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Induced *Acidic chitinase* Expression and Scab-Resistant in Wheat Under Field Condition

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Abstract

Fusarium head blight (FHB) caused by *Fusarium graminearum* is responsible for billions of dollars in agriculture losses. The goal of the present study was evaluation the expression of *acidic chitinase,* one of PR proteins, in wheat defense response against different FHB induced treatments in 'Falat' as a highly susceptible and 'Sumai3' as a tolerant cultivar. These treatments contained fungi extract, spore suspension, DON and salicylic acid (SA). Infected spikes are sampled in 0, 3, 6, 12, 24, 36, 72 hours and 7 days after artificial inoculation and subjected to total RNA extraction and cDNA synthesis. Quantitative Real-Time PCR was performed with specific primer to detect expression differences between tolerant and susceptible cultivars. Output data was analysed using REST software, indicated differences in expression level of cultivars during the disease expansion cycle. These results support the emerging role of *acidic chitinase* gene in salicylic acid signaling pathway against necrotrophic fungal pathogen and demonstrate the effect of *acidic chitinase* gene in SAR signaling cascade against fungal extract treatment as PR protein inducer, as compare with these DON can't act as a elicitor for *acidic chitinase* gene according to its expression profiling in this experiment. Results may serve as a foundation for dissecting mechanisms and regulations underlying FHB and SAR elicitors resistance reactions in wheat and revealing functions of the plant *acidic chitinase* gene in defense.

Keywords: Chitinase; Elicitor; FHB; QRT-PCR; REST

Introduction

Fusarium head blight (FHB) is economically one of the most important fungal diseases of wheat throughout the world. It also is an important disease of wheat in different areas of Iran, such as Mazandaran, Gorgan, Gonbad and Moghan regions [13]. One of the major hurdles in the production of high quality and yield of food crops is the difficulty in control of plant diseases, an aspect that concerns the producer and consumer as well. This is because many fungal pathogens have developed resistance against the active ingredients of a

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wide spectrum of fungicides, and there is a common perception that pesticides are undesirable. It is in this context that the application of biotechnology would be a better choice to minimize the incidence of disease in agricultural crops [12]. One such approach would be through the induction and enhancement of the plant's own defense mechanisms rather than the application of toxic compounds. The induction of such a plant defense strategy seems logical as the induced disease resistance in plants is likely to offer protection against different pathogens, the attractive alternative which is natural, safe, effective and sustainable in controlling plant diseases [31].

The induced resistance mechanism may involve not only some preformed components but also a cascade of induced responses [20]. These include novel antimicrobial compounds (phytoalexins), proteins and physical barriers to penetration. This cascade of resistance factors is induced only when a plant recognizes the presence of a potential pathogen, and the compounds capable of triggering such responses are termed elicitors [5].

Plants can defend themselves against different pathogens through a wide array of mechanisms that may be local, systemic, inducible or constitutive. There are several mechanisms like hypersensitive reactions (HR), production of phytoalexins and pathogenesis-related (PR) proteins, and simple physical barriers to penetration through deposition of lignin, etc. Already, a number of fungal elicitors have been extracted and characterized [7]. Cultured plant cells and fungal elicitors, fungal cell-wall fragments that elicit defense-related gene expression, have been extensively used to detect defense-related gene products in plant–pathogen interactions [21]. A number of fungal elicitors have been isolated and characterized from culture medium of fungi grown *in vitro* and from intracellular washing fluids of infected plants [19]. Acidic and basic chitinases are located in intercellular spaces and in vacuoles, respectively. Tissue specific expression of chitinase isoenzymes has been observed in maize seedlings [4,6].

Arabidopsis mutants affected in the production or action of the signaling compounds salicylic acid (SA), jasmonic acid (JA), or ethylene (ET) likewise show an enhanced disease susceptibility phenotype upon infection by specific pathogens, indicating that these regulators play a role in the basal resistance against these pathogens [11,28]. The same regulators have also been implicated in certain types of nonhost resistance and in *R* gene-mediated resistance, suggesting that expression of these different types of resistance involves activation of partly similar defensive mechanisms [8,29].

Whether or not a plant turns out to be susceptible or resistant is likely determined by the Quantitative Real-Time PCR with which these method is performed by their effectiveness against individual pathogens with different modes of attack, but it has been observed that few PR proteins are up-regulated, earlier, faster and/or more in resistant genotypes than in susceptible genotypes [27]. Between various quantification methods of measuring gene expression, QRT-PCR is the most sensitive and flexible and can be used to compare the levels of mRNAs in different sample populations, characterize patterns of mRNA expression, discriminate between closely related mRNAs and analyze RNA structure.

