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Cloning and Sequence Analysis of *bZIP* and *WRKY* Transcription Factor Genes in Einkorn, Emmer and Modern Wheat Cultivars

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ABSTRACT

Wheat is an annual herbaceous plant whose breeding is done all over the world. *Triticum monococcum* with 2n chromosomes is known as the oldest wheat. *T. turgidum* has four set of chromosomes and used for pasta making. Emmer wheat (*T. turgidum* ssp. dicoccoides) is wild form of nearly all the domesticated wheats. *T. aestivum* has six set of chromosomes and used for bread making. bZIP transcription factors have DNA binding motifs and form the leucine zipper dimerization. Most of WRKY transcription factors control regulation of important functions for the development of plants. In this study, *bZIP* and *WRKY* genes were firstly cloned and compared between different wheat cultivars. For this, genes were amplified in PCR and transferred to pENTR™/D-TOPO® vector. Plasmids were sequenced and aligned. *bZIP* gene with 450 bp and *WRKY* gene with 672 bp in length were successfully cloned. DNA sequence of genes were translated to protein sequence. A total of 150 amino acids for bZIP and 224 amino acids for WRKY proteins were determined. With this study, sequences of *bZIP* and *WRKY* genes were firstly identified in ancestral and modern wheat cultivars. Obtained results from this study may be used for development of abiotic stress resistance plants.

Introduction

Wheat is a single-year herbaceous plant that has been rehabilitated all over the world. Wheat is a basic nutrient used for the production of flour and feed. Wheat has constituted the most important foodstuff of the people in almost every period. Turkey is among the top ten countries in the world for wheat production. As modern wheat cultivars, *T. aestivum* with six sets of chromosomes (2n = 42) and *T. turgidum* ssp. durum with four sets of chromosomes (2n = 28) are used in bread making and pasta making, respectively. Siyez (*Triticum monococcum*) which is cultivated first known wheat species has 2n chromosome structure. It was first discovered 10,000 years ago in the Near East and is still produced in Kastamonu by traditional methods. The emmer wheat (*T. turgidum* ssp. dicoccoides), which is called as Gernik or Kavlıca wheat, is still being cultivated around Kars today. Gernik is both a wild progenitor of tetraploid wheat and a genetic transmitter of A and B genomes of wheat. To date, studies have shown that Gernik is an excellent source of genes for durum wheat and bread wheat (Cattivelli et al., 2002).

Wheat is one of the three most important grains along with rice and maize and is used by mankind as a food and feed source for more than 5,000 years. According to FAO (2015) statistics, wheat production was approximately 671 million tons in the 215-million-hectare area. Turkey is among the top ten countries in the world in wheat production. However, abiotic and biotic factors cause a loss of about 29% in wheat production (Manickavelu et al., 2012). Therefore, studies on new molecular biology techniques and sequence information of genes obtained from plant genome projects and the development of such stress-tolerant wheat cultivars have gained importance in view of both literature and economic.

Transcription factors (TF) play an important role in controlling genes and gene clusters in plants. TFs such as bZIP (Uno et al., 2000), MBF1 (Zhang et al., 2009), WRKY (Mare et al., 2004), MYB (Jin and Martin, 1999) and NAC (Xue et al., 2009) genes, are known to play a direct role in many metabolic events in plants. It is known that drought and desertification are expected to increase gradually in the 21st century due to reasons such as global warming and wrong land use. For this reason, it is great importance to characterize these transcription factors at the molecular level in the development of plant cultivars that can be grown in low water or arid regions. bZIP transcription factors have a region of DNA that binds to leucine to form the dimerization motif. The bZIP domain consists of two different structures. The α-helix structure is composed of 16 amino acids and constitutes the core localization signal. The N-x7-R / K motif contains leucines consisting of seven repeats that are linked to DNA. Binding of the 2 subunits to the hydrophobic regions of DNA results in a structure known as leucine zipper.Proteins with the bZIP domain are found in all eukaryotes. bZIP proteins are linked to ACGT core regions in DNA. These are known as A-box (TACGTA), C-box (GACGTC) and G-box (CACGTG) (Jakoby et al., 2002). WRKY type transcription factors were first discovered by Ishiguro and Nakamura (1994) in sweet potato. Then gene families of WRKY type transcription factors were characterized in different plants. The WRKY domain consists of 60 amino acid residues

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specific for the WRKY protein. The WRKYGQK sequence is preserved in this domain and followed by the zinc finger motif. The WRKY domain which identified as W box ([T][T]TGAC[C/T]) binds to the target gene promoter and controls transcription (Chen et al., 2012). WRKY type transcription factors are composed of 3 different groups according to WRKY number and domain type. Group I WRKY type transcription factors have two WRKY domains. In Group II WRKY type transcription factors, there is only one WRKY domain. C_2H_2 zinc finger motif is found in Group I and Group II WRKY type transcription factors whereas C_2HC motif is found in Group III WRKY type transcription factors. WRKY type transcription factor family is involved in biotic and abiotic stress regulation in plants. With this study, it is aimed to determine the sequences of genes belonging to bZIP and WRKY type transcription factors found in modern and ancestral wheats. Thus, by identifying the single nucleotide polymorphisms (SNPs) in these genes, we have informed about the structural differences of the proteins encoded by these genes.

