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Cellular and Molecular Interactions of *Magnaporthe oryzae S3* in Rice

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The interaction between *Magnaporthe oryzae S3* and rice was elucidated at the cellular and molecular level. The interactions at the cellular level was observed under SEM where the level of inter and intracellular proliferation of the microbe in three rice varieties was tested yielding Pongsu Seribu as the most resistant. Through DAB assays we detected reactive oxygen species (ROIs) being expressed at the location of pathogen proliferation. In Pongsu Seribu the location of proliferation and the neighboring cells showed elicitation of ROIs. As ROIs are known to trigger defense response, the expression of a defense gene, Pathogenesis related gene(*PR-1b*), was quantified in rice varieties in response to infection. Pongsu Seribu showed the highest level of *PR-1b* gene expression that is in line with the variety being the most resistant.

Key words: *Magnaporthe oryzae*, DAB staining, ROI, Pathogenesis related protein.

Magnaporthe grisea is an heterothallic Ascomycete fungus which causes blast disease in rice [Khang *et al*., 2005; Silue and Notteghem, 1990]and leads to 10-40% yield losses globally [Collemare *et al*, 2008; Kumar *et al*., 1999]. Recently a newly branched out species of *M. grisea* (anamorph: *Pyricularia grisea* Sacc.), *M. oryzae* (Hebert; Barr) has been reported from analysis of field isolates [Couch & Kohn 2002; Filippi *et al*., 2011; Forlani, 2010; Lee *et al*. 2006; Wang 2012].

Dean *et al*., (2005) reported that the blast disease symtoms were largely attributed to the complex secretomesof up to 739 secreted proteins disease produced by *M. grisea* producing[Valente *et al*., 1991; Yamada *et al*., 1976]. In the initial process of infection, *Magnaporthe spp* uses these exudates on the leaf surface that includes a complex mixture of hydrophobic materials to assist with host recognition, induction of spore germination and appressorium formation [Knogge, 1996; Kollatukudy, 1995]. The appressoria exerts turgor pressure as a result of aggregation of many soluble materials including glycerol [Zaira & Talbot 2008]. The appresorium is important in the penetration of host cell through the high intracellular, potential of melanin which is very important in establishing pathogenicity and virulence in *Magnaporthe isolates* [Howard *et al* 1989; Mares *et al*., 2006].

However the resistance reported by any cultivar is short-lived as there is resistance breakdownthree to four years into use [Cai-Lin *et al*., 2011; Corre-Victoria *et al*., 2004; Jian-yuan *et al*., 2008; Kim & Kim 2009; Liang-fen *et al*., 2010]. Lesion mimic mutants spontaneously form localized or expanding lesions on their leaves and therefore are thought to have defects in regulatory components that lead to cell death. As spontaneous lesion formation in these mutants is

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often accompanied by the accumulation of reactive oxygen intermediates (ROIs), predominantly superoxide anion (O2) and hydrogen peroxide(H_2O_2) [Brodersen *et al*., 2002; Devadas *et al*., 2002; Jabs *et al*., 1996], it is reminiscent of the hypersensitive response (HR)which occurs in cells in response to pathogen-derived elicitor molecules. HR includes the oxidative burst, a transient increase of ROIs, and programmed cell death, and inhibits pathogen proliferation at the infection site. ROIs play pivotal roles in the antimicrobial responses during the HR. Furthermore, ROIs, mainly H_2O_2, might serve as diffusible signals that activate systemic defense responses in distant tissues [Alvarez *et al*., 1998; Levine *et al*., 1994]. On the other hand, excessively accumulated ROIs are toxic. Therefore, the generation of ROIs must be accurately regulated to ensure the efficient activation of defense related genes as well as to ensure that radicals and protein products as from the activation of these pathways do not accumulate to toxic levels within the host.

This study aims is to lookinto the proliferation of pathogen in different rice host and determined level of invasion and ROI activation. As ROI is associated with activation of defense related genes, we monitored the level of expression of *PR-1b* in plants post infection. The results of this study are presented below.

