Research Article

Uluhan, E., E.N. Keleş, and F. Tufan, Analysis of WRKY Transcription Factors in Barley Cultivars Infected with *Fusarium culmorum* International Journal of Life Sciences and Biotechnology, 2019. 2(3): p. 165-174.

Analysis of WRKY Transcription Factors in Barley Cultivars Infected with *Fusarium culmorum*

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ABSTRACT

One of the most critical problems of cereal breeding is *Fusarium* crown rot disease caused by various *Fusarium* species. *Fusarium culmorum* is one of the predominant pathogen in Turkey and causes serious product losses. In this study, the early response of barley cultivars upon *F. culmorum* infection were analyzed by disease severity and gene expression patterns of WRKY transcription factors. In that context, firstly, disease severities of 9 barley cultivars (*Hordeum vulgare* L. cvs. Epona, Escadre, Gazda, Oliver, Avcı 2002, Burakbey, Tarm 92, Manava, and Ramata) infected with *F. culmorum* were determined with disease index percentages. After 7 days of infection, Epona was more sensitive than the other cultivars while the lowest disease index was observed in Gazda. Expression analysis of *HvWRKY6*, *HvWRKY9*, *HvWRKY24*, *HvWRKY25*, *HvWRKY33*, *HvWRKY34*, *HvWRKY42*, and *HvWRKY46* genes were conducted by qPCR at 72 hours after infection in Epona and Gazda. As a result of pathogen stress, it was observed that the transcript level of *HvWRKY33* was significantly upregulated in both cultivars. *HvWRKY6*, *HvWRKY34* and *HvWRKY46* genes were increased in Epona while upregulation of *HvWRKY25* and *HvWRKY34* genes were detected in Gazda. No significant decreases were detected in any cultivars. This study is important in terms of providing an association between *WRKY* genes and pathogen stress response.

ARTICLE HISTORY
Received
08 July 2019
Accepted
21 August 2019

KEY WORDS
Barley, Fusarium culmorum, Fusarium crown rot, WRKY

Introduction

When the pathogens breach the physical barriers of plants, microbial molecules and cell wall derivatives called pathogen/microbe associated molecular patterns (PAMP/MAPM) are recognized by plant membrane pattern recognition receptors (PRR) and plants initiate pattern-triggered immunity (PTI). In order to inhibit PTI, pathogens secrete virulence effectors into the cell and in turn can be recognize by intracellular plant receptors and this activates effector triggered immunity (ETI). The second level of immunity often leads hypersensitive response (HR), a localized form of programmed cell death (PCD)

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preventing pathogen spread and systemic acquired resistance (SAR) [1]. Also, following pathogen infection, the recognition of pathogen results in the generation of reactive oxygen species (ROS). ROS accumulation is closely associated with the induction of defense response. The inducible defense response often allows to expression of a large number of defense related genes include many different types of proteins such as cell wall proteins, hydrolytic enzymes and pathogenesis related proteins, transcription factors (TF), protease inhibitors and signaling compounds (ethylene, jasmonic acid, salicylic acid etc.), enzymes associated with the synthesis of lignin and phytoalexins, and hypersensitive response [2, 3, 4]. TFs play an important role in controlling transcriptional regulation of gene expression in response to stress conditions, in cooperation with other proteins. Transcriptional reprograming is crucial for the plant defense system and help plants overcome different stresses [5]. *WRKY, bZIP, MYB, bHLH, AP2/ERF, NAC* and homeodomain TFs have been shown to participate in the regulation of stress responses [6, 7, 8, 9, 10]. WRKYs have vital roles in plant defense against abiotic and biotic stresses as well as involved in many developmental processes such as seed development, dormancy, leaf senescence, and trichome development and some signal transduction processes mediated by plant growth regulators [11, 12, 13]. WRKYs have conserved 60 amino acid regions comprising of the highly conserved *WRKYGQK* peptide sequence and a zinc finger like motif. WRKYs bind specifically to the [(C/T)TGAC(T/C)] sequence also known as W-box elements and are able to regulate expression of target genes containing these sequences in promoter regions [5]. WRKYs can act as positive or negative regulators in defense response. Numerous studies have shown that WRKYs play important role in PR gene expression and SAR-associated process [14, 15, 16, 17, 9, 18]. By the phylogenetic and comparative gene expression analysis, 45 *WRKY* family members were identified in barley [19].

