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Research Article

Assay of *NPR1* gene expression in wheat under powdery mildew stress

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Abstract: One of the effective plant disease management strategies is based on the employment of resistance inducers. In the present study, to assay, the effects of Salicylic acid (chemical inducer) and *Piriformospora indica* (biological inducer) on wheat powdery mildew (*Blumeria graminnis* f. sp. *tritici*), the expression rate of *Non-expresser of pathogenesis-related genes1* (*NPR1*) gene was evaluated using qPCR. For this purpose, Falat and Tajan cultivars were selected as susceptible and resistant genotypes to powdery mildew, respectively. To evaluate the rate of gene expression, the *P. indica* colonized Falat along with mock plants were inoculated with Powdery mildew. In another experiment, Falat treated with SA and control plants were inoculated with Powdery mildew 48 h after treatment with SA. Gene expression was assayed in Falat compared with resistant cv. Tajan. Sampling was carried out at 0, 6, 12, 24 and 48 hours after infection. Comparisons of gene expression patterns showed that after infection, the expression levels of *NPR1* increased in induced and non-induced Falat and Tajan cultivars. The maximum gene expression levels were observed at 24 hours post infection. But the expression levels of the gene at this time were much higher in induced treatments compared with control. The current study showed that *NPR1* can be involved in resistance strategy. Thus, using *NPR1* gene as a desired gene in genetic engineering for increasing the potential of plant resistance to pathogens can be considered. Moreover, the high response of *NPR1* gene in induced plants indicated that both SA and *P. indica* play a critical role in inducing resistance.

Keywords: Wheat, Resistance inducer, *Piriformospora indica*, Salicylic acid, *NPR1* gene

Introduction

Powdery mildew (PM) is an economically important disease in wheat (*Triticum aestivum*) caused by the obligate biotrophic fungus *Blumeria graminis* f. sp. *tritici* (*Bgt*). Under low infestation, the disease damage ranges from 13 to 34% but 50 to 100% loss could be recorded in a field when a severe infestation occurs (Li *et al.,* 2011; Alam *et al.,* 2013).

Plants have evolved the sophisticated immune system in challenge with microbial pathogens. Each plant genome encodes hundreds of so-called resistance (R) proteins that allow the plant to recognize specific pathogen-derived molecules known as avirulence (avr) factors (Dong, 2001). Among these resistance proteins,

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Non-expresser of pathogenesis-related genes1 (*NPR1*) is essential for transduction of the salicylic acid (SA) signal to activate *Pathogen Related* (*PR*) genes and induction of systemic acquired resistance (SAR), which confers long-lasting broad-spectrum disease resistance in plants (Dong, 2001). The antagonistic effects of SA on Jasmonic acid (JA) signaling require also *NPR1* expression (Spoel *et al*., 2007). Moreover, homologues of *NPR1* have been cloned and characterized in several crop plants (Yuan *et al*., 2007; Zhao *et al*., 2009; Zhang *et al.,* 2008). Several studies also indicated that overexpression of *AtNPR1* in *Arabidopsis*, and its expression in rice, tomato, and wheat caused enhancement in pathogen resistance via increasing *PR* genes expression (Fitzgerald *et al.,* 2004; Makandar *et al*., 2006). It has been reported that silencing of *NPR1* gene in rice exhibited enhanced susceptibility to pathogens and over-expression of this gene conferred disease resistance to bacterial blight in rice (Yuan *et al.,* 2007). In addition, researches conducted by Stein *et al.* (2008) and Molitor *et al.* (2011) showed that *NPR1* gene has an important role in resistance to Powdery mildew in Arabidopsis. Makandar *et al.* (2006) also confirmed that increasing *NPR1* gene in wheat causes resistance to *Fusarium graminearum*.

Several reports indicated that SA and its analogues such as Benzothiadiazole (BTH) act as inducers for *NPR1* (Durrant and Dong, 2004). This leads to induction of both local and SAR in a wide range of plant species when challenged with invaders (Durrant and Dong, 2004). SAR is an inducible defense mechanism that confers resistance to a broad spectrum of pathogens (Durrant and Dong, 2004; Makandar *et al*., 2006).