MATERIALS AND METHODS

Plant Material

Three genotypes of *Oryza sativa* L. indica were identified for this experiment under greenhouse conditions: cv MR219, Pongsu seribu and Maswangi. These three rice varieties have different disease severity levels against *Magnaporthe oryzae*. MR219 and Maswangi are common local cultivars that are used widely in paddy fields. The rice seeds were placed on filter paper in petri dishes and incubated at 40 ^{0}C for 48 hours to break their dormancy after which the filter paper was then moistened and the seeds were left for 3 days at 28 ^{0}C to germinate. The germinated seeds were transferred to pots containing sterilized soil and allowed to grow for two weeks.

DNA extraction of *Magnaporthe oryzae*

The genomic rDNA was extracted using DNeasy Plant Mini Kit (QIAGEN)**.** Fresh fungal mycelium was harvested from YPG grown fungal culture and 100 mg tissue was weighed, and ground in liquid nitrogen. The finely ground mycelia was transferred immediately into sterilized 1.5 mL tubes and the extraction protocol proceded according to manufacturer's recommendations.

Amplification of rDNA Internal Transcribed Spacers (ITS) of *M. oryzae*

The ribosomal DNA (rDNA) of internal transcribed spacer regions was amplified using ITS1-5'-TCTGTAGGTGAACCTGCGG-3' and ITS4-5'-TCCTCCGCTTATTGATATGC-3'(Chakraborty *et al*., 2010; White *et al*.,1990). The thermal cycling conditions used is as follows: 94 ºC initial denaturation cycle for 4 minutes followed by 35 cycles of 94 ºC for 1 minutes, 53 ºC for 1 minutes and 72 ºC for 2 minutes, and then a final extension at 72 ºC for 10 minutes. PCR products were purified by QIAquick PCR purification kit (Qiagen). The purified PCR products were sequenced and the sequence was converted into consensus sequence using BioEdit Program ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The resulting consensus sequence was blasted against the whole nucleotide sequences in GenBank via NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This provided the genus and species level identities for the organism.

Phylogenetic analysis of *M. oryzae*

All the DNA sequences were aligned using the ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) option available in BioEdit Sequence Alignment Editor Version 7 followed by manual adjustment (Hall 1999). The sequences were analyzed in MEGA version 6 (Tamura *et al*., 2013) using the Partition homogeneity test.The molecular phylogenetics used a maximum parsimony (MP) and maximum likelihood (ML). The MP with the smallest number of nucleotide changes to fit the observed sequence data is chosen to represent the true tree (Nakhleh *et al*., 2005). The ML inference of phylogeny hypothesizes the evolutionary history in terms of tree with the highest probability or likelihood to give rise to the observed data set.

Plant inoculation

The plantlets were then inoculated with 5 mm fungal plugs of *M. oryzaeS3* cultured and grown on potato dextrose agar (PDA). The plugs were placed at the base of the stem and wrapped

with cotton moistened with distilled water and 0.5% gelatin. The humidity and moisture was maintained by covering the site of infection with aluminum foil. The disease symptoms appeared after 72 hours post inoculation (hpi), and the aluminum foil was removed and the leaves were harvested [Park *et al*., 2008; Plodpai *et al*., 2013].

3, 32 - Diaminobenzidine staining of infected tissues

Leaves of 2 week-old plants were inoculated with *M. oryzae S3* and harvested 72 hours post-inoculation (hpi), and incubated in 1 g L^{-1} DAB-HCl solution (pH 3.8), and left in the dark for 8 hours. The leaves were then clarified from the chlorophyll content by treatment with ethanol:chloroform (4:1) solution at room temperature for 2 days. The samples were stored in 50% (v:v) glycerol and later examined under compound microscope (Olympus CX21LED, Olympus Australia Pty. Ltd.) at 100 x magnification.