In current study, we have employed QRT-PCR technique for investigation of expression pattern of *acidic chitinase* gene of wheat in response to FHB. In particular, we address progress in the identification of the role of *acidic chitinase* gene in SAR and progress in surveying *acidic chitinase* transcripts against SA treatment to demonstrate *acidic chitinase* effect in the SAR signaling cascade. Also with the use of pattern expression of *acidic chitinase* gene against SA treatment in comparative with the result from *acidic chitinase* transcripts against fungal treatment and DON treatment revealed that DON hadn't elicitor effect on *acidic chitinase* gene after inoculation in resistant variety relative to susceptible variety.

Materials and Methods

Two wheat (*Triticum aestivum L.*) genotypes with contrasting levels of resistance and susceptibility to Fusarium head blight (FHB) were tested at the experimental field. An Iranian spring wheat cultivar, Falat, as a highly susceptible to FHB along with a Chinese originated FHB tolerant cultivar, Sumai3 which known for Type I and II FHB resistance [2]. have been employed. To prepare inoculums, fungal isolate was collected from 2009 field trap nursery and cultured on potato dextrose agar medium. About 5 gr straw powders were added to 125 ml of distilled water in to 250 ml flask. Mixtures were autoclaved at 120°C and 1 atmosphere for 30 minutes two times during 48 hours. Then, each flask was inoculated with an agar plug from a clean *F. graminearum* isolate under a laminar flow hood. The flasks were swirled gently at 120 rpm at 25°C for 96 hours.

Inoculation and Data Collection

The number of conidiospores per ml was determined by counting spores using a hemacytometer and adjusted

Table 1. Properties and Nucleotide sequences of primers used in QRT-PCR

Gene	Gene description	Accession No	Sequences	Amplified fragment(bp)
GAPDH	glyceraldehydes-3-phosphate dehydrogenase	EF592180	3'-TCACCACCGACTACAATGACC-5'	121
			3'-ACAGCAACCTCCTTCTCACCG-5'	
Chitinase	acidic chitinase	AF112966	3'-TTCTGGTTCTGGATGACCAAC-5'	151
			3'-ACTGCTTGCAGTCCGTGCCAC-5'	

to the desired spore concentration of 10^5 conidia spores/ml with distilled water. Plants were grown in field at Gorgan Agricultural Research Station in 2010 and inoculation was conducted in 6 to 7 weeks after germination at anthesis according to Zadoks stages 65-69 [32]. Wheat spikes inoculated with suspension of *F. graminearum* spores that served as one treatment. This treatment was injected between lemma and palea of 10 central spikelets per each spike. The severity of FHB was visually estimated using a 0–100% scale [25].

To evaluate defense reaction for the efficacy to induce resistance, 10 ml of fungal extract, 20 ppm of toxin (DON), 200ppm of salicylic acid and distilled water as control was injected between the palea and lemma of 10 central spikelets per each spike. The inoculated spikes were covered with plastic bag.

Sampling

Glumes were collected for RNA isolation at 0, 3, 6, 12, 24, 36, 72 hours and 7 days after inoculation (DAF). The mock inoculation was made by distilled water in both 'Sumai3' and 'Falat' for all time points. Immediately, the sampled spikes were placed on liquid nitrogen and transferred into a -80 °C freezer for storage until RNA extraction. The lemma, palea and subtending section of the rachis were pooled and ground into fine powder in liquid nitrogen using sterile mortar and pestle.

RNA Extraction, cDNA Synthesis and QRT-PCR

Total RNA was isolated from the inoculated samples (testers) and mock inoculated (control) glumes of 'Sumai3' and 'Falat' using RNX-PLUS kit (Cinagen, Iran). Extracted RNA was quantified by spectrophotometer and its quality was verified by 1.5% agarose gel electrophoresis. RNA was treated with *DNase* I (FermentaseTM, Germany) to remove DNA contamination before cDNA synthesis according to manufactures instructions. The first strand of cDNA was synthesized from 2 µg of total RNA as the template using M-MuLV Reverse Transcriptase (Fermentase, Germany) and oligo $(dT)_{18}$ primer. The forward and reverse primers for *acidic chitinase* and reference gene (*GAPDH*) were designed by Primer3 online software based on 3′-UTR region of each gene [23].

Table 1 shows properties and sequences of primers for the *acidic chitinase* gene and also for the reference gene, GAPDH. The relative expression pattern of *acidic chitinase* gene in sampling time points was evaluated by SYBR Green method using SYBRBIOPARS Kit (Gorgan University of Agricultural Sciences and Natural Resources, Iran). After an initial activation step of the DNA polymerase at 95°C for 3 min, samples were subjected to 35 cycles of amplification (denature at 95 °C for 10 sec, annealing at 60°C for 10 sec and extension at 72 °C for 10 sec).

