Study on Differentially Lipase Gene Expression against Different Inducers of Defense Response in Scab-Resistant and Susceptible Wheat Cultivars

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*J Rec Adv Agri* 2012, 1(1): 6-14
Study on Differentially Lipase Gene Expression against Different Inducers of Defense Response in Scab-Resistant and Susceptible Wheat Cultivars

Bagheri Bajestani F., *2Ramezanpour S. S., 2Soltanloo H., 3Vakili Bastam S.

1Plant breeding and Biotechnology Department, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
2Plant breeding and Biotechnology Department, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
3Plant breeding and Biotechnology Department, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

Abstract

*Fusarium graminearum* is the pathogenic agent of Fusarium head blight (FHB), which is a destructive disease on wheat and barley, thereby causing huge economic loss and health problems to human by contaminating foods. Chinese cultivar, sumai3, is one of the few wheat cultivars with resistance to scab. The expression pattern of *lipase* gene has been studied during wheat defense reaction to fungal extract, spore suspension, toxin (DON) and salicylic acid treatments in Scab-tolerant (Sumai3) and scab-susceptible (Falat) wheat cultivars. Infected spikes are sampled during 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 disease expansion cycle. We compared result from four expression pattern against different treatment for obtain the role of *lipase* gene in SAR cascade. Particulary observed aggregation of *lipase* transcripts in 6 hours to 7 days against *F. graminearum* spores suspension and they increased in 24 to 7 days against fungal extract in resistant variety. Also *lipase* transcripts increased in 6 and 72 hours against DON treatment in resistant variety. These increases observed whereas accumulate of *lipase* gene decreased in susceptible variety. This evidence can use for discover the SAR element in systemic reaction and can be base of other similar experiment to clear this mechanism.

Key words: Artificial inoculation, DON, *lipase*, REST software, SAR element

Corresponding Author: ramezanpours@gau.ac.ir
Received on: 07 Mar 2012
Accepted on: 13 Apr 2012
Online Published on: 28 Apr 2012
Introduction

The recent increase in prevalence and severity of Fusarium head blight (FHB) on wheat in different areas of Iran, such as Mazandaran, Gorgan, Gonbad and Moghan regions (Moosawi et al., 2007), has caused hardship and economic loss to producers and the grain industry (Gilbert and Tekauz, 2000). FHB is more common in warm and humid areas of the world (Parry et al., 1995). Several severe FHB epidemics have occurred in North America with losses in excess of one billion US dollar a year. Comparable losses due to FHB outbreaks have occurred in other wheat-growing regions of the world. Infection by F. graminearum ascospores is initiated in wheat florets at anthesis, from where the fungus spreads to other spikelets within the spike. Eventually, the fungus-infected spikelets become necrotic and bleached (McMullen et al., 1997). Grain from F. graminearum-infected plants accumulate the mycotoxin deoxynivalenol (DON), a vomitoxin, which further limits grain quality. In wheat and barley, monogenic, gene-for-gene resistance to F. graminearum has not been identified. Resistance to FHB in some cultivated wheat cultivars is derived from the Chinese cv. Sumai 3 and its derivatives. Sumai3 derived resistance to FHB is a complex, quantitative trait which confers type II resistance that limits fungal spread from the site of infection. The best control methods combine the planting of cultivars that are partially resistant to FHB with fungicide application and rotation with non host crops (Bai and Shaner 2004). The genetic manipulation of components of defense signaling pathways offers an alternative strategy for controlling plant diseases. Regulatory genes that control the expression of multiple defense genes are excellent targets for developing broad spectrum and durable resistance against pathogens (Stuiver and Custers 2001). Systemic acquired resistance (SAR) is an inducible defense mechanism that confers resistance to a broad spectrum of pathogens. SAR is associated with accumulation of elevated levels of salicylic acid (SA) and expression of the pathogenesis-related (PR) group of genes, some of which encode antimicrobial proteins (Durrant and Dong 2004). The role of SA in SAR has been well characterized (Shah, 2003).

