Expression of the pathogenesis related proteins, NH-1, PAL, and lipoxygenase in the iranian Tarom and Khazar rice cultivars, in reaction to *Rhizoctonia solani* – the causal agent of rice sheath blight

*Mohammad Sayari**, *Valiollah Babaeizad*, *Mohammad Ali Tajick Ghanbari*, *Heshmat Rahimian*

Department of Plant Protection, Sari Agricultural Sciences and Natural Resources University, Mazandaran, Sari, P.O. BOX 578, Iran

Received: March 30, 2013
Accepted: January 20, 2014

Abstract: Pathogenesis related (PR) genes of rice are among the most important defense genes in the interaction of rice with pathogens. In this study, the role of NH-1, several PR genes, phenylalanine ammonia-lyase (PAL), and lipoxygenase in the defense responses of rice against *Rhizoctonia solani*, the causal agent of rice sheath blight disease, was evaluated. The Tarom and Khazar cultivars (cvs), as resistant and susceptible genotypes, respectively, were used. The expression rate of defense genes in two-week-old seedlings inoculated with a virulent isolate of *R. solani* AG-I-1 A was investigated. The lesions in the Tarom cv were less than half the size of those on the Khazar cv. The expression scripts of the genes were calculated by quantative Real-Time PCR (RT-PCR). Results showed that the expression rate of all genes in the resistant cultivar was higher than that of the susceptible genotype, post inoculation. Analysis of data by the t-Student test also indicated significant differences in the expression level of the genes between Khazar and Tarom. The results of this study suggest that the investigated genes are involved in the resistance responses of rice against the sheath blight agent. For the first time, the induction of PR-5, PR-9, PR-10, PR-12, PR-13, and NH-1 was observed in this study in the resistant and susceptible Iranian cultivars of rice following attacks by *R. solani*.

Key words: jasmonic acid, PR proteins, *Rhizoctonia solani*, rice, salicylic acid

Introduction

Rice (*Oryza sativa* L.) is susceptible to several disease agents during its growth. Rice sheath blight, caused by *Rhizoctonia solani* Kuhn [sexual stage: *Thanetophorus cucumeris* (Frank) Donk] is one of the most damaging factors in rice production throughout the world. The most effective method for the control of this disease is the application of commercial fungicides. Breeding programs for the developing rice varieties resistant to the sheath blight agent has not, thus far, been promising (Kawasaki *et al.* 1999). There is still a demand for the development of new strategies for controlling this pathogen. In general, the plant's responses to stressful situations are signals associated with salicylic acid (SA) accumulation (Jones and Dong 2006). In plant response against necrotrophic pathogens, other pathways, mainly, jasmonic acid (JA) and ethylene pathways, are engaged. However, these straightforward responses cannot be applied to pathogens, such as *Fusarium* and *Rhizoctonia*, that have a complicated lifestyle (Bouarab *et al.* 2009). In rice, the defense responses to pathogens is co-activation/suppression of both SA- and JA-mediated defense signaling pathways (Zhao *et al.* 2008). Previous studies have shown that in rice, the defense mechanism to the necrotrophic fungus *R. solani*, is a combination of diverse defense responses (Zhao *et al.* 2008). Nonexpresser of pathogenesis related genes 1 (NPR-1) also known as NIM1 (non-inducible immunity) and SAI1 (salicylic acid insensitive), is essential for transduction of the SA signal and acts downstream of SA in this pathway to activate PR genes (Shah and Klessing. 1999). The overexpression of *Arabidopsis* NPR-1 in genetically manipulated rice plants causes an increased expression of PR genes and eventually enhances resistance to fungal diseases (Chern *et al.* 2005). Shah and Klessing (1999) also showed that overexpression of NH-1 in rice plants increased the level of resistance to *Xanthomonas oryzae* pv. *oryzae* (bacteria leaf blight pathogen). Pathogenesis related proteins (PRs) are different types of proteins with putative protective functions. So far, plant PR proteins have been classified into 18 different families based on their amino acid sequences, serological relationships, and enzymatic activities (Van Loon 1997). Some groups of PR proteins including PR-3, PR-4, PR-8, and PR-11 have chitinase activity and so can affect the growth or survival of many fungi because of the presence of β-1,3-glucans or chitin in their cell walls (Kauffmann *et al.* 1987). Chitinase class-1 (PR-3) increased resistance in rice to *R. solani* and *Magnaporthe grisea* (Herbert) M.E. Barr (Datta *et al.*

