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cDNA-AFLP Analysis of Plant Defense Genes Expressed in Wheat (cv. Chamran) Infected with *Mycosphaerella graminicola*

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ABASTRACT

Differential induction of genes in wheat (cv. Chamran) in response to *Mycosphaerella graminicola* was studied using cDNA-AFLP analysis. The three weeks old wheat seedlings were inoculated by the fungal pathogen, then the samples were collected at six time points (0, 12, 24, 48, 72, 96 hours) after inoculation with pathogen. By comparing the expression patterns of induced (infected) with non-induced (uninfected) plants, 276 differentially expressed fragments were identified and sequenced. Blast search of wheat genes assigned these sequences to different functional categories including defense, metabolism, energy, transcription, transport, signal transduction, stress response, secondary metabolism, and unknown sequences. Eight defense-related genes including *lipoxygenase, peroxidase (PR 9), chitinase (PR 2, 4, 8) , PR-1, thaumatin-like protein (PR5), Phenylalanine Ammonia Lyase (PAL), β-1-3 glucanase (PR3), disulfide isomerase*, and *methionine sulfoxide reductase* were induced 12 to 24 hours after inoculation in this cultivar of wheat. Induction of *glucosyltransferase, Catalase,* and *putative xylanase inhibitor* genes were observed around 48 h after inoculation with pathogen. Expression patterns of the other three genes, i.e., chalcone synthase, EXECUTER 1 protein, and nonspecific lipid transfer protein showed that these genes were induced later after inoculation (72-96 hours). Our data showed that expression of the PR- proteins were enhanced 24 h after inoculation, suggesting that they may play a role in the defense against *M. graminicola*. Expression of lipoxygenase, glycosyltransferase, thaumatin like protein, putative xylanase inhibitor, EXECUTER 1 protein and Nonspecific lipid transfer protein are reported for the first time in this interaction.

Keywords*:* Blast search, Differential induction, Leaf blotch, PR- proteins, Resistance genetics.

INTRODUCTION

Leaf blotch, caused by the fungal pathogen, *Mycosphaerella graminicola* (asexual stage: *Zymoseptoria tritici*) (Quaedvlieg *et al*., 2011), is a serious destructive foliar disease of wheat in several temperate and subtropical regions of the world (Eyal *et al*., 1985).

The observation of host responses to *M. graminicola* such as hydrogen peroxide accumulation (Shetty *et al*., 2003), callose accumulation (Cohen and Eyal, 1993) and accumulation of autofluorescing substance (Duncan and Howard, 2000) with no distinct disease symptoms in the early stages after pathogen inoculation led to investigations of

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the wheat- *M. graminicola* interaction (Ray *et al*., 2003; Shetty *et al*., 2003).

Up to now, some efforts have been made to identify resistance genes against *M. graminicola*. Eighteen major resistance genes (Stb1-18) have been identified (Tabib Ghaffary *et al*., 2011). Detection of resistance genes are very important steps in studying the resistance genetics against the pathogen, however our knowledge about defense genes that are induced against *M. graminicola* after inoculation is still negligible (Adhikari *et al*., 2007).

Analysis of plant-pathogen molecular interactions may be suitable for ascertaining the difference between compatible and incompatible responses. Expression profiles of cultivar Tadinia (containing the Stb4 gene) inoculated with *M. graminicola* during the first four days after inoculation showed that some genes such as *chitinase, phenylalanine ammonia lyase, pathogenesis-related protein PR-1,* and *peroxidase* were up-regulated at early stages (Adhikari *et al*., 2007). Protein disulfide isomerase, which is well known as a component of signal transduction pathways, and three Pathogenesis Related (PR) proteins, PR-1, PR-2, PR-5, were highly induced in inoculated wheat leaves during 3-12 hours after inoculation (Ray *et al*., 2003).

The results of another study showed that four genes including *chitinase, phenylalanine ammonia lyase, PR1,* and *peroxidase* were up regulated within 3 hours-1 day after inoculation. Nine other genes had bimodal up regulated patterns; the first peak was at 1-3 and the second at 12-24 days after inoculation. The remaining gene (a serine carboxypeptidase) had a trimodal pattern (Goodwin *et al*., 2003).

Screening based on cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) has been used as an efficient method for isolation of differentially expressed genes (Durrant *et al*., 2000; Yin *et al*., 2010). It is a powerful and sensitive tool to detect low-abundance transcripts (Breyne *et al*., 2003). This method is based on selective amplification of cDNA fragments generated by restriction fragment endonucleases, electrophoretic separation of the products and comparison of the band pattern between treated samples and controls (Weiberg *et al*., 2008).

We carried out a cDNA-AFLP analysis to identify new genes associated with the infection and defense processes in an Iranian resistant cultivar (Chamran), which is widely used in a large area of Iran and no confirmed resistance genes against *M. graminicola* has been reported (Abrinbana *et al*., 2012).