Microscopic evaluation of plant-pathogen interaction sites

The DAB stained and clarified tissue were used to observe the fungal infection structures within the rice plant cells using by epifluorescence microscopy (excitation filter 485 nm, dichroic mirror 510 nm, barrier filter 520 nm, TCS SP; Leica Microsystems, Wetzlar, Germany). Three seedlings from each rice variety were used to examine the fungal mycelium penetration and growth within the plant cells. The interaction between the pathogen and the rice leaf epidermal cells was divided into four according to the scoring system as stated in Odile *et al*., (2008).Type 1: appressoria established; Type 2: showed epidermal infected cells and formation of appressorium with H_2O_2 production; Type 3: infected epidermal cells and extended into the neighbouring epidermal cells and produced high levels of H_2O_2; and Type 4: presence of intracellular and intercellular hyphae in and around epidermal cells.

Extraction of total RNA from infected rice tissue

Seventy two hours post infection leaves (third leaf only) were harvested and subjected to total ribonucleic acid (RNA) extraction. The total RNA was extracted using an RNeasy Plant Mini Kit (QIAGEN Company) according to manufacturer's recommendation and the concentration was quantified spectrophotometerically via NanoDrop 1000(Thermo Scientific, USA).

Two-step Real-Time RT-PCR

RNA (generated from 5 individual plants per experiment point) was reverse transcribed to cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) using random hexamers. Real-time PCR was performed using Fast SYBR Green Technology in the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, USA). A master mix for each PCR run was prepared with SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, USA). Final concentrations, in a total volume of 20 mL, were: 10 mL fast SYBR Green Master Mix, 10 ng of cDNA and 300 nM of each specific sense and anti-sense primer. The Primer 3 Programme was used to design specific primers for cDNA amplification of full length *Oryza sativa* pathogenesis-related protein class 1b(*PR-1b*) gene by using the complete cds sequence of the gene (Accession Number U89895) from the NCBI database as template. The following primer pairs were generated: PR1b-F-5'-CGAGAAGAGCGACTAGGAGTAC-3' (22mers) and PR1b-R-5'-GCCTCTGTCCGACGAAGTTG-3' (20 mers). This primer set was used to quantify PCR of the *PR1* inpre-infected and post infected *M. oryzae* plants. Actin gene from *Oryza sativa OsRAC1* mRNA (Accession Number AB047313; complete cds), was used as the control housekeeping gene to ensure all samples were dispensed equally and observations were viable [Odile *et al*., 2008].The primers designed for the housekeeping gene are: 52 F-GCGTGGACAAAGTTTTCAACCG-32 and 5'R-TCTGGTACCCTCATCAGGCATC-3'. Reverse transcription mixture (cDNA) was added to the PCR mixture and the **PCR**reaction was carried out as follows: denaturation at 95^0C for 20 s, followed by40 cycles at 95^0C for 3 s followed by 60^0C for 30 s. All samples were amplified in triplicate from the same RNA preparation and the mean value was calculated from these three values. The amplification efficiency determined for each housekeeping gene was calculated using the slope of the regression line in the standard curve. Amplified products were verified on a 2% agarose gelfor their correct sizes and melting curve experiments showed whether a single amplification had been obtained for each gene.

The RNA amplification levels for reference gene and for all samples were determined as Ct (cycle threshold) values, which are the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of the PCR reaction.We applied an alternative approach which involved a comparative ""CT method[Livak & Schmittgen, 2001] to analyze the relative changes in gene expression from real-time quantitative PCR experiments as follows; the "C_T value for each sample was determined by calculating the difference between the CT value of the target gene and the CT (Critical Threshold) value of the endogenous reference gene. the "C_T value for each controls was determined by calculating the difference between the CT value of the target gene and the CT value of the endogenous reference gene. The —"C_T value calculated the difference between the "C_T value of the target gene (sample) and the "C_T value of the endogenous reference gene (calibrator). The normalized target gene expression level in sample (Fold change) = $2^{-(—"CT)}$.

