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Characterizing Resistance Genes in Wheat-Stem Rust Interaction

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ABSTRACT

Stem rust, caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*), is one of the most important diseases of wheat with devastating epidemics in Iran and the world. In this study, we evaluated some Iranian wheat landraces in a greenhouse at the seedling stage against a new pathotype related to Ug99 of *Pgt*, which was collected from Iran and designated as TTSSK. Marker analysis was done on resistant landraces. Molecular markers for detecting some *Sr* genes were used. The results showed that *Sr22, Sr35* and *SrWeb* provided resistance against TTSSK in the resistant landraces. In addition, some of the susceptible landraces that were resistant at adult stage were used for *Sr2* analysis. The results showed that some of these landraces were carrying other adult plant resistance gene/genes except *Sr2*. To evaluate the defence gene expression in compatible and incompatible interactions, cv. Morocco (susceptible) and KC-440 landrace (resistant) were used. Sampling was done at 0, 12, 18, 24, and 72 hours post inoculation (hpi) with stem rust isolate and water as mock treatment. *β-1,3 glucanase* gene expressions were studied using *qGLU-S* and *qGLU-AS* primers. Also, *18srRNA*, *β-tubulin* and *EF1-α* genes were used as internal control. The results showed that in incompatible interactions, the defence gene expression was increased at 24 hpi, but in compatible interactions, expression level reached the peak at 12 hpi and it significantly decreased at 18 hpi. The results revealed that the expression of defence genes such as *β*-1,3 glucanase was earlier in compatible interactions than in incompatible interactions, but the quantity of expressed gene was less than in incompatible interactions.

Keywords: *β*-1,3 glucanase, Real-time PCR, *Sr* genes, SSR marker, Ug99.

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INTRODUCTION

Wheat is the second source of calories after rice for consumers in developing countries (Braun *et al.,* 2010). *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn. (*Pgt*) is the causal agent of stem or black rust and is one of the most important fungal diseases of wheat. The disease's symptoms include blister-like pustules or uredinia on the leaf sheaths of wheat plants, and also on the stem tissue, leaves, glumes and awns (Singh *et al.*, 2008). There are several primary hosts for the pathogen including hexaploid common bread wheat (*Triticum aestivum*), tetraploid durum wheat (*Triticum turgidum* var. *durum*), barley (*Hordeum vulgare*), triticale (X *Triticasecale*), and wheat progenitors (Roelfs *et al.,* 1992). *Pgt* also survives on common barberry and some other species of *Berberis, Mahoberberis* and *Mahonia*. The disease causes severe yield

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losses. Affected susceptible cultivars will turn to broken stems and shrivelled grains in three weeks before harvest (Singh *et al.,* 2008).

In 1998, stem rust infections occurred in Uganda that showed virulence to *Sr31* (Pretorius *et al.,* 2000). This race was nominated as TTKS (Ug99) by Wanyera *et al.* (2006) by using the North American nomenclature system (Roelfs and Martens, 1988) and then as TTKSK (Jin *et al.,* 2008). Subsequently, Ug99 was reported in Kenya and Ethiopia in 2005 (Wanyera *et al.,* 2006) and in Sudan and Yemen in 2006 (Singh *et al.,* 2008). A new race of Ug99 with pathogenicity against *Sr24* was identified in Kenya in 2006 (Jin *et al.,* 2007). It was predicted that these races could immigrate to North Africa, the Middle East, and Asia and attack the most susceptible varieties that were currently grown in these areas (Singh *et al.,* 2008). Subsequently, Ug99 was confirmed in Iran in March 2008 (FAO, 2008) and the TTKSK pathotype was collected from Broujerd and Hamedan in Northwestern Iran in 2007 (Nazari *et al.,* 2009). Due to drought conditions, there were no reports from Iran in 2008, but in 2009, it was found in Khuzestan (Singh *et al.,* 2011). Stem rust was also reported in Pakistan in 2009, and based on phenotyping and DNA analysis, another important race (RRTTF) was identified (Mirza *et al.,* 2010).

Heretofore, about 58 to 60 resistance genes to *Pgt* have been specified in wheat (Chen *et al.,* 2018; Getie *et al.,* 2016; McIntosh *et al.,* 2014). Many of them are race specific genes and expressed in both seedling and adult stages, except *Adult Plant Resistance* (APR) genes, and can easily be employed in breeding programs, but they frequently overcome by new pathotypes (Park 2008; Singh *et al.,* 2009; Herrera-Foessel *et al.,* 2011). Although Polygenic adult plant resistance to rust is considered as durable and non-specific resistance, *Sr2/Yr30*, *Lr34/Yr18/Sr57*, *Lr46/Yr29/Sr58*, *Lr67/Yr46/Sr55* and *Sr56* are some examples of existing *APR* genes in durable resistance (William *et al.,* 2003; Herrera-Foessel *et al.,* 2011; Singh *et al.,* 2012; Bansal *et al.,* 2014). Some of the race-specific genes, including *Sr5*, *Sr17*, *Sr27* and *Sr36*, are responsible for developing only microscopic or macroscopic sensitive reactions. Also, *Sr6* is effective at cool temperatures (Jin *et al.,* 2007). Significant changes have occurred in the pathogen through its distribution in Africa. Virulence for resistance genes, e.g. *Sr24* and *Sr36*, occurred in Kenya during 2006 and 2007, respectively (Jin *et al.,* 2008; Jin *et al.,* 2009). Changes in pathogen population are rapid and thirteen different variants have been recognized as belonging to the TTKS race lineage (Xu *et al.,* 2018; FAO, 2017).

