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The host pathogen interactions observed between *Fusarium* sp. F2 and rice

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Abstract

An ITS verification was conducted on a fungal isolate (*Fusarium* sp. F2) obtained from diseased culms of MR219. The ITS sequence analysis determined the isolate be a member of the *Fusarium incarnatum-equisetti* complex (FIEC). This isolate was then used to study cellular interactions in three rice varieties (Pongsu Seribu, Maswangi and MR219) where the microscopic observation of DAB stained infected tissues displayed inter and intracellular proliferation in all varieties with low to high levels of ROIs detected in and around infected tissues. Further to the cellular observation, we studied the variation in expression levels of a defense related gene, *PR-1b* where the level of ROIs expressed within the infected tissue corresponds directly to the levels of gene expressed. Pongsu Seribu was the most resistant rice variety to *Fusarium* sp. F2 infections based on disease severity, ROI levels, gene expression profiles and fold increase.

Keywords: *Fusarium* sp., ITS analysis, reactive oxygen intermediates (ROI), Pathogenesis Related (PR) protein.

Introduction

Plant pathogens are found in most ecosystems in nature. However the presence of effective defense within the host negates the ability of the pathogen to cause disease (Jones and Takemoto, 2004; Jones and Dangl, 2006). The defense mechanisms that exist within plants include processes such as hypersensitive response (HR), and the production of a plethora of antimicrobial agents such as phytoalexins and pathogenesis-related (PR) proteins (Narasimhan et al., 2001). The activation of these response mechanism are mediated through signal molecules such as reactive oxygen species (ROS), salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Devadas et al., 2002; Dong, 1998; Lee et al, 2004). Reactive oxygen intermediates (ROIs), such as superoxide anion (O_2) and hydrogen peroxide (H_2O_2) (Brodersen et al., 2002; Devadas et al., 2002; Jabs et al., 1996), have been reported to be produced in hypersensitive response (HR) which results in a pathogen derived reaction. ROIs transiently increase within the host in response to HR and eventually result in programmed cell death, which inhibits pathogen proliferation. These molecules have been shown to have a pivotal role in activating the systemic defense response where defense related genes such as *PR-1*, *PR-2*, and *PR-5* have either independently or interdependently activated the the systemic defense mechanism *in planta* (Alvarez et al., 1998; Levine et al., 1994; Dangl and Jones, 2001; Jing et al. 2007).

The genus *Fusarium* (Nectriaceae, Ascomycota) has a wide distribution and may colonize biomes which include forests, deserts, littoral, agricultural as well as aquatic and manmade environments (Leslie and Summerell 2006). *Fusarium* sp. negative effects on agriculture, forest (Windels 2000, Wingfield et al. 2008), human and animal health (Schroers et al. 2009) has been mainly attributed to the production of one or more toxins, such as fumonisins, moniliformin, zearalenone and enniatins (Bottalico and Perrone 2002, Logrieco et al. 2002). The presence and distribution of individual *Fusarium* spp. in noncultivated and cultivated soils may be influenced by a number of factors, including organic content, pH, temperature and moisture, (Burgess and Summerell 1992). The differentiation of this genus with regards to species, host and geospatial structure has not been conducted extensively until the recent years where molecular phylogeny has demonstrated that morphologically defined *Fusarium* species often comprises of multiple phylogenetically diagnosable species (O'Donnell et al. 1998b, 2000, 2004, 2008, 2009) and that the previously morphologically defined *F. oxysporum*, *F. solani* and *F. equiseti*, are now known to represent species complexes (O'Donnell et al. 1998a, 2000, 2009). Here we describe the molecular and cellular characterization of the infection process of a species of *Fusarium* that produced wilt like symptoms in rice. The physically observed disease severity in rice plants was correlated to the level of inter and intracellular infiltrations caused by *Fusarium* sp. F2. In addition the interaction between the host-microbe which was detected by the production of ROIs and the activation of a defense related gene was observed to find a correlation between the resistance levels of the rice varieties used and the elicitation of ROIs and their relationship to the activation of systemically induced resistance.