*Corresponding address:
mohammad_sayari_1985@yahoo.com

2001). Many functions have been attributed to PR-5 genes including protection against osmotic stress (Kononowicz *et al.* 1992), and freezing tolerance (Hon *et al.* 1995). Antimicrobial activity is also shown, probably by disrupting the fungal plasma membrane permeability (Datta *et al.* 1999). The overexpression of PR-5 in manipulated rice and orange plants resulted in the increased resistance to *R. solani* and *Phytophthora citrophthora* (Smith & Smith), respectively, in these plants (Datta *et al.* 1999). In a study by Muthukrishnan *et al.* (2001), the plants with high expression rate of PR-5 were considerably more resistant to *R. solani* compared with those with no expression of this gene. PR-9 is a special form of peroxidase (POX) which could take action in cell-wall strengthening by catalyzing lignification and also is expressed in plants during pathogen infections (Passardi *et al*. 2004). In rice plants, POX activity was induced after inoculation with *M. grisea* and *X. oryzae* pv. *oryzae*, especially in resistant rice cultivars (Jaishree *et al.* 1997). The widespread occurrence of PR-10 proteins in both monocotyledonous and dicotyledonous plants has been reported (Jwa *et al.* 2001). In a study by Kim *et al.* (1999), the level of PR-10 and probenazole-inducible protein 1 (PBZ1) elevated in rice plants in response to inoculation with *M. grisea* or treating with JA. Members of PR-10 protein family, the only PR family which has antiviral activity, are stimulated in fungal invasions too (Somssich *et al.* 1986). Defensins are a group of low molecular weight defence proteins with cysteine-rich repeats which have been found in many monocotyledonous and dicotyledonous plants, mammals, fungi, and insects (Lay and Anderson 2005). Inoculation of *Arabidopsis* plants with *Alternaria brassicicola* (Schwein.) Wiltshire resulted in enhanced resistance of plants, associated mainly with activation of the plant defence gene PDF1.2. It has been demonstrated that overexpression of this gene in the transgenic plants, challenges many pathogens including *Alternaria* and *Fusarium* (Epple *et al.* 1997). The expression of PR-12 caused resistance of potato plants to *Verticillium* under field conditions (Gao *et al.* 2000). The increased resistance to seed-transmitted bacterial diseases in the transgenic rice seedlings, over-expressing the oat thionin (PR-13) gene, has been reported (Iwai *et al.* 2002). Transcripts of PR-12 and PR-13 classes of PR proteins in the transgenic potato plants were evaluated under field conditions. Plants were inoculated with some fungal pathogens including *Fusarium*, *Alternaria*, and *Plasmodiophora*. The development of diseases caused by the above mentioned pathogens was noticeably reduced (Gao *et al.* 2000). Phenylalanine ammonia-lyase (PAL) is the first enzyme in phenylpropanoid and flavonoid pathways, which catalyzes the first reaction in biosynthesis of a broad range of phenylpropanoid natural products like lignin, pigments, flavonoid, and phytoalexins. The PAL activity increased after inoculation of rice plants with *R. solani* (Bera and Purkayastha 1999). Furthermore, ZB8 (a member of PAL group) proved to be activated following wounding and treatment of rice plants with fungal elicitors (Zhu *et al.* 1995). Lipoxygenase (LOX) is the first enzyme in the production of JA, and catalyzes the formation of JA from linolenic acid. A drastic increase in the LOX activity has been observed in rice leaves after infection with *M. grisea* (Ohta *et al.* 1991). Previous studies have proved the role of LOX in enhanced resistance of rice plants inoculated with necrotrophic pathogens (Rance *et al.* 1998). Zhao *et al.* (2008) were the first researchers who analyzed the response of rice plants to *R. solani*. In our study, we attempted to evaluate the transcription level of some defense genes, including OsNH1, PR proteins (PR-3, PR-5, PR-9, PR-10, PR-12, and PR-13), and two enzymes: PAL and LOX, in resistant (Tarom) and susceptible (Khazar) Iranian cultivars of rice in response to inoculation with *R. solani* AG-I-1 A. We also attempted to evaluate the possible role of these defense related genes in the resistant response of rice plants to sheath blight disease.

Materials and Methods

Plant material

Rice cultivars Tarom and Khazar, as resistant and susceptible cultivars, respectively, were used in this study. The seeds were initially surface-disinfected by washing in 0.5% Carboxine-Tiram for 4 h. The seeds were then rinsed 3 times with distilled deionized (DD) water. Next, the seeds were plated on wet filter papers in a Petri dish. The plates were incubated at 25°C for 3–4 days till the seeds germinated. The sprouted seeds were transferred to plastic pots containing a clay : humus mixture (2 : 1, v/v), and incubated in a growth chamber [16 h of light, 265 μmol/m^2/sec; 21°C (dark period) to 27°C (light period); 70% (light period) to 95% (dark period) relative humidity, RH]. The pots were soaked with a solution of iron fertilizer (GESAL Pflanzen Tonic, CIBAGeigy, Basel, Switzerland) and kept at 100% RH during the first 7 days after sowing.

Inoculation and sample collection

For inoculation, a virulent isolate of the fungus (SB4099) belonging to the AG-1-I A group of *R. solani* was used. Two-week-old seedlings were used for treatment. Inoculation was conducted with the mentioned isolate, by placing slices of the margins of 5-day-old mycelial plugs (1–2 cm in diameter) grown on Potato Dextrose Agar (PDA), beneath the leaf sheath. The inoculated seedlings were covered immediately with plastic bags to let the pathogen develop. The pots were returned back again to a climate chamber at 27°C/20°C (day/night) with 80% RH and a photoperiod of 16 h with 240 μmol/m^2/sec photon flux density. The plastic bags were removed after 3 days. The leaves were collected at 0, 12, 24, 48, 72, and 100 hours post-inoculation (hpi), frozen in liquid nitrogen, and stored at −80°C until the RNA extraction.

Disease development

Six-week-old seedlings were used for inoculation. The lesion length of each inoculated plant was measured to estimate sheath blight resistance or susceptibility of the rice cultivars. Twenty-five replications were used for each cultivar in the comparison study. The cultivar assay was repeated three times. Separation of cultivar means was done using a Student's t-test (p values of < 0.001).

Table 1. Primer pairs used in the present study and their nucleotide sequences

Genes	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size [bp]	Accession number
Ubiquitin	GGCAAGACCATCACCCTTGAGG	TGGACTCCTTCTGGATGTTGTA	234	CA763279
PR-3	TACTGTGTCCAGAGCTCGCAGTGG	TCTGGTTGTAGCAGTCCAAGTTGG	165	D16221
PR-5	ACCTCTTCCGCTGTCCTC	GAAGACGACTTGGTAGTTGC	241	X68197
LOX	AGATGAGGCGCGTGATGAC	CATGGAAGTCGAGCATGAACA	185	D14000
PR-10	CATGCTACTGCTCACCTTTGA	TCACTCTAGGTGGGATATACT	320	AF274850
Defensin	CCGGCGAACTGCGTGTAC	GGCGTCGAGCAGAATTGG	230	CA759867
Thionin	AGGGTGGTGCTTCAGCTTGT	GGTGTTCTGCGAGGTGATGA	300	CA763250
PAL	GGTGTTCTGCGAGGTGATGA	AGGGTGGTGCTTCAGCTTGT	180	X16099
POX	CATGCTACTGCTCACCTTTGA	TCACTCTAGGTGGGATATACT	220	AF014467
NH-1	GAACCCGGGATGGACACCACCATTG	AAGGATCCTCAAGGTACCTCCAAACCAAG	325	DQ450948

