2010) noted that 27 cultivars were clustered in six groups in consent with their known origin. Our results of ISSR dendrogram were consistent with this observation. Overall, from the previous results we concluded that the studied genotypes had a high level of polymorphism between each other. In addition, ISSR markers showed higher polymorphism than SCoT markers, contrary (Abdein *et al.,* 2018) found that SCoT marker revealed higher polymorphism than ISSR marker between tomato genotypes. The high polymorphism percentage between the two markers is normal because each marker targets different genome sequences, differ in their ability to differentiate individuals, the mechanism of detecting polymorphism. But in the same time, they can be complementary to each other, as it is shown in the present study. Moreover, (SCoT 1 & 12) and (ISSR-HB-11,15 and 98-A) primers possessed highest values than all other studied primers thus they considered to be the most informative primers for distinguishing the genotypes. An important trait of an appropriate marker system is the ability to differentiate between various accessions and abundance of genotype-specific markers. Thus, ISSR and SCoT markers considered to be a good DNA-marker for distinguishing the tested genotypes because of high genetic variation detected among studied genotypes. This highlights the significance of ISSR and SCoT markers to detect polymorphism and genetic relationships. The same observation is mentioned by many investigations carried out on wheat germplasm (Pakseresht *et al.,* 2013; Zamanianfard *et al.,* 2015; Etminan *et al.,* 2016).

Expression profiles of *TaWRKY* genes under drought stress

The main aspect of this part is to evaluate *TaWRKY* genes expression levels under long drought conditions in two Egyptian wheat genotypes. The results indicated that all studied genes were up regulated. These data are similar to previous reports of functional WRKY genes in wheat, (Zhu *et al.,* 2013; Okay *et al.,* 2014; He *et al.,* 2016) suggesting that *TaWRKY* involved in response to drought stress. From Figure (3A), it is evident that the expression of Shandaweel 1 for *TaWRKY44* had the highest mRNA levels under 25 % PEG but the same gene showed the lowest expression under 25 % PEG in Misr 3. These discrepancies may be due to the utilization of wheat genotypes with different genetic backgrounds. In the same context, Zhu *et al.* (2013) found that *TaWRKY7* transcripts were more abundant in PEG conditions in cv. SR3 compared with

cv. JN177 plants. In addition, *TaWRKY32* and *TaWRKY34* transcripts were higher in SR3 plants stressed by PEG and ABA treatment. *TaWRKY20* levels were higher in cv. JN177 plants exposed to PEG and ABA treatments. Likewise, Okay *et al.* (2014) showed that *TaWRKY19* expression was reported to be up-regulated in leaves in Sivas 111/33 genotype albeit it was down-regulated in leaves of Atay 85 genotype. Based on prior studies, it is evident that genotypes that have better performance under stress conditions are those maintaining higher transcript levels of the studied genes. Accordingly, Shandaweel 1 had better performance under drought conditions because it was the most upregulated genotype for all genes under all PEG concentrations. Similar results were found by Abd El-Moneim *et al*. (2020) when studied the expression of four *TaNAC* genes under different concentrations of PEG6000 stress. Studied genes/ genotypes showed high variation in the transcript expression response to PEG6000. These results corroborated previous studies analyzed the transcriptome of emmer wheat (*T. turgidum*) after exposed to water deficit and showed that *TaWRKY29, TaWRKY40*, and *TaWRKY90* were differentially expressed (Ergen *et al.,* 2009). *TaWRKY44* was most upregulated gene under all studied concentrations of PEG. In accordance with these results, Wang *et al*. (2015) found that the expression of *TaWRKY44* were upregulated by drought stress which implied that this gene plays important roles in plant drought stress response. Moreover, *TaWRKY50* dramatically downregulated under all PEG concentrations / genes except for Shandaweel 1 at 15 % PEG. Similarly, Niu *et al*. (2012) and Okay *et al.* (2014) Found that expression of *TaWRKY16* and *TaWRKY24* were down-regulated in leaves of Xifeng 20 and Sivas 111/33 cultivars under drought stress. One of the important factors that complicate the improvement of crops upon exposure to stresses is the intensity of PEG (Munns and Tester, 2008). The results of genes expression confirm this idea because both studied genotypes exhibited different expression with different concentrations intensity of PEG. Also, most of highest and lowest relative expressions for the studied genes were at 25 % and 5% PEG respectively.

