

A PROTEOMIC APPROACH TO IDENTIFYING DEFENSE  
RELATED PROTEINS IN RICE CHALLENGED  
WITH THE FUNGAL PATHOGEN *RHIZOCTONIA SOLANI*

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## ABSTRACT

Sheath blight caused by the fungal pathogen fungus *Rhizoctonia solani* Kuhn, is an economically important disease of rice in the southern United States. The overall goal of this study was to identify proteins that were affected by sheath blight development by comparing protein expression patterns between the susceptible, wild-type cultivar Labelle and the resistant, mutant line LSBR-5. Protein samples were extracted from inoculated and non-inoculated rice leaf sheaths after 24 hrs and then loaded onto a Bio-Rad 2-DE gel system. Approximately 1,000 protein spots stained with Sypro-Ruby were reproducibly resolved in all gels used in the comparison analysis. The comparison analysis of relative abundances of protein spots between inoculated and non-inoculated samples was carried out with PDQUEST image analysis software. With MS/MS spectra produced by ESI-Q-TOF analysis, 27 out of a total of 36 protein spots were identified through NCBI nr and NCBI EST database searching with Mascot MS/MS Ion Search Engines (Matrix Sciences). Twenty two protein spots were detected in response to inoculation of both susceptible and resistant plants where 21 protein spots were up-regulated and 1 protein spot was down-regulated. Sixteen of the 22 proteins were identified. The presumed functions of the identified proteins were related to antifungal activity, energy metabolism, photosynthesis, protein degradation, and antioxidation. Eight of 16 identified proteins showed higher expression ratios in the inoculated LSBR-5 than in the inoculated Labelle. An additional 14 protein spots were detected in the response of the resistant LSBR-5. Eleven of 14 protein spots were identified with presumed functions relating to antifungal activity, signal transduction, energy metabolism, photosynthesis, molecular chaperone, protein degradation, and antioxidation.

This study is the first to monitor protein expression patterns of the rice leaf-sheath responding to challenge by *R. solani* and to detect response differences between resistant mutant and susceptible parental material. The information and detected proteins in this study will serve as a solid foundation for future studies to elucidate induced defense mechanisms of rice when infected with *R. solani*.

## CHAPTER 1: GENERAL INTRODUCTION

Rice (*Oryza sativa* L.) is an important food crop, providing 23% of the total calories consumed worldwide (Brar and Khush, 2002). In the Asian countries Korea, China, and Japan rice accounts for ~ 60% of daily caloric intake. Although 90 % of the world rice is produced and consumed in Asia, rice is a minor crop in the United States. Nevertheless, the annual per capita consumption of rice in the United States has increased three-fold to 27 pounds (12.3 kg) in the last forty years (<http://www.riceonline.com/USRPA120602.html>). U.S. rice is mainly produced in California, Texas, Arkansas, Mississippi, Missouri, and Louisiana, which ranks third in overall output, but produces the lowest yield per acre of any state (<http://deltafarmpress.com/news/012505coats-article>).

The sheath blight disease, caused by fungus *Rhizoctonia solani* Kuhn, causes significant yield reductions in Louisiana (Lee and Rush, 1983). This disease was not given much attention before the advent of semi-dwarf varieties in the 1980's. The modern semi-dwarf varieties are normally grown at high densities that create a favorable environment for development of sheath blight, one of the most important constraints to high grain yield and quality in the midsouth rice production areas (Groth and Lee, 2002; Lee and rush, 1983). *R. solani* is believed to secrete various toxins, the most notable is the RS phyto-toxin, a carbohydrate molecule containing glucose, mannose, N-acetylgalactosamine, and N-acetylglucosamine, (Vidhyasekaran et al., 1997). The general symptoms of Sheath blight include necrotic dark, reddish-brown elliptical or oval-shaped areas on the leaf sheath, leaf blade, and culm. The fungus produces sclerotia, a source of primary inoculum for *R. solani* that survives in the soil during the winter and than establishes initial infections on the lower leaf sheaths during the late spring or early summer. Environmental conditions play an

important role with optimum relative humidity at ~ 96 % and optimum temperature from 30 to 32°C (Hashiba, 1985; Shi and Cheng, 1995). The fungus can spread to the leaf blade and panicles under highly favorable conditions and that can cause up to 50% yield loss in a susceptible cultivar (Lee and Rush, 1983).

Currently, the best method for controlling sheath blight is applying commercial fungicides, but development of resistant varieties would significantly decrease the cost of production for the rice farmer (Meena et al., 2000). Even though resistant wild relatives (Bonman et al., 1992) and somaclones (Rush et al., 1992) can be used as breeding material in sheath blight resistance breeding programs, adapted resistant germplasm sources have yet to be incorporated into modern commercial cultivars. One alternative to increase resistance is the insertion and expression of a gene or genes encoding antifungal activity. Datta et al. (1999) produced enhanced levels of sheath blight resistance in the *indica* cultivar ‘Chisura Boro II’ by insertion and expression of the thaumatin-like protein which showed antifungal activity and has been shown in other research to be induced in plants by inoculation with plant pathogens or treatment with elicitors produced by pathogens (Bryngelsson and Green, 1989; Van et al., 1987).

Plants respond to various environmental conditions, such as pathogen attack or improper growing conditions, by modulating their gene expression patterns (Scheideler et al., 2002). Defense-related proteins (Bowles, 1990) regulated by pathogen attack or treatments such as elicitors secreted by pathogens are referred to as pathogenesis-related proteins (PR-proteins). In addition to the pathogen treatments, PR-proteins have been shown to be induced by various stresses such as wounding, fungal cell wall elicitors, ethylene, UV light, or heavy metals (Grover et al., 2003). The induction of PR proteins has been detected in various plant

species and PR-proteins have been classified into 17 families based on sequence similarity and enzymatic properties (Van et al., 1999). Various genes that encode defense-related proteins have been inserted into plants and crops to enhance resistance to pathogens (Grover et al., 2003). Broglie et al. (1991) developed transgenic tobacco showing resistance to *R. solani* by constitutively expressing a bean chitinase gene. Constitutive expression of transgenes *Cht-2* or *Cht-3*, genes that encode the chitinase protein, was shown to confer resistance against two different races of the rice blast pathogen in two rice varieties (Nishizawa et al., 1999).

Recently, DNA microarray and proteomics techniques have been used to analyze expression patterns of highly complex mixtures of mRNA or proteins and to explore gene functions at the genomic level in different tissues or stress conditions. These approaches examine whole gene expression patterns in certain stressed environments that provide a 'global' snapshot of genetic factors involved or associated with plant defense mechanisms. The proteomic approach possesses certain advantages over the microarray technique in examining whole gene expression patterns. For example, the genome contains the encoded information that allows the rice plant to develop and reproduce, but proteins, not the genes themselves, are the key components that actually perform essential functions in the cell. The DNA genomic sequence has been considered by some as a basic framework for further investigations into mechanisms of gene action (Whitelegge, 2002). Because the amount of protein is often not predictable from the amount of mRNA produced in the cell, and post-translational modifications such as phosphorylation and glycosylation can be vital for cellular functions, the study of proteins is crucial to judiciously examine their levels and activities at a given time in a tissue, or in response to a certain environment of condition (Zivy et al., 2000).

Although the concept of the "proteome" is currently receiving considerable attention, identification of specific proteins is an established technique whereby isolated proteins are first separated by their isoelectric point (pI) in the first dimension of electrophoresis and then separated by their molecular weight in the second dimension using SDS-PAGE. With two dimensional electrophoresis (2-DE), ~ 2000 proteins can theoretically be separated and displayed in a single gel (O'Farrel, 1976). In the 1990s, new protein identification methods based on mass spectrometry dramatically improved efficiency (Yates, 1998). For this method, the protein of interest, displayed in the 2-DE gel, is either enzymatically or chemically cleaved into several peptides and the mixture is analyzed by mass spectrometric techniques. This group of peptides is referred to as a peptide mass fingerprint (PMF). Results obtained from mass spectrometric analysis is compared to virtual fingerprints obtained from theoretical cleavage of protein sequences stored in data bases for candidate protein identification. Using computer technology, proteins can be identified with peptide mass fingerprinting with the sequenced protein compared with known proteins in appropriate databases. This technique of protein identification, referred to as peptide mass fingerprinting (PMF), is particularly applicable for proteins from organisms whose genome or cDNAs are sequenced (Gervaeert, 2000). Recently, the DNA sequence from more than 30 organisms including *Escherichia coli*, *Arabidopsis thaliana*, and *Oryza sativa* has been determined (Gibson and Muse, 2002; Goff et al., 2002).

Tandem mass spectrometry (MS/MS) is the preferred instrument for high – throughput protein identification (Havilo et al., 2003). An ElectroSpray Quadrupole Time of Flight (ESI-Q-TOF) instrument determines sequence information of the peptide from trypsin digestion by providing MS/MS of peptide mass and fragmented ion mass of individual

peptides. Two methods are used in analysis of protein identification with the MS/MS spectrum. The first method converts the MS/MS spectrum into a probable amino acid sequences (de novo sequencing) while the second method involves searching databases containing theoretical MS/MS spectra. The MS/MS analysis method can be used in various organisms whose DNA sequence data base are not well developed because this analysis method relies on amino acid sequence of peptides. Moreover, the quality of protein identification of MS/MS analysis is generally regarded to be more acceptable than that of PMF whose method relies only on mass similarity of peptide fragments of a protein (Heazlewood et al., 2003).

Since the original description of two-dimensional gel electrophoresis of proteins by O'Farrell (1975), plant biologists have used this technique for various purposes, including genetic and physiological studies. While DNA-based markers have been and still are intensively utilized for measuring genetic distances in populations, proteins revealed by 2-DE can also be used for this purpose. For example, David et al. (1997) used two-dimensional electrophoresis to study genetic differentiation of 11 wheat populations originating from a single population that evolved independently during 8 years of cultivation in different locations. Of the 48 proteins used to investigate population differentiation, 15 showed significant differences at  $P < 0.05$  level. Populations that evolved independently at the same location showed similar differentiation, even though their cultivation methods were different. They concluded that natural selection acted strongly on the genetic composition of the populations. Variations in gene expression have been studied during development and in response to various treatments, and this has led to identification of proteins whose expression is regulated under the conditions described above. Imin et al. (2000) displayed over 4,000

proteins of rice anthers at the young microspore stage, representing 10% of the estimated total genomic output. They constructed rice proteome reference maps that could be used as the model of anther proteomes for related species. Konishi et al. (2001) compared protein expression patterns in rice after inoculation with the blast fungus, caused by *Magnaporthe grisea*, under different levels of nitrogen fertilization. They found that RuBisCO (rubulose -1, 5-bisphosphate carboxylase/oxygenase) increased proportional to the amount of nitrogen applied, and the rice thaumatin-like protein (PR-5) was induced 120 hours after blast fungus inoculation. The advantage of displaying several hundreds or more gene products on a single gel can be used to examine differences in protein expression patterns between mutants and their corresponding wild types. By comparing 2-DE patterns of the wild type and the mutant, the effect of the mutation can be evaluated and characterized at the protein level. Damerval et al. (1998) examined the effect of the *o2* mutation on maize protein expression during grain development by 2-DE in seven different pairs of near-isogenic lines. The abundance of 36 polypeptides was found to be modified in the seven backgrounds. Protein products of the known target genes, such as zeins, b-32 protein and pyruvate orthophosphate dikinase were present in greater amounts in normal lines than in mutants. Moreover, enzymes belonging to various metabolic pathways were tentatively identified, most of which were not previously known to be affected by the *o2* mutation. They concluded that the *O2* gene could act as a connecting regulatory gene for different pathways of grain metabolism.

With developed technologies in proteomics, rice researchers have detected and identified specific proteins that are expressed in certain conditions such as biotic and abiotic stress, different cells, tissues, and developmental stages (Koller et al., 2002; Konishi et al., 2001). Salekdeh et al. (2002) analyzed changes in the rice leaf proteome during drought stress.

More than 2,000 proteins were separated reproducibly and 50% of the total were quantified by gel image software. Forty two proteins showed significant changes in their abundance in response to drought stress. Previously known functions of these proteins were involved in photosynthesis, cell elongation, anti-oxidant metabolism, or lignification.

The overall goal of the proposed research is to use proteomic methods to detect and identify rice proteins that are associated with infection by *R. solani*. The first step involves establishment of greenhouse conditions for optimal growth of *R. solani* after inoculation of resistant and susceptible rice lines. The second step will decide harvesting area of infected leaf sheath and extraction of protein samples taken from resistant and susceptible lines inoculated with *R. solani*. The third step will establish optimized conditions for 2-DE gel analysis of rice leaf sheath proteins. Finally, with the established protocols, changes of rice leaf sheath proteome of LSBR-5 (a somaclonal mutant line showing resistance to *R. solani*) (Xie et al., 1992) and Labelle (the susceptible parental line of LSBR-5) will be monitored after challenge by *R. solani*. This research represents the first step to understand the response of rice to *R. solani* infection at the proteome level. Results from this study will serve as a foundation for further investigations into rice defense mechanisms against *R. solani* and the breeding of new rice varieties expressing high levels of resistance against the rice sheath blight disease.

# **CHAPTER 2: TWO-DIMENSIONAL ELECTROPHORESIS PROTEOMIC ANALYSIS OF RICE LEAF SHEATH TO THE CHALLENGE BY THE FUNGAL PATHOGEN *RHIZOCTONIA SOLANI***

## **2.1 Introduction**

### **2.1.1 Two-Dimensional Electrophoresis Method in Rice Proteomics**

Rice is considered a model species in plant molecular genetics due to its small, diploid genome that has been completely sequenced to reveal a 466 mega-base size with an estimated 46,022 to 55,614 genes (Goff et al., 2002). However, due to post transcriptional and post translational modifications, it is not always possible to predict the exact function of genes involved in responses to different environments, including biotic or abiotic stress. Thus, the genome sequence should be considered as one component within the overall framework of “functional genomics and proteomics” that attempts to determine gene and protein function by monitoring and profiling expression patterns in different cells, tissues, organs, and individuals. Profiling changes of proteomes and comparison analysis of proteomes are important research areas in proteomics (Heazlewood et al., 2003).

In the 1970s, the two-dimensional electrophoresis (2-DE PAGE) technique was developed to separate protein mixtures according to their charge (pI) by isoelectric focusing (IEF) in the first dimension and according to their molecular size (Mr) by SDS-PAGE in the second dimension (O’Farrell, 1975). With certain advances of this technique, 2-DE PAGE has been a main component in proteomics coupled with a Mass Spectrometry for protein identification. The disadvantage of pH gradient instability and irreproducibility during the IEF procedure was a barrier for 2-DE PAGE to be used in proteomic research. After the development of immobilized pH gradients (IPG) for the IEF procedure (Bjellqvist et al., 1982), the 2-DE method was the most common method in protein separation. Moreover,

various ranges of IPGs are now available from different commercial sources. A relatively narrow range of IPGs allows the separation of protein mixtures at high resolution (Bio-Rad). Comparison analysis of protein expression patterns can be performed by comparing gel images which contain protein spots having different intensity and area. The analysis of 2-DE gels, the essence in profiling changes of proteomes, relies on image analysis software which has complex algorithms for protein spot matching and quantification. These programs enable one to analyze a highly complex gel which contains more than 1000 protein spots. The analysis of spot detecting and spot matching can be conducted by automated algorithms of these programs. However, the results need to be confirmed and edited manually. Thus, the image analysis procedure is a bottle-neck in high-through put proteomics and new, efficient algorithms are needed to overcome this disadvantage.

Even though proteomic analysis has been applied in various plants, studies involving *Arabidopsis thaliana* and rice are the most prevalent due to the availability of DNA and protein databases (Heazlewood et al., 2003). Recently, one of the most comprehensive rice proteomic studies was published by Koller et al. (2002). A total of 25,426 peptides and 2,528 unique proteins from leaf, root, and seed in rice were detected using the completed rice genomic database constructed by the Syngenta Company (Goff et al., 2002). By classifying the identified proteins, they found that most enzymes involved in the central metabolic pathways (glycolysis, gluconeogenesis, citric acid cycle, oxidative pentose phosphate pathway, amino acid biosynthesis) were produced in the three tissues. Moreover, they confirmed that biosynthesis and breakdown of starch was tissue-specific via compartmentalized isoforms of ADP-glucose pyrophosphorylase.

