Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: P. Vosough-Mohebbi, M. Zahravi, M. Changizi, S. Khaghani, Z.-S. Shobbar (2020) Identification of the differentially expressed genes of wheat genotypes in response to powdery mildew infection. *Caryologia* 73(2): 63-72. doi: 10.13128/caryologia-752

Received: December 3, 2019

Accepted: April 3, 2020

Published: July 31, 2020

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Identification of the differentially expressed genes of wheat genotypes in response to powdery mildew infection

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Abstract. Bread wheat (*Triticum aestivum* L.) is the most widely grown crop worldwide. Powdery mildew caused by fungal pathogen *Blumeria graminis* is one of the most devastating diseases of wheat. The present study aimed to identify differentially expressed genes and investigate their expression in response to *B. graminis* in susceptible (Bolani) and resistant (KC2306) wheat genotypes, using publicly available microarray data set and qRT-PCR analysis. A total of 5760 and 5315 probe sets were detected which 5427 and 4630 by adjusted P-value < 0.05 and 168 and 144 genes based on e-value < 1×10^{-5} cut-off were differentially expressed in susceptible and resistant wheat genotypes, respectively. Among exclusively up regulated genes in the resistant genotype 12 hpi compared to its control, fifteen potential genes that may be responsible for *B. graminis* inoculation resistance were detected. The results of real time PCR for the candidate genes showed that the genes were upregulated in the resistant genotype 12 hpi compared to its control, which validated the results of microarray analysis. The *bZIP*, *ERF*, and *ARF1* genes may play an important role in *B. graminis* resistance. The powdery mildew responsive genes identified in the present study will give us a better understanding on molecular mechanisms involved in *B. graminis* resistance in different wheat genotypes.

Keywords: wheat, genotype, powdery mildew, microarray, qRT-PCR.

INTRODUCTION

Bread wheat (*Triticum aestivum* L, AABBDD 2n = 42) is the most widely grown crop in the world, belongs to Poaceae family (Chen S *et al.* 2018). It occupying 17% of all the cultivated land and providing approximately 20% of globally consumed calories (Gill *et al.* 2004; Vetch *et al.* 2019). In

Iran, more than 39% of all cultivated lands belongs to wheat, as the most important human food (Abdollahi 2008). There are many biotic and abiotic stresses which affecting the quality and quantity of crops in the country (Sheikh Beig Goharrizi *et al.* 2016; Sheikh-Mohamadi *et al.* 2018; Sanjari *et al.*, 2019). Powdery mildew caused by fungal pathogen *Blumeria graminis* is one of the most devastating diseases of wheat, occurring in regions with cool and humid climate that particularly is very conducive for the development of this disease (Chang *et al.* 2019; Liu N *et al.* 2019). Iran is one of the most important primary centers of the wheat distribution, thereby it has one of the richest wheat germplasm worldwide. Therefore, the wild wheat relatives in the area could be a source of novel resistance genes to be transferred into wheat cultivars (Pour-Aboughadareh *et al.* 2018). Also, this rich wheat germplasm can be used as a valuable material for better understanding of the molecular mechanisms involved in the wheat-pathogen interaction (Brunner *et al.* 2012). Traditional breeding has been used to transmitting resistant genes to susceptible wheat cultivars, by this method some powdery mildew resistant genes were discovered (Xin *et al.* 2012). Traditional breeding is based on the phenotype, therefore less information can derived and some traits, such as disease resistance, cannot be observed easily (Chen H *et al.* 2014). The microarray technology provides us a lot of information about the genes. Recognizing new genes and analyzing their expression in response to powdery mildew will provide a valuable molecular information for enhancing disease resistance in the plant and microarray analysis could provide a plethora of gene expression profiles (Xin *et al.* 2011). Microarray has been widely used to detect the pathogen-resistant genes in wheat responding to different plant diseases. Li *et al.* (2018) identified 36 Lr39/41-resistance related differentially expressed genes at 48 h post inoculation (hpi) in leaf rust resistant and susceptible wheat isogenic lines. Foley *et al.* (2016) investigated the differentially expressed wheat genes in response to the *Rhizoctonia solani* isolate (AG8) and identified a significant number of genes involved in reactive oxygen species production and redox regulation. Erayman *et al.* (2015) examined the early response to *Fusarium* head blight in moderately susceptible and susceptible wheat cultivars at 12 hpi using microarray technology. The authors reported that 3668 genes were differentially expressed at least in one time comparison, which the majority of them were associated with disease response and the gene expression mechanism. Putative transcription factor (TF) genes constitute 7% of all plant genes, they are proteins which play a major role in gene expression regulation (Yazdani *et al.*, 2020). Xin *et al.* (2012) examined the leaves transcriptomes before and after *B. graminis* inoculation in a susceptible and its near-isogenic wheat line and compared the result of microarray with qRT-PCR analyses. Since Iran has one of the richest wheat germplasm in the world however, limited studies has performed regarding pathogen resistant genes in the germplasm. Therefore the present study aimed to identify differentially expressed genes and investigate their expression in response to *B. graminis* in both susceptible and resistant wheat genotypes, using publicly available microarray data set and qRT-PCR analysis.