Results

Molecular analysis of Fusarium sp. *F2*

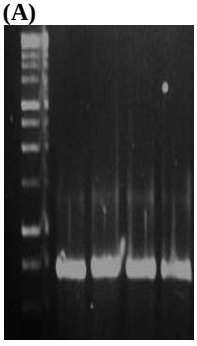
ITS-rDNA analysis was conducted on the isolate using ITS1 and ITS4 respectively. An amplicon of ~ 800bp was obtained with this primer pair (Fig 1A) which was then sequenced, assembled and blasted against the nucleotide database in

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

Accession (GI)	Sequence	Host	Country	Ident (%)
	Fusarium solani F2			
430824858	*Fusarium* sp. AL-21	*Citrus limon* var. *Eureka*	Mexico	100
323434976	*Fusarium equiseti* Fe2		China	100
434184516	*Fusarium oxysporum* CM54	*Nothapodytes nimmoniana*	India	99
430824859	*Fusarium* sp. AL-22	*Citrus limon* var. *Eureka*	Mexico	99
317383326	*Fusarium* sp. 1	*Saccharum officinarum*	USA	99
417353586	*Fusarium chlamydosporum* 12213	*Brickellia rosmarinifolia*	China	99
430824851	*Fusarium* sp. AL-14	*Citrus limon* var. *Eureka*	Mexico	99
440546826	*Fusarium equiseti* JH04	*Artemisia annua* L.	India	99
559783702	*Fusarium equiseti* MYS-1	maize	India	99
562759532	*Fusarium* sp. G646	plant root	China	99
434184532	*Fusarium chlamydosporum* C22	*Nothapodytes nimmoniana*	India	99
217314863	*Fusarium equiseti* T34		China	99
325563878	*Fusarium* sp. LH60	*Camellia sinensis*	China	99
312434277	*Fusarium* sp. ATT015	*Atta texana* nest	Brazil	99
334855389	*Fusarium* sp. TA26-56	*Actiniaria* sp.	China	99
343170156	*Fusarium chlamydosporum* strain C25		China	99
346464445	*Fusarium equiseti* 18 Fusa	*Phaseolus vulgaris* L. cultivar INTA Rojo	Finland	99
346464446	*Fusarium chlamydosporum*	*Phaseolus vulgaris* L. cultivar INTA Rojo	Finland	99
346654935	*Fusarium* sp. P37E2	*Eleocharis atropurpurea*	Venezuela	99
294714334	*Fusarium* sp. CB-2	manufactured-gas plant-contaminated soil	Mexico	99
294805293	*Fusarium* sp. Z13-09	mangrove plants	China	99
283099669	*Fusarium chlamydosporum* Z6-14		China	99
134290455	*Fusarium incarnatum* GT3		China	99
228481048	*Fusarium* sp. SLP 015	*Paphiopedilum bellatulum*	Thailand	99
126131206	*Fusarium* sp. P002	*Peltogyne purpurea*	USA	99
586829668	*Fusarium* sp. UOM V	*Vitex negundo*	India	99
608606094	*Fusarium equiseti* NH3982	wheat	India	99
134303359	*Fusarium equiseti* bxqLGT	corneal scraps from fungal keratitis	China	99
164654195	*Fusarium incarnatum* FKCB-031		China	99
283099666	*Fusarium equiseti* Z3-49		China	99
429472654	*Fusarium chlamydosporum* JL-26	*Tribulus terrestris* L.	China	99
594703775	*Fusarium chlamydosporum* CEF-016		China	99
164470845	*Fusarium* sp. LD-13	*Hippophae rhamnoides*	China	99
255709842	*Fusarium* sp. 56GP/F	*Garcinia mangostana*	Malaysia	99
256915163	*Fusarium* sp. 168DZ/F	*Durio zibethinus*	Malaysia	99
404211721	*Fusarium lacertarum* R3	*Phoenix dactylifera*	Tunisia	99