Each sample was evaluated in 3 technical and two biological replications. Relative gene expression was calculated by Pfaffl formula [17]. The ratio between the target genes and reference gene was analyzed by the REST software [18]. Melting curve was used to check primer specifity.

Results

Effect of F. graminearum Spores as Inoculum on Acidic chitinase Gene Expression

Two wheat genotypes named as Sumai3 and Falat, respectively as FHB tolernt and susceptible, were investigated to determine the correlation between infection in spike tissues and timing of transcript accumulation of defense response gene including *acidic chitinase* against *F. graminearum* spores (tester). The y-axis values indicate the relative expression of *acidic chitinase* gene in 'Sumai3' and 'Falat' glumes inoculated with *F. graminearum* spores (tester) compared to control (mock inoculated) at each time point after inoculation (x axis).

As shown in Figure 1, in susceptible variety 'Falat', *acidic chitinase* transcripts was accumulated significantly in glumes at 3 hours after inoculation, but gene expression had down-regulated at 6 hours lower than control. There were increases at 12, 24, 36, 72

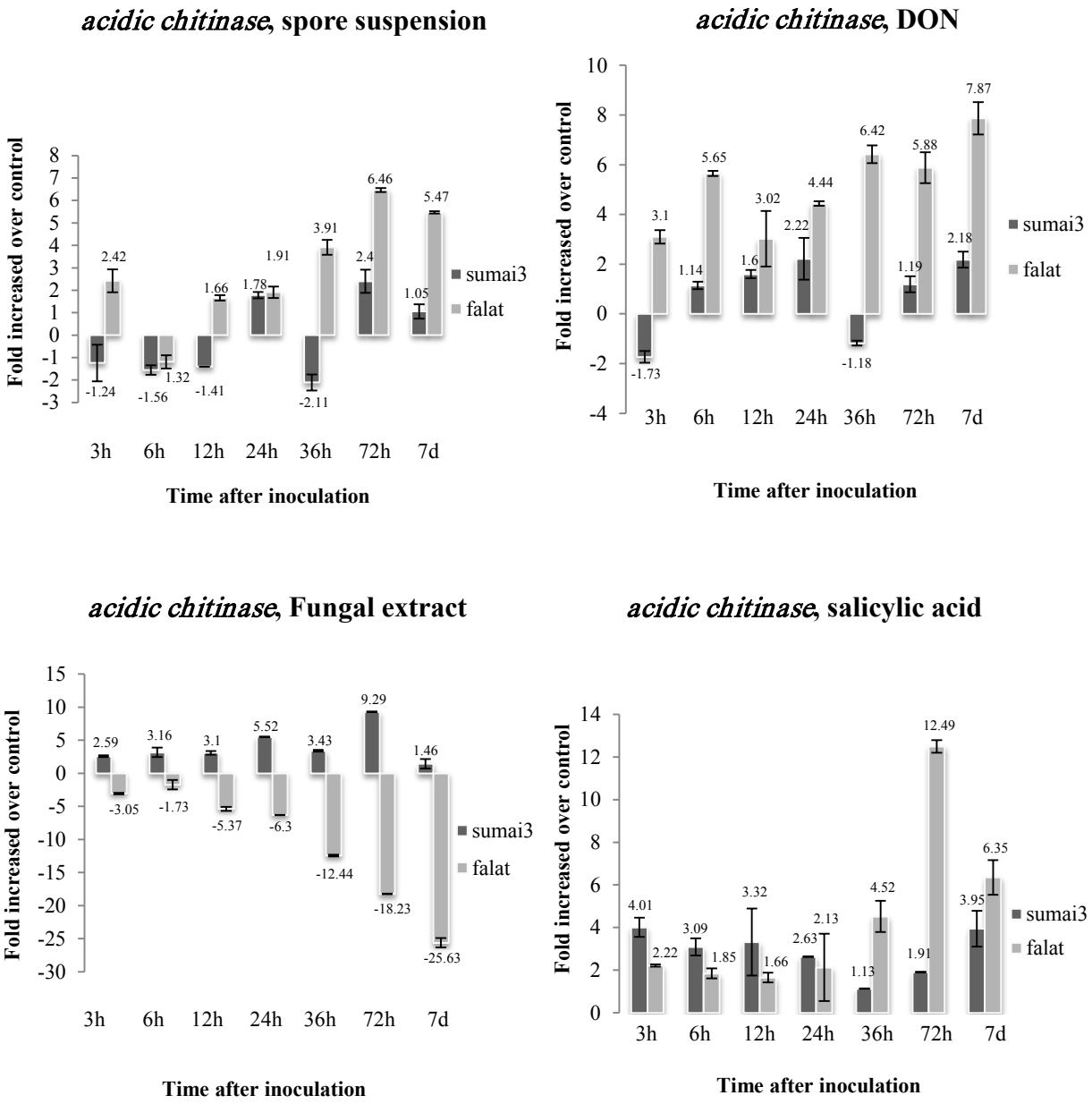

Figure 1. Fold changes in accumulation of *acidic chitinase* transcripts in FHB-tolerant ('Sumai3') and FHB-susceptible ('Falat') wheat cultivars at different times after treatment with fungal extract, *F. graminearum* spore suspension, DON, and salicylic acid. The relative fold change of target gene transcripts was calculated using the comparative cycle threshold method. The infected samples were quantified relative to the controls (mock inoculated) at the same time points. GAPDH was used as an endogenous control to normalize the data for input RNA difference between the various samples. Mean values and standard deviation (S.D.) are shown.

hours after inoculation. The highest level of expression existed at 72 hours after inoculation in susceptible variety.

In tolerant variety 'Sumai3', the expression of gene was decreased at 3, 6, 12 hours after inoculation lower than control, but there was increase in transcript level of *acidic chitinase* gene at 24 hours after inoculation. Observed down-regulation *acidic chitinase* transcripts at 36 hours after inoculation lower than control, but had significant increased at 72 hours after inoculation that followed with decrease at the last time.

In this case against our results, up-regulation