Fusarium crown rot (FCR) is a destructive disease of cereals including wheat and barley. Researchers conducted in Turkey revealed that *F. culmorum* shows high prevalence among the *Fusarium* species causing the disease [20, 21]. This pathogen significantly reduces product yield and quality as well as produce mycotoxins such as deoxynivalenol and zearalenone that are harmful to human and animal health [22, 23]. In this study, firstly, we aimed to investigate the early phenotypic response of 9 barley cultivars to *F. culmorum* infection with disease index (DI) (%). Secondly, we comparatively analyzed

the expression profiles of 8 *WRKY* genes (*HvWRKY6*, *HvWRKY9*, *HvWRKY24*, *HvWRKY25*, *HvWRKY33*, *HvWRKY34*, *HvWRKY42* and *HvWRKY46*) in root tissues of Epona and Gazda cultivars at 72 hours after infection (hai).

Materials and Methods

Plant and fungal material

Seeds of barley cultivars (*Hordeum vulgare* L. cvs. Avcı 2002, Burakbey, Epona, Escadre, Gazda, Manava, Oliver, Ramata and Tarm 92) used in the study were obtained from Istanbul Yeni Yüzyıl University and commercial companies. *F. culmorum* F16 isolate obtained from the culture collection of the Department of Plant Protection, Faculty of Agriculture, Ondokuz Mayıs University, was used in pathogen stress experiments.

Pathogen infection of barley cultivars and assessment of FCR

Pathogen infection was conducted according to Covarelli et al. [24]. Briefly, seeds of barley cultivars were disinfected with 0.64 % sodium hypochlorite and 10 % ethanol for 5 min and washed three times with ddH_2O. Then, seeds were placed between filter papers soaked with ddH_2O and germinated for 2 days in dark (at 4 °C for one day then at 25 °C for 2 days). Prior to infection applications, F16 was grown for 7 days in PDA medium. The main roots of 3-day-old barley seedlings were infected with the discs of F16 and the pathogen-free discs were used as control. Seedlings were grown under controlled conditions for 7 days (25 ºC, 6/8 h of day/night cycle). Sixteen seedlings were used in each experiment with three replicates. Barley cultivars were examined phenotypically for FCR disease with browning index scale ranged from "0" to "4". DI percentages of barley cultivars were calculated using the formula DI (%) = Σ(Rn x X / 4N) x 100 (Rn: number of plants in the category; X: scale value of each plant; N: assessed for each cultivar).

Molecular analysis

Epona and Gazda cultivars were selected according to the DI% for further molecular analysis. Roots of six seedlings were sampled at 72 hai and were flash-frozen and powdered in liquid nitrogen. 50 mg of roots were used for total RNA extraction with NucleoSpin® RNA kit (Macherey-Nagel). The quantities and purities of total RNAs were determined by spectrophotometer (Multiskan GO, Thermo Scientific). First strand

cDNAs were synthesized from 500 ng of total RNA (NEB; E6300S). cDNAs were diluted 1:2 in nuclease free water for further analysis by qPCR and stored at −20 °C. qPCR was conducted using SensiFast™ SYBR No-Rox Kit (Bioline, UK) on BioRad CFX Connect™ Real-Time PCR Detection System. qPCR reactions contained 10 µl SensiFast™ SYBR No-Rox mix (1X), 0.4 µM of each primer, and 2 µL of cDNA corresponding to 25 ng total RNA. qPCR was preceded by a polymerase activation step at 95 °C for 2 min, followed by 40 cycles of 5 sec at 95 °C, 10 sec at 58 °C and 10 sec at 72 °C. Melting curve analysis was performed at the end of cycling. Two technical and two biological replicates were performed in the experiments. ADP-ribosylation factor 1-like protein and actin were examined for expression stability under pathogen stress as housekeeping genes. Primer sets of *WRKY* genes were listed in Table 1. Fold changes in gene expressions were determined by $2^{-\Delta\Delta Cq}$ method [25].

