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Isolation and Characterisation of *O. rufipogon Pib* Gene

Kalaivani Nadarajah[1]*, Amatul Samahah Md. Ali[1] and R. Wickneswari[2]

[1]School of Biosciences and Biotechnology, [2]School of Environment Sciences and Natural Resources, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi Selangor Darul Ehsan, Malaysia.

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***Magnaporthe grisea* is known to be a major problem in the rice industries. *Pib gene* codes for resistance against the pathogenic fungi. *Oryza rufipogon* is a wild type variety of paddy. It is able to live under extreme condition and resistant towards a lot of rice diseases. In this study a ~ 6000 bp (6062bp) *Pib* gene was isolated from *O.rufipogon via* PCR using specific set of primers designed from the *Pib* gene of *O. sativa japonica* cv Nipponbare adopting the primer walking strategy. In addition to the above, the complete cDNA sequence of *Pib* was obtained via RACE-PCR. The cDNA is 3978 bp (ORF 3426; 5'UTR 307; 3'UTR 245) and has a predicted molecular weight of 52 kDa and a pI of 6.2. Meanwhile, Southern hybridisation studies showed that the *Pib* gene is a member of a small gene family where two to four bands were produced when enzyme fractionated genomic DNA was screened with the NBS-LRR region of the *Pib* gene. When the translated protein sequence was subjected to a motif analysis, three NBS sites; GGVRSLVVQ, LVVLNDL and GSRVLV (240 to 327) and fifteen leucine-rich repeats were detected between and 328 to 1088 amino acid of the gene. The NBS sites are believed to be involved in signal transduction, while the LRR sites are important in resistance specificity. In addition to the above, the *Pib* gene contained the 2Fe-2S ferredoxin and EFG-like protein motif. Multiple sequence alignment analysis using ClustalW, showed that the amino acid sequence of the putative *Pib* protein of *O. rufipogon* is highly identical to the amino acid sequences of *Pib* protein from *O.sativa japonica* (92.0 %), *O.sativa indica* (94.0%), *Zea mays* (68%), *Triticum aestivum* (55%) and *Arabidopsis thaliana* (42%). Phylogenetic analysis of the *Oryza* genus, cereals and *Arabidopsis thaliana* indicates that these plants originate from the same ancestor, but have diverged through million years of evolution. The expression profiles of *Pib* gene was examined in *Oryza* species, post infection where the expression of the 1.1kb fragment of NBS-LRR region was detected in *O.rufipogon* and the transgenic lines.**

Key words: *Pib* gene, *Oryza rufipogon*, disease resistance, characterisation.

Magnaporthe grisea is an airborne foliar fungus that causes blast disease in rice. The fungus infects leaves during early growth stages of rice plant, giving lesions which are followed by premature leaf senescence of infected tissue, especially in case of heavy infections. After heading, the pathogen infects the panicles or the neck node. Rice blast has been the most important diseases in rice (*Oryza sativa* L.) in Japan and in other rice-growing countries, where it is responsible for more than 50% of loss due to disease (Agrios, 2005; Smith, 1999). The use of resistant cultivars has been the most economical and effective way of controlling this disease. However, the useful lifespan of many cultivars is only a few years, due to the breakdown of the resistance in the face of high pathogenic variability of the fungus (Babujee and Gnanamanickam, 2000). The inheritance of host resistance to rice blast has been studied extensively (Lin *et al.,* 2007; Chada and Gopalakrishna, 2005; Chen *et al.,*

* To whom all correspondence should be addressed.
E-mail: vani@ukm.my

2003). At least 30 resistance loci have been identified in rice (Wang *et al.,* 2009; Wang *et al.,* 2001; Wang *et al.,* 1999; Miyamoto *et al.,* 1996). Several genes for blast resistance have recently been mapped by using restriction fragment length polymorphism (RFLP) markers (Babujee and Gnanamanickam, 2000; Rybka *et al.,* 1997; Miyamoto *et al.,* 1996;). At present, however, these loci are being characterised at a molecular level (Kellogg, 1998; Sasaki, 1998).

The rice blast resistance genes confer a high race specific resistance to blast fungus races. The specific interaction between resistance genes in rice plant and avirulence genes in the fungal pathogen can be well explained by the gene-for-gene interaction. The molecular analysis of the corresponding avirulence genes in blast fungus and its genome analysis are the most advanced in the pathogenic fungi. Several avirulence genes from the fungus have been cloned (Kang *et al.,* 1995; Sweigard *et al.,* 1995). Identification and isolation of both resistance genes from the host and avirulence genes from the pathogen can be expected to help clarify the molecular mechanisms underlying specific host-pathogen recognition in plants. The accumulation of information regarding resistance gene-mediated host defense mechanisms will facilitate engineering of genes conferring durable resistance to a broad spectrum of pathogens. Plant resistance genes have been extensively studied in recent years, and more than 20 resistance genes in various plants have been cloned by map-based cloning or transposon tagging strategies (reviewed in Baker *et al.,* 1997).

