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USE OF MULTIPLEX PCR TO DETECT DROUGHT-TOLERANT GENES *DREB1* IN SOME GENOTYPES OF BREAD WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract

Genotypes of 13 soft wheat (*Triticum aestivum*) L. were grown in the (Tikrit Meteorological Station) during growth season 2017-2018 by planting the seeds in plastic pots. DNA was isolated from the young leaves. PCR reactions were performed using five specialized primers to identify the *Dreb1* genes found in the three genomes of wheat (A, B and D). Previous studies have indicated their significant role in giving plants resistance against stress Abiotic stress and in particular drought stress. The P21F / P21R and P25F / PRa primers were designed to detect the downstream and upstream regions of the *Dreb1* gene in genome A. The P18F / P18R primer was specifically designed for the *Dreb1* gene in genome B. The pair of P20F / P20R and P22F / PRa primers were designed to amplify *Dreb1* sequences in genome D. The results were positive with most of the primer interactions where the required bands were found in most genotypes, except for the P21F / P21R primer reactions were negative, Multiplex PCR reaction was also applied to all genotypes and more results were obtained for this interaction.

***Keywords*:** Multiplex PCR, genotypes, *Triticum aaestivum L.*, *Dreb1*.

Introduction

Triticum aestivum L. is one of the most widely cultivated crops in the world and is the basis for food security in addition to maize and rice. (FAO, 2016). Wheat is one of the most important food crops in the world, which is negatively affected by drought. More than 50% of the world's 237 million hectares under the cultivation of wheat is affected by drought periodically (Rajaram, 2001; Shao *et al,* 2005*;* Kirigwi *et al*., 2007). The wheat crop is grown in various agro-ecological conditions ranging from temperate to semi-tropical climates. Thus, there are significant climatic differences in temperature and relative humidity where the wheat crop undergoes large seasonal changes (Kaur *et al*., 2016). The selection of plant crops with drought resistance is an important solution to the problem of drought and water scarcity due to the large variation between rainfall periods, without affecting the quantity and quality of production (Ramanjulu and Bartels, 2002). Drought is one of the most important environmental stresses it is defined as the case where the rate of loss of water by transpiration and evaporation is greater than the rate of absorption of the soil (Vannozzi *et al*., 1999; Abogadallah, 2014). Recent advances in molecular biology, genomics, proteomics and metabolomics, have given insight into the plant gene network system, consisting mainly of inducible genes (environmental factors and developmental cues), expression programming, regulatory elements (*cis*-element and *trans*-element), corresponding biochemical pathways, and various signaling factors (Zhu, 2003; Munns, 2005). In general, gene transcription is controlled directly by a network of Transcriptional Factors (TFs) (Chaves and Olivera, 2004). Transcription factors are proteins with a DNA domain that are linked to the cis-acting element of the target gene. They are either induce (activator) or repress (repressor) RNA polymerase activity and thus regulates gene expression (Riechmann *et al*., 2000; Ciarmiello *et al.,* 2011). DRE elements (cis-acting element) have been found in promoter's areas for many induced Genes by drought and low temperatures (Shinozaki and Yamaguchi-shinozaki, 2007).Dehydration Responsive Element Binding (DREB) proteins form a large family of TFs that stimulate the expression of a large number of functional genes that give plants the ability to endurance stress (Agarwal *et al*., 2006). Many molecular methods have been used to characterize a series of regulatory genes for the DREB family involved in many different pathways, including genes related to dehydration, cold, high salinity (Peng *et al*., 2013). Functional or gene-specific Markers FMs have been developed and are derived from the polymorphism of the allele variants found in locus of the functional genes within the genomic DNA, which are directly related to the phenotypic variation, these markers have the ability to accurately detect the alleles in one locus within the target gene is therefore an ideal molecular marker for Marker Assisted Selection (MAS) in the breeding. FMs also outperforms random DNA markers by diagnosing the desired allel trait (Andersen and Lübberstedt, 2003; Gupta and Rustgi, 2004; Varshney *et al*., 2005; Bagge *et al.*, 2007; Liu *et al*., 2012). Multiplex PCR was also been applied in order to reduce the time and effort involved in the traditional polymerase reaction PCR. Five specialized primers were used to determine *dreb1* genes in 13 genotypes of soft wheat plant