Among the several race specific resistance genes to stem rust (Ug99 race lineage) that were identified, only *Sr22*, *Sr26*, *Sr35* and *Sr50* were found to be most effective against all the current important races and had the capability to be used successfully (Singh *et al.,* 2015).

Many researchers have studied resistance at seedling stage extensively (Jin and Singh, 2006; Jin *et al.,* 2007; Njau *et al.,* 2010). Furthermore, Pretorius *et al.* (2012a) evaluated *Sr2*, *Sr24* and *Sr31* resistance genes in some wheat cultivars and lines in South Africa. Landraces are simultaneous with traditional farming and are believed to be a useful source of genetic diversity for breeding (Villa *et al.,* 2006; Warburton *et al.,* 2006; Newcomb *et al.,* 2013). Baranova *et al.* (2016) evaluated seventeen known *Sr* genes (*Sr2, Sr22, Sr24, Sr25, Sr26, Sr32, Sr35, Sr36, Sr39, Sr40, Sr44, Sr47, Sr9a, Sr15, Sr17, Sr19*, and *Sr31*) in 6 sources of resistance to Ug99 by applying molecular markers in Russia. Some of these genes were identified in the analyzed lines and may be recommended as donors of resistance gene in breeding programs.

Following the emergence of Ug99, virulence factors and effective genes' investigation revealed that resistance genes including *Sr5, Sr6, Sr7a, Sr7b, Sr8a, Sr8b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9f, Sr9g, Sr9h, Sr10, Sr11, Sr12, Sr16, Sr17, Sr18, Sr19, Sr20, Sr21, Sr23, Sr24, Sr30, Sr31, Sr34,* *Sr36, Sr38, Sr41, Sr49, Sr54, SrMcN* and *SrWld-1* are no longer effective. Since local races can migrate to new areas and become predominant there, virulence for single genes or gene combinations may be still indistinctive (Singh *et al.*, 2015).

Higher plants have protective reactions called ‘defence responses’ against microbial pathogens. The inducible or constitutive defence mechanisms are conserved among different plant species to some extent (de Jonge *et al.,* 2011; Balasubramanian *et al.,* 2012). Numerous classes of proteins, called Pathogenesis-Related (PR) proteins, are involved in reaction to the invasion of various microbial pathogens such as viruses, bacteria, viroids and fungi (Leubner-Metzger and Meins, 1999). Also, PR-proteins are induced by chemicals like plant hormones such as ethylene, jasmonic acid and salicylic acid. For the first time, PR-proteins were reported from tobacco leaf extracts that showed hypersensitive reaction to tobacco mosaic virus (van Loon and van Kammen, 1970). The PR-proteins that are isolated from plants are classified into 17 families according to characteristics such as sequence similarities, serological or immunological relationships, and enzymatic characteristics (Leubner-Metzger and Meins, 1999; Balasubramanian *et al.,* 2012). β-1,3 glucanase enzymes are members of PR-2 family. Studies have shown that plant β-1,3 glucanases are involved in defence along with chitinase isozymes. It degrades β-1,3/1,6 glucans of the hyphal cell wall and leads to hydrolysed cell walls in fungal pathogens (Mohammadi and Karr, 2002). PR-proteins release β-1,3 glucan that act as an elicitor and induce plant defence mechanism (Somssich and Hahlbrock, 1998). The expressions of β-1,3 glucanase in plant-pathogen interactions and their wide range of antimicrobial activities have been assessed widely (Balasubramanian *et al.,* 2012). The induction of β-1,3 glucanase in monocot cereals such as wheat (Anguelova-Merhar *et al.,* 2001), barley (Xu *et al.,* 1992), rice (Romero *et al.,* 1998), and corn (Jondle *et al.,* 1989; Nasser *et al.,* 1988) have been studied. Also, the expression of this defence gene was evaluated in tomato-*Alternaria solani* interaction (Salim *et al.,* 2011).

Among the different methods for assessing gene expression profile, quantitative PCR or Real time PCR is one of the most widely used techniques and plays a prominent role in biological researches (Skovgaard *et al.,* 2006; Cossio-Bayugar *et al.,* 2008; Kavousi *et al.,* 2009; Long *et al.,* 2011). This technique has more benefits than the other quantitative methods of gene expression (Morrison *et al.,* 1998). Some of these advantages are: allows the detection of PCR amplification during the primary stages of the reaction, increases the dynamic range of detection, no-post PCR processing, and diagnosis is possible down to a 2-fold change, and so on. This method is used for the evaluation of gene expression in different pathosystems such as peanut leaf spot (Luo *et al.,* 2005), wheat stripe rust (Bozkurt *et al.,* 2007), and wheat leaf blotch (Oliver *et al.,* 2008) diseases.