Total RNA extraction and DNase treatment of the samples, and cDNA synthesis

The total RNA from collected upper leaf samples was extracted at the different above-mentioned time courses using TRIZOL reagent (Invitrogen), as described by Triant and Whitehead (2008). Next, the RNAs were treated with RQ1 RNase-free DNase (Promega, Madison, WI) as described in the Promega Technical Bulletin No. 518. To check for genomic DNA in the RNA samples, a Differential Display PCR (DD-PCR) reaction was performed with "cDNA" produced without reverse transcriptase as a control template. The first strand of the cDNA of each sample was synthesized using RevertAid first strand cDNA Synthesis Kit (MBI Fermentas, St. Leonrod, Germany) according to the manufacture's instructions.

Quantitative RT-PCR

Quantitative Real-Time PCR (RT-PCR) was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) for evaluating nine rice defense related genes (Table 1). Syber green quantitative PCR (qPCR) Master Mix kit was used for quantitative RT-PCR, applied in 96-well plates. The 2 µl diluted cDNA (50 ng) of each sample, as the template, was used with the cycle parameters were as follows: initial denaturation at 94°C for 4 min, followed by 40 cycles consisting of denaturation at 94°C for 5 sec, and 30 sec at 60°C (annealing/extension). In all applications, the Ubiquitin gene was applied as the housekeeping gene. The PCR reactions were done in duplicate.

Data analyzing

The rate of gene expression was calculated using the delta-delta CT (ΔΔCT) method (Livak and Schmittgen 2001). At first, the threshold cycles (CT) of the duplicate PCR results of each gene were averaged and used for quantification of the transcripts. Then, the average of the CT value of the Ubiquitin gene was subtracted from the average of the CT value of the target gene to obtain the ΔCT value. The 2ΔΔCT value was given to estimate the relative expression rate of each gene. Each value was obtained from two independent experiments. A standard deviation was given to each value and the results were analyzed by the Student's t-test.

Results

Disease development

The rice cultivar Tarom showed a higher degree of resistance to *R. solani* than the Khazar cv. The determination of lesion length indicated that the Khazar cv, is more susceptible to rice sheath blight agent than the Tarom cv. The size of the lesions in the Tarom cv was half as high as those on the Khazar cv. Analysis of data by the Student's t-test showed significant differences between the two cultivars (p values of < 0.001).

Expression pattern of some pathogenesis related genes and NH-1 during fungal infection

The rice cultivars, Tarom and Khazar, were examined to determine the association between the inoculation in the sheath tissues and the timing of the transcript elevation of NH-1 and 6 other different defense genes encoding PR-3 (chitinase), PR-5 (thaumatin-like protein), PR-9 (peroxidase), PR-10 (ribonuclease), PR-12 (defensin), and PR-13 (thionin). To analyze the expression profile of these genes, RT-PCR analyzing was carried out. The total RNA from 2-week-old seedlings was extracted at six different time courses (0, 12, 24, 48, 72, 100 hpi) post-inoculation with *R. solani.* The RNA then turned into first-strand cDNA and was used as templates in RT-PCR amplification. The expression of all the PR genes investigated in this study were enhanced significantly in the Tarom cv after inoculation with *R. solani*. Almost in all cases, expression elevation commenced at 12 hours after inoculation (hai) or continued enhancing up to 24–48 hai after initial elevation. The basal levels of all PR genes at 0 hai in both cultivars used in this study, were low to unnoticeable.

The NH-1 expression in both genotypes was greatly elevated at 24 hai and obtained its maximum level at this

time point. Then the NH-1 expression reduced slightly till 100 hai (Fig. 1). The expression level of NH-1 in the Tarom cv at 24 hai was 10 fold higher than the cv. Khazar The transcripts accumulation of NH-1 at 24 hai in the Tarom and Khazar cvs was about 15 and 2 fold, respectively, compared to the control sample.

In response to infection at 24 hai, PR-3 transcripts elevated dramatically, then diminished by 48–100 hai in the Tarom cv, while the expression level of this gene in the Khazar cv was not noticeable (Fig. 1). The maximum levels of PR-3 in the Tarom cv at the peak time point (24 hai) was 14 fold higher when compared to the control samples (0 hai). The expression level of the Tarom cv was 5 fold higher when compared to the Khazar cv in the peak time. In both genotypes, PR-5 transcript accumulation became evident by 12 hai and continued till 48 hai and then lessened (Fig. 1). The maximum level of PR-5 was observed at 48 hai and was 15 and 4 fold higher for the Tarom and Khazae cvs, respectively, compared to the control. The transcript levels of PR-5 in resistant genotype at peak time was approximately 8.3 fold higher than the Khazar cv. PR-9 transcripts in both cultivars were induced at 24 hai and continued till 48 hai and then lessened till 100 hai (Fig. 2). Compared to the Khazar cv, the expression level of this gene in the Tarom cv at peak time was about 4 fold. The transcript levels of POX at 48 hai were 12 and four times of the control at their peak in cvs. Tarom and Khazar, respectively. The PR-10 expression level in the Tarom cv elevated significantly at 12 hai and reached its highest level at this time point, and then lessened. In contrast, the

