



RESEARCH PAPER

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Differentially expressed proteins in suspension culture of rice induced by blast disease

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Abstract

Rice (*Oryza sativa* L.) is an important cereal for more than half of the human population in the world. *Magnaporthe grisea* is the agent of the most important rice fungus diseases in the world. This experiment was conducted to investigate the effect of blast fungus on expression of proteins in suspension culture of rice. Suspension culture of rice inoculated with conidial suspension (1×10^5 conidia/mL) races of *M. grisea* for 48 hours. For identification of proteins involved in resistance to magnaporth disease, proteins profile of suspension culture was investigated after inoculation. Comparing proteome pattern of susceptible rice cultivar, Tarom, under control and stress conditions lead to classification differentially expressed proteins into two functional categories, defense and metabolism/biosynthesis. Metabolism and biosynthesis related proteins include triose phosphate isomerase, Isoflavone reductase and Transketolase were as the most important defense protein was pathogen related protein.

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Introduction

Rice is one of the most important cereals in the world which fed more than 50% of the world population. Completing the genome project sequencing of rice made it a suitable source for protein expression profile studies in various tissues (Yang *et al.* 2007). Furthermore, rice because of small genome size compared with other grains such as corn and barley selected as a plant model among the monocot plants for functional gene research (Chao *et al.* 2010).

Fungi, important group of plant pathogenic, are responsible for the most percentage of economic damages due to biological factors (Panstruga 2003). Rice blast disease caused by *Magnaporthe grisea*, is one of the most important fungal diseases of rice in most parts of the world and 20 to 30 percent of the world's rice production is lost due to this disease (Skamnioti and Gurr 2009). Filamentous ascomycetes fungi is a model system for studying the molecular basis of plant and pathogen interaction due to its genetic, economic and sequencing (Ebbole 2007).

Proteomics due to identification of new proteins is a useful approach for understanding regulatory mechanisms in the interaction between plants and pathogens complex and dynamic. The ultimate goal of proteomics would be evaluation of protein expression at the cellular level in response to various physiological changes (Thurston *et al.* 2005). Proteomics will facilitate identification of proteins and details of the messages cascade during plant-pathogenic interaction (Quirino *et al.* 2010).

Rice proteomics initiated with separation specific protein of different tissues in the early 1990, and in last few years much research performed about different aspects of rice proteome, including proteome analysis of specific tissue and specific response against various biotic and abiotic factors (Rakwal and Agrawal 2003). Among the few proteomics studies regarding plants and pathogens interactions less attention were given to the interaction between plants and fungi compare with

bacteria and virus (Afroz *et al.* 2011). Protein expression changes in rice leaves (Kim *et al.* 2004) and cell suspension cultures (Kim *et al.* 2009) were investigated after inoculation with blast fungus.

The aim of this experiment is evaluation of protein expression in rice cell suspension cultures, due to lacking callus tissue of chloroplasts, interacting with blast fungus.

Material and methods

Preparation of cell suspension cultures of rice

Rice seeds, Tarom *cv*, were sterilized in 70% ethanol for 90 seconds and then in 5% sodium hypochlorite (commercial bleach) for 20 minutes. The seeds were washed three times with distilled water placed on callus induction medium in petri dishes until callus induction was maintained in the dark at 27°C. After callus induction, suitable callus was transferred to liquid medium with shaking at 125 rpm in the dark at 25°C. Rice suspension cells were grown in *erlenmeyer* flask containing 125 ml MS medium supplemented with vitamins and 30g/l sucrose and 2mg/l 2,4D. Suspension-cultured cells were subcultured every three week.

Blast fungus growing conditions

Isolate blast fungus Gil-804 obtained from Rasht Rice Research Center. Mycelium of *M. grisea* was grown on potato dextrose agar (Sigma, USA) at 24°C and in darkness. After 10 to 14 days, when the fungal mycelium covered the PDA medium in petri surface was gently scraped using a sterile scalpel. Chopped mycelia transferred and spread on spore medium (20g rice bran + 16g agar + 20g dextrose agar + 200g potato per liter), then for spore production were kept under N-UV for 10 days.

Preparation of the fungal suspension

For this purpose an amount of sterile distilled water containing 0.05% (v/v) Tween-80 was poured into a petri dish containing spores. Then with a sterile scalpel gently scrape in surface area and then passed through two layers of sterile glass wool and then suspensions were obtained with a concentration of

1×10^4 . Spore concentration was measured by Hemacytometer Lam.