Due to high level of variation in wheat rust populations, wheat breeders and pathologists should continually look for finding new effective resistance genes and deploying them in new cultivars. Hence incorporating multiple resistance genes in host-plant resistance is still the most beneficial and sustainable management strategy (McCallum *et al.,* 2016; Figlan *et al.,* 2017), especially in developing countries where fungicide application is not usually affordable (Getie *et al.,* 2016). Despite Iran being considered as the centre of origin of bread wheat, there is little information on the resistance of Iranian wheat landraces against *P. graminis* f. sp. *tritici* members of the Ug99 lineage. Since wheat production has a long history in Iran, and also other researchers (Newcomb *et al.,* 2013) mentioned the high rate of resistance to Ug99 in the studied landraces from Iran and Afghanistan, we hypothesized that Iranian wheat landraces were potential sources for identifying new resistance genes against upcoming destructive races like Ug99 for utilizing in wheat breeding programs.

This study aimed to identify the resistance genes that are involved in resistant landraces at the seedling stage in the greenhouse. Also, we evaluated the plant expression of the defence gene, *β-1,3-glucanase,* in compatible and incompatible interactions in order to prepare *β-1,3-glucanase* gene expression patterns in wheat-stem rust interactions.

MATERIALS AND METHODS

Plant Material and Infection Type Evaluation

An isolate of *P. graminis* f. sp. *tritici* (*Pgt*), which was collected from Dasht-e Azadegan, was obtained from the Department of Cereal Research, Seed and Plant Improvement Institute Karaj, Iran, and identified as TTSSK (Mojerlou *et al.*, 2013) by tests on both the differential sets received from CIMMYT and ICARDA for identification of *Pgt*'s race.

Also, 62 Iranian wheat landraces were obtained from the Department of Genetics and National Plant Gene-Bank of Iran, Seed and Plant Improvement Institute Karaj, Iran, and were tested with the *Pgt* isolate. These landraces were selected from among 700 accessions that were evaluated against local races of stripe rust at adult stage. They showed resistance to local races of stripe rust in the previous studies (unpublished). The urediniospores of stem rust were suspended and sprayed onto the quite enlarged primary leaves of 7 to 9 days old seedlings. The inoculated seedlings were incubated at 18°C in a dew chamber in darkness for 14 hours. Later, the plants were transferred to a greenhouse bench at 18±2°C with a photoperiod of 16 hours (Jin *et al.*, 2007). Infection Types (ITs) described by Stackman *et al.* (1962) were assessed for 14 days post inoculation. Infection types 0-2 or combinations were supposed as low ITs and ITs 3 to 4 were assumed high. In each test, five seedling plants were evaluated and each seedling test was repeated thrice. The landraces that conferred to low ITs were utilized for marker analysis. Some of the susceptible landraces, which were resistant at adult stage in previous experiments, were analysed for detection of the *Sr2* gene using molecular markers.

DNA Extraction and Marker Analysis

Genomic DNA was extracted from frozen leaves using Dellaporta *et al.* (1983) method. The quality and quantity of the extracted DNA were assessed by using agarose gel (1% w/v) electrophoresis and spectrophotometer (Eppendorf 6131) measurement. The DNA samples were diluted to 50 ng μL^{-1}.

Polymerase Chain Reaction (PCR) assays were performed according to reported protocols for *Sr2* (CsSr2; Mago *et al.,* 2011), *Sr22* (WMC633 and BARC121; Olson *et al.,* 2010; Yu *et al.*, 2010), *Sr24* (barc71; Mago *et al.*, 2005), *Sr25* (BF145935 and Gb; Liu *et al.*, 2010; Yu *et al.*, 2010), *Sr26* (Sr26#43 and BE518379; Mago *et al.*, 2005; Liu *et al.*, 2010), *Sr35* (cfa2193; Zhang *et al.*, 2010), *Sr36* (gwm319; Tsilo *et al.*, 2008), and *SrWeb* (GWM47; Hiebert *et al.*, 2010).

Treatments, RNA Extraction and cDNA Synthesis

In order to study plant defence gene expression in wheat-stem rust interaction, *Pgt* pathotype, TTSSK, Morocco cultivar and Kc-440 landrace were used as compatible and incompatible interactions, respectively. The wheat plants were grown and kept in a greenhouse. Fresh urediniospores were collected and sprayed onto the 7-day-old seedlings. The inoculated plants were placed in a humid and dark place at 18˚C for 14 hours, and, subsequently, transferred to a growth chamber at 25±2˚C with a 16 hour photoperiod (McIntosh *et al.,* 1995). Mock-inoculated seedlings with water were used as control, and were subjected to the same process as the inoculated seedlings. Leaf samples were taken at 0, 12, 18, 24 and 72 hours post-inoculation (hpi) and stored at -80˚C for further analysis. Three replications were considered for each treatment at every time point. The symptoms were recorded for 14 days after inoculation based on the methods by Stackman *et al.* (1962) and McIntosh *et al.*, (1995).

