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ORIGINAL ARTICLE

# Proteomics Analysis of Rice Proteins Up-regulated in Response to Bacterial Leaf Streak Disease

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**Abstract** Bacterial leaf streak (BLS), caused by the pathogen *Xanthomonas campestris* pv. *Oryzicola*, is a major rice disease in tropical and subtropical regions of Asia. Rice proteins responsive to BLS are still not well characterized. We took a proteomics approach to identify the proteins that are up-regulated in rice leaves after infection. Approximately 1,500 protein spots were detected on each 2-D gel after silver-staining; those with increased protein levels were selected for MALDI-TOF-MS analysis. We identified 32 up-regulated proteins that might be involved in disease resistance signal transduction, pathogenesis, and regulation of cell metabolism. By using publicly available microarray data, we determined the mRNA transcripts of 23 proteins expressed in the leaves. Seven genes were analyzed by northern blots, which demonstrated that transcript levels were increased after bacterial infection. Our findings help elucidate the molecular mechanisms underlying BLS and provide a solid foundation for further research on the functions of relevant genes.

**Keywords** Proteomics, Rice bacterial leaf streak, Up-regulated proteins

## Introduction

Rice bacterial leaf streak (BLS), caused by the pathogen *Xanthomonas campestris* pv. *Oryzicola*, has become a serious threat to rice production in tropical and subtropical regions of Asia. Yield losses from BLS are normally 10 to 20% but can reach 40 to 60% in severe cases (Chen et al. 2007). Breeding disease-resistant rice appears to be the most economic and efficient means of control. However, despite many studies of this disease and its associated pathogen, resistant cultivars have not yet been obtained. Thus, it is important to identify rice genes that are responsive to this infection, and further characterize their functions in pathogenesis. Molecular markers are useful tools for breeding disease-resistant rice and reducing losses in yield.

Rice resistance to BLS is thought to be a typical quantitative trait (Tang et al. 2000; Chen et al. 2006). Although some progress has been made in genetics studies toward achieving resistant plants, no major quantitative trait loci (QTLs) have been fine-mapped and cloned (Tang et al. 2000; Chen et al. 2004; Zheng et al. 2005; Chen et al. 2006). Although there is a lack of well-characterized genetic markers, nevertheless some proteins in rice cultivars Jiafuzhan and Minghui 63 have been shown to be associated with bacterial infection (Huang et al. 2006; Chen et al. 2007). However, the molecular mechanisms underlying rice BLS are still poorly understood.

We followed a proteomics approach in systematically identifying the up-regulated proteins in rice '9311' after infections with BLS. This cultivar is widely grown because of its favorable agronomic traits, such as high grain yield and quality. Moreover, its genome has already been sequenced (Yu et al. 2002). Our study objective was to obtain data through proteomics analysis that could be used to improve our understanding of the pathogenesis of BLS and to reveal the molecular pathways involved in the disease response. Such proteomics efforts might also provide an important bridge between genetics and genomics studies of BLS.

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## Materials and Methods

### Material Preparation

Seeds of rice (*Oryza sativa*) cultivar 9311 were kindly provided by Professor Mengliang Cao (China National Hybrid Rice Research and Development Center). A strong pathogenic strain (89773-1-1) of *Xanthomonas campestris* pv. *Oryzicola* from southern China was isolated at the Fujian Agriculture and Forestry University College of Plant Protection. After the seeds germinated, plants were grown in pots containing field soil. When the sixth leaf of the main shoot was completely extended, we used the pricking inoculation method (Tang et al. 2000; Huang et al. 2006) to infect the fourth and fifth leaves with the bacterium, at a concentration of $9\times10^8$ cfu $mL^{-1}$. For comparison, control plants were mock-inoculated with sterile water only. At 48 h post-inoculation, the first four leaves from the top were collected from the treated shoots. They were either stored in liquid nitrogen or immediately used for protein extraction as described by Damerval et al. (1986) and Li et al. (2010). The total protein concentrations in samples were determined with a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc city, country---Rockford, USA).

### 2-Dimensional Gel Electrophoresis and Spot Selection

Two-dimensional gel electrophoresis (2-DE) was performed as described by Li et al. (2010). The first dimension -- isoelectric focusing (IEF) -- was carried out with 3.5% polyacrylamide gels in glass tubes (20 cm long, 1 mm diam.). For each pairing of infected and control samples, equal amounts of total proteins (100-150 μg) were loaded onto the gel. The second dimension, SDS-PAGE, was performed with 12% polyacrylamide gels at 15°C. Equipment for 2-DE was obtained from the Beijing WoDeLife Sciences Instrument Company.