Relation between markers polymorphism and WRKY genes expression analysis

The analysis of ISSR and SCoT markers showed their effectiveness by generating several specific bands that can be used in marker-assisted breeding for drought tolerance in wheat. Meanwhile, the association of molecular markers with gene expression levels is one of the important factors to understand and investigate the genetic role of tolerance by prediction the genomic regions that affect the plant's response. Identification of molecular markers associated with genes expression under stress is the most important step in selecting genotypes having tolerance to such trait at the early stages of growth. For this reason, it might be possible that one or more of the detected unique bands are responsible for the noted higher expression of *TaWRKY*. For example, Shandaweel 1 genotype represented high diversity (18 unique bands). This would reflect high adaptation to environment, which is beneficial to its propagation and the screen of specified locus. In the same time, Shandaweel 1showed highest expression patterns for all studied *TaWRKY* genes under drought stress. It worthy to mention, that SCoT marker is created from the functional region of the genome while SCoT primers were designed to amplify from the short conserved region surrounding the ATG translation start codon (Joshi *et al.,* 1997; Sawant *et al.,* 1999; Collard and Mackill, 2009; Xiong *et al.,* 2009). Moreover, members of the WRKY family regulate gene expression by exclusively binding to the W-box (TTGACC/T), which is a *cis*-element in the promoter region of target genes (Bakshi and Oelmüller, 2014; Ulker and Somssich, 2004). These findings might suggest that may be the genotype-specific markers revealed by SCoT markers (7 markers) responsible for the high expression of studied genes in this genotype; however, purification, sequencing and analysis of these bands might be necessary in the proximate research work.

Table (1): List of the primers names and their nucleotide sequences used in the study for ISSR procedure

S. No	Name	Sequence 5` → 3`
1	44-A	CTCTCTCTCTCTCTCTTG
2	98-A	CACACACACACACA
3	44-B	CTCTCTCTCTCTCTCTAG
4	HB-11	GTGTGTGTGTGTTGTCC
5	HB-15	GTGGTGGTGGC

Table (2): List of the primers names and their nucleotide sequences used in the study for SCoT procedure

S. No	Name	Sequence 5` → 3`
1	SCoT 1	ACGACATGGCGACCACGC
2	SCoT 9	ACAATGGCTACCACTGCC
3	SCoT 10	ACAATGCTACCACCAAGC
4	SCoT 11	ACAATGGCTACCACTACC
5	SCoT 12	CAACAATGGCTACCACCG

Table (3): Sequence of primers used in real-time PCR

S.No	Name	Primer sequence 5` → 3`
1	*TaWRKY2*	F GTAACAGTGACTTCCTCGCCGTA R GGTAGCAGCATCGGTAGTAGCA
2	*TaWRKY4*	F AAGAGCAGTGAGCATCCAAGGA R GGCAAAGGGTGATTGTGAGAACTC
3	*TaWRKY8*	F GTCTCGTCAACGCTGTCCAATG R GGTGGTCGCAGTAGGAATGGTA
4	*TaWRKY20*	F CACCACCACCACCACCTC R AGCAGCGACGACGACATC
5	*TaWRKY31*	F GCACACCACCACCACCTC R AGCAGCGACGACGACATC
6	*TaWRKY44*	F CCAACGGCGGTGATAACTACAT R GCTACTGGATGCTGCCTTCTG
7	*TaWRKY50*	F GCGGCGCTGACAGAGGGGAGA R TTGGGTACTTGGCGCCGAGGA
8	*TaActin*	F CTTGTATGCCAGCGGTCGAACA R CTCATAATCAAGGGCCACGTA

