

Characterization of differentially expressed genes induced by virulent and avirulent *Magnaporthe grisea* strains in rice

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Abstract

To identify differentially expressed genes of rice induced by virulent and avirulent of pathogen *Magnaporthe grisea*, an *indica* rice Zhong156 with race-specific resistance to leaf blast, was used to establish two subtractive cDNA libraries. For this purpose a Suppression Subtractive Hybridization was used after inoculation with an avirulent strain ZC15 and a virulent strain ZB1 of *Magnaporthe grisea*. Then differential screening, sequencing, function annotation and RT-PCR were carried out and 25 cDNA clones showing differential expression (during 72 h of infection) between the avirulent and virulent strains were identified. The expression profile showed that (1) these differentially expressed genes were induced or suppressed after inoculation with the two strains, but the changing trends, degree and time of expression varied in different interactions. These differences were due to the different strains of the pathogen infection; (2) the genes induced or repressed by both strains were considered participating in the fundamental defense responses and (3) genes induced by the avirulent strain which repressed by the virulent strain (or opposite trends) might play a pivotal role in regulating the race-specific resistance.

Keywords: Race-specific resistance; leaf blast; defense genes; suppression subtractive hybridization (SSH); cDNAs libraries; *Oryza sativa* L.

Abbreviations: *M. grisea* *Magnaporthe grisea*; SSH_Suppression Subtractive Hybridization; RL-SAGE_Robust-Long Serial Analysis of Gene Expression.

Introduction

Rice blast, caused by the fungus *Magnaporthe grisea* (*M. oryzae*) is the main cause of yield loss in rice worldwide (Ou, 1985). The number of rice cultivars with durable resistance to *Magnaporthe grisea* is very limited. Besides, newly released varieties always undergo resistance breakdown shortly after cultivation (Ballin et al., 2008). The lack of durably resistant cultivars is due to the evolution of pathogens toward virulence as well as our limited knowledge of resistance mechanisms. Since 1922, rice blast field isolates were first distinguished in cultivar specificity by Sasaki (Yamada, 1985). Hundreds of pathogen races have been identified based on their infection spectra on different rice cultivars (Orbach et al., 2000). The gene-for-gene model was proposed to interpret the interaction between plants and their microbial pathogens (Flor, 1971). The avirulence (AVR) genes in the pathogen show a functional correspondence with particular R genes in rice (Zeigler et al., 1994). The rice blast system is a classical gene-for-gene system (Silué et al., 1992). Several fungal AVR genes as well as a variety of disease resistance genes (R genes) have been identified yet (Böhnerta et al., 2004; Deltail et al., 2010; Ashikawa et al., 2012). Preformed defense systems likely play a role in basal resistance in limiting the growth of a normally virulent pathogen (Deltail, et al., 2010; Deltail, et al., 2012). To date, different methods, such as suppression subtractive hybridization (SSH) and robust-long serial analysis of gene expression (RL-SAGE) have been utilized to isolate the

defense-related genes involving in the interaction between *M. grisea* and rice (Xiong et al., 2001; Lu et al., 2004; Hu et al., 2006; Gowda et al., 2007). Most of these works have, however, utilized different rice accessions with various resistance or susceptibility to the same pathogen race at seedling growth stages only. Few studies have been made to examine the interactions with different pathogen races on the same germplasm.

Our earlier studies suggested that the high yielding rice cultivar Zhong 156 carries blast resistance gene *pi-24(t)* and displays race-specific resistance to *M. grisea* (Zhuang et al., 2002). However, no attempt has been taken yet to investigate resistance gene expression in this cultivar. We employed the SSH method to generate subtracted libraries from Zhong 156 after infection with avirulent and virulent strains of *M. grisea*. In addition, we detected the expression patterns of selected genes after inoculation with these two strains, managing to reveal the molecular basis of the race-specific resistance.

Results and Discussion

Construction and differential screening of SSH cDNA libraries

Both forward and reverse subtractive cDNAs libraries were made for isolating differential expressed genes involved in

Table 1. The results of BLAST search and annotation of cDNA Clones of rice selected with differential expressions after infection by avirulent and virulent strains of *M. grisea*.