MATERIALS AND METHODS

Wheat Cultivar and Greenhouse Condition

In this study, one Iranian wheat cultivar (Chamran) resistant to *M. graminicola* was used for gene-expression analyses and two susceptible (Darab2 and Tajan) cultivars were used as susceptible controls, but not included in gene expression analyses, to confirm successful establishment of the pathogen. Chamran is a spring wheat with the potential of carrying effective *Stb* genes, may be *Stb15* (Abrinbana *et al*., 2012). Seeds of the cultivars were sown individually into 10-cm-diameter pots filled with a 1:1 mixture of peat moss and perlite (N, P_2O_5, K_2O ml^{-1}, pH 5.2-6, EC 0.4-6 $mS.cm^{-1}$, MIKSKAAR).The Experiment was conducted in the green house of the Agricultural and Natural Resources Centre of Khuzestan, Iran. Pots were placed on greenhouse benches at day and night temperatures of approximately 22 and 18°C, respectively, with 16-hours photoperiod before inoculation by *M. graminicola* (Adhikari *et al*., 2007).

Fungal Inoculum and Method of Inoculation

Inoculum was prepared from a mixture of fourteen highly virulent isolates of *M. graminicola*. These isolates were collected from naturally infected bread wheat fields

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from Iran and had previously been shown to be virulent on wheat (Ghaneie *et al*., 2011). The isolates were cultured by streaking a small piece of a frozen (-80°C) stock of spores onto plates containing YMDA (4 g of Yeast extract+4 g of Malt extract+10 g of Dextrose+15 g of Agar in 1 L of distilled water). To prevent bacterial contamination, 10 mg L^{-1} Gentamycin as an antibiotic were added to the warm medium (approximately at 45-50˚C) after autoclaving. The plates were incubated at 18-20˚C. To prepare spore suspension, 1-2 mL of distilled water was added to YMDA plates colonized with the fungal pathogen and followed by shaking horizontally. The resulting spore suspensions were added to 250 mL Erlenmeyer flasks containing 150 mL of YMGB (4 g of Yeast extract +4 g of Malt extract+10 g of Glucose in 1 L of distilled water). The antibiotic was added to liquid medium in the same way as solid medium. The flasks were kept in a shaker-incubator at 18-20˚C and 160 rpm with a photoperiod of 12 hours light and 12 hours darkness for 5-7 days. The prepared inoculum suspensions were adjusted to ≈ 3×10^6 spores mL^{-1} by hemocytometer. One drop of Tween 20 was added per 150 mL of spore suspension. Spore mixture was sprayed with a hand–operated sprayer onto each plant 25 days after sowing. For each cultivar, two treatments including inoculation with spores and mock inoculation (sprayed by water) were carried out in three replications. Inoculated seedlings were put under polyethylene cover and relative humidity near 100% was provided by a humidifier for 72 hours. Three days after inoculation (72 hours), plants were transferred to the greenhouse. Greenhouse temperature varied from 20 to 25°C during the day and from 18 to 20°C during the night and relative humidity was about 75%. The pots were watered daily or as needed. To confirm successful infection, the inoculated susceptible seedlings were kept until symptoms fully developed on leaves. The symptoms appeared 21 days after inoculation and fully developed 30 days after inoculation. The reactions of the resistant cultivar to *M. graminicola* isolates were as limited necrotic lesions on the leaf tips, bearing few pycnidia.

Sampling Procedures

To analyze the differential expression profiles, plants were sampled at 6 time points (0, 12, 24, 48, 72, 96 hours) after inoculation. Three plants for each sample were collected by scissors at the base of the stem, placed in a labeled bag, then frozen in liquid nitrogen immediately and stored at -80°C.

RNA Extraction and cDNA Synthesis

Wheat leaves were ground to a fine powder in a sterile mortar and pestle under liquid nitrogen. Approximately 100 mg of ground leaf powder was transferred into sterile 1.5- mL microtubes. Total RNA was extracted using RNX-Plus (Cinnagen, Iran) following the manufacturer's instruction. RNA was treated with RNase-free DNase I (Vivantis, Malaysia) and incubated at 37°C for 20 minutes. The DNase I (Vivantis, Malaysia) was inactivated at 65°C for 10 minutes. The mRNA was isolated from 120 μg of total RNA by mRNA extraction kit (Macherey Nagel, Germany). The first- and second- strand cDNA was generated using the kit (QIAGEN, Germany) and RNase H, DNA polymerase I (Vivantis, Malaysia) according to the manufacturer's protocol. The concentration and quality of RNA and DNA were measured with a spectrophotometer and staining with SafeStain (Cinnagen, Iran) after agarose-gel electrophoresis.

Template Preparation, cDNA-AFLP Reaction and PAGE Analysis

The template for cDNA-AFLP analysis was prepared according to standard

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protocols (Vos *et al*. 1995; Bachem *et al*. 1996). cDNA was digested by *EcoR* I and *Mse* I (Vivantis, Malaysia) and *EcoR* I and *Tru9* I (Vivantis, Malaysia) and then double-stranded adaptors (Bioneer, korea) were ligated to the digested cDNA. Sequences of primers and adaptors used for AFLP reaction were as follows (N can be any nucleotide) (Table 1).