(F) Forward primer; (R) Reverse primer

Table (4): Number and types of the amplified DNA bands as well as the polymorphism percentage generated by the ISSR primers form the nine wheat genotypes

ISSR	MB	UB	PB	TAB	FS (larger)	FS (smaller)	PIC	EMR	MI	P (%)	Rp
44-A	3	5	9	12	1970	280	0.6	6.75	4.07	75	9.53
98-A	1	9	11	12	2160	270	0.83	10.08	8.40	91.67	4
44-B	1	1	9	10	1315	290	0.67	8.1	6.13	90	4.86
HB-11	2	7	20	22	1495	210	0.73	18.18	13.26	90.91	11.9
HB-15	2	3	14	16	1690	175	0.59	12.25	7.19	87.5	13.21
Total	9	25	63	72			3.51	55.37	39.06	87.5	43.51
Average	1.8	5	12.6	14.4			0.7	11.07	7.81	0.87	8.7

MB monomorphic band, **UB** unique band, **PB** polymorphic band, **TAB** total amplified bands, **FS** fragment size, **PIC** polymorphic information content, **EMR** effective multiplex ratio, **%P,** percent of polymorphism, **Rp** resolving power.

Table (5): Number and types of the amplified DNA bands as well as the polymorphism percentage generated by the SCoT primers form the nine wheat genotypes

SCoT	MB	UB	PB	TAB	FS (larger)	FS (smaller)	PIC	EMR	MI	P (%)	Rp
Scot-1	1	4	22	23	1820	150	0.77	21.04	16.17	95.65	10.64
Scot-9	4	3	4	8	695	260	0.42	2	0.84	50	9.28
Scot-10	2	6	10	12	975	225	0.74	8.33	6.14	83.33	6.32
Scot-11	2	6	10	12	760	120	0.74	8.33	6.19	83.33	6.17
Scot-12	4	5	10	14	695	230	0.59	7.14	4.22	71.43	11.46
Total	13	24	56	69			3.26	46.85	33.56	0.81	43.88
Average	2.6	4.8	11.2	13.8			0.65	9.37	6.71	0.77	8.78

MB monomorphic band, **UB** unique band, **PB** polymorphic band, **TAB** total amplified bands, **FS** fragment size, **PIC** polymorphic information content, **EMR** effective multiplex ratio, **%P**, percent of polymorphism, **Rp** resolving power.

Table (6): Positive and negative genotype-specific markers and their molecular sizes (bp) and total number of markers for each genotype using ISSR and SCoT analysis.

Marker type	Number (and MW in bp) of Genotype-specific markers										
	Primer	**Misr2**	**Misr3**	**Sids 12**	**BaniSeuf 7**	**Sohag 4**	**Shandaweel 1**	**Sakha 95**	**Giza 168**	**Misr 1**	**Total**
Positive ISSR	44A	1(649)					2 (1456, 682)		2 (1263, 974)		5
	44B						1 (1316)				1
	98A	1(438)		1(277)			7(2162,1773,1606,1428,1022,909,838)				9
	HB-11	1(404)		1(1115)	1(1227)	1(244)		1(258)	1(230)	1(724)	7
	HB-15		1(216)			1(185)	1(1686)				3
Negative ISSR	HB-15	2(481,341)									2
Total		5	1	2	1	2	11	1	3	1	27
Positive SCoT	SCoT 1						1(1816)			3(236,189,152)	4
	SCoT 9	1(695)			1(259)			1(328)			3
	SCoT 10	4(615,371, 322,223)		1(707)			1(790)				6
	SCoT 11			1(288)		1(463)	4(758,694,579,116)				6
	SCoT 12				1(910)	2(693,794)	1(832)			1(813)	5
Negative SCoT	SCoT 1							1(806)			1
	SCoT 10		1(529)								1
	SCoT 12	1(401)									1
Total		6	1	2	2	3	7	2	0	4	27
Total		11	2	4	3	5	18	3	3	5	

Table (7): Similarity index of ISSR analysis of nine wheat genotypes.