Silver-staining (Mortz et al. 2001) was used to visualize the protein spots in the SDS-PAGE gels, which were scanned with a UMAX POWERLOOK scanner in transparency mode. The images were then analyzed with ImageMaster 2D Platinum software. Parameters were optimized for detecting the spots (saliency 8, smooth 5, and minimum area 50). To compensate for variations in staining between gels, spot intensities were normalized relative to total intensities. Each pairing of infected and control images was compared to identify the proteins that were up-regulated in response to BLS infection. Three biological replicates were analyzed, and proteins that were consistently up-regulated were selected. Those chosen spots were excised manually from the gels for protein identification. For spots with strong silver-staining, we collected proteins from one or two gels. For spots that were weak after staining, proteins were collected from three gels for each spot. If we could not determine the protein identity of a selected spot, we ran additional gels.

### MALDI-TOF-MS Analysis

Methods for gel de-staining, in-gel digestion of protein spots, and peptide extraction have been described previously (Shevchenko et al. 1996; Gharahdaghi et al. 1999). For MALDI-TOF-MS, 1 μL of protein sample was mixed with 1 μL of matrix solution and analyzed over the mass range of 800 to 3500 Daltons with a Bruker III ReFlexTM Mass Spectrometer (Bruker, Germany). The spectrometer was calibrated with external standard proteins. Flexanalysis 2.0 software was used to analyze the mass spectra, which were internally calibrated with known trypsin autocleavage peptide masses.

### Database Search and Protein Identification

Peptide mass fingerprints (PMFs) that were revealed by MALDI-TOF-MS were analyzed for protein identification with MASCOT 2.2 software (http://www.matrixscience.com). This database search was based on the non-redundant protein set from NCBI (NCBInr)/MSDB/SwissPort. The following parameters were used: *Oryza sativa* as taxonomy, one missed cleavage, 0.2-Dalton peptide mass tolerance, monoisotopic peptide masses, oxidation of methionine and carbamidomethylation of cysteine as variables, and fixed modification allowed. The criteria for a positive match included at least three independent peptides matched, with coverage of those matched peptides being at least 10% of the full-length protein. In addition, we considered the biochemical attributes of the proteins, including molecular weight and isoelectric point, for matching purposes.

### Protein Domain Analysis

To annotate the functions of these up-regulated rice proteins, we obtained their amino acid sequences from NCBI Entrez (http://www.ncbi.nlm.nih.gov/) or UniProtKB (http://www.pir.uniprot.org/). The sequences were used to search for the conserved protein domains documented in the Pfam database (http://pfam.sanger.ac.uk/). Significant domain matches (E-value <0.00001) were utilized for functional annotation.

### Microarray Data Collection and Processing

To examine the transcriptional expression patterns of the identified proteins, we obtained 343 microarray profiles of rice from NCBI's Gene Expression Omnibus (GEO, available at http://www.ncbi.nlm.nih.gov/geo/). These patterns had

been generated in 25 microarray studies using the Affymetrix rice genome array (documentation available at http://www.affymetrix.com/support/technical/byproduct.affx?product=rice). Here, we selected rice microarray profiles with both hybridization intensity values and detection calls ('Present', 'Marginal', or 'Absent') that are publicly available at the GEO database. The profiles were sorted into six sample types, including root (72 profiles), leaf (41), shoot (143), whole seedling (24), flower (39), and seed (24). To combine these profiles into a single dataset, we divided the intensity values of each by the array median, and then transformed the results by a base-10 logarithm. Thus, each profile in the integrated microarray dataset had a median expression value of zero. Array probes corresponding to the up-regulated rice proteins were identified by applying the annotation file from Affymetrix. Gene expression patterns were visualized with TM4 MeV software (http://www.tm4.org/mev/).