Materials and Methods

Genotypes of 13 soft wheat *Triticum aestivum* L. were used. The genotypes were grown in The Tikrit Meteorological Station on (2017-12-1). The soil used was the mixture (clay loam) and was packaged in a plastic pots. The seeds were also planted for all the genotypes with about 20 seeds per pot and 3-4 cm deep, the soil was fertilized in in two batches, first batch at a rate of 5 g of dap fertilizer per pot and the second batch of 2.5 g of urea after 45 days of planting.

Table 1 : Wheat genotypes and their pedigree

Pedigree	Genotypes	No.
PLo - RuFT Gtos – RheL (M1.2904) – 1M – SM – 14 – osk - GAP	sham6	1
Cementa Elena, (SLR 64 x Lermacho).	Aras	2
	Florkwa	3
ACSAD 875 /5/ Cs/E.Gig//Cs/3/3*Pvo's'/4/K134(60)/ Vee's'	Acsad5	4
Ousis/ Kauz //4* BNC	Oasis	5
	Klak 2	6
	Rizgary	7
	Adana99	8
	Hidhab	9
Ajeeba x lnia 12 x Mexico24	Abu ghraib	10
Ures / Rows /3/ Jup/ B/ S// Ures	API 99	11
	Site Mall	12
Kauz*2/ Yaco// Kauz /3/ Ousis	Kauz	13

DNA extraction

The DNA was isolated from the soft leaves using CTAB, which consists of 100 mM Tris-HCl, 1.4 NaCl, 20 mM Na2 EDTA, 2% CTAB, PH 8, in the way mentioned by Weigand *et al*. (1993) Based on the foundations developed by Saghai-Maroof *et al*. (1984).The concentration and purity of extracted DNA is measured using a Nano drop device.

To ensure the presence of DNA, the samples extracted on the 1% agarose gel are passed, the gel is then exposed and photographed under UV light.

PCR Method: To determine polymorphism in the DNA sequence of *Dreb1* gene of each genome, five pairs of genome-specific primers were used (Wei *et al*., 2009; http://www.premierbiosoft.com) (Table 2).

The master reaction mixture was prepared by mixing the reaction components in a 0.2 ml sterile Eppendorf tube, to be a final reaction volume 20 µl consist of (2 µl DNA with 50 ng / µl concentration, 1 µl for both the forward and reverse primer, 6 µl Distilled water, 10 µl of mastermix 1X supplied by promega).

Table 2 : Genome-specific primer used for detect of the wheat *Dreb1* genes

Ann. temp. (ºC)	Chromosome location	Sequence	Primer
50	3B	CCCAACCCAAGTGATAATAATCT	P18F
		TTGTGCTCCTCATGGGTACTT	P18R
		TCGTCCCTCTTCTCGCTCCAT	P20F
63	3D	GCGGTTGCCCCATTAGACATAG	P20R
		CGGAACCACTCCCTCCATCTC	P21F
63	3A	CGGTTGCCCCATTAGACGTAA	P21R
63	3D	CTGGCACCTCCATTGCCGCT	P22F
57	3A	CTGGCACCTCCATTGCTGCC	P25F
		AGTACATGAACTCAACGCACAGGACAA	PRa*
	PRa is a public primer matched with P22F and P25F, respectively		

The PCR was carried out as follows: initial denaturation at 94 °C for 3 min; 34 cycles of 94 °C for 1 min, an annealing step at variable annealing temperatures depending on the primer pairs for 1 min, 72 °C for 1.5 min; and a final extension at 72 °C for 10 min and then held at 4 °C prior to analysis. The PCR products were electrophoresized on 2% agarose gels, and visualized under UV light by Gel Documentation System.

Multiplex PCR reactions: The master reaction mixture was prepared by mixing the reaction components in a 0.2 ml sterile Eppendorf tube, to be a final reaction volume 50 µl consist of (2 µl DNA with 50 ng / µl concentration, 4 µl for both the forward and reverse primers, 14 µl Distilled water, 25 µl of master mix 1X supplied by promega).