597453977	*Fusarium incarnatum* FQ13091302	*Solanum lycopersicum*	China	100
430824857	*Fusarium* sp. AL-20 IRH-2012g	*Citrus limon* var. *Eureka*	Mexico	99
432140623	*Fusarium* sp. AP1-5-1	*Fortunearia sinensis*	China	99
432140624	*Fusarium* sp. AP1-5-2	*Fortunearia sinensis*	China	99
432140625	*Fusarium* sp. TC1-6	*Fortunearia sinensis*	China	99
389567255	*Fusarium oxysporum* f. sp. *Ciceris*		India	99
429472659	*Fusarium chlamydosporum* JL-T3	*Tribulus terrestris* L.	China	99
429472660	*Fusarium chlamydosporum* strain JL-T6	*Tribulus terrestris* L.	China	99
430824853	*Fusarium* sp. AL-16 IRH-2012c	*Citrus limon* var. *Eureka*	Mexico	99
388252480	*Fusarium equiseti* isolate FZ7	melon plant	China	99
388252484	*Fusarium equiseti* isolate KA	melon plant	China	99
393804113	*Fusarium incarnatum* RH4	salt-tolerance plant	China	100
401802586	*Fusarium* sp. AD-2012a	chilli	India	99
401802585	*Fusarium* sp. AD-2012a cbe1	chilli	India	99
401871126	*Fusarium equiseti* F3RS1	Aleppo pine seedlings	Algeria	99
421975896	*Fusarium* sp. 1-4-1	cotton	China	99
367461102	*Fusarium chlamydosporum* DHMJ22		China	
373809859	*Fusarium chlamydosporum* F0817	*Malus x domestica Fuji*	Japan	
379642783	*Fusarium* sp. LF239 isolate LF239	*Tethya aurantium*	Germany	
379642796	*Fusarium* sp. LF252 isolate LF252	*Tethya aurantium*	Germany	

(A)



(B)

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GTTGCCTCGG CGGATCAGCC CGCGCCCCGT AAAACGGGAC GGCCCGCCCG AGGACCCCTA AACTCTGTTT 70
TTAGTGGAAC TTCTGAGTAA AACAAACAAA TAAATCAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA 140
TCGATGAAGA ACGCAGCAAA ATGCGATAAG TAATGTGAAT TGCAGAATTC AGTGAATCAT CGAATCTTTG 210
AACGCACATT GCGCCCGCCA GTATTCTGGC GGGCATGCCT GTTCGAGCGT CATTTCAACC CTCAAGCTCA 280
GCTTGGTGTT GGGACTCGCG GTAACCCGCG TTCCCCAAAT CGATTGGCGG TCACGTCGAG CTTCCATAGC 350
GTAGTAATCA TACACCTCGT TACTGGTAAT CGTCGCGGCC ACGCCGTAAA ACCCCAACTT CTGAATGTTG 420
ACCTCGGATC AGGTAGGAAT ACCCGCTGAA CTTAAGCATA TCAATAAGGC GGATCAGCCC GCGCCCCGTA 490
AAACGGGACG GCCCGCCCGA GGACCCCTAA ACTCTGTTTT TAGTGGAACT TCTGAGTAAA ACAAACAAAT 560
AAATCAAAAC TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA CGCAGCAAAA TGCGATAAGT 630
AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA ACGCACATTG CGCCCGCCAG TATTCTGGCG 700
GGCATGCCTG TTCGAGCGTC ATTTCAACCC TCAAGCTCAG CTTGGTGTTG GGACTCGCGG TAACCCGCGT 770
TCCCCAAATC GATTGGCGGT CACGTCGAGC TTCCATAGCG TAGTAATCAT ACACCTCGTT ACTGGTAATC 840
GTCGCGGCCA CGCC
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Fig 1. (A) Amplicon obtained with ITS1 and ITS4 for *Fusarium* sp. F2: Lane 1 1Kb Ladder, Lane2-4: ITS amplicon of >750bp. (B) Sequence of ITS amplicon of *Fusarium* sp. F2, size 854bp.

Table 2. Disease severity of *Fusarium* sp. F1 and F2 scored on MR219 rice variety.

Isolate	Disease Severity Scored on Inoculated MR219 rice variety (5 plantlets inoculated)					
	1	2	3	4	5	Average
Fusarium sp. F1	2	2	3	2	2	2 (2.2)
Fusarium sp. F2	4	5	5	4	4	4 (4.4)

Score: 0 = No disease symptoms detected; Score of 1 least symptomatic to score 5 dead or severely infected.