Moreover, some scholars have observed that root colonization of various plants by the endomycorrhizal fungus *Piriformospora indica* protected symbiotic plants, including cereals, against abiotic and biotic stresses (Deshmuckh *et al*., 2006; Stein *et al.,* 2008). The *P. indica* confer resistance reminiscent induced systemic resistance (ISR) in plants (Stein *et al*., 2008; Molitor *et al*., 2011). ISR strategies based on *P. indica* could provide an approach to enhance resistance against a broad range of pathogens without high metabolic cost (Molitor *et al*., 2011).

In spite of several results with *NPR1* gene in diverse plants, there is little information on the function of *NPR1* homologue in wheat. Therefore, it was necessary for isolation, characterization, and identification of *NPR1* gene from wheat. In the present study *NPR1*gene, designated as *TaNPR1*, was isolated and its partial length characterized in wheat cultivar 'Falat', due to importance of *NPR1* gene in disease resistance induction. Then, *NPR1* gene expression profiles under treatments with inducers SA and endomycorrhizal fungus *P. indica* on the susceptible plant were also examined.

Materials and Methods

Experimental materials

Seeds of the two wheat cultivars, including Falat and Tajan were obtained from seed and plant improvement institute. These two wheat genotypes were selected as susceptible and resistant cultivars to powdery mildew, respectively. These genotypes were grown in a greenhouse at 20 °C/18 °C (day/night) with 60% relative humidity and a photoperiod of 16/8 h light/dark with 240 $\mu molm^2s^{-1}$ photon flux density in 3 replicates. The biotrophic causal agent of powdery mildew, *Blumeria graminis* f. sp. *tritici* (*Bgt*), race Karaj, was propagated in the same conditions on susceptible wheat cultivar 'Bolani'.

Salicylic acid treatment

Two weeks later, susceptible plants were treated with SA (3 mM) that was dissolved in 30% ethanol. Only 30% (v/v) ethanol with no SA was used in control (Muchembled *et al.*, 2006). All plants were inoculated with *Bgt* spores (50 conidia per mm^2) at 48 hours post treatment and incubated in a growth chamber (Renard-Merlier *et al*., 2009).

Piriformospora indica treatment

Roots of 3 days old seedlings of Falat were immersed into a suspension of *P. indica* spores

with 5 × 10^5 spore/ml for 12h. The colonized plants were then transplanted into the soil (perlite, clay and ceramic) and kept in growth chamber and irrigated weekly with Hoagland solution. To prove root colonization with *P. indica*, the roots of some plants were rinsed with tap water three weeks after inoculation and stained by vierheilig *et al*. (1998) method, then observed by light microscope. Colonized and control plants were inoculated with *Bgt* spores (50 conidia per mm^2) and incubated in the same conditions.

Inoculating resistant and susceptible wheat cultivars with *Bgt*

Two-weeks old seedlings of resistance and susceptible cultivar were inoculated with *Bgt* spore (50 conidia per mm^2) and incubated in a growth chamber.

RNA extraction and gene expression analysis

The first leaves of ten seedlings were harvested at 0, 6, 12, 24 and 48 hours post-infection (hpi) with *Bgt* in treated and control plants and Tajan cv. Then, plant tissues (100 mg) were ground in liquid nitrogen. Total RNA was extracted by RNX-plus kit (Sinaclon, Cat, No: RN7713C). The extracted RNA was incubated with RNase-free DNase (Fermentas, Cat. No: EN0525). The first-strand cDNA of each sample was synthesized using Revert Aid™ First Strand cDNA Synthesis kit (Fermentas, Germany) according to the manufacturer instructions. Quantitative Real-Time PCR (qRT-PCR) was performed in 15µl of SYBR Green Quantitative PCR Master Mix kit (Fermentas, Cat. No: K0221) in 96-well plates of C1000™ Thermal Cycler (BioRad). The synthesized cDNA was diluted 1:5 with nuclease- free water and 2 µl of the diluted cDNA (300 ηg) was used as a template. The qRT-PCR program included 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 30s. The expression was determined using delta-delta Ct method (Livak and Shmittgen 2001). For normalization, the *Actin* gene was applied as the housekeeping gene. Experiments were performed in 3 replicates and standard value and the results were analyzed by the student's T-test.