Pre-amplification PCR conditions were as follows: 30 seconds denaturing at 94°C , 1 minute annealing at 56°C and 1 minute extension at 72°C for 25 cycles (Zhang *et al*., 2003) followed by 5 minutes at 72°C (Polesani *et al*., 2008).

Pre-amplification PCR products were diluted 1 to 10 and selective amplification was performed with the following program: 1 cycle of 45 seconds denaturing at 94°C, 30 seconds annealing at 65°C and 1 minute extension at 72°C; 13 cycles of 30 seconds denaturing at 94°C, 30 seconds annealing at 65°C (scale down of 0.7°C per cycle) and 1 minute extension at 72°C; 22 cycles of 30 seconds denaturing at 94°C, 30 seconds annealing at 56°C and 1 minute extension at 72°C (Zhang *et al*., 2003) followed by 10 minutes at 72°C. Selective amplification products of inoculated and non- inoculated plants were separated on a 6% polyacrylamide gel (Vivantis, Malaysia) run at 100W until the bromophenol blue (Vivantis, Malaysia) reached the bottom. The gel was stained for detection of separated DNA fragments by silver nitrate (Vivantis, Malaysia) (Wu, 2010). The highly reproducible cDNA patterns were obtained for two independent sampling of the same individuals for all primer pairs.

Isolation and Sequencing of Fragments

Differentially expressed bands were cut out from the gel with a razor blade, soaked in 50 µL of sterile distilled water and incubated overnight at 4°C. DNA fragments were amplified by PCR under the same conditions as used for selective amplification. PCR products were checked on a 1% agarose gel (Zhang *et al*., 2003), purified using a Vivantis kit (Vivantis, Malaysia) and were sent for sequencing. DNA sequences were compared with the sequences in the GenBank non-redundant database using the Basic Local Alignment Search (BLASTx) program.

RESULTS

cDNA- AFLP Aanalysis

Overall, 81 primer combinations were used and 9 primer pairs with high efficiency were selected. The products ranged in size from approximately 100 to 800 bp. A total number of 276 differentially expressed fragments for the six time points were identified by comparing the expression patterns of infected with uninfected samples. Figure 1 shows a typical cDNA- AFLP profile with one of the primer pairs.

Sequence Analysis of Differentially Expressed cDNA Fragments

Two hundred thirty-one differentially

Table 1. Sequences of primers and adaptors used for AFLP reaction.

Primer Names	Primer Sequences
EcoR I adaptor top strand,	*5′-CTGCTAGACTGCGTACC-3′*
EcoR I adaptor bottom strand,	*5′-AATTGGTACGCAGTC-3′*
Tru9 I adaptor top strand,	*5′-GACGATGAGTCCTGAG-3′*
Tru9 I adaptor bottom strand,	*5′-TACTCAGGACTCAT-3′*
EcoR I pre-amplifying primer,	*5′-GACTGCGTACCAATTC-3′*
EcoR I selectively amplifying primer,	*5′-GACTGCGTACCAATTCNNN-3′*
Tru9 I pre-amplifying primer,	*5′-CATCTGACGCATGGTTAA-3′*
Tru9 I selectively amplifying primer,	*5′-CATCTGACGCATGGTTAANNN-3′*

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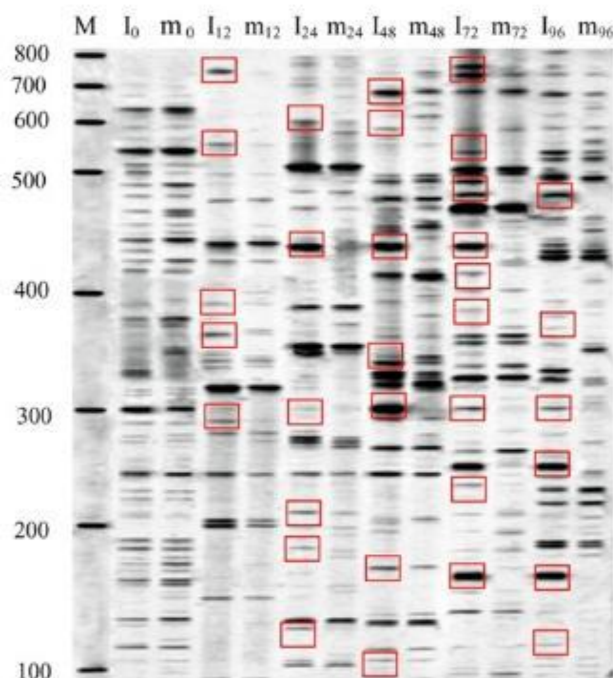

Figure 1. Expression pattern of wheat transcripts after inoculation with *M. graminicola* displayed by cDNA-AFLP in Chamran cv. An example shows selective amplification with *EcoR* I-AGT+*Tru* 9 I-CCA. (I: *M. graminicola*- infected leaves 0, 12, 24, 48, 72, 96 hours after inoculation; m: Mock inoculation 0, 12, 24, 48, 72, 96 hours after inoculation; M: Molecular weight marker. The red boxes indicate differentially expressed bands).

amplified cDNA fragments were extracted, of which 201 fragments showed significant similarity to genes with known function using BLASTx alignment (http:/www.ncbi.nlm.gov/BLAST) and differentially expressed wheat genes were assigned to different functional categories (Table 2).