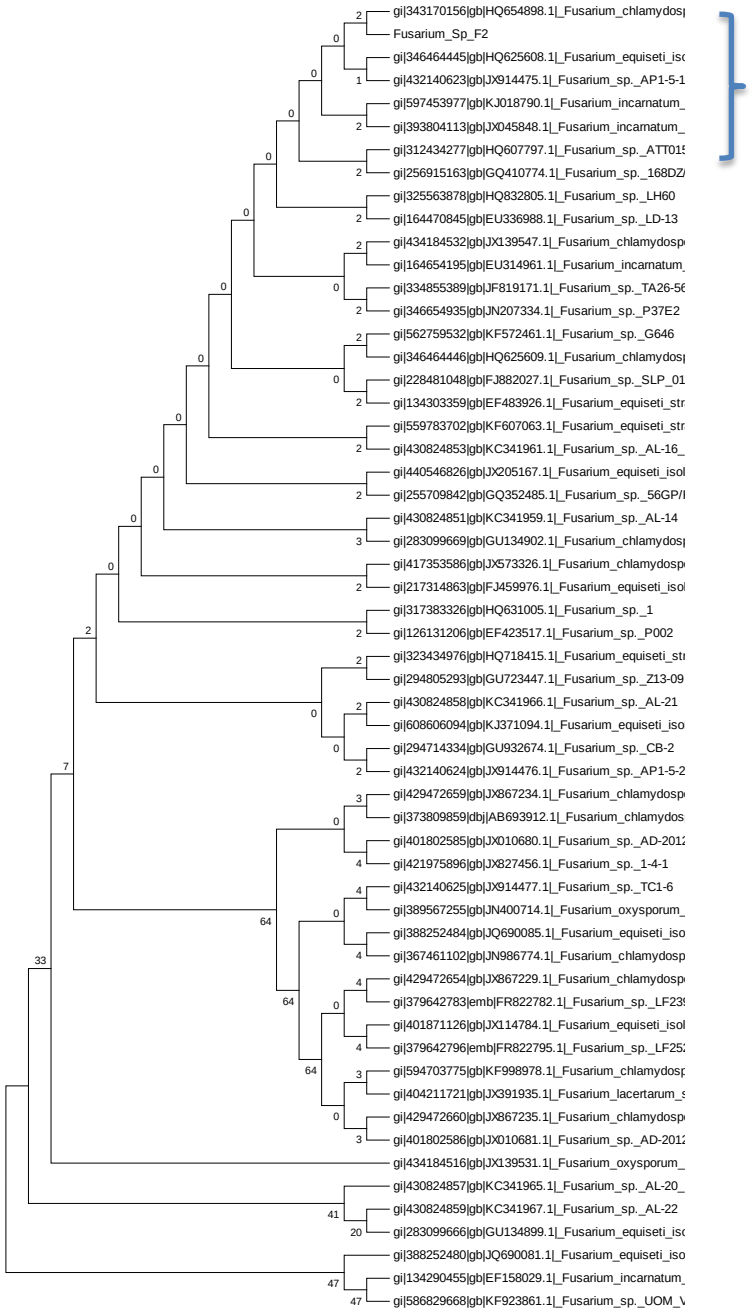

Fig 2. Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [Tamura & Nei 1993]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [Felsenstein 1985]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [Felsenstein 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 57 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 464 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011]. The bracket indicates the clade within which our isolate clusters.

A B

Fig 3. The influence of *Fusarium* sp. F1 and F2 on rice plants (MR219 variety) under greenhouse conditions. (A) *Fusarium* sp. F1. (B) *Fusarium* sp. F2. *Fusarium* sp. has been known to cause causes stem canker around the culm as indicated by arrows and wilt of the rice plant (B).

NCBI (Fig 1B). A Maximum Likelihood tree was generated via MEGA 6 using 56 nucleotide sequences of *Fusarium* spp available in NCBI and the sequence of our isolate (Fig 2). The values presented next to the branches shows the percentage of replicate trees in which the associated taxa clustered together. The data was edited to eliminate all positions containing gaps and missing data. The phylogenetic analysis showed that our isolate showed 100% homology to *Fusarium* sp. AL-21 IRH-2012h and *Fusarium equiseti* strain Fe2 (Accession no: GI:430824858 and GI:323434976) and a high level of identity to other isolates as shown in Fig 2. and Table 1. Although the *Fusarium* isolates closely related to *Fusarium* sp. F2 were derived from different host they were mostly from the Asian region (China and India) (Fig 2, and Table 1). As shown by the phylogenetic analysis and the wilt symptoms exhibited by the source tissue, *Fusarium* sp. F2 is most likely a member of a species complex that is made up of *F. incarnatum, F equiseti,* and *F. chlamydosporum* (Fig 2.).