The dominant gene *Pib*, which confers high resistance to most blast races, has been mapped to the distal end of the long arm of chromosome 2 (Shinoda et al. 2001) and the *Pib gene locus is between* RFLP markers i.e. RZ 123, RZ 213 and G 1234. In previous studies the gene has been isolated via map-based cloning and the analysis of the gene showed that it produced several resistance providing products such as detoxification enzymes, kinases and NBS/LLR proteins (Shang *et al.,* 2009; Lin *et al.,* 2007; Wang *et al.,* 1999). The NBS site is essential in the signal transduction involved in the process of recognition and response towards the invading pathogen (Young & Innes 2006). The leucine rich repeats contained within the leucine rich domain on the other hand is responsible for the resistance specificity between host and pathogen. Any change within these sites will result in conformational changes that will result in either the activation or the silencing of the resistance mechanism towards the pathogen (Shang *et al.,* 2009; Lin *et al.,* 2007; Wang *et al.,* 1999).

Here we report the isolation and characterisation of the *Pib* gene from *O. rufipogon* and then proceed to transform and analyse the efficiency of this gene in affording resistance towards *Magnaporthe grisea* infections in the transformed cultivated rice variety MR219.

MATERIAL AND METHODS

Plant material and inoculation of *M. grisea*

The *O. rufipogon* harboring the blast resistant allele against *M.grisea* var. *oryzae* was selected for this study. Seeds of rice cultivar MR219, *O. sativa japonica* Nipponbare and *O.rufipogon* were kindly provided by Malaysian Agricultural Research Institute. The *indica* rice accession MR219 and *O. sativa japonica* Nipponbare were used as the susceptible varieties in comparison with *O.rufipogon* when inoculated. Seeds were germinated and plants were grown under greenhouse conditions at 24–30 ^{0}C with a light and dark cycle of 16 and 8 h, respectively. Plants grown in greenhouse for approximately 2–4 weeks (four-leaf seedling stage) were used for disease evaluation. For fungal inoculations, conidia of *M. grisea* was collected at a concentration of 10^5 conodia ml^{-1} and sprayed onto plants that were at 3rd or 4th leaf stages. Inoculated plants were kept moist by leaving pots in water trays and spraying the chamber to keep it moist for the initial 24 hrs. The plants were then removed and allowed to grow in normal growth environment. Tissue samples were harvested for a time course analysis post inoculation to examine expression profiles of the *Pib* genes in the three rice species.

Nucleic Acid Isolation

Rice genomic DNA was prepared from leaf samples that were harvested and immediately frozen in liquid nitrogen and stored at -80 ^{0}C until processed. DNA was isolated using DNeasy Plant Mini Kit (Qiagen, Valencia, CA) from 0.1 g of leaf tissue following the manufacturer's instructions. For Southern blot analysis, genomic DNA was

extracted using a conventional CTAB protocol. Total RNA was extracted from 0.1 g of leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) in accordance to the protocol supplied by the manufacturer. Total RNA was obtained from leaf samples collected from plants at the four-leaf seedling stage, immediately frozen in liquid nitrogen and stored at -80 ^{0}C until processed. Plant tissue was kept frozen and ground in liquid nitrogen.

Primer design for amplification of *Pib* gene from *Oryza rufipogon*

As *O. rufipogon* is from the same ancestry as *O. sativa japonica* Nipponbare (Bautista et al. 2001), we used the genomic sequence of *O. sativa* to design primers for the amplification of *Pib* from *O. rufipogon.* The *Pib* sequence of *O. sativa* var *japonica* (EF642429.1) was obtained from NCBI (*National Centre of Bioinformatics Technology*) at http://www.ncbi.nlm.nih.gov/ . This sequence was then fed into the *Primer3Plus* at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi . The *Primer3Plus* at the end of the analysis provided several primer pairs with the stipulated GC content, product size, annealing temperature and primer length.

PCR Amplification of *Pib* Gene

PCR amplifications were carried out in a 100 µl reaction containing 100 ng genomic DNA, 0.2 µM of each primer , 0.25 mM each of the four dNTPs , 2 mM $MgCl_2$, 1 x *Taq* buffer and 2.5 U Finnzymes – DyNAzyme EXT™ system. Primer pairs were designed using the above programme for use in PCR reaction to amplify the *Pib* gene from both *O.rufipogon* and *O.sativa indica* (MR219). Additional primer sets were designed via primer walking strategy based on the sequences obtained and the sequence derived from NCBI (Table 1). PCR was performed in PTC-0200 DNA Engine (MJ Research, USA) with initial heating and denaturation conducted at 94º for 25 sec. This was followed by annealing at 50°C for 10 sec and elongation at 72°C for 1.5 min. This was conducted in 35 cycles and the last elongation reaction was

Table 1. Primer Pairs Obtained from the Primer3Plus Programme

Primer	Sequence	Tm (°C)	GC (%)
Pib1(20 bp)	5' CTGGGCAAATCTAGCAAATG 3'	63	45
Pib2(20 bp)	5' CAATGCCGAGTGTGCAAAGG 3'	60	55
Pib3(20 bp)	5' CAACGATCCAATCATGCACG 3'	58	50
Pib4(20 bp)	5' ATGACACCCTGCGATGCAAG 3'	61	55
Pib5(20 bp)	5' ATGACACCCTGCGATGCAAG 3'	56	55
Pib6(20 bp)	5' TCAGGTTGAAGATGCATAGC 3'	58.5	45