Induction of Defense-Related Genes in Response to *M. graminicola*

According to Table 2, the eight defense-related genes lipoxygenase, peroxidase (PR 9), chitinase (PR 2, 4, 8), PR-1, Phenylalanine Ammonia Lyase (PAL), disulfide isomerase, B-1-3 glucanase (PR 3), thaumatin like protein (PR 5), and methionine sulfoxide reductase were induced 12 to 24 hours after inoculation in cultivar Chamran. Induction of glycosyltransferase, catalase, and putative xylanase inhibitor genes were observed around 48 hours after pathogen inoculation. Expression patterns of three other genes such as chalcone synthase, EXECUTER 1 protein, and non-specific lipid transfer protein showed that these genes were induced later after inoculation (72-96 hours).

DISCUSSION

The cDNA-AFLP technique has been applied to identify resistance genes in wheat against some pathogens such as stem rust and leaf rust (Yin *et al*., 2010; Zhang *et al*., 2003).

In this study, we used cDNA-AFLP to investigate the gene expression profiles of wheat cv. Chamran infected with *M. graminicola* in comparison with water-inoculated cultivar, as the control, at six-time points after inoculation. Based on Table 2, some differentially expressed transcripts derived from different time points were similar to known defense genes.

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Table 2. List of selected wheat transcripts induced in Chamran cultivar in response to inoculation with *M. graminicola* relative to mock infection (water-inoculated control).