DAB assays on infected tissues

Three isolates of *Fusarium* were used to screen for the most virulent of strains and *Fusarium* sp. F2 was selected on the basis of the highest level of virulence shown by the isolate on the MR219 rice variety (Fig 3, Table 2). To further characterise the host-pathogen interactions at the cellular level, DAB staining was used to examine the occurrence of ROIs (i.e. H_2O_2) in rice leaves three days post inoculation (Fig 4). The presence of ROIs in tissue is seen by the formation of a red precipitate. MR219, Maswangi and Pongsu Seribu were the three rice varieties used in this study where MR219 and Maswangi are two cultivated rice varieties in Malaysia while Pongsu Seribu is a traditional rice variety with lower yield and good disease resistance. The rice plants were inoculated with *Fusarium* sp. F2 as and subjected to DAB staining and subsequent microscopic analysis.

Our microscopic observations of 72 hr old DAB stained infected tissue showed that spores had germinated and the fungal mycelium was visible within the cells (Fig 4). ROIs were produced both at the inter and intracellular level where the mobility of the pathogen in host cell leaves a trail of oxidative burst. Based on these observations it would seem that all three rice varieties showed compatible host interactions with *Fusarium* sp. F2. However the three varieties varied in levels of cellular proliferation and ROIs where Maswangi showed the highest level of cellular proliferation followed by MR219 and Pongsu Seribu. Based on the low level of cellular proliferation seen in the tissue, it would seem that Pongsu Seribu is the most resistant among the three varieties (Fig 4). All observations of *Fusarium* sp. F2 using the DAB staining profiles placed this fungus under the Type 3 and Type 4 scale where the fungus was attempting or has established itself within the host through the proliferation of infection hyphae into the infected and neighboring epidermal cells (Fig 4) (Odile et al., 2008). 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 is toxic to the pathogen. In the resistant variety (Pongsu Seribu), the ROIs produced is higher and results in reduced disease symptoms and proliferation as seen at the cellular level in infected plants (Fig 3 and Fig 4). In addition to the variation in resistance level of the host, the virulence level of the pathogen also plays an integral role at determining the level of oxidative bursts generated.

Expression profiling of PR-1b in infected plants

ROIs have been implicated as agents that induce or signal the defense response *in planta*. Hence we proceeded to examine the results of elevated ROIs in cell to the expression of a defense related gene, *PR-1b*. The expression profile of this gene in all three rice varieties infected with *Fusarium* sp. F2 was quantified via RT-qPCR with Actin as reference gene.

Variation in the expression level of *PR-1b* gene 72 hours post inoculation with *Fusarium* sp. F2 is shown in Fig 5. The highest expression level was observed in Pongsu Seribu while the lowest was in Maswangi. The expression levels and the fold-change were calculated and both values showed the highest turnaround in Pongsu Seribu (Fig 5B). However expression level (Δ CT) was not significantly different for MR219 and Maswangi. There was however a significant difference between both these varieties and Pongsu Seribu (Fig 5B). A significant difference in fold change ($2^{-(-\Delta\Delta CT)}$) was recorded for all varieties (Fig 5C). As ascertained in Fig 5, the *PR-1b* gene is activated in response to *Fusarium* sp. F2. The ROI production is believed to trigger the activation of systemic resistance by means of defense activation. The higher levels of expression observed in the resistant line (Pongsu Seribu) indicates the potency of the defense machinery to safeguard the plant from invasion.