During the initial association with their hosts, plant pathogens encounter the epicuticular waxes and cuticle covering host epidermal cells. The cuticle is a continuous layer of lipid material that consists of insoluble polymeric material called cutin. The cutin polymer matrix with embedded waxes forms an efficient barrier against desiccation and decreases the vulnerability of plants to pathogen attack by providing both mechanical disease resistance and cellular signals for resistance responses (Kolattukudy, 1985). Lipase enzymes may contribute to pathogenesis by degrading wax, cuticle and cell walls, thus aiding in spore germination and infection. Furthermore, they can act as elicitors of host defense reactions and may also play a nutritional role during certain stages of the fungal life cycle.

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 (Steiner et al., 2008). 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 wheat lipase gene in response to FHB. The goal of
the present study was evaluation one of famous PR proteins in wheat defense response to different Fusarium treatments in tolerant and susceptible cultivars and evaluated the role of lipase gene in SAR cascade. Several comprehensive literature reviews about SAR had been published (Chen et al., 1995; Hunt and Ryals, 1996; Neuenschwander et al., 1995) Salicylic acid (SA) has been identified as a key signal for the expression of PR proteins during LAR and SAR in tobacco, cucumber, and Arabidopsis. After pathogen infection, levels of SA increase in infected and uninfected leaves, and SA accumulation is essential for the expression of PR proteins and for resistance during LAR and SAR (Hunt and Ryals, 1996).

Materials and Methods

Plant materials

The following two wheat (Triticum aestivum L.) cultivars were used for the expression analysis: Sumai 3 is a Chinese cultivar known as being resistant to FHB which known for Type I and II FHB resistance (Bai and Shaner, 1994) and Falat is an Iranian spring wheat cultivar known as being susceptible to FHB was tested in the comparative pattern of lipase transcripts experiment.

Fungal materials

The isolate of Fusarium graminearum used for inoculation was obtained from infected wheat cluster collected from 2009 field trap nursery and cultured on potato dextrose agar medium. The single-spore isolation was developed on potato dextrose agar (PDA) medium. The inoculum was obtained by growing the isolate on an autoclaved straw powder mixture in flask. This straw powder mixture was prepared by soaking about 5 gr the healthy wheat straw powder in to 125 ml of distilled water in to 250 ml flask, then autoclaving at 120 °C and 1 atmosphere for 30 minutes two times during 48 hours. After transfer of an agar plug from a clean F. graminearum isolate under a laminar flow hood to wheat straw powder mixtures, the flask was incubated for 96 hours at 25 C, and were shaken thoroughly 120 rpm. Abundant sporation on the surface of straw powder appeared, The fungal spore suspension was filtered through layers of cloth to remove fungal mycelia and the spore concentration quantified using a haemocytometer. The number of conidiospores per ml was determined by counting spores using a hemacytometer and adjusted 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 (Zadoks et al., 1974). Wheat spikes inoculated with suspension of F. graminearum that served as one treatment (tester). 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 (Stack and McMullen, 1995).

Preparation of other treatments

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.

RNA extraction, cDNA synthesis and QRT-PCR;

Field samples were obtained 0, 3, 6, 12, 24, 36, 72 hours and 7 days after inoculation. The mock inoculation was made by distilled water in both ‘Sumai3’ and ‘Falat’ for all time points. Immediately, infected wheat heads from each of the four treatments (inoculation with F. graminearum suspension, DON, SA, fungal extract or water) 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.

All of the inoculated samples (testers) and mock inoculated (control) glumes of ‘Sumai3’ and ‘Falat’ used in the extraction procedure 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 total RNA as the template using M-MuLV Reverse Transcriptase (Fermentase, Germany) and oligo (dT) 18 primer. The forward and reverse primers for QRT-PCR were designed by Primer3 online software (Rozen and Skaletsky, 2000).

Table 1 shows properties and sequences of primers for the lipase gene and also for the reference gene, GAPDH. The relative expression pattern of lipase 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) and a terminal extension step at 72 °C for 5 min.