***TaNPR1* gene isolation and bioinformatic assay**

A pair of degenerated primers (*Tainpr1*) were designed on the basis of conserved nucleotide sequences of cereals *NPR1* cDNA using Bio Edit 7.0.9.0 and Oligo Explorer V1.4 software (Table 1). PCR amplification program was as follows: 94 °C for 3min; 35 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min and 72 °C for 20 min. The PCR products of the predicted size (about 930 bp) were purified from the agarose gel and then sequenced by Bioneer Company. Then, on the basis of *TaNPR1* gene, a pair of sense primer (*TaNPR1*) was designed for RT-PCR that amplified a fragment length of 157 bp (Table 1). Identification of purified nucleotide sequences was performed using BLAST program. Sequence alignment and comparison with sequences were conducted using ClustalW program from Bio Edit 7.0.9.0. The phylogenetic tree was generated from deduced nucleotide sequences for *TaNPR1* and *NPR1* homologues from other species using MEGA 5.0 software, maximum likelihood clustering method.

Results

Assessment of susceptible wheat colonized with *P. indica*

Microscopic studies on Falat cultivar's roots inoculated with *P. indica* showed the ability of this endomycorrhizal fungus in root colonization. Dense growth of *P. indica* hyphae were observed on the outer surface and in cortex sections of roots. Within the root cortex, fungal hyphae developed as a mass of spherical to pear shaped chlamydospores inside the root cells (Fig. 1). The microscopic studies indicated the appropriate establishment of *P. indica* in wheat roots.

In order to assay the role of *P. indica* and SA in the induction of resistance to *Bgt*, the rate of mildew colonies formed per unit area (2.5 cm^2) on leaves of Falat cv. (control) were compared with those of Falat treated with SA and *P. indica*, 7 days post inoculation. The number of *Bgt* colonies was found to be 47%

and 42% less than control plants in *P. indica* and SA treated plants, respectively. Moreover, the size of established colonies on leaves was decreased in both treated plants compared to that in control plants.

Table 1 Nucleotide sequence of primers used for isolation and gene expression of *TaNPR1*.

Primer name	Forward primer (5'→3')	Reverse primer (5'→3')
Ta inpr1	GCA ACA AAT CTT GCR TGA AAC TG	TCA TCR TCC ATG ATC TTG TC
Actin	GGA AAA GTG CAG AGA GAC ACG	TAC AGT GTC TGG ATC GGT GGT
Ta NPR1	GCT TGT CAG GAT GCT GCT	GAA CAG TAT AAC CTC TTG GGT TTC

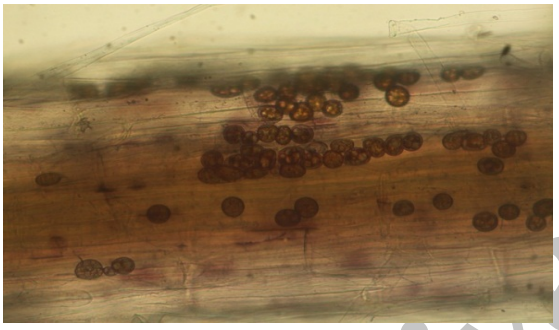

Figure 1 Formation of *Piriformospora indica* chlamydospores inside colonized wheat root of Falat cultivar.

The rate of *NPR1* transcription in Falat colonized with *P. indica*

Inspection of gene expression pattern showed that in the absence of a pathogen challenge, expression of *NPR1* gene was similar between *P. indica* colonized and non-colonized plants. While the *NPR1* gene expression levels increased in both plants at early hours after infection by *Bgt*. *NPR1* transcripts level elevated 1.5 fold more in 6 and 12 hpi in *P. indica* colonized plants compared to non-colonized plants. The maximum level of *NPR1* gene expression was observed at 24 hpi in both non- and colonized plants (Fig. 2a). At this time, the expression rate of *NPR1* in non- and colonized plants increased 5.1 and 8.9 folds, respectively. So that, it was revealed that *NPR1* gene transcription levels in *P. indica* colonized plants was 1.7 fold higher than that of the non-colonized plants.