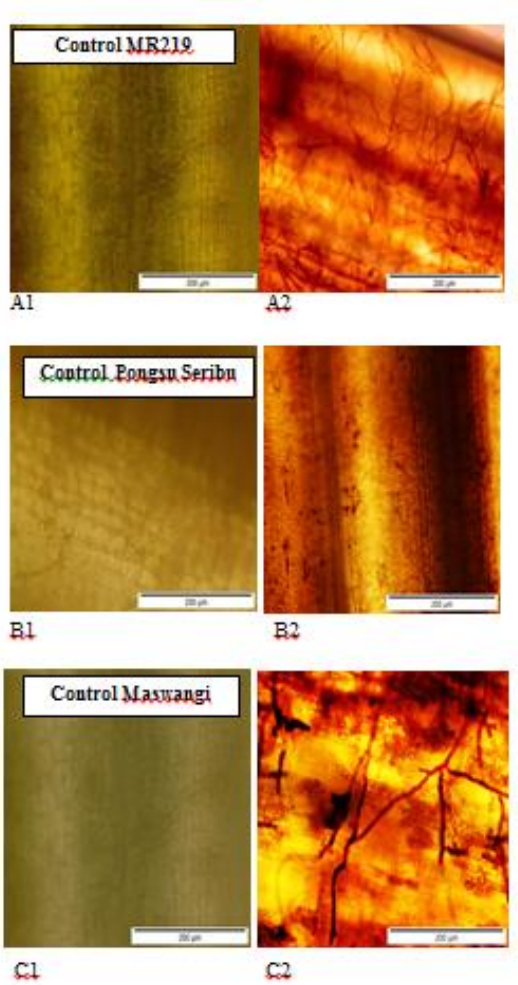

Fig 4. Interaction between plant and pathogen and colonization leaves MR219, Pongsu Seribu and Maswangi by *Fusarium* sp. F2. (A1) Control MR219 Non-infected. (A2) *Fusarium* sp. F2 showed the cellular proliferation of the fungus into the host. *(*B1) Control Pongsu Seribu non-infected. (B2) *Fusarium* sp. F2 shows the cellular proliferation of the fungus into the host. (C1) Control Maswangi non-infected. (C2) *Fusarium* sp. F2 in Maswangi showed high levels of cellular proliferation of the fungus into the host compared to the other two varieties.

Besides, the extent of defense proteins proteins expressed, the speed, at which these proteins are expressed also determines the extent of resistance.

Materials and Methods

Host inoculation

MR219, Pongsu Seribu and Maswangi were three rice varieties used in the molecular and cellular experiments. Tissues of these rice varieties were inoculated with 5mm fungal plugs of *Fusarium* sp. (F1 and F2 species) by placing the plug at the base of the stem and then wrapping this infection site with cotton moistened with distilled water and 0.5% gelatine. Humidity and moisture at the site of infection was maintained by covering the site with aluminum foil. The plants were monitored and disease symptoms were observed 72 hours post inoculation.

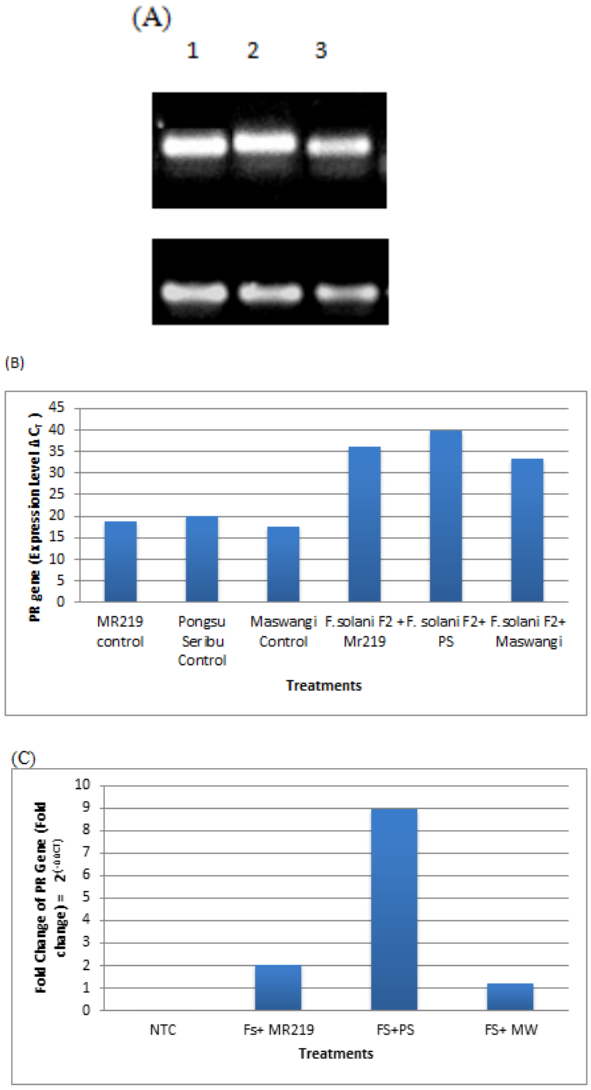

Fig 5. Expression level of *PR-b1* gene at 72 hours post inoculation with *Fusarium* sp. F2 in rice. (A) Expression level observed via RT-qPCR for post inoculation in MR219 (1), Pongsu Seribu (2) and Maswangi (3). Ç-actin gene was used as a control housekeeping gene in this assay. (B) *PR-1b* gene Expression level (Δ C_T) values obtained from calculating the expression levels in three rice varieties 72 hours post inoculation with Fusarium sp. F2; and (C) Fold Change ($2^{-(\Delta\Delta CT)}$) of *PR-1b* as calculated for all three rice varieties 72 hours post inoculation with *Fusarium* sp. F2.