Japanese and Australian proteomic research groups have independently constructed

rice proteome reference maps, depicted in a 2-DE PAGE database where all proteins are separated, and identified by their pI value and molecular mass, (<http://gene64.dna.affrc.go.jp/RPD/main.html>; <http://semele.anu.edu.au/2d/2d.html>). The Japanese rice proteome website provides various rice tissues and organelle proteome maps, and even though common proteins occur in different tissues, the database also provides information on specific proteins (Komatsu et al., 2004). In the Austrian rice anther proteome map, over 4,000 protein spots were presented in the 2-DE gel within the pI range 4-11 and molecular weights 6-122 kDa, corresponding to ~ 10 % of the rice genome. These databases represent previous rice proteomic studies from various groups and will provide a reference for the further research in rice proteomics using the 2-DE PAGE methodology (Imin et al., 2001).

Most rice proteomic research to date has focused on detection and characterization of the rice proteome in response to biotic and abiotic stresses. Rakwal et al. (2000) investigated the rice seedling proteome response to treatment by jasmonate (JA) or jasmonic acid, a key molecule in the octadecnoid signaling pathway with diverse roles including plant defense. JA treatment was reported to reduce the amount of the ribulose biphosphate carboxylase (RuBisCo) subunit that in turn induced necrosis in the JA-treated rice tissue. In contrast, PR-5 (PR protein: pathogenesis related protein), acidic 17 kDa PR-1, and a basic 28 kDa BBPIN (Bowman-Bric Proteinase Inhibitor Protein) protein were induced after JA treatment. Previous research has shown that PR-5 and PR-1 proteins exhibited antifungal activity, and proteinase inhibitors increased at a wound site in maize (Rohrmeier et al., 1993).

Shen et al. (2003) monitored changes in the rice leaf sheath proteome responding to physical wounding. Over 400 protein spots were detected on a 2-DE gel visualized with coomassie brilliant Blue (CBB). They observed down-regulation of calreticulin, histidine H1,

hemoglobin, and putative peroxidase proteins while up-regulated proteins were observed such as Bowman-Brick trypsin inhibitor, putative receptor-like protein kinase, calmodulin-related protein, RuBisCO small subunit, and the mannose-binding rice lectin. This report was the first to observe modulated protein changes in the rice leaf sheaths associated with signal transduction, photosynthesis, and stress-response categories.

Using the 2-DE PAGE method, Konishi et al. (2001) observed that the PR-5 protein was induced by infection of the leaf blade by the pathogen *Magnaporthe grisea*, and the level of RuBisCO proteins produced was modified depending on the specific amount of nitrogen fertilizer applied. Kim et al. (2003) analyzed protein expression patterns in suspension-cultured rice cells after inoculation with *Magnaporthe grisea* and defense-inducing elicitors JA, salicylic acid (SA), and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). In this study they detected the following up-regulated proteins: OsPR-10 (rice pathogenesis related protein, class 10), isoflavone reductase like protein,  $\beta$ -glucosidase, putative receptor-like protein kinase, six isoforms of PBZ1 (robenazole-induced protein), and two isoforms of SalT (salt induced protein). Different expression patterns of PR-10, PBZ1, and SalT via Weston blots showed that 2-DE gels could be used to successfully characterize the differential response between disease compatible and incompatible interactions.

The 2-DE PAGE method was also used to investigate protein expression patterns during cell developmental stages. Kerim et al. (2003) observed changes in the rice proteome of pollen proteins after extraction from anthers at six different developmental stages. Approximately 2,500 protein spots were separated on 2-DE gels with a total of 150 proteins differently expressed during anther development. The 40 identified proteins were presumably associated with metabolic and signaling pathways, light mediated signal transduction,

carbohydrate metabolism, and cell wall and cytoskeleton synthesis during development of pollen mother to mature pollen cells.

### **2.1.2 Plant Defense Systems against Disease**

Plants resist pathogen attacks by preexisting defense systems and/or induced defense systems. Preexisting defense systems include structural defenses that prevent pathogen attachment and penetration into the host cell by hair on the surface of the leaf, thick wax, or cuticle layers. Some phenolic and fatty acid-like compounds are present in high concentrations that help resist pathogen attack. The induced defense system also includes structural and chemical defenses, but is only activated after pathogen attack (Agrios, 1997). The induced defense system is generally activated by recognition of elicitors which are molecules secreted from pathogens (Hammond-Kosack and Jones, 2000). The recognition of elicitors activates the following series of induced defense systems; hypersensitive response (HR), induction of pathogenesis related protein (PR- protein), and systemic acquired resistance (SAR) (Hammond-Kosack and Jones, 1996).

The plant–pathogen interaction, an important research area to elucidate plant defense systems, has been intensively studied in the last 15 years (Reymond, 2001). The resistance relationship between the avirulence *avr* gene in the pathogen and resistant *R* gene in the host plant has been studied extensively in plant pathology. Flor (1955) firstly demonstrated this relationship with the “gene for gene concept”. According to this hypothesis, the host plant contains an *R* gene corresponding to a specific elicitor (ligand) encoded by a pathogen *avr* gene wherein the interaction produces an “incompatible” or resistant reaction to the pathogen. Since Flor’s research was first reported, numerous *R* gene mediated plant –pathogen interactions have been described (Dangl, 2001). Most *R*-gene mediated resistance shows high

specificity to the elicitors, which is supported by the fact that most *R* genes can recognize only one specific elicitor and a few *R* genes can recognize two elicitors (Nimchuk et al., 2003). The majority of R proteins have common features that include variable-length leucine rich repeats (LRR) domains whose functions are assumed to mediate protein-protein interactions (Jones and Jones, 1996). However, the direct interaction of an R protein with a receptor for an Avr protein has been reported in a few cases. For example, rice plants that are resistant to the rice blast disease contain the *Pi-ta* gene corresponding to the *avr Pi-ta* gene of the fungus. Using the yeast two hybrid system, Jia et al. (2000) showed that *avr Pi-ta* protein did bind to the *Pi-ta* protein of the resistant rice plant.

In the early infection stage of plant disease, recognition of the pathogen is an important event for the resistant plant. *R*-gene mediated recognition of the elicitor triggers a highly effective resistant defense system leading to prevention of pathogen growth and spread to other adjacent cells, which is termed as an incompatible interaction. On the other hand, the absence of an *R* gene corresponding to the specific elicitor of the pathogen allows pathogen growth and spread, which is termed as a compatible interaction. However, Maleck et al. (2000) reported that expression patterns of many defense related genes were similarly changed in incompatible and compatible interactions between *A. thaliana* and the fungus *Peronospora parasitica*. Thus, they suggested that the resistance in the incompatible interaction triggered by recognition of the elicitor may result from more rapid and higher amounts of defense gene expressions than in the compatible interaction in which the susceptible plant failed to stop pathogen growth due to late and/or low levels of defense gene expression.

*R* gene mediated recognition initiates the effective resistant defense response called

hypersensitive response (HR) in the early infection process. HR is considered localized sudden cell death of the pathogen in the infected areas to prevent further growth (Goodman et al., 1994). Several biochemical functions are changed in the cell undergoing the HR process. The recognition by the *R* gene triggers increasing extracellular pH and  $K^+$  modifications (Orlandi et al., 1992), while taking up  $H^+$  and  $Ca^{2+}$  into the cell (Goodman et al., 1994). Reactive oxygen species (ROS; oxide anions, hydrogen peroxide, and hydroxyl radicals) are molecules related to cell senescence that are rapidly generated during the defense response (Baker et al., 1993). However, this oxidative burst occurs not only in the incompatible interaction, but also in the compatible interaction (Goodman et al., 1994). The ROS triggers the hydroperoxidation of membrane phospholipids, producing toxic mixtures of lipid hydroperoxides that disrupt cell membranes (Agrios, 1996). Cells adjacent to the localized damaged cell by HR also undergo changes of biological significance. For example, antimicrobial substances referred to as phytoalexins, are synthesized and accumulated in healthy cells surrounding damaged cells, while callose and lignin are deposited in the cell wall to prevent pathogen penetration (Agrios, 1996).

Systemic acquired resistance (SAR), a distinct defense strategy from HR, includes a signal transduction pathway, gradual spread of resistance to the whole plant, and prolonged nonspecific resistance against various pathogens (Lamb and Dixon, 1997). SAR is activated by a necrotic lesion resulting from either the response of HR or the cell damages caused by the pathogen attack. Contrary to the fact that HR is restricted to localized infected cells, SAR is gradually spread from infected to non infected cells, resulting in immunizing the whole plant to additional pathogen attack (Hunt and Ryals, 1996). SAR produces a broad spectrum of pathogen protection and reduces disease severity caused by virus, bacteria, and fungi.

However, the level of protection is not always effective against pathogens (Friedrich et al., 1995). Salicylic acid (SA) is considered to be involved in the SAR defense system, and the correlation between increased concentration of SA and the enhancement of disease resistance has been reported in various plants (Malamy et al., 1990; Dempsey et al., 1993; Cameron et al., 1994). In addition, the reports that SAR was induced by the treatment of exogenous SA (White, 1979; Ward et al., 1991) suggested the essential role of SA in SAR induction. The role of SA was supported by the report of transgenic tobacco plants that were not able to accumulate free SA or induce the SAR response to pathogens due to expression of salicylic hydroxylase, an enzyme that converts SA to catechol (Friedrich et al., 1995).

The mechanism of SAR induced by SA is unknown, and the mobility of SA from the infected area to whole plant cells located far from the infected cell has not been proved. Rasmussen et al. (1991) reported that they detected systemic increase of SA and SAR response in the cucumber whose infected primary leaf had been removed before SA accumulated in the phloem. Chen et al. (1993, 1995) proposed H<sub>2</sub>O<sub>2</sub> as a possible messenger of SA to carry SAR signaling. They identified a soluble SA binding protein that may be involved in SAR signaling by inhibition of a catalase enzyme bound with SA, thus increasing levels of H<sub>2</sub>O<sub>2</sub> that in turn induced expression of the PR-1 gene. However, Bi et al. (1995) reported that establishment of SAR was not correlated with the accumulation of H<sub>2</sub>O<sub>2</sub> in the uninfected leaves of inoculated *A. thaliana*.

Recent reports profiling gene expression patterns with microarray and proteomic technology showed that a dramatic change of gene expression occurred in plant-pathogen interactions. Those changes included pathogen defense, abiotic stress, and housekeeping metabolism (Scheideler et al., 2002). The expressions of genes which were historically

suggested to be involved in the induced defense system were screened with microarray and proteomic techniques. Induction of *Xa1*, a *R* gene in resistant rice to bacteria blight disease caused by *Xanthomonas oryzae*, was detected by Yoshimura et al. (1998). However, expression of various *R* genes in the plant response to pathogens have not been detected (Wang et al., 2002), although *R* genes have an important role in the early response. This conflict may be explained by the limitation of the sensitivity of microarray and proteomic techniques or the possible scenario that *R* genes are constitutively expressed in cells regardless of pathogen attack. Nevertheless, regulation of ion pump and channel genes, being involved in the changes of ion fluxes and membrane permeability in the early defense response, were detected. For example, a plasma membrane H<sup>+</sup>-ATPase was up-regulated in the *Arabidopsis* constitutive immunity (*cim*) mutant which shows constitutive SAR (Maleck et al., 2000). Gene expression profiles obtained from microarray data with the treatment of SA in *Arabidopsis* showed the activation of PR- proteins, supporting the role of SA in SAR (Maleck et al., 2000). Several profiles of gene expression patterns by the treatment of defense signal molecules, such as SA, JA, and ethylene, were reported, where inductions of various defense related genes or proteins were detected. By categorizing the expressed genes or proteins into similar functions and summarizing the overlapped genes or proteins through the treatments, various defense signaling pathways and their cross talk were suggested (Schenk et al., 2000; Verberne et al., 2000; Rakwal et al., 2000). With microarray technology, many transcriptional factor genes were detected in plant- pathogen interactions, implying their involvement in the defense system. Chen et al. (2002) reported detection of expression of 74 transcriptional factor genes from *Arabidopsis* in response to the bacterial pathogen (*Pseudomonas syringae*). With the outputs results that these transcriptional factors were

reduced in the mutant plants which have defects in the SA, JA, or ethylene signaling pathway, they suggested associated relationships of these transcriptional factors to the SA, JA, and ethylene signaling pathway

In summary, the plant defense response is a quite complex event. In historical research of plant-parasite interaction revealed that the defense system is activated by the recognition of pathogen attack followed by signaling events and expressions of defense related proteins. Even though traditional molecular genetic and biochemical methods allowed us to elucidate specific defense mechanism, the acquired knowledge is fragmented not to be suitable to characterize the global snapshot of the defense system and the networking of each specific defense pathway. With recently developed microarray and proteomic techniques, it is now able to monitor the gene or protein expression patterns in genomic wide level, verifying the historically proposed models of defense mechanism and discovering new candidate genes and proteins. The 2-DE method coupled with mass spectrometry has been applied in the rice proteomics to monitor protein expression pattern under certain stress conditions. Rice is an adequate model plant to be used in the proteomic research due to its completed genome sequences, and accumulated transcript and protein database.

Limited information is available in the rice response to *R. solani* infection even though this fungus causes significantly economic loss in the southern United States rice industry. With the 2-DE based proteomic method, we will monitor the protein expression patterns of rice leaf sheath by *R. solani* infection. The information obtained from this research will serve to increase our understanding of plant defense mechanisms and provide strategies for development of adapted sheath blight-resistant germplasm.

## **2.2 Materials and Methods**

### **2.2.1 Plant Materials**

The Louisiana cultivar “Labelle” was used as a susceptible line to sheath blight, and LSBR-5, selected from Labelle in somaculture (Xie et al., 1990), was used as a resistance source that has shown high levels of resistance under greenhouse and field conditions. Genetic control of resistance in LSBR-5 was reported to be controlled by a single recessive gene (Xie et al., 1992).

Seeds from Labelle and LSBR-5 were sterilized by 50 % bleach, rinsed with water and germinated in Petri dishes at 28°C in the dark. Three individual germinated seeds per line were placed in a single pot with 16 cm diameter on February 1<sup>st</sup>, 2003 and on March 7<sup>th</sup>, 2003, and then grown in the greenhouse under natural light conditions for 6 weeks before inoculation. The temperature of the greenhouse was ~ 30°C during the day and ~ 22°C at night. Six pots were prepared for each line where three pots were used for the inoculated treatment and the remainders were used for the non-inoculated treatment.

### **2.2.2 Inoculation Method**

The inoculation method suggested by Singh et al. (2002) was modified where *R. solani* strain LR 172 was grown on 3.9 % potato dextrose agar (PDA) (DIFCO) at 28°C in the dark (Fig. 2.1). The immature sclerotia were white in color with condensed mycelia on the surface. A rice leaf sheath of a six-week old plant at the later tillering stage was opened slightly to expose a stem. A white, compact, immature sclerotium derived from four-day old mycelia was placed underneath the leaf sheath. Approximately 50 µL of sterilized water were then added (Fig. 2.2.). Leaf sheaths and stems were not wounded during inoculation. Leaf sheaths of non-inoculated plants also were opened slightly, and only sterilized water was

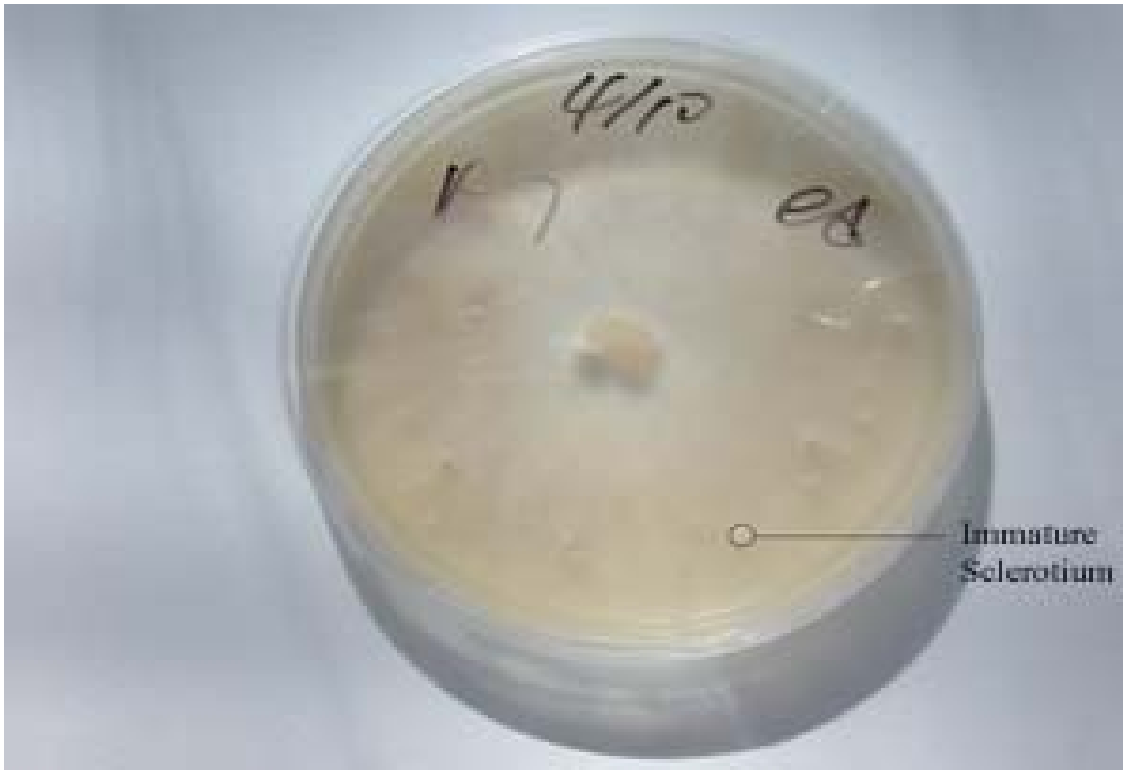


Fig. 2.1 Immature sclerotia developed on 4-day-old mycelia of *R. solani* strain LR172 grown on potato dextrose agar (PDA) medium.