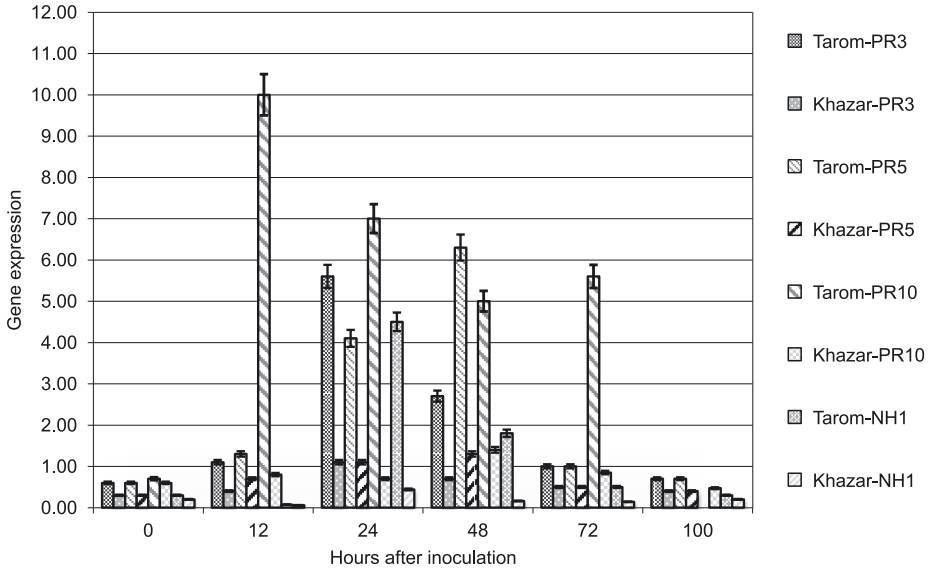

Fig. 1. Activity of defense-related genes in rice seedlings inoculated with *R. solani*. Total activity of PR-3, PR-5, PR-10, and NH-1 was determined in rice seedlings. The rice seedlings were inoculated at six different time course with *R. solani*

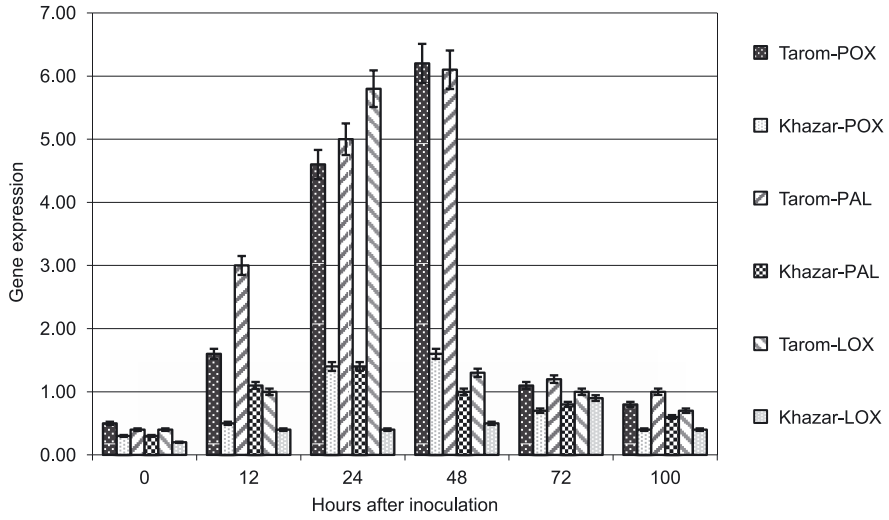

Fig. 2. Activity of defense-related genes in rice seedlings inoculated with *R. solani*. Total activity of peroxidase, phenylalanine ammonia-lyase, and lipoxygenase was analyzed in rice seedlings. The rice seedlings were inoculated at six different time course with *R. solani*

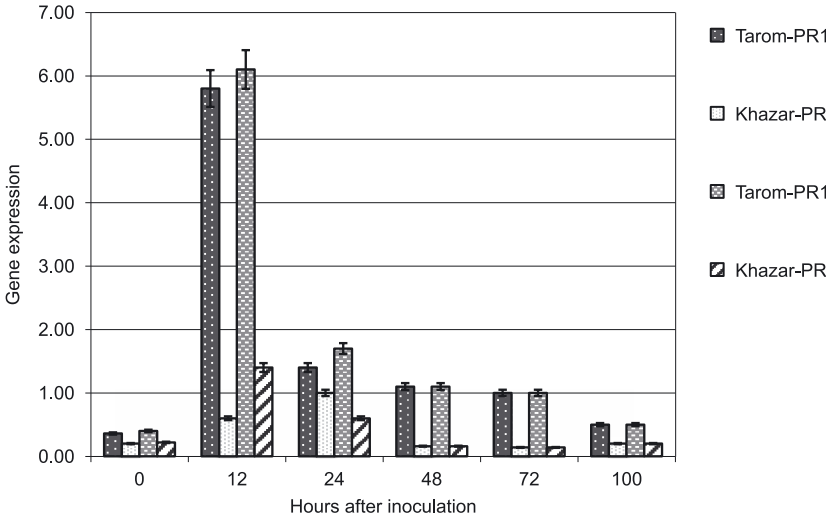

Fig. 3. Activity of defense-related genes in rice seedlings inoculated with *R. solani*. Total activity of PR-12 and PR-13 was determined in rice seedlings. The rice seedlings were inoculated at six different time course with *R. solani*

transcript levels of PR-10 in the Khazar cv were elevated at 48 hai (Fig. 1). The expression level of this gene in the Tarom cv at 12 hai was approximately 10 fold higher in comparison with the Khazar cv. The PR-12 transcripts increased significantly at 12 hai in the Tarom cv and the peak of expression observed at this time course (Fig. 3). The expression level of PR-12 at this time point in the Tarom cv was about 10 fold when compared to the Khazar cv. The transcript accumulation of this gene in the Khazar cv rised at 24 hai and was about 5 fold higher than the control. The PR-13 activity showed a 4 fold higher level of activity in the Tarom cv at 12 hai in contrast to the Khazar cv, and then the activity lessened till 100 hai (Fig. 3). The expression rate of this gene at 12 hai in the Tarom and Khazar cvs was approximately 15 and 6 fold higher, respectively, in contrast to the control. Our results suggest that PR-3, PR-5, PR-9, PR-10, PR-12, PR-13, and NH-1 play an important role in the stage resistance to sheath blight in the Tarom cv.