Table 1 List of primer sequences used for WRKY expression analysis by qPCR

Genes	NCBI accession	Primer sequences (5'-3')	Amplicon size (bp)
HvADP-ribosylation factor 1-like protein F	AJ508228.2	GACATCTGGTGAAGGGTTGT	95
HvADP-ribosylation factor 1-like protein R		CATTCCTCGAAGCAGTCCTC	
HvActin F	AY145451.1	GGCACACTGGTGTCATGGT	90
HvActin R		GCGCCTCATCACCAACATA	
HvWRKY6 F	EF488106.1	CGAAGGTCATTGTGCTGTTG	101
HvWRKY6 R		CTGTACCCATCGCTCATCTT	
HvWRKY9 F	DQ840408.1	AGGTTTCAGCTCATGCACCA	106
HvWRKY9 R		TGACACCCTTGCCACCACTA	
HvWRKY24 F	DQ863108.1	CATGAGCAGAGCACCATCT	110
HvWRKY24 R		GACATCATCCGCACCTGTAT	
HvWRKY25 F	DQ863109.1	CATCATGGAGGTCCAAGCAA	114
HvWRKY25 R		ACCCGACAATGTCCTTCTGG	
HvWRKY33 F	DQ863117.1	CTGCAACTTTCCCAGGTACT	96
HvWRKY33 R		GGGTCGCTGTGATCTTTCT	
HvWRKY34 F	DQ863118.1	AACCAACAGAGCGACATAGG	98
HvWRKY34 R		CTGTCGGTCTCCATCTTGAC	
HvWRKY42 F	DQ863125.1	AGTGAAGGACAGTGCTGATG	104
HvWRKY42 R		GGTCTTCCTCGTTCTCTTCC	
HvWRKY46 F	AY323206.1	ATTCGCCTGGTATGGTTGAG	106
HvWRKY46 R		TCCTCCTCCTCAGTAGCATC	

Statistical analysis

The statistical analyses related to DI % were performed using the one-way analysis of variance (ANOVA) with least significance difference (LSD) test function at $P \leq 0.05$ in R 3.1.3 statistical software with RStudio (Version 0.98.1103) and the package agricolae (**P < 0.05, ** P < 0.01, *** P < 0.001*).

Results

Pathogen infection of barley cultivars and assessment of FCR

F.culmorum hyphal growth on the root surfaces of the seedlings and necrotic formations of FCR disease were observed in all cultivars at 7 day after infection (dai). Infected seedlings were significantly suppressed in the roots and shoots compared with control groups. Among the barley cultivars infected with *F.culmorum*, Epona was selected as relatively sensitive cultivar according to the DI with 80.8 %. With a rate of 53.3 %, Gazda was a contrast cultivar with a lower DI than the other 8 cultivars (Fig. 1). A significant difference was found in Epona in terms of DI compared with other cultivars and Epona and Gazda were selected for further molecular studies.