### Northern Blot Analysis

Total RNA was extracted from leaves with TRIZOL reagent (Invitrogen). RNA concentrations were measured by UV spectrophotometry. For northern blot analysis, equal amounts of total RNA (10 μg) were used for each sample, and the procedure followed the protocol of Sambrook et al. (1989). The hybridization probes were amplified by PCR from cDNA templates. Reverse transcription was performed with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences). The following primer pairs (forward and reverse) were used for the amplifications: 5'-GCATGGGGAAATCCACCTT-3' and 5'-CCAAATGTCGTCCAACACAAG-3' for protein Spot 1; 5'-GTTTGGGAAGGTCTACGATGG-3' and 5'-CCAATAGGACAACACCAAAGC-3' for Spot 2; 5'-CAGGAGGATTTGGAAAGAGTGTA-3' and 5'-GCGAAGACATCGGTGAGGG-3' for Spot 5; 5'-TGGAGAAGGTGGGTTTGGG-3' and 5'-CGCAGTATGATAATGCCAAATG-3' for Spot 11; 5'-TGGTGCATTTGGAACTGTATAT-3' and 5'-CAAAGCTGTAGACATCCGACT-3' for Spot 13; 5'-GGGGGTTTCGGTAAAGTGTA-3' and 5'-AAGAAGCATTATGCCATAACCA-3' for Spot 14, and 5'-ATGGCTCCGGTCAGCATCTC-3' and 5'-TTAAGCATACTCGGTAGGGTGAGC-3' for Spot 38. The amplified gene fragments were sequenced for validation. Hybridization probes were labeled with $^{32}P$ using a Random Primer DNA Labeling Kit (Takara Bio, Inc).

## Results and Discussion

### Identification of Up-regulated Proteins in Response to Bacterial Leaf Streak

Typical leaves from pathogen- and mock-inoculated leaves

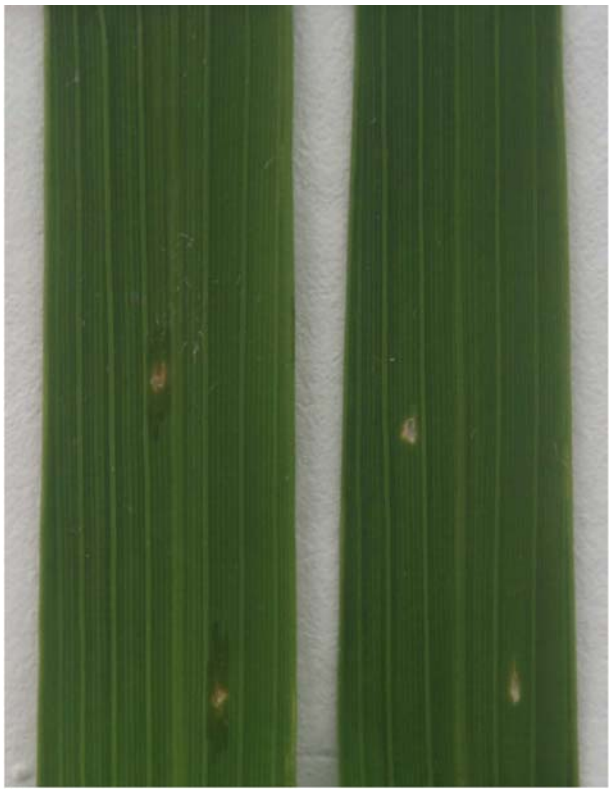

**Fig. 1.** Typical leaves at 2 d after inoculation with either sterile water only (right) or *Xanthomonas campestris* pv. *Oryzicola* (left).

are shown in Fig. 1. To understand the molecular mechanisms of the rice response to BLS disease, we used a proteomics approach for identifying the proteins up-regulated in leaves after infection. High-resolution maps were obtained for both infected and control tissues. Approximately 1,500 protein spots were detected on each gel. Based on their spot intensities, we selected 39 proteins that were consistently up-regulated in three biological replicates. These spots were excised manually from the gels, and analyzed with mass spectrometry.

In all, 32 proteins showed an increase in abundance of 1.5-fold or greater in the infected leaves when compared with the controls. Fig. 2 provides a representative map of leaves at 2 d post-inoculation, with up-regulated proteins indicated by spot numbers. Table 1 shows their functional annotations, sequence accessions, identification results, and relative levels in the inoculated leaves. Most of these up-regulated proteins may play important roles in the disease response, including disease resistance signal transduction, pathogenesis, and cell metabolism. [We note here that, because we consider down-regulated proteins to be as important as those that are up-regulated, we plan to study them separately.]

