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Functional Identification of Genes Encoding Effector Proteins in *Magnaporthe oryzae*

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1. Introduction

In the course of coevolution of plants and pathogens for many millions of years, plants possessed many kinds of recognition and resistance mechanisms to prevent or limit pathogen infection. At the same time, pathogen also initiated many pathogenicity mechanisms, such as development of specialized infection structures, secretion of hydrolytic enzymes, production of host selective toxins, and detoxification of plant antimicrobial compounds (Idnurm and Howlett, 2001; Talbot, 2003; Randall et al., 2005), to avoid or overcome plant resistance mechanism. However, filamentous pathogen including fungi and oomycete could secrete a diverse array of effector proteins into the plant cell to manipulate the plant innate immunity, which facilitates the pathogen to successfully colonize and reproduce (Birch et al., 2006; Chisholm et al., 2006; Kamoun, 2006; O'Connell and Panstruga, 2006; Catanzariti et al., 2007; Kamoun, 2007). Several studies have shown that effector proteins could play dual role as both toxins and inducers of host resistance. Effector proteins were regarded as functioning primarily in virulence, but they also could elicit innate immunity in plant varieties carrying corresponding resistance protein.

Rice blast caused by *Magnaporthe oryzae* (Couch and Kohn, 2002) is the most devastating fungal disease of rice (*Oryza sativa*; Zeigler et al., 1994; Talbot, 2003). Functional identification of *M. oryzae* effectors can elucidate some pathogenicity mechanisms of the blast fungus, providing a clue to better manage blast disease. Several *Avr* genes have been cloned and characterized from *M.oryzae*, such as *Avr-Pita* (Orbach et al., 2000; Valent et al., 1991), *Avr1-CO39* (Farman and Leong, 1998), *Ace1* (Bohnert et al., 2004; Collemare et al., 2008) and the *Pwl* effectors (Kang et al.,1995; Sweigard et al., 1995). The *Avr-Pita* effector appears homologous to fungal zinc-dependent metalloproteases and is dispensable for virulence on rice (Jia et al., 2001; Orbach et al., 2000). *Avr-Pita* interacts with the cognate resistance protein *Pi-ta* (Jia et al., 2000). *Avr-Pita1* (*Avr-Pita*) including *Avr-Pita2* and *Avr-Pita3* are *Avr-Pita* family (Khang et al., 2008). *Avr-Pita2* acts as an elicitor of defense responses mediated by *Pi-t*a, while *Avr-Pita3* does not. Members of *Avr-P*ita family are detected among blast isolates isolated from different kinds of hosts by PCR-based method.

Ace1 effector is a putative cytoplasmic fusion polypeptide containing a polyketide synthase (PKS) and a nonribosomal peptide synthetase (NRPS), two distinct classes of enzymes that are involved in the production of microbial secondary metabolites (Bohnert et al., 2004;

Collemare et al., 2008). *Ace1* is thought to function as avirulence indirectly by producing a secondary metabolite that activates Pi33. *Ace1* is only expressed in appressoria, suggesting that the secondary metabolite produced might have a role in virulence (Bohnert et al., 2004; Fudal et al., 2005).The *Pwl* effectors, encoded by the *Pwl* (pathogenicity on weeping lovegrass, *Eragrostis curvula*) gene family, are rapidly evolving, and they are small glycine-rich secreted proteins that commonly distributed in *M.oryzae*. Presently, four genes such as *Pwl1-Pwl4* have been determined in *M. oryzae*, and the four genes confer species-specific avirulence on weeping lovegrass and finger millet, but not on rice (Kang et al., 1995; Sweigard et al., 1995). Yoshida *et al*. (2009) examined DNA polymorphisms of 1032 putative secreted proteins from the genome sequence of isolate 70-15 among 46 isolates, and found no association with Avr function on a set of differential rice cultivars carrying different *R* genes, indicating that the isolate 70-15 might have lost several functional *Avr* genes through sexual recombination.

After fungal effector proteins are secreted into plant cells, the question arises: do they mediate virulence or avirulence on host? To discover pathogenicity mechanism of the pathogen, it is indispensable to identify the function of effector proteins. Here we will introduce our studies on functional identification of effector proteins from *M. oryzae*.

2. Screening candidate effector-encoding genes from *M. oryzae*

Whole-genome sequence of fungal pathogens has provided an enormous amount of data that can be analyzed for mining putative secreted effector proteins. *M. oryzae* genome sequence has been available online, which contribute many novel effector-encoding genes. Some online softwares could be aided to predict some features such as secretion, domain and homology of effector proteins. Presently, secreted proteins are categorized into two classes based on their secreted pathway, one is classically secreted proteins, with N-terminal signal peptide, and the other is non-classically secreted proteins, whose secreted pathway is known as leaderless secretion (Nickel, 2003). Combination of SignalP v3.0, TargetP v1.01, big-PI predictor and TMHMM v2.0 (http://www.cbs.dtu.dk/ services/) are used to predict classically secreted effector proteins. Non-classical secreted proteins were further predicted using SecretomeP 2.0 Server (http://www.cbs.dtu.dk/services/).