Fig. 2.2 Inoculated rice leaf sheath. An immature sclerotium was placed underneath the leaf sheath with  $\sim 50 \mu\text{L}$  of sterilized water. The circled area indicates location of the sclerotium.

added without sclerotium. After inoculation, plants were placed in a humidified chamber (130 cm long, 115 cm wide, 115 high, containing 150 liters of water, and covered with 0.03 mm thick plastic) at 5 PM for 24 hrs to maintain ~100 % humidity on the leaves (Fig.2.3). To reduce plant stress caused by constant high heat and humidity, the plastic was removed from the chamber the following morning at 8 AM for 10 minutes. Non-inoculated Labelle and LSBR-5 served as negative controls for this experiment.

### 2.2.3 Replication

Two separate experiments or “biological” replications were carried out in this study where plants for the first replication were grown in February 2003 and the second in March



Fig. 2.3. Inoculated and non-inoculated plants were placed in a humidified chamber beginning at 5 PM for 24 hrs. The humidifier inside the chamber was turned on for ~ 15 minutes in the beginning of the incubation period to maintain ~100 % humidity on the leaves.

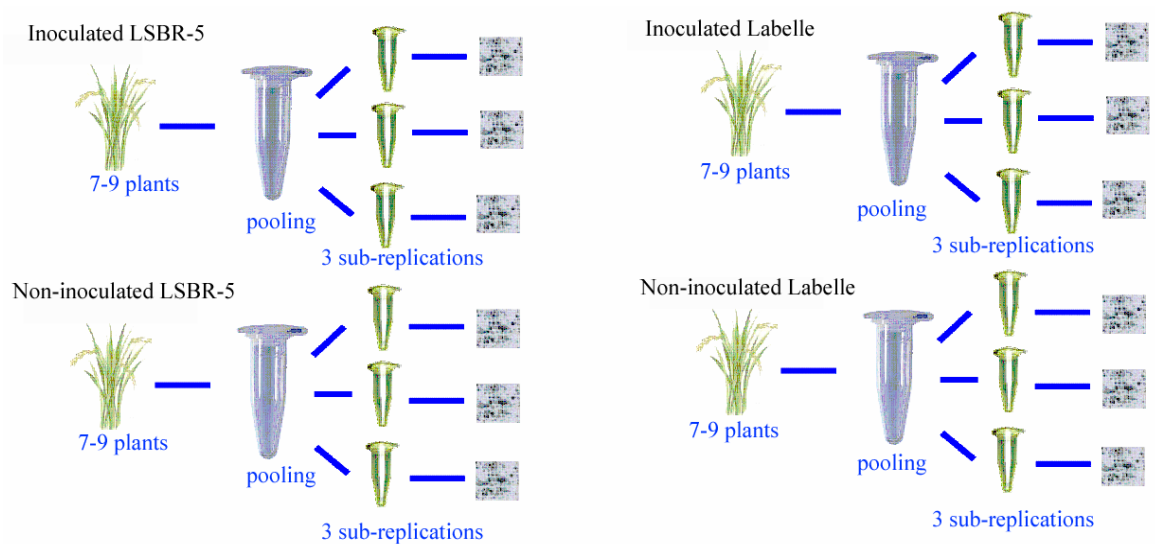
2003. Each replication consisted of four treatments: inoculated LSBR-5, non-inoculated LSBR-5, inoculated Labelle, and non-inoculated Labelle. For each treatment, one pooled protein sample was created by extracting and combining proteins from treated leaf sheaths of 7 to 9 plants. The pooled protein sample was distributed equally among three aliquots which served as sub-replications of the pooled protein sample. Thus, this experimental design consisted of two biological replications, four treatments, and three sub-replications nested to the treatment. Fig. 2.4 shows the experimental setup.

#### **2.2.4 Leaf Sheath Harvest**

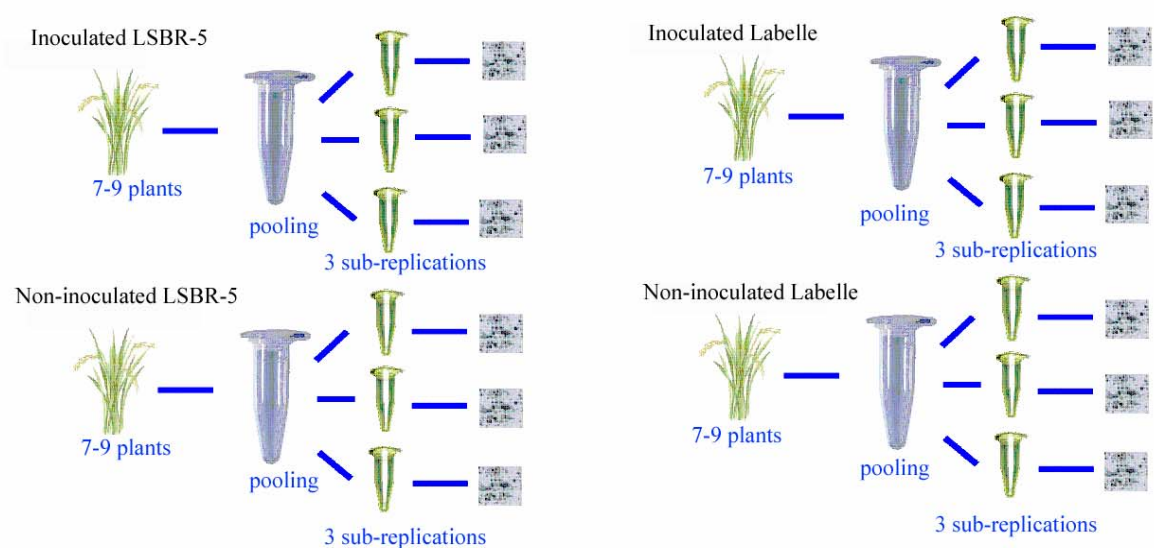
After 24 hour incubation in the plastic chamber, inoculated leaf sheathes showed typical symptoms of sheath blight disease (Fig. 2.5.). To determine the appropriate tissue for harvest that did not contain mycelia of *R. solani*, inoculated leaf sheaths were stained in five separate experiments with Trypan Blue (Frye et al., 1998) that showed mycelia were not detected ~ 2 cm above the lesion formed 24 hrs after inoculation (Fig. 2.6.) Therefore, to avoid contamination with *R. solani*, ~ 3 cm of leaf sheath material was harvested 2 cm above the lesion. The harvested leaf sheath samples were immediately placed on dry ice and then stored at -80°C before protein extraction. For each treatment, leaf sheaths were harvested from 7 to 9 plants and pooled to obtain sufficient protein extracts.

#### **2.2.5 Protein Extraction and 2DE PAGE**

Frozen leaf sheaths were ground in a mortar with liquid nitrogen and suspended in 10 % trichloroacetic acid (TCA) in acetone with 0.07 % dl-dithiothreitol (DTT) at -20°C for 1h, followed by centrifugation for 15 min at 35,000 g. The pellets were washed once with ice-cold acetone containing 0.07 % DTT at -20°C for 1hr and centrifuged again for 15 min at 35,000 g. This washing step was repeated four to five times until the supernatant was clear.



Biological replication #1



Biological replication #2

Fig.2.4 The experimental design of the comparison analysis included 2 biological replications, 4 treatments, and 3 sub-replications nested to the treatment.



Fig. 2.5 Lesion formation on rice leaf sheath of Labelle cultivar 24 hours after inoculation with immature sclerotium of *R. solani*

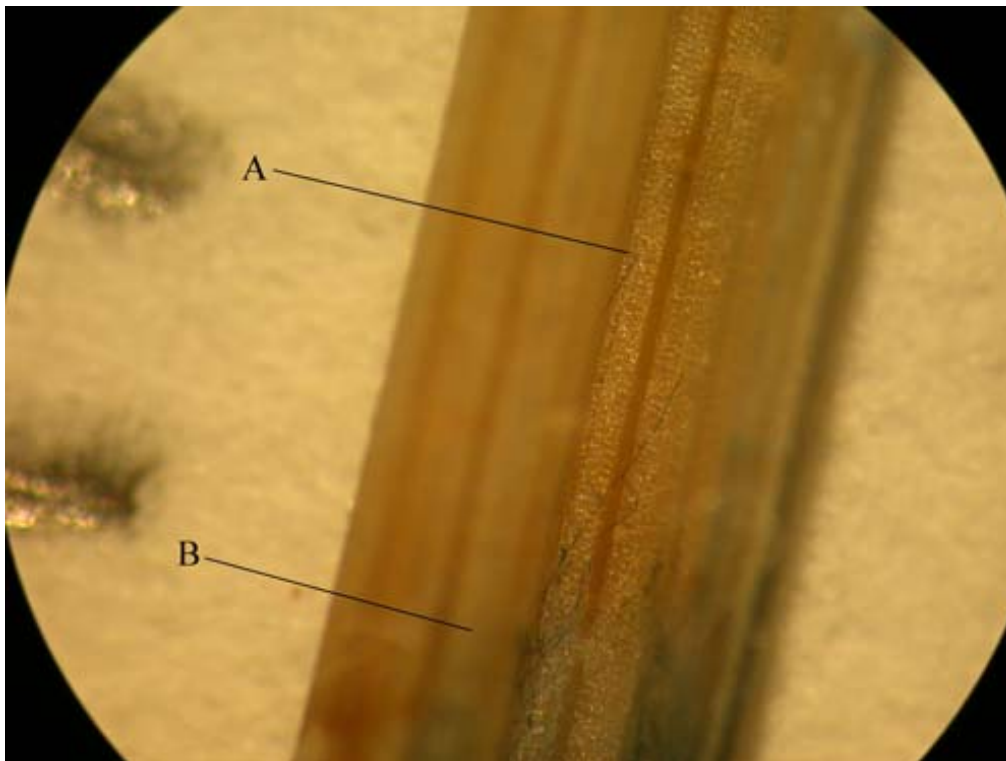


Fig. 2.6 Mycelia of *R. solani* in the rice leaf sheath stained with Trypan Blue. A: upper limit of mycelial growth, 24 hr post inoculation. B: upper boundary of the lesion, 24 hrs post inoculation; length between A and B = 2cm. A sclerotium was inoculated beneath the B level.

The final precipitated pellets were placed in a -80°C freezer until frozen, ~ one hour, and then placed in an AdVantage Freeze Dryer (VirTis) in which the temperature gradient between the shelf ( -40°C) and the condenser (-78°C) induced sublimation to remove moisture from the frozen pellets. The freeze-dry procedure was carried out under vacuum overnight until the pellet was a dry powder. A total of 10 mg of the dried powder was dissolved in 350 µL of sample buffer containing 7 M urea, 2 M thiourea, 4 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5 % ampholytes (Bio-Rad), and 0.7 % DTT ( it is basic buffer but no one report this buffer's pH). The powder dissolved in the extraction buffer was gently shaken for 1 hr then centrifuged for 30 min at 35,000 g at room temperature. The supernatant was distributed in 100 µL aliquots and kept at -80°C before 2-DE PAGE analysis. A total of 100 µg of protein, assayed by PlusOne™ 2-D Quant Kit (Amersham Biosciences), were loaded onto a 17 cm immobilized gradient (IPG) strip (Bio-Rad). The first dimension electrophoresis (isoelectric focusing) was carried out with the Bio-Rad Protein Isoelectric Focusing Unit according to the manufacturer's recommendation. Prior to running the second dimensional electrophoresis, the IPG strips were immersed in equilibration buffer #1 which contained 6M Urea, 0.375 M Tris, pH 8.8, 2 % lithium dodecyl sulfate (LDS), 20 % glycerol, 2 % (w/v) DTT for 10 minute, then placed in equilibration buffer #2 which contained 6 M Urea, 0.375 M Tris, pH 8.8, 2 % LDS, 20 % glycerol, 2.5 % (w/v) iodoacetamide for 10 minutes. The equilibrated IPG strips were washed in sterile, distilled water, then placed on top of a 12 % non-gradient and 18 x 20 cm polyacrylamide-bis gel where the ratio of acrylamide to bis-acrylamide was 37.5:1. No stacking gel was used. The IPG strip was sealed with 1 % agarose containing bromophenol blue (BPB). In the second dimension electrophoresis, lithium dedecyl sulfate (LDS) was used instead of sodium dodecyl

sulfate (SDS) because electrophoresis was conducted at a relatively low temperature of 6°C. The Bio-Rad Protein II XL Gel Cell was used in the second dimension electrophoresis with running conditions of constant 16mA for 30 min followed by constant 30 mA per gel until the BPB dye reached the bottom of the gel.

### **2.2.6 Gel Staining and Image Analysis**

The 2 D-gels were stained with Sypro-Ruby (Bio-Rad), and images were created by an FX scanner (Bio-Rad). Spot detection and matching analysis, first conducted by the PDQUEST program (Bio-Rad), were reviewed manually for each gel and then combined across all 24 gels of the study. The quantity of a protein spot was measured by area and intensity factors by PDQUEST and normalized across all 24 gels. Normalization was performed by converting raw quantities into the relative quantities over the reference spot whose quantity was not affected by the inoculation. The consistency of the reference spot was conformed by pre-comparison analysis among the treatments where the quantity of the reference protein spots was calculated by dividing raw quantity of reference spot by the sum of total detected spot quantities. The Student's t-statistic was used to identify protein spots in both sample sets showing variation in their abundance between treatments. Thus, the protein spots described in this study showed statistical significance and reproducibility across experiments.

### **2.2.7 Protein Identification**

#### **2.2.7.1 Protein Digestion**

Protein spots were excised from six preparative gels with the ProteomeWork gel cutter (Bio-Rad). A total of 300 µg of protein extracted from inoculated LSBR-5 was loaded in the preparative gel then the 2-DE was conducted with the same protocol for the analytical 2DE

gel which is used in the comparison analysis. The preparative gels were stained with Sypro-Ruby (Bio-Rad). The excised protein spots were digested with trypsin using the MassPREP station (Waters). The excised spots were de-stained with 50  $\mu$ L of 50 mM ammonium bicarbonate and 50  $\mu$ L of 50 % acetonitrile, washed once with 50  $\mu$ L of 100 mM ammonium bicarbonate and 50  $\mu$ L of dehydrated acetonitrile. Digestion was conducted with 6 ng/ $\mu$ L trypsin in 25 $\mu$ L of 50 mM ammonium bicarbonate for 5 hrs at 37°C. The digested protein was extracted twice (first with 1 % formic acid (30  $\mu$ L), and second with 1% formic acid (12  $\mu$ L) / 50 % acetonitrile (12  $\mu$ L), then the digested proteins were combined and maintained in a PCR plate at 4°C until further analysis.

