Insertion of transposable elements in AVR-Pib of Magnaporthe oryzae

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Abstract

Background: Rice blast is a very serious disease caused by *Magnaporthe oryzae*. The avirulence (*AVR*) genes of rice blast are perceived by the corresponding rice-blast resistance (*R*) genes and prompt specific resistance. A mutantion in *AVR* is a major force for new virulence. Exploring mutations in *AVR* among *M. oryzae* isolates from rice-production fields could aid assessment of the efficacy and durability of *R* genes. We studied the probable molecular-evolutionary patterns of *AVR-Pib* alleles by assaying their DNA-sequence diversification and examining their avirulence to the corresponding *Pib* resistance gene under natural conditions in China.

Results: PCRs detected results from *M. oryzae* genomic DNA revealed that 162 out of 366 isolates collected from Yunnan Province contained *AVR-Pib* alleles. Among them, 36.1–73.3% isolates from six different rice-production areas of Yunnan contained *AVR-Pib* alleles. Furthermore, 36 (28.6%) out of 126 isolates had a transposable element (TE) insertion in *AVR-Pib*, which resulted in altered virulence. The TE insertion was identified in isolates from rice rather than from *Musa nana* Lour. Twelve *AVR-Pib* haplotypes encoding three novel *AVR-Pib* variants were identified among the remaining 90 isolates. *AVR-Pib* alleles evolved to virulent forms from avirulent forms by base substitution and TE insertion of Pot2 and Pot3 in the 5' untranslated region of *AVR-Pib*.

Conclusions: Our results revealed that *AVR-Pib* alleles suffered neutral evolution and divergences that led to overcome specific resistant *Pib* alleles under field conditions. The TE insertion in *AVR-Pib* was selected and adapted to rice and other Gramineae species.

Background

In the coevolution of plants and pathogens, the latter can adapt to the host and environment, and selection is the major evolutionary force. Up to now, the “arms race” and “trench warfare” hypotheses of coevolution between host resistance (*R*) genes and pathogen avirulence (*AVR*) genes have been proposed. In the principal hypothesis of the arms-race, the mutation of *R* genes and *AVR* genes is derived by directional selection. Contrarily, it is derived by undirectional selection in the trench-warfare hypothesis.

Rice blast is one of the most serious diseases in rice worldwide, which caused by the fungus *Magnaporthe oryzae*. Application of rice varieties with multiple resistant genes is the most important method governing the disease by an economical, environmentally and friendly manner. Up to now, more than 26 rice blast *R* genes have been cloned in rice: *Pita*, *PiCO39*, *Pish*, *Pi1*, *Pik*, *Pikp*, *Pikh/Pi54*, *Pikm*, *Pb1*, *Pid3*, *Pia*, *Pib*, *Pid2*, *Pit*, *Pizt*, *Pi2*, *Pi5*, *Pi9*, *pi21*, *Pi25*, *Pi36*, *Pi37*, *Pi56*, *Pi63* (www.ricedata.cn/gene/gene_pi.htm), *Pi64* [1] and *Pigm* [2].

Based on the gene-for-gene theory, rice *R* gene(s) can discern the corresponding *AVR* of *M. oryzae* and trigger the defense response to prevent invasion. So far, 12 *AVR* genes have been cloned in *M. oryzae*: *AVR-Pib* [3], *AVR-Pi54* [4], *AVR-Pi9* [5], *AVR-Pia* [6], *AVR-Pik/km/kp* [6], *AVR-Pii* [6], *AVR-Pizt* [7], *ACE1* [8], *AVR1-CO39* [9], *AVR-Pita* [10], *PWL1* [11], and *PWL2* [12]. The *AVR-Pib* gene of *M. oryzae* predicts the resistance efficacy of the rice *R* gene *Pib*. *AVR-Pib* encodes a putative secreted protein with 75 amino acids. *AVR-Pib* is perceived by the host as a Pib resistance protein and prompts the innate immune response [3]. Transposable element (TE) insertion, segmental deletion, absence and point mutations have been identified in *AVR-Pib* in Chinese rice-blast isolates, which has resulted in loss of avirulence [3]. Only TE insertion has been observed in *AVR-Pib* in 248 *M. oryzae* isolates from the Philippines [13].