TDF[a]	Pimer comb	Length (bp)	Exp time	copy	Homology	Blastx score	% S[b]
					Defense		
CH-7	CA-CA	360	12	3	Lipoxygenase2 *Triticum aestivum* (ACS3498.1)	2e-42	80
CH-35	AC-TG	150	12,24	7	PAL- *Hordeum vulgare* subsp *vulgare*(CAA89005.1)	2e-18	93
CH-13	AC-TG	230	48,72,96	5	Glycosytransferase *Triticum aestivum* (ACB47884.1)	1e-27	85
CH-16	AC-TG	180	12,48	2	PR-protein 1A/1B *Hordeum vulgare* (P32937.1)	2e-48	87
CH-5	CA-TG	680	12,24	3	PR-protein 1 *Triticum aestivum* (ACQ41880.1)	4e-62	87
CH-18	AC-TG	260	12,72,96	3	Chitinase *Hordeum vulgare* (AAA56787.1)	2e-21	73
CH-4	AC-AG	670	12,48	2	Chitinase I *Triticum aestivum* (BAB82471.1)	5e-06	95
CH-6	GT-TG	510	24,48	2	Chitinase II (PR3) *Hordeum vulgare* (AJ276226.1)	3e-12	92
CH-1	AC-TG	780	48,72,96	5	Catalase *Triticum aestivum*(Q43206.1)	3e-07	77
CH-8	CA-CA	540	12,24	3	Peroxidase5 *Triticum monococcun* (AAW52718.1)	2e-06	87
CH-29	CA-AG	150	12,24	3	Peroxidase1 *Triticum monococcun* (CAA59486.1)	1e-61	79
CH-6	CT-AG	570	24	1	Peroxidase3 *Triticum monococcun* (AAW52717.1)	1e-40	79
CH-3	CA-AG	680	12,72,96	3	Thaumatin like protein *Triticum aestivum*(AF389883.1)	1e-26	96
CH-12	CA-TG	290	72	1	Lipoxygenase1 *Hordeum vulgare*(AAP04432.1)	8e-14	94
CH-2	CA-AG	750	48,72	3	Putative xylanase inhibitor *Triticum durum*(CAC87260.1)	3e-10	92
CH-21	AC-CA	270	24,48,72	4	B-1-3 glucanase *Hordeum vulgare*(AAU11328.1)	6e-21	71
CH-7	CT-TG	520	12,24	3	Disulfide isomerase *Triticum durum* (CAC21229.1)	1e-86	91
CH-9	GT-CA	490	72	1	Peptydyl prolyl isomerase *Hordeum vulgare* (BAK02378.1)	1e-40	86
CH-17	CA-TG	330	24	1	Germin like protein2 *Triticum aestivum* (CAB55558.1)	1e-15	86
CH-26	GT-CA	260	96	1	Chalcone synthase *Triticum aestivum* (AAQ19320.1)	5e-19	57
CH-1	GT-AG	790	96	1	EXECUTER 1 protein *Ricinus communis* (XP_002532765.1)	7e-19	65
CH-4	GT-TG	640	12,24,48	3	Methionine sulfuxide reductse *Secale cereale* (CAE92372.1)	2e-11	75
CH-8	CA-AG	600	96	1	Nonspecific lipid transfer protein *Triticum aestivum* (CAH69201.1)	5e-10	86
					Metabolism		
CH-25	AC-TG	250	48,96	2	Obtusifoliol 14 aplha demethylase Brachypodium *distachyon*	6e-07	79
CH-8	AC-AG	420	12,48,72	3	(XP_ 003573829)	2e-04	59
CH-11	AC-CA	440	72	2	Patatin like protein *Hordeum vulgare* (BAK09816.1)	2e-70	79
CH-17	AC-AG	280	48	2	Phospholipase *Solanum lycopersicum* (NP_001234509.1)	3e-79	95
CH-1	AC-CA	750	96	1	4-Cumarate-CoA- Ligase *Lolium perene* (CAP08804.1)	2e-61	95
CH-1	CA-TG	790	24	2	Sucrose synthase *Triticum aestivum* (AAA34304.1)	1e-04	82
CH-15	GT-GT	420	48,96	2	Cystein synthase *Zea mays*(NP_001105469.1)	6e-12	83
CH-22	GT-AG	160	96	2	Dnaj – like protein *Sorgum bicolar* (XP_002460134.1)	3e-30	53
CH-13	CA-AG	450	24,48	1	Tubulin alpha chain *Oryza sativa* (ABG78593.1)	4e-31	92
CH-4	AC-TG	580	96	2	Ribulose phosphate 3 epimerase *Zea mays* (NP_001149850.1)	2e-18	69
CH-37	AC-TG	130	96	1	Carbonic anhydratase *Triticum turgidum* (ADD65763.1)	7e-16	57
CH-7	GT-AG	390	12	2	Beta ketoacyl reductase *Zea mays* (NP_001105466.1)	6e-18	86
CH-16	GT-AG	410	48	1	Glycine rich protein *Triticum aestivum* (CAB88804.1)	8e-15	82
CH-5	CA-AG	600	72,96	1	Protein translation factor SUI 1 *Zea mays* (NP_0011.4887.1)	6e-22	85
CH-17	GT-TG	250	48	4	40S ribosomal protein *Triticum aestivum* (Q517K3.1)	5e-72	87
CH-5	GT-AG	380	24	1	Long chain acyl-CoA oxidase *Triticum aestivum* (Q9AUDB)	2e-56	75
CH-12	CA-AG	420	96	1	Ribosomal L10 protein *Oryza sativa* (Q874B2)	6e-34	82
CH-22	AC-CA	180	48	1	Gamma- glutamyl cystein synthetase *Zea mays* (Q6F4IB)	6e-33	71
CH-11	CA-AG	210	12	1	Lipase class3- like *Oryza sativa* (Q6K2K7)	1e-27	80
CH-33	CA-CA	170	24,48,72	4	Sedoheptulose- biphosphate aldolase *Hordeum vulgare* (Q94OFB)	6e-60	78
CH-10	AC-TG	440	12,24,96	3	Cystein protease *oryza sativa* (BAB63884.1)	2e-13	65
					Energy		
CH-10	AC-TG	400	12,96	2	Putative NADH dehydrogenase *Arabidiopsis thaliana* (CAB79624.1)	2e-67	70
CH-30	AC-TG	210	48,72,96	3	Vacuolar ATPase *Triticum aestivum* (ABN54974.1)	3e-32	76
CH-8	CA-AG	550	12	2	Vacuolar ATP synthase B chain *Hordeum brevisubulatum* (AER42623.1)	1e-17	76
CH-19	AC-AG	240	24,48,96	5	Cytochrome P450 *Oryza sativa* (CAH66020.1)	4e-09	74
CH-16	AC-CA	230	48,72	4	Cytochrome f *Oryza sativa* (AAS46130.1)	3e-21	89
CH-4	GT-CA	680	72	3	Photosystem II D1 *Triticum aestivum* (AAO31798.1)	2e-35	75
CH-9	CA-CA	340	12	3	Chlorophyll a/b binding protein *Triticum aestivum* (P04784.1)	2e-10	72
CH-12	AC-TG	430	48	1	NADH- Cytochrome P450 oxyoreductase *Hordeum vulgare* (Q2L953)	1e-29	79

[a] TDF= Transcript-Derived Fragment, [b] S= Similarity.

Table 2 continued…

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Continued of Table 2. List of selected wheat transcripts induced in Chamran cultivar in response to inoculation with *M. graminicola* relative to mock infection (water-inoculated control).