Clone	Annotation	e-value	identities	ZB1 ^a	ZC15 ^a	accn ^b
Cell rescue, defense and virulence						
<i>FA05</i>	contains similarity to aromatic rich glycoprotein	4e-14	97%	+	+	JK999629
<i>FB06</i>	Putative TOC159	2e-12	71%	-	NC	JK999634
<i>FE03</i>	Metallothionein-like protein type-1	2e-07	95%	+	+	JK999640
Transcription						
<i>FA11</i>	RRM-containing RNA-binding protein-like	4e-17	46%	+	+	JK999632
<i>FD01</i>	Putative G-box binding factor	5e-08	93%	-	-	JK999638
<i>RG03</i>	RNA polymerase beta	1e-32	67%	+	-	JK999648
Protein synthesis/protein fate						
<i>FB10</i>	Putative poly (A)-binding protein	9e-31	79%	+	+	JK999635
<i>FD05</i>	Ubiquitin carrier protein	7e-45	100%	+	+	JK999639
<i>RE06</i>	60S ribosomal protein	6e-24	100%	-	+	JK999641
<i>RG11</i>	Putative Bowman-Birk serine protease inhibitor	1e-30	98%N	-	-	JK999651
Metabolism/energy						
<i>FA02</i>	Putative chlorophyll a/b-binding protein precursor	1e-59	100%	-	-	JK999627
<i>FC06</i>	Fructose-bisphosphate aldolase-1	9e-76	89%	-	NC	JK999636
<i>FC09</i>	Glycine dehydrogenase P protein	1e-64	79%	-	NC	JK999637
<i>RF02</i>	Putative cytidine deaminase	1e-14	100%	+	+	JK999642
Signal transduction mechanism						
<i>FA10</i>	Putative cryptochrome 2	2e-94	94%N	+	+	JK999631
<i>RF11</i>	Hypothetical protein OmiCp102	7e-35	100%	NC	-	JK999646
Cellular transport/biogenesis of cellular components						
<i>FA03</i>	Putative glycine-rich cell wall structural protein precursor	4e-04	58%	+	+	JK999628
<i>FB03</i>	Ferredoxin I	3e-19	100%	-	-	JK999633
Transposable elements, viral and plasmid proteins						
<i>RF09</i>	Putative retrotransposon protein	4e-07	43%	+	-	JK999645
<i>RG07</i>	Putative retrotransposon protein	3e-16	93%	+	-	JK999649
<i>RG10</i>	Putative transposon protein	2e-10	86%	+	+	JK999650
Unkown						
<i>FA06</i>	P0684C02.5	3e-45	98%	+	+	JK999629
<i>RF05</i>	P0455H0310.30	2e-07	83%	+	-	JK999643
<i>RF08</i>	Putative fimbriata	2e-15	100%	-	NC	JK999644
<i>RG02</i>	Unknown protein	6e-19	62%	+	+	JK999647

Note: The clones beginning with an “F” were selected from the forward subtractive library), with an “R” were selected from the reverse subtractive library. “a” the expressions of 25 genes in zhong 156 after inoculation with ZB1 and ZC15 strains based on RT-PCR: “+” means induced, “-” means suppressed, “NC” means no change. “b” the genebank accession number.

race-specific resistance to rice leaf blast. Although SSH revealed the efficient and prompt cloning of differentially expressed transcripts, these sequential procedures cannot be totally free of false positive clones due to the PCR. Screening by Northern analysis is labor-intensive and reverse Northern analysis is not sensitive enough to detect low-abundant transcripts reliably. To overcome these major drawbacks, we used the cDNA microarray technique adopted as a form of reverse Northern blot analysis to eliminate false positive clones and to increase selection efficiency (Yang et al., 1999).

We randomly picked 1152 clones from two libraries for differential screening by reverse Northern blot analysis. Comparing the hybridization signals between probes from unsubtracted tester and driver cDNAs, and between probes from subtracted tester and driver cDNAs, clones showing obviously similar differences of hybridization signal between the unsubtracted pair and subtracted pair were selected (Fig 1). Then all of 64 cDNA clones with an obvious differential hybridization signal were chosen for sequencing.

BLAST search of selected clones

RT-PCR analysis was used to further confirm the cDNA clones selected from the subtractive libraries. Expression difference

was observed in 25 out of 64 clones between the incompatible and compatible reaction in plant after inoculation with avirulent and virulent strains. The result of RT-PCR was consistent with that of differential screening except for 3 clones, *FA06*, *FE03* and *FD05*, which exhibited the different changing trends. Sequences were submitted to the NCBI database for sequence alignment and functional annotation and their functional categorizations of the cDNAs were performed by aligning translated amino acid sequences of top-hit homology with the Munich Information Center for Protein Sequences (MIPS) database (Frishman et al., 2001). All of 25 unique genes were identified.