Total of 12,595 putative proteins including 1,486 small proteins from *M. oryzae* genome database were predicted. Of which, 1,134 putative proteins were predicted for classically secreted proteins with N-terminal signal peptide. Here, we will focus on small secreted proteins (amino acid length <100), there were 119 classically secreted proteins among 1,486 small proteins. Among 119 effector proteins, 116 effectors had a Sec-type signal peptides, and had common A-X-A motif, X stand for any amino acid residue, C-domain of the signal peptide could be cleaved by one of the various type I SPase of *B.Subtilis* (Tjalsma et al., 1997; 1998; 1999). In C-domain, uncharged residues were present at the -1 and -3 positions, high frequency of leucine (29%) was at -2 position. Frequency of alanine at -1 position was 71%, and the other 19 amino acid residues occurred at +1 position except cysteine (C).Most of secretory proteins with this signal peptide are secreted into the extracellular environment. Length of signal peptides of 116 secretory proteins centralized in 16~22 amino acid residues, signal peptides with 18 amino acid residues reached the highest amounts, the second was signal peptide with 19 amino acid residues. Signal peptide with the most length was composed of 36 amino acid residues, and the shortest signal peptide was composed of 15 amino acid residues (Figure 1).

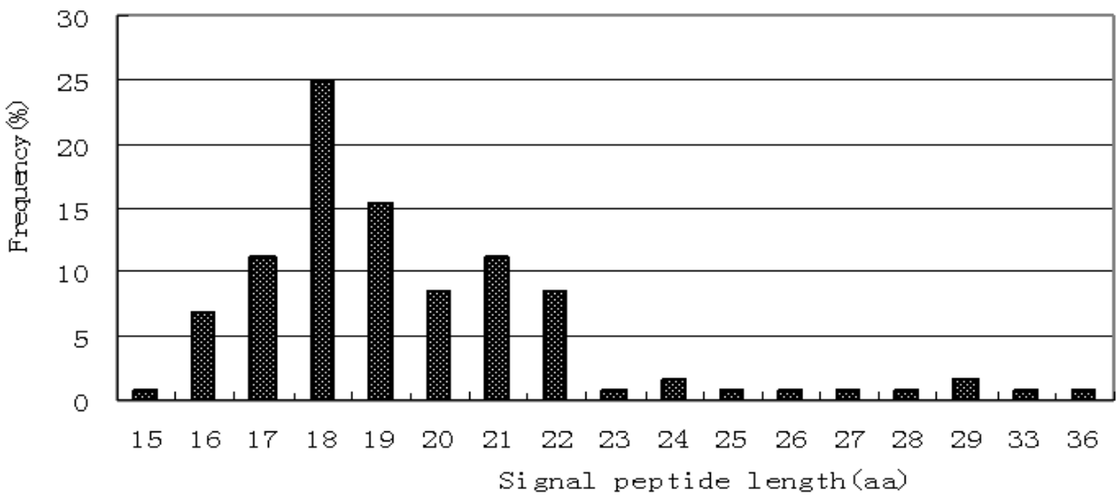


Fig. 1. Length distribution of predicted secretory (Sec-type) signal peptide of short protein in *M. oryzae*.

Because signal peptide has common application value in exogenous gene expression, it was necessary to analyze their composition and structure. We analyzed amino acid composition of 116 Sec-type signal peptides sequence. Frequency of 20 kinds of amino acids in 116 signal peptides sequences of secreted proteins were analyzed (Figure 2). Result showed that nonpolar amino acids such as alanine, leucine, proline and valine have the highest frequency(45.79%), followed by negatively charged acidic amino acids including aspartic acid, glutamic acid, phenylalanine, histidine, isoleucine, threonine, methionine, tryptophan and tyrosine (33.49%). The frequency of polar amino acids (glycine, asparagines, glutarnine, serine) positively charged basic amino acids such as arginine and lysine had the lowest frequency (15.83%).