Each sample was evaluated in 3 technical and two biological replications. Relative gene expression was calculated by Pfaffl formula (Pfaffl, 2001). The ratio between the target genes and housekeeping gene was analyzed by the REST software (Pfaffl et al., 2002). Melting curve was used to check primer specificity.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene description</th>
<th>Accession No</th>
<th>sequences</th>
<th>Amplified fragment(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>glyceraldehydes-3-phosphate dehydrogenase</td>
<td>EF592180</td>
<td>TCACCACCGACTACAATGACC-3'</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>ACAGCAACCTCCTTCTCACC-3'</td>
<td></td>
</tr>
<tr>
<td>PR protein</td>
<td>lipase</td>
<td>TaBs117A2</td>
<td>CACAAAATATCCGACCCACC-3'</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>5'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TTCTGGTTCTGGATGACCAAC-3'</td>
<td></td>
</tr>
</tbody>
</table>

Results and Discussion

Effect of F. graminearum spores as inoculum on lipase gene expression

Two wheat genotypes named as Sumai3 and Falat as FHB resistant and susceptible, respectively were investigated to determine the correlation between infection in spike tissues and timing of transcript accumulation of defense response gene including lipase against F. graminearum spores (tester). The y-axis values indicate the relative expression of lipase 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’, lipase transcripts was accumulated significantly in spikes at 3 hours after inoculation, but it has down-regulation at 6 hours and at 12 hours the lipase transcripts were lower than control. The expression of lipase gene in other time point showed a constant pattern between 24 hrs to 7 days after inoculation. The highest level of lipase expression was observed at 3 hours after inoculation by FHB spore suspension in susceptible variety, Can say this response is for higher sensitive in this variety than resistance one that follow with tensive reaction in Falat.

The transcripts of lipase gene was reduces in resistance variety ‘Sumai3’ at 3 hours after inoculation but its up-regulation was started at 6 hours and 12 hrs with down regulations at 24 and 36 hrs and reached to highest level at last time point. The highest level of lipase expression was observed at 7 days after inoculation in resistance compared to susceptible variety. Suggested GLIP1, in association with ethylene signaling, may be a critical component in resistant to A. brassicicola in Arabidopsis (oh et al., 2005).
**Effect of DON on accumulation of lipase**

In susceptible variety at 3 hours, gene expression had increased with down-regulations at 6 hrs and 12hrs after inoculation when the *lipase* transcripts were lower than control. There were two increases in Lipase transcript level at 24, 36 hrs after inoculation. Observed down-regulation of lipase expression at 72 hrs after inoculation in falat profiling that continued with up-regulation at 7 days after inoculation in susceptible variety.

As compared, in resistance variety, up-regulations was observed at 3 hrs and 6 hrs after inoculation and some decreases at 12 hrs, 24 hrs, 36 hrs after inoculation lower than control. Lipase transcript was accumulated at 72 hours after inoculation against DON treatment, but there was down-regulation at 7 day after inoculation in resistance variety. Our finding in this experiment was consistent with the result reported by (Nishiuchi et al., 2006), they 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.

We observed increment of lipase transcripts at 3, 6 and 72 hrs in resistant variety, the early reaction in susceptible cultivar is more intensive than resistant cultivar. Considered with this matter that increase lipase in initial time in resistant cultivar was more important for the detoxified and acetylated with plant element affect on DON in the further time after infection (Nichelson, 2009).

Effect of fungal extract on accumulation of lipase

In susceptible variety, expression of lipase gene were up-regulated in 3 hours and at 6 hours time points after inoculation with fungal extract treatment and decreased at 12 hrs after inoculation. There were decreases in transcript level of lipase gene lower than control at 24, 36, 72 hrs and in this profile increasing observed at 7 days after inoculation. The highest level of lipase transcripts was observed at 6 hours after inoculation in susceptible variety. There was more intensive reaction in early response in susceptible cultivar against fungal extract.

In resistance variety down-regulations were observed at 3, 6 and 12 hrs after inoculation lower than control. There were up-regulations of gene expression at 24, 36, 72, hrs and 7 days after inoculation with fungal extract treatment in resistance variety.

The heightened FHB resistance in the transgenic AtNPR1 -expressing wheat is associated with the faster activation of defense response when challenged by the fungus PR1 expression is induced rapidly to a high level in the fungus-challenged spikes of the AtNPR1-expressing wheat (Makandar et al., 2006).