Inoculation of suspension cultured with M. grisea

2–3 days following subculture suspension culture of rice in fresh medium, it directly inoculated with conidial suspension (1×10^5 conidia/mL) races of *M. grisea* for 48 hours. For mock inoculation, suspension culture of rice was similarly treated with distilled water containing 0.001% (v/v) Tween 80.

Protein extraction

48 hours after infection, fresh samples (500mg) gently homogenized in 2ml of a phosphate buffer and a little quartz sand in a pestle and mortar in ice. This homogenates after transfer to microtube centrifuged for 10 min at 15,000 in 4 °C. This step was repeated twice. For precipitation of proteins, 50% trichloroacetic acid added to the supernatant to a final concentration of 10% followed by incubation on ice for 30 min. Centrifuge was performed (15000g) for 10 min at 4 °C. Resulting pellets were washed with 200 μ l pure ethanol and centrifuged at 4 °C for 2 min. This step was repeated two times and then pellets were dried at room temperature. The dried powder then solubilized in lysis buffer [CHAPS, urea, thiourea, dithiothreitol (DTT), and Triton and ampholine pH 3-10, the solution was centrifuged at 15000 g for 10 min in room temperature, and supernatant was loaded onto isoelectric focusing (IEF).

Isoelectric focusing

Tube gels electrophoresis in the first dimension with 11 cm in length and 3 mm in diameter prepared using urea, ultrapure water, NP-40, acrylamid solution 30%, ampholine 3 -10, APS 10% and TEMED. Then Protein samples loaded onto gel tubes. Isoelectric focusing carried out in the three stages, 200 V for 30 min, 400 V for 16 hours and 600 V for one hour. IEF gels equilibrated twice in SDS sample buffer containing Tris, Glycerol, SDS and β -mercaptoethanol for 15 minutes.

Sodium dodecyl sulfate polyacrylamide gel

The second dimension was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis of 12%. After completing the electrophoresis in the first dimension, tube gels were assembled on the second dimension gel by agarose gel. Electrophoresis was carried at 35 mA/per gel for 3 h.

Gel staining and scanning

After electrophoresis in the second dimension, gels stained with Coomassie Brilliant Blue [methanol, acetic acid and Coomassie Blue] for two hours on a shaker. Then destaining performed overnight in a destaining solution (methanol, acetic acid). Finally gels were scanned using densitometer GS-800 scanner.

Gel analysing

Following scanning, images were analyzed with the PDQuest software (version 8.2) by identifying the weakest and smallest spots on the gels. Detected spots in all the gels matched with each other followed with manual match for the spots that were not detected by the software. After spots finding, normality test performed and volume percentage of spots obtained for all replications. Comparison of the normalized relative protein intensity of infected and a control group from three independent biological replicates was performed using t test. Spots with significant changes in expression were identified. Minimum and maximum pH in the range of 5 to 8 and molecular weight of each protein spots were determined using a ladder. Identification of proteins spots were performed by searching the protein databases in ExPasy and rice proteome databases using the pI and molecular weight of spots.

Results

Two-dimensional electrophoresis and gels analyzing

To identify proteins involve in resistance to rice blast fungus, proteins extracted from cell suspension cultures 48 hours after infection and differentiated by two-dimensional electrophoresis. Experiment performed with three independent replicates and resulting two-dimensional gels were analyzed by PDQuest software. Totally 119 common and

repeatable protein spots were detected in control and fungus stress conditions. Four protein spots with significant expression changes were identified in the susceptible cultivar, Tarom, by t-test. Two out of four proteins decreased in expression but two proteins

indicated increment in the expression. Figure 1 represent the relative position of protein spots with significant expression changes in Tarom cultivar under control and stress conditions.

Table 1. Characteristics of changed proteins identified in susceptible rice cultivar to blast disease.

Number of spot	Specie	PI		MW		Description	Accession number	Database	Expression changes
		theory	experiment	theory	experiment				
1314	<i>Oryza sativa</i>	51/38	19/9	59/38	18/9	Putative pathogenesis related	BAC83017	Expasy	increase
5601	<i>Oryza sativa</i>	1/61	48/6	88/68	43/5	Putative transketolase1	XP-550612	NCBI	increase
7204	<i>Oryza sativa</i>	84/24	28/5	27/48	6/5	Triose phosphate isomerase	gi 34908172	RUE 2009	decrease
4402	<i>Oryza sativa</i>	99/45	88/6	41	44/6	Isoflavone reductase	AY071920	NCBI	decrease

These Proteins can be categorized in to some distinct groups based on biological role, including proteins involved in metabolism and energy (Triose phosphate isomerase, and isoflavone reductase and trans ketolase), defense proteins (putative pathogenesis related). Most of the changed proteins have been contributed in metabolic pathways except pathogenesis related protein (Fig. 2 and 3). Responsive proteins to fungus blast along with other characteristics are listed in Table 1.