RACE-PCR and RT-PCR PRIMER Pairs

PRIMER	Primer sequence	Tm	GC %	Size of Fragment
5'NBSLRR23'	F2-ATGGTCCTTTATCTTTATTG R2-TTGCTCCATCTCCTCTGTT	58	45	2.0
5'NBSLRR3'	F1–GTAGGTACATCAAGGACGAG R1-AGGTGTTCGCCCCGCAGGT	62	55	1.6
NBSLRR3'	F6-TCGTCACAGAATAATAATCAA R6-GGTTTCCCACTCTCTTACA	57	55	1.5
NBSLRR3'	F3-AGATGTTAGTAGCAAGTTCC R3-TGTCAGTTATGTCCAAAGTG	60	45	1.3
NBSLRR	F2-CACTGTTGTAGCGGAGGAGA R2-CAGTACGCGATTTTCATTGTTC	63	56	1.1
NBSLRR4	F4-ACTTTGTTGTGCTTGATAAC R4-ATGGTGAACGGTATCTGTAT	57	40	1.0
LRR53'	F5-AGAAAACTGGCTGGCTGTAG R5-TCACGTAGAGGAAAGAAAACC	60	40	0.7

at 72ºC for 10 mins. Amplified PCR products were electrophoresed in 1% w/v agarose gels (Sigma-Aldrich, USA), stained with ethidium bromide and product size determined using a DNA 1kb Plus marker (Promega, USA) under UV transilumination. PCR fragments of interest were excised under UV illumination and purified using QIAquick Kit (QIAGEN, Hilden).

RACE-PCR

The 5' and 3' rapid amplification of cDNA ends (RACE) experiments were conducted using the GeneRacer Kit (Invitrogen, CA) according to manufacturer's instructions. First strand cDNA was synthesised from both total and messenger RNA obtained from non-inoculated MR219 plants. Gene specific and nested gene-specific primers used for amplification are listed. 5' and 3' RACE products were cloned into vector pTZ57R/T using InsT/AcloneTM PCR Product Cloning Kit (Fermentas Life Sciences, Canada), followed by transformation into One Shot Chemically Competent *E. coli* cells (Invitrogen, Carlsbad, CA). Plasmid DNA of individual positive colonies was extracted, purified and confirmed by sequence analysis.

RT-PCR

RT-PCR reactions from samples collected during the time course assay were performed using a two-step RT-PCR method which allows the amplification of multiple fragments from a single RNA source. To eliminate genomic DNA contamination, total RNA samples were treated with TURBO DNA-free (Ambion, Austin, TX) according to the manufacturer's recommendations. For gene expression analysis by one-step RT-PCR, 5 mg of DNase-treated total RNA was reverse transcribed to cDNA and PCR amplified with SuperScript III One- Step RT-PCR Kit with Platinum *Taq* (Invitrogen, Carlsbad, CA) using gene-specific primers (Table 1). A control reaction (minus RTase) was included to exclude spurious amplifications due to potential presence of contaminating DNA.

Cloning of DNA Fragments

The purified PCR fragment of *O. rufipogon* and *Oryza sativa indica* was cloned into plasmid vector pTZ57R/T using InsT/AcloneTM PCR Product Cloning Kit (Fermentas Life Sciences, Canada) and transformed into One Shot Chemically Competent *E. coli* cells (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The transformed colonies were screened and confirmed for the presence of insert by colony PCR. Plasmid DNA was isolated by adopting the protocol described by Birnboim and Doly (1979) with necessary modifications. Sequencing of the purified plasmid was done in First Base, Malaysia using the ABI – PRISM 377 (Versi 3.4.1).

Bioinformatic Analysis of Isolated *Pib* Gene

The nucleotide sequences of the cloned fragments were translated into amino acid sequences using ExPASy (Expert Protein Analysis System) Translate Tool, a proteomics server of Bioinformatics (http://www.ebi.ac.uk/emboss/transeq/). The deduced amino acid sequences were subjected to multiple alignment analysis via the ClustalW Program (http://www.ebi.ac.uk/Tools/clustalw) or T-coffee (http://www.ebi.ac.uk/t-coffee) (Thompson et al. 1997). Motif analysis was conducted using the InterProScan Program (http://www.ebi.ac.uk/Tools/InterProScan/). The amino acid sequences of the *Pib* gene of *O.rufipogon's* was compared with protein sequences deposited in the GenBank using BLASTP algorithm (http://www.ncbi. nlm.nih. gov/BLAST/) (). Pair wise comparison of gene sequences with *O.sativa japonica* var Nipponbare was made using BL2SEQ algorithm (Tatusova and Madden, 1999). In addition to these, amino acid sequences of the *Pib* gene isolated from *O. sativa indica* and *O.rufipogon* was compared to the sequences *Pib* like genes of *Arabidopsis thaliana, Triticum aestivum* and *Zea mays* that were retrieved from the GenBank. These sequences were used in the phylogenetic analysis of this gene via the Pyhlip programme at (http://evolution. genetics. washington.edu/phylip.html).