### Proteins Related to Disease Resistance Signal Transduction

Three of the up-regulated proteins (Spots 1, 4, and 11) may

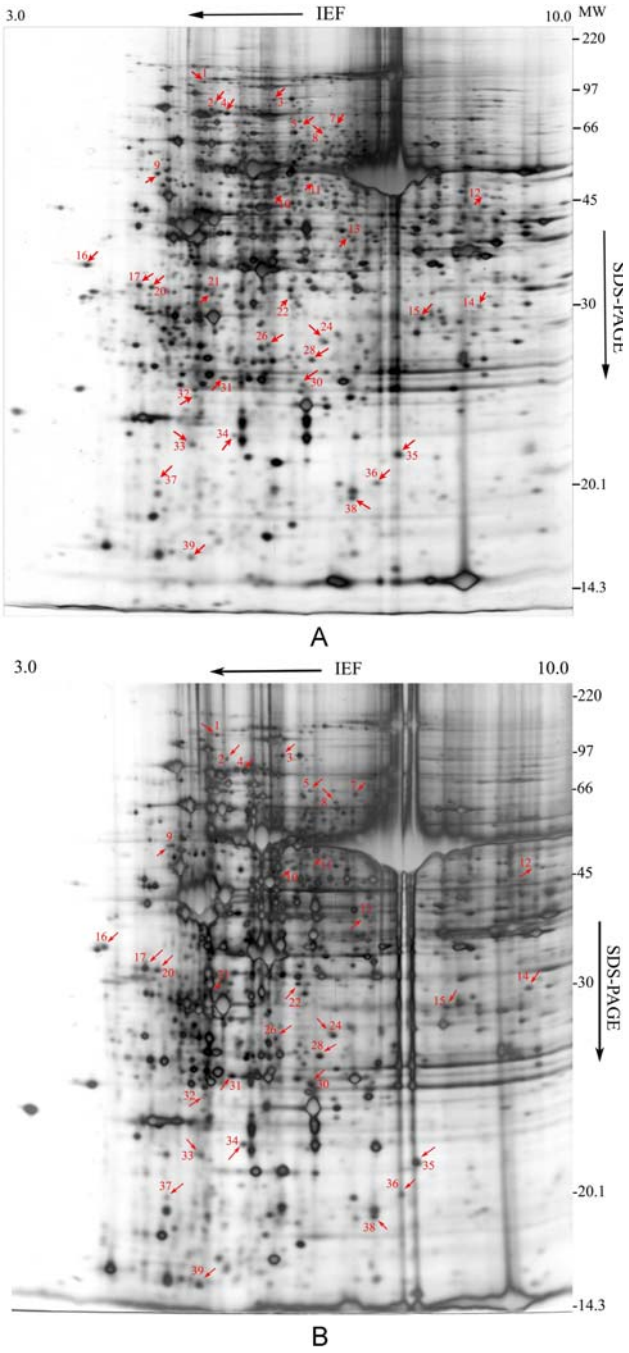


**Fig. 2.** Representative maps of proteins from leaves at 2 d after inoculation with either sterile water only (A) or *Xanthomonas campestris* pv. *Oryzicola* (B). Up-regulated proteins are indicated with spot numbers.

function as disease resistance (R) genes. Spot 1 was identified as a putative R protein that contains a nucleotide binding site (NBS) and C-terminal leucine-rich repeat (LRR) domains. Plant NBS-LRR proteins are involved in pathogen detection (DeYoung and Innes 2006). Spots 4 and 11 are putative serine/threonine protein kinases that have kinase and U-box domains. These two may function as STK-type R genes (McDowell and Woffenden 2003). The recognition of pathogen avirulence gene products by plant R proteins is important for activating the defense signal transduction pathway. Although R genes often mediate qualitative resistance, they can also serve as quantitative resistance loci (Wisser et al. 2005). Resistance to BLS in rice is possibly a typical quantitative trait. For example, Tang et al. (2000) have detected 11 QTLs underlying resistance to BLS on six chromosomes (1, 2, 3, 4, 5, and 11) in rice. A QTL on the short arm of Chromosome 5, *qBlsr5a*, has been mapped more precisely (Han et al., 2008), while Chen et al. (2006) have identified a new major QTL on the short arm of Chromosome 11. Among the three putative R protein identified here (Spots 1, 4, and 11), two (4 and 11) were found on Chromosome 10 while Spot 1 was found on the short arm of Chromosome 5. We must still determine whether these three are involved in quantitative resistance.

Five proteins on Spots 2, 5, 13, 14, and 24 may function as receptor-like kinases (RLKs), which are key components of signal transduction pathways in plants. Many studies have shown that RLKs can be involved in stress responses and defense-related processes (Song et al. 1995; Thomas et al. 1998; Jung et al. 2004; Lee et al. 2004; Wang et al. 2004; Sasabe et al. 2007). Here, Spot 3 was identified as a mitogen-activated protein kinase. These MAPKs are involved in responses to biotic and abiotic stresses (Jiang and Song 2005). We also noted that Spot 10 carries an aspartic proteinase that is possibly involved in programmed cell death and disease resistance signal transduction (Mutlu and Gal 1999; Xia et al. 2004).