Based on the BLAST search results, all the sequences showed high protein homology in rice (Table1). The putative functions of 25 clones from the SSH library were categorized eight groups including cell rescue/defense and virulence, transcription, protein synthesis/protein fate, metabolism/energy, signal transduction mechanism, cellular transport/biogenesis of cellular components, transposable elements/viral and plasmid proteins and unknown.

The 8 functional categories determined were similar to data obtained by Lu (Lu et al., 2004). Several previously reported defense-related genes encode for Metallothionein (Ma et al., 2003), Ubiquitin- conjugating enzyme (Wu et al., 2003; Catala

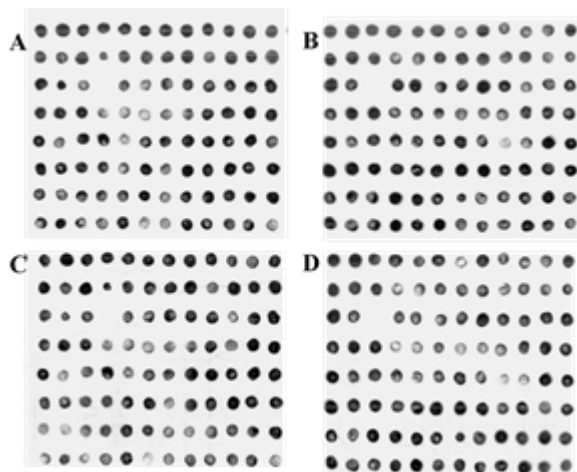


Fig 1. The representative example of reverse Northern blot analysis. The inserts of cDNA clone from the forward library were amplified by PCR and equal amount of PCR products were spotted onto nylon membranes. Four duplicate membranes were then hybridized with un-subtracted tester probes-cDNA of Zhong156 after inoculation with an avirulent strain ZC15 (A) and un-subtracted driver probes-cDNA of Zhong156 after inoculation with a virulent strain ZB1 (B); subtracted tester probes-PCR products of SSH PCR using cDNA of Zhong156 after inoculation with an avirulent strain ZC15 as the template (C) and subtracted driver probes-PCR products of SSH PCR product taking the cDNA template of Zhong156 after inoculation with a virulent strain ZB1 as the template (D), respectively.

et al., 2007; Conti et al., 2008), Bowman-Birk serine protease inhibitor (Qu et al., 2003), Glycine-rich cell-wall protein were also identified in our investigation (Kevei et al., 2002, Lei and Wu, 1991, Brady et al., 1993). There are some genes selected from libraries, such as, *FA06* showed differential expression in different interaction, may play roles in signaling or other aspects of the defense response, but their function needs to be further studied.

Expression of clones selected during rice infected by avirulent and virulent strains of *M. grisea*

In previous research, most of the studies used the same pathogenic strain to infect different varieties to find pathogen responsive genes (Lu et al., 2004, Xiong et al., 2001). In this paper, however, the same rice variety was used to be infected by different pathogen strains to analyze genes involved in race-specific resistance. Through this method, we could mine the different molecular base of the identical host responding to different pathogens. Consequently, the differentially expressed genes caused by the different strain infection were detected. A large part of the difference between incompatible and compatible interactions can be explained quantitatively by gene expressions (Tao et al., 2003). However, a few genes showed major differences in expressions in incompatible and compatible interactions, even in different directions during *M. grisea* infection (Kim et al. 2001). To understand the expression pattern of selected genes in rice seedling we undertook the RT-PCR analysis at five time points (0, 12, 24, 48 and 72 h) after inoculation (Fig 2, Table1) with different strains of *M. grisea*. The results showed that the expression of the selected genes were either induced or suppressed at various levels and times with responding to different strains.