Rate of *NPR1* transcriptome in Falat treated with SA

At 48 h after spraying plants with SA, the expression rate of *NPR1* increased significantly in treated plants in comparison with control plants (Fig. 2b). In addition, at different hpi an increase in *NPR1* gene expression level in both groups of plants was observed. However, in treated plants, *NPR1* transcripts increased significantly by 1.7 fold over that of the control plants at 12 hai. The *NPR1* expression reached its peak value in both groups at 24 hpi. Comparison of transcript levels at this time indicated that level of transcription in treated plants was about 2.1 fold higher than in control plants. Also, both groups of plants at 48hpi showed a reduced level of expression, but in treated plants, this rate was drawn lower than control plants.

The rate of *NPR1* transcriptomes in susceptible and resistant cv.

The gene expression increased 3.5 and 2.3 fold at 6 hpi, in cv. Tajan and Falat, respectively (Fig. 2c). Although, gene expression in Tajan increased about 1.5 fold more than Falat at 12 hpi. The *NPR1* expression at 24 hai in both cultivars demonstrated its maximum value. At this time, the rate of *TaNPR1* gene expression in Tajan was almost 1.7 fold higher than Falat. Also, Gene expression at 48 hpi in both groups decreased dramatically, but the expression level was higher in Tajan cultivar.

Isolation and sequence analysis of wheat *NPR1* gene

A partial-length cDNA of *TaNPR1* gene containing about 913 bp, which was registered in GenBank by accession No, KM017012 was used in this experiment. The *TaNPR1* is formed of 267 adenine (A), 184 cysteine (C), 212 guanine (G) and 250 thymine (T) nucleotides. This cDNA encoded a predicted polypeptide of 303 amino acids with an estimated molecular mass of 34.09 kDa and a theoretical pI of 7.8

and 100.7 aliphatic indexes. A BLAST search of *TaNPR1* in GenBank revealed that this gene from wheat shared the highest identity of 89% with wheat *TdNPR1* (JX424315), 88% with barely *HvNPR1* (AM050559) and 84% with Rice *OsNPR1* (DQ450947, AY923983).

Assaying the type and amount of amino acids in the TaNPR1 protein suggests that this protein contained all amino acids but tryptophan (Fig. 3). In the structure of this protein, leucine amino acid with over 40 molecules was the most abundant amine acid, while, tyrosine, cysteine, and glutamine with less than 7 molecules were the least abundant amino acids. Also, results of maximum likelihood phylogenetic tree indicated that TaNPR1 protein was grouped with the proteins encoded by Ta-NPR1, TdNPR1, HvNPR1 and OsNPR1. Furthermore, GhNPR1 and NtNPR1 were clustered together and AtNPR1, GmNPR1 and ZmNPR1 formed the third group (Fig. 4).