#### **2.2.7.2 Tandem Mass Spectrometry**

The digested protein samples were placed into the tandem mass spectrometer, Q-TOF micro (Waters) with the CapLC system (Waters) using the PepMap C18 analytical column (LCPackings) at an 8  $\mu$ L/min flow rate. The linear gradient was initiated from 95 % A (H<sub>2</sub>O/0.05 % HCOOH) to 65 % B (acetonitrile/0.05 % HCOOH) in 36 min followed by a linear gradient to 90 % B in 2 min. Tandem mass (MS/MS) spectra were acquired by MassLynx software (Waters) through Q-TOF micro (Waters) whose internal parameters were set with the electrospray capillary voltage at 3.0 kV, the cone voltage at 30 V, and the source temperature was 80°C. The MS survey scan was  $m/z$  300–1600 with a scan time of 0.9 s and an interscan time of 0.1 s. The minimum threshold intensity of a peak was 10 counts.

#### **2.2.7.3 Database Searching with MS/MS Spectra**

MS/MS spectra were used to search against the NCBI non- redundant protein database using MS/MS Ion Search Engine, a computer software program conducting protein identification based on matching the MS/MS spectra of a protein with a protein or DNA

sequence data base ([http://www.matrixscience.com/search\\_form\\_select.html](http://www.matrixscience.com/search_form_select.html)). The rice (*Oryza sativa*) protein database was searched first, then green plant (Viridiplantae) database (<ftp://ftp.ncbi.nih.gov/blast/db/FASTA/>) was searched if the selected rice proteins were classified as unnamed, unknown, or hypothetical. The significance of the protein match with the ion score was based on the Mouse scoring algorithm (Pappin, 1993). The ion score was calculated as  $-10 \times \text{LOG}_{10}(P)$ , where P is the absolute probability that the observed match is a random event. Thus, a relatively small P value means that the match of identified protein and the MS/MS spectra is not a random event. A significant specific match increases the ion score, so a high score means highly significant matching (MASCOT Help; [http://www.matrixscience.com/help/scoring\\_help.html#PBM](http://www.matrixscience.com/help/scoring_help.html#PBM)). A single protein having a higher score than the minimum score for the significance level ( $p < 0.05$ ) was judged as a significant match. In each MASCOT search output result, the minimum score for significance level was provided, based on the absolute probability and the size of the sequence database being searched.

## **2.3 Results**

### **2.3.1 Sheath Blight Disease Development in the Inoculated Leaf Sheath**

After 24 hr incubation, a yellowish water soaked elliptical lesion was observed in the inoculated leaf sheath. Staining the infected tissue with Trypan Blue confirmed that the lesion was produced by inoculation with *R. solani* (Fig. 2.6). Lesions occurred on both the resistant LSBR-5 and susceptible Labelle with no significant difference in lesion size between the two lines 24 hr after inoculation (Fig. 2.7).

### **2.3.2 Protein Separation on 2-DE Gels**

To determine optimum 2-DE gel conditions, a pilot experiment was conducted where a

protein sample was extracted and separated on a broad range (pH 3-10) IPG strip for the first dimension and a 12 % linear poly-acrylamide gel for the second dimension. The majority of the protein spots were detected in the center of the gel (Fig. 2.8.), indicating that a narrower pH range for the IPG strip was necessary for increased resolution. However, proteins were well separated from top to bottom by their molecular weight. Thus, the 2-DE gel with pH 4-7 range and a 12 % linear poly-acrylamide gel were used in all subsequent experiments (Fig.2.9).



Fig. 2.7. Detection of lesions resistant line LSBR-5 and susceptible Labelle 24 hrs after inoculation. The circled areas indicate location of the lesions.

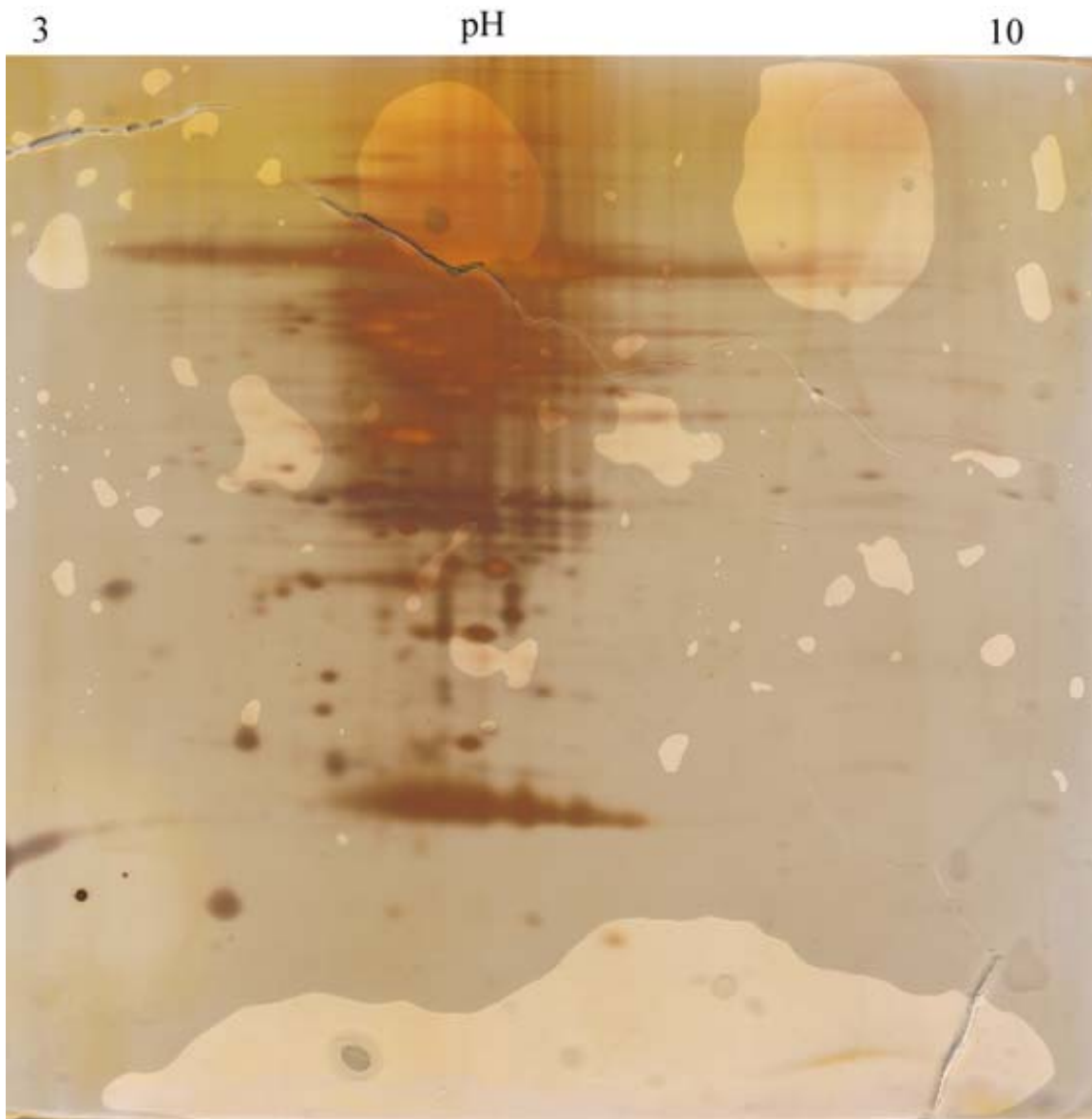


Fig. 2.8. Silver stained 2-DE gel of rice leaf sheath proteins. Proteins were separated in the broad pH range IPG strip (pH 3-10) and 12 % linear poly-acryl amide LDS-PAGE. Gel image was acquired with HP scanner.

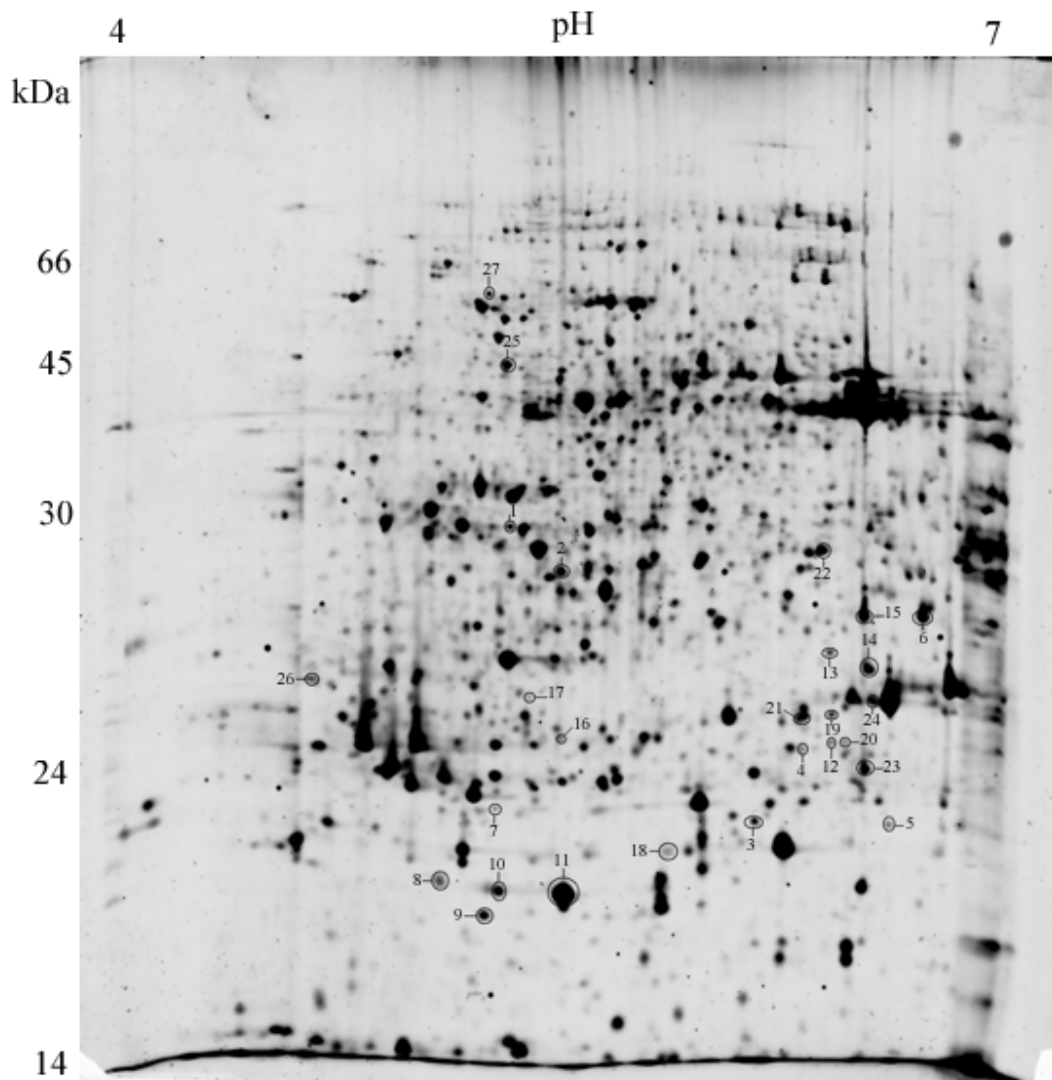


Fig. 2.9 Sypro-Ruby stained 2-DE gel of rice leaf sheath proteins extracted from inoculated LSBR-5. Proteins were separated in the pH4 to 7 IPG strip and 12 % linear poly-acrylamide LDS-PAGE. Gel image was acquired with FX scanner (Bio-Rad). Circled proteins were identified by ESI-Q-TOF mass Spectrometry.

With our established 2-DE protocol, ~ 1,000 protein spots stained with Sypro-Ruby were reproducibly resolved on all 2-DE gels used in the comparison analysis (Fig. 2.9). Some proteins spots assumed to be basic proteins were condensed at the end in the pH 7 range. However, most of the individual protein spots placed within the pH 4 to pH 7 range were well separated except for two protein groups located in the upper right and lower left areas of the gel. These protein groups have been reported in previous research to high abundant RuBisCO proteins (Kim et al., 2001).

### **2.3.2 Comparison Analysis of Protein Expression Patterns**

A total 24 2-DE gels were completed for comparison analysis that consisted of three sub-replications, two treatments, two biological replications, and two rice lines. Protein spots were reproducibly resolved across all 24 gels, resulting in similar protein spot locations across replications (Fig. 2.10). In a comparison analysis between inoculated LSBR-5 and non-inoculated LSBR-5, 36 protein spots were detected whose relative abundance varied significantly in a quantitative manner. Among the 36 proteins, 32 were up-regulated and four were down-regulated in the inoculated LSBR-5. The changes of the proteome in susceptible Labelle to *R. solani* were also investigated by comparison analysis between inoculated Labelle and non-inoculated Labelle. Twenty two proteins showed significant variation in their relative abundance. Twenty one proteins were up-regulated while one was down-regulated. Interestingly, all 22 proteins detected both in Labelle and LSBR-5 and they showed similar expression patterns (Fig. 2.11). The relative abundance of 14 protein spots significantly varied only in the response of inoculated LSBR-5 (Fig. 2.12). In summery for the 36 detected protein spots, 22 were detected in the response of both resistant and susceptible lines and 14 protein spots were detected in only the response of resistant line (Fig. 2.13). The expression patterns