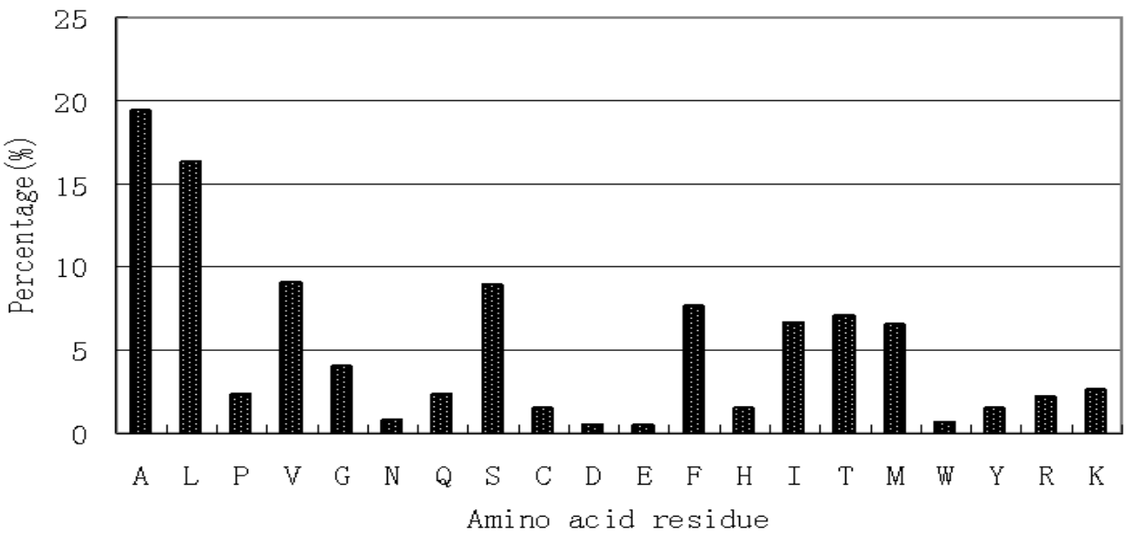


Fig. 2. Frequency of amino acid in predicted signal-peptide-containing short proteins.

Subcellular location of secreted effector proteins can provide important information to explain plant pathogen interactions. SubLoc v1.0 was used to predict subcellular location of 116 secreted effector proteins. Result showed that 50 proteins were secreted extracellularly, 30 proteins were transported into nucleus, 25 effectors were transferred into mitochondria and 11 were translocated into cytoplasm.

3. Polymorphism of effector-encoding genes in blast isolates from Yunnan, China

To analyze polymorphism distribution of effector-encoding genes in blast isolates from Yunnan, 45 ones from 116 genes were selected as candidates for analyzing polymorphism in 21 isolates from Yunnan. The result showed that each gene appeared in different distribution among 21 isolates, For example, MGS0001.1 was presented in 16 isolates, but not in five isolates. MGS0011.1 was distributed in 21 isolates. Although MGS0351.1 was distributed in 21 isolates with the PCR product size ranging from 350 to 400 base pairs, the reference sequence size of the gene was 412 bp. To explain sequence difference between PCR product and reference sequence of gene, PCR products of MGS0001.1 and MGS0351.1 from three different isolates were cloned and sequenced, respectively, the sequence analysis showed that PCR products sequences of MGS0001.1 from three different isolates appeared high identical with the reference sequence. While PCR product sequences of MGS0351.1 from three different isolates showed fragment- deletion of GTTGTTTTGTTGTT and GTTGTT, comparing with reference sequence, but the deletion occurred in intron region of the gene.

There was three-type polymorphism distribution of 45 genes in 21 blast isolates. The type I included 18 ones among 45 genes, which distributed in 21 isolates, the type II consisted only of MGS0351.1 which was present in 21 isolates, but PCR products showed fragment deletion comparing to reference sequence. The type III consisted of 26 genes that were randomly present in 21 isolates, while not all genes were distributed in 21 isolates. Among 45 effector-encoding genes, MGS0123.1 had the lowest frequency of 52.4%. Many genes could be examined in each isolate, except in isolate 21. Nineteen genes were not determined in the isolate 21. More than 40 genes could be determined in other 44 isolates, and all the 45 genes distributed in isolate 7, 14 and 15 (Table 1).

The results indicated that 45 effector-encoding genes not only had the polymorphism distribution but also appeared conserved in 21 blast isolates. Some genes were not determined in isolates, the reason might be the result of gene evolution during plant-pathogen interaction. Thus, it is essential to analyze their function for conserved or varied genes.

4. Effector-encoding gene cloning and *in vitro* functional identification

We selected 10 effector genes as candidates to clone and functionally identify. The cloned nucleotide sequence of MgNIP04 from Y99-63c was aligned with the short protein, MGS0004previously sequenced.MgNIP04 was identical to MGS0004.MgNIP04 encoding a 96 amino acid protein with unknown domains or motifs. MgNIP04 contained a signal peptide of 20 amino acids at the N-terminus. Subcellular localization prediction suggested that it was a cytoplasmic protein. However, it is not homologous to Nep1-like proteins, a novel class of necrosis-inducing proteins found in a variety of taxonomically unrelated

microorganisms (Clare et al., 2004). The plasmids of pMALMgNIP04 and pMAL were induced to express of fusion proteins of MBP-MgNIP04 andMBP.

Gene code	Type	Twenty-one isolates																				Fre-quency (%)
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
MGS0011.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0253.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0255.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0274.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0338.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0662.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0703.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0718.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0992.1	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0997.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS1033.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS1035.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS1195.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS1242.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS1298.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS1344.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS1382.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS1473.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0351.1	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
MGS0001.1		+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+	-	76.2
MGS0004.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	95.2
MGS0074.1		+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	90.5
MGS0123.1		+	-	+	-	+	+	+	-	+	-	-	-	-	+	+	-	-	+	+	-	+	52.4
MGS0140.1		+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	90.5
MGS0149.1		+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	90.5
MGS0398.1		+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	95.2
MGS0415.1		+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	95.2
MGS0431.1		+	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	85.7
MGS0621.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	95.2
MGS0698.1		-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	85.7
MGS0879.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	95.2
MGS1011.1	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	85.7
MGS1041.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	95.2
MGS1070.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	90.5
MGS1078.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	95.2
MGS1117.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	76.2
MGS1172.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	90.5
MGS1276.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	90.5
MGS1322.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	95.2
MGS1361.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	95.2
MGS1392.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	90.5
MGS1439.1		+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	90.5
MGS1460.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	95.2
MGS1470.1		+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	90.5
MGS1477.1		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	95.2
Frequency (%)		97.7	97.7	93.3	93.3	95.6	95.6	100	93.3	95.6	93.3	97.7	95.6	95.6	100	100	97.7	95.6	95.6	97.7	82.2	57.8	-

"+" mean gene was examined in isolates, "-"mean gene was not examined in isolates.