The total RNA was isolated from about 200 mg of the frozen wheat leaves using RNX-plus solution (Sinaclon Co., www.sinaclon.com) according to the manufacture's data sheet. The RNA quality and integrity were determined by running agarose gel, and the quantity of total RNA was determined by spectrophotometer (Ependorph 6131). The first-strand cDNA synthesis was done by using Revert Aid First Strand cDNA Synthesis Kit (Fermentas) with the Oligo (dT) 18 primer following the manufacturer's instruction.

To assess the accuracy of cDNA synthesis, PCR was performed by using the listed primers in Table 1 in an Eppendorf gradient thermocycler (Germany) with the following cycling conditions: 5 minutes at 95ºC, 30 cycles of 15 seconds at 95ºC, 30 seconds at 62ºC and 20 seconds at 72ºC, and a 5 minutes final extension step at 72ºC. The PCR product was separated on a 2% (w/v) agarose gel and visualized under UV light.

Real-Time Quantitative PCR Analysis

β-1,3-glucanase expression in wheat leaves were analyzed by 5X HOT FIREPol® EvaGreen®HRM Mix (ROX) based real-time quantitative PCR (Q-PCR) assays of 0, 12, 18, 24 and 72 hpi incompatible and compatible interactions, and in mock inoculated control plants. Three independent biological replications were performed for each time point.

Q-PCR was performed on a real-time PCR step one (ABI) machine. Specific primers qGlu-S/qGlu-AS (Table 1) was used to quantify induction of *β-1,3 glucanase* transcripts. Also, *18SrRNA*, *beta tubulin* and *EF-1α* were used to normalize the amount of cDNA samples (Table 1). The PCR reactions were carried out in a 10 µL volume containing 2 µL 5X Eva Green Mix (ROX), 0.25 µL (10 pmol) of each primer and 2 µL template (1:10 diluted cDNA from leaf samples).

Amplifications were performed by using the following programs: 95˚C for 5 minutes; 40 cycles of 95˚C for 15 seconds and 62˚C for 30 seconds. For each sample, the reactions were set up in triplicates to ensure the reproducibility of the results, and three non-templates were included as negative controls. The products were analyzed by melt curve, which was obtained at the end of amplification, as well as agarose gel electrophoresis to ensure that a single product was being amplified. The $2^{-(\Delta\Delta CT)}$ method was applied to quantify the relative gene expression (Livak and Schmittgen, 2001).

RESULTS

Seedling assessment was performed in the

Table 1. Primers used for Q-PCR amplification of *β*-1,3 glucanase.

Gene	Primer	*Primer sequence*	Reference
β-1,3- glucanase	qGLU-S	*5'-AGGATGTTGCTTCCATGTTTGCCG-3'*	Liu *et al.* (2010)
	qGLU-AS	*5'-AAGTAGATGCGCATGCCGTTGATG-3'*	
Beta tubulin	-	*5'-GGACCGTACGGGCAGATCT-3'*	Mohammadi *et al.* (2007, 2008)
	-	*5'-CACCAGACTGCCCAAACACA-3'*	
EF-1α	-	*5'-CAGATTGGCAACGGCTACG-5'*	Lopato *et al.* (2006)
	-	*5'-CGGACAGCAAAACGACCAAG-5'*	Crismani *et al.* (2006)
18SrRNA	q18-S	*5'-AACACTTCACCGGACCATTCA-3'*	Liu *et al.* (2010)
	q18-AS	*5'-CGTCCCTGCCCTTTGTACAC-3'*	

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greenhouse on 62 Iranian landraces. Among the 62 tested landraces, 28 landraces that showed low Infection Types (ITs) against race TTSSK were selected for marker analysis. The infection type data and marker analysis results are shown in Table 2. The infection type test was replicated three times in the greenhouse, and when variation was observed between the replicates, the highest infection type was used. During all the tests, differential lines and a susceptible cultivar were used to support the identities of the races used.

The results showed that none of the resistant landraces carried the *Sr2* gene. Additional tests were performed on 21 landraces that were susceptible against TTSSK at the seedling stage (Table 3). These landraces were evaluated against local races in the field at adult plant stage and were resistant (unpublished). Based on these results, some of

Table 2. Infection type induced by the race TTSSK of *Puccinia graminis* f. sp. *tritici* on 28 Iranian wheat landraces and marker analysis results for *Sr2, Sr22, Sr24, Sr25, Sr26, Sr35, Sr36* and *SrWeb*.