## Defense-related Proteins

Two pathogenesis-related (PR) proteins -- Spot 31 (PR1-like) and Spot 38 (PR10) – were up-regulated in our inoculated leaves. These two may be involved in the plant defense against BLS infection. Overexpression of PR proteins can enhance disease resistance to a variety of pathogens (Xiong and Yang 2003). Spot 9 is an allene oxide synthase that contains the cytochrome P450 domain. This enzyme is involved in the biosynthesis of jasmonic acid, which has been implicated as a signaling molecule in plant defenses. Spot 17 is probably an ascorbate peroxidase that detoxifies peroxides, e.g., hydrogen peroxide, using ascorbate as a substrate. Ascorbate peroxidases may be important for controlling the $H_2O_2$ concentration during intracellular signaling under stress conditions or pathogen attack (Shigeoka et al. 2002). Spot 26 is a patatin-like protein. Patatins, a group of storage glycoproteins with lipid acyl hydrolase activity, can be stimulated by drought stress (Matos et al. 2000), and may also be involved in plant defenses against parasites (Strickland et al. 1995).

**Table 1.** Up-regulated rice proteins identified after searching NCBInr/MSDB/SwissPort databases with peptide mass fingerprints

| Spot no. | Protein description | Sequence accession/ gene locus | Theoretical MW/pI | Peptides matched/ unmatched | Sequence coverage (%) | Probability -based Mowse scores | Relative protein level±SE[a] | *P*-value[b] |
|---|---|---|---|---|---|---|---|---|
| 1 | Putative disease resistance protein | Q75IT4_ORYSJ/ LOC_Os05g30220.1 | 139069/6.97 | 10/29 | 10 | 38 | 1.45±0.08 | 0.013 |
| 2 | Putative receptor-like kinase | gi\|52353491/ LOC_Os05g44970.1 | 104567/6.25 | 9/29 | 14 | 59 | 1.98±0.11 | 0.006 |
| 3 | Mitogen-activated protein kinase 6 | MPK6_ORYSJ/ LOC_Os10g38950.1 | 43088/5.96 | 6/49 | 23 | 40 | 1.70±0.24 | 0.050 |
| 4 | Putative protein kinase, expressed | Q336U7_ORYSJ/ LOC_Os10g40060.1 | 73877/6.26 | 8/37 | 16 | 56 | 1.85±0.34 | 0.064 |
| 5 | Putative receptor-type protein kinase LRK1 | Q6ZFS6_ORYSJ/ LOC_Os08g03150.1 | 74938/6.37 | 5/36 | 14 | 45 | 1.63±0.17 | 0.033 |
| 7 | Putative DegP2 protease | gi\|51038169/ LOC_Os05g05480.1 | 65771/5.73 | 7/34 | 15 | 42 | 1.75±0.12 | 0.012 |
| 8 | Hypothetical protein | gi\|115461911/ LOC_Os05g04160.1 | 72166/8.37 | 8/38 | 21 | 48 | 1.48±0.05 | 0.005 |
| 9 | Allene oxide synthase 3 | C74A3_ORYSJ/ LOC_Os02g12680.1 | 54518/6.52 | 3/15 | 11 | 25 | 2.27±0.50 | 0.062 |
| 10 | Aspartic proteinase | ASPRX_ORYSJ/ LOC_Os05g04630.1 | 54910/5.78 | 5/23 | 11 | 38 | 1.83±0.30 | 0.056 |
| 11 | Putative receptor kinase | Q94LU6_ORYSJ/ LOC_Os10g40100.1 | 98054/5.90 | 7/27 | 11 | 35 | 1.57±0.16 | 0.035 |
| 12 | Ribulose bisphosphate carboxylase large chain | RBL_ORYSA/ LOC_Os10g21268.1 | 53418/6.22 | 6/16 | 10 | 54 | 1.71±0.18 | 0.028 |
| 13 | Receptor-like kinase | Q9LL55_ORYSA/ LOC_Os01g02400.1 | 74669/8.38 | 7/30 | 12 | 56 | 1.74±0.13 | 0.015 |
| 14 | Putative receptor-like kinase | Q75I96_ORYSJ/ LOC_Os03g49620.2 | 67285/5.36 | 7/27 | 18 | 53 | 1.55±0.07 | 0.009 |
| 15 | Putative Rpp14/Pop5 family protein | gi\|38343977/ LOC_Os04g22870.1 | 17536/9.04 | 8/33 | 49 | 81 | 2.24±0.85 | 0.141 |
| 16 | Hypothetical protein | gi\|115459660/ LOC_Os04g45490.1 | 42108/5.47 | 9/41 | 34 | 66 | 2.57±0.65 | 0.068 |
| 17 | Probable L-ascorbate peroxidase 3 | APX3_ORYSJ/ LOC_Os04g14680.1 | 32198/8.25 | 4/18 | 15 | 31 | 1.67±0.13 | 0.018 |
| 20 | Putative transcription elongation factor | gi\|14589372/ LOC_Os10g10260.1 | 31552/9.81 | 4/20 | 16 | 39 | 1.71±0.16 | 0.024 |
| 21 | ATP synthase subunit beta | Q75HQ5_ORYSJ/ LOC_Os05g35320.1 | 43111/5.66 | 6/11 | 18 | 62 | 3.36±0.50 | 0.021 |
| 22 | Putative short-chain dehydrogenase reductase | gi\|115459604/ LOC_Os04g44980.1 | 33367/5.56 | 4/15 | 15 | 31 | 1.47±0.17 | 0.053 |
| 24 | Receptor protein kinase-related protein-like | gi\|34394257/ LOC_Os07g26180.1 | 60581/7.19 | 4/16 | 13 | 35 | 1.55±0.16 | 0.039 |
| 26 | Patatin-like phospholipase | gi\|222625063/ LOC_Os03g27610.1 | 53638/9.37 | 6/28 | 18 | 51 | 1.66±0.25 | 0.061 |
| 28 | Hypothetical protein | Q5VP13_ORYSJ/ LOC_Os01g28510.1 | 26657/6.82 | 4/31 | 15 | 34 | 1.45±0.03 | 0.003 |
| 30 | Putative cyclophilin | gi\|115469870/ LOC_Os06g49470.1 | 22268/9.12 | 3/25 | 22 | 28 | 1.82±0.12 | 0.010 |
| 31 | PR1-like protein | Q6Z1E1_ORYSJ/ LOC_Os06g37999.1 | 24933/6.73 | 3/14 | 18 | 36 | 1.44±0.03 | 0.003 |
| 32 | Hypothetical protein | gi\|116317774/ LOC_Os08g13500.1 | 67797/7.76 | 7/27 | 17 | 56 | 2.43±0.30 | 0.020 |
| 33 | Ribonuclease P-related protein | gi\|115457596/ LOC_Os04g22870.1 | 19951/8.72 | 5/32 | 27 | 48 | 2.11±0.09 | 0.003 |
| 34 | Hypothetical protein | gi\|115470893/ LOC_Os03g58230.1 | 24365/8.86 | 3/21 | 16 | 37 | 2.33±0.09 | 0.002 |