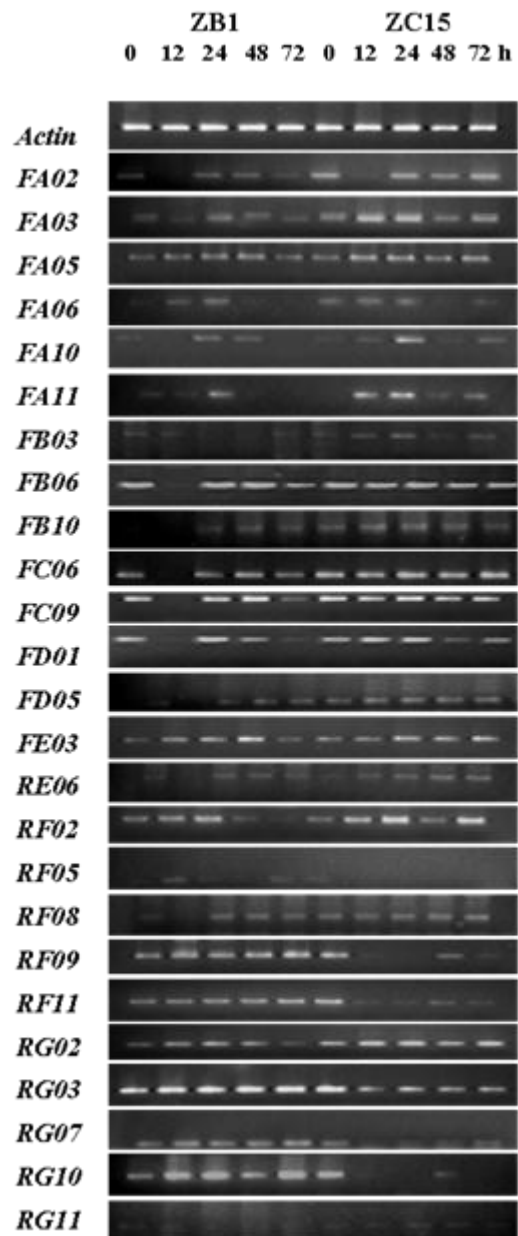


Fig 2. RT-PCR analysis of some selected differential expression genes in zhong 156 seedling plants after inoculation with avirulent (strain ZC15) and virulent strains of *M. grisea*. The equal amounts of total mRNAs of Zhong156 leaves were isolated 0, 12, 24, 48 and 72h after inoculation with ZC15 and ZB1 strains and then were converted into cDNAs, respectively. All of equal amounts cDNAs were used as template for RT-PCR. The rice actin gene was used as an endogenous control.

Some genes, such as *FA11* encoding RNA-binding protein, were induced by both strains, but with stronger induction in the incompatible interaction (between the avirulent strain and rice) than that in the compatible interaction (between the virulent strain and rice). By contrast, such *FD01* encoding G-box binding factor, were repressed with different degree at 12h after inoculation by both two strains. We considered that these genes participated in the defense responses as the fundamental resistant genes. This result was similar to that of Kim (Kim et al., 2001). However, some genes, such as *FD05* (encoding

ubiquitin carrier protein) were induced from 24h after infection by ZB1 while showing a faint increase after infected by ZC15. Some other genes like *RE06* (encoding 60s ribosomal protein) were induced by ZC15 but suppressed by ZB1. For example genes like *RG07* were suppressed by ZC15 and induced by ZB1. Genes like *RF11* were suppressed by ZC15 and showed no significant change after inoculation with ZB1. We suggest that these genes might play pivotal roles in regulating the race-specific resistance.

After infection, the resistance reaction was characterized by up-regulation of defense genes. The susceptible interaction was characterized by large-scale down-regulation of gene expression related to plant growth (Swarbrick et al., 2008). Nevertheless, the present study indicated that most of genes involved in cell rescue and defense, protein synthesis/protein fate were induced by both avirulent and virulent strains, whereas most of genes related to metabolism/energy were repressed by two strains. The genes related to transposable elements showed stronger induction by the virulent strain and repressed by the avirulent strain.

The expression analysis showed that all of 25 genes selected in this study were regulated by infection with different strains of *M. grisea*. They may involve in race-specific resistance to leaf blast in rice. Further study of the function of these genes will allow the formulation of hypotheses to explain the race-specific blast resistance.

Materials and methods

Plant materials

The rice cultivar Zhong156 and two strains, ZC15 and ZB1, of *M. grisea* were used in this study. The cultivar Zhong156 carrying a race-specific resistance gene *Pi-24(t)* is susceptible to race ZB1 (virulent) and resistant to ZC15 (avirulent) at the seedling stage of rice (Zhuang et al., 1997; Zhuang et al., 2002).