Table 1. Frequency of 45 genes in 21 isolates of M. oryzae from Yunnan, China.

To determine if MgNIP04 could interact with rice proteins, *E. coli*-expressing MBP-MgNIP04 was inoculated on wounded rice leaves. MBP expressed from pMAL-c2X was used as a control. The concentration of expression products of MBP-MgNIP04 and MBP were determined following the procedure of Bradford (1976) using bovine serum albumin as a standard. The expressed products of pMAL-MgNIP04 and pMAL-c2X at a concentration of 2.0 μg/μl were inoculated on wounded rice seedling leaf tissues. Necrotic specks formed around the inoculation site of leaf tissues that were inoculated with fused MBP-Mg04, while no necrotic specks appeared when inoculated with MBP. The results demonstrated that the protein encoded by MgNIP04 could directly interact with rice proteins. We also performed other experiments such as MgNIP04 *in vitro* induced H_2O_2 production, induced callose deposition in rice leaves and roots which could be automatically transported in rice root cells, when rice suspension-cultured cells were treated by MBP-MgNIP04.

The vector of pCAMbia-MgNIP04 was transformed into blast isolate of Y98-16. The differences of conidiation, germination, appressorium and pathogenicity between wild type strain of Y98-16 and transformant harboring MgNIP04::GFP were identified. The result revealed =no obvious difference in conidiation, germination and appressorium between Y98-16 and the transformant.The pathogenicity was further assayed for Y98-16 and transformant, rice cultivar Lijiangxintuanheigu that was almost susceptible to all races of blast fungus was challenged with blast strains. The result showed the transformant caused less symptom on Lijiangxintuanheigu than Y98-16. These data indicated *MgNIP04* did not influence blast fungus pathogenicity qualitatively but quantitatively, and virulence level of blast fungus decreased along with increasing of copy number of *MgNIP04* (there was one more copy at least in transformant than in Y98-16 although the gene copy number was not analyzed).

In order to test infecting ability difference of Y98-16 and transformant to rice roots, we used Y98-16 and transformant to inoculate the roots of Lijiangxintuanheigu. Brown symptom appeared on rice roots when blast fungus infected rice roots for 7 days. Brown symptom on the roots caused by Y98-16 regardless of areas and amounts of brown lateral roots regardless of areas and amounts of brown lateral roots was more apparent than by transformant. To determine whether *MgNIP04* was expressed in mycelia colonizing the roots, the roots infected by transformant and Y98-16 were observed using laser scanning confocal microscopy, respectively. The result showed that MgNIP04::GFP was observed in mycelia colonized roots. Along with infecting time elongation, the colonizing mycelia gradually increased. The frozen slices from brown- and no brown-root tissue were observed using laser scanning confocal microscopy. The results showed that mycelia not only had infected the epidermal cell but also colonized along root cell intervals.

5. Expression pattern of effector protein-encoding genes from *M. oryzae*

Many studies have used quantitative polymerase chain reaction (PCR) to evaluate fungal growth during the infection process (Hu et al., 1993; Mahuku et al., 1995; Groppe and Boller, 1997; Judelson and Tooley, 2000).Therefore, we detected the expression pattern of candidate novel genes *MGNIP10*, *MGNIP18*, *MGNIP24*, *MGNIP34*, *MGNIP38*, *MGNIP53*, *MGNIP74*, *MGNIP97* and *MgNIP04* in different isolates from Yunnan, China, the same isolate grown under nitrogen-starvation medium and complete medium and different time points when Lijiangxintuanheigu was challenged with blast fungus using real-time fluorescence quantitative PCR.

All expression levels of candidate genes were normalized by *actin* housekeeping gene and quantified by both the comparative threshold method and standard curve method. The results showed that expression levels of all candidate genes were significantly different in isolates of 94-64-1b Y99-63, 95-23-4a, Y98-16 and 94-64-1b. When two isolates of Y98-16 and Y99-63 grown under complete medium and nitrogen-starvation medium, relative expression quantity of genes was different. Expression of more genes was detected when two isolates grew under nitrogen starvation for 24 h, than when the two isolates grew under complete medium (Figure 3).