KC no [a]	Collection location	IT [b]	*Sr2*	*Sr22*	*Sr24*	*Sr25*	*Sr26*	*Sr35*	*Sr36*	*SrWeb*
55	Tabriz	2/2+	-	-	-	-	-	-	+	+
106	Tabriz	2+	-	-	-	-	-	+	+	+
107	Tabriz	2+/3	-	-	-	-	-	+	+	+
118	Khoy	2+	-	+	-	-	-	+	+	+
121	Khoy	1+	-	-	-	-	-	+	+	-
127	Khoy	2	-	+	-	-	-	+	+	-
129	Khoy	1+	-	-	-	-	-	+	+	+
130	Khoy	1+	-	+	-	-	-	+	+	+
131	Khoy	2+	-	-	-	-	-	+	+	+
136	Khoy	0	-	-	-	-	-	+	+	-
140	Khoy	0	-	-	-	-	-	-	+	-
142	Khoy	2+	-	-	-	+	-	-	+	+
144	Khoy	2+/3	-	-	-	+	-	-	+	-
151	Khoy	0	-	-	-	+	-	-	+	-
158	Khoy	0	-	-	-	+	-	-	+	-
433	Broujerd	0	-	-	-	+	-	-	+	+
440	Broujerd	2	-	-	-	+	-	-	+	+
553	Kermanshah	2+	-	-	-	+	-	-	-	-
653	Khoram abad	2	-	-	-	-	-	+	+	-
977	Khoram abad	1+	-	-	-	+	-	+	+	+
1013	Khoram abad	2	-	-	-	+	-	-	+	-
1033	Unknown	2+	-	-	-	-	-	-	-	+
1048	Unknown	2	-	-	-	+	-	+	+	+
1058	Unknown	2	-	-	-	-	-	-	+	-
1082	Torbat jam	0	-	-	-	+	-	-	+	-
1085	Unknown	2+	-	-	-	+	-	+	+	+
1100	Unknown	0	-	-	-	+	-	-	+	+
1104	Unknown	2+	-	-	-	+	-	+	+	+
Sr22TB	-	0, ;	-	+		-	-	-	-	-
LcSr24Ag	-	;, 1	-	-	+	-	-	-	-	-
LcSr25Ars	-	2+/3	-	-	-	+	-	-	-	-
Eagle	-	2	-	-	-	-	+	-	-	-
Mq(2)5*G2919	-	3	-	-	-	-	-	+	-	-
W2691SrTt-1	-	3	-	-	-	-	-	-	+	-
Morocco	-	4	-	-	-	-	-	-	-	-

[a] Gene bank accession number of the landraces. [b] Infection Types (ITs) according to 0 to 4 scale. Within line variation is indicated by '/'.

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Table 3. Infection type induced by the race TTSSK of *Puccinia graminis* f. sp. *tritici* on 21 susceptible Iranian wheat landraces at seedling stage and results of a survey using the marker linked to *Sr2* resistance.

KC no[a]	Collection location	Infection type[b]	*Sr2*
42	Tabriz	3	-
59	Tabriz	3	Null
101	Tabriz	3/3+	-
109	Tabriz	3	-
112	Khoy	3/3+	Null
120	Khoy	3	-
137	Khoy	3	+
138	Khoy	3	+
139	Khoy	2+/3	+
180	Urmia	2+/3	-
190	Urmia	2+/3	-
231	Miandoab	2+/3	?
324	Sarab	2+/3	-
352	Moghan	2+/3	-
439	Broujerd	2+/3	Null
545	Kermanshah	3/3+	?
655	Khoram abad	3	-
717	Gulpaigan	2+/3	Null
764	Moghan	3/3+	-
886	Hamedan	2+/3	Null
1198	Unknown	3/3+	-

[a] Gene bank accession number of the landraces. [b] Infection types according to 0 to 4 scale. Within line variation is indicated by '/'.

the landraces (KC 137, KC 138 and KC 139) that were susceptible at the seedling stage, tested positive for the *Sr2* gene that is responsible for adult plant resistance; the resistance may also be due to additional genes/QTL as well. Some landraces that were susceptible at the seedling stage showed resistance at the adult plant stage, suggesting another resistance gene(s) in addition to *Sr2*.

Based on the results, the landraces KC 118, KC 127 and KC 130 carried the *Sr22* gene. Since the TTSSK race is avirulent on *Sr22* (Mojerlou *et al.*, 2013), this gene may justify the resistance reaction of these landraces that needs inheritance studies. However, these genotypes may contain other resistance genes. Meanwhile, none of the tested landraces was positive for *Sr24* or *Sr26* markers (Table2). It suggests that they carry resistance genes other than *Sr24* and *Sr26*. Moreover, the TTSSK race was virulent on *Sr26* and avirulent on *Sr24*. Thus, the *Sr26* gene will not be effective against TTSSK.

In summary, it has been suggested that *Sr22*, *Sr35* and *SrWeb* provided resistance against TTSSK in the landraces in this study. Based on marker data, the landrace KC 151 (IT= 0) carries none of the *Sr22*, *Sr35* and *SrWeb* genes, suggesting that it carries another resistance gene(s), and this needs further studies.

In this study, *Pgt*-Morocco and *Pgt*- KC440 pathosystems were used as compatible and incompatible interactions due to their susceptible and resistance interactions, respectively. Three reference genes, including *18SrRNA*, *beta tubulin*, and *EF-1α*, were considered in qRT-PCR assessments.