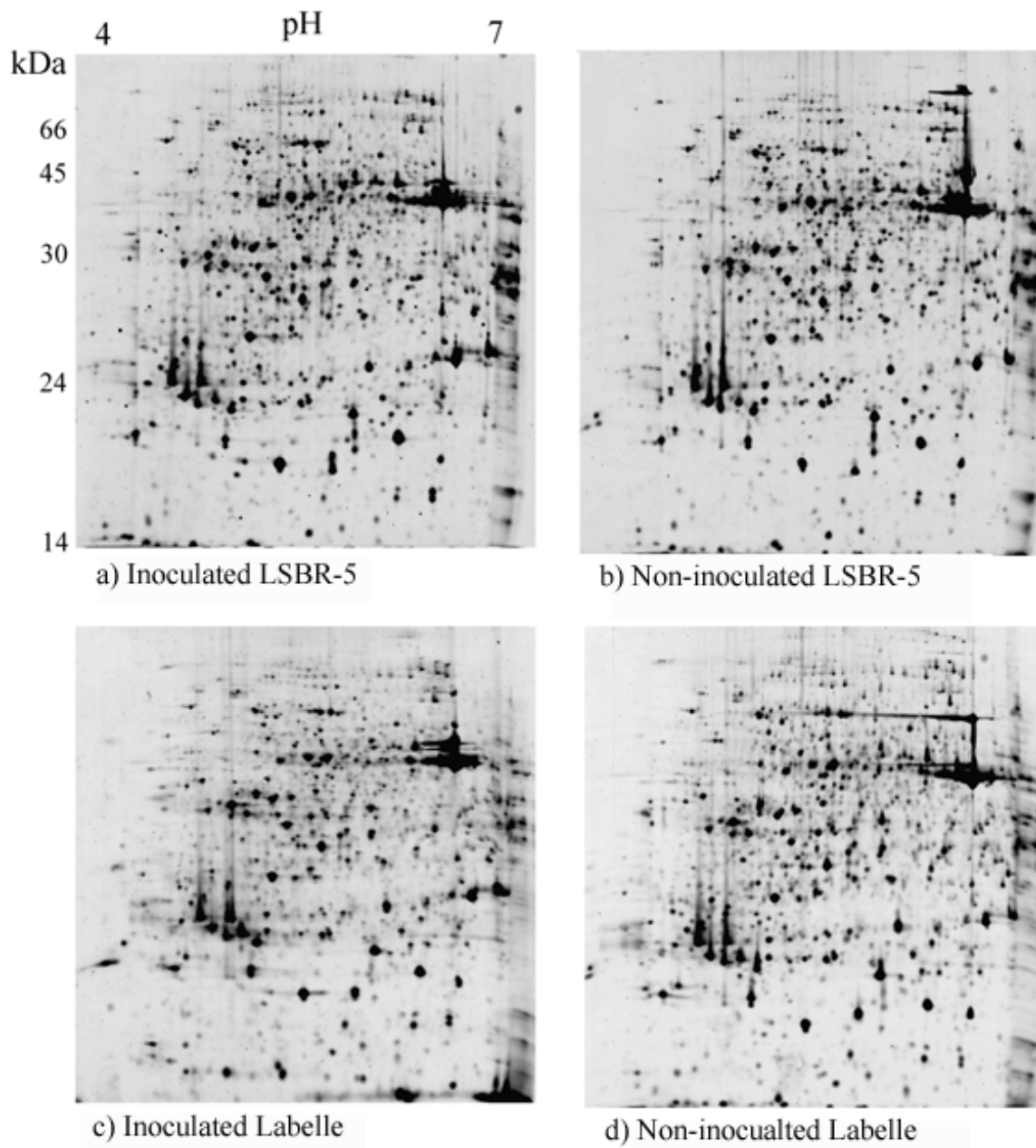


Fig. 2.10 Sypro-Ruby stained 2-DE gels of inoculated and non-inoculated Labelle

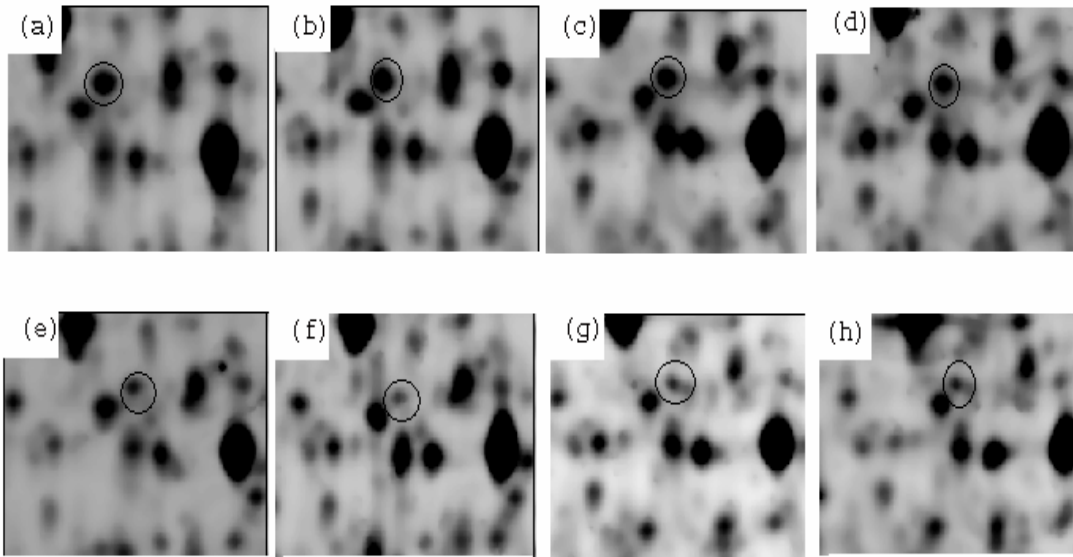


Fig. 2.11 Details of Sypro-Ruby Stained 2DE gels. The relative abundance of the circled protein spot was significantly higher in both inoculated LSBR-5 and inoculated Labelle than in non-inoculated LSBR-5 and non- inoculated Labelle, respectively. (a) (b) inoculated LSBR-5, (c) (d) inoculated Labelle (e) (f) non-inoculated LSBR-5, (g) (h) non-inoculated Labelle

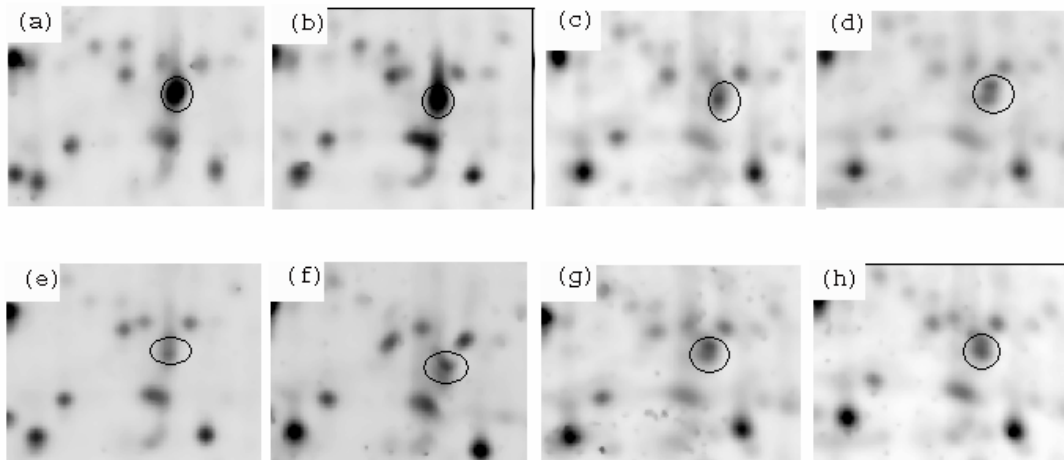


Fig. 2.12 Details of Sypro-Ruby stained 2DE gels. The relative abundance of the circled protein spot was significantly higher in the inoculated LSBR-5 than in non-inoculated LSBR-5. The relative abundance of the circled proteins spot did not vary in the inoculated Labelle compared to the relative abundance in the non-inoculated Labelle. (a) (b) inoculated LSBR-5, (c) (d) inoculated Labelle (e) (f) non-inoculated LSBR-5, (g) (h) non-inoculated Labelle

of the detected 36 proteins were compared between the response of LSBR-5 and the response of Labelle in detail by comparing their relative abundance between inoculated LSBR-5 and inoculated Labelle. Among the 22 proteins detected in the response of LSBR-5 and Labelle, 12 proteins were up-regulated in both responses with no significant difference in expression ratios between inoculated LSBR-5 and inoculated Labelle (Fig. 2.14.a) Nine proteins were up-regulated in the responses with a higher expression ratio observed in the inoculated LSBR-5 than in the inoculated Labelle (Fig.2.14.b) Eleven proteins were up-regulated only in the response of LSBR-5, showing significantly higher relative abundance in the inoculated LSBR-5 than in the inoculated Labelle (Fig. 2.14.c) All down-regulated proteins exhibited distinctive expression patterns. For example, all were down-regulated in the response of LSBR-5 and/or Labelle, but the relative abundance in non-inoculated LSBR-5 was significantly higher than in non-inoculated Labelle (Fig. 2.14.d)

### 2.3.3 Protein Identification

Twenty seven proteins out of 36 detected protein spots were identified through the

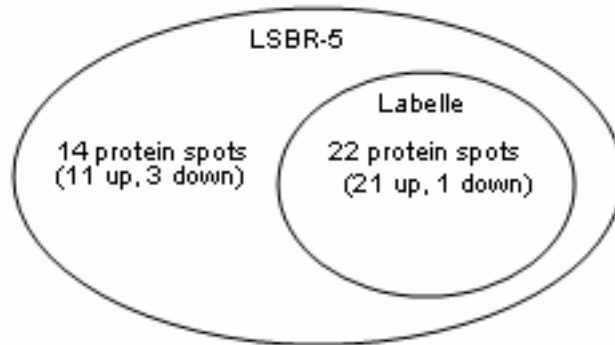


Fig. 2.13 Venn diagram of detected protein spots in the response of resistant LSBR-5 and susceptible Labelle infected by *R. solani*. Twenty two protein spots were detected in the response of inoculated resistant LSBR-5 and susceptible Labelle where 21 protein spot were up-regulated and 1 protein spot was down-regulated. Fourteen protein spots were detected only in the response of inoculated resistant LSBR-5 where 11 protein spots were up-regulated and 3 protein spots were down-regulated.

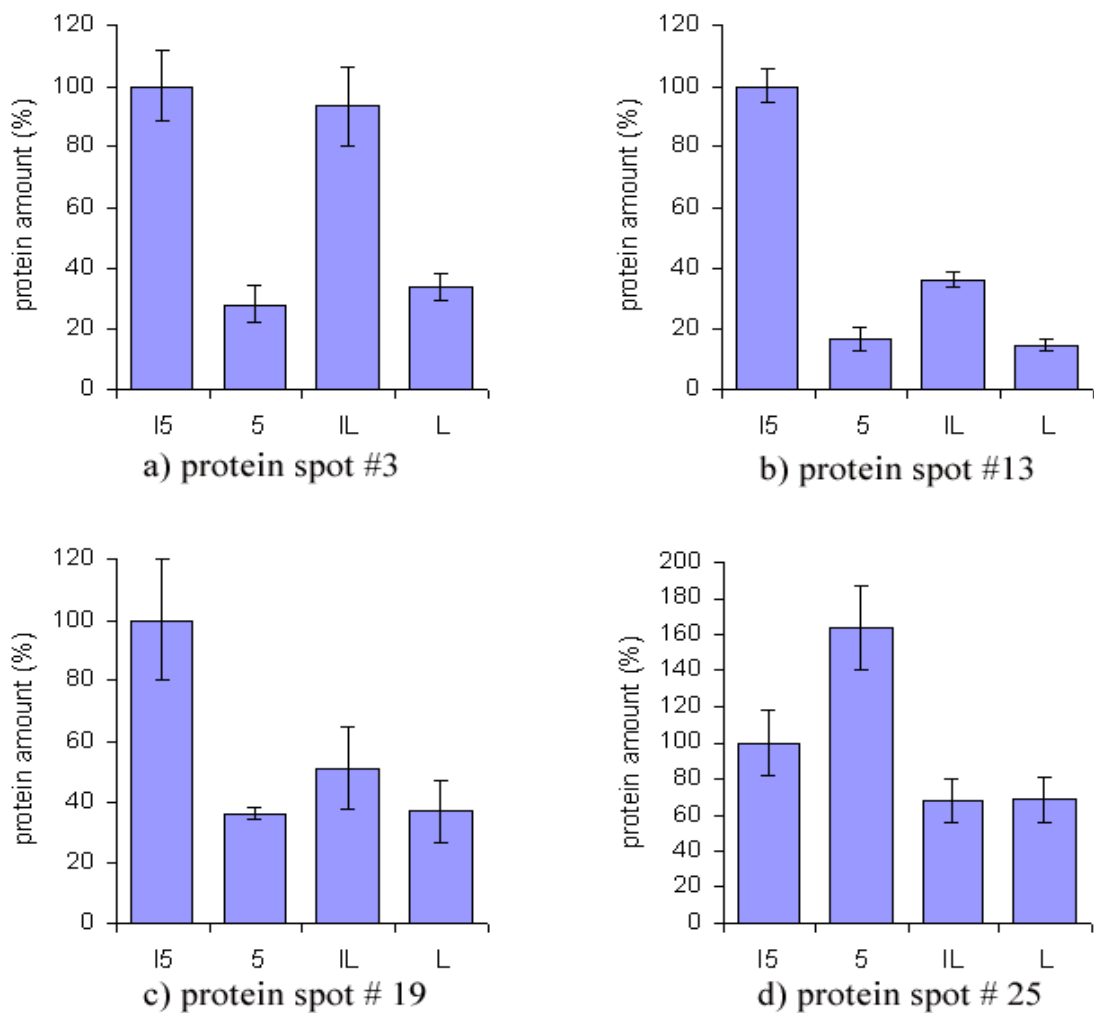


Fig. 2.14 Histogram of protein expression patterns of LSBR-5 and Labelle produced in response to challenge by *Rhizoctonia solani*. Relative protein amounts were calculated by converting the spot quantity into the percent value over the spot quantity of inoculated LSBR-5. I5: Inoculated LSBR-5, 5: Non-inoculated LSBR-5, IL: Inoculated Labelle, L: Non-inoculated Labelle.

MASCOT MS/MS Ion search engine with MS/MS spectra produced by Q-TOF micro (Waters). These 27 proteins were identified with a significantly high ion score which is calculated as  $-10 \times \text{LOG}_{10}(P)$ , where P is the absolute probability that the observed match is a random event. Thus, the higher score indicates identity or extensively homology. Fig. 2.15, an example of an identified protein, represents MS/MS spectra of a fragmented ion of a peptide from protein spot # 14 identified as beta-1-3-glucanase and ion scores of identified peptides which are significantly higher so that identified peptides were accepted. The remainder of the unidentified proteins failed to be matched against the NCBI non-redundant protein and NCBI EST databases. All of the unidentified proteins matched as RuBisCO from various plants, various kinds of proteins, or hypothetical proteins with low ion scores below the threshold, implying that the match occurred randomly. Twenty five out of 27 proteins were identified as rice proteins with the majority originating from *japonica* cultivars. The remaining two proteins were identified from *A. thaliana* sources. Twenty five percent of the proteins were identified as RuBisCO proteins, which is consistent with the source of the crude protein extract used in this study. Putative chitinase was identified in two different spots (spot #19 and spot #21). The multiple spots of an identical protein are frequently reported in proteomic studies, presumably due to post translational modification and existence of different isoforms. All identified proteins are listed in Table 1 and 2 with protein name, NCBI accession number, score, and the relative abundance compared with each treatment.

### **2.3.3.1 Identified Proteins Detected in Response of LSBR-5 and Labelle to *R. solani***

Sixteen out of the 22 proteins were identified (Table 2.1). According to the literature, the presumed functions of the identified proteins are related to antifungal activity, energy metabolism, photosynthesis, protein degradation, and antioxidation (Umeda and Uchimiya,

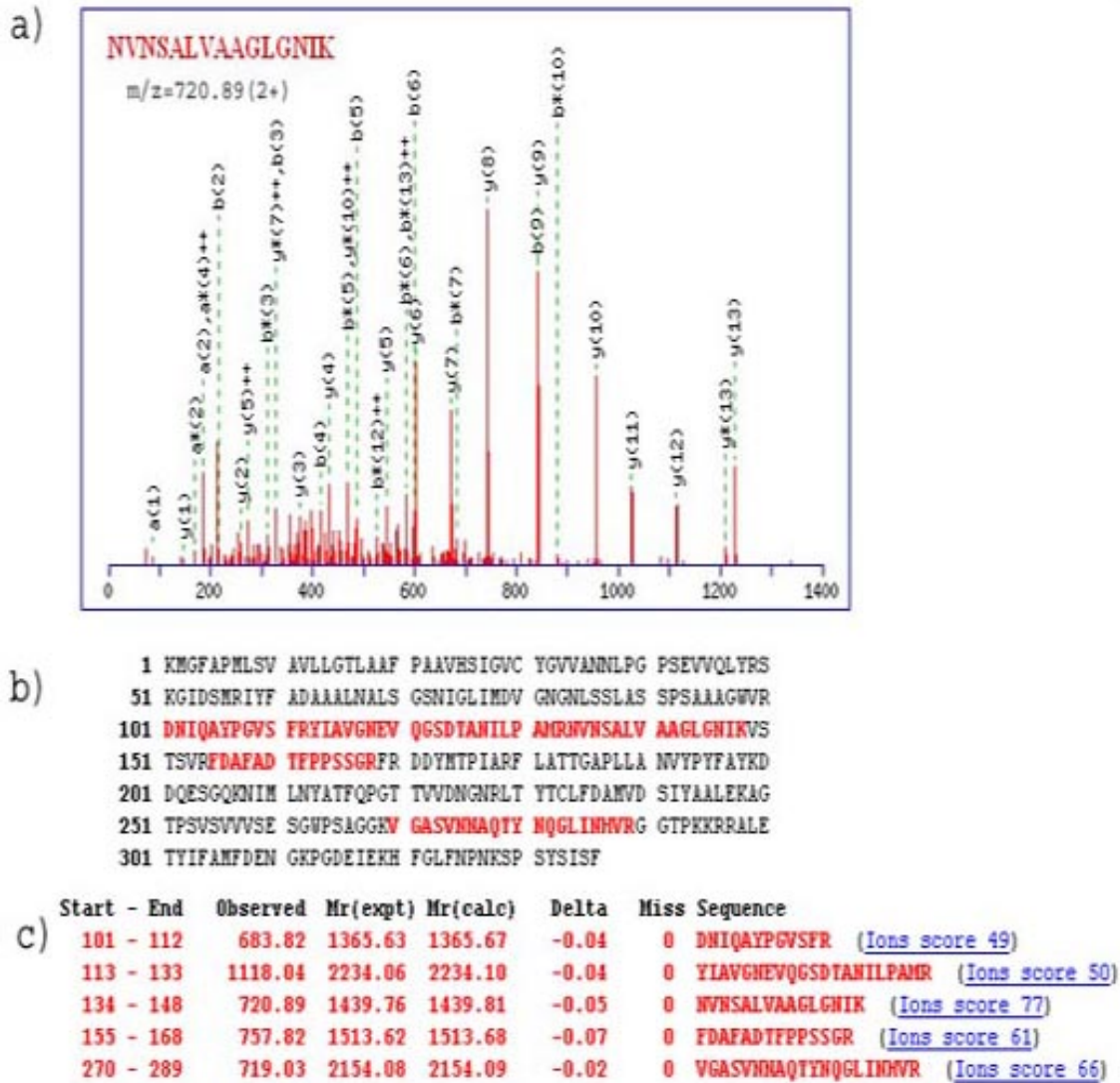


Fig.2.15 Protein identification with MS/MS spectra using Mascot MS/MS Ion Search.

a) MS/MS spectra of fragmented ion of a peptide from protein spot # 14. These ions. “a, b, and y” represent a fragmented ion carrying a charge. In the type of “a, b, and c” ion, the charge is retained on N terminal. In the type of “x, y, and z” ion, the charge is retained on the C terminal. The number in parenthesis indicates the number of residues in the fragment.

b) Amino acid sequences of beta-1,3-glucanase. The bold red indicates five identified peptides.

c) Peptide summary of beta-1,3-glucanase produced by Mascot MS/MS Ion Search with MS/MS spectra of protein spot # 14. Start-End: location of the identified peptide in the amino acid sequences of the protein. Observed: observed m/z of peptide ion. Mr(expt): peptide mass acquired from experiment. Mr(calc): peptide mass acquired from amino acid sequences. Delta: the difference between Mr(expt) and Mr(calc). Miss: number of missing sequences.