Southern Blot Analysis

Ten micrograms of genomic DNA from rice cv. MR219, *O.sativa japonica* and *O.rufipogon* was digested with three restriction enzymes (*Eco* RI, *Bam* HI, and *Sac* I) and fragments were electrophoresed in 0.8% agarose and transferred to a positively charged nylon membrane Hybond-N+ (GE Healthcare, Piscataway, NJ). The NBS-LRR *Pib* gene probe was random-prime labelled with digoxigenin (DIG) labelling mixture (Roche, Mannheim, Germany). For the preparation of the digoxigenin-

labelled probe, the amplified *Pib* gene product obtained from *O.rufipogon* DNA using specific primers was recovered using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into the pJET1.2/blunt vector using the CloneJET PCR Cloning Kit (Fermentas, Glen Burnie, MD). Subsequently, the recombinant plasmids were directly used as template for PCR synthesis of the DNA probe. The probe was 1100 bp long, corresponding to nucleotides 3196–4201 of genomic *Pib* sequence from Nipponbare. The colorimetric method was used for the detection of the DIG-labeled nucleic acids. All test procedures and buffer preparations were carried out according to the instructions of the manufacturer.

RESULTS AND DISCUSSION

Isolation of *O.rufipogon* and *O. sativa indica Pib* Gene

One of the challenges faced by the agricultural sector is to reduce loss of produce through diseases (Maruthasalam *et al.* 2007). Loss of yield due to disease has been a major problem encountered by countries producing rice. Therefore a number of research groups have initiated work in generating diseasse resistance to the various rice diseases through the conventional approach as well as the use of transgenics (Tanksley & McCouch 1997). The wild rice, *O.rufipogon* is used as a source of disease resistance genes as this rice species has been shown to have resistance against both biotic and abiotic stresses.

As there has always been a high level of homology between the *Oryza* species, the sequence of the *Pib* gene sequence of *O.sativa* var japonica (EF642429.1) was used to design specific primers to amplify the *Pib* gene from the genome of *O.rufipogon* and *O.sativa indica.* The *O.sativa indica* sequence was obtained from NCBI (*National Centre of Bioinformatics Technology*) at http://www.ncbi.nlm.nih.gov/ and analysed through the *Primer3Plus* at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi . The *Primer3Plus* provided several primer pairs with the stipulated GC content, product size, annealing temperature and primer lengths. As it was not possible to obtain the entire gene through the use of one primer set, the primer walking strategy was adopted to produce the entire gene from *O.rufipogon* genome. Since the sequence for *Pib* gene in *O.sativa indica* was incomplete i.e. only partial cds was available, we decided to obtain the complete sequence of the gene in our local elite rice cultivar, MR219, for use in this study.

The genomic DNA of *O. rufipogon and O.sativa indica*, was subjected to PCR reactions with the primer pairs listed in Table 1 to obtain the genomic sequence of *Pib* gene. The gene product obtained from this reaction was in the range of ~ 6kb as estimated by the Primer3Plus Programme. Similarly the total RNA from the rice species was used as template to obtain the cDNA sequence of the gene. Using specific primer and nested primer sets, the cDNA containing the complete ORF sequence of the *O.rufipogon* and *O.sativa indica Pib* cDNA was obtained using RACE PCR. The purified PCR products of both rice varieties was sequenced at First BASE Laboratories Sdn. Bhd., and the sequence reads were obtained; 3978 bp for *O.rufipogon* and a 3776 bp sequence read for the *O.sativa indica* Pib cDNA.

Sequence Analysis of *Pib Protein in O. rufipogon*

The motif analysis on the *Pib* gene was conducted via the InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) bioinformatic tools. Two motifs were detected through this analysis; they are the 2Fe-2S ferredoxin and EGF (epidermal growth factor) like motif.

Ferredoxin is a protein that is involved in the transfer of electrons in metabolic reactions (Masson & Cammack 1992). There are various groups of feredoxins; one of these groups is found in the membrane of chloroplast, where it functions as the electron transporting protein in photosynthesis. EGF (epidermal growth factor) however is usually found in animals. The highly conserved EGF gene produces a protein that is thirty to forty amino acids in size (Appella et al. 1988). The function of this protein is unclear; however, this motif has been identified in the domains of extracelular membrane proteins and thus has been suggested to be bound to secretory proteins. Both these motifs have no known function in resistance (Fig. 1C).

The ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) analysis conducted however showed that the *Pib* gene has the NBS-LRR regions that have been linked as signature

(A)

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                        p-loop
PibORUF          HPINPHGGVRSLVVQFYANSRQGQLPAATFALSGVSVLYKKQRDAGSSSEAQDDLLKDF      240
PIBOSIND         HSFDANGGIGSLVRQFKANSREGTGKTPQGTPSGVSVLN------G-EMEAQDYLLHDF
PIB_OSJAP        HPFSPRGGVQSLVNQLHANQGVEALLEK-F---------------G-EKEAQDYLAKKF
PIBSORBI         HPFSPKDGVQSLVNQLHANQGVEALLEKQF---------------G-EKTEQDYLLMEF
PIBTEASTIVUM     CPFNSVGGARSIIRQFYINLLQDPVENMDFQVL-----------RGMGMKKENDLVDEF
LRR-PK_ATHAL     TGSIPEGIVNCTAFQVLDLSYNQLTGEIPFDIG-----------G---FLQVATLSLF