RESULTS AND DISCUSSION

ITS and phylogenetic analysis of *Magnaporthe oryzae*

The ITS sequence of the *Magnaporthe sp* S3 was obtained using ITS1 and ITS4 primer pairs. The ~ 500bp product was sequenced, assembled via BioEdit and Blasted against the nucleotide database in NCBI. Figure 1 provides the Maximum Likelihood tree generated with 38 nucleotide sequences of *Magnaporthe grisea*, *Magnaporthe oryzae* and *Pyricularia* sp. sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 409 positions in the final dataset. The sequence showed 99 % homology to *M. oryzae* Ken54-20 (Accession no:114842256) and high level homology to other *M. oryzae*used in this analysis(isolates PO-02-7306,Ina 72,and ken54-04).

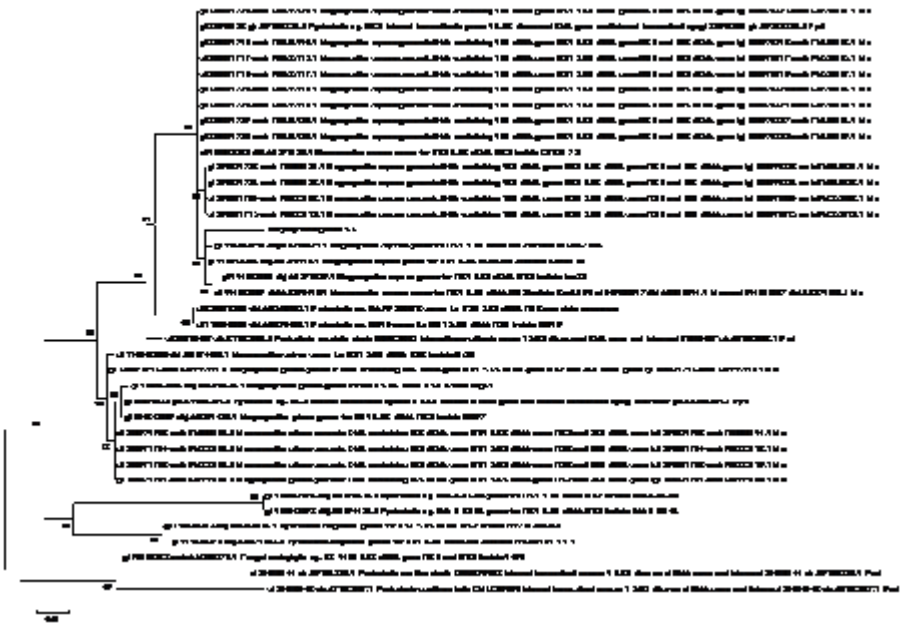

Fig. 1. Molecular phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1651.7483) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach

The *M. oryzae* isolates obtained from *Oryza sativa* as host segregated into a monoplyletic clade. This therefore indicates the probability that our isolate is a *M.oryzae*.

The tree showed strong relatedness to host where the isolates segregated neatly according to host i.e *Eleusina sp, Oryza sativa, Digitaria sp, Zingiber sp*. However as for the geographical relatedness, most segregated according to their country or region of origin, while we observed some evolutionary stages of relatedness between species from different localities (Fig. 1, Fig. 2 and Table 1).Therefore isolates clusteredpredominantly according to their host. A similar tree was generated using Maximum Parsimony (Figure 2) where the isolates obtained from *O. sativa* sp formed a monophyletic cladeas in ML. Figure 2 has three clear clades (A, B, and C) with evolutionary stages above and between the *M. oryzae/ O. sativa sp*clade.