The rice resistance gene of *Pib* is located on the long arm of chromosome 2 [14–15]. The cDNA length of the *Pib* gene contains 306 bp of 5' untranslated regions (UTRs), 3753 bp of open reading frames (ORFs) (containing 3 exons), and 229 bp of 3' UTRs, and encodes a NBS-LRR protein with 1251 amino acids [16]. In China, 16 out of 204 (7.8%) varieties have been detected hoarding the *Pib* gene in a mini-core collection of Chinese rice germplasm using their functional markers [17]. Also, 11 varieties have been identified with the *Pib* gene among 58 leading rice cultivars or hybrid rice parents in China using functional DNA markers [18]. *Pi-b* has been detected in 33 landraces among 176 landraces (18.8%) from Yunnan Province in China [19]. *Pib* gene homologs (87-bp deletion in exon 1 of *Pib*, leading to loss of the resistance function of *Pib*) have been identified in Yunnan Yuanjiang type of common wild rice (*Oryza rufipogon* Griff) [20]. In the Philippines, 32 out of 52 commercial rice varieties have been shown to contain *Pib* as detected by polymerase chain reaction (PCR) primers specific to *Pib* [13]. *AVR-Pib* has been detected in 463 among 621 (74.6%) isolates of *M. oryzae* collected from Jiangsu Province in China [21], whereas TE insertion was found in the 5' UTR of *AVR-Pib* [3, 13].

The effectiveness of resistance of *Pib* has been examined in different rice-production provinces in China. *Pib* exerts a high level of resistance to *M. oryzae* from Heilongjiang Province, and can be applied as a parent for resistance breeding in Heilongjiang Province [22]. *Pib* is moderately resistant in Fujian Province [23], but *Pib* exhibits low resistance in Guangdong, Sichuan and Guizhou Provinces [24–25]. Different resistance spectra of *Pib* were detected in 282 blast isolates collected from *Indica* rice- and *Japonica* rice-production regions in Yunnan Province [26]. Those results showed that the *Pib* gene exhibits different resistance to Chinese rice blast from different rice-growing regions. There were 42 out of 54 varieties containing *Pib* in Chinese elite hybrid rice varieties, two haplotypes of *Pib* were identified, and 9 different haplotypes of *AVR-Pib* were found among 27 *M. oryzae* [27]. The reactions of differential isolates on 54 rice varieties between the frequency of *AVR-Pib* haplotypes in the differential isolates showed a good correlation [27]. Those showed that the *Pib* gene widly distributed in rice varieties in China, and the adapt variation of *AVR-Pib* of *M. oryzae* are occurred.

Further clarification of the diversification and evolution of the *AVR* gene is useful for prediction of the effectiveness and durability of *R* genes for resistance breeding. Here, we wished to: (i) detect the diversification of nucleotide sequences of *AVR-Pib* alleles of *M. oryzae* under field conditions; (ii) determine the virulence function of *AVR-Pib* variations against the *Pib* gene; (iii) reveal the diversification and molecular-evolutionary principles of *AVR-Pib* alleles in *M. oryzae* in Yunnan Province.

Results

Effectiveness of the Pib **gene and frequency of** AVR-Pib **alleles**

The efficacy of the *Pib* gene was examined by pathogenicity assays. A total of 223 of the 366 *M. oryzae* isolates (4000 isolates were collected from six rice growing regions from 1997 to 2012 in Yunnan Province, and total of 366 isolates are selected from six rice growing regions as representative isolates.) tested were avirulent to the *Pib* gene-containing rice monogenic line IRBLb-B (Table 1). The percentage of avirulent isolates to *