Proteins related to disease resistance

Protein spot 1314 with significant expression changes under fungal stress was belong to defense group and is probably a member of PR protein family (Fig. 1; Table 1). Relative expression of this protein increased in Tarom under stress condition at the 10 % probability level. This suggests the ability of suseptible cultivar for production and accumulation of inductive PR proteins to acquire resistance in response to stress condition.

Proteins involved in biosynthesis and metabolism

Protein spot 5601 identified as protein trans ketolase which is related to biosynthesis and metabolism of molecules (Fig. 1; Table 1). Increment of expression in this protein spots under stress condition at the 5%

probability level indicates increased biosynthesis and metabolism of molecules in response to the blast disease in susceptible cultivar.

Protein spot 4402 which affected by blast fungus treatment is probably isoflavone reductase (Fig. 1; Table 1). This protein showed reduced expression in susceptible cultivar at the 5% probability level after treatment with the rice blast fungus. Decreased expression of this protein reflects inability of the susceptible cultivar to produce desired protein and induce an effective defense response against the rice blast fungus. It seems this protein is effective in defense responses against biotic stress.

Another protein from this group with significant expression changes is 7204 protein spot, which probably belongs to Triose phosphate isomerase (Fig. 1; Table 1). Expression of this protein was reduced in the susceptible cultivar under fungus stress condition at the 10% probability level. Triose phosphate isomerase through cellular respiration provides energy for starch reserves and is necessary carbon sources for the production of amino acids and proteins. Therefore, down regulation of this protein in susceptible cultivar shows low performance of Tarom against blast fungus disease.

Discussion

Proteins related to disease resistance *pathogen related protein*

The first step in plant defense responses against infection with of pathogens often begins by induction of plant resistance genes and their products which are classified as PR proteins (Meyers *et al.* 2005). Pathogen related proteins (PR) are a broad term for all plant protein induced by microbes usually present in plant tissue and only overexpressed during infections (Ryals *et al.* 1996). General role of these proteins is adaptation of plant to stress biologically (Sticher *et al.* 1997).

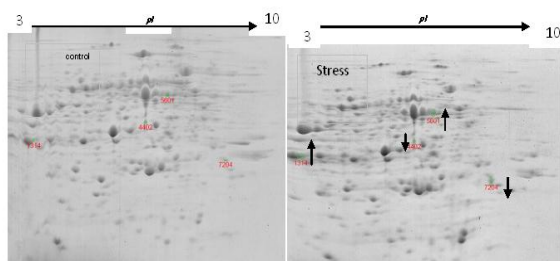


Fig. 1. Relative position and expression changes of protein spots in susceptible cultivar, Tarom, under control and blast fungus stress conditions.

PR proteins first time was identified in tobacco leaves treated with tobacco mosaic virus and then various proteins belonging to this group were identified in other families (Van Loon and Van Kammen 1970). PR Proteins, first time, were classified into five groups based on molecular weight, isoelectric point, storage location and type of activity (Van Loon 1985). Recently more than 17 different PR proteins were identified based on structural and functional characteristics in monocot plants, and two cotyledons. More of these proteins due to hydrolysis activity have antimicrobial activity and thereby participate in the defense mechanism. Often PR proteins may be involved in inhibit growth and proliferation and spread of pathogens and causing resistance to pathogens. When these proteins are expressed in transgenic plants they reduce only a limited number of diseases depending on the type of plant and pathogens (Ryals *et al.* 1996). Several

isoforms of PR proteins identified in rice, which kind inducible protein depending on the type of stress and tissue examined. For example reported, induction of PR proteins alkaline in leaves of the rice inoculated with blast fungus and jasmonic acid (Jwa *et al.* 2006). Kim *et al.* (2004) reported induction of Protein PR₁₀ in leaves and cell suspension cultures of rice interaction with the blast fungus and elicitor. Many proteins PR₁₀ activate in plants upon pathogen attack or after treatment with elicitor. role of PR₁₀ proteins is unknown, although recommended these proteins have ribonuclease activities (Bantignies *et al.* 2000).