TDF[a]	Pimer comb	Length (bp)	Exp time	copy	Homology	Blastx score	% S[b]
					Secondary metabolism		
CH-26	GT-TG	280	12,48	2	Putative flavanone-3-hydrolase *Oryza sativa* (AAL58118.1)	8e-06	87
CH-28	AC-CA	210	48,72,96	5	Coaffeoyl CoA-3-O-methyl transferase *Avena sativa* (BAC78632.1)	6e-18	90
CH-15	CA-TG	270	72	1	Stibene synthase *Oryza sativa* (Q8SDT8)	5e-17	72
					Stress response		
CH-2	CA-AG	780	12,48,72	4	Wali 3 *Triticum aestivum* (AAA50848)	7e-09	92
CH-23	GT-CA	310	48,72	2	Mtn 19-like protein *Zea mays* (ACG30313.1)	4e-34	69
CH-31	GT-AG	640	24,48,96	6	Metallothionein like protein *Zea mays* (ACG32329.1)	3e-08	81
CH-27	CA-AG	220	48	3	Thioredoxin *Oryza sativa* (BAB20886.1)	4e-21	69
CH-12	CA-TG	320	48	1	Glutathione S-transferase GST 24 Hordeum vulgaris (Q9FQDA)	7e-81	86
					Signal transduction		
CH-14	AC-AG	270	24,72,96	4	Serine/Theronine kinase *Hordeum vulgare* (BAC65051.1)	7e-23	65
CH-10	CA-AG	450	24	2	Receptor like protein kinase *Hordeum vulgare* (BAJ97053.1)	2e-60	78
CH-21	GT-AG	220	12,72	2	Cystein-rich receptor like protein kinase *Oryza sativa* (EAY77642.1)	2e-51	86
CH- 7	GT-CA	540	72,96	2	Map kinase phosphatase *Triticum aestivum* (ACB05479.1)	2e-21	71
CH-28	GT-CA	230	48,72	3	Putative protein phosphatase type 2c *Triticum aestivum* (ABS1109301)	2e-41	86
CH-26	AC-TG	110	48	1	Protein tyrosine phosphatase *Oryza sativa* (AAX96301.1)	3e-37	82
CH-8	GT-AG	350	24	1	NAC family protein *Oryza sativa* (Q2Z1Y1)	2e-13	89
CH-15	CA-CA	450	72	1	So2- like protein *Avena fatua* (Q8LK24)	1e-12	72
CH-12	GT-TG	320	72	1	Calcium transporting ATPase *Hordeum vulgare* (CCA05936.1)	2e-25	78
					Transcription		
CH-31	AC-CA	140	48,72	3	RNA binding protein S1 *Oryza sativa* (CA746073.1)	4e-24	67
CH-18	GT-CA	290	12,48	2	WRK 10 transcription factor *Triticum aestivum* (ACD80371.1)	2e-75	90
CH-9	AC-TG	350	24,48,96	4	DNA binding protein *Avena fatua* (CAA88331.1)	1e-74	79
CH-16	AC-AG	490	12,24,48	3	Nucleolycine TIAR- like *Hordeum vulgare* (BAK00528.1)	4e-18	85
CH-26	GT-CA	220	24,48,96	4	AGL6- like MADS box transcription factor *Triticum monoccocum*(ACY71519.1)	7e-08	76
CH-14	CA-TG	180	24	1	H-protein promoter binding factor 1 *Triticum aestivum* (AAC24592.1)	2e-28	89
CH-18	AC-CA	250	48	1	RGA protein *Hordeum vulgare* (CAA72178.1)	5e-12	72
CH-15	GT-TG	320	48	1	Putative bZIP transcription factor *Oryza sativa* (AAD12004.1)	8e-22	78
CH-8	CA-AG	580	96	1	Scarecrow like 21 *Zea may* (AAF21044.1)	5e-15	78
CH-16	GT-AG	220	72	1	Ethylene responsive element binding factor *Triticum monoccocum* (BAA32422.1)	6e-20	65
					Transporter		
CH-20	CA-TG	770	12,24,72	4	PDR- type- ABC transporter *Triticum aestivum* (ACP19711.1)	1e-47	85
CH-25	AC-CA	240	12,72,96	3	Suger transporter *Oryza sativa* (BAC79742.1)	2e-14	64
CH-14	GT-TG	300	12,72,96	4	Synthaxin 132 *Glycin max* (XP_003555337.1)	2e-10	76
CH-42	CA-AG	130	48,72	2	ATP sulfurylase *Brassica junca* (CAA11416.1)	6e-20	84
CH-15	AC-AG	350	48,72	2	14- 3- 3- like protein *Zea mays* (Q01526.2)	3e-14	78
CH-10	GT-AG	430	72,96	3	ADP- ribosylation factor Medicago truncatula (XP_003618209.1)	7e-19	83
					Unknown (16%)		

[a] TDF= Transcript-Derived Fragment, [b] S= Similarity.