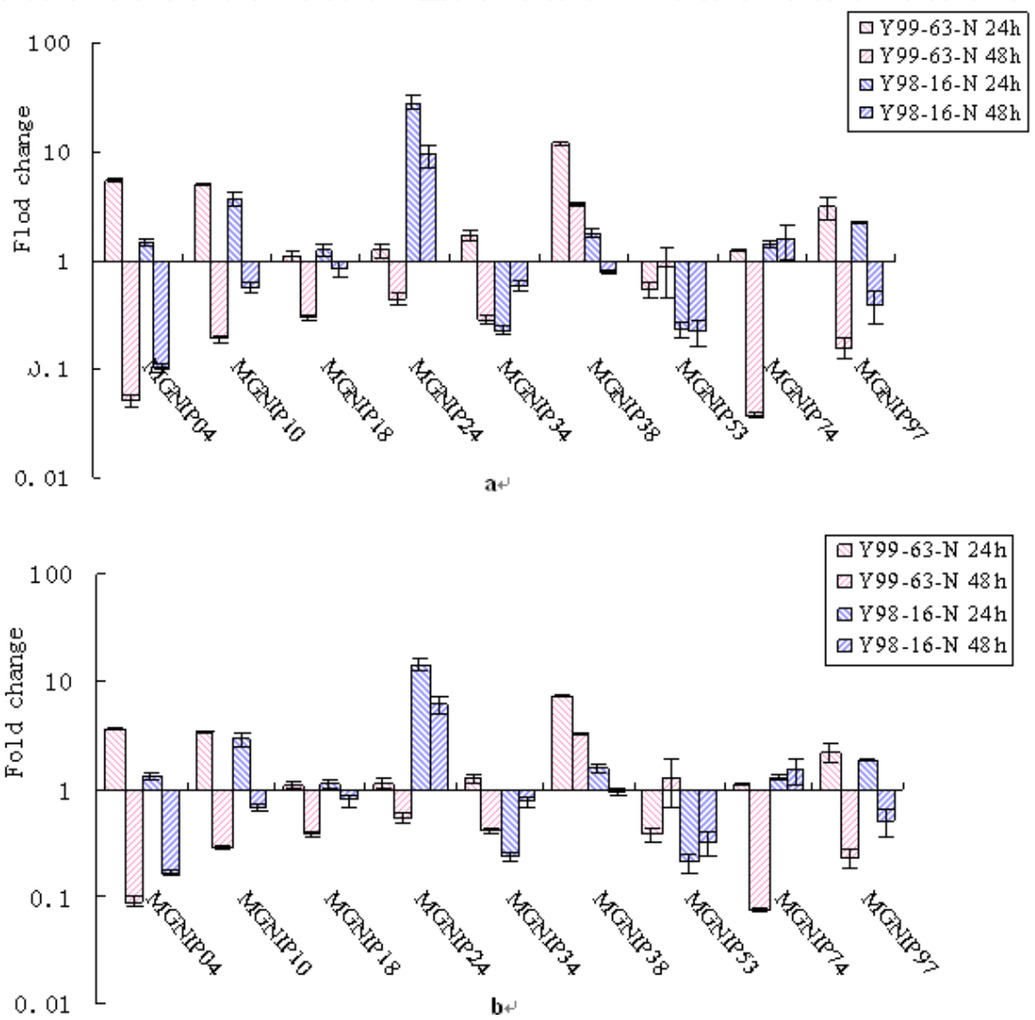
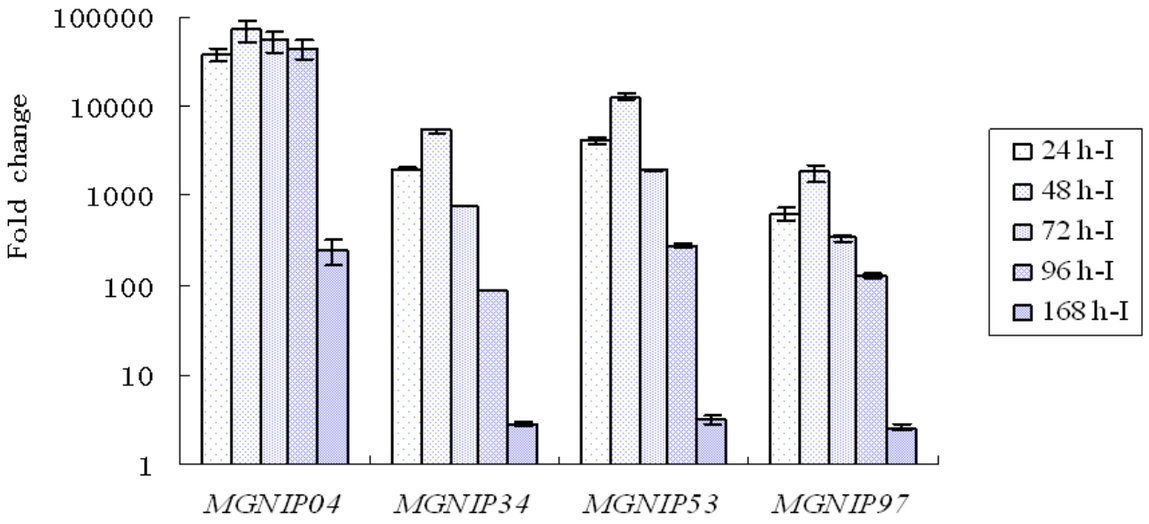
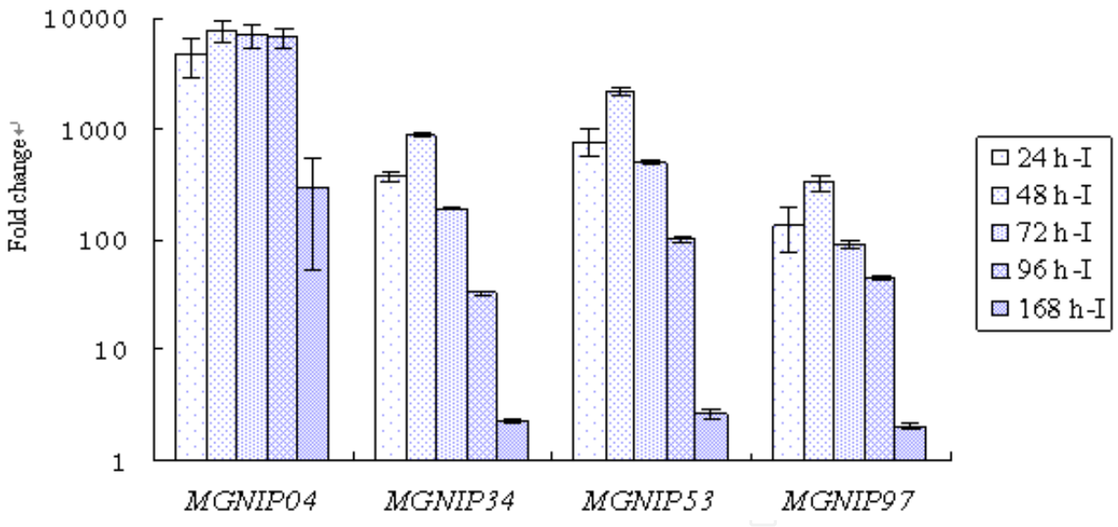