occurred of defense-related genes in resistance relative to cultivar susceptible during early fungal stress (3-12 h) [3]. The highest level of *acidic chitinase* gene expression at 72 hours after inoculation in tolerant variety same as susceptible variety, but this increase in susceptible variety was significantly more than resistance variety. We can say that sensitivity reaction against fusarium suspension in susceptible variety was higher than tolerant variety and for this reason gene expression was more in susceptible variety. In this treatment peers that some other member of chitinase family such as basic or neutral are acting in signaling pathway.

Effect of DON on Accumulation of Acidic chitinase

In susceptible variety defense reaction was started with increases *acidic chitinase* transcripts at 3, 6 hours after inoculation against toxin DON treatment. There was reduction in gene expression at 12 hours after inoculation, but observed two significant increases at 24, 36 hours after inoculation. There was dow-regulation at 72 hours after inoculation that continued with increase at 7 days after inoculation.

As compared, in tolerant variety, down-regulation was observed at 3 hours after inoculation lower than control. Gene expression increased at 6, 12, 24 hours after inoculation against DON treatment, but there was significant decrease at 36 hours after inoculation lower than control that continued with increase at 72 hours and 7days after inoculation. Unlike our experiment [15] showed that pure trichothecene have an elicitor-like activity, including activation of MAPKs in *Arabidopsis thaliana*, induction of defense genes, accumulations of SA and reactive oxygen species, and lesion formations, but in this experiment peers that DON can't act as elicitor of *acidic chitinase* in surveying the pattern of its expression profile.

Effect of Fungal Extract on Accumulation of Acidic chitinase

In tolerant cultivar 'sumai3'against fungi extract treatment, there were up regulation in *acidic chitinase* gene transcripts at 3 hours to 24 hours after inoculation, there was decrease at 36 hours but increased at 72 hours that followed with decrease *acidic chitinase* gene expression at 7 days. The highest level of *acidic chitinase* gene expression was observed 72 hours after inoculation.

Against fungi extract treatment as shown in Figure 1, the expression of *acidic chitinase* gene showed significant reduction at all time points after inoculation in 'Falat' suseptible cultivar lower than control. This evidence showed effective role of fungi extract to product disease in inoculated cultivars. This evidence was same as Kong *et al*. (2005).

Effect of Salicylic Acid on Accumulation of Acidic chitinase

In tolerant cultivar, there were increases and constant pattern of *acidic chitinase* expression in 3, 6, 12, 24 hours after inoculation, but there was significant decrease at 36 hours, observed increases at 72 hours and 7 days after inoculation. The highest level of *acidic chitinase* expression was observed at 3 hours after inoculation.