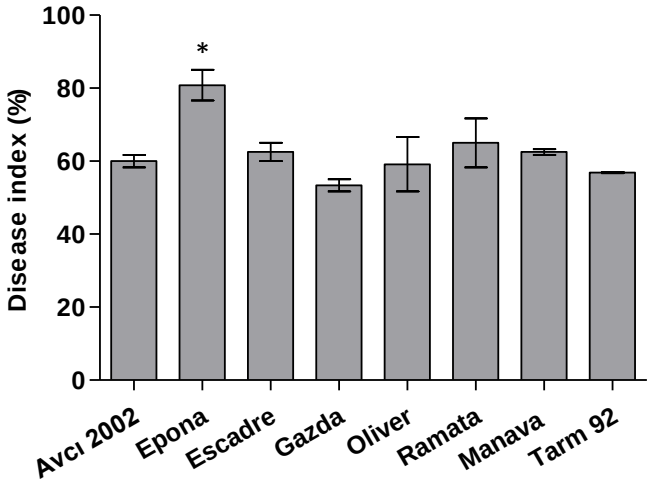

Fig 1 Determination of DI % at 7 dai in barley cultivars (**P < 0.05*)

Molecular Analysis

We have analyzed the gene expression profiles of selected *WRKY* genes at 72 hai in Epona and Gazda roots with qPCR. We compared the expression stability of two housekeeping genes (actin and ADP-ribosylation factor 1-like protein) as a reference and we found that ADP-ribosylation factor 1-like protein was more suitable in FCR for normalization of qPCR [26]. According to qPCR results, significant increases were detected in expression of *HvWRKY6* (4.6 fold), *HvWRKY33* (7.9 fold), *HvWRKY34* (2.7 fold) and *HvWRKY46* (2.6 fold) in Epona upon infection while there was a small but not significant decreases in expressions of *HvWRKY9*, *HvWRKY24* and *HvWRKY42*. In terms of Gazda, the transcript levels of *HvWRKY9, HvWRKY42, and HvWRKY25* showed a slight, but not a significant decrease in stress groups compared to control. However, *HvWRKY25* (7.7 fold) and *HvWRKY33* (5.7 fold) transcript levels were significantly increased in Gazda (Fig 2). Transcript level of *HvWRKY33* was significantly upregulated in both cultivars.

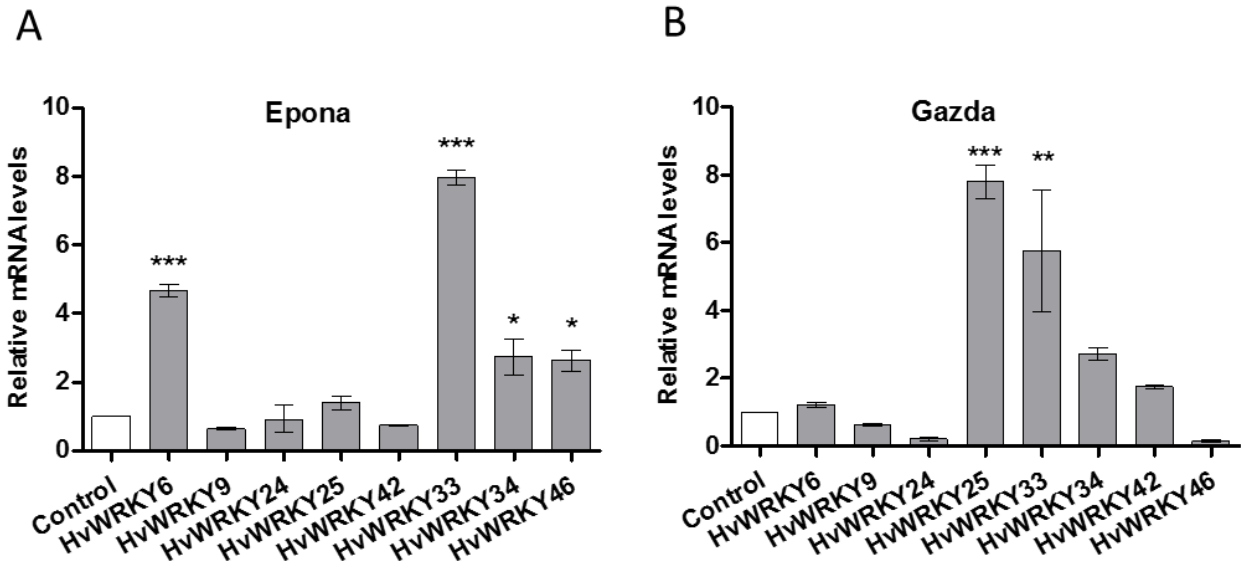

Fig 2 Determination of relative mRNA levels of *HvWRKY* in root tissues of Epona (A) and Gazda (B) at 72 hai (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Discussion