MATERIALS AND METHODS

Plant material and growth condition

Two susceptible (Bolani) and resistant (KC2306) wheat genotypes to powdery mildew, were obtained from the National Plant Gene-bank, Karaj, Iran. The seeds of these two genotypes were planted in 10 cm diameter pots, at greenhouse condition. At the first fully expanded leaf stage, the plants inoculated with a pathotype (*B. graminis*) collected from Moghan, Ardabil, Iran (a local pathotype). Inoculation was performed by dusting or brushing conidia from neighboring sporulating susceptible seedlings onto the test seedlings. Leaf samples of each genotype were collected at 0 and 12 hpi. The samples were stored in liquid nitrogen for qRT-PCR analysis.

In silico powdery mildew gene expression survey

In the present study we used a publicly available microarray data set published by Xin *et al.* (2011), available at GEO (http://www.ncbi.nlm.nih.gov/gds/) with GSE27320 accession number. The authors used a susceptible wheat cultivar '8866' and it's near isogenic line with single powdery mildew resistance gene. The differentially expressed sequences were homology searched against wheat transcription factor (TF) database (http://planttfdb.cbi.pku.edu.cn/), and then BLAST analysis with a strict cutoff e-value $<1 \times 10^{-5}$ was performed. Venn diagram was generated to find the differentially expressed genes between the two genotypes and the TFs which up-regulated in the resistant genotype were selected. To confirm the authenticity of the selected TFs, Pfam proteins family database (https://pfam.xfam.org/) was used. Also, the hierarchical cluster algorithm was performed on the differentially expressed genes among the samples.

Table 1. List of primer pairs using for validation of gene expression using qRT-PCR.

PID	Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
TaAffx.51406.1.S1_at	*GATA*	GAAGTCAAACCCTCCCTCAAG	GCAAACAAACATCTCACATTTCC
Ta.9390.1.A1_at	*ARF*	GAGATCGCCCGTCTTTAGC	ACCAACACTACATTCAAACAAAG
TaAffx.78909.1.S1_at	G2	GGTCTCTCGCTCGGTCTC	CTCATCCACTTGTTCTTCATCG
TaAffx.85891.1.S1_at	bHLH	AAGGTGCTGGAGAATCAAGG	CTCATTGTTCGCTGGGTTC
Ta.25219.1.A1_at	ARF	ACTACTACAACATTTCCTCGTATC	GACAACTGACACTGTATTCTGG
Ta.4054.2.S1_at	ARR	GCTGTTACTGTTTGTCCTTCTG	TCTTGTCTCATTCCACCATCC
TaAffx.36896.1.A1_at	MYB	CAATGTCGTCAAGAAGGAAGA	CCGTCGTGCTGAGAAACC
TaAffx.37068.1.S1_at	GRF	CGGAACCTACTACGACCATC	GATTCAGATTGCCTCAACATAG
TaAffx.120915.1.S1_at	FAR1	TTTACCAGTGATGTTCTTTTCT	CTCCAGGGTGTCCAATGC
TaAffx.129201.1.S1_s_at	C3H	AAATGGGAAATTGGACAGATACC	CATAGAAAGAGACCACATAAAGG
Ta.7033.1.S1_s_at	HB	AATGAAGCACATGACGACAAG	ACCGACAATCCAACACTCTG
Ta.21124.1.S1_x_at	ERF	TCCGCCAACCAACTGTTAG	CAGTCATCGTCGCCAAAGC
Ta.6443.2.S1_at	bZIP	AAACGGCGAACAACACAGG	ACCATCAAGGAGAACAACAAC
Ta.4828.1.S1_a_at	C3H	ACTGCTCGTCGTCTCCTAC	TGCGTAATGCTACTACTGATTC
Ta.2023.1.S1_at	bHLH	GCCATAACGCACATCACTG	ATTACACGAACAAGAACCTCA
Reference gene	actin	GTGTACCCTCAGAGGAATAAGG	GTACCACACAATGTCGCTTAGG