Expression profile of PAL and LOX after inoculation with *R. solani*

The sheath blight agent was able to elevate the expression rate of PAL and LOX, the two important enzymes in the phenylpropanoid and octadecanoid pathway, respectively. The rate of expression of LOX, a crucial enzyme in the octadecanoid pathway, leading to the synthesis of JA, was elevated in rice plants after inoculation with *R. solani*. The elevation of the expression of this gene was considerably higher in the resistance cultivar when compared with the susceptible one.

The PAL gene showed induction at 12 hai and continued to elevate till 48 hai in both cultivars (Fig. 2). The maximum level of PAL transcripts in infected tissues of the Tarom cv was 6 fold higher than the level observed in the Khazar cv at this time point. The Tarom and Khazar cvs constituted about a 15 and 3 fold higher expression rate of PAL, when compared with the control samples, respectively. In cv. Tarom, accumulation of LOX transcripts was observed at 12 hai and continued to increase up to 24 hai (peak point), and then declined until 100 hai. The expression rate of LOX in the Khazar cv experienced its maximum level at 72 hai (Fig. 2). The expression rate of this gene at 24 hai was 5.6 fold in the Tarom cv when compared with the Khazar cv. The expression rate of LOX in the Tarom cv at 24 hai was about 14.5 fold in comparison with the control, while this rate in Khazar cv at 72 hai was approximately 4 fold.

Discussion

The expression level of six PR proteins, PR-3, PR-5, PR-9, PR-10, PR-12, and PR-13, and also of some defense related genes, namely PAL, LOX, and OsNH-1, was compared in two Iranian rice cultivars. The cultivars were Tarom, as resistant and Khazar known as a susceptible genotype to the sheath blight disease after inoculation with a virulent isolate of the fungus. The Student's t-test showed the existence of a significant difference in the expression rate of those genes in the two cvs (p values of < 0.001). The transcripts of PR-10, PR-12, and PR-13, in the Tarom cv accumulated at 12 hai, in response to infection. At this time point, the hyphae of the fungus showed profuse growth on the surface of the rice stem and some hyphae had penetrated into the epidermal cells (Zhao *et al*. 2008). In the resistant genotype (Tarom), transcripts of PR-3, PAL, PR-9, LOX, PR-5, and NH-1, continued to accumulate up to 24–48 hai. The high level of defence related agents at this time period accompanies development of mycelium along the parenchyma tissue (Zhao *et al*. 2008). In many dicotyledonous plants such as cucumber (*Cucumis sativus* L.), tobacco (*Nicotiana tabacum* L.), and *Arabidopsis thaliana* (L.) Heynh, the SA level elevates after pathogen attacks. This leads to expression of the defense responses, including induction of PR genes (Malamy *et al.* 1990). Rice contains a two fold higher basal level of SA, in comparison with the dicotyledonous plants (Raskin *et al.* 1990). The SA level did not rise following attacks on rice of fungal pathogens like *M. grisea* and *R. solani* or with the bacterial pathogen *Pseudomonas syringae* (Silverman *et al.* 1995).