Some defense genes such as lipoxygenase, Glycosyltransferase, Thaumatin-like protein, Putative xylanase inhibitor, EXECUTER 1 protein, and Non-specific lipid transfer protein were isolated for the first time in this interaction. The 32 derived fragments with similarity to known defense genes were induced 12-24 hours after inoculation. The results confirmed that, possibly, earlier responses were required for an effective resistance of wheat to infection by M. graminicola, and suggested involvement of a generalized resistance response to M. graminicola. Putative defense genes in wheat were activated very early in response to M. graminicola, even before the fungus penetrated the host. Therefore, transcript levels of the defense- response genes such as

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PR-1, PR-2, PR-3, and Protein Disulfide Isomerase (PDI) increased three hours after inoculation by M. graminicola and reached maximal levels by 12 h after inoculation, confirming the result of Ray *et al*.(2003) and Adhikari *et al*.(2007). Four transcript fragments were isolated at 24, 48, and 72 hai (hours after inoculation) in our research and had similarities to β-1-3-glucanases genes (PR 2), which contributed to cell wall hydrolysis or production of elicitors that drive the plant defense response (Tuzun *et al*., 1999; Shetty *et al*., 2009). Isolation of a transcript-derived fragment of a thaumatin-like protein gene in a resistant cultivar at 24 hai with M. graminicola in the present study confirmed rapid induction and suggested a defense role for thaumatin-like proteins. Thaumatin-like proteins (PR 5) is involved in response to biotic stresses, causing the inhibition of hyphal growth and reduction of spore germination (Thompson *et al*., 2006; Niderman *et al*., 1995; Stevens *et al*., 1996). Several isozymes of peroxidase were expressed during infection of wheat by Septoria tritici, and a high accumulation of transcript 12 hai was found (Shetty *et al*., 2003). Therefore, the induction of different peroxidase genes, 12 and 24 hai in this study confirmed the results of Shetty *et al*. (2003). It seems that peroxidase genes have an important role in wheat defense against M. graminicola.

Shetty *et al*. (2009) showed that resistance was associated with an early accumulation of chitinase transcripts in wheat against *Septoria tritici.* We observed that expression of *chitinase* genes were started 24 hai and continued in all-time points, and this finding also showed rapid induction of PR-proteins and is in agreement with other studies (Anand *et al*. 2003; Adhikari *et al*. 2007; Shetty *et al*. 2009).

Our data showed that expression of the PR-proteins were enhanced 24 hai, suggesting that induction of PR proteins at early stages of contact may help the resistant cultivar Chamran to minimize colonization by *M. graminicola*. Meanwhile, expression of some PR proteins and secretion of their proteins into intercellular spaces at other time points may be involved in controlling of development of the pathogen by antimicrobial properties. Phenylalanyl Ammonia-Lyase (PAL) is a central enzyme of the phenylpropanoid pathway, which leads to the production of phytoalexin (Van Loon and Van Strien, 1999). Seven transcript-derived fragments belonging to *PAL* genes were recognized in the resistant cultivar Chamran at 12 and 24 hai. Our results showed rapid induction of *PAL* genes in an incompatible interaction of wheat with *M. graminicola*, similar to the results of Adhikari and coworkers (Adhikari *et al*., 2007) who showed that *PAL* genes were expressed at early stages (3 to 24 hai). However, PAL was up-regulated 3, 9, 11, and 15 dai (days after inoculation) in resistant genotypes (Shetty *et al*., 2009).

Several other genes, for example, lipoxygenase, disulfide isomerase, germin-like protein, and methionine sulfoxide reductase, were induced early in the present study.

Plant Lipoxygenase (LOX) involved in the defense response to pathogens, wounding and stress (Akram *et al*., 2008; Feussner and Wasternack, 2002; Shah, 2005; Ray *et al*., 2003). The rapid but only low to moderate induction of LOX in the response to inoculation with *M. graminicola* in a resistant line was observed and it may prevent or retard fungal colonization (Ray *et al*., 2003). It has been illustrated that the enzyme activity was maximum at 18 hai (Babitha *et al*., 2006). Their results are in agreement with our present data.

Protein Disulfide Isomerase (PDI) is well known as a molecular chaperone, and component of signal transduction pathways and antioxidant properties of PDI could limit potential cell damage by reactive oxygen species generated during the wheat-*M. graminicola* interaction (Ray *et al*., 2003). PDI was induced within 3 hours after inoculation with the highest induction in a resistant cultivar, which was treated with *M. graminicola* (Ray *et al*., 2003). Therefore,

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our findings in the present work are in agreement with their results.