RNA extraction and qRT-PCR analysis

In order to extract the total RNA of the samples, Trizol reagent (Invitrogen, USA), was used. In addition, total RNA was treated using RNase-free DNase (Geneall, Korea) and reversely transcribed to double-stranded cDNAs using oligo $(dT)_{18}$ primers by cDNA synthesis kit (Takara, Japan). Oligo software was used to design gene-specific primers for 15 selected genes (Table 1). The qRT-PCR was performed on a Bio-Rad, MiniOpticon Real-Time PCR detection system using SYBR Green Supermix (Takara, Japan). The reactions were performed using the following program: 95 °C for 5 min and 40 cycles (95 °C for 30 s, 57 up to 61°C for 30 s and 72 °C for 30 s). Wheat *Actin* gene was used as the internal control. For each data point, the C_T value was the average of C_T values derived from three biological and three technical replicates, were normalized based on the Ct of the control products (*Ta actin*). The relative quantitative analysis preformed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008) then subjected to a complete random design (CRD) and least significant difference (LSD) test using SAS software package (SAS Institute Inc.). The correlations were visualized as a colored heat map. The heat map and the bi-plots were created by MetaboAnalyst (Xia and Wishart 2016). Also, the graphs were performed using GraphPad Prism software. In addition, the gene expression of powdery mildew-sensitive genotype under normal condition was used as calibrator for calculating the relative gene expression.

RESULTS AND DISCUSSION

Microarray analysis

In order to identify the differentially expressed genes in susceptible and resistant wheat genotypes responding to *B. graminis*, the publicly available microarray data set published by Xin *et al.* (2011) was used. The reproducibility of the microarray data was confirmed by the authors. Based on the BLAST of the differentially expressed sequences against wheat transcription factor (TF) database, 5760 and 5315 probe sets were detected which 5427 and 4630 were differentially expressed by adjusted P-value <0.05, and 168 and 144 genes were differentially expressed based on e-value <1 × 10–5 cut-off in susceptible and resistant wheat genotypes, respectively (Fig. 1). Among 168 and 144 DEGs, 70 and 56 DEGs were upregulated and 98 and 88 DEGs were downregulated, respectively (Fig. 2). In previous study, of the total of 61,127 probe sets, 44.57 and 42.43% were detected as expressed genes in susceptible and resistant wheat genotypes at 12 hpi by *B. graminis*, respectively (Xin *et al.* 2011). According to results of present and previous studies, many genes are involved in wheat resistance to this disease. In another study, Bruggmann *et al.* (2005) studied the epidermis-and mesophyll-specific transcript accumulation in powdery mildew-inoculated wheat leaves. The authors reported that out of 17000, 141 transcripts, were found to accumulate after *B. graminis* f. sp. *hordei* inoculation using microarray hybridization

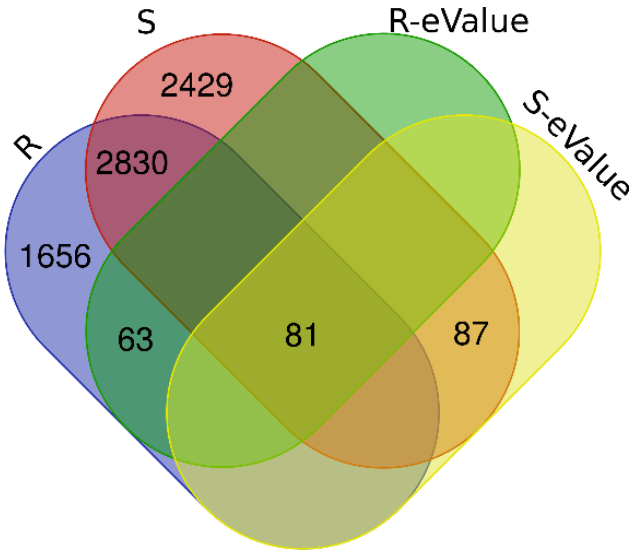

Figure 1. Venn diagrams showing the common and unique differentially expressed genes in S (red) and R (blue) genotypes detected (P-value < 0.05) and S-evalue (yellow) and R-evalue (green) based on e-value <1 × 10^{-5} cut-off.