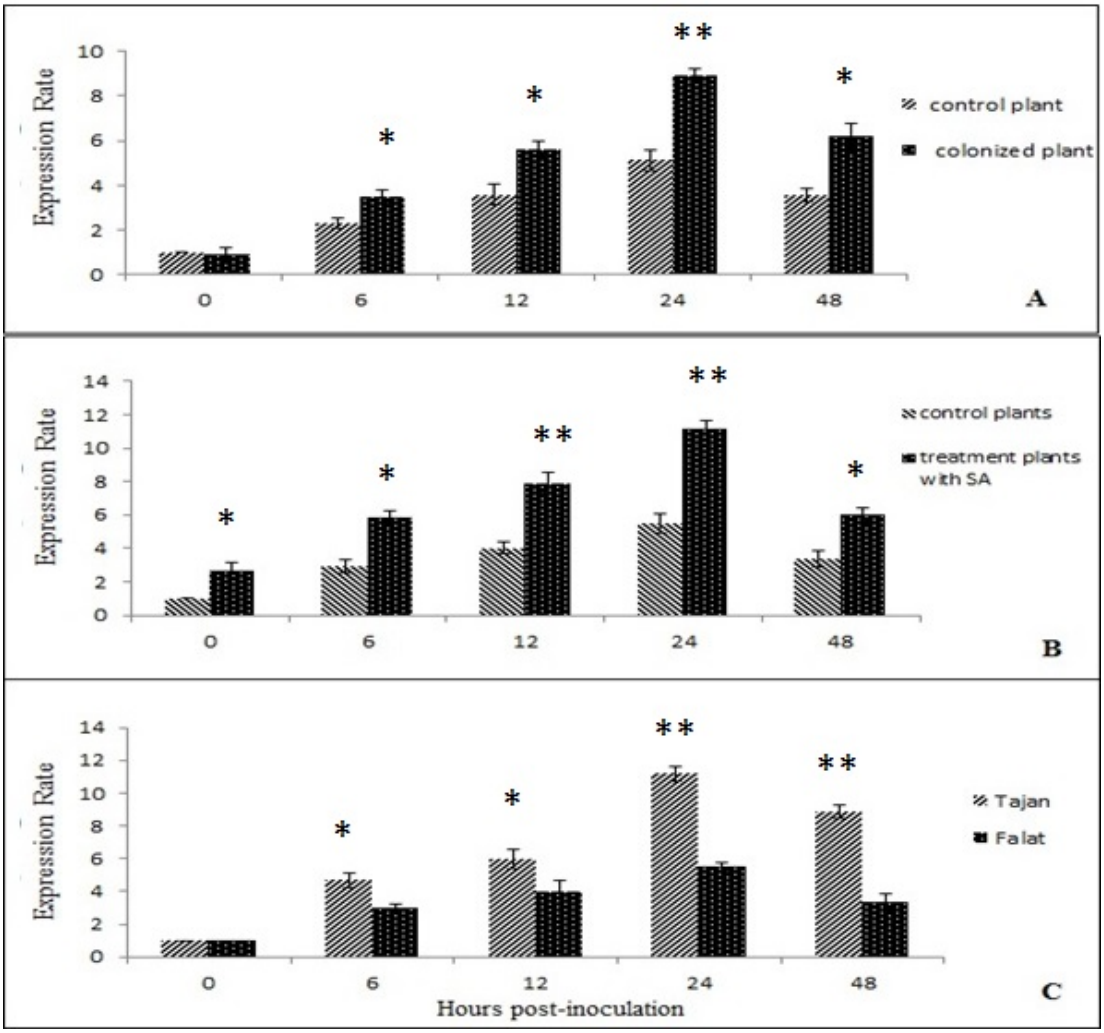

Figure 2 Expression levels of *NPR1* gene in *P. indica* colonized and control 'Falat' (A), treated with SA and control 'Falat' (B) and Resistant ('Tajan') and Susceptible ('Falat') plants challenged with *B. graminis*(C). * and ** = indicate significant difference at 0.05 and 0.01 levels, respectively.

1 NKSCMKLLERCLEMVVQSNLDMITLEKVLP PDVVKQITDLRLSFGLASSEDMGFPNKHVR

61 RILRALDPDD VELVRMLLKE GQTNLDDAFA LHYAVEHCDSKITTELLDIA LADVSLRNPR

121 GYTVLHIAAR RKDPKIIVSL LTKGARPSDF TFDGRKAVHISKRLTKHGDY FANTGEGKPY

181 PNDKLCIEIL EQAERRDSHF GKAYVSLALD GDVLRGRLLY LENRVALSRI LFPVEARVAT

241 DIAQVDGTSE FALGHLVAIDLNGTPTKMKDEHLARMRALSKTVELGKRYFPRCSNVLDKI

301 MDD

Figure 3 Sequence of amino acids in an ORF +3 protein of wheat TaNPR1.

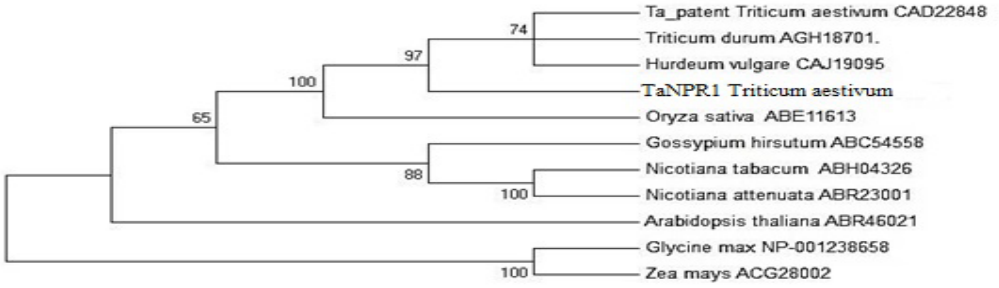

Figure 4 Maximum likelihood phylogenetic tree of NPR1 sequences from various plant species. Branches are labeled with plant species names followed by the protein data bank accession number. Bootstrap value is shown at every node (1000 replicates). Branches over 70 percent are confirmed.