The Multiplex PCR was carried out as follows: initial denaturation at 94 °C for 3 min; 34 cycles of 94 °C for 30 sec, an annealing step at 61 °C for 1.5 min, 72 °C for 1.5 min; and a final extension at 72 °C for 10 min and then held at 4 °C prior to analysis. The Multiplex PCR products were electrophoresized on 2% agarose gels, and visualized under UV light by Gel Documentation System.

Result & Discussion

The process of adding the incubation solution at a temperature of 65 °C to the crushing product, which is at 0 °C or less, allows for the dissolution and decomposition of the proteins associated with the DNA to be replaced by CTAB, which has the ability to bind to the DNA, it also preserves the DNA in the water phase and is not deposited with other components and with the help of the high concentration of sodium chloride (Weigand *et al.,* 1993).

Three *Dreb1* genes were amplified in particular in each genome (A, B and D) from the wheat plant by using Five pairs of genome-specific primer sets.

The P25F / PRa primer pair was designed to amplify a DNA fragment (596-bp) in the downstream region of the DREB-A1 gene in the genome A, the pair of P21F / P21R primers selected to amplify DNA (1113-bp) in the Upstream region of the same gene on genome A.

The P18F / P18R primer was designed to amplify a DNA fragment ranging from 717-789 bp for *Dreb1* gene on genome B. The P22F / PRa primers was designed to amplify DNA (596-bp), and primer P20F / P20R Was selected to amplify a DNA fragment (1193-bp) in *Dreb1* gene sequencing regions on the D genome.

The results were as follows:

Results of specific-primers reactions for *dreb1* gene in A genome, the primer (P25F / PRa) showed good results in the studied genotypes, the required band appeared in 11 genotypes while absent in the genotypes Aras and Klak2 Figure 1, The P21F / P21R primer reactions did not show the desired result with all the genotypes used.

While the P18F / P18R primers specific for Dreb1 gene in B genome showed good results with all studied genotypes Figure 2.

Results of specific-primers reactions for *dreb1* gene in D genome, The P22F/PRa primer reactions showed good results in the studied genotypes, the required band appeared in 12 genotypes while absent in the genotype ACSAD Figure 3, While the P20F / P20R primer reactions showed required band with 11 genotypes and was absent from the genotypes (ACSAD, API 99) Figure 4.

In Multiplex interactions, four pairs of primers were used (P25F, P18F, P20F, P22F) which showed good results in a separate reaction, and the P21F / P21R primer was excluded because it did not show a result, the result was the band appearance (596 bp) in most genotypes, and the band (717- 789 bp) appeared in some genotypes, the band 1193 bp was clearly shown in the sham 6 genotype and appeared lightly in other genotypes, two bands approximately 230-290bp were found in all genotypes Figure 5.