1994; Mars, 1996; Asada, 1992; Agrios, 1997; Versta, 2003; Van et al., 1999). All of these identified proteins were previously reported (Abeles et al., 1992; Laxalt et al., 1996; Bartling et al., 1993; Shen et al., 2003; Kim et al., 2004; Giulivi et al., 1994; Linthorst et al., 1990) as being involved in the plant response to various environmental or biotic stresses, suggesting that common defense responses exist in plants and some defense related proteins are expressed even in the susceptible plants. Eight out of 16 identified proteins showed higher expression ratios in the inoculated LSBR-5 than in the inoculated Labelle, implying a quantitative differential response between LSBR-5 and Labelle when challenged with *R. solani*.

### **2.3.3.2 Classification of Proteins Detected in Inoculated LSBR-5**

Eleven out of the 14 proteins were identified (Table 2.2) with presumed functions relating to antifungal activity, signal transduction, energy metabolism, photosynthesis, molecular chaperone, protein degradation, and antioxidation (Grune et al., 1995; Agrios, 1997; Lin et al., 1995; Luu-The et al., 1991, Roberts, 2003, Lorimer, 1996) Thus, the functions of these proteins uniquely detected only in the response of LSBR-5 were similar to the functions of the proteins detected in both LSBR-5 and Labelle. Moreover, some proteins, such as RuBisCO, ascorbate peroxidase, and glyceraldehyde 3-phosphate dehydrogenase, were identified in the both categories, implying that the response of LSBR-5 differs with the response of Labelle with the additional quantitative expression of the defense related proteins.

## **2.4 Discussion**

### **2.4.1 Rice Leaf Sheath Protein Separation with the 2-DE Method**

With our established protein extraction and 2-DE method, ~ 1,000 rice leaf-sheath proteins were resolved on 2-DE gels. This is the highest resolution of protein separation when

Table 2.1 Identified proteins whose relative abundance varied significantly in the resistant line LSBR-5 and the susceptible line Labelle with infection by *R. solani*

Spot	Name	Accession number	Score <sup>a)</sup>	Ratio of Relative abundance <sup>b)</sup>			
				IL/NL <sup>c)</sup>	I5/N5 <sup>d)</sup>	I5/IL	N5/NL
1	putative ACC oxidase [Oryza sativa (japonica cultivar-group)]	gi 52353464	100	1.71 ± 0.14	1.53 ± 0.03	N.S	N.S <sup>e)</sup>
2	glyceraldehyde-3-phosphate dehydrogenase [Oryza sativa]	gi 2331137	195	2.02 ± 0.65	2.78 ± 0.33	N.S	N.S
3	Putative glutathione S-transferase [Oryza sativa (japonica cultivar-group)]	gi 50919661	119	2.22 ± 0.77	3.18 ± 0.54	N.S	N.S
4	glutathione S-transferase II [Oryza sativa (japonica cultivar-group)]	gi 34909798	103	2.55 ± 0.51	3.52 ± 0.61	N.S	N.S
5	thylakoid-bound ascorbate peroxidase [Oryza sativa (japonica cultivar-group)]	gi 29616952	50	2.99 ± 0.80	5.17 ± 1.70	N.S	N.S
6	ribulose-bisphosphate carboxylase (EC 4.1.1.39) large chain precursor - rice chloroplast	gi 7436576	130	2.00 ± 0.67	3.27 ± 1.46	N.S	N.S
7	Putative ascorbate peroxidase [Oryza sativa (japonica cultivar-group)]	gi 50920595	55	2.72 ± 0.90	3.65 ± 0.39	N.S	N.S
8	putative mitochondrial F0 ATP synthase D chain [Oryza sativa (japonica cultivar-group)]	gi 50946875	110	3.24 ± 1.36	4.89 ± 2.87	2.10 ± 1.35	N.S
9	Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit)	gi 131978	120	2.22 ± 0.66	3.52 ± 1.13	1.95 ± 0.50	N.S
10	rubisco large subunit	gi 476752	113	2.00 ± 0.14	5.16 ± 1.13	2.07 ± 0.87	N.S
11	rubisco large subunit	gi 476752	190	1.68 ± 0.01	2.11 ± 0.05	2.27 ± 0.01	N.S
12	20S proteasome beta subunit [Oryza sativa (japonica cultivar-group)]	gi 50933089	83	1.53 ± 0.05	3.28 ± 1.13	1.50 ± 0.05	N.S
13	putative 26S proteasome non-ATPase regulatory subunit 14 [Oryza sativa (japonica cultivar-group)]	gi 54287494	142	2.28 ± 0.26	4.43 ± 1.26	2.39 ± 0.52	N.S
14	beta-1,3-glucanase [Oryza sativa]	gi 4884530	253	2.16 ± 0.07	3.90 ± 1.20	2.50 ± 0.27	N.S
15	ribulose-bisphosphate carboxylase (EC 4.1.1.39) large chain precursor - rice chloroplast [Oryza sativa]	gi 7436576	128	2.03 ± 0.48	4.13 ± 0.98	2.30 ± 0.18	N.S
16	putative germin protein [Oryza sativa (japonica cultivar-group)]	gi 34904670	53	0.64 ± 0.07	0.70 ± 0.05	2.12 ± 0.40	1.90 ± 0.27

a) The Score is given as  $S = -10 \times \log(P)$ , where P is the probability that the observed match with a given MS/MS spectra is a random event. Thus, a high score indicate identity or extensively homology.

b) Ratios of relative abundance are presented as means ± standard deviation (n=2). The ratios were calculated by dividing the relative abundance of a protein spot in one treatment by the relative abundance of a protein spot in another treatment. A value greater than 1 represents an up-regulated protein, whereas a ratio less than 1 represents a down-regulated protein.

c) IL : inoculated Labelle, NL: non-inoculated Labelle.

d) I5 : inoculated LSBR-5, N5: non-inoculated LSBR-5.

e) non-significant

Table 2.2 Identified proteins whose relative abundance varied significantly only in response of resistant LSBR-5 with infection by *R. solani*.

Spot	Name	Accession number	Score <sup>a)</sup>	Relative abundance <sup>b)</sup>			
				IL/NL <sup>c)</sup>	I5/N5 <sup>d)</sup>	I5/IL	N5/NL
17	ribulose-1,5-bisphosphate carboxylase/oxygenase activase [Oryza sativa]	gi 1778414	70	N.S	3.91 ± 1.01	3.03 ± 0.94	N.S <sup>e)</sup>
18	Ribulose bisphosphate carboxylase large chain (RuBisCO large subunit)	gi 6093918	53	N.S	5.19 ± 0.10	3.12 ± 0.18	N.S
19	putative chitinase [Oryza sativa (japonica cultivar-group)]	gi 55168113	55	N.S	4.79 ± 2.44	2.77 ± 1.70	N.S
20	Unknown protein [Oryza sativa (japonica cultivar-group)] (putative 3-beta hydroxysteroid dehydrogenase/isomerase protein Arabidopsis thaliana).AAM61751	gi 34897418	213	N.S	2.76 ± 0.03	1.95 ± 0.18	N.S
21	putative chitinase [Oryza sativa (japonica cultivar-group)]	gi 55168113	179	N.S	2.91 ± 0.20	2.00 ± 0.30	N.S
22	glyceraldehyde 3-phosphate dehydrogenase A subunit [Arabidopsis thaliana]	gi 166702	168	N.S	2.76 ± 0.60	1.87 ± 0.50	N.S
23	Putative alpha 1 subunit of 20S proteasome [Oryza sativa (japonica cultivar-group)]	gi 50920359	173	N.S	3.98 ± 0.98	2.44 ± 0.06	N.S
24	Stromal ascorbate peroxidase [Oryza sativa (japonica cultivar-group)]	gi 32879781	45	N.S	5.41 ± 0.87	3.27 ± 1.27	N.S
25	putative chaperonin 60 beta precursor [Oryza sativa (japonica cultivar-group)]	gi 34897924	687	N.S	0.67 ± 0.10	1.94 ± 0.20	3.20 ± 0.20
26	14-3-3-like protein [Oryza sativa]	gi 7271253	190	N.S	0.60 ± 0.06	N.S	1.55 ± 0.08
27	endospERM lumenal binding protein [Oryza sativa]	gi 2267006	299	N.S	0.65 ± 0.01	1.82 ± 0.17	2.30 ± 0.30

a) The Score is given as  $S = -10 \times \text{Log}(P)$ , where P is the probability that the observed match with a given MS/MS spectra is a random event. Thus, a high score indicate identity or extensively homology.

b) Ratios of relative abundance are presented as means ± standard deviation (n=2). The ratios were calculated by dividing the relative abundance of a protein spot in one treatment by the relative abundance of a protein spot in another treatment. The ratio value higher than 1 represents an up-regulated protein. The ratio value lower than 1 represents a down-regulated protein.

c) IL : inoculated Labelle, NL: non-inoculated Labelle

d) I5 : inoculated LSBR-5, N5: non-inoculated LSBR-5

e) non-significant

compared with recently published rice leaf-sheath proteomic research. For example, Shen et al. (2003) identified a rice leaf sheath proteome with only 400 proteins resolved on a 2-DE gel. Moreover, when our 2-DE gels were stained with silver instead of Sypro-Ruby, which was used in our comparison analysis of gels, a much higher resolution was obtained (Fig 2. 16). Thus, it is recommended to use silver staining method for construction of high-resolution 2-DE maps. In the comparison analysis of protein expression patterns, however, the Sypro-Ruby staining method showed a linear response to protein amount over a large dynamic range, even though it is known to be less sensitive than silver staining (Yan et al., 2000). According to the completed rice genome sequence, rice is estimated to have ~ 46,022 to 55,614 genes (Goff et

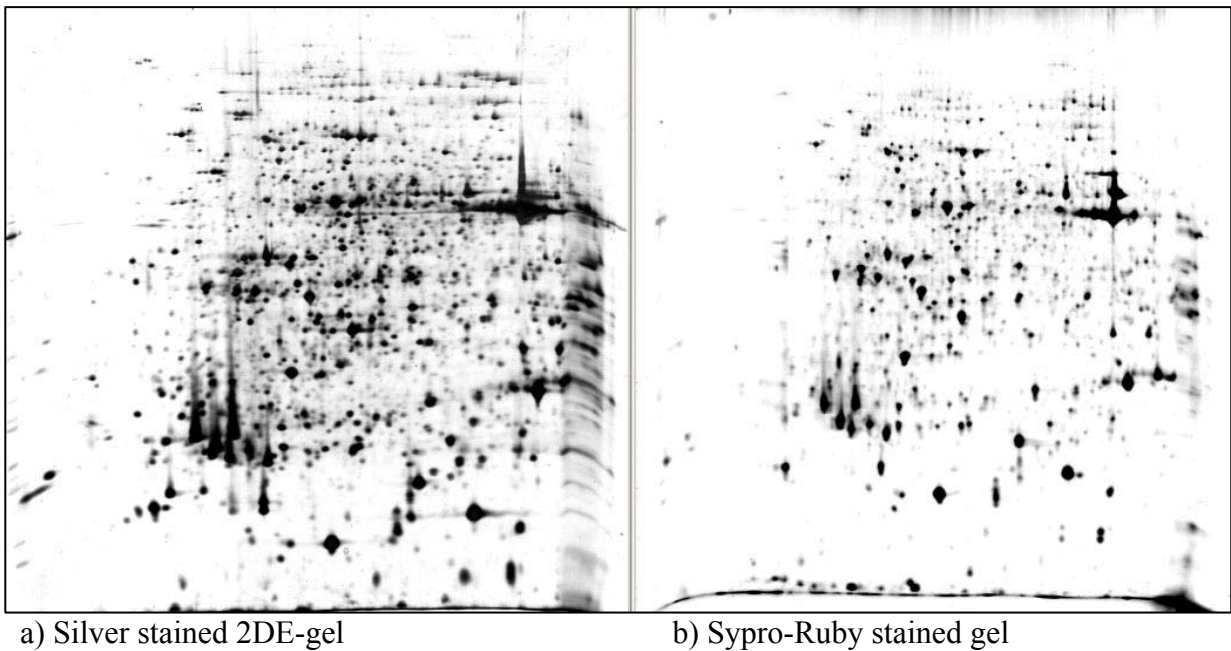


Fig. 2.16. Comparison between silver and Sypro-Ruby staining of 2-DE gels. Both 2-DE gels were of rice leaf sheath proteins extracted from LSBR-5 24 hrs post inoculation. Proteins were separated in the pH 4 to 7 IPG strip and 12 % linear poly-acrylamide LDS-PAGE. The silver stained gel image was acquired with a GS-700 densitometry (Bio-Rad) and the sypro-Ruby stained gel image was acquired with an FX scanner (Bio-Rad).

al., 2002). Our 2-DE gel roughly covers 20% of the rice leaf-sheath proteome based on an assumption that 10% of the total genome is expressed in the leaf sheath. This large gap may be due to limitations of protein loading capacity and staining sensitivity of the 2-DE gel system. According to 2-DE PAGE evaluation in the yeast proteome by Gygi et al. (2000), proteins occurring in more than 51,200 copies per cell can be detected in a 2-DE gel with silver staining method when 40  $\mu$ g of whole yeast lysate is loaded, whereas detection of less abundant proteins at 1,000 copies per cell requires a minimum of 0.5 mg of protein sample. Limitations of loading capacity in the IPG gel and high abundant RuBisCO proteins in the plant cells mask the detection of low abundant regulatory and signal transduction proteins (Rakwal et al., 2003). Kim et al. (2001) reported a pre-fractionation method to overcome this 2-DE disadvantage where rice leaf crude proteins were extracted with Mg/Nonidet P-40 (NP-40) and precipitated into 10% polyethylene glycol (PEG), 10 to 20% PEG, and supernatant fractions precipitated with acetone. Approximately 972, 672, and 1026 protein spots were resolved on silver-stained 2-DE gels in the 10% PEG, 20% PEG, and supernatant fractions, respectively. A total of 2670 protein spots were separated from the three fraction samples, producing less than 1.2% overlapping proteins. RuBisCO proteins were enriched in the 20% PEG fraction.

Another disadvantage of the 2-DE system is that the extracted protein samples are biased in the pH 4-7 range. Many basic and hydrophobic proteins are difficult to be resolved in the general 2-DE gels due to electrophoretic interference caused by basic proteins and the reducing agent DTT used in the extraction buffer (Bae et al., 2003). Recently, a liquid-phase separation HPLC technique coupled with mass spectrometer has been developed as an alternative for reducing complexity of complex protein extracts (Wagner et al., 2002). Unlike

the 2-DE method, this technique can analyze a broad pH range of proteins, broad molecular mass range of proteins, hydrophobic proteins, membrane proteins, and low abundant proteins. In addition, this technique can be automated by on-line coupling to mass spectrometry. With multidimensional protein identification technology (MudPIT) developed by Link et al. (1999), a complex protein mixture is digested into a peptide fraction before injection into a micro capillary column for mass spectrometry analysis. Specifically, the peptide complex is separated by a biphasic microcapillary column consisting of a strong cation exchange phase in the first dimension and reverse phase chromatography in the second dimension. The peptides are directly eluded off the biphasic microcapillary into an on-line coupled tandem mass spectrometer (MS/MS). A tandem mass spectrum of a peptide generated by MS/MS is interpreted into amino acid sequences of the peptide, leading to the identification of the protein from which the peptide was derived (Witelegge, 2002; Washburn et al., 2002). Koller et al. (2002) presented the most comprehensive proteome work to date on rice tissue using both MudPIT and 2-DE methods. These techniques allowed detection and identification of 2,528 unique proteins; 556 protein by 2-DE and 2,363 proteins by the MudPIT procedure.