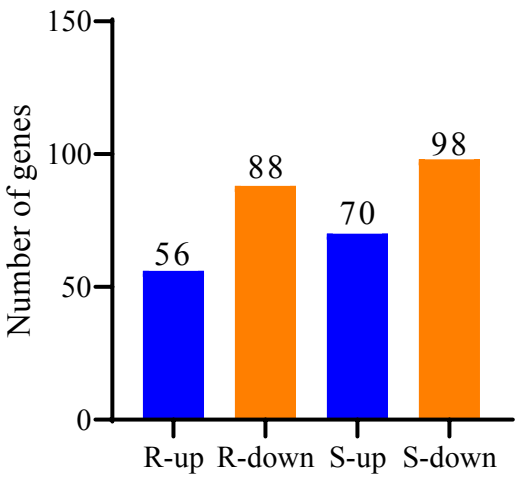

Figure 2. Number of up- and down-regulated genes responsive to powdery mildew in susceptible and resistant genotypes.

analysis. Our results were consistent with the previous studies that pathogen infection activates a wide range of genes and pathways in the transcriptional networks in wheat plant (Bolton *et al.* 2008; Coram *et al.* 2008; Bozkurt *et al.* 2010). Among exclusively up regulated genes in the resistant genotype 12 hpi compared to its control, potential genes that may be responsible for *B. graminis* infection resistance were detected (Table 2). For example, *G2* (TaAffx.78909.1.S1_at) was the top upregulated gene with a $\log_2$ fold change of 3.39, and its expression might has a function in stress and disease resistance (Liu F *et al.* 2016; Zeng *et al.* 2018). The *bHLH1* (TaAffx.85891.1.S1_at) was also in the top fifteen upregulated genes with a $\log_2$ fold change of 3.08, the plant *bHLH* transcription factors family have key function in regulation of developmental processes and environmental stresses (Wang *et al.* 2015). However, limited information is available on their roles in wheat disease caused by pathogens infection. Another bHLH family member, *bHLH2*, was also revealed to be upregulated in resistant genotype.

Table 2. Top 15 exclusively upregulated genes in the resistant genotype 12 hpi.

Probe set ID	$\log_2$ (fold change)	Adj. P-Value	P-Value
TaAffx.51406.1.S1_at	1.4868	0.0354	0.0023
Ta.9390.1.A1_at	1.2165	0.0171	0.0006
TaAffx.78909.1.S1_at	3.3896	0.0329	0.0021
TaAffx.85891.1.S1_at	3.0839	0.0149	0.0004
Ta.25219.1.A1_at	2.2206	0.0198	0.0008
Ta.4054.2.S1_at	1.0219	0.0383	0.0027
TaAffx.36896.1.A1_at	1.0314	0.0462	0.0037
TaAffx.37068.1.S1_at	1.7241	0.0449	0.0036
TaAffx.120915.1.S1_at	1.2725	0.0151	0.0004
TaAffx.129201.1.S1_s_at	1.1133	0.0411	0.0031
Ta.7033.1.S1_s_at	1.1329	0.0184	0.0006
Ta.21124.1.S1_x_at	1.7122	0.0143	0.0004
Ta.6443.2.S1_at	1.5355	0.0107	0.0002
Ta.4828.1.S1_a_at	1.0126	0.0397	0.0029
Ta.2023.1.S1_at	1.3044	0.0158	0.0005

Gene expression analysis

Fifteen potential genes that may be responsible for *B. graminis* infection resistance were selected for gene expression analysis by real-time PCR. The genes including *ARF1*, *HB*, *C3H2*, *C3H1*, *bZIP*, *MYB*, *ARR*, *bHLH1*, *G2*, *FAR1*, *bHLH2*, *GATA*, *ERF*, *ARF2* and *GR* were selected among exclusively up regulated genes in the resistant genotype 12 hpi. The result of gene expression indicated that all the selected genes were upregulated in the resistant genotype, and *HB*, *C3H2*, *C3H1*, *MYB*, *ARR*, and *bHLH1* genes were downregulated in the susceptible genotype at 12 hpi compared to their controls (Fig 3). Based on the results of cluster analysis, the top fifteen genes were classified in four main groups (Fig. 4). The *bZIP* and *ERF* genes formed the first group, which their expressions were significant at 12 hpi in both genotypes compared to their control conditions (before inoc-

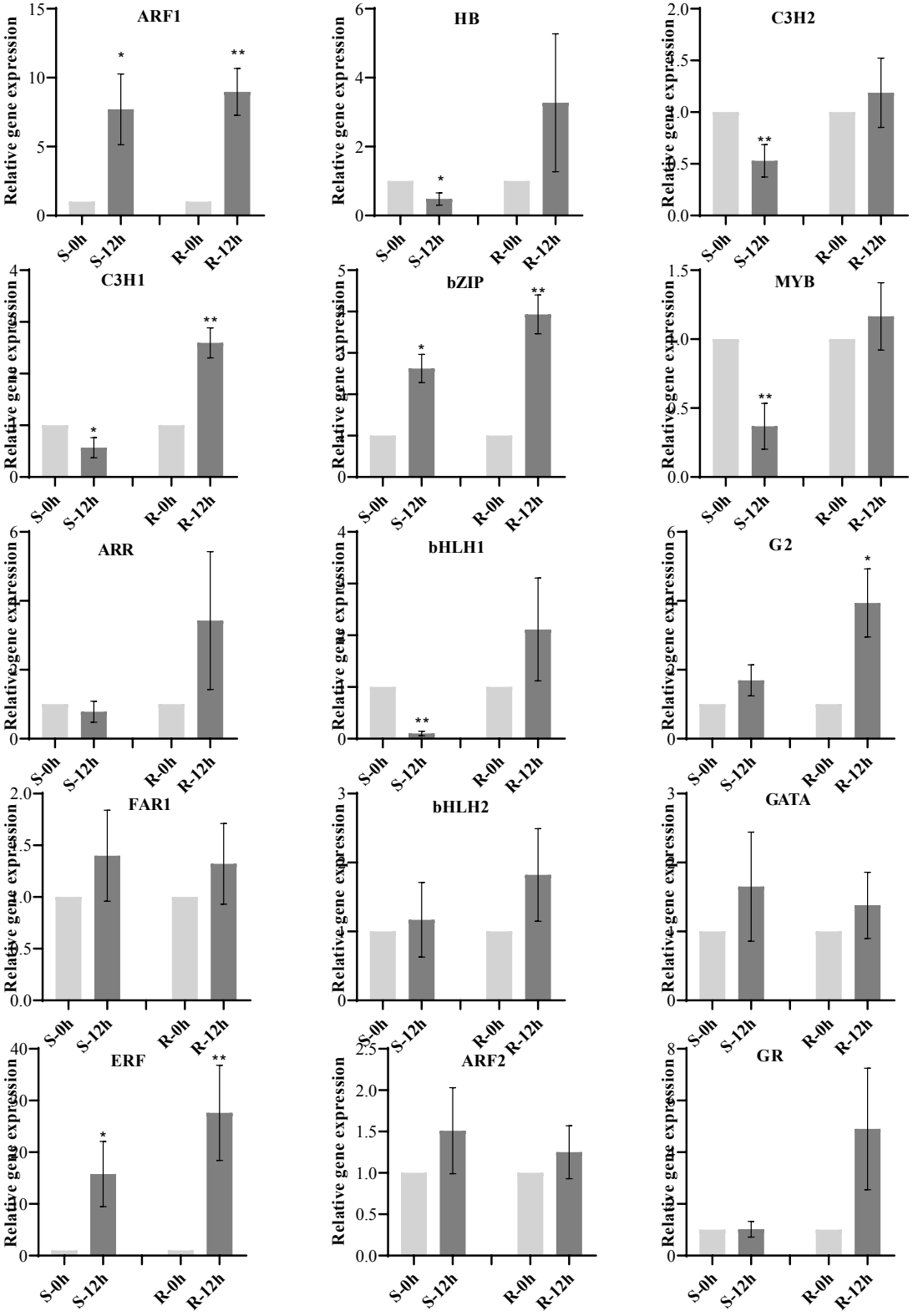

Figure 3. Expression patterns of the top 15 genes by qRT-PCR. Student's t-test was performed to analyze the changes in the gene expression 12 h after powdery mildew infection compared to 0 h in respective genotype. * and ** are statistically significant at 0.05 and 0.01 levels, respectively.

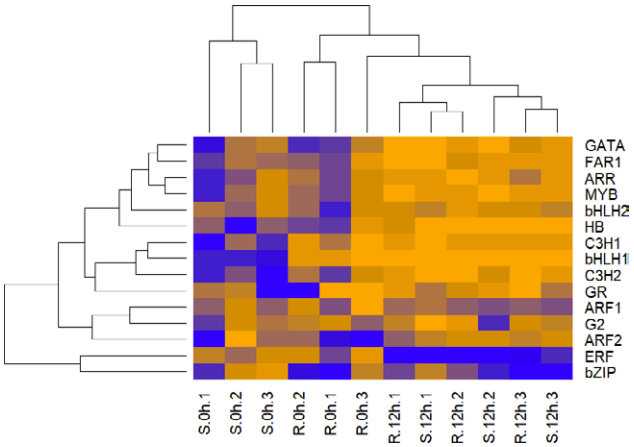

Figure 4. Hierarchical clustering analysis of the top 15 differentially expressed genes among the susceptible and resistant samples.

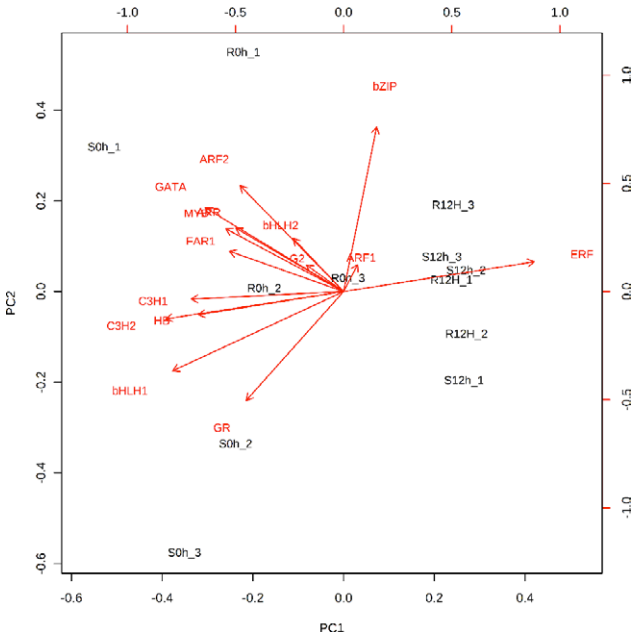

Figure 5. Bi-plot derived from PCA analysis based on the top 15 genes.

ulation). There are different pathways including salicylic and jasmonic acids, and ethylene, which involved in the plant resistance against pathogens (Yuan *et al.* 2019). The ERF and bZIP transcription factors are two main families responding to pathogen attack due to their importance, abundance, and availability of functionally well-characterized (Amorim *et al.* 2017). The result of present study showed that the expression of *bZIP* and *ERF* genes were upregulated in the genotypes, however this upregulation in resistant genotype was higher than the susceptible genotype. Many studies have shown that up-regulation and activation of bZIP and ERF transcription factor families are common as part of plant defense mechanism to response to pathogen attack (Tateda *et al.* 2008; Amorim *et al.* 2017; Tezuka *et al.* 2019). For examples, the *bZIP60* gene was significantly up-regulated in *Nicotina benthamiana* in response to *Pseudomonas cichorii* inoculation, showing an involvement of the bZIP in the plant innate immunity (Tateda *et al.* 2008). An ERF transcription factor in *Oriza sativa* (*OsERF83*) was expressed in leaves in response to blast fungus infection and led to blast resistance by regulating the expression of defense related genes (Tezuka *et al.* 2019). The result of PCA analysis, based on the first two main components showed that these two genes were far from the other genes (Fig 5), indicating different expression patterns for the genes. This result was consistent with the cluster analysis. The high regulation and different expression patterns of these genes, indicating their important roles in responding to pathogen attack. Also, the result of correlation analysis showed a low and positive correlation coefficient between these two genes.

The second group consisted of three genes including *ARF1*, *ARF2*, and *G2* genes. *ARF1* gene similar to the genes in the first group was significantly upregulated in the susceptible and resistant genotypes 12 hpi compared to their control, however the upregulation in resistant genotype was higher than the susceptible genotype. This result was confirmed by PCA analysis, which had similar expression pattern with *bZIP* and *ERF* genes. In the present study the expression level of two auxin response factors were investigated. Recently, the ARFs were introduced as an active actor in plant resistance mechanism against different pathogens attack (Bouzroud *et al.* 2018). Similar to our results, two ARFs were detected in rice upon *Magnaporthe grisea* and *Striga hermonthica* infections (Ghanashyam and Jain 2009). Differential expression of ARF genes were observed in cotton in response to *Fusarium oxysporum f. sp vasinfectum* infection (Dowd *et al.* 2004). Our results showed that the expression of both ARF genes were upregulated in the genotypes 12 hpi compared to their controls, indicating the importance of auxin pathway in wheat resistance mechanism against the *B. graminis*. The result of gene expression revealed that *G2* gene was significantly upregulated in the resistant genotype, while it was not significant for susceptible genotype 12 hpi compared to their controls. The result of real time PCR confirmed the result of microarray analysis, in both analyses *G2* was highly upregulated in the resistant genotype. Previous studies have shown that G2 plays an important role in disease defense mechanism

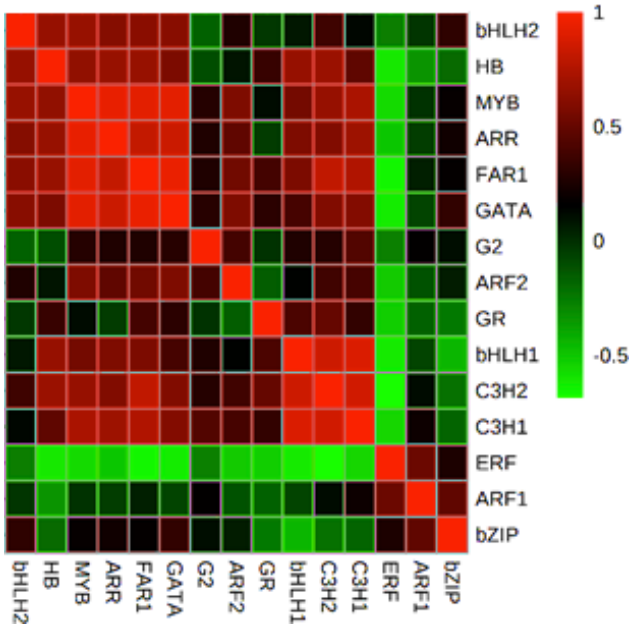

Figure 6. Heat map of the correlations among the top 15 genes.

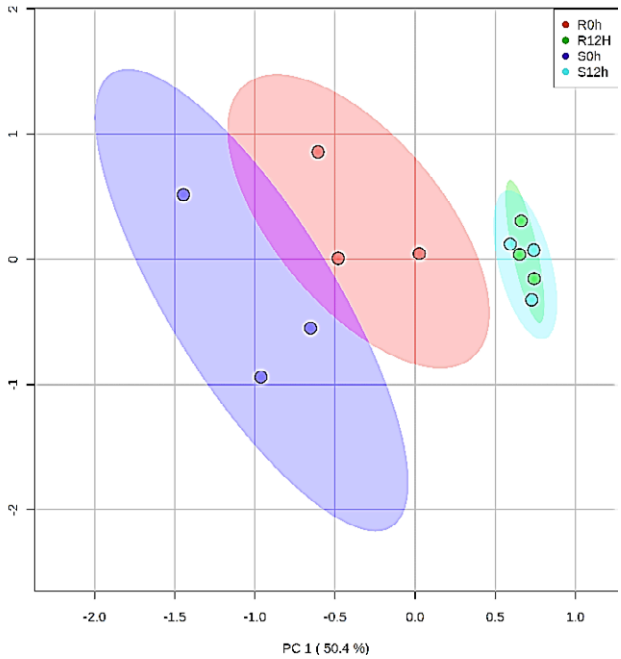

Figure 7. Bi-plot derived from PCA analysis based on susceptible and resistant samples.

in different plants such as *Arabidopsis* (Murmu *et al.* 2014) and rice (Nakamura *et al.* 2009). Over expression of a G2-like family (*AtGLK1*) in *Arabidopsis* resulted in significant up-regulation of some genes involved in the defense mechanism and salicylic acid signaling pathway, which displayed stronger resistance to *Fusarium graminearum* (Savitch *et al.* 2007).

The third group contained four genes, i.e. *C3H1*, *C3H2*, *GR*, and *bHLH1*. The results of PCA analysis based on the two first main components confirmed the result of dendrogram, which these genes were close to each other than the other genes (Fig. 5). The expression levels of *C3H1*, *C3H2*, and *bHLH1* genes were significantly downregulated in the susceptible genotype, while the four genes were upregulated in the resistant genotype 12 hpi compared to their controls. Also, the result of correlation analysis revealed that there were positive and significant correlation coefficients among these three genes ($P < 0.01$). *OsC3H12* and *OsDOS* genes (C3H family) positively and quantitatively regulates rice resistance to different diseases, which are likely associated with the jasmonic acid pathway (Kong *et al.* 2006; Deng *et al.* 2012). The bHLH transcription factors are important signaling components with dual roles in the regulation of defense responses thorough jasmonic acid pathway (Wild *et al.* 2012; Hu *et al.* 2013). The results of gene expression showed that the *GR* (*GRF*) gene was upregulated in resistant genotype, however no change was observed in the susceptible genotype compared to their controls. GRF-regulated genes are involved in some hormone biosynthesis pathways such as jasmonic acid, salicylic acid, ethylene, and auxin, which can activate the plant defense mechanisms and coordinate between developmental process and plant defense mechanisms (Liu J *et al.* 2014). It seems that the genes in the third group caused to wheat resistance to the disease thorough jasmonic acid pathway. Our result showed that the expression of *C3H1*, *C3H2*, and *bHLH1* genes were downregulated in the susceptible genotype 12 hpi, which could be due to the process called stress-induced transcriptional attenuation (SITA). In response to pathogens infections, the plant cells interrupt their daily routines to protect themselves from damage, cells start the production of new proteins to help damaged proteins and at the same time, many normally expressed genes rapidly downregulate in the SITA process (Aprile-Garcia *et al.* 2019).

The fourth group consisted of six genes, namely *HB*, *bHLH2*, *MYB*, *ARR*, *FAR1*, and *GATA*. The expressions of *HB* and *MYB* genes were significantly downregulated in the susceptible genotype 12 hpi compared to the control. Also, there was a significantly positive correlation coefficient between them ($P < 0.05$). It seems that the downregulation of these two genes in the susceptible genotype were due to the SITA process. Cominelli *et al.* (2005) reported that an *Arabidopsis* transcription factor

(*AtMYB60*) involved in stomata movement, which rapidly downregulated by stress. One of the plants defense mechanism to pathogen attack, is closing their stomata to prevent pathogen entry (Arnaud and Hwang 2015). It seems that the susceptible genotype downregulated the *MYB* gene to close the stomata and preventing pathogen entry, which can be a part of SITA process. The results demonstrated that all genes in the fourth group were up regulated in the resistant genotype 12 hpi compared to the control, however none of them was statistically significant. The roles of these transcription factors in plant pathogen resistant were reported in different studies. For example, Zhang *et al.* (2018) studied the expression pattern of TFs in resistant (*Vernicia montana*) and susceptible (*V. fordii*) tung trees responding to *Fusarium* wilt disease. The authors reported that the MYB and bHLH families had the largest number of TFs among 59 different families in both *V. fordii* and *V. montana* species during the four infection stages. According to the result of correlation analysis all the genes in the fourth group had a significant correlation coefficient with each other ($P < 0.05$ or $P < 0.01$), although the highest correlation coefficient among the studied genes was between GATA and FAR1 ($r=0.94$, $P < 0.01$). According to the PCA analysis based on the genotypes, the two first main components confirmed more than 68% of total variance (Fig. 7). It was obvious from the figure 7, the main variance of PC1 (50.4%) and PC2 (17.8%) were explained by infection treatment (0 and 12 hpi) and genotype factors, respectively. Usually under similar conditions, genetic factors are the main source of variances among the samples (Hassanabadi *et al.* 2019; Farajpour *et al.*, 2017). Finally, the result of PCA confirmed the results of microarray and real time PCR analyses.

CONCLUSIONS

In the present study, the publicly available wheat microarray data set and the real time PCR analysis were used to study the transcriptomes and gene expression of susceptible (Bolani) and resistant (KC2306) wheat genotypes in response to powdery mildew. A total of 5760 and 5315 probe sets were detected which 5427 and 4630 were differentially expressed by adjusted P-value < 0.05, in susceptible and resistant wheat genotypes, respectively. Among exclusively up regulated genes in the resistant genotype 12 hpi compared to its control, fifteen potential genes that may be responsible for *B. graminis* inoculation resistance were detected. The results of real time PCR for the candidate genes showed that the genes were upregulated in the resistant genotype 12 hpi compared to its control, which validated the results of microarray analysis. Our results illustrated aforementioned genes positively regulated resistance mechanism to powdery mildew infection. Also, they may be good candidate genes for studying and improving resistance to powdery mildew in wheat. The powdery mildew responsive genes identified in the present study will give us a better understanding on molecular mechanisms involved in B. graminis resistance in different wheat genotypes.

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