                           Kinase2                Kinase3a
PibORUF          NGYVDTKKYLVVLNDLSTIEEWDAIKPYLRKNHNGSRVLVCTQQHEVASCCTCTDDK---
PIBOSIND         TGYVTNKKYLVVLNGLSTIEEWDWIKTYLPNNHNGSRVLVCTQQAEVASCCTCTDDKYKV
PIB_OSJAP        NGCVNDKKCLIVLNDLSTIEEWDQIKKCFQKCRKGSRVIVSSTQVEVASLCAGQESQ---
PIBSORBI         NGYVNGSKFLIVLNNLSTIEEWDQIKKCFPNNKKGSRVLVSSTQVEVASLCAGQESQ---
PIBTEASTIVUM     KSYLNDKSYLIVIDGMSTTEEWDQIKPCFPNNKRGSRVIVSTKQVEVASLCAGPQNV---
LRR-PK_ATHAL     QGNQLSKKIPIVINNMQALAVLDLSGNLLSGSIPGSIVTVTEKLYLHSNKLTGSIP--
                                                                          327
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NBS

328 amino acid to 1088 amino acid (*Pib* gene in *O.rufipogon*)

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LDQLRMLRVLDLEDVTFLLTQKRFD
ALLCHKYLSIGYSSIYSLPPR
IGKLOGQILNMLYIAAPSE
ISKQCLRCSRKVYDNFSLNIIP
IAELHMATKSCWSESFGVKVPG
IEKLSSQSLVNAALPLGA
AELHMATSCWSESGVKPKG
LGHLSKLRKVITKGSKEKKCKILYAA
LASIETCLDSISSPPL
LRTLGLNGSLEEMPNW
LRTLGLNGSLEEMPNWLGA
LPNLLYLYLGEKLVFKNLTLR
IYELDQLREMRFGSSPLLEKIELSCC
HGHHLPRKESLVPRYKSSK
VLRMDSDRRHDLGAEAGGLV
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Leucine Rich Domain

LHMSQAKQSHKLVVIQFTTSRCPASRYIAPAFTEYAKEFAGAVFIKVNVDSDE
ESVTDWYDIEGIVPTFFFVKDGEKIDKIPGANKELLRAKIRRHTASPYFLR

TRX Domain

(B)

ID No	Organism	% identity (amino acid)
EF642429	*O. sativa japonica*	92 %
EF642428	*O. sativa indica*	94 %
AY217116	*Triticum aestivum*	55%
AY986485	*Zea mays*	68 %
BT005790	*Arabidopsis thaliana*	42 %

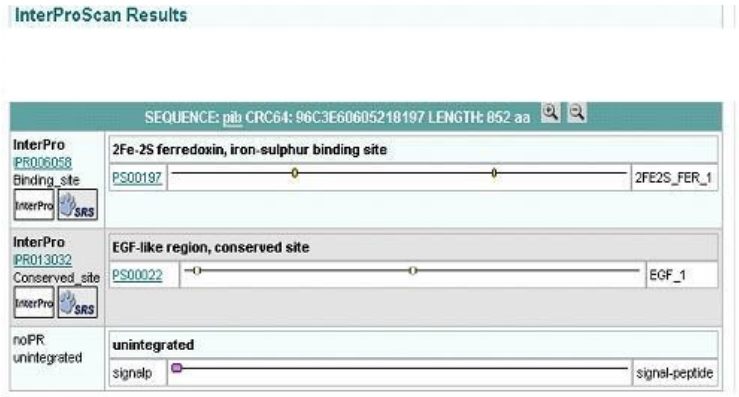

Fig. 1(A). Deduced Amino Acid Sequence From the Pib Proteins of *O.rufipogon* in Comparison with Other *Oryza* Species, Cereals and *A.thaliana*. The NBS domain contains three conserved motifs (kinase 1a (P-loop), kinase 2 and kinase 3a) that are shown underlined. The C-terminal leucine-rich domain (LRD), shown separately from the rest of the sequence contains 15 imperfect repeats, which are aligned according to the potential consensus LxxLxxL. At the C-terminal region the thioredoxin (TRX) domain is shown in bold face. **(B)** percentage of similarity between species at the amino acid level. **(C)** Motifs detected in addition to NBS-LRR and TRX.

motifs for disease resistant genes. All other sequences of *Pib* or *Pib* like genes in cereal used in this analysis were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Previous studies by Wang *et al.* (1999), had classified the *Pib* gene as a NBS-LRR (nucleotide binding site-leucine-rich repeat) containing resistance gene. Both these sequences were subjected to bioinformatic analysis and the results of BlastN and ClustalW analysis showed that the putative *Pib* gene from *O.rufipogon* has 91% identity to *O. sativa japonica* (Accession No EF642429.1) and 93 % identity to *Oryza sativa indica* MR219 sequence (Table 2). However the level of homology between the sequences of *Pib* genes in other cereals and *Arabidopsis thaliana* was relatively low i.e. where the *O. rufipogon Pib* protein was 62.9% identical to *Triticum aestivum,* 46.9% to *Zea mays* and 35.4 % to *Pib* protein of *Arabidopsis thaliana.*

Table 2. Blastn Analysis on the Nucleotide Sequence of the Putative *O. rufipogon Pib* Gene with Other *Oryza and Cereal Pib* Gene Sequences

Organism	Accession	% identity	E value
O. sativa indica		93%	0.0
O.sativa japonica	EF642429	91%	1e-151
Triticum aestivum	AY217116	62.9%	1e-231
Zea mays	AY986485	46.9%	2e-105
A. thaliana	BT005790.1	35.4 %	3e-149