The experimental conditions were optimized to remove non-specific products and to obtain accurate results. These parameters were confirmed by the single peak of melt curve and specific band on agarose gel. Based on the normalized data, *β-1,3-glucanase* gene expression enhanced and reached the peak (6 folds) at 12 hpi in compatible interaction.

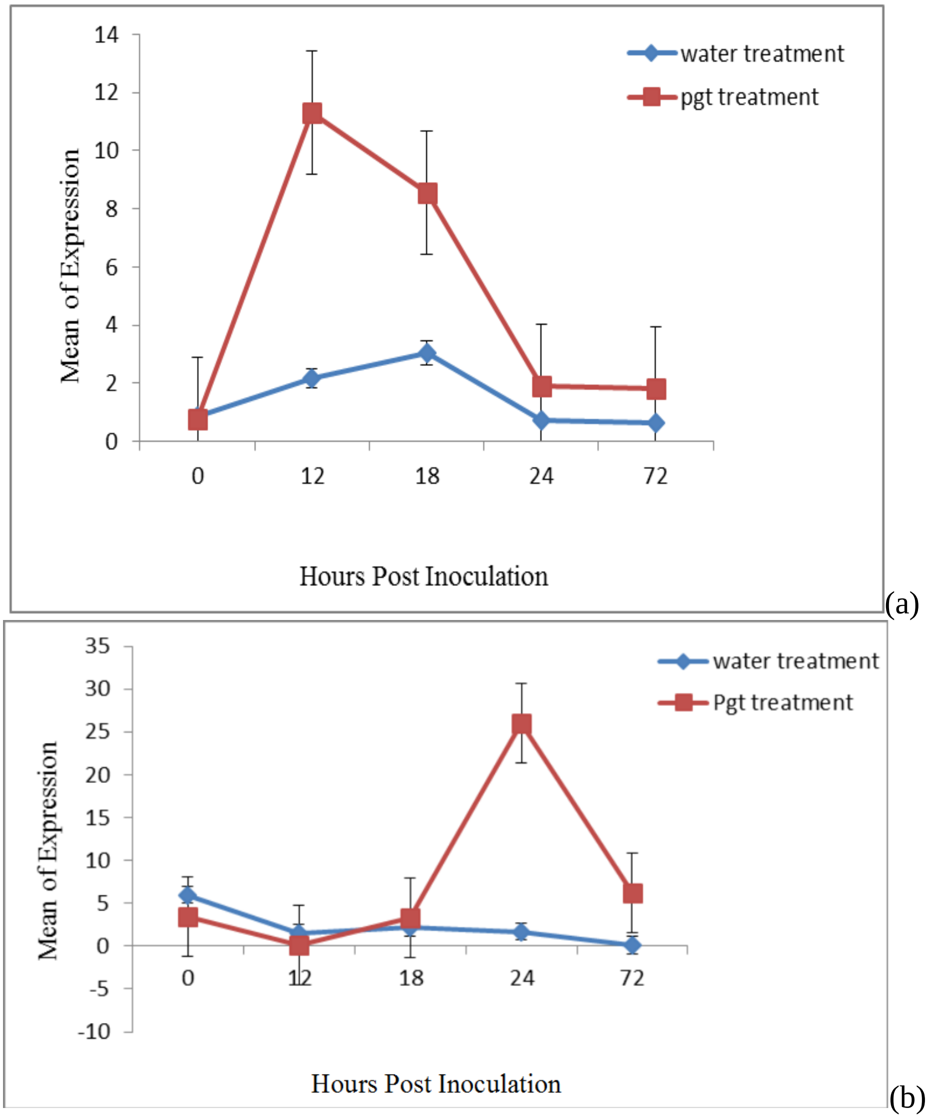

Figure 1. *β-1,3 glucanase* gene expression in compatible interaction (a) and incompatible interaction (b) *β*-tubuline is used as internal control to normalize the data.

Then, it decreased rapidly at 18, 24 and 72 hpi (Figure 1a). On the other hand, in incompatible interaction, the gene expression compared with mock treatment increased at 24 hpi (12 folds) and dropped dramatically at 72 hpi (Figure 1b). Melt curve analysis showed that the peak of the curve occurred around 80 ˚C for all the genes that stated specific amplification of the Q-PCR products.

Based on the results, in compatible interactions, defence gene expression such as β-1,3-glucanase induced and increased after inoculation. After 18 hours, due to host susceptibility and suppression of signal transduction pathways, defence gene expression decreased and led to host susceptible reaction. In contrast, in incompatible interaction, the biggest value of expressed gene was observed at 24 hpi. This period is essential for penetration and establishment of the pathogen. Therefore, at 12 hpi, defence gene expression induced and reached the highest level after pathogen establishment.