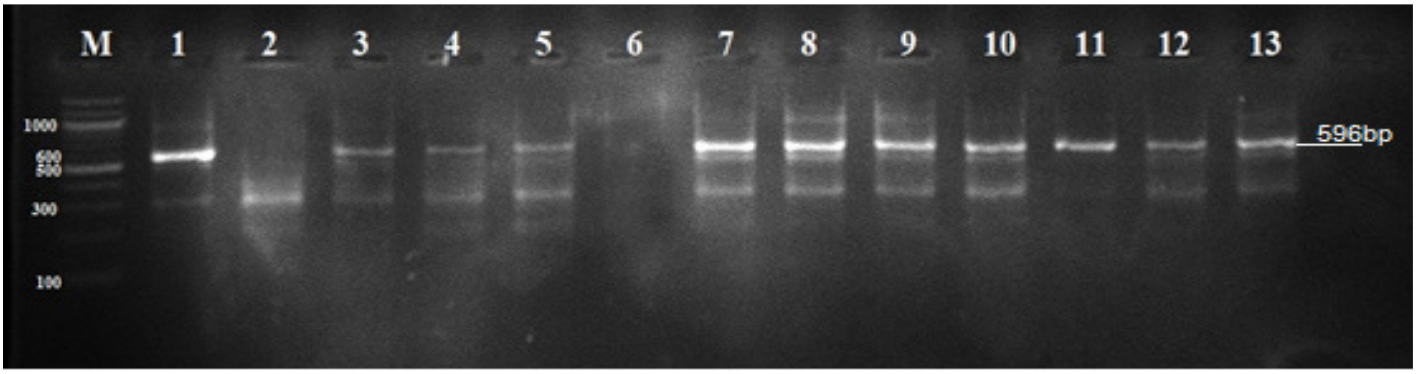

Fig. 1. PCR-based chromosome assignments of the *Dreb1* genes in wheat *Triticum aestivum* L. genotypes using an A genome-specific primer pair P25F/PRa. Arrow shows a 596-bp DNA fragment. M – DNA ladder 100-bp. 1- sham6, 2- Aras, 3- Florkwa, 4- Acsad5, 5-Oasis, 6-Klak2, 7- Rizgary, 8- Adana, 9- Hidhab, 10- Abu ghraib), 11- API99, 12- Site mall, 13- Kauz.

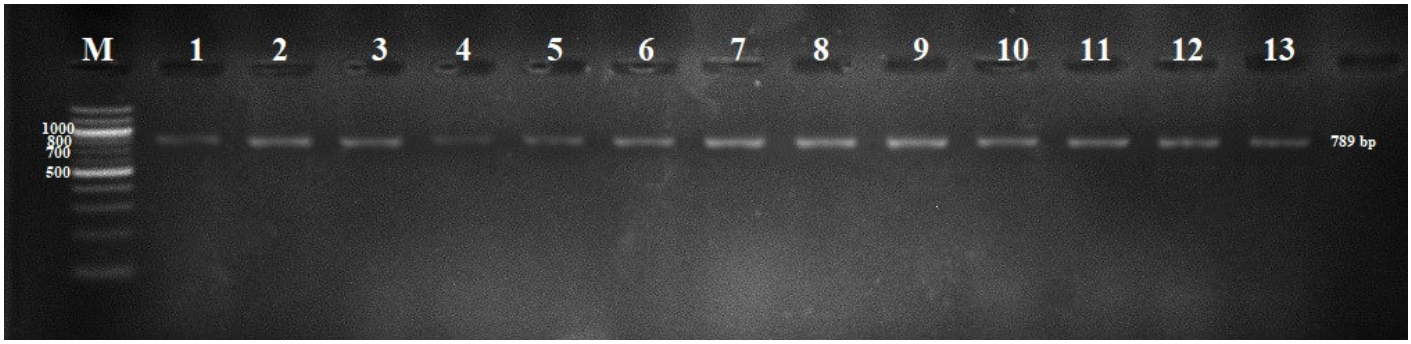

Fig. 2. PCR-based chromosome assignments of the *Dreb1* genes in wheat *Triticum aestivum* L. genotypes using an B genome-specific primer pair P18F/P18R. Arrow shows a 789-bp DNA fragment. M – DNA ladder 100-bp. 1- sham6, 2- Aras, 3- Florkwa, 4- Acsad5, 5-Oasis, 6-Klak2, 7- Rizgary, 8- Adana, 9- Hidhab, 10- Abu ghraib), 11- API99, 12- Site mall, 13- Kauz.

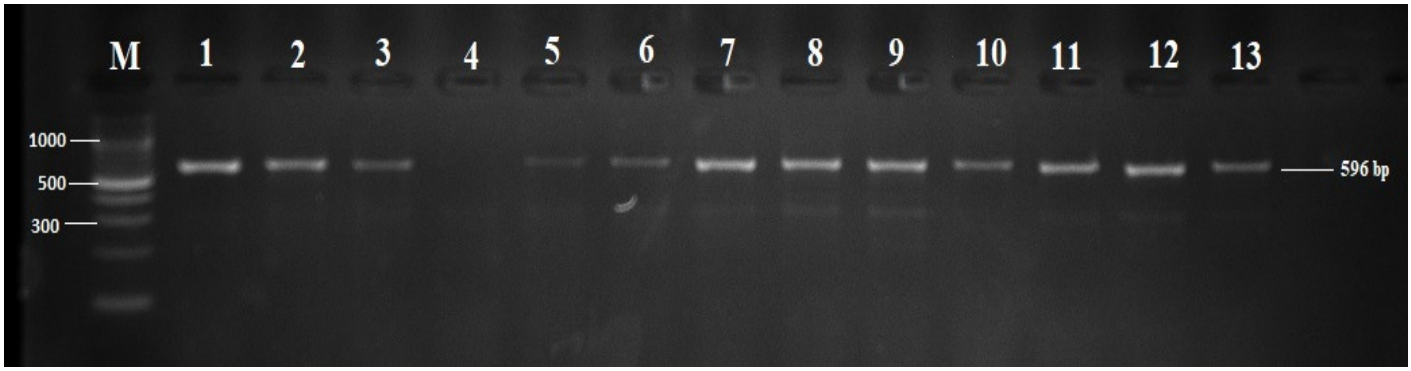

Fig. 3 : PCR-based chromosome assignments of the *Dreb1* genes in wheat *Triticum aestivum* L. genotypes using an D genome-specific primer pair P22F/PRa. Arrow shows a 596-bp DNA fragment. M – DNA ladder 100-bp. 1- sham6, 2- Aras, 3- Florkwa, 4- Acsad5, 5-Oasis, 6-Klak2, 7- Rizgary, 8- Adana, 9- Hidhab, 10- Abu ghraib), 11- API99, 12- Site mall, 13- Kauz.

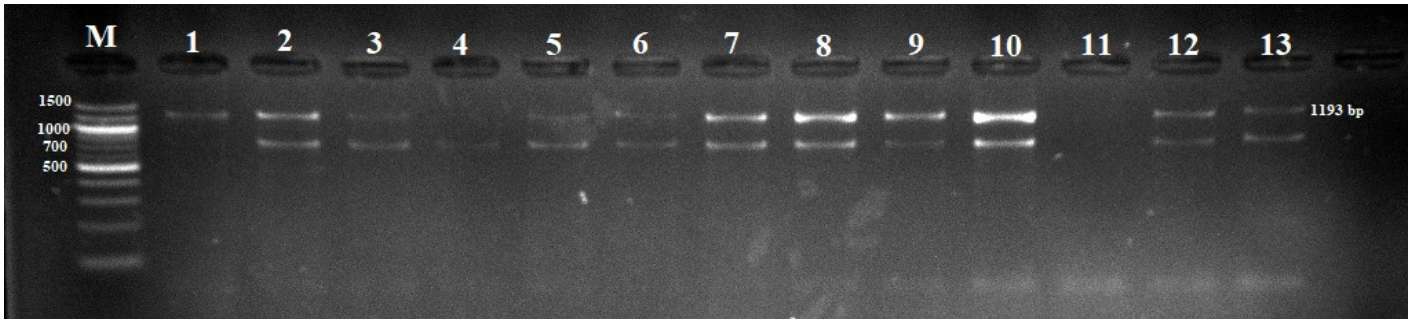

Fig. 4 : PCR-based chromosome assignments of the *Dreb1* genes in wheat *Triticum aestivum* L. genotypes using an D genome-specific primer pair P20F/P20R. Arrow shows a 1193-bp DNA fragment. M – DNA ladder 100-bp. 1- sham6, 2- Aras, 3- Florkwa, 4- Acsad5, 5-Oasis, 6-Klak2, 7- Rizgary, 8- Adana, 9- Hidhab, 10- Abu ghraib), 11- API99, 12- Site mall, 13- Kauz.

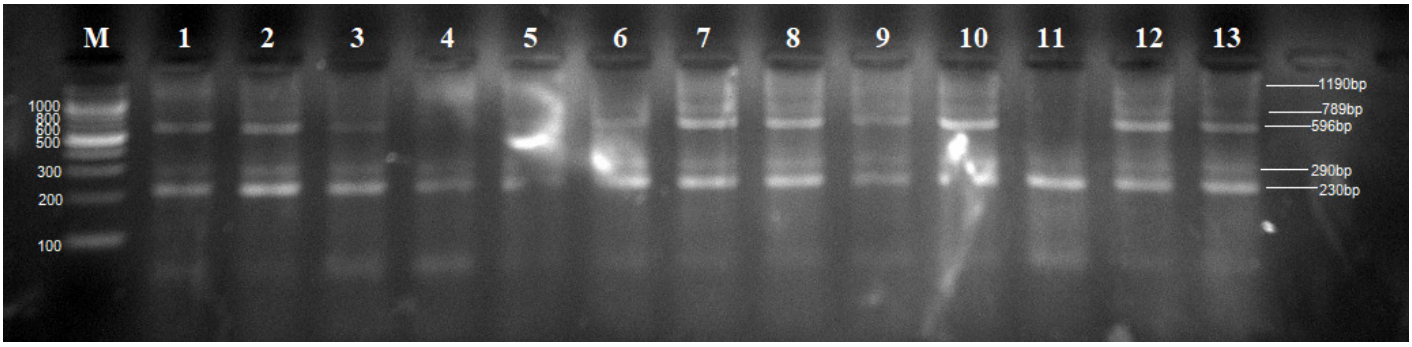

Fig. 5 : Multiplex PCR-based chromosome assignments of the *Dreb1* genes in wheat *Triticum aestivum* L. genotypes, using genomes-specific primer pairs (P25F, P18F, P22F, P20F). M – DNA ladder 100-bp. 1- sham6, 2- Aras, 3- Florkwa, 4- Acsad5, 5-Oasis, 6-Klak2, 7- Rizgary, 8- Adana, 9- Hidhab, 10- Abu ghraib), 11- API99, 12- Site mall, 13- Kauz.

Functional or gene-specific markers have been used extensively to detect resistant genes and desirable traits in plants, it was used in the molecular characterization of resistance genes (L34, Yr18, PM38) in wheat (Wu *et al*., 2010) and was used in the field of Conventional plant breeding and in wheat breeding (Gupta *et al*., 2009; Gupta *et al*., 2010), and in this study was used to detect tolerance genes for drought *Dreb1* in wheat.

This results obtained indicate that most of the genotypes in which the required bands have been appeared with specialized primers contain *Dreb1* genes in the genome (A, B and D), This may indicate that these genotypes are genetically or molecularly capable of resisting stresses as Drought and salinity and cold (Peng *et al.*, 2013).

The absence of bands may indicate a change in the sequence of the wheat genome because of the mutations, the complementary sites of the primers on the genome may have several mutations in their sequences (Huseynova *et al*., 2013), As confirmed by the results of Wei *et al*. (2009). Three amino acid mutations were detected in genome A (47, 151, 184 for common wheat), as well as three single amino acids mutation in genome D (156, 178, 233) in the Areas rich in Ser and Thr.

Also the absence of bands may indicate that the specific sequence and complementary for primers on DNA is not found (Al-Khafaji and Abu Al-Ma'ali, 2013).

About multiplex PCR, we have not got the ideal result due to the sensitivity of multiplex PCR reaction, this reaction requires several conditions where the used primers should have similar melting temperatures as well as their GC content (Garg *et al.,* 2008). Also, they should be specifically designed for this type of reaction to ensure an increase interaction efficiency (Xu and Shang, 2017).

The sequencing of large Cascading genomic regions and by multiplex PCR can generate cross-reaction due to primers overlap, which affects the amplification result. This may require the separation of convergent primers into several multiplex pools (Nikiforova *et al.*, 2015).

Describing and identifying useful genes such as those responsible for granting resistance to the plants as drought-tolerant genes is a key objective to enhance crop productivity. Identification of these genes is very useful and important in detecting the desired genes in new varieties for effective and meaningful selection (Semagn *et al*., 2006; Bibi *et al.*, 2010).

The results obtained may provide us with excellent opportunities to develop stress tolerant crops in the future. These results may also be useful to breeders in wheat breeding programs aimed at improving drought tolerance or resistance.

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