So, the role of SA in induction of some pathogenesis related proteins is unclear. On the other hand, the NH-1 expression rate increased dramatically at 24 hai in the resistant cultivar (cv. Tarom) compared to the susceptible cultivar (cv. Khazar). Overexpression of NH-1 has been shown to enhance resistance of rice plants to *X. oryzae* pv. *oryzae*. It has also been shown, that the NH-1 level contributes greatly in the resistance of sugar beet and *Arabidopsis* to several diseases (Chern *et al.* 2005). These findings suggest that defense mechanisms in rice against pathogens are mediated by some signaling pathways similar to those acting in dicotyledonous plants (SA-dependant pathways). Chitinase is the mediator enzyme in the phenylpropanoid pathway which is responsible for biosynthesis of flavonoid and isoflavonoid. In our study, the PR-3 level increased substantially in the Tarom cv after being faced with *R. solani*, possibly, because of the presence of chitin in the *Rhizoctonia* cell wall. It has been recently reported, that PR-3 transcripts increase in rice plants inoculated with *R. solani* after treatment with leaf extracts of *Zizyphus jujuba* Mill. overexpression (Kagale *et al.* 2011). The antifungal activity of PR-3 product in *in vitro* studies on *Fusarium solani* and *R. solani* has also been proven (Broglie *et al.* 1991; Sela-Buurlage *et al.* 1993). Thaumatin-like PR-5 protein has been shown to permeabilize fungal membranes. The role of thaumatin-like PR-5 protein in the defense mechanism against the sheath blight disease to rice has been demonstrated (Grover and Gowthaman 2003). Our finding on the rise in the number of PR-5 transcripts in rice plants after being faced with *R. solani* is consistent with those reported by other researchers (Velazhahan *et al.* 1998). Recent studies on rice and wheat plants have demonstrated the noticeable effect of PR-5 on resistance of these plants to *R. solani* (Lin *et al.* 1995) and *Magnaporthe oryzae* (Hao *et al.* 2012), respectively. The role of osmotin (PR-5) in boosting the resistance of tobacco plants to *Bipolaris*, *Fusarium*, and *Phytophtora* has been demonstrated (Broglie *et al.* 1991). The expression rate of peroxidase which was elevated in 24 and 48 hai, suggests the possible role of this enzyme in the induction of resistance in rice to *R. solani*. Cloning of peroxidase isolated from *R. solani* infected rice plants and its introduction to Indica rice cultivars, has been shown to increase the resistance of rice plants to sheath blight disease (Datta *et al.* 2001). Lignin is one of the defense responses of plants. The role of this enzyme in resistance of rice plants to the bacterial blight caused by *X. oryzae* pv. *oryzae,* and its interference in the production of lignin has been widely studied (Hilaire *et al.* 2001). The level of expression of the PR-10 gene in Tarom was considerably higher than that in the Khazar cv, which suggests the role played by this gene in the resistance of rice genotypes to sheath blight. One possible explanation for the activation of PR-10 after *R. solani* challenge of rice plants, is destruction of parts of the fungal cell walls by enzymes like chitinase and β-1,3-glucanase and the subsequent release of monomers of chitin and glucan, as defense signal molecules, in the plant cell (Sela-Buurlage *et al.* 1993). Results obtained on the active role of PBZ1; a homologue of PR-10, in rice plants in response to *R. solani*, supports the function attributed to PR-10 (Midoh and Iwata 1996). Proteins PR-12 and PR-13, known as defensins and thionins, respectively, are capable of permeating pathogen membranes (Edreva 2005). Both proteins have been found to exert strong antifungal activity. Overexpression of the barley thionin gene in rice plants has enhanced resistance to *P. syringae* (Florack *et al.* 1993). Silencing of PR-13 in tobacco has led to elevated susceptibility of the plants to *P. syringae* pv. *tomato* (Pst) DC 3000 (Rayapuram *et al.* 2008). The *in vitro* antifungal activity of defensin against *R. solani* has been demonstrated (Olli and Kirti 2006). Overexpression of a radish defensin increased resistance of tobacco (*N. tabacum*) to *Alternaria longipes* (Terras *et al.* 1995), and of tomato to *A. solani* (Parashina *et al.* 2000). As a marker, PR-12 has been used for the induction of the JA and ET-dependent defense-signaling pathway (Lay and Anderson 2005). In our present report, accumulation of defensin and thionin transcripts in the resistant genotype substantially elevated after being faced with *R. solani* compared to those in the susceptible genotype. This finding demonstrates the contribution of these genes in the resistance response of rice to the pathogen. The increased number of transcripts of defensins and thionin strengthens the possible contribution of JA in their expression. The first enzyme in SA biosynthesis is considered to be PAL. The elevated expression of this gene in the inoculated rice plants may suggest that SA-dependant pathways in the challenged rice plants interfere with some defense mechanisms that could be effective against *R. solani*. Contrary to this, it has been demonstrated that INA, the functional analog of SA, elevates JA levels and induces the expression of JA-responsive genes in rice (Schweizer *et al.* 1997) and thionin gene in barley (Wasternack *et al.* 1994). Hence, it appears that the high basal level of SA in rice plants may lead to a rise in the JA level and can consequently cause enhanced expression of JA- dependant genes, such as PR-3, PR-4, and PR-12. Our data on PAL are in agreement with those of Taheri and Tarighi (2009), who found that PAL expression rate rose in riboflavine-treated rice plants after inoculation with *R. solani*. The level of expression of LOX increased in the resistant plants (Tarom cv) after these plants were faced with *R. solani*. This has also been the case in the studies of Dong and Beer (2000). The lipoxygenase enzyme is the product of the LOX gene. The activity of the lipoxygenase enzyme is associated with the plant defense mechanisms against pathogens and could be elevated by many chemicals which function as the plants' defense activators, *e.g.*, INA (2,6-dichloroisonicotinic acid) in rice (Schaffrath *et al.* 2000) and BABA (β-aminobutyric) in grape (Hamiduzzaman *et al.* 2005). A rise in the expression rate of LOX may lead to production of some molecules such as hydroperoxides, free radicals, antimicrobial compounds, and some signal molecules like JA (Croft *et al.* 1993). In tobacco plants inoculated with *P. parasitica* var. *nicotianae,* the expression of LOX was considered crucial in plant defense mechanisms (Rance *et al.* 1998). The principal enzyme in the octadecanoid pathway leading to biosynthesize of JA is LOX (Ohta *et al.* 1991). These results are in agreement with the previous findings which suggest that jasmonates initially produced by LOX, accumulate rapidly after treatment of plants with some chemicals, or following in-

oculation of plants with pathogens, and eventually lead to enhanced resistance of plants (Creelman and Mullet 1997). The results obtained implicate the possible role of the jasmonic acid signaling pathway in induction of defense responses in rice after attack with the sheath blight pathogen. After inoculation of rice plants with *R. solani*, the LOX level was increased. Therefore, this gene appears to interfere in rice resistance mechanisms. Results of the present study are in complete agreement with those obtained by Dong and Beer (2000). In our experiments, the expression rate of LOX in rice seedlings rose after inoculation with *R. solani.* This finding implicates the phenomenon of induced resistance acting in this pathosystem. The expression profile of LOX was in accord with those reported by other researchers (Creelman and Mullet 1997). It appears that in the *R. solani*-rice pathosystem, Systemic Acquired Resistance (SAR) is not the principal defense pathway in which SA acts as the main signal molecule. Zhang *et al.* (2004) demonstrated that benzothiadiazole (BTH), a structural analog of SA, can activate rice resistance responses against blast (*M. grisea*), sheath blight (*R. solani*), and bacterial blight (*X. oryzae* pv. *oryzae*) diseases (Ge *et al.* 1999; Zhang *et al.* 2004). Since in the present study, both of the genes' JA and SA pathways were activated considerably in the resistant line, it can be concluded that in the rice-*R. solani* pathosystem, the contribution of different defense pathways are, in effect, to restrict pathogen.

Acknowledgements

Support for this research was provided by the Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari, Iran.

References

Bera S., Purkayastha R.P. 1999. Multicomponent coordinated defence response of rice to *Rhizoctonia solani* causing sheath blight. Curr. Sci. 76 (34): 1376–1384.

Bouarab K., Brisson N., Daayf F. 2009. Molecular Plant-Microbe Interactions. CAB International, Cambridge, 337 pp.