**Table 1.** Cotninued

| Spot no. | Protein description | Sequence accession/ gene locus | Theoretical MW/pI | Peptides matched/ unmatched | Sequence coverage (%) | Probability -based Mowse scores | Relative protein level±SE[a] | *P*-value[b] |
|---|---|---|---|---|---|---|---|---|
| 35 | Hypothetical protein | gi\|125597246/ LOC_Os06g28700.1 | 17876/11.30 | 4/29 | 40 | 49 | 2.08±0.15 | 0.010 |
| 36 | GTP-binding protein Rab 6 | Q8H4Q9_ORYSJ/ LOC_Os07g31370.1 | 23045/7.68 | 3/31 | 20 | 38 | 1.96±0.10 | 0.005 |
| 37 | Putative alternative oxidase | gi\|222612438/ LOC_Os02g47200.1 | 15261/5.08 | 4/42 | 45 | 61 | 2.51±0.74 | 0.089 |
| 38 | Pathogenesis-related protein 10 | Q75T45_ORYSJ/ LOC_Os12g36830.1 | 17004/4.88 | 5/44 | 29 | 44 | 1.65±0.19 | 0.039 |
| 39 | Hypothetical protein | gi\|125544481/ LOC_Os03g33520.1 | 16062/9.04 | 4/36 | 33 | 37 | 2.36±0.16 | 0.007 |

[a]Relative protein level was calculated as the ratio of spot intensities between pathogen-inoculated leaves and mock controls. Means and standard error (SE) from three biological replicates are shown.
[b]*P*-value, computed with a one-sample t-test and SAS 8.0 software, represents the probability of the mean relative protein level being ≤ 1, given the observed measurements.