Material and Method

Plant Materials and Growth of Plants

In this study, winter wheat variety, Kiziltan-91 (*Triticum turgidum* spp.); summer wheat variety, Yüreğir-89 (*Triticum aestivum* L.); Siyez (*Triticum monococcum*) and Gernik (*T. turgidum* ssp. dicoccoides) were used as plant material. Wheat seeds were kindly obtained from the Ministry of Agriculture and Rural Affairs Central Research Institute (Ankara) and İhsangazi Municipality (Kastamonu). The seeds of Kiziltan-91, Yüreğir-89, Siyez and Gernik wheat cultivars were germinated after surface sterilization. Plants were irrigated with Hoagland solution (Hoagland and Arnon, 1950) in aquaculture environment under controlled environmental conditions (16s light, 8s dark photoperiod, 20/18 °C) for 10 days. Watering of the plants was continued for 3 days with Hoagland solution containing 250 mM NaCl or 20% polyethylene glycol 6000 (PEG-6000) to increase the expression levels of the genes of interest. At the end of the stress, the leaves of the control and stressed plants were collected, frozen in liquid nitrogen and stored at -80 °C for use in RNA isolation.

Total RNA Isolation and cDNA synthesis

Total RNA isolations were made using Trizol (Invitrogen) reagent according to our previous studies (Celik Altunoglu et al., 2017; Celik Altunoglu et al., 2016; Yer et al., 2016; Baloglu et al., 2015; Baloglu et al., 2014a; Baloglu et al., 2014b). RNA concentration and protein contamination were checked by spectrophotometric measurements at 260 and 280 nm with a MultiscanGO nano-spectrophotometer. RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific Fermentas was used for cDNA synthesis from RNA samples. According to this method, 1 µl oligo $(dT)_{15}$ primer (100mM) was added to 1 µg total RNA followed by the addition of nuclease-free DEPC-distilled water to a volume of 12 µl. The tube was incubated at 37 °C for 5 minutes after adding 4 µl 5xM-MuLV reaction buffer, 1 µl RiboLock ™ (20 u/µl) (Fermentas, USA), 2 µl 10 mM dNTP mix. Then, 1 µl of M-MuLV RT (200 u/µl) was added and the cDNA synthesis reaction was performed in the PCR device for 1 hour at 42 °C. For reverse transcriptase process, cDNA samples were incubated at 70 °C for 5 minutes after being cooled on ice and then stored at -80 °C until RT-PCR runs.

Cloning of transcription factor genes into Gateway compatible entry vector

The primers specific for the bZIP-type transcription factor gene, *WLIP19* and the WRKY type transcription factor gene, *TaWRKY10* were designed by the CLC Genomics Workbench version 10.1 bioinformatics program (Table 1). The initial sequence of the forward primers are designated as CACC, which allows the entrance towards the pENTR ™ / D-TOPO® entry vector. In addition, Tm, GC% and ΔG values of the primers were also checked for their suitability.

Table 1. Primer sets to be used in the cloning of genes.

Genes	Gene bank No.	Primers	Product size
TaWLIP 19	AB193552	F: 5`-CACCATGTCGTCGCCGTCGC-3` R: 5`-TCAGAACTGGAACGCGTCAGGC-3`	453 bp
TaWRKY10	HQ700327	F: 5`-CACCATGGCGGCTTCGCTA-3` R: 5`-CTAGGACTCCGAGGAGTGC-3`	669 bp