Fig. 3. Expression pattern of some predicted effector protein-encoding genes from *M.oryzae.*
a: Relative expression quantity of target gene of Y99-63 and Y98-16 cultured in different mediums using $2^{-\Delta\Delta Ct}$ method
b: Relative expression quantity of target gene of Y99-63 and Y98-16 cultured in different mediums using the standard curve method.

We detected expression level of all candidate genes at 24 hpi, 48 hpi, 72 hpi, 96 hpi and 168 hpi. Results revealed that all gene expression levels were apparently up-regulated, achieved the maximum at 48hpi, but decreased after 72 hpi (Table 2 and Figure 4).



a



b

Fig. 4. Expression pattern of some predicted effector protein-encoding genes in infected rice leaves.
a: Relative expression amount of target gene in infected rice leaves at different stages post inoculation using $2^{-\Delta\Delta Ct}$ method
b: Relative expression amount of target gene in infected rice leaves at different stages post inoculation using stand curve method.

Isolates Y99-63 / Targeted gene	Control	24 h-I		48 h-I		72 h-I		96 h-I		168 h-I	
		$2^{-\triangle\triangle Ct}$ Fold change	Standard curve Fold change	$2^{-\triangle\triangle Ct}$ Fold change	Standard curve Fold change	$2^{-\triangle\triangle Ct}$ Fold change	Standard curve Fold change	$2^{-\triangle\triangle Ct}$ Fold change	Standard curve Fold change	$2^{-\triangle\triangle Ct}$ Fold change	Standard curve Fold change
MGNIP04	1	37602± 6231	4800± 854	71139± 19038	7660± 1802	54388± 14248	7101± 1626	44878± 10524	6812± 1360	243± 76.8	291± 237
MGNIP10	1	4.88± 0.22	2.24± 0.08	8.92± 1.04	3.63± 0.37	0.92± 0.04	0.6± 0.02	0.29± 0.01	0.25± 0.00	0.1± 0.01	0.13± 0.02
MGNIP18	1	1799± 201	334± 28.1	2386± 743	414± 95.7	359± 36.3	95± 7.37	76± 4.36	29± 1.26	5± 0.48	3± 0.24
MGNIP24	1	236± 37.5	77± 15.49	287± 14.8	77± 3.26	68± 9.84	25± 2.95	19± 0.89	10± 0.36	1± 0.06	0.6± 0.04
MGNIP34	1	2012± 68.3	376± 11.7	5401± 297	879± 44.2	773± 8.74	192± 1.99	88± 1.51	32± 0.51	3± 0.11	2± 0.08
MGNIP38	1	1323± 121	263± 21.4	1959± 512	354± 79.7	282± 20.7	78± 4.98	100± 17.0	36± 5.35	8± 0.57	5± 0.35
MGNIP53	1	4117± 306	776± 56	12888± 1242	2198± 209	1931± 87.3	501± 22.5	277± 14.2	101± 5.10	3± 0.27	3± 0.22
MGNIP74	1	9± 2.00	5± 0.82	17± 5.22	8± 1.87	5± 0.39	3± 0.19	3± 0.17	2± 0.09	0.1± 0.01	0.1± 0.01
MGNIP97	1	634± 98.7	135± 18.5	1825± 385	325± 59.7	337± 34.3	89± 7.86	130± 9.00	45± 2.65	3± 0.19	2± 0.13

Table 2. Relative expression quantity of target gene in infected rice leaves at different stages post inoculation.