The nucleotide sequence of the Pib cDNA encoded a 3426 bp ORF that was flanked by 5'- and 3'-untranslated regions of 307 bp and 245 bp, respectively. Fig. 1A shows the derived 1142 amino acid sequence for the *Pib* gene. The protein contains a predicted NBS. The sequences GGVRSLVVQ (187±196), KKYLVVLNDL (247±256) and GSRVLV (275±284) correspond to the kinase 1a (P loop), 2 and 3a consensus motifs, respectively (Grant *et al.,* 1995). The C-terminal region of the Pib protein is composed of 15 imperfect leucine-rich repeats (LRRs), from amino acids 327±1088 (Fig. 1). These vary in length from 16 to 26 (Fig. 1A and B). The LRRs show a consensus which is a reasonably good match to the consensus motif LxxLxxLxxLxLxx (N/C/T)x(x) LxxIPxx observed in all R genes encoding proteins containing cytoplasmic LRRs (Jones and Jones, 1997). These structural characteristics suggest that the *Pib* gene product interacts with other proteins in a defense-reaction signal transduction pathway (Baker *et al.,* 1997).

In addition to the conserved NBS-LRR domain, the Pib protein contains a thioredoxin (TRX) domain. While we have no evidence to support a hypothesis that the Pib protein with a TRX domain plays a role in pathogen recognition, there have been reports that have implicated the role of this domain in pathogen recognition (Costanzo & Jia, 2009). In plant defense responses to biotic and abiotic stresses in tomato *Cf-9,* TRXs has been shown to interact with the NBS-LRR proteins in mediating the resistance response (Nekrasov *et al.,* 2006; Rivas *et al.,* 2004). In this report TRX domain has been shown to act as an independent adaptor protein recruiting a protein kinase (ACIK1) upon elicitation with the Avr9 peptide. To date no strong evidence has been provided to characterise NBS-LRR R proteins by the presence of a thioredoxin domain at their C terminus.

The cDNA sequence of *O.rufipogon* was compared against genomic sequence that revealed the location and size of the three introns in the genomic sequences. Fig. 2 shows the diagrammatic representation of the full cDNA sequence of *Pib*. The ORF Finder program in NCBI was used to predict the ORFs and location of introns and exons of the *Pib* gene. The programme provided us with the structure as in Fig. 2 where there are 4 exons and 3 introns, with the largest intron being intron 1 which is 1465 bp. Introns 2 and 3 are ; 147 bp and 472 bp respectively.

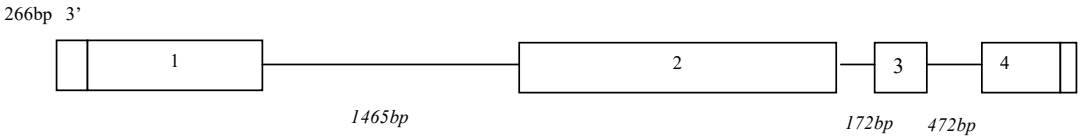

Fig. 2. The *O.rufipogon* Genomic Sequence of *Pib* Gene was Compared to the cDNA Sequence. The above diagramme illustrates the location of the introns based on gaps present in cDNA sequences when compared against the genomic sequence of *Pib* indicating the presence of intron sequences at the indicated locations

Phylogenetic Analysis

The results that were obtained from the ClustalW analysis (http://www.ebi.ac.uk/Tools/clustalw2/index.html), was used to determine the phylogenetic relationship between the rice, cereal and *A. thaliana* Pib proteins (Fig. 3). The phylogram obtained through Phylip (http://evolution. genetics. washington. edu/phylip. html), exhibited the there where two main clades, one for the Oryza species (clade 1) and the other for the cereals (clade 2). The distance analysis of the three rice species in clade 1 shows that *O. rufipogon Pib* gene is closely related to the gene in *O. indica* as shown by both these species being part of the same arm. Both these species are also related to *O. sativa japonica* but at a slightly lower level. The phylogram shows that *Triticum aestivum* and *Zea mays* exhibit a lower level of relatedness to the *Oryza* species (Fig. 3) where these two species have been separated into a separate clade indicating that these two species are more related to each other then they are to the *Oryza* species. However, they do share some degree of relatedness to the *Oryza* species, and in fact have been reported as to share a common ancestry with *Oryza* but are separated through the natural process of evolution. *Oryza* and *A. thaliana* likewise also share a common ancestry but have been separated by millions of years in evolution. It is believed that the Genus *Oryza* existed 130 million years ago in Gondawanaland (NAPPO 2003).