#### **2.4.2 Comparison Analysis between the Response of LSBR-5 and the Response of Labelle**

With the 2-DE proteomic approach, we detected reproducible proteome differences between the host-parasite response of LSBR-5 and that of Labelle. This study revealed that 22 proteins out of the 36 proteins showed significant variation in relative abundance after pathogen challenge of the resistant LSBR-5 and the susceptible Labelle. It is concluded that a common stress response system is activated in both resistant and susceptible plants regardless of the resistance level against *R. solani*. Moreover, all identified proteins from the 22 proteins were previously reported as being involved in disease response (Abeles et al., 1992; Laxalt et

al., 1996; Bartling et al., 1993; Shen et al., 2003; Kim et al., 2004; Giulivi et al., 1994; Linthorst et al., 1990). For example,  $\beta$ -1-3 glucanase which has antifungal activity by degrading the fungus cell wall was up-regulated in the response of both resistant and susceptible lines. This phenomenon of the defense related gene expression in the resistant and susceptible plants was previously reported in the interaction between *A. thaliana* and the fungus *Peronospora parasitica* (Maleck et al., 2000). In the current study, nine proteins out of the 22 detected in both responses were up-regulated to a significantly higher level in LSBR-5, than in Labelle, and an additional 14 proteins whose quantities did not vary in the response for Labelle showed significant quantitative variation in the response of LSBR-5. Proteins identified with higher induced levels in LSBR-5 than in Labelle were also reported as being involved in disease response (Mauch et al., 1988; Laxalt et al., 1996; Michalowski and Bohnert, 1992; Nam et al., 2003; Holland et al., 1998; Vidal et al., 1997). From the different protein expression patterns detected in this study, it is postulated that the resistance of LSBR-5 resulted from the ability to produce greater amounts of defense proteins than the susceptible Labelle, and this ability was possibly controlled by key mutant regulatory protein(s) acquired during *in vitro* somaclonal culture of the resistant mutant.

Putative regulatory proteins would not be detected in this study if they were low abundant, basic, hydrophobic, or membrane-bound proteins that could not be separated in our 2-DE gel system. Because the protein samples were harvested 24 hours post inoculation, the key protein(s) may have been expressed much earlier and then rapidly degraded. Thus, high resolution in protein separating techniques, new protein extraction methods for various types of proteins, and various time points for harvesting will be required for the future studies in detecting key defense regulatory protein(s).

### 2.4.3 Identified Proteins

Various proteins were identified in this study that are known to be involved in antifungal activity, signal transduction, energy metabolism, photosynthesis, protein folding and degradation, signal transduction, and antioxidation (Umeda and Uchimiya, 1994; Mars, 1996; Asada, 1992; Agrios, 1997; Roberts, 2003; Lorimer, 1996; Versta, 2003; Van et al., 1999). This suggests that the plant response to *R. solani* consisted of various defense pathways. Moreover, many of the proteins detected in the current study were previously reported to be expressed by various pathogen treatments as well as various abiotic stresses, suggesting that some common response pathways exist in the rice plant defense system. Based on the identified proteins from this study, the detected response was similar to systemic acquired resistance (SAR). As typically expressed proteins during SAR, PR-proteins (glucanase and chitinase) were increased in this response. Moreover, 14-3-3 like protein and germin protein, whose activity is increased in the HR to increase H<sub>2</sub>O<sub>2</sub>, were decreased, whereas glutathione transferases and peroxidases, antioxidant proteins, were increased. This response which is similar to the SAR phenomenon may be explained with the harvested leaf sheath sample and the harvesting time. As mentioned in the Materials and Methods section, leaf sheath samples were harvested 24 hours post inoculation, so the harvested leaf sheath area may not have participated directly in the early hypersensitive response to *R. solani*.

#### 2.4.3.1 $\beta$ -1,3-glucanase

$\beta$ -1,3-glucanases were up-regulated in both the resistant and susceptible lines of this study with expression ratios in the mutant line higher than in the susceptible line.  $\beta$ -1,3-glucanase, referred to as PR-2, is a hydrolytic enzyme commonly found in plants that hydrolyzes  $\beta$ -1,3-linked glucans, the major component of fungal cell walls (Van Loon et al.,

1999; Yamaguchi et al., 2002). For enhancing resistance against fungal pathogens, the genetic engineering technique has been applied in over-expressing this antifungal enzyme in various plants such as alfalfa (*Medicago sativa*), tobacco (*Nicotiana. tabacum*), and tomato (*Lycopersicon esculentum*) (Masoud et al., 1996; Yoshikawa et al., 1993; Jongedijk et al., 1995). Consistent with this study, Bera and Purkayastha (1997) showed that  $\beta$ -1,3-glucanases and chitinase were induced upon infection with *R. solani*. Besides pathogen attack,  $\beta$ -1,3-glucanase was reported to be induced by various defense-signaling molecules such as salicylic acid, methyl jasmonate, and ethylene (Linthorst et al., 1990; Rickauer et al., 1997; Mauch and Staehelin 1989). Thus, the induction of ACC oxidase in our results may be associated in the induction of  $\beta$ -1, 3-glucanases.

#### **2.4.3.2 Chitinase**

Two different protein spots were identified as putative chitinases that were up-regulated only in the resistant line. Chitinase, referred as PR-3, catalyzes the hydrolysis of  $\beta$ -1-4-linkage of the N-acetylglucosamine polymer of chitins, a major component of fungal cell walls (Lin et al., 1995). Like  $\beta$ -1,3-glucanase, chitinase has been genetically introduced and over-expressed in various plants such as rice (*Oryza sativa*), tobacco (*Nicotiana. tabacum*), and tomato (*Lycopersicon esculentum*) (Lin et al., 1995; Broglie et al., 1991; Tabaeizadeh et al., 1999). Chitinase and  $\beta$ -1,3-glucanase can synergistically inhibit fungal growth *in vitro*, and the co-expression of these two enzymes significantly enhanced resistance against *R. solani* in transgenic rice (Mauch et al., 1988; Kim et al., 2003). This synergetic effect may contribute to the high resistance levels of LSBR-5. It is interesting that only  $\beta$ -1,3-glucanase increased in the response of the susceptible Labelle, whereas  $\beta$ -1,3-glucanase and two chitinases increased in the response of resistant LSBR-5. Moreover, the  $\beta$ -1,3-glucanase

showed a higher expression ratio in LSBR-5 than in Labelle.

#### **2.4.3.3 Glutathione S-Transferases**

Glutathione S-transferase (GST) was up-regulated in both the resistant and susceptible plants with similar expression ratios in the current study. GST plays a role in detoxification that catalyzes the conjugation of the tripeptide glutathione (GSH) to a variety of hydrophobic, electrophilic, and cytotoxic substrates (Mars, 1996). This enzyme was initially identified and studied in plants as an enzyme for detoxifying herbicides. Plants with high levels of GST activity can tolerate herbicides by conjugating herbicide-GSH, this converting a toxic herbicide to a non-toxic form. In contrast, weeds are susceptible to herbicides because of their low GST activity (Gronwald and Plaisance, 1998). GST has numerous roles in cellular processes with a common function, namely the recognition and transport of a broad spectrum of reactive electrophilic compounds from both exogenous and endogenous origins (Mars, 1996). GST is known to be involved in tagging toxic endogenous substrates with GSH conjugation to transport toxic substrates into the vacuole through a glutathione pump (Ishikawa, 1992). For example, the maize bz-2 mutant whose BZ-2 gene was shown to be a GST, showed localized necrosis caused by failure to transport the anthocyanin precursor cyaniding-3-glucoside (C3G) into the vacuole resulting in an inappropriate accumulation of C3G in the cytoplasm (Marrs et al., 1996). Many plant GST genes were reported to be auxin-inducible where GTS binds auxin at the noncatalytic site or catalytic site, depending on different auxins, suggesting that GTS plays different roles in auxin function. For the non-catalytic binding auxin, such as IAA and  $\alpha$ -NAA, GST is associated with temporary storage, transport, or uptake of auxin, whereas for the catalytic binding auxin, such as 2,4-D and 2,4,5-T, GST plays a role of detoxification of auxins ( Jones, 1994; Droog et al., 1993; Bilanz and

Strum, 1995). GST has an important role in plant defense from oxidative damages caused by various biotic or abiotic stresses such as heavy metal, wounding, ethylene, ozone, and pathogen attack (Mars, 1996). For example, by conjugation with GSH, the induced GST detoxifies membrane lipid peroxides such as 4-hydroxyalkenals or 13-hydroperoxylinoleic acid which are toxic substrates causing oxidative damage in cell (Bartling et al., 1993). From the known roles of GST, it is postulated that GST detected in this study may have an essential role in the defense response by detoxifying RS phyto-toxins produced by *R. solani* and protecting cells from oxidative damage caused by *R. solani* attack.

#### **2.4.3.4 Ascorbate Peroxidase**

Putative ascorbate peroxidase and thylakoid-bound ascorbate peroxidase were up-regulated in both the resistant and susceptible plants with similar expression ratios, whereas stromal ascorbate peroxidase was up-regulated only in the resistant line. Ascorbate peroxidase (APX), scavenges superoxide, hydroxyl radicals, and singlet oxygen in the cytosol, chloroplast, and mitochondria of higher plants. APX uses two molecules of ascorbate, the most important antioxidant substrate in plants, to reduce  $H_2O_2$  to water (Asada, 1992). The active oxygen species causing oxidative damages in cells can be produced not only by biotic or abiotic stresses, but also by normal metabolic processes including chloroplast, mitochondrial, and plasma membrane electron transport systems (Foyer et al., 1994). The decline of the cytosolic APX protein level was detected in viral infected tobacco during programmed cell death (PCD), known as the hypersensitive response (HR) in plants. This decline of cytosolic APX level was due to post-transcriptional suppression of APX where the transcripts of the cytosolic APX gene were increased under high levels of  $H_2O_2$  during PCD. Suppression of APX supposedly contributes to PCD by allowing  $H_2O_2$  accumulation in the

cells (Mittler et al., 1998). The opposite results in our study where APX proteins were up-regulated in LSBR-5 and Labelle may imply that the harvested leaf sheaths were not undergoing PCD. Moreover, the harvested leaf sheath in our study did not show any necrosis or cell death. The 14-3-3 and germin proteins inducing oxidative burst were down-regulated in this study, whereas the proteins involved in defense of oxidative stress were up-regulated, such as glutathione S-transferase, ascorbate peroxidase, and 20S proteasome. Production and removal of H<sub>2</sub>O<sub>2</sub> must be strictly controlled during SAR because H<sub>2</sub>O<sub>2</sub> acts not only as an antimicrobial compound and a signal molecule inducing defense proteins, but also as a toxic molecule to the host plants (Wang et al., 2002). Thus, in the response of the resistant and susceptible line in this study, the APX proteins were possibly involved in reducing H<sub>2</sub>O<sub>2</sub> concentrations to protect cells from oxidative damage.

#### **2.4.3.5 26S Proteasome**

The 26S proteasomes were up-regulated in both the resistant and susceptible plants with the expression ratio in the mutant line higher than in the susceptible line. The 26S proteasome has an essential role in the ubiquitin/26S proteasome (Ub/26S) mediated ATP dependant proteolysis system in which proteolysis is very specific and strictly controlled (Versta, 2003). The 26S proteasome, composed of two particles, the 20S core protease and the 19S regulatory particle, recognizes and degrades a polyubiquitylated protein which is formed by covalent linkage between ubiquitin and lysine residues in the targeted protein (Sullivan et al., 2003). Plants respond to the various environmental changes by expressing new proteins as well as degrading regulatory proteins, damaged proteins, and proteins that become useless in a new environment. The ability to switch from one physiological mode to another is essential for the growth of plants that cannot move away from an unfavorable environment (Basset et

al., 202; Sullivan et al., 2003). The ubiquitin /26S proteasome system has an important role in the plant growth by being involved in numerous physiological and developmental events such as flower development, plant hormone regulated processes, proteolysis and light-regulated development (Callis and Vierstra, 2000; Hellmann and Estelle, 2002; Hershko and Ciechanover, 1998). The importance of the ubiquitin/26S proteasome system is also supported by the large number of identified genes encoding ubiquitin/26S proteasome components in plants. More than 1,300 genes of these components have been identified in *A. thaliana*, which is approximately 5% of the total genome (Verstra et al., 2003). The induction of the 26S proteasome in both LSBR-5 and Labelle implies that the physiological status of both plants was shifted to a defense mode.

#### **2.4.3.6 20S Proteasome**

The 20S proteasomes were up-regulated in both the resistant and susceptible lines of our study where the expression ratio in the mutant line was higher than in the susceptible line. A putative alpha 1 subunit of 20S proteasome was up-regulated only in the resistant line. The 20S proteasome, the proteolytic core in the 26 proteasome, is known to be involved in the degradation of proteins modified by oxidation (Grune et al., 1995). In mammalian cells, the 20S proteasome has been shown to recognize and selectively degrade oxidatively damaged proteins, such as hydrogen peroxide-modified hemoglobin (Giulivi et al., 1994). Amino acids of proteins can be modified by the oxygen radicals or other activated oxygen produced as by-products of cellular metabolism or from the response to abiotic or biotic stress. Subsequently, oxidatively modified proteins can undergo chemical fragmentation or form aggregates due to covalent cross-linking reactions and increased surface hydrophobicity (Dean et al., 1997). The up-regulation of 20S proteasome in this study may be involved in the defense against

oxidative stress which is consistent with the up-regulation of several antioxidant proteins (APX and GST).

#### **2.4.3.7 ACC Oxidase**

ACC oxidases were up-regulated in both the resistant and susceptible plants with similar expression ratios. ACC oxidase plays a key role in ethylene biosynthesis by oxidative cleavage of 1-aminocyclopropane-1-carboxylic acid (ACC) to form ethylene which is a plant hormone involved in accelerating senescence and fruit ripening (Kim et al., 2003; Abeles et al., 1992). Ethylene is also regarded as a signal molecule to induce plant defense responses to environmental stress such as drought, mechanical wounding and pathogen attack (Abeles et al., 1992; Diaz et al., 2002). For example, Diaz et al. (2002) reported that in the tomato cv Moneymaker, disease symptoms caused by the fungal pathogen *Botrytis cinerea* were significantly increased by pre-treatment of the strong, irreversible ethylene perception inhibitor, 1-methylcyclopropene (MCP), suggesting that ethylene perception was required for defense response signaling. In a recent report, ethylene was reported to mediate the methyl jasmonate (MJ) induced defense response in Douglas fir (*Pseudotsuga menziesii*) (Hudgins et al., 2004). Exogenous MJ treatment induced formation of an additional xylem traumatic resin duct (TD), a defense structure induced by herbivory, pathogen invasion, or wounding, and increased ACC oxidase concentrations in Douglas fir, whereas the pre-treatment of MPC, ethylene response inhibitor, reduced TD formation induced by the MJ treatment (Hudgins and Franceschi, 2004)

#### **2.4.3.8 Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)**

Glyceraldehyde-3-phosphate dehydrogenases of rice (*Oryza sativa*) were up-regulated in both the resistant and susceptible plants with similar expression ratios, and a protein spot

identified as glyceraldehyde 3-phosphate dehydrogenase, with homology to an A subunit isoform from *Arabidopsis*, was up-regulated only in the resistant line. GAPDH plays an important role in glycolysis and gluconeogenesis by reversibly catalysing the oxidation and phosphorylation of glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglycerate (BPG), a high energy intermediate used for the synthesis of ATP with a co-substrate of  $\text{NAD}^+$  (Umeda and Uchimiya, 1994). GAPDH is known to be induced by anaerobic conditions (Sachs et al., 1996). Ricard et al., (1989) suggested that rice seedlings in an anaerobic environment can maintain high metabolic rates by increasing GAPDH. Umeda and Uchimiya (1994) detected a significant increase in the amount of GAPDH transcripts in submergence-tolerant rice compared to an intolerant cultivar. GAPDH was induced in potato leaves and stems by infection of the late-blight fungal pathogen *Phytophthora infestans*, the treatment of eicosapentaenoic acid (an elicitor found in *P. infestans*), or the treatment of exogenous salicylic acid (SA), a signal molecule inducing SAR (Laxalt et al., 1996). These reports of GAPDH induction in various environmental conditions including abiotic and biotic stresses suggests that the GAPDH metabolism pathway has multiple roles in the plant cell or related multiple defense pathways are regulated by similar signal transduction pathways (Laxalt et al., 1996).