Broglie K., Chet I., Holliday M., Cressman R., Biddle P., Knowlton S., Mauvais C.J., Broglie R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254 (5035): 1194–1197.

Chern M.S., Fitzgerald H.A., Canlas P.E., Navarre D.A., Ronald P.C. 2005. Overexpression of a rice *NPR1* homolog leads to constitutive activation of defense response and hypersensitivity to light. Mol. Plant-Microbe Interact. 18 (6): 511–520.

Creelman R.A., Mullet J.E. 1997. Biosynthesis and action of jasmonates in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 48 (1): 355–381.

Croft K., Juttner F., Slusarenko A.J. 1993. Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv. *phaseolicola*. Plant Physiol. 101 (1): 13–24.

Datta K., Tu J.M., Oliva N., Ona I., Velazhahan R. 2001. Enhanced resistance to sheath blight by constitutive expression of infection-related rice chitinase in transgenic elite indica rice cultivars. Plant Sci. 160 (3): 405–414.

Datta R.,Velazhahan N., Oliva I., Ona T., Mew GS., Kush S. 1999. Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease. Theor. Appl. Genet. 98 (2): 1138–1145.

Dong H., Beer S.V. 2000. Riboflavin induced disease resistance in plants by activating a novel signal transduction pathway. Phytopathology 90 (8): 801–811.

Edreva A. 2005. Pathogenesis-related proteins: Research progress in the last 15 years. Gen. Appl. Plant Physiol. 31 (1–2): 105–124.

Epple P., Apel K., Bohlmann H. 1997. Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. Plant Cell 9 (4): 509–520.

Florack D.E.A., Visser B., De Vries Ph.M., Van Vuurde J.W.L., Stiekema W.J. 1993. Analysis of the toxicity of purothionins and hordothionins for plant-pathogenic bacteria. Eur. J. Plant Pathol. 99 (5–6): 259–268.

Gao A.G., Hakimi S.M., Mittanck C.A., Wu Y., Woerner B.M., Stark D.M., Shah D.M., Liang J., Rommens C.M.T. 2000. Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat. Biotechnol. 18 (12): 1307–1310.

Ge X., Song F., Zheng Z. 1999. Systemic acquired resistance to *Magnaporthe grisea* in rice induced by BTH. Acta Agric. Zhejiangensis 11 (4): 311–314.

Grover A., Gowthaman R. 2003. Strategies for development of fungus-resistant transgenic plants. Curr. Sci. 84 (3): 330–340.

Hamiduzzaman M.M., Jakab G., Barnavon L., Neuhaus J.M., Mauch-Mani B. 2005. Beta-Aminobutyric acidinduced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. Mol. Plant-Microbe Interact. 18 (8): 819–829.

Hao Z., Wang L., Huang F., Tao R. 2012. Expression of defense genes and antioxidant defense responses in rice resistance to neck blast at the preliminary heading stage and full heading stage. Plant Physiol. Biochem. 57 (2): 222–230.

Hilaire E., Young S.A., Willard L.H., McGee J.D., Sweat T., Chittoor J.M., Guikema J.A., Leach J.E. 2001. Vascular defense responses in rice: Peroxidase accumulation in xylem parenchyma cells and xylem wall thickening. Mol. Plant-Microbe Interact. 14 (12): 1411–1419.

Hon W.C., Griffith M., Mlynarz A., Kwok Y.C., Yang D.S.C. 1995. Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. Plant Physiol. 109 (3): 879–889.

Iwai T., Kaku H., Honkura R., Nakamura S., Ochiai H., Sasaki T., Ohashi Y. 2002. Enhanced resistance to seed-transmitted bacterial diseases in transgenic rice plants over producing an oat cell-wall-bound thionin. Mol. Plant-Microbe Interact. 15 (6): 515–521.

Jaishree M., Chittoor J., Leach E., Frank F. 1997. Differential induction of a peroxidase gene family during infection of rice by *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interact. 10 (7): 861–871.

Jones J.D.G., Dangl J.L. 2006. The plant immune system. Nature 444 (2): 323–329.

Jwa N.S., Agrawal G.K., Rakwal R., Park C.H., Agrawal V.P. 2001. Molecular cloning and characterization of a novel jasmonate inducible pathogenesis-related class 10 protein gene,

JIOsPR10, from rice (*Oryza sativa* L.) seedling leaves. Biochem. Biophys. Res. Commun. 286: 973–983.

Kagale S., Marimuthu T., Kagale J., Thayumanavan B., Samiyappan R. 2011. Induction of systemic resistance in rice by leaf extracts of *Zizyphus jujuba* and *Ipomoea carnea* against *Rhizoctonia solani*. Plant Signal Behav. 6 (7): 919–923.

Kauffmann S., Legrand M., Geoffroy P. 1987. Biological function of "pathogenesis-related" proteins: Four PR proteins of tobacco have 1,3-*β*-glucanase activity. EMBO J. 6 (11): 3209–3212.

Kawasaki T., Henmi K., Onstenk J., Hatakeyama S., Iwano M., Satoh H., Shimamoto K. 1999. The small GTP-binding protein Rac is a regulator of cell death in plants. Proc. Natl. Acad. Sci. USA 96 (19): 10922–10926.

Kim S.T., Lim D.S., Canman C.E., Kastan M.B. 1999. Substrate specificities and identification of putative substrates of ATM-kinase family members. J. Biol. Chem. 274 (3): 37538–37543.

Kononowicz A.K., Nelson D.E., Singh N.K., Hasegawa M., Bressan R.A. 1992. Regulation of the osmotin gene promoter. Plant Cell 4 (5): 513–524.

Lay F.T., Anderson M.A. 2005. Defensins-components of the innate immune system in plants. Curr. Protein Pept. Sci. 6 (1): 85–101.

Lin W., Anuratha C.S., Datta K., Potrykus I., Muthukrishnan S., Datta S.K. 1995. Genetic engineering of rice for resistance to sheath blight. Biotechnology 3 (7): 686–691.