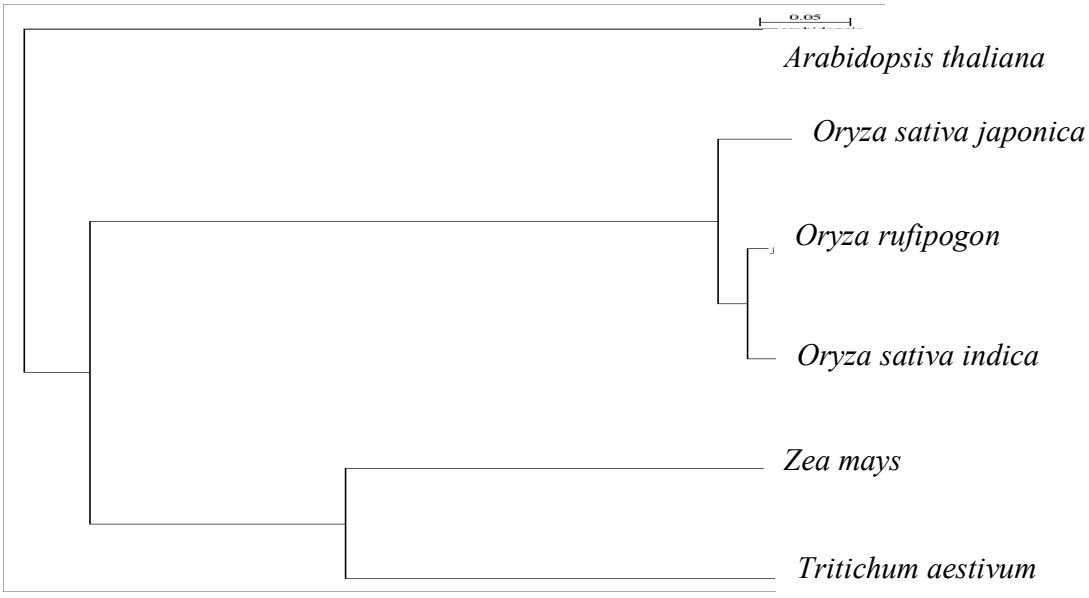

Fig. 3. Phylogenetic Tree Comparing the Pib Protein. Samples incle comparison between the *Pib* gene of *O. rufipogon, O. sativa japonica, O. sativa indica, Triticum aestivum, Zea mays* and *Arabidopsis thaliana.* The phylogram was built using the Phylip program

The *Pib* Gene is Member of a Small Gene Family

Several isolated R genes have been shown to be members of clustered gene families (Anderson *et al.,* 1997; Meyers *et al.,* 1998; Simons *et al.,* 1998; Song *et al.,* 1995). Southern hybridisation analysis using the above mentioned probe derived from the coding region of the *Pib* gene clearly indicated that the *Pib* gene is a member of a small gene family (Fig. 4). This is in accordance with previous reports that have shown this gene to contain between two to four copies of the gene.

Complementation of a Susceptible Rice Variety in Transgenic Plants

To examine the gene function of *Pib*, we performed a genetic complementation test. The complete cDNA of *Pib* was transformed into *Oryza sativa indica* MR219 calluses via *Agrobacterium* mediated transformation. A total of 50 kanamycin resistant plantlets were obtained and inoculated with *M. grisea*. Of these, 8 plants showed a resistant reaction indicating that *O.sativa indica*

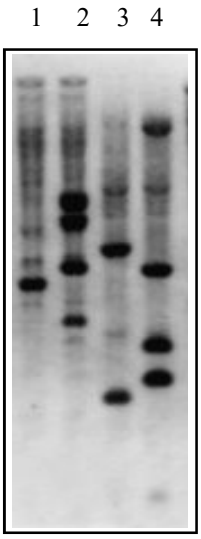

Fig. 4. Determination of Copy Numbers of *Pib* in *Oryza sativa japonica* and *Oryza rufipogon.* Lane 1 and 2; *Oryza sativa japonica* digested with *Bam* HI *and Eco*RI respectively; and lane 3 & 4; *Oryza rufipogon* digested with the same enzymes

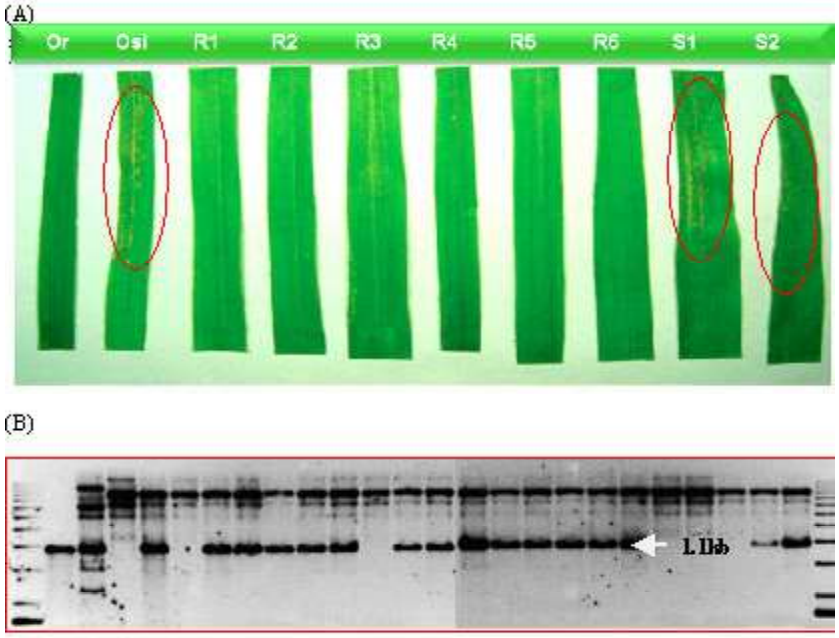

Fig. 5. Complementation Study Was Conducted on transformed *Oryza sativa indica* MR219. (A) shows a comparison of the leaves obtained from *O.rufipogon, O.sativa indica* MR219 wild type, putative positive transformants and negative transformants. The circles indicate the lesions observed on diseased leaves. (B) Southern hybrodisation to detect presence of *Pib* cDNA in transformed MR219 rice. DNAs (2 mg/lane) from susceptible line and resistant lines were digested with *Eco*R1 and subsequently blotted and probed with DIG labeled NBS-LRR region of *Pib* gene

MR219 was complemented by the 3978 bp fragment corresponding to the *Pib* gene (Fig. 5 - representing response to rice blast assay in MR219, transgenic MR219 and *O.rufipogon*). Fig. 5 shows that the lesions that are linked to the fungal infestations were seen clearly in the wildtype MR219 variety and in the susceptible transgenic lines (i.e. unsuccessful transformants). The wild rice and resistant transgenic lines of MR219 showed no disease symptoms post-inoculation (Fig. 5A). No disease symptoms were observed even one month post inoculation. The presence of the transgene in transgenic plants was done via Southern hybridization performed on digested DNA obtained from putative transgenic *Pib* plantlets (Fig. 5B).