Observation of fungal infections caused by *M. oryzae*in rice leaves

3,3'-Diaminobenzidine was used to detect hydrogen peroxide (H_2O_2) and reactive oxygen intermediate (ROIs) production [Odile *et al*., 2008; Thordal *et al*., 1997]. 3, 32 - Diaminobenzidine (DAB) forms a reddish-brown precipitate in the presence of H_2O_2 and endogenous ROIs in plant cells.For this experiment *M.oryzae S3*was applied on MR219, Maswangi and Pongsu Seribu as the rice host to observe the host pathogen interactions at the cellular level. MR219 and Maswangi are two cultivated rice varieties in Malaysia and Pongsu Seribu is a traditional rice variety which has lower yield than the cultivated lines but has been reported to have good resistance to disease. The rice plants were inoculated with the pathogen and these experiments were carried out in triplicate.

Figure 3 shows that the spores of the pathogen had germinated within 72 hours and the

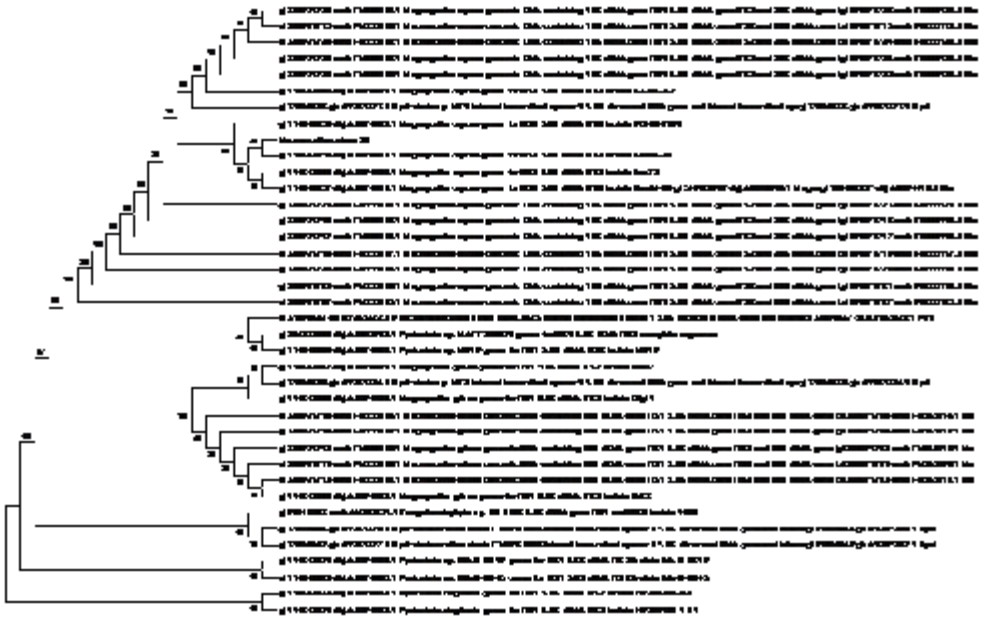

Fig. 2. Maximum Parsimony analysis of taxa. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 7 most parsimonious trees (length = 188) is shown. The consistency index is (0.636905), the retention index is (0.834239), and the composite index is 0.563555 (0.531331) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [1]. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated. The three clades indicated by brackets

mycelial structures were visible at the cellular level. As a result of conducting these assays on our three rice varieties, we were able to conclude that all three varieties showed compatible host interactions with *M. oryzae S3*. However, the three varieties varied in their compatibility level which directly affected the disease severity shown by the pathogen. Figure 3also shows *M. oryzae S3* caused the most prominent disease symptoms with Maswangi (C1). This was closely followed by cultivar MR219 and finally Pongsu Seribu (Figure 3A1 and B1). Observation at the cellular level under the Leica Diaplan Microscope, showed *M. oryzae S3* hypha had proliferated the epidermal cells of all rice varieties. The order of infiltration observed at the cellular level matched the physical observations of disease in plants. The cellular invasion was extensive in Maswangi (Type 4)followed by MR219 (Type 3)and Pongsu Seribu(Type 2). Therefore we can conclude that Pongsu Seribu is the most resistant of the three cultivars. In addition the reddish brown precipitate formation that indicates ROIs and H_2O_2 release, Pongsu Seribu had the highest level of DAB stainingthat formed not just