Genotypes	Misr 2	Misr 3	Sids 12	Bani Seuf 7	Sohag 4	Shandaweel 1	Sakha 95	Giza 168	Misr 1
Misr 2	1.0								
Misr 3	0.42	1.0							
Sids 12	0.73	0.40	1.0						
Bani Seuf 7	0.64	0.71	0.15	1.0					
Sohag 4	0.59	0.87	0.17	0.00	1.0				
Shandaweel 1	1.00	0.80	0.36	0.52	0.60	1.0			
Sakha 95	0.81	0.35	0.29	0.60	0.48	0.47	1.0		
Giza 168	0.89	0.59	0.30	0.38	0.18	0.56	0.23	1.0	
Misr 1	0.92	0.65	0.20	0.47	0.43	0.09	0.31	0.31	1.0

Table (8): Similarity index of SCoT analysis of nine wheat genotypes.

Genotypes	Misr 2	Misr 3	Sids 12	Bani Seuf 7	Sohag 4	Shandaweel 1	Sakha 95	Giza 168	Misr 1
Misr 2	1.0								
Misr 3	0.96	1.0							
Sids 12	0.94	0.00	1.0						
Bani Seuf 7	0.74	0.21	0.28	1.0					
Sohag 4	1.00	0.42	0.46	0.35	1.0				
Shandaweel 1	0.89	0.74	0.75	0.56	0.24	1.0			
Sakha 95	0.68	0.61	0.57	0.41	0.17	0.17	1.0		
Giza 168	0.84	0.45	0.29	0.49	0.46	0.36	0.31	1.0	
Misr 1	0.97	0.60	0.53	0.63	0.40	0.21	0.24	0.34	1.0

Table (9): Similarity index of SCoT and ISSR combination analysis of nine wheat genotypes.

Genotypes	Misr 2	Misr 3	Sids 12	Bani Seuf 7	Sohag 4	Shandaweel 1	Sakha 95	Giza 168	Misr 1
Misr 2	1.0								
Misr 3	0.63	1.0							
Sids 12	0.80	0.13	1.0						
Bani Seuf 7	0.64	0.43	0.09	1.0					
Sohag 4	0.75	0.42	0.19	0.04	1.0				
Shandaweel 1	1.00	0.80	0.44	0.54	0.42	1.0			
Sakha 95	0.71	0.39	0.30	0.47	0.26	0.31	1.0		
Giza 168	0.72	0.48	0.20	0.35	0.21	0.46	0.17	1.0	
Misr 1	0.46	0.60	0.22	0.48	0.48	0.00	0.19	0.11	1.0