Genes were firstly amplified in PCR using *Pfu* Polymerase enzyme. PCR conditions of the genes are listed below; denaturation at 95 °C for 30 seconds, annealing at 50 °C to 60 °C for 30 seconds (optimized for gene specific primers) and extension at 72 °C for 60 seconds for 35 cycles and finally at 72 °C 10 minutes for last extension. The PCR product of the genes was run on a 1% agarose gel and bands seen in the expected size were cut and separated by QIAquick Gel Extraction Kit. DNA bands containing purified genes were then transferred to the entry vector pENTR ™ / D-TOPO® from Invitrogen. The recombinant vectors were transferred to competent *E. coli* cells with heat shock. For this, 5 µL of the ligation mixture was added to 50 µL competent *E. coli* cells and this mixture was kept in ice for 30 min. The cells were held in a water bath for 90 seconds at 42 °C for heat shock. Finally, the cells were incubated in the ice for 5 min. This bacterial mixture was then grown at 200 rpm for 60 min in a shaking incubator with the addition of 950 µL SOC medium. 50 µL of bacterial culture was grown on selective LB (Luria Broth, Sigma) agar plates containing kanamycin at 37 ° C for one day. It is expected that resistance to kanamycin in cells where transformation has taken place. Therefore, the grown colonies on the LB medium with kanamycin were selected as positive colonies. Colony PCR was performed with the gene specific primers and the PCR products were run on a 1% agarose gel. Plasmid isolation was performed from the positive colonies and sent to sequencing for both directions. Finally, gene sequences were aligned using the CLC Genomics Workbench version 10.1 bioinformatics program. Sequences of the genes were compared and searched for SNPs.

Results and Discussion

Cloning of *bZIP* and *WRKY* Genes

Table 2. Quantity and purity of RNA samples.

Wheat cultivars	A_{260}	A_{280}	A_{260}/A_{280}	ng/µl
Kızıltan-91	4,507	2,107	2,14	180,3
Yüreğir-89	25,581	11,957	2,14	1023,3
Siyez	12,673	6,01	2,11	506,9
Gernik	6,183	2,956	2,09	247,3

RNAs used in gene cloning and sequencing analysis were isolated using Trizol reagent. The amount and purity of the isolated RNAs are shown in Table 2. Gel electrophoresis was also performed to confirm the quality of the RNA. The appearance of the 25S and 18S bands on the gel proved that the RNA samples did not disintegrate. Fig. 1 shows the quality of RNA samples run on 1% gel. The purity of the RNA samples was evidenced by the appearance of 25S and 18S bands without breaking down. The results obtained from agarose gel electrophoresis were found to be consistent with the spectrophotometric results. As it can be seen in Table 2, no DNA contamination was found.

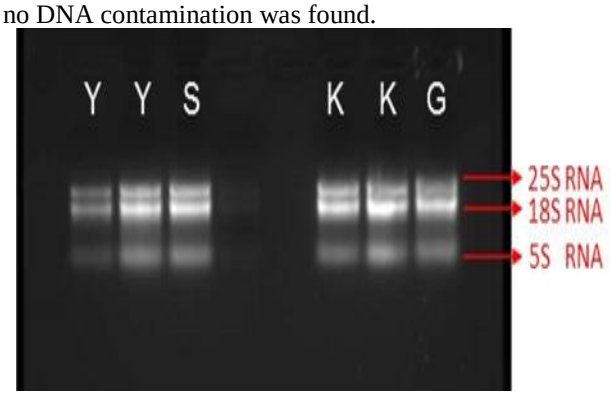

Figure 1. 1% agarose gel electrophoresis results of total RNA samples isolated from K: Kiziltan-91 (*T. turgidum* spp.); Y: Yüreğir-89 (*T. aestivum* L.); S: Siyez (*T. monococcum*) and G: Gernik (*T. turgidum* ssp. dicoccoides) cultivars. The gel was run at 100 V for 40 minutes in 1X TAE solution. Each sample was loaded with 1 µg of total RNA sample.

Firstly, cDNA samples were synthesized from RNA samples. Optimal PCR conditions for cloning of the *bZIP* and *WRKY* genes were determined for each gene separately. All genes were amplified in PCR using *Pfu* Polymerase enzyme with the help of gene specific primers from the cDNAs of Yüreğir-89, Siyez, Gernik and Kızıltan-91 cultivars. The results obtained from the PCR amplification of *bZIP* and *WRKY* genes are shown in Fig. 2 and 3, respectively. *bZIP* gene size cloned from all wheat cultivars was 450 bp. *WRKY* genes had 670 bp. Like *bZIP* gene, there was no change in gene size of *WRKY* genes obtained from ancestral and modern wheat cultivars.