The Expression Pattern of the *Pib* Gene

The expression of *Pib* was investigated by semi-quantitative analysis using the RT-PCR method. In this analysis, the transgenic lines, *O. rufipogon* and MR219 were tested using a primer set (NBSLRR primer set) that was to amplify the NBS-LRR region of the gene which is ~ 1kb in size (Fig. 6a). As shown in Fig. 6a, Pib was transcribed in the transgenic lines and *O. rufipogon* where the ~1kb band was observed. In the non-transformed and MR219, the ~1kb band was not evident. Each experiment was repeated at least three times.

In addition to the complementation studies mentioned above, further confirmation that the resistance was due to the presence of transgene was determined via PCR using the self (T1) progenies of one independent resistant and susceptible transformant each as template that post *M.grisea* inoculation. A semi-quantitative analysis was conducted via RT-PCR, where total RNA extracted at 24 hour time intervals for 3 days was used as template with the previously designed NBS-LRR specific primer for *Pib* (Fig. 6b). The resistance specificity of transformants was tested by spray inoculation of plantlets with *M. grisea*. A 3:1 (resistant to susceptible) segregation was observed for the four T1 lines. The resistant progeny produced a single band that was consistent in size and intensity from 0hrs to 72hrs. This showed that the expression of the incorporated *Pib* cDNA caused a constitute expression of the gene in the plant and thus was not regulated by the infection process of fungi. Thus, we conclude that we have managed to isolate and transform the functional *Pib* gene into MR219, and the *Pib* gene

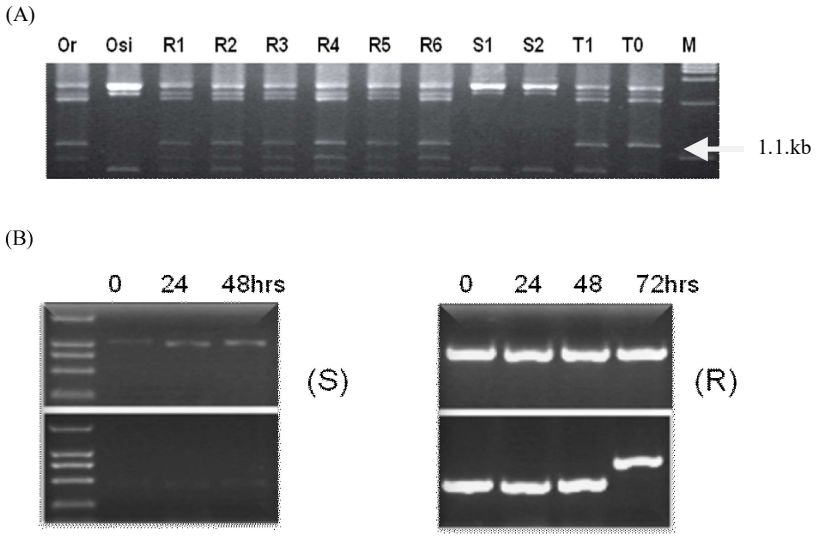

Fig. 6. Semi-Quantitative RT-PCR Analysis of *Pib* Gene Expression in Transgenic Lines Transformed with Complete Pib cDNA. (A) RT-PCR conducted on eight transgenic lines that were selected from the putative positive transformants post kanamycin screening. All resistant lines (R) showed the presence of a 1.1kb band. This band is absent in *O.sativa indica* and in susceptible lines.(B) One susceptible and one resistant line from (A) was selected for the semi- quantitative expression of *Pib* gene in transgenic lines post inoculation with fungi

did confer resistance to *Oryza sativa indica* MR219 but was independent of the fungal infection process as a trigger. Future research with the transformants will include testing against other pathogens of rice such as *Xanthomonas oryzae*, and Rice Tungro Bacilliform Virus as the *Pib* gene has been reported to be involved in generating broad spectrum resistance (Wang *et al.,* 2007).

In conclusion, the complete cDNA of *Pib* from *O.rufipogon* this simple isolation and characterisation studies show that this gene was sufficient in affording resistance to the local MR219 cultivar of rice. Through the genetic map that was generated in our studies with crosses between MR219 and *O.rufipogon,* we have identified a location on chromosome 8 for the *Pib* gene clusters. In this study we believe that we have only managed to isolate and characterise one out of the four genes in this cluster. In our future studies we hope to isolate the remaining 3 genes and to compare the resistance afforded by all four genes in the cluster. There will also be a study directed to determine if the entire cDNA is needed to provide resistance. We believe this gene also holds promise in providing broad spectrum resistance against diseases in rice.

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