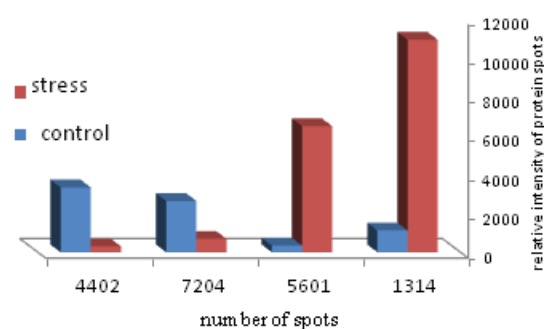


Fig. 2. Classification of proteins involved in resistance to blast fungus in susceptible cultivar of rice, Tarom.

Proteins involved in biosynthesis and metabolism

Trans Ketolase

Trans Ketolase is one of key enzymes in Kelvin cycle. This pathway which is the main source for production of phenolic compounds is correlated with the defense mechanism in plants (Agrios 2005). Cells under stress conditions change their secondary metabolites to produce defense compounds and communicated among defense pathway and signal transduction (Schenk *et al.* 2000). This protein for production of sugar intermediate in oxidation pathways and rehabilitation in tri carboxylic acid cycle catalyzes reverse transfer two groups katool carbon of the five-carbon sugars keto to five-carbon sugars aldo (Lee *et al.* 2007). Other role of this protein is transfer of carbon groups of sedoheptulose 7 phosphate or fructose 6-phosphate to glyceraldehyde 3 phosphate and produce ribulose 1,5-bisphosphate. This enzyme involved in both Kelvin cycle, and oxidative pentose phosphate precursors and products of this protein is starting point for other metabolic pathways.

Therefore, reduced activity and amount of this protein not only effects Kelvin cycle but also influence the production of carbohydrates, amino acids and other products of the metabolic pathway (Stefan *et al.* 2001). Proteome analysis of rice leaf tissue in response to elicitor (Liao *et al.* 2009) indicated increased level of expression in trans ketolase, and role of this protein in plant responses to elicitor through carbon and nitrogen metabolism, but Mahmood *et al.* (2006) reported decreased level of this protein expression in rice plants treated with bacteria Blight. These findings suggest that trans ketolase have effective role in metabolism of rice while interacting with bacterial and fungal diseases (Lee *et al.* 2007). Change expression of trans ketolase in arabisopsis apoplast have also been reprted by Casasoli *et al.* (2008) in response to elicitor. Cluzet *et al.* (2004) reported significant increase expression of this protein in treatment of alfalfa with elicitor. Over expression of this protein was reported under heat stress in rice plants (Lee *et al.* 2007).

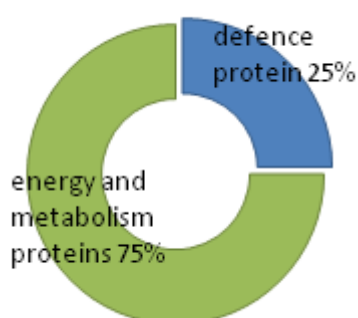


Fig. 3. Relative intensities of differentially expressed protein in rice suspension culture of susceptible rice cultivar, Tarom, after fungus treatment.

isoflavone reductase

Flavonoids and isoflavone reductase are diverse group of compounds in plants have an important role in growth, development and defense response of plants against pests and diseases, often have antioxidant activities as well (Rice-Evans *et al.* 1997). Isoflavone reductase is a key enzyme for production and accumulation of phytoestro isoflavone in legume plants in response to pathogen attack, fungal elicitor and abiotic stresses. Since, in the present study,

expression of isoflavonoid reductase was reduced in susceptible line after infection with the blast fungus, these proteins would be good candidates to be studied in details as effective factors for hypersensitivity reaction and systemic resistance against fungal infection (Kim *et al.* 2003). Kim *et al.* (2004) have reported that expression of defense proteins in resistant varieties would be faster and more than that of susceptible varieties and accumulation of these proteins would cause resistance. They studied induction of isoflavone reductase in leaves and cell suspension cultures of rice interacting with blast fungus and elicitor.

Triose phosphate

Triose phosphate isomerase is an essential enzyme catalyzes conversion of dihydroxy acetone phosphate to diglycerinaldehyde 3 phosphate in the glycolytic pathway (Curto *et al.* 2006). As our result, the expression of tryose phosphate isomerase enzyme in susceptible variety was decreased after treatment with blast fungus (Ryu *et al.* 2009). Increased expression and accumulation of this protein has also been reported in rice plants infected with bacteria Blight under salt and drought stresses (Mahmood *et al.* 2006). High expression of this protein provides a carbon source for production of amino acids and proteins (Dorion *et al.* 2005). Thus reduced expression of these proteins in the susceptible cultivar, Tarom, and other founding suggest that this protein is effective in strengthen defense response against biotic and abiotic stresses (Ryu *et al.* 2009).

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