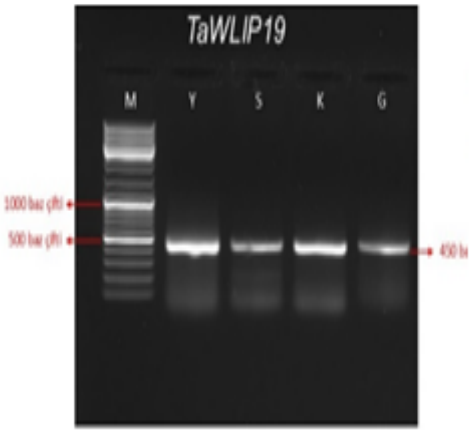

Figure 2. Cloning of *TaWLIP19* gene with the size of 450 bp. K: Kiziltan-91 (*T. turgidum* spp.); Y: Yüreğir-89 (*T. aestivum* L.); S: Siyez (*T. monococcum*) and G: Gernik (*T. turgidum* ssp. dicoccoides) cultivars. The gel was run at 100 V for 60 minutes in 1X TAE solution. Each sample was loaded with 10 µL.

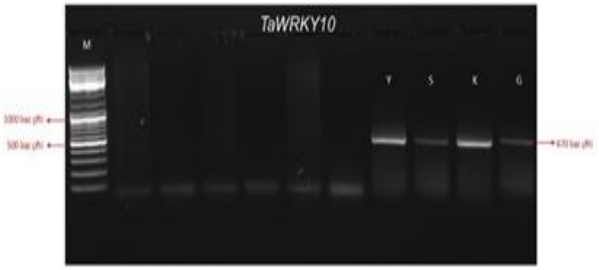

Figure 3. Cloning of *TaWRKY10* gene with the size of 670 bp. K: Kiziltan-91 (*T. turgidum* spp.); Y: Yüreğir-89 (*T. aestivum* L.); S: Siyez (*T. monococcum*) and G: Gernik (*T. turgidum* ssp. dicoccoides) cultivars. The gel was run at 100 V for 60 minutes in 1X TAE solution. Each sample was loaded with 10 µL.

Sequence Analysis of *bZIP* and *WRKY* Genes

Sequence analyzes of the *bZIP* and *WRKY* genes were performed in the form of service retrieval. Sequence analyzes were carried out with both forward and reverse primers for each gene to detect SNPs. Bi-directional readings of the obtained genes were firstly analyzed by CLC Genomics Workbench version 10.1 bioinformatics program. Fig. 4 presents the sequence information of the *bZIP* gene and its comparison with *TaWLIP19* gene. According to gene sequences data from Yüreğir-89, Siyez, Gernik and Kızıltan-91 cultivars, a total of six SNPs were detected in *bZIP* gene.

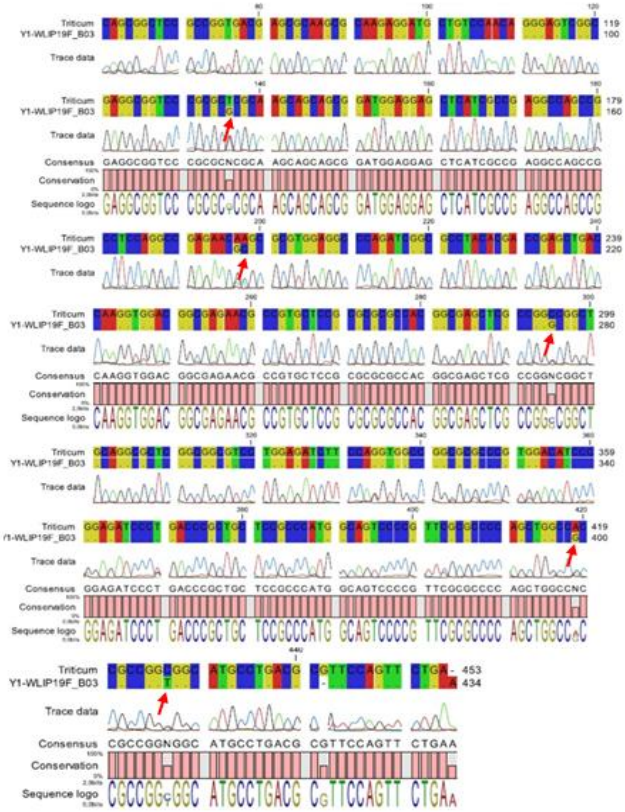

Figure 4. Sequence analysis of *TaWLIP19* genes cloned from *T. turgidum* spp, *T. aestivum* L., *T. monococcum*, *T. turgidum* ssp. dicoccoides cultivars. Red arrows indicate SNPs in the gene sequence.