DISCUSSION

In our study, most of the landraces that were susceptible against TTSSK at the seedling stage showed chlorosis in a phenotyping test. However, three of these lines tested positive for the *Sr2* gene based on molecular data. Pretorius *et al.* (2012a) compared the seedling chlorosis test with the CAPS marker and revealed that only scores of 4 and 5 clearly suggested the presence of *Sr2*. Brown (1997) had already mentioned that seedling chlorosis varied between cultivars and lines when they carry *Sr2*, but high temperatures like 35 ºC are needed to give the appearance of this phenotype in some cases. Also, the CAPS marker could not produce the expected fragment in some of the Iranian landraces. Our data are in concordance with findings of Mago *et al.*, (2011), who mentioned that the presence of *Sr2* in some backgrounds was not validated by the CAPS marker. As *Sr2* gene has been transferred to ‘Hope’ cultivar and other hexaploid wheat cultivars during modern breeding, the absence of *Sr2* gene in Iranian landrace accessions was expectable. The presence of *Sr2* in three landrace accessions revealed misclassification of these accessions as landraces. Misclassified landraces were found among accessions from Ethiopia and Switzerland and have been reported by other researchers (Newcomb *et al.*, 2013).

Some Iranian landraces are postulated to carry the *Sr22* gene, which may confer resistance in these landraces individually or in combination with other gene(s). *Sr22* was originally designated in the diploid wheat species (Kerber and Dyck, 1973) and, afterwards, introduced to tetraploid and hexaploid wheat. Therefore, care must be taken while explaining the results of different genetic backgrounds (Olson *et al.*, 2010). None of the landraces carried *Sr24* or *Sr26* genes. The *Sr26* gene was introgressed to chromosome 6A of hexaploid wheat from *Agropyron elongatum* (Knott, 1961). Therefore, the absence of *Sr26* in Iranian wheat landraces may be due to their genetic backgrounds. The effectiveness of *Sr26* against the TTKSK lineage and its low frequency among modern cultivars makes it ideal for use by breeders. The absence of *Sr24* and *Sr26* genes in Iranian durum cultivars has been reported by Mohammadi *et al.* (2013). Besides, the lack of *Sr26* gene in Iranian commercial bread wheat genotypes has been reported by Patpour (2013). Our results were in accordance with these researchers.

Besides, pathotype identification using differential lines indicated that the TTSSK pathotype was virulent on *Sr25* and *Sr36* genes. Therefore, these genes were not responsible for the resistance reaction of these landraces against this pathotype. Also, the virulence of this gene has been reported for stem rust races in some areas (Jain *et al.*, 2009; Safavi and Afshari, 2017; Patpour *et al.*, 2017). It should be mentioned that diagnostic markers barc121 and Gb could not provide more reliable results for the *Sr22* and *Sr25* genes than wmc633 and bf145935 markers in the studied landraces, respectively.

This pathotype of *Pgt* showed an avirulence reaction to *Sr35*, indicating that this gene may confer resistance in the landraces under study. Contrary to the other pathotypes of *Pgt* in the Ug99 lineage, the TTSSK pathotype showed virulence against *Sr36*. Accordingly, *Sr36* is not responsible for resistance in the studied landraces. *SrWeb* gene was identified in most of the studied landraces. Hence, it may contribute to resistance to TTSSK. *SrWeb* is an Ug99 resistance gene from cultivar Webster, which is temporarily designated as *SrWeb* and is an allele at the *Sr9* locus, which was recently designated as *Sr9h* (Rouse *et al.*, 2014). However, the reaction of TTSSK on *SrWeb* needs to be examined on proper differential lines.

In our study, 14 out of the 28 landraces carried the *Sr25* gene (Table 2). Patpour (2013) showed that 89 Iranian bread wheat genotypes had *Sr25*. The present study is consistent with this finding. In our study, *SrWeb* (82.14%), *Sr25* (50%) and *Sr35* (50%)

had the highest frequencies in the studied landraces.

PR-proteins are one sort of proteins that are produced in plants due to pathogens' invasion or signal molecules related to pathogens (Van Loon *et al.*, 1994). The expression of these proteins occurs in compatible and incompatible plant interactions, locally and systematically. Also, the expression occurs specifically. For example, gene clusters that code PR proteins in plant infection with fungal-like pathogen *Peronospora parasitica* are completely different from those that are involved in *Alternaria brassicola* infection (Thomma *et al.*, 1998). Liu *et al.* (2010) studied the role of *β*-1,3-glucanase in resistance reaction of wheat against stripe rust. The results showed that defence gene expression increased at 18 hpi, and the highest level of gene expression was observed at 24 hpi in compatible interaction. In contrast, in incompatible interaction, gene expression did not increase till 24 hpi. Also, gene expression reached the highest level at 12 hpi, and it was 8 folds compared to compatible interaction. Tadayon *et al.* (2010) showed that in wheat-*Septoria tritici* interaction, *β-1,3-glucanase* gene expression decreased by 34-fold, 10 days after start of the infection in susceptible cultivar. The evaluation of *chitinase* and *β-1,3-glucanase* gene transcripts in interaction of citrus seedlings with the bacterial causal agent of citrus canker revealed that the transcripts of these genes increased by 4-fold compared to the control at 24 hpi, and decreased significantly at 96 hpi (Mansouri *et al.*, 2010).