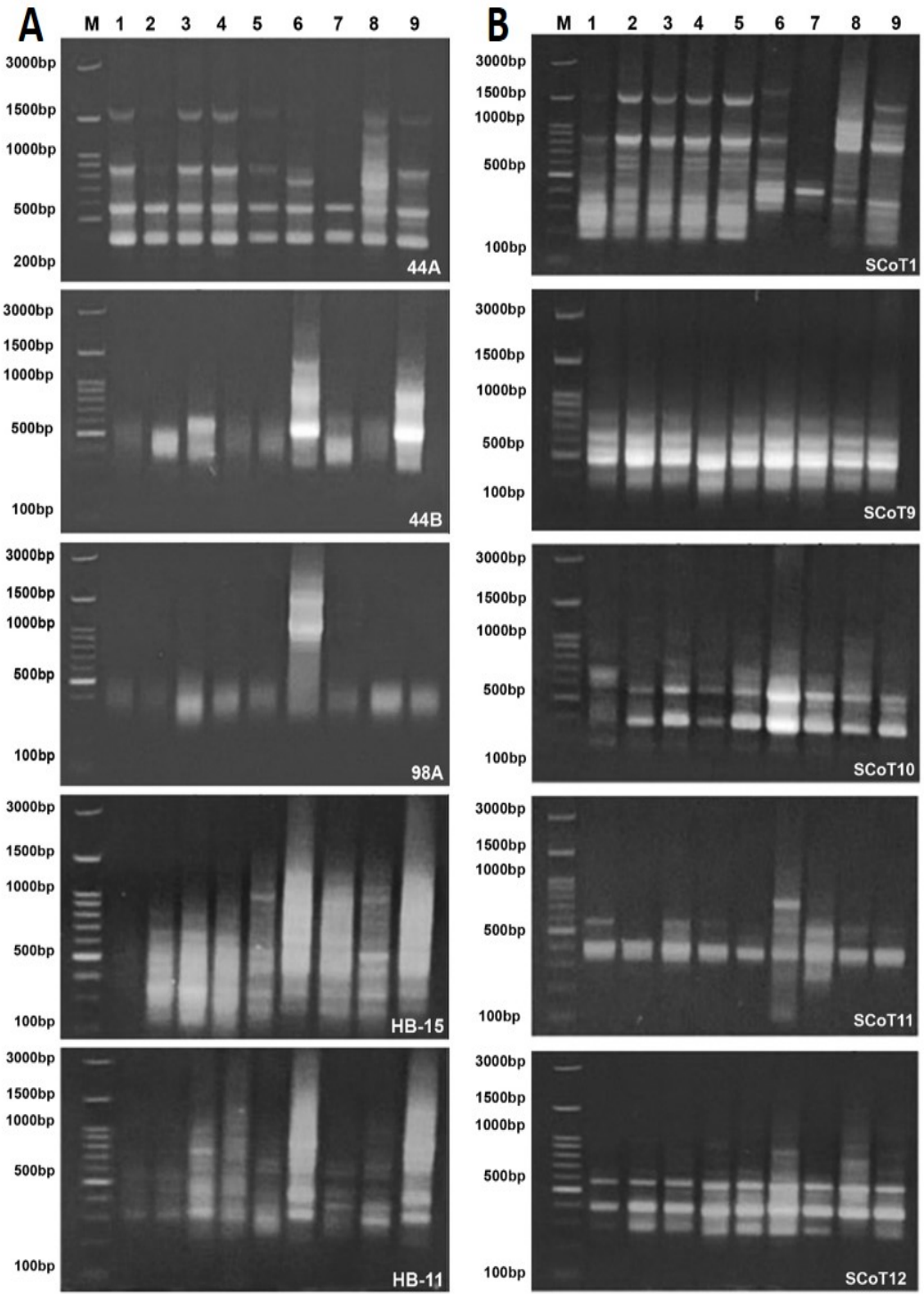


Figure (1): (A) ISSR fingerprinting of wheat genotypes: M; DNA marker, lanes 1-9; Misr-2, Misr-3, Sids-12, Bani Suef-7, Suhag-4, Shandaweel1, Sakha-95, Giza-168 and Misr-2, respectively. (B) SCoT fingerprinting of wheat genotypes: M; DNA marker, lanes 1-9; Misr-2, Misr-3, Sids-12, Bani Suef-7, Suhag-4, Shandaweel1, Sakha-95, Giza-168 and Misr-2, respectively