Subsequently, in susceptible variety against salicylic acid treatment, *acidic chitinase* gene expression was lower than resistant cultivar transcripts and constant at 3 hours to 24 hours after inoculation. Observed significant increases at 36, 72 hours and continued with decrease at 7 days after inoculation. The highest level of *acidic chitinase* gene expression was observed 72 hours after inoculation against salicylic acid treatment in susceptible variety. This evidence was same as Anand et al., (2003) that there were the increase in conjugated SA levels that observed in hemizygous and homozygous *chi/glu* transgenic wheats. This pattern demonstrate that *acidic chitinase* gene envolved in SAR reaction, we examined inducible effect of SA treatment on *acidic chitinase* transcripts accumulation, the result revealed that this gene is member of SAR cascade. So SA treatment can induce *acidic chitinase* gene in LAR and then in SAR.

Discussion

In this study we tried to analyze the role of defense gene in response to different inducers inoculation by applying the QRT-PCR method. The results showed rapid accumulation of *acidic chitinase* in tolerant cultivar comparing to the susceptible cultivar against fungal extract and SA treatments due to its role in host defense to these elicitors. Gene encoding inducible plant defense related proteins, particularly PRs, comprise broad, evolutionarily conserved families with individual members differing widely in occurrence and, where known, activity. Therefore, they likely have an ancient origin with subsequent diversification to serve different functions. Those proteins that are expressed during plant development in specific stages or organs may, through their specific hydrolytic activities, contribute to the generation of signal molecules that can act as morpho-genetic factors, such as Chitinase in somatic

embryogenesis [16, 22], Plants contain a whole array of cellular mechanisms to defend themselves against invading pathogens. In many cases, pathogen recognition by a host activates the so-called hypersensitive response (HR), which is a resistance response characterized by localized cell death.

Due to symptom of this disease, the SAR pathway is activated. SAR activation results in the development of a broad-spectrum, systemic resistance [9, 14] as well as application of external SA treatment is used to find out whether the induction of *acidic chitinase* gene correlates with the onset of SAR in these species.

All these responses are deployed between hours and days after infection. Besides this acute defense at the infection site, some necrotizing pathogens also induce defense responses in distal parts of host plants. *acidic chitinase* gene expression pattern of another treatments such as *f. graminearum* spore suspension, DON, fungal extract in challenged part induction of these local responses is compared with distal responses (induce *acidic chitinase* gene) or use of SA treatment as testimony for induce *acidic chitinase* gene that is one PR protein. In certain plant species, this induced ‘‘immunity’’ develops only in non-challenged parts of infected leaves (local acquired resistance or LAR), whereas in others such as tobacco, *Arabidopsis*, and cucumber, it is also seen in upper non infected leaves (systemic acquired resistance or SAR) [24].

In this study, we have evaluated expression pattern of a pathogenesis related protein of two wheat cultivars including *acidic chitinase*, during several time points after infection by different treatments, causal agent of fusarium head blight disease. The artificial inoculation has been conducted under field condition on 'Sumai3', as a well-known FHB-tolerant cultivar and 'Falat', as highly FHB-susceptible check. Quantitative real-time PCR analysis showed that studied PR protein gene was regulated specifically and distinctively in tolerant and susceptible cultivars. The expression profile of studied PR gene showed accumulation of related mRNA and sometime higher up-regulation in tolerant cultivar in response to salicylic acid, fungi extract infection.

In previous experiment, most *acidic chitinases* were induced by Fusarium fungi. The inducible PR proteins are mostly *acidic chitinases* that are secreted into the intercellular space. Location of the major, acidic PR proteins in the intercellular space seems to guarantee contact with invading fungi or bacteria before these are able to penetrate. In contrast to the acidic PR proteins, which are mostly intercellular, the basic PR proteins are mostly localized intracellularly in vacuoles [30].

In conclusion, we demonstrated that general expression of *acidic chitinase* gene was clearly increased and/or decreased under field condition. However elicitors had different particular patterns and induced significantly in resistant cultivar at 3 hours to 24 hours after inoculation in response to SA. Evidence indicated that *acidic chitinase* gene was involved in defense reaction in SAR reaction in a dependent SA pathway. The early up-regulation of *acidic chitinase* gene in tolerant variety led to activate of PR-protein genes to initiate SAR and tolerant reaction, but late increase of this gene in response to SA in susceptible cultivar resulted to inability of this cultivar to activate PR-proteins immediately after inoculation which led to susceptible response. Fungal extract could activate the accumulation of *acidic chitinase* transcripts just in tolerant variety no in susceptible cultivar. It seems DON treatment was more effective on the susceptible variety and with effect close to the tolerant cultivar levels.

Probably, the other *chitinase* gene (basic or neutral) can have effective role as PR-protein under two other inducers containing DON and spore suspension to turn on SAR pathway in SA-dependent or SA-independent cascade same as Soltanloo *et al*. (2010).

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