#### **2.4.3.9 Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase ( RuBisCO)**

Twenty five percent of the identified proteins in this study were Ribulose 1,5-bisphosphate carboxylase/oxygenases (RuBisCO) which were up-regulated in both the resistant and susceptible plants. The level of RuBisCO proteins, one of the important enzymes in photosynthesis, is known to be reduced in infected plant cells because pathogens attack chloroplasts that lead to their degradation (Agrios, 1997). In recent rice proteomic research,

however, RuBisCO proteins were reported to be increased in the rice leaf sheath by wounding stress (Shen et al., 2003). The induced level of RuBisCO proteins in the response of LSBR-5 and Labelle may be explained by the unique area of harvested rice leaf sheath samples. As mentioned in the materials and methods, the protein samples were extracted from the cells adjacent to the infected cells to avoid contamination with fungal mycelia. Because the sampled leaf sheaths were not chlorotic and did show green color, it is likely that the chloroplasts of the sampled cells were not yet affected directly by the infection, and photosynthetic activity was increased to compensate for loss in adjacent infected cells.

#### **2.4.3.10 Mitochondrial F<sub>0</sub> ATP Synthase D Chain**

Putative Mitochondrial F<sub>0</sub> ATP synthase D chain proteins were up-regulated in both the resistant and susceptible plants where expression ratios in the mutant line were higher than in the susceptible variety. ATP synthase catalyzes the production of ATP by attachment of inorganic phosphate (PO<sub>4</sub>) group to adenosine diphosphate (ADP), storing the energy produced during respiration or photosynthesis into the bond between ADP and PO<sub>4</sub>. This energy stored in ATP is used by all types of cellular work, including activation of enzyme, mobilization of compounds, synthesis of proteins, cell growth, and defense reactions (Agrios, 1997). Mitochondrial ATP synthase consists of an F<sub>1</sub> catalytic domain and F<sub>0</sub> domain in the inner membrane (Stock et al., 1999). The transcripts of mitochondrial F<sub>0</sub> ATP D chain were increased in wild rice (*Oryza minuta*) by the infection of the fungal pathogen *Magnaporthe grisea* (Shim et al., 2004). However, it is not known whether mitochondrial F<sub>0</sub> ATP synthase D chain has a specific role in the rice defense mechanism. Because various pathways require energy to activating enzymes involved in the defense response (Agrios, 1997), induction of this protein in our study is consistent with this hypothesis.

#### **2.4.3.11 3-Beta Hydroxysteroid Dehydrogenase/Isomerase**

The 3-beta hydroxysteroid dehydrogenase/isomerase proteins were up-regulated only in the resistant line. 3-beta hydroxysteroid dehydrogenase/isomerase is known to be involved in the biosynthesis of steroid hormones in mammals (Luu-The et al., 1991). In plants, this enzyme was suggested to be involved in the formation of cardenolides in Grecian foxglove (*Digitalis lanata*) (Finsterbusch et al., 1999). Milkweed produces cardenolide which is toxic to insects in the leaf as a defense mechanism against sucking insect herbivores (Martel et al., 2004). To our knowledge, however, the occurrence of cardenolide in rice has not been reported. The occurrence of 3-beta hydroxysteroid dehydrogenase/isomerase enzyme activity in *Solanum tuberosum* and *Nicotiana tabacum* plants, which are unable to form cardenolides, implies that this enzyme may be involved in other steroid metabolic functions (Seidel et al., 1990). In recent proteomic research of ginseng (*Panax ginseng*), this enzyme was reported to be induced by high light-stress conditions (Nam et al., 2003). However, the specific role of this enzyme in the rice defense response is unknown.

#### **2.4.3.12 Germin Proteins**

Putative germin proteins were down-regulated in both the resistant and susceptible lines. Germin proteins are induced in the embryos of cereal seeds during germination (Grzelczak and Lane, 1984). Germins have been identified as an oxalate oxidase which catalyzes the oxidation of oxalate by molecular oxygen, yielding CO<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> (Lane et al., 1993). From the fact that germin is localized in cell walls in wheat and the H<sub>2</sub>O<sub>2</sub> produced by the germins can be utilized by peroxidases in the oxidative cross-linking of cell-wall polymers, germin is likely involved in initiation and termination of cell wall expansion (Olson and Varner, 1993; Lane, 1994). In addition to cell wall developmental role in cereals, germin was

reported to be induced by abiotic and biotic stress such as salt stress and infection of powdery mildew (*Erysiphe graminisi*) (Michalowski and Bohnert, 1992; Zhang et al., 1995). In contrast to previous research, germin was slightly down-regulated in the response of LSBR-5 vs. Labelle. This contrary result may be explained by the role of germin in plant defense. The oxalate oxidase activity of germin induces H<sub>2</sub>O<sub>2</sub>, a reactive oxygen species leading to an oxidative burst. As mentioned earlier in the discussion, however, some proteins increased in the response have antioxidant functions to remove the oxidative damage to the host plant. Thus, in the aspect of the global snapshot of the response of LSBR-5 and Labelle, the reduction of germin is consistent with the induction of antioxidant proteins.

#### **2.4.3.13 14-3-3 Protein**

The 14-3-3 proteins were down-regulated only in the resistant line. The 14-3-3 protein is a phosphoserine-binding protein that regulates various target proteins by direct protein-protein interactions. In animal cells, targets of this protein are involved in metabolism, signal transduction, and ion transport (Roberts, 2003). In plants, the 14-3-3 protein affects the activity of various enzymes and ion channels. For example, nitrate reductase and plasma membrane H<sup>+</sup> pumping ATPase are inhibited and activated, respectively, by the direct interaction with 14-3-3 proteins (Comparot et al., 2003; Kinoshita and Shimazaki., 1999).

The 14-3-3 protein has an important role in hypersensitive cell death in the plant defense response. In the incompatible interaction in barley with avirulent powdery mildew fungus, 14-3-3 protein and plasma membrane H<sup>+</sup> ATPase were induced in epidermal cell layers where the hypersensitive cell death occurred (Finnie et al., 2002). Zhou et al. (2000) proposed the model of HR in barley with powdery mildew. H<sup>+</sup> ATPase is activated in the epidermal cell by the detection of the avirulent pathogen, causing acidification in the apoplast.

This low pH is optimum to oxalate oxidase, generating H<sub>2</sub>O<sub>2</sub>. The direct interaction of 14-3-3 protein and ankyrin repeated containing proteins (ARK2) from *A. thaliana* was detected by yeast-two hybrid screening. The transfer and expression of antisense AKR2 in *A. thaliana* did induce hypersensitive cell death accompanied by increased levels of H<sub>2</sub>O<sub>2</sub> and accumulation of defense related PR1 and GST6 transcripts, suggesting that the 14-3-3 protein did induce levels of H<sub>2</sub>O<sub>2</sub> and increase pathogen resistance by acting as a suppressing regulator of ARK2 (Yan et al., 2002). The down- regulation of 14-3-3 protein in our study suggests that the response of Labelle and LSBR-5 are not consistent the with the hypersensitive cell death. Another possible explanation for this result is that the harvesting time of 24 hours might be too late to investigate an early response, up-regulating function of the 14-3-3 proteins.

#### **2.4.3.14 Chaperonin 60 Beta Precursor**

Chaperonin 60 beta precursors were down-regulated in this study only in the resistant line. Chaperones are known as stress-related proteins that bind particularly to denatured proteins to prevent degradation and to assist in protein refolding of ATP (Rochester et al., 1986). In eubacteria and eukaryotic organelles, chaperonin 60 is presumably involved in numerous enzyme-folding functions (Lorimer, 1996). In plant chloroplasts, the level of chapronin 60, being involved in assembly of RuBisCO holoenzyme, is normally coordinated with that of RuBisCO (Avni et al., 1989). However Holland et al. (1998) reported that the accumulation of chapronin 60 was detected in *N. tabacum* seedlings by salt, cold, or prolonged darkness while the RuBisCO large subunit was decreased. This negative correlation suggests the possible role of chapronin 60 in stress responses. In the present study, a slight reduction of the putative chaperonin 60 beta precursor was detected with induction of RuBisCO proteins in the response of LSBR-5 to *R. solani*. This result may be explained by

the deduction ratio (quantity in inoculated LSBR-5/ quantity in non-inoculated LSBR-5) was less than two-fold, so this small amount of deduction of chaperonin 60 beta precursor may not have influenced total chaperonin 60 activities.

#### **2.4.3.15 Lumenal Binding Protein (BIP)**

Lumenal binding proteins (BIP) were down-regulated in this study only in the resistant line. The lumenal binding protein (BIP), an endoplasmic reticulum (ER) – resident chaperone, is known to be induced in the defense response by being involved in the synthesis of defense related proteins which are synthesized by the rough ER (Edith et al., 1999). In tobacco, BIP was induced rapidly in response to attack by *Erwinia carotovora*, reaching a maximum accumulation of BIP transcripts after just two hours of incubation, whereas  $\beta$ -1-3 glucanase, which was the most rapidly expressed PR protein, reached a maximum accumulation eight hours after incubation (Vidal et al., 1997; Jelitto-Van Dooren et al., 1999). Shen et al., (2003) reported that calreticulin in the endoplasmic reticulum, a  $\text{Ca}^{2+}$  binding protein that has molecular chaperone function, was down-regulated in the rice leaf sheath by wounding stress. It is not known how the down-regulation of ER chaperones is related to the defense response in rice. However, the result of down-regulation of BIP in the response of LSBR-5 may not correspond to an early defense response.

#### **2.4.4 Proteome Analysis of the Rice Response to *R. solani* – a Summary**

Based on results from this study, the rice response to *R. solani* may be described where energy required to induce a defense was supplied by proteins involved in energy metabolism (GAPDH, RuBisCO, ATP synthase), and the physiological status of infected rice was shifted to a defense mode via induction of proteolytic enzyme(s). This was accomplished by induction of antioxidant proteins (GST, APX, 20S proteasome) and accompanied by a

slight reduction in H<sub>2</sub>O<sub>2</sub>- inducing proteins that protected plant cells from oxidative stress caused by *R. solani* infection. The antifungal proteins were used directly to attack and degrade fungal cell walls.

As mentioned previously in this discussion, the highly abundant proteins identified in this study were biased in the pH 4-7 range. Thus, the proteins identified in this study represent a useful starting point for future research, but the results reflect only a portion of the total rice defense strategy. To monitor the complete rice leaf sheath proteome, non gel-based high resolution proteomic approaches, such as MudPIT, and pre-fractionation of protein samples for increasing resolution of the 2-DE approach should be used in future analysis of rice-*R. solani* interactions.

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### CHAPTER 3: SUMMERY AND CONCLUSIONS

The 2-DE based proteomic research was conducted to detect and identify proteins in rice that are associated with defense responses to *R. solani*, causal agent of sheath blight disease. In this study, the objectives were to: (1) establish an optimum infection method under greenhouse conditions (2) establish a robust protein extraction method for the rice leaf sheath, (3) establish optimum 2-DE running conditions for analysis of the rice leaf sheath proteome and (4) conduct comparison analysis of protein expression patterns in the resistant mutant line LSBR-5 and the susceptible parental line Labelle after infection with *R. solani*.

After 24 hr incubation in greenhouse humidified chamber with ~100 percent relative humidity, lesions occurred in the rice leaf sheath inoculated with immature sclerotia. By staining the fungal lesion with Trypan Blue, growth of mycelia from the surface of the sclerotia was detected. As Singh et al (2002) reported, this inoculation method was simple, rapid, and reproducible under the greenhouse conditions. Moreover, the home-made plastic chamber was able to maintain adequate humidity for rapid fungal growth.

For whole protein extraction related to Objective 2, ground rice leaf sheaths were suspended in acetone containing 10% TCA and 0.07 DTT and then lyophilized. Proteins were extracted from the dried powder with buffer containing 7M urea, 2M thiourea, 4% CHAPS, 0.5 % ampholytes, and 0.7 % DTT. The majority of extracted proteins were detected in the pH 4-7 range in the 2-DE gel. With this extraction buffer which contains 0.2 % more DTT than that suggested by Koller et al. (2002), horizontal streaks on the 2DE-gel were reduced in our study that resulted in improved 2-DE gel image analysis.

Proteins were separated in the 2-DE gel with first dimensional electrophoresis was conducted in the 17 cm, pH 4-7 IPG strip and second dimensional electrophoresis (LDS-

PAGE) was conducted in the 12 % linear poly acylamide gel. In the Sypro-Ruby stained gel, ~ 1000 protein spots were detected, showing reproducibility for all 24 gels used in the comparison analysis. Even though the total resolved proteins in the 2-DE gel do not cover the whole proteome of rice leaf sheath, our established 2-DE method produced the highest resolution in the recently published rice leaf-sheath proteomic research.

In the comparison analysis of protein expression patterns, 36 proteins showed significant quantitative variation in response of the resistant line LSBR-5 to *R. solani*. Among 36 protein spots, 22 protein spots displayed significant variation in the response of the susceptible line Labelle as well. A total of 26 out of the 36 detected proteins spots were identified through the MS/MS mass spectrometry. The presumed functions of the identified proteins included antifungal activity, energy metabolism, photosynthesis, protein degradation, signal transduction, molecular chaperone, and antioxidation.

With the 2-DE proteomic approach, we detected different protein expression patterns between the resistant mutant and the susceptible parental variety. Regardless of the resistance level, some defense related proteins were increased in both lines, whereas additional defense related proteins were increased only in LSBR-5 in a quantitative manner. Based on results of this study, the resistance of LSBR-5 is likely due to higher expression levels of defense related proteins than in Labelle in a quantitative manner that rescues cells from stress caused by the *R. solani* infection.

Using a proteomic approach based on the 2-DE coupled with MS/MS mass spectrometry, this is the first study to monitor protein expression patterns in the rice response to *R. solani*. In this study, detected proteins involved in various metabolic pathways were likely involved in the rice defense system against *R. solani*. In spite of the detection of various

defense related proteins, we could not detect the key regulatory protein(s) which control the expression of the detected defense related proteins in the resistant mutant line. The key regulatory proteins are possibly a low abundant, basic, hydrophobic, or membrane-bound protein that could not be separated in our 2-DE gel system or expressed at the sampling of 24 hours post inoculation. In future studies for detecting key regulatory protein(s), it is clear from this study that new protein separating methods which ideally enable detection of low abundant proteins, new protein extraction methods for various types of proteins, and various time points for harvesting need to be implemented

## VITA

Joohyun Lee was born at Incheon of Korea (ROK) on December 10<sup>th</sup> 1972. He attended Song-Doh High School at Incheon from 1988 to 1991. He entered Seoul National University in 1991 to start his bachelor's degree in the agronomy department. He served in the Korean Air Force from 1993 to 1994. During his military service, he received an award for outstanding soldier in the tournament of Antiaircraft Shooting. He returned to the university to receive his bachelor's degree on February 1997. During his entire bachelor's study, he received a scholarship for his outstanding GPA. Under the direction of Dr. Koh, he received his master's degree in rice breeding from Seoul National University. He learned various breeding techniques from conventional methods to molecular methods by participating in several research projects including genetic analysis of epistasis among rice endosperm mutant genes, developing RILs (Recombinant inbred lines) for QTL analysis, genotyping with DNA markers, rice anther culture. From 2001 to 2005, he studied his doctoral degree under direction of Dr. Oard at Louisiana State University, Department of Agronomy and Environmental Sciences. He optimized the two dimensional electrophoresis (2-DE) method for rice leaf-sheath proteomics and conducted rice proteomic research with the 2-DE method to detect and identify defense related proteins in rice. He has presented one oral presentation in the 2004 Rice Technical Working Group conference and has two poster presentations during the Fourth International Rice Genetics Symposium in 2002, and the Plant and Animal Genome Conference in 2005.