Several evidences suggest that Germin-Like Proteins (GLP) are involved in general plant defense responses and, possibly, in cell wall reinforcement (Lane, 2002; Manosalva *et al*., 2009; Shetty *et al*., 2009). Our results confirmed the observation of Godfrey *et al*. (2007) and Segarra *et al*. (2003) that expression of germin-like protein was enhanced after inoculation with the pathogen. Finally, Methionine Sulfoxide Reductase (MSR) plays a critical role in protecting the plant against pathogens and the activity of oxidative stress (Zhao *et al*., 2003). In another experiment, the *MSR* gene in wheat had a bimodal expression pattern with both early (1-3 dai) and late (12-24 dai) in response to *M. graminicola* (Adhikari *et al*., 2007). Our data confirm the enhanced expression of the *MSR* gene at the initiation of the wheat defense response. Therefore, *LOX, PDI, GLPs,* and *MSR* genes may have a role in the resistance of wheat to *M. graminicola*.

Some other genes including non-specific lipid transferase, EXECUTER 1 protein, chalcone synthase, peptidyl prolyl isomerase, glucosyltransferase, putative xylanase inhibitor and catalase were induced in wheat at 48, 72, and 96 hai. Some of these genes may be involved in decreasing fungal biomass (Adhikari *et al*., 2004) and prevention of change in growth from the biotrophic to the necrotrophic stage of fungus after induction in wheat (Adhikari *et al*., 2007). They also may be involved in plant defense in wheat against M. graminicola. Our results showed that chalcone synthase was induced four dai and this result was not in parallel with the study of Shetty *et al*.(2009). This was because chalcone synthase levels in the resistant cultivar were up regulated at 1 and 5 dai and generally reduced (3,7,9 and 13,15 dai) whereas no significant differences in its level were observed at the remaining time points (Shetty *et al*., 2009). In addition, its difference could have occurred because of cultivar reaction. Therefore, this gene should be the subject of further studies.

One of the main goals of this study was an investigation of defense response genes and identification of some new defense genes in Iranian wheat cultivar Chamran. Analysis of inoculated plants with cDNA- AFLP 12 hours after inoculation helped us to identify some new defense genes against *M. graminicola,* which could be used in quantitative methods like Real-time PCR for further study. Due to the large genome size of wheat, cloning of STB resistance genes is difficult (Somasco *et al*., 1996; Adhikari *et al*., 2003). Thus, the defense-related genes and other introduced genes may serve as useful molecular markers, but further study is needed.

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تجزیه و تحلیل ژن های دفاعی در گندم (رقم چمران) آلوده به *Mycosphaerella graminicola* بوسیله روش cDNA-AFLP

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چکیده

القای افتراقی ژن ها در گندم (رقم چمران) در پاسخ به *Mycosphaerella graminicola* با استفاده از روش cDNA-AFLP مورد مطالعه قرار گرفت. گیاهچه های سه هفته ای گندم توسط بیمارگر قارچ تلقیح شد، سپس در شش نقطه زمانی (۰ ، ۱۲ ، ۲۴ ، ۴۸ ، ۷۲ ، ۹۶ ساعت) پس از تلقیح با بیمارگر نمونه برداری از گیاهان انجام شد. با مقایسه الگوی بیان گیاهان تلقیح شده (آلوده) با گیاهان تلقیح نشده (غیر آلوده) ، ۲۷۶ قطعه با بیان متفاوت شناسایی و توالی یابی شدند. با بررسی توالی های بدست امده در NCBI این توالی به دسته های مختلف عملکردی از جمله دفاع ، متابولیسم ، انرژی ، رونویسی ، انتقال ، انتقال سیگنال ، پاسخ استرس ، متابولیسم ثانویه و توالی ناشناخته دسته بندی شدند. هشت ژن مربوط به دفاع از جمله لیپوکسیژناز ، پراکسیداز (PR 9) ، کیتیناز2 (PR 2 ، 4 ، 8)، PR-1 ، پروتئین شبه توماتین (PR5) ، فنیل آلانین آمونیاک لیاز (PAL) ، 3-1-β گلوکاناز (PR3) ، دی سولفید ایزومراز و متیونین سولفوکسید ردوکتاز ۱۲ تا ۲۴ ساعت پس از تلقیح در این رقم گندم القا شدند. القای گلوکوزیل ترانسفراز ، کاتالاز و ژنهای مهارکننده زایلاناز حدود ۴۸ ساعت پس از تلقیح با پاتوژن مشاهده شد. الگوهای بیان سه ژن دیگر ، یعنی کالکون سنتاز ، پروتئین EXECUTER 1 و پروتئین انتقال چربی غیر اختصاصی نشان داد که این ژن ها ۷۲الی ۹۶ ساعت پس از تلقیح القا شده اند. داده های ما نشان داد که بیان پروتئین های PR ۲۴ساعت پس از تلقیح افزایش یافته و نشان می دهد که آنها ممکن است در دفاع در برابر *M. graminicola* نقش داشته باشند. بیان لیپوکسیژناز ، گلیکوزیل ترانسفراز ، پروتئین مانند توماتین ، مهار کننده زیلاناز قلمداد ، پروتئین EXECUTER 1 و پروتئین انتقال چربی غیر اختصاصی برای اولین بار در این واکنش گزارش شده است.

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