Table 1. The ITS sequences of *Magnaporthe spp*used in the phylogenetic analysis

Accession (GI)	Sequence	Host	Country
114842256	*Magnaporthe oryzae*Ken54-20	*O. sativa*	Japan
114842259	*Magnaporthe oryzae*PO-02-7306	*O. sativa*	Japan
114842257	*Magnaporthe oryzae* Ken54-04	*O. sativa*	Japan
549429917	*Magnaporthe oryzae* 0903-4	*O. sativa*	Japan
114842260	*Magnaporthe oryzae* GFSI1-7-2	*Setaria italica*	Japan
114842258	*Magnaporthe oryzae*Ina 72	*O. sativa*	Japan
31616438	*Pyricularia* sp. MC1		Thailand
308071727	*Magnaporthe oryzae*K60-131P	*Eleusine coracana*	Kenya
308071723	*Magnaporthe oryzae*K33-184	*Eleusine coracana*	Kenya
308071722	*Magnaporthe oryzae*K33-189	*Eleusine coracana*	Kenya
308071721	*Magnaporthe oryzae*K44-111P	*Eleusine coracana*	Kenya
308071720	*Magnaporthe oryzae* K48-115N	*Eleusine coracana*	Kenya
308071719	*Magnaporthe oryzae*K55-124P	*Eleusine coracana*	Kenya
308071718	*Magnaporthe oryzae*K56-125N	*Eleusine coracana*	Kenya
308071717	*Magnaporthe oryzae*G22	*Eleusine sp*	UK
308071726	*Magnaporthe oryzae*K1-15	*Eleusine sp.*	Kenya
308071725	*Magnaporthe oryzae*K23-123	*Eleusine coracana*	Kenya
308071724	*Magnaporthe oryzae*K13-67	*Eleusine coracana*	Kenya
308071715	*Magnaporthe oryzae*D2-S26	*Eleusine coracana*	Uganda
254272368	*Pyricularia sp.* MAFF 306672	*Panicum maximum (Guinea grass)*	Japan:
114842264	*Pyricularia sp.* NI919	*Leersia oryzoides*	Japan
31616437	*Pyricularia angulata*NBRC9625		Thailand
308071713	*Magnaporthe grisea*D10-S73	*Digitaria sp.*	Uganda
308071714	*Magnaporthe grisea*D10-S38	*Digitaria sp.*	Uganda
308071712	*Magnaporthe grisea*D15-S37	*Digitaria sp.*	Uganda
308071711	*Magnaporthe grisea*D15-S42	*Digitaria sp.*	Uganda
114842267	*Magnaporthe grisea*NI907	*Digitaria sanguinalis*	Japan
31616439	*Pyricularia sp.* MC2		Thailand
308071716	*Magnaporthe grisea*D1-S49	*Digitaria sp.*	Uganda
114842266	*Magnaporthe grisea*Dig41	*Digitaria sanguinalis*	Japan
114842268	*Magnaporthe grisea*Br33	*Digitaria horizontalis*	Japan
114842272	*Pyricularia zingiberis* HYZiM201-0-1	*Zingiber mioga*	Japan
114842271	*Pyricularia zingiberis* HYZiM101-1-1-1	*Zingiber mioga*	Japan
114842274	*Pyricularia sp.* INA-B-93-19	*Phyllostachys bambusoides*	Japan
114842273	*Pyricularia sp.* INA-B-92-45	*Sasa sp.*	Japan
31616444	*Pyricularia costina* CMUZP0003		Thailand
31616442	*Pyricularia costina* CMUZE0003		Thailand

around where the fungal mycelium was seen but also in the neighbouring cells indicating a high level of ROI production in and around the point of entry (Figure 3 A3, B3 and C3).