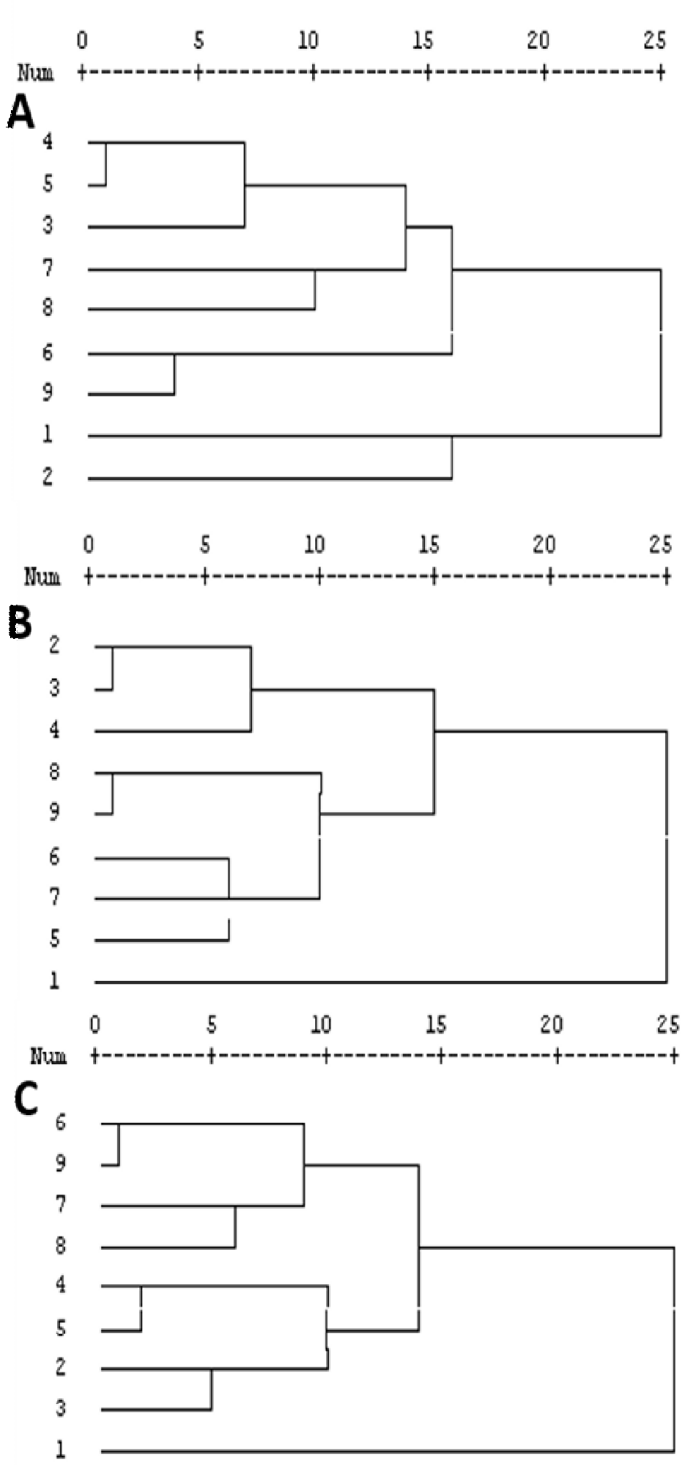
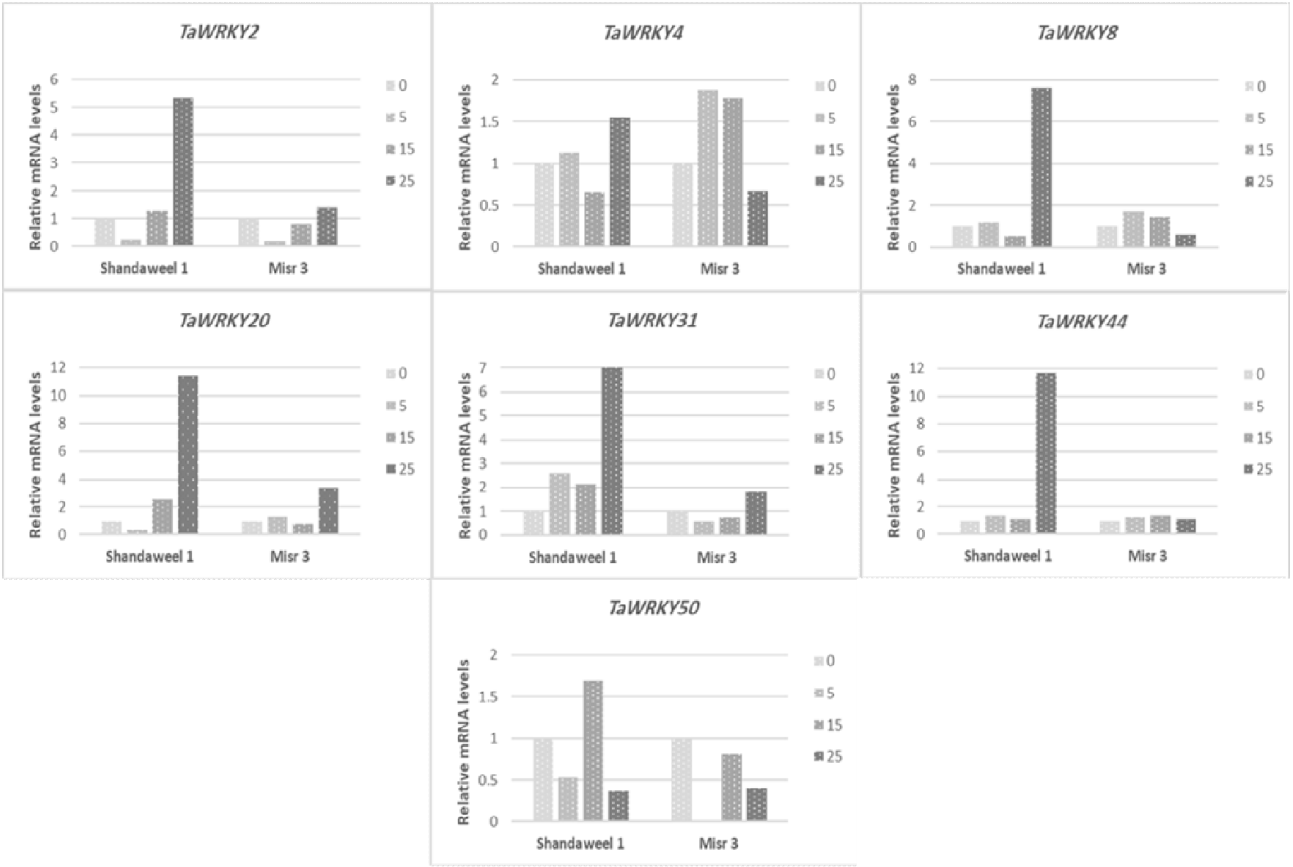