### Proteins Involved in Cell Metabolism

Ten of the up-regulated proteins (Spots 7, 12, 15, 20, 21, 22, 30, 33, 36, and 37) may be involved in cell metabolism. Spot 7 is a putative DegP2 protease that may be related to the repair mechanism of Photosystem II. The level of DegP2 is increased in response to a high concentration of NaCl, desiccation, or illumination with high intensity light (Haussühl et al. 2001). Spot 12 was identified as part of the large chain of ribulose bisphosphate carboxylase (RuBisCO). Many proteins corresponding to the RuBisCO large and small chains are significantly up-regulated by salt stress in rice (Kim et al. 2005) or maize (Zörb et al. 2004). Under such conditions, oxygenase activity is enhanced and the carboxylase activity of RuBisCO is suppressed (Sivakumar et al. 2000). The accumulation of RuBisCO also increases photorespiration (Martino et al, 2000; Kim et al, 2005).

Spots 15 and 33 contain the Rpp14/Pop5 family domain and, thus, may be active in tRNA processing. Further identifications showed that Spot 20 is a putative transcription elongation factor, Spot 21 is part of the ATP synthase beta chain, Spot 22 is probably a short-chain dehydrogenase/ reductase SDR family protein, and Spot 30 is a putative cyclophilin with a conserved peptidyl-prolyl cis-trans isomerase domain. Cyclophilins facilitate protein-folding by catalyzing the cis-trans isomerization of the peptide bonds that precede proline residues (Wang and Heitman 2005).

Spot 36 was identified as the GTP-binding protein Rab 6, which is involved in retrograde Golgi transport (Bischoff et al. 1999). Spot 37 is probably an alternative oxidase that transfers electrons from reduced ubiquinol to oxygen, forming water in the mitochondria. Under cold stress, the transcript levels of alternative oxidase genes are increased in rice (Ito et al. 1997). Most of the proteins mentioned above are already known to function as enzymes in the metabolism of nucleic acids, proteins, or carbohydrates. However, we must still investigate how these metabolic pathways are affected by BLS.

### Proteins with Unknown Functions

We concluded that the remaining seven spots (8, 16, 28, 32, 34, 35, and 39) are hypothetical proteins with unknown functions. Although their involvement in the disease response is unclear, some of them contain conserved domains or motifs. For example, Spot 8 has seven pentatricopeptide repeat (PPR) motifs. This 35-amino acid motif, with no known function, occurs as multiple tandem copies in members of the PPR gene family that are greatly expanded in plants (Small and Peeters 2000). Another hypothetical protein, Spot 16, shows sequence similarity with the bacterial translational elongation factor EF-G. Spot 32 contains a sequence region resembling the transposase 28 domain, while Spot 39 has an Exo70 domain. In yeast, that Exo70 protein comprises one subunit of the exocyst complex that is involved in the late stages of exocytosis (TerBush et al. 1996). Finally, Spots 28, 34, and 35 are hypothetical proteins with no conserved domains.

### Transcriptional Expression Patterns of Identified Proteins

Although our proteomics analysis identified 32 proteins with increased levels of expression after bacterial inoculation, their sequence coverage and probability-based Mowse scores were relatively low to ensure proper protein identification (Table 1). To provide additional evidence, we investigated their expression patterns, which included detecting mRNA transcripts and then monitoring their changes in levels after

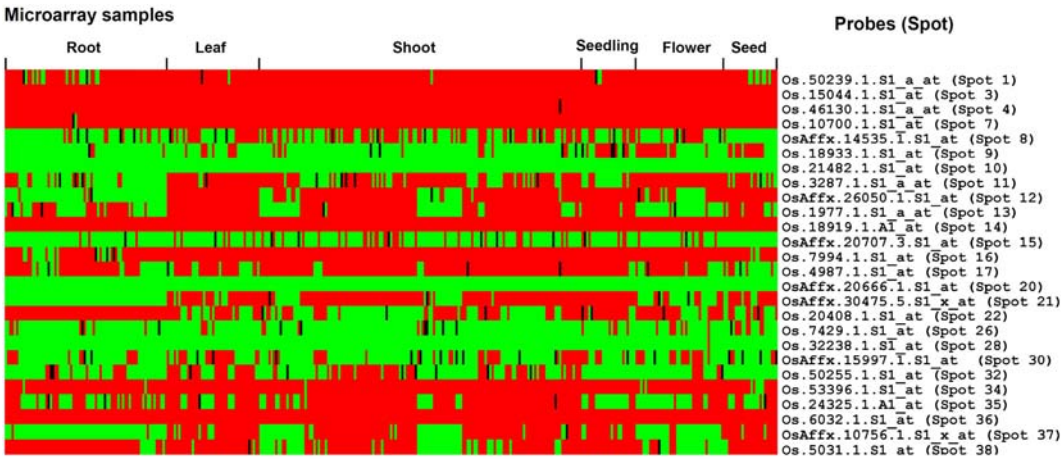


**Fig. 3.** Transcriptional expression patterns of identified proteins based on microarray data. Heat map shows probe detection calls, with 'Present' in red, 'Marginal' in black, and 'Absent' in green.

inoculation.