Discussion

Powdery mildew would be one of the most damaging wheat diseases without the extensive use of conventional fungicides. Disease resistance strategies using the components of the JA and SA-signaling pathways have the potential to be effective and versatile. However, their success depends on managing between pathogen and plant defense mechanism. In this article, we have discussed the effect of Salicylic acid and *P. indica* which are classically described as inducers of disease resistance, in *NPR1* gene expression and reducers disease severity.

NPR1 is a novel protein with ankyrin repeats that is important in the induction of *PR* genes (such as *PR1*, *PR2*, and *PR5*) and eventually induced resistance to biotic stresses such as bacterial disease caused by *Xanthomonas oryzae* pv. *oryzae* and fungus *Magnaporthe oryzae* in rice (Takatsuji, 2014) and *Fusarium graminearum* disease in wheat (Makandar *et al.,* 2006) and abiotic stresses such as enhanced oxidative stress tolerance in transgenic tobacco (Srinivasan *et al.*, 2009) and reduced tolerance to heat and salt in Mutant npr1 Arabidopsis (Larkindale *et al.*, 2005). Partial-length of *TaNPR1* gene isolated from Falat leaves contained a 913 bp. the BLAST search of *TaNPR1* in GenBank revealed that this gene shared the highest identity with *T. durum*, *H. vulgar* and *O. sativa*. Also, results of maximum likelihood phylogenetic tree indicated a high level of conservation of TaNPR1 protein from different plant species, especially among the monocots such as wheat, barley, and Rice. To now many researchers have cloned homologues of *NPR1* in several crop plants (Yuan *et al*., 2007; Zhao *et al*., 2009; Zhang *et al.,* 2008).

Inspection of gene expression pattern showed a significant increase in *NPR1* gene expression in early hours after infection by *Bgt* in treated Falat and also in cv. Tajan compared

with control plant. Results of previous investigations indicated that *P. indica* and SA confer induced resistance (IR) in plants (Stein *et al*., 2008; Molitor *et al*., 2011). Meanwhile, based on the results, in absence of a pathogen (zero time), *NPR1* gene were undistinguishable between non- and colonized plants by P. indica. So, the *P. indica* by skillful systemic effects caused priming plant to the rapid increase in defense gene upon infection occurred by *Bgt.* Results of previous investigations indicated that *P. indica* confers resistance reminiscent of ISR in plants (Molitor *et al*., 2011). Also, significantly increased *NPR1* gene in plants treated with SA, indicated that SA has positive effects on *NPR1* increasing, SAR induction, elevation of *PR* genes transcription and inducing disease resistance in plants. The contribution of SA and *NPR1* on plant defense response were also reported (Yuan *et al*., 2007; Maier *et al*., 2011). Previous studies indicated that mutations in *NPR1* and SA-responsive genes lead to suppress cell death and increase susceptibility to biotrophic pathogens (Rate *et al.,* 1999; Fitzgerald *et al*., 2004).

The plant resistance mechanisms against pathogens, amount, and rate of defense genes expression play a central role. So, the SA and *P. indica* with higher and earlier induction of defense genes may cause an earlier induction of IR in treated plants during the penetration phase to prevent the growth of fungal hyphae in plant cells (Eichmann and Hückelhoven, 2008). So that, earlier and higher expression of *NPR1* gene have been observed in treated plants at 6 to 12 hpi. This overexpression has occurred preceding the formation of haustorium at 12 to 24 hpi (Eichmann *et al*., 2006). These results were also reported by stein *et al*., (2008), Molitor *et al*. (2011) and Li *et al*., (2011) in *P. indica* colonized plants.

The expression of *NPR1* gene in Falat was delayed and significantly lower than that in Tajan after infection. So, it seemed that delaying expression of *NPR1* gene provides an opportunity for the fungus to spread on Falat. We obtained a high potential of plant defense mechanisms through *NPR1* gene in cv. Tajan. Thus, if *NPR1* gene expression is increased at the right time, then it would induce resistance to pathogens that coincides with results by Sugano *et al.* (2010) and Feng *et al* (2011).