DAB (3, 3'-Diaminobenzidine) assay and microscopic evaluation technique

Two-week-old young rice plants were monitored for any infections or pest prior to introduction of pathogen. Plug cultures were inoculated as mentioned in host inoculation technique and diseased leaves were harvested 72 hours post-inoculation (hpi) and incubated in 1 mg mL^{-1} DAB-HCl solution (pH 3.8), and left in the dark for 8 hours. This was then followed by a clarification protocol to remove chlorophyll content. The leaves were placed in clearing solution of Trichloroacetic acid (0.15% (w: v) plus ethanol–chloroform (4:1, v: v) for 2 days. Infected tissues were observed under epifluorescence microscopy (excitation filter 485 nm, dichroic mirror 510 nm, barrier filter 520 nm, TCS SP; Leica Microsystems, Wetzlar, Germany). In scoring the

level of interaction between host-pathogen, a scale was developed by Odile et al., (2008). According to this scale when appresoria was established and cellular response was observed in infected epidermal tissue with hypersensitive response (HR), this would be recognized as a Type 1 interaction. Type 2 interactions however would involve the production of ROIs which results in cells staining positively in the infected epidermal cell post HR. Type 3: DAB staining is observed in infected epidermal cell and extends into the neighboring epidermal cells and produces multiple HR in cells. Finally Type 4 would entail DAB staining which correlates to the presence of fungal intracellular infection where hyphae is observed inside infected epidermal cells.

ITS-rDNA analysis of Fusarium* sp. *F2

DNeasy Plant Mini Kit (Qiagen) was used to extract the genomic rDNA according to manufacturer's recommendation. ITS1-5'-TCTGTAGGTGAACCTGCGG-3' and ITS4-5'-TCCTCCGCTTATTGATATGC-3' were selected for use in this ITS-rDNA analysis (Chakraborty et al., 2010; White et al., 1990). PCR amplification of the ribosomal DNA of internal transcribed spacer regions was conducted using the following parameters: the initial denaturation for 4 minutes at 94 ºC, followed by 35 cycles of 94 ºC for 1 minute, 53 ºC for 1 minute and 72 ºC for 2 minutes and a final extension at 72 ºC for 10 minutes. The PCR products were purified via QIAquick PCR purification kit (Qiagen). The purified amplicon was then sequenced and the consensus sequence (BioEdit Program ClustalW-http://www.ebi.ac.uk/Tools/msa/clustalw2/) was served as a query against all the 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 relationship of Fusarium* spp.**

Fifty-six nucleotide sequences of *Fusarium* spp. was downloaded from NCBI for the purpose of this analysis. These sequences together with the sequence of *Fusarium* sp. F2 (57 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 of sequences (Hall, 1999). The sequences were then fed into MEGA6 (Tamura et al., 2011) using the Partition homogeneity test. This programme was used to obtain the most robust tree. For this purpose Neighbor Joining, Maximum Parsimony and Maximum Likelihood analysis were conducted. However in studying the molecular phylogenics of *Fusarium* spp, the Maximum Likelihood tree was chosen to represent the true tree, with the smallest number of nucleotide changes to fit the observed sequence data (Nakhleh et al., 2005).

RNA extraction from infected rice tissue

RNeasy Plant Mini Kit (Qiagen) was used to extract total RNA from the infected rice tissue according to manufacturer's recommendation.

Real-time RT-PCR of PR-1b gene

High Capacity cDNA Reverse Transcription Kit was used to reverse transcribe RNA to cDNA (Applied Biosystems) using random hexamers. The primer pair PR1b-F-5'-CGAGAAGAGCGACTAGGAGTAC-3' (22mers) and PR1b-R-5'-GCCTCTGTCCGACGAAGTTG-3' (20 mers) was generated to amplify the *PR-1b* gene while 5′F-GCGTGGACAAAGTTTTCAACCG-3′ and 5'R-TCTGGTACCCTCATCAGGCATC-3' was used to amplify the *Oryza sativa OsRAC1* (Accession Number AB047313; complete cds) Actin gene which was used as the housekeeping gene (Odile et al., 2008). Real-time PCR was performed using Fast SYBR Green Technology in the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems). The reverse transcription mixture (cDNA) was added to the PCR mix and the PCR reaction was carried out as follows: initial denaturation at 95^0C for 20 s followed by 40 cycles at 95^0C for 3 s and 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 for housekeeping gene was calculated using the regression line in the standard curve. The RNA amplification levels for reference gene and for all samples were determined as Ct (cycle threshold) values.