Inoculation with *M. grisea*

Rice plants were grown in a greenhouse under 80% relative humidity with 12 h of light period ($500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) at 25°C followed by 12 h of darkness at 20°C. Four-week-old seedlings were sprayed with conidium suspension (2×10^5 spores mL^{-1}) containing 0.02% Tween-20 of ZC15 and ZB1, respectively. The inoculated plants were placed in plastic bags for 24 h at 28°C with 100% relative humidity, and subsequently transferred to the growth chamber under the same conditions mentioned above. Leaf tissue was collected at 0, 12, 24, 48 and 72h after inoculation. After harvesting, all leaf samples were immediately frozen in liquid nitrogen and stored at -70°C. Total RNA was extracted and prepared poly (A)⁺ RNA using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions.

Suppression subtractive library construction and differential screen

SSH was carried out using the PCR-Select cDNA Subtraction kit and following the manufacturer's protocol (Clontech, Palo Alto, CA). For the subtraction part of the methodology, the double-stranded cDNAs were synthesized from poly (A)⁺ RNAs, pooled equally of 12, 24, 48 and 72h post-inoculation samples with two strains, respectively. In the "forward subtractive cDNA library" cDNAs of Zhong156 inoculated with an avirulent strain ZC15 was used as "tester", and that cDNA with a virulent strain ZB1 infection was used as "driver".

In the "reverse subtractive cDNA library" cDNA that extracted from Zhong 156, inoculated with a virulent strain ZB1, was used as a "tester", and that cDNA with the avirulent strain ZC15 was used as a "driver". Two rounds of SSH PCR were carried out following the PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA). The final products were inserted into pGEM-T easy vector (Promega) and transformed into *E. coli* JM109 competent cells (Promega), then cultured in LB medium with IPTG and X-Gal.

For differential screening, individual white clones from two libraries were randomly picked and used as PCR templates. Following amplification of the template insert cDNA with nested primer 1F and primer 2R provided in the PCR-Select cDNA lib Kit, the PCR products were transferred to Hybond-N⁺ nylon membrane for hybridization. Four identical blots were prepared for hybridizations with both subtracted and un-subtracted tester and driver probes, respectively. For the forward library differential screen the un-subtracted tester probe was cDNA of zhong156 after inoculation with ZC15. The un-subtracted driver probe was cDNA of zhong 156 after inoculation with ZB1. The subtracted tester probe is forward SSH PCR product of the tester, and the subtracted driver probe is forward SSH PCR product of the driver. Conversely, for the reverse library differential screen the cDNA of zhong 156 after infection with ZB1 was used as the tester probe, and the cDNA of zhong156 after infection with ZC15 was used as driver probes, respectively. The probes were prepared by labeling the subtracted and un-subtracted of tester and driver cDNAs following the protocol of ECL Labeling Kit (Amersham Pharmacia Biotech Ltd, UK). Each hybrid experiment was conducted in triplicate with a newly labeled cDNA probe of the corresponding tester and driver.

BLAST search and sequence analysis

The nucleotide sequences of the selected cDNA clones were sequenced using M13-F (5'-GTTTCCAGTCACGAC-3') and M13-R (5'-CAGGAAACAGCTATGAC-3') primers (Technical manual of the pGEM-T easy vector, Promega) determined flowing an AutoCycle sequencing kit and an ALFexpress DNA Analysis System (Amersham Pharmacia Biotech, UK). Each of cDNAs was sequenced in triplicate times by both forward and reverse primers. The sequences were submitted to the National Center for Biotechnology Information (NCBI) database, and the BlastX or BlastN programs were used to search for known proteins or nucleic acid homology to the cDNA sequences.

RT-PCR analysis

The total mRNAs were isolated and converted in cDNAs of 0, 12, 24, 48 and 72h after inoculation with ZC15 and ZB1, respectively. All of equal amounts cDNAs were used as template for RT-PCR. The primers for RT-PCR were designed according to the cDNA sequence. The rice actin gene was used as a control. The amplifications were performed at 94°C for 2 min and followed by 25 cycles at 94°C for 30s, 62°C for 30s, 72°C for 1min, and a final elongation at 72°C for 8 min. The annealing temperatures and number of cycles might be modified with different primers.

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