6. Rice defense-related gene expression pattern

Based on the results, we knew that *MgNIP04 in vitro* expression products could induce callose deposition of rice suspension cells, up-taken by rice root without presence of pathogen and quantitatively influence virulence level of blast fungus Y98-16. Are there differences of defense-related gene expression pattern between Y98-16 and transformant infecting Lijiangxintuanheigu, respectively? We used RT-PCR to analysis gene expression of selected defense-related genes when rice cultivar of Lijiangxintuanheigu was inoculated by Y98-16 and transformant, respectively. The genes of *APXa* (AY254495.1), *Chia4a* (AB096140.1), *CHS* (AB058397.1), *OsPAL* (AX16099.1), *OsPHGPX* (AJ270955.1), *OsPR1a* (AJ278436.1) and *PR-10a* (AF274850.1) were expressed from 24hpi to 168hpi when Lijiangxintuanheigu was challenged with Y98-16 and transformant, respectively, and rice β-actin gene (CT831215.1) was control. The expression of *OsGST2* (AJ486976.1), *PR-10c* (AF274852.1) and *Npr1* (DQ450949.1) was not detected at any time points regardless of Y98-16 or transformant inoculating Lijiangxintuanheigu. The expression of *PR-10b* was detected at all selected time points during transformant infecting rice, but it was not detected during Y98-16 infecting rice. The expression of the ethylene synthesase gene was only detected at 0, 24, 48 and 96hpi when Lijiangxintuanheigu was challenged with Y98-16, while the gene

expression was detected at all time points when transformant inoculating rice. The expression of *OsPR4* was only detected at 168hpi during transformant inoculating rice. Based on these data, the most tested defense-related genes expression pattern was not any different between Y98-16 and transformant of MgNIP04::GFP, which indicated *MgNIP04* quantitatively influenced pathogenicity of blast fungus.

7. Expression difference of effector-encoding genes from blast isolates with different virulence determined using two-dimensional gel electrophoresis

Fungi maintained their cell living and even growth through material reutilization when they were in nutrition-stress environment. Some research showed that expression quantity of pathogenicity-related genes increased when rice blast strains grew under nitrogen-starvation medium, which enhanced the pathogenicity of blast strains (Talbot et al., 1997).

The two isolates of Y99-63 and Y98-16 were from Yunnnan, China. Virulence test of two isolates of Y98-16 and Y99-63 on rice isogenic lines of IRBL1-24 had been previously performed in our lab, and virulence of Y99-63 was more intensive than Y98-16. To analyze the virulence of extracellularly secreted proteins on rice varieties such as susceptible variety of Lijiangxintuanheigu, resistant variety of Tetep and rice isogenic lines of IRBL1-24, we separated the extracellularly secreted proteins when Y98-16 and Y99-63 grew under nitrogen starvation for 48h, and the wounded rice leaves were inoculated with extracellularly secreted proteins. The result showed that necrosis speck occurred around the wounded leaves and wounded stems of rice when secreted proteins were inoculated on leaves or stems for 48h, and speck diameter of leaves or stems treated with secreted proteins was 2 to 4 fold larger than leaves or stems treated with sterilized water.

We compared difference of extracellularly secreted proteins from Y99-63 and Y98-16 growing under nitrogen-starvation medium for 48h using two-dimensional electrophoresis technology (Figure 5). The result showed that more proteins spots were detected from Y99-63 growing under nitrogen-starvation medium than Y98-16 (Table 3). And pI and molecular weight of secreted proteins had an apparent difference between Y99-63 and Y98-16 (Figure 6 and Figure 7).

Strain	Replicate group	Protein spots	Protein matched spots	Match Rate 1	Match Rate 2
*					
Y98-16	3	253±10	253±10	100%	100%
Y99-63	3	262±10	132±8	50.4%	52.2%

Note: * mean master reference gel; Match rate 1 for the match-point block of gel protein spots representing the ratio; Match rate 2 is the ratio of match point to total master.