Plant pathogens attack the plants and attempt to establish themselves within the hosts as we can see clearly from **Figure3.** Their invasion of the host cell however seemed to initiate the oxidative burst which results in the production of ROIs in the host cells which are toxic to the pathogen. In resistant varieties, the ROIs levels were high (as seen by the reddish precipitate form throughout tissue) and resulted in reduced disease symptoms and proliferation as seen at the cellular level.The oxidative burst that is seen in this experiment is a key factor in the elicitation of the defense response in host and we believe this is achieved through the activation of defense pathways as well as disease resistance genes [Gong *et al*., 2006; Lamb & Dixon 1997; Lee *et al*., 2004; Moore *et al*., 2011; Namdani *et al*., 2012; Yang *et al*., 2009; Yanjun & Shiping, 2010].

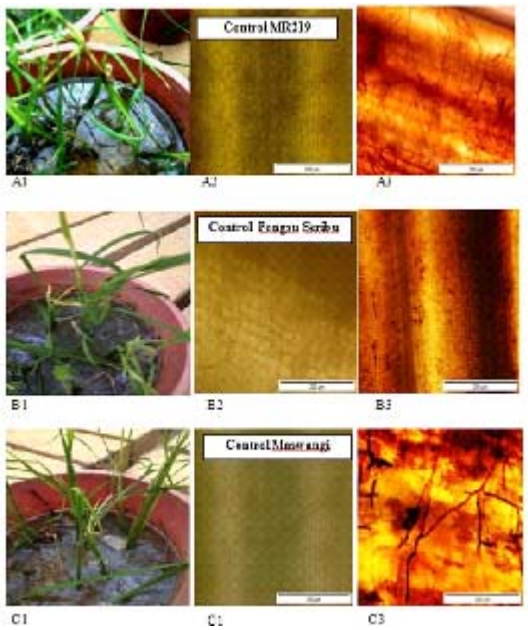

Fig. 3. Interaction between plant and pathogen and colonization of MR219, Pongsu Seribu and Maswangi leaves by *M. oryzae*. A1, Inoculated leaves of MR219 with *M. oryzae*. A2, Control Non-infected MR219. A3, Cellular proliferation of *M. oryzae*into the host (Type 3). B1, Inoculated leaves of Pongsu Seribu with *M. oryzae*. B2, Control Non-infected Pongsu Seribu. B3, Cellular proliferation of *M. oryzae*into host (Type 2). C1, Inoculated leaves of Maswangi with *M. oryzae*. C2, Control Non-infected Maswangi. C3, Cellular proliferation of *M. oryzae* into the host (Type 4).

PR-1b gene expression profiling in *M. oryzae*infected plants

Since the previous experiments showed that ROIs were being expressed within the infected tissues, therefore we proceeded to examine how effectively ROIs are involved in triggering the