F. culmorum is a destructive pathogen which causes FCR especially in small grain cereals such as barley and wheat. FCR reduces product yield and quality, as well as contamination of grains with mycotoxins pose serious threats to human and animal health. The use of fungicides in the chemical prevention of FCR is not an effective approach

because of the fact that pathogenic fungi have large populations and are highly immune to these fungicides. At the same time, these fungicides can cause problems such as environmental pollution and phytotoxicity [27]. *Fusarium* tolerant cultivars are the most effective and economical approach. However, there are limited reports on germplasm screening [28, 29]. Development of tolerant cultivars requires time and resistance is not permanent as pathogen often evolves to overcome host resistance. WRKYs can be successfully used in marker assisted selections to develop new cultivars with improved FCR tolerance. In this study, we firstly analysed the early responses of barley cultivars to *F. culmorum* with the infection of roots via plate assay. The assay is successfully used in several studies including wheat, barley and oat [30, 31, 32, 33].

WRKYs play important roles by controlling the expression of genes involved in various biological processes and biotic and abiotic stress responses [34, 9, 35]. There are limited reports on WRKYs on biotic stress response in barley. In a previous study by Meng et al. time-course expression profiles of 26 *HvWRKY*s including *HvWRKY6*, *HvWRKY9*, *HvWRKY42* and *HvWRKY46* were analyzed to investigate their role in mildew locus a (Mla)-mediated immunity to *Blumeria graminis* f. sp. Hordei. They found that 12 *HvWRKY*s were differentially expressed: with 10 highly upregulated and 2 significantly downregulated [36]. They conducted loss- and gain-of-function studies and demonstrated that *HvWRKY10*, *HvWRKY19* and *HvWRKY28* positively regulate the barley transcriptome in response to *B. graminis* infection. Liu et al. demonstrated that *HvWRKY1* and *HvWRKY2* repress the activity of the powdery mildew-induced promoter of *HvGER4c* [37]. In another study on *Fusarium* head blight (FHB) in barley, *HvWRKY23* was shown to modulate defense response and enhance resistance against FHB [38]. We analyzed *F. culmorum* related biotic stresses on the relative mRNA levels of 8 WRKYs in two barley cultivars Epona and Gazda. Regarding to our qPCR results, *HvWRKY33* was significantly upregulated in both cultivars. There are no previous reports regarding the effect of the *HvWRKY33* on stress response. In a previous study by Gao et al. *HvWRKY6*, *HvWRKY40* and *HvWRKY70* have exerted positive effects on wheat resistance to *Puccinia triticina* [39]. In our study, *HvWRKY6* was significantly increased upon infection in a relatively sensitive cultivar Epona while no significant change was detected in Gazda. *OsWRKY82* is orthologous with *HvWRKY6* and in a previous report was shown to induced by inoculation with *M. grisea* and *Rhizoctonia solani* [40].

Conclusion

Pathogen tolerance responses involved in complex transcriptional networks and the underlying mechanisms are largely unclear. These results may be helpful for defense response regulation by *WRKY*s upon pathogen infection in barley and determination of FCR tolerant cultivars. Further gene silencing and over-expression studies will contribute our understandings on the role of *WRKY*s in defense response to pathogen tolerance.

Acknowledgements
This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) 2209-A (Project no: 1919B011702097).

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