Livak K.J., Schmittgen T.D. 2001. Analysis of relative gene expression data using realtime quantitative PCR and the $2^{\Delta\Delta}$C (T) method. Methods 25 (4): 402–408.

Malamy J., Carr J.P., Klessig D.F., Raskin I. 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. Science 250 (4983): 1002–1004.

Midoh N., Iwata W. 1996. Cloning and characterization of a probenazole-inducible gene for an intracellular pathogenesis-related protein in rice. Plant Cell Physiol. 37 (1): 9–18.

Muthukrishnan S., George H., Liang H., Trick N., Bikram S. 2001. Pathogenesis-related proteins and their genes in cereals. Plant Cell, Tissue and Organ Culture 64 (2–3): 93–114.

Ohta H., Shida K., Peng Y.L., Furusawa I., Shishiyama J., Aibara S., Morita Y. 1991. A lipoxygenase pathway is activated in rice after infection with the rice blast fungus *Magnaporthe grisea*. Plant Physiol. 97 (1): 94–98.

Olli S., Kirti P.B. 2006. Cloning, characterization and antifungal activity of defensin Tfgd1 from *Trigonella foenum-graecum* L. J. Biochem. Mol. Biol. 39 (3): 278–283.

Parashina E.V., Serdobinskii L.A., Kalle E.G., Lavorova N.V., Avetisov V.A., Lunin V.G., Naroditskii B.S. 2000. Genetic engineering of oilseed rape and tomato plants expressing a radish defensin gene. Rus. J. Plant Physiol. 47 (3): 417–423.

Passardi F., Penel C., Dunand C. 2004. Performing the paradoxical: How plant peroxidases modify the cell wall. Trends Plant Sci. 9 (11): 534–540.

Rance I., Fournier J., Esquerre-Tugaye M.T. 1998. The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences. Proc. Natl. Acad. Sci. USA. 95 (11): 6554–6559.

Raskin I., Skubatz H., Tang W., Meeuse B.J.D. 1990. Salicylic acid levels in thermogenic and non-thermogenic plants. Ann. Bot. Lond. 66 (4): 369–373.

Rayapuram C.W., Haas C., Baldwin I.T. 2008. PR-13/Thionin but not PR-1 mediates bacterial resistance in *Nicotiana attenuata* in nature, and neither influences herbivore resistance. Mol. Plant-Microbe Interact. 21 (7): 988–1000.

Schaffrath U., Zabbai F., Dudler R. 2000. Characterization of RCI-1, a chloroplastic rice lipoxygenase whose synthesis is induced by chemical plant resistance activators. Eur. J. Biochem. 267 (19): 5935–5942.

Schweizer D.B., Buchala A., Métraux J.P. 1997. Gene expression patterns and levels of jasmonic acid in rice treated with the resistance inducer 2,6-dichloroisonicotinic acid. Plant Physiol. 115 (1): 61–70.

Sela-Buurlage M.B., Ponstein A.S., Bres-Vloemans S.A., Melchers L.S., Van den Elzen P.J.M., Cornelissen B.J.C. 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and *β*-1,3-glucanases exhibit antifungal activity. Plant Physiol. 101 (2): 857.

Shah Kachroo P., Klessig F.D. 1999. The *Arabidopsis ssi1* mutation restores pathogenesis-related gene expression in *npr1* plants and renders defensin gene expression salicylic acid dependent. Plant Cell 11 (2): 191–206.

Silverman P., Seskar M., Kanter D., Schweizer P., Metraux J.P., Raskin I. 1995. Salicylic acid in rice (biosynthesis, conjugation, and possible role). Plant Physiol. 108 (2): 633–639.

Somssich I.E., Schmelzer E., Bollmann J., Hahlbrock K. 1986. Rapid activation by fungal elicitor of genes encoding "pathogenesis-related" proteins in cultured parsley cells. Proc. Natl. Acad. Sci. USA 83 (20): 2427–2430.

Taheri P., Tarighi S. 2009. A study on the effect of riboflavin as a defense activator in rice against *Rhizoctonia* diseases. J. Plant Prot. 23 (1): 68–80.

Terras FR., Eggermont K., Kovaleva V., Raikhel N.V., Osborn R.W., Kester A., Rees S.B., Torrekens S., Van Leuven F., Vanderleyden J. 1995. Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell **7** (5): 573–588.

Triant D., Whitehead A. 2008. Simultaneous extraction of high-quality RNA and DNA from small tissue samples. J. Hered. 100 (2): 246–250.

Van Loon L.C. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. Eur. J. Plant Pathol. 103 (9): 753–765.

Velazhahan R., Chen-Cole K., Anuratha C.S., Muthukrishnan S. 1998. Induction of thaumatin-like proteins (TLPs) in *Rhizoctonia solani*-infected rice and characterization of two new cDNA clones. Physiol. Plant 102 (1): 21–28.

Wasternack C., Atzorn R., Jarosch B., Kogel K.H. 1994. Induction of a thionin, the jasmonate-induced 6-kDa protein of barley by 2,6-dichloro-isonicotinic acid. J. Phytopathol. 140 (2): 280–284.

Zhang W., Ge X., Song F., Zheng Z. 2004. Systemic acquired resistance of rice against sheath blight disease induced by benzothiadiazole. Acta Phytophylacica Sin. 30 (2): 171–176.

Zhao C.G., Wang A.R., Shi Y.J., Wang L.Q., Liu W.D., Wang Z.H., Lu G.D. 2008. Identification of defense-related genes in rice responding to challenge by *Rhizoctonia solani.* Theor. Appl. Genet. 116 (4): 501–516.

Zhu B., Chen T.H.H., Li P.H. 1995. Activation of two osmotin-like protein genes by abiotic stimuli and fungal pathogen in transgenic potato plants. Plant Physiol. 108 (3): 929–937.