Table 3. Comparison of 2-DE maps of two strains in *M.oryzae.*

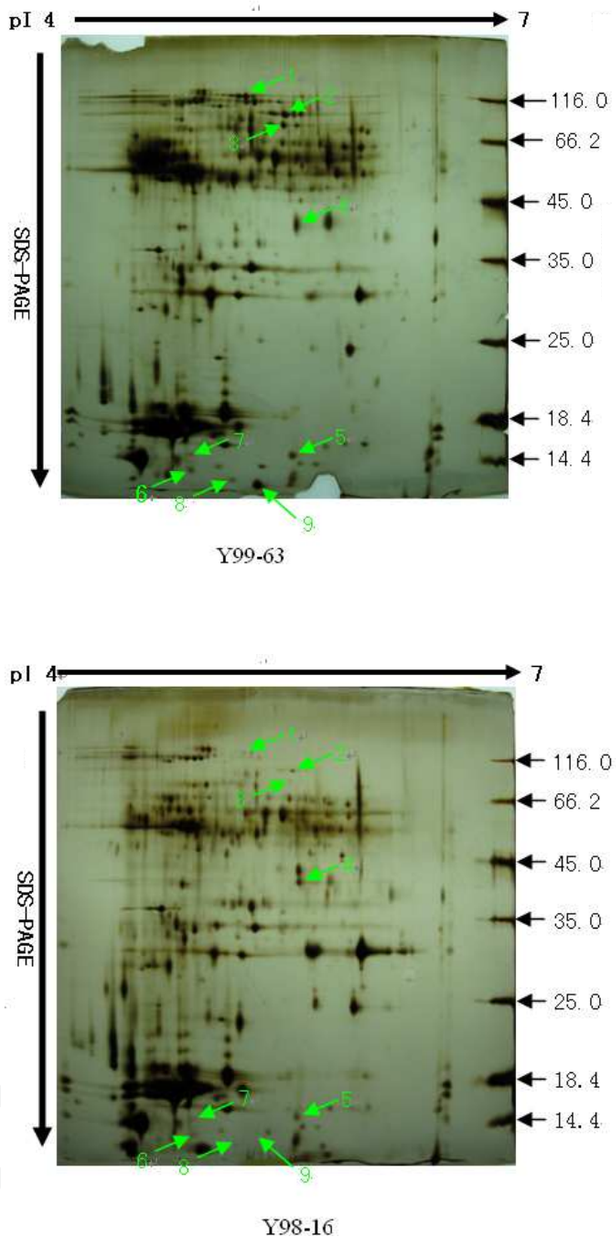


Fig. 5. 2-DE maps of Y99-63 and Y98-16.Protein spots labeled with arrow (1 to 5)are co-expressed in two strains of Y99-63 and Y98-16 with the pI ranging from 5.5 to 6.0, but the expression level of Y99-63 is over five times more than Y98-16. Protein spots indicated by arrow (6 to 9) are proteins which are specifically expressed in the strain Y99-63, with their MW ranging from 10 to 20 kDa and pI from 5.0 to 6.0. MW is indicated on the right side in KD. IEF is abbreviation for isoelectric focusing and SDS-PAGE is for SDS-polyacrylamide gel electrophoresis.

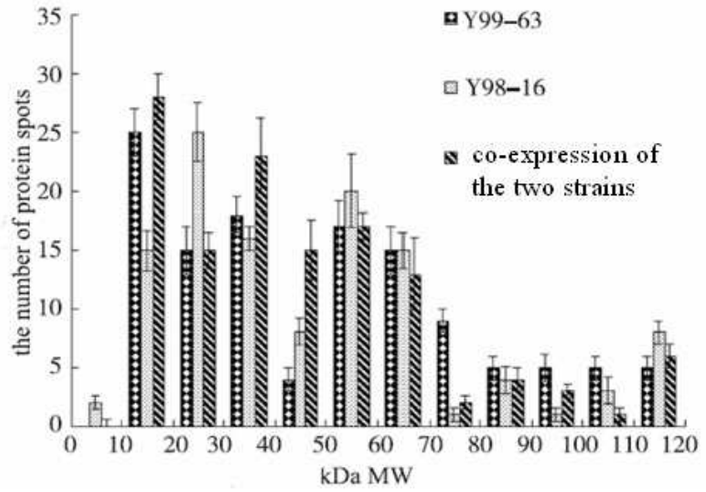


Fig. 6. Comparison of the distribution of the proteins spots based on molecular weight.

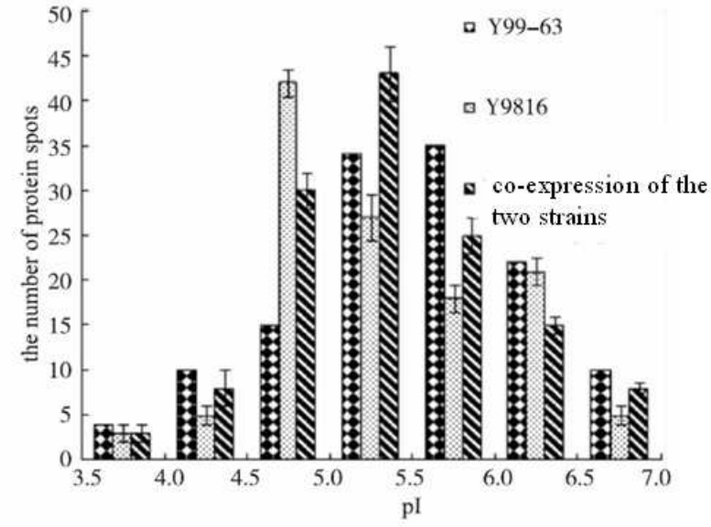


Fig. 7. Comparison of the distribution of the proteins spots based on pI.

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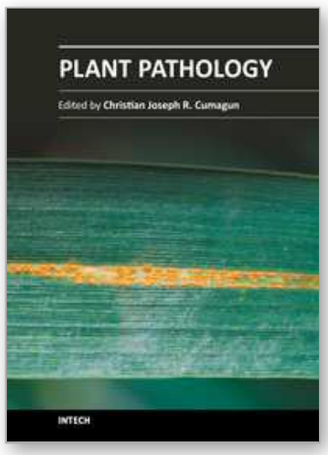

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