The results of our study showed that *β-1,3-glucanase* gene expression was earlier in compatible interactions than in incompatible interactions, but the quantity of expressed gene was less in the compatible interactions. Our data are in line with those of Liu *et al.* (2010). On the other hand, in susceptible genotypes, the expression of defence genes increased immediately after inoculation and declined sharply after establishment of the pathogen. In contrast, defence gene expression in resistant genotypes began to increase after the establishment of the pathogen. Moreover, *EF-1α* and *β-tubulin* genes were used in this study as internal control for data normalization and the results were similar for both. The results revealed that *beta tubulin* and *EF-1α* genes were more efficient than *18SrRNA*. Researchers have argued that the *EF1α* gene is a suitable gene for gene expression data normalization due to its stability in biotic and abiotic stresses (Nicot *et al.*, 2005; Jain *et al.*, 2006). Also, *18SrRNA* was not an efficient gene for data normalization. The results of the current study are in accordance with other researchers (Jain *et al.*, 2006; Feng *et al.*, 2012).

The Ug99 race group threatens wheat production worldwide due to its fast evolution and migration, and susceptibility of over 70% of global wheat cultivars (Jin *et al.*, 2007; Singh *et al.*, 2008; Steffenson *et al.*, 2009; Singh *et al.*, 2011; Pretorius *et al.*, 2012 a, b). Most varieties that are grown in Africa, the Middle East, and Asia are 10 to 15 years old and, as Singh *et al.* (2011) suggests, it is advisable that they be replaced by new ones. In addition, most commercial wheat cultivars are susceptible to the new race Ug99 and its derivatives; therefore, it is advisable to promote varieties with adult plant resistance in combination with race specific resistance genes to confer resistance against new races. In this study, we evaluated some of the Iranian wheat landraces against the new pathotype, TTSSK, which was collected from Iran and was virulent to most of the resistance genes including *Sr31*. Since wheat production has a long history in Iran, it seems that Iranian wheat landraces are potential sources for identifying new resistance genes against upcoming destructive races, and their utilization is suggested in wheat breeding programs to achieve resistant cultivars. Finally, it is suggested that more efforts be made to strengthen this aspect of cultivar development.

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شناسایی ژن های مقاومت دخیل در تعامل گندم- زنگ ساقه

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چکیده

زنگ سیاه که توسط قارچ *Puccinia graminis* f.sp. *tritici* (*Pgt*) ایجاد می‌شود، یکی از بیماری‌های مهم گندم با اپیدمی های مخرب گزارش شده در ایران و جهان می‌باشد. در تحقیق حاضر برخی از نمونه‌های ژنتیکی گندم بومی ایران در گلخانه و در مرحله گیاهچه‌ای نسبت به پاتوتیپ جدیدی از نژاد Ug99، TTSSK، که از ایران جمع آوری شده بود، مورد ارزیابی قرار گرفتند. تجزیه و تحلیل با استفاده از نشانگرهای مولکولی به منظور شناسایی تعدادی از ژن‌های مقاومت موجود در نمونه‌های ژنتیکی مقاوم انجام شد. نتایج نشان داد که ژن‌های مقاومت *Sr22*، *Sr35* و *SrWeb* عامل بروز مقاومت در ژنوتیپ‌های مقاوم بررسی شده می‌باشند. سپس، ژنوتیپ‌های حساس که در مرحله بلوغ مقاومت نشان داده بودند، به منظور ردیابی حضور ژن *Sr2* مورد بررسی قرار گرفتند. نتایج این بررسی نشان داد که برخی از این نمونه‌های ژنتیکی حامل ژن(های) مقاومت در مرحله بلوغ دیگری به جز *Sr2* می‌باشند. در بخش دیگری از تحقیق به منظور ارزیابی تغییرات بیان ژن دفاعی در تعاملات سازگار و ناسازگار، از رقم موروکو (حساس) و نمونه ژنتیکی KC-440 (مقاوم) استفاده شد. نمونه برداری صفر، ۱۲، ۱۸، ۲۴ و ۷۲ ساعت پس از مایه زنی با جدایه *Pgt* و آب به عنوان شاهد، انجام شد. بیان ژن بتا–۱ و ۳ گلوکاناز با استفاده از آغازگرهای *qGLU-S* و *qGLU-AS* و همچنین ژن‌های *18SrRNA*، بتا توبولین و *EF1-α* به عنوان کنترل داخلی مورد بررسی قرار گرفت. نتایج نشان داد در تعامل ناسازگار بیان ژن دفاعی، ۲۴ ساعت پس از مایه‌زنی افزایش یافت اما در تعاملات سازگار، سطح بیان ژن ۱۲ ساعت پس از مایه‌زنی به حداکثر میزان خود رسید و سپس ۱۸ ساعت پس از مایه‌زنی به شدت کاهش یافت. بر این اساس، بیان ژن دفاعی بتا–۱ و ۳ گلوکاناز در تعاملات سازگار نسبت به تعاملات ناسازگاز سریع‌تر اتفاق می افتد، اما مقدار بیان این ژن نسبت به تعاملات ناسازگار کمتر است.

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