In addition, the evaluation of gene expression indicated that, at 48 hpi, the transcription level of *NPR1* gene dampened, indicating an effective suppression of defense-associated genes upon haustorium establishment (Spanu *et al*., 2010; Molitor *et al*., 2011). However, reduction of expression level in non-treated plants was higher than that of the treated plants, So, the treated plants with increased expression levels try to prevent the growth of fungal secondary hyphae.

Previous studies represented that unlike SAR, ISR requires only cytosolic but not nuclear localization of *NPR1* (Stein *et al*., 2008; Molitor *et al*., 2011). According to the significant increase in *NPR1* gene expression in treated plants we believe that this gene if located in the nucleus and/ or cytosol, plays a positive role in the induction of resistance responses during biotic stress. The role of *NPR1* gene in resistance to *Fusarium graminearum* in wheat (Makandar *et al*., 2006), and resistance to Powdery mildew in Arabidopsis (Stein *et al*., 2008; Molitor *et al*., 2011) has been proven. As a result, wheat *NPR1* gene can be used as the desired gene for induced resistance to the pathogen, that high response of this gene to inducers and its high gene expression in Tajan cv. after infection is in agreement with this hypothesis. Thus, using *NPR1* gene in genetic engineering for increasing the potential of plant resistance to pathogens can be considered. As well as, high expression of *NPR1* gene in treated Falat indicated that SA-dependent pathway and *P. indica* play important roles in IR against *Bgt*. Therefore, application of these two inducers is proposed for inducing resistance processes.

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بررسی الگوی بیان ژن *NPR1* گندم تحت بیماری سفیدک سطحی

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چکیده: یکی از استراتژی‌های مدیریت بیماری براساس استفاده از القاکننده‌های مقاومت می‌باشد. در مطالعه اخیر، به‌منظور بررسی اثر سالسیلیک اسید (القاکننده شیمیایی) و قارچ *Piriformospora indica* (القاکننده بیولوژیکی) در القای مقاومت به بیماری سفیدک سطحی گندم (*Blumeria graminnis* f. sp. *tritici*) بیان ژن (*NPR1*) *Non-expresser of pathogenesis-related genes1* با استفاده از تکنیک qPCR مورد بررسی قرار گرفت. در ابتدا دو رقم فلات و تجن به‌ترتیب به‌عنوان حساس‌ترین و مقاوم‌ترین ژنوتیپ به سفیدک سطحی انتخاب شدند. سپس به‌منظور بررسی الگوی بیان ژن، فلات هم‌زیست شده با *P. indica* به‌همراه گیاهان کنترل در معرض آلودگی با سفیدک سطحی قرار گرفتند. سپس در آزمایش جداگانه‌ای فلات تیمار شده با اسید سالسیلیک بعد از ۴۸ ساعت به‌همراه گیاهان کنترل با عامل بیماری مایه‌کوبی شد. علاوه براین میزان بیان ژن *NPR1* در رقم فلات نیز در مقایسه با رقم تجن مورد بررسی قرار گرفت. سپس نمونه‌برداری در بازه‌های زمانی ۰، ۶، ۱۲، ۲۴ و ۴۸ ساعت پس از آلودگی در تمامی تیمارها انجام گرفت. مقایسه الگوی بیان ژن نشان داد که پس از اعمال آلودگی سطح بیان ژن *NPR1* در فلات القا شده و کنترل و هم‌چنین رقم تجن افزایش یافت. بیش‌ترین سطح بیان ژن در ۲۴ ساعت پس از آلودگی مشاهده گردید. اما سطح بیان ژن در این بازه زمانی در گیاهان القا شده و رقم تجن به‌طور معنی‌داری بیش‌تر از کنترل بود. نتایج این بررسی بیانگر این است که ژن *NPR1* می‌تواند در استراتژی مقاومت دخیل باشد. لذا ژن *NPR1* را می‌توان به‌عنوان ژن مطلوب در مهندسی ژنتیک برای افزایش پتانسیل القای مقاومت به پاتوژن درنظر گرفت. علاوه براین، پاسخ بالای *NPR1* در گیاهان تیمار شده نشان‌دهنده این است که دو القاکننده SA و *P. indica* نقش مهمی را برای القای مقاومت ایفا می‌نمایند.

واژگان کلیدی: گندم، القای مقاومت، *Piriformospora indica*، سالسیلیک اسید، ژن *NPR1*