The relative changes in gene expression from real-time quantitative PCR experiment were determined via an alternative approach that involved a comparative ΔΔCT method (Livak and Schmittgen, 2001). The difference between the CT value of the target gene and the CT (Critical Threshold) value of the endogenous reference gene was used to calculate ΔC_T. The difference between the CT value of the target gene and the CT value of the endogenous reference gene was used to determine the ΔC_T value for each control. The $\Delta\Delta C_T$ value calculated the difference between the ΔC_T value of the target gene (sample) and the ΔC_T value of the endogenous reference gene (calibrator). The target gene expression level was normalized in samples (Fold change) = $2^{-(-\Delta\Delta CT)}$.

Discussion

Fusarium sp. F2 was isolated as the causative agent in rice stem tissue exhibiting wilt symptoms. The ITS-rDNA sequence of *Fusarium* sp. F2 when blasted against the NCBI database showed relatedness to non-FOSC/FSSC species isolates such as *F. chlamydosporum, F. incarnatum* and *F. equiseti*. There are three major species complexes of *Fusarium* sp. which are *Fusarium oxysporum* species complex (FOSC), *Fusarium incarnatum-equiseti* species complex (FIESC) and *Fusarium solani* species complex (FSSC). Based on the sequence relatedness, our isolate is most likely a FIESC (Balmas et al., 2010). In addition to being a complex, our isolate showed no relatedness is source of tissue but exhibited some relatedness to geographical distribution. The geospatial structure of *Fusarium* spp. has not been evaluated critically until recent discoveries of more complex species diversity was reported based on phylogenics which demonstrated that *Fusarium* spp. often comprise of multiple diagnosable species (O'Donnell et al. 1998b, 2000, 2004, 2008, 2009). Morphological species most commonly recovered from rice such as *F. oxysporum, F. solani* and *F. equiseti*, are now known to represent species complexes (O'Donnell et al. 1998a, 2000, 2009). Cryptic species, such as *F. oxysporum, F. equiseti* and *F. solani* have been reported as cosmopolitan and seemingly independent of climatic factors, while other species, such as *F. sambucinum, F. acuminatum, F. compactum,* and *F. longipes* were reported to exhibit limited distribution correlated with certain environmental factors (Backhouse and Burgess 1995, Sangalang et al. 1995). Following identification, the cellular level interaction between host-pathogen was observed via DAB assay where the production of ROIs was observed in

and around the infected epidermal cells. ROIs are known to be toxic to microbes (Thordal et al., 1997) where the oxidative burst that is seen in this experiment is a key factor in the elicitation of the defense response in host which results in the activation of defense pathways as well as disease resistance genes (Harman 2000; Jing et al., 2007; Lamb and Dixon 1997; Lee et al., 2004; Moore et al., 2011; Yang et al., 2009; Yanjun and Shiping, 2010). In this study the effect of ROI on plant defense was monitored through the expression profiles of *PR-1b* gene where a direct correlation was found between ROI levels and defense gene expression. Previous reports have indicated that the efficient activation of PR genes results in improved resistance to disease (Tao et al., 2003; Vergne et al., 2007; Wen et al., 2003). This is in agreement with several studies where it has been proven that specialised defense mechanisms that respond to biotic stresses are known to induce the expression of broad spectrum antimicrobial agents such as pathogenesis-related proteins (Dong, 1998; Dangl and Jone, 2001; Glazebrook, 2001) which facilitates the elicitation of systemic acquired resistance (SAR) in plants (Harman 2000; Hayata et al., 2010; Jing et al., 2007). As observed here, the PR-1b protein is expressed variably in different rice varieties and this is largely correlated to the level of compatibility or incompatibility of the host-pathogen interaction.

Conclusion

In conclusion, the *Fusarium* sp. isolated from the wilt symptom exhibiting rice tissue was identified as a member of the FIESC through sequence and phylogenetic analysis. The complex showed no source tissue relatedness but was geographically related. *Fusarium* sp. F2 showed compatibility to all rice varieties used in this study which was shown by DAB assay where ROIs was produced at and around site of infection resulting in the activation of the defense response *in planta*. However the level of compatibility of host differed with the highest levels of ROI and gene expression being linked to the most resistant rice variety, Pongsu Seribu followed by MR219 and Maswangi.

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