Figure (2): Phylogenic relationship as detected by cluster analysis using (A) ISSR (B)SCoT(C) combine data between studied genotypes. (1) Misr 2 (2) Misr 3 (3) Sids 12 (4) Bani Seuf 7 (5) Sohag 4 (6) Shandaweel 1 (7) Sakha 95 (8) Giza 168 (9) Misr 1. Dendrograms calculated by using Jaccard's similarity coefficients and UPGMA algorithm

A



B

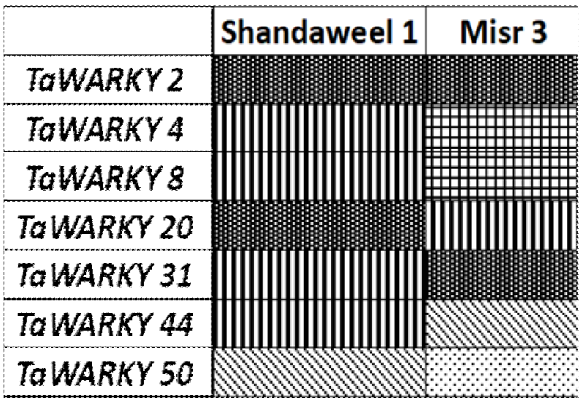


Figure (3): (A) Expression patterns of seven *TaWRKY* genes, bar graphs represent leaves expression of two wheat genotypes. Three weeks seedlings were treated for one week with 0, 5,15,25 % of PEG6000. (B) Summary of different expression patterns for all studied genes and genotypes. First pattern colored with , second pattern colored with , third pattern colored with , fourth pattern colored with and fifth pattern colored with