We used 343 microarray expression profiles compiled from the NCBI GEO database to determine whether the identified genes are expressed in rice leaves. Fig. 3 shows the expression patterns of 26 genes with probes in the Affymetrix rice genome array. The other six genes were not represented by any probes. The heat map generated here was based on probe-detection calls -- Present, Marginal, or Absent. Values for hybridization intensity of those probes are shown in Supplemental Fig. 1. Our results suggest that 23 of these genes are apparently expressed in leaves as well as other tissues. RuBisCO (Spot 12) was highly expressed in leaves but not in roots; the same pattern was found with Spots 21 and 37. The mRNA transcripts for Spots 10, 20, and 28 were not detected by microarray probes in any tissue type.

To examine the changes in mRNA levels at 0, 12, and 48 h post-inoculation, we selected seven genes, including two putative R proteins (Spots 1 and 11), four RLKs (2, 5, 13, and 14) and the PR protein 10 (38), for northern blot analyses (Fig. 4). In particular, expression for Spots 2 and 5 was not demonstrated by those microarray data. Consistent with those data, however, mRNA transcripts of Spots 1, 11, 13, 14, and 38 were detected in the mock-inoculated leaves. Transcript was also found for Spot 2 but not immediately for Spot 5. However, mRNA for the latter was expressed in rice leaves at 12 and 48 h post-inoculation. More importantly, the mRNA transcripts of five genes (Spots 1, 2, 5, 13, and 38) were significantly increased after infection whereas those of Spots 11 and 14 were increased only slightly. Therefore, our northern blotting results were consistent with the proteomics data, suggesting that these identified genes are truly responsive to BLS.

In conclusion, among the 32 proteins identified here, 10 are believed to function in disease resistance signal transduction;

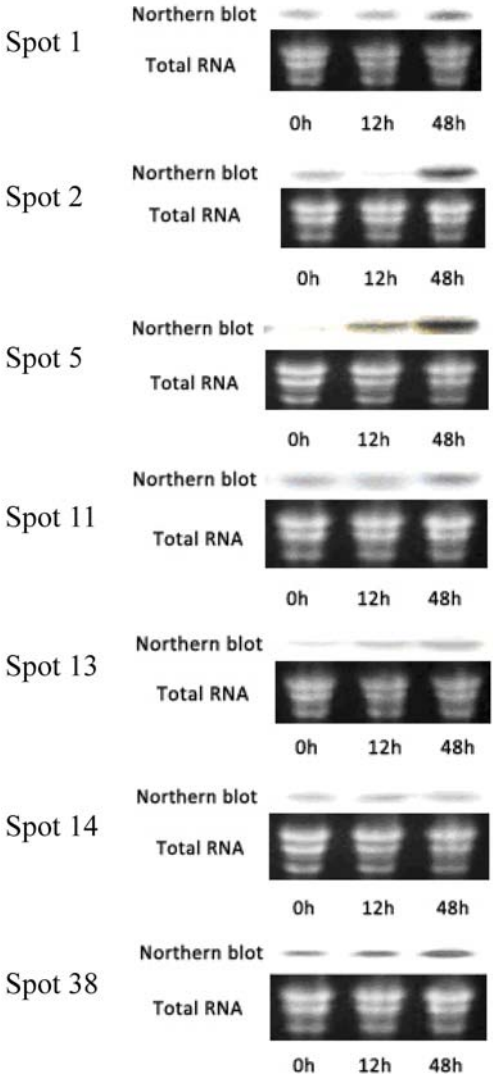


**Fig. 4.** Northern blots to examine changes in mRNA levels in leaves after infection. Seven selected genes were analyzed at 0, 12, and 48 h post-inoculation.

five are related to pathogen defense; 10 appear to be involved in cell metabolism; and seven have unknown functions. The mRNA transcripts of 25 identified proteins were expressed in rice leaves and most were increased after infection occurred. This demonstrates that multiple rice genes are up-regulated in the disease response. Further characterization of them may provide valuable information for elucidating the molecular mechanisms of BLS and enhancing current efforts to breed disease-resistant rice cultivars.

## Acknowledgments

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