Effect of salicylic acid on accumulation of lipase

In susceptible variety, transcripts of lipase gene was increase significantly at 3 hrs after inoculation with salicylic acid treatment and decreased at 6 hrs followed with increase at 12 hrs. There were down-regulations at 24, 36 hrs after inoculation lower than control. Observed increases at 72hrs and 7 days after inoculation with salicylic acid treatment. The highest level of lipase transcripts was observed at 3 hrs after inoculation.

In resistance variety, there was reduction in transcript level at 3 hours after inoculation. But it was increased at 6 hours after inoculation. One depletion occurred in 12 hours after inoculation that followed by up-regulation at 24 hours after using salicylic acid. Happened constant level of lipase transcripts at 36 and 72 hours, and it decreased at 7 day lower than control. Tobacco mosaic virus, induction of pathogenesis-related 1 (PR-1) lipase gene expression by SA, and development of systemic acquired resistance in Arabidopsis (Kumar et al., 2003). Showed accumulation of SA in fungus-infected spikes was correlated with elevated expression of the SA-inducible PR1 gene and FHB resistance (Makandar et al., 2011).

Accumulation of lipase in response to external treatment of SA applied for demonstrating that lipase is one of the components of SAR cascade. We observed increasing in lipase transcripts at 6 hrs to 72 hrs in resistant cultivar was higher than susceptible one, then lipase can be one member of SAR cascade in gene expression accumulative. So SA treatment can induce lipase gene in (LAR) and then in (SAR).

In this study we tried to analyze the role of lipase defense gene in response to different inducers in response to FHB in wheat by applying QRT-PCR method. The results showed accumulation of lipase transcript at 6 hrs to 72 hrs in resistant cultivar against SA treatment comparing to susceptible one, this treatment demonstrate that lipase gene had role in SAR cascade. 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.
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 disease, the SAR pathway is activated. SAR activation results in the development of a broad-spectrum, systemic resistance (Hunt and Ryals, 1996; NeuenSchwander et al., 1996). Application of external SA treatment is used to find out whether the induction of lipase 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. lipase 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 lipase gene) or use of SA treatment as testimony for induce lipase 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) (Ryals et al., 1996).

Induced resistance (IR) has emerged as a potential alternative, or a complementary strategy, for crop protection. IR signifies the control of pathogens and pests by prior activation of plant defence pathways. A molecular understanding of IR in cereals, including the most important global crops wheat and rice, This definition also covers what has been termed ‘priming’, defence systems are alerted so that responses are expressed upon challenger attacks (Conrath et al., 2002). In Arabidopsis, the SAR marker genes are PR-1, PR-2, and PR-5 (Uknes et al., 1992). The genes encoding these SAR marker proteins have been cloned and characterized and have been used extensively to evaluate the onset of SAR (Ward et al., 1991; Uknes et al., 1992).

In conclusion, we demonstrated that general expression of lipase gene was increased and/or decreased under field condition. Evidence indicated that lipase gene was involved in defense reaction pathways of resistant cultivar during 24 hrs until 7 days against fungal extract treatment. Our results revealed that lipase gene could use for induce defense reaction in SAR pathways in resistance variety. The expression of this gene in resistant cultivar was an evidence of its role in defense reaction to elicitors in SAR pathways; this pattern was useful to produce safety variety. We observed effective expression at 6 hrs and 72 hrs against DON treatment (chemical treatment) in resistance variety comparative with susceptible one; The expression of lipase gene is most important in initial time after infection, so DON can act as elicitor for PR proteins. In this research we demonstrate lipase role in elicited reaction with SA treatment and DON inducible effect in lipase expression.

In particular, we address progress in the identification of the role of lipase gene in SAR and progress in surveying lipase transcripts against SA treatment to demonstrate lipase effect in the SAR signaling cascade. Also with the use of pattern expression of lipase gene against SA treatment in comparative with the result from lipase transcripts against fungal treatment and DON treatment revealed that DON showed elicitor effect on lipase.

Conclusions

In this study, we have evaluated expression pattern of a pathogenesis related proteins, lipase in two wheat cultivars including Falat and Sumi3, 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-resistance 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 resistance and susceptible cultivars. The expression profile of studied PR gene showed accumulation of related mRNA and sometime higher up-regulation in resistance cultivar in response to F. graminearum suspension, DON, salicylic acid and fungal extract infection.
gene after inoculation in resistant variety relative to susceptible variety.

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