CONCLUSION

This study highlights the genetic variability between some Egyptian wheat genotypes using ISSR and SCoT markers. The results revealed polymorphic and reproducible profiles for the studied genotypes. ISSR markers showed greater level of genetic polymorphism than SCoT markers. SCoT primers 1&12 and ISSR primers HB-11,15 and 98-A, revealed the highest values of PIC, EMR, MI and Rp than all other studied primers. Across the two types of markers, a total of 54 genotype-specific markers were observed. Some of these markers can be associated with drought tolerance. Gene expression of some *TaWRKY* TFs under drought stress was studied by real time PCR. The expression of Shandaweel 1 genotype was higher than Misr 3 for all studied genes except the expression of *TaWRKY4* was the opposite. However, *TaWRKY44* represented highest mRNA transcript levels for Shandaweel 1. Generally, ISSR and SCoT markers showed its effectiveness in discriminating the tested genotypes by generating several unique and specific bands. These bands could be identified as markers associated with drought tolerance in wheat. Shandaweel 1 genotype revealed the highest number of unique markers (18) and had high *TaWRKY* expression. Therefore, these markers can be considered as positive markers for drought tolerance and indicating the high genetic distance between it and the other wheat genotypes. While, the lowest number of markers (2) was revealed by Misr 3 and had low *TaWRKY* expression, which are considered as negative markers for drought tolerance.

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توصيف الاختلافات الوراثية باستخدام الواسمات الجزيئية ISSR، SCoT و دراسة التعبير الجيني *TaWRKY* في بعض أصناف القمح المصري تحت ظروف الجفاف

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تم استخدام عشرة بادئات من الواسمات الجزيئية (ISSR)و (SCoT) بغرض تقييم الاختلافات الوراثية بين بعض أصناف من القمح المصرية. أظهرت النتائج أن عدد الحزم الكلية الناتجة من كلا النوعين (١٤١) وكان منهم (٧٢) حزمة ناتجة من تقنية (ISSR) منها (٨٧.٥ %) أليلات متباينة بينما أظهرت تقنية (SCoT) (٦٩) حزمة منها (٨١.١%) أليلات متباينة. وقد أظهرت تلك النتائج القدرة العالية لتقنية (ISSR) في الكشف عن التباين الوراثي بين الأصناف المدروسة. وعلى جانب آخر تم دراسة مجموعة من المؤشرات الوراثية التي تساعد على التمييز بين التقنيات المستخدمة. وقد أظهرت قيم معامل التعددية الشكلية (PIC) و (Rp) عدم وجود أي اختلافات بين التقنيات المستخدمة بينما أظهرت المؤشرات (EMR) و (MI) قيم أعلى عند استخدام تقنية (ISSR). وأوضحت النتائج أن البادئ SCoT1 أظهر أعلى نسبة في الآليات المتباينة (P%) , (MI) و EMR بينما أظهر البادئ SCoT12 أعلى القيم في المؤشر (Rp). وأظهر البادئ HB-11 أعلى القيم في المؤشرات (MI) و (EMR)بينما أظهر البادئ 98A أعلى القيم في المؤشرات (P%) و PIC . وقد أظهرت النتائج (٥٤) حزمة مميزة للأصناف المدروسة وأظهر صنف شندويل1 أعلى عدد من تلك الحزم المميزة. ومن ناحية أخرى أظهرت دراسة التعبير الجيني لعدد سبع جينات *TaWRKY* اختلاف في التعبير الجيني بين الأصناف المدروسة تحت ظروف الجفاف باستخدام PEG6000. وقد أظهر الصنف شندويل ١ تعبير جيني أعلى من الصنف مصر ٣. بوجه عام فقد أظهرت الدراسة اختلافات وراثية عالية باستخدام الواسمات الجزيئية والتعبير الجيني للأصناف المدروسة.