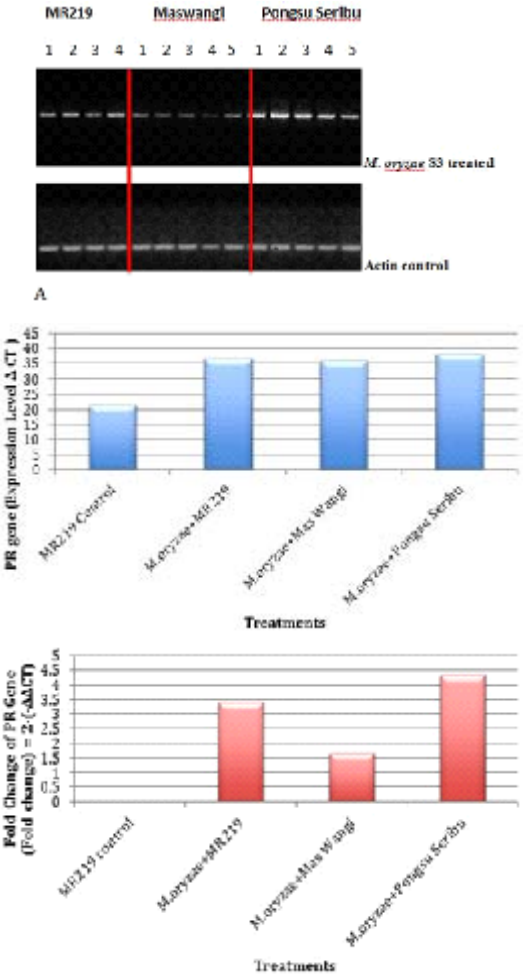

Fig. 4. Expression level of *PR-b1 gene* at 72 hours post inoculation with *M oryzae S3* in rice. (A) Expression level observed via RT-qPCR for post inoculation in MR219, Maswagi and Pongsu Seribu. ²-actin gene was used as a control housekeeping gene in this assay. (B) *PR-1b* gene Expression level (" C_T) values obtained from calculating the expression levels in three rice varieties 72 hours post inoculation with *M. oryzae*; and (C) Fold Change ($2^{-(\text{—}\check{S}CT)}$) of *PR-1b* as calculated for all three rice varieties 72 hours post inoculation with *M. oryzae*

defense genes *in planta*.Here we quantified the expression of a defense related gene, *PR-1b*, in response to infection by *M.oryzae S3* via RT-qPCR with Actin as reference gene.

Figure 4(A) exhibits the variation in the expression level of *PR-1b* gene 72 hours post inoculation with *M.oryzae S3*. The highest level of expression is observed in Pongsu Seribu and the lowest in Maswangi. We further calculated the expression levels and the fold change in the espression of the *PR-1b* gene and found in both cases the best turnaround of the gene was exhibited in Pongsu Seribu as the host (Figure 4B and C). Expression Level (" CT) detected for *PR-1b* gene in MR219 and Maswangi did now show significant difference while there was a significant difference between both these varieties and Pongsu Seribu (Figure 4B).However there was a significant difference in fold change ($2^{-(—dCT)}$) for all varieties (Figure 4C).

As observed in Figure 4, the *PR-1b* gene is activated in response to proliferation by *M.oryzae S3*. The fungal penetration of cell that resulted in ROI production is believed to have triggered the activation of systemic acquired resistance by means of defense gene activation. The higher levels of expression observed in the resistant variety (Pongsu Seribu)indicates the efficiency of the defense machinery to protect the plants from invasion. In addition to the level of defense proteins expressed, the speed at which they are expressed also determines the level of resistance. The faster and higher the response, the more resistant the variety [Bae *et al*., 2009; Tao *et al*., 2003; Vergne *et al*., 2007; Wen *et al*., 2003;Zheng & Shetty, 2000].

These results are in agreement with several studies where specialized defense mechanisms in response to biotic stresses are known to induce theexpression of broad spectrum antimicrobial agents (such as pathogenesis-related proteins) [Dong, 1998; Dangl & Jone, 2001Glazebrook, 2001]which facilitate theelicitation of systemic acquired resistance (SAR) in plants[Guo *et al*., 2007; 2009; Harman 2000; Hayata *et al*., 2010; Jing *et al*., 2007].

CONCLUSION

The ITS analysis showed that our isolate is most probably a*Magnaporthe oryzae*with high homology to isolate ken54-20. The phylogenetic analysis showed that the isolates clustered together predominantly by host specificity followed by geographical relatedness.

The DAB assays showed mycelial infiltration at the cellular level with ROIs production in and around the cells. As a consequence of ROIs production, *PR-1b*gene expression was detected 72 hours post inoculation and the levels corresponded to the level of resistance shown by the host. The assays showed that Pongsu Seribu was the most resistant variety with the lowest disease symptoms and the highest ROI and H_2O_2 production. Therefore it can be concluded that the production of ROIs and the release of H_2O_2 is essential in reducing disease symptoms through the activation of the defense mechanism.

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