Although there were SNPs in *bZIP* gene, there was no change in protein sequences of bZIP for each wheat cultivars. In plants, bZIP transcription factors are responsible for the regulation of light and stress signaling, seed ripening, pathogen defense and flower development (Jakoby et al., 2002, Rahaie et al., 2011). It has been reported that the

expression level of the *TaWLIP19* gene, one of the bZIP transcription factors, is increased by low temperature, drought and ABA treatment (Kobayashi et al., 2008). In our previous study, expression profile of *bZIP* gene was examined after drought and salt stress applications in ancestral and modern wheat cultivars (Baloglu et al., 2014a). In detail, *bZIP* genes from Yüreğir-89, Siyez and Kızıltan-91 cultivars were upregulated in 12h time period under the drought condition. In salt stress, *bZIP* genes indicated similar pattern with drought stress. Yüreğir-89, Siyez and Kızıltan-91 *bZIP* genes exhibited gradually increased expression from 3 h to 24 h time point (Baloglu et al., 2014a). In this study, DNA and protein sequences of *bZIP* gene were extracted from Yüreğir-89, Siyez, Gernik and Kızıltan-91 cultivars and compared to each other. These results support our previous research into expression profile of *bZIP* genes in different wheat cultivars which correlate expression profiles and their sequences. It is possible to hypothesize that SNPs in *bZIP* genes of Yüreğir-89, Siyez, Gernik and Kızıltan-91 cultivars did not affect the expression profile of this gene under various abiotic stress conditions. The possible explanation of this is that SNPs did not influence protein sequences of *bZIP* genes.

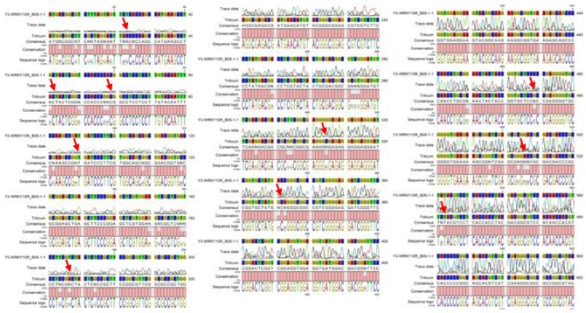

Figure 5. Sequence analysis of *TaWRKY10* genes cloned from *T. turgidum* spp, *T. aestivum* L., *T. monococcum*, *T. turgidum* ssp. dicoccoides cultivars. Red arrows indicate SNPs in the gene sequence.

Fig. 5 indicates the sequence information of the *WRKY* gene and its comparison with *TaWRKY10* gene. According to gene sequences data from Yüreğir-89, Siyez, Gernik and Kızıltan-91 cultivars, a total of ten SNPs were detected in *WRKY* gene. Although there were SNPs in *WRKY* gene, there was no change in protein sequences of WRKY for each wheat cultivars. Many of the WRKY-type transcription factors control the regulation of important functions in plant development such as embryo development in plants, fruit ripening, tan biosynthesis in seed coat, maturation of stem cells, senescence and dormancy. Wang et al. (2013) identified 10 different WRKY-type transcription factors and determined that the expression level of *TaWRKY10* gene was increased after salt, drought, cold and H_2O_2 stress treatments. In our previous study, expression profile of *WRKY* gene was examined after drought and salt stress applications in ancestral and modern wheat cultivars (Baloglu et al., 2014a). It was reported that a gradual increase in expression of *WRKY* genes from Yüreğir-89, Siyez and Kızıltan-91 cultivars was observed under the drought condition. With similar to drought stress expression pattern, expression of *WRKY* genes exhibited upregulation in both ancestral and modern wheat cultivars after salt stress. In addition, the most interesting finding was the expression profile of *bZIP* and *WRKY* genes showed similar expression patterns under both drought and salt stress conditions in Siyez, Yuregir-89 and Kiziltan-91(Baloglu et al., 2014a). In this study, DNA and protein sequences of *WRKY* gene were extracted from Yüreğir-89, Siyez, Gernik and Kızıltan-91 cultivars and compared to each other. These results match those observed in our earlier studies which associate with expression profile and sequences of *WRKY* gene. It is therefore likely that such connections exist between expression profile and SNPs in which *WRKY* genes of Yüreğir-89, Siyez, Gernik and Kızıltan-91 cultivars did not impress the expression profile of this gene under various abiotic stress conditions. The possible explanation of this is that SNPs did not affect protein sequences of *WRKY* genes.

In our study, *bZIP* and *WRKY* genes were firstly cloned to obtain DNA and protein sequence information. Different SNPs regions in the *bZIP* and *WRKY* genes obtained from einkorn, emmer and modern wheat cultivars were detected. However, there were no change in 3D structure of bZIP and WRKY proteins because of no alternation in the protein sequences. Similar expression profile of these TF genes in different wheat species is also proof of this situation. This study provides background information on the characterization of these cloned transcription factor genes and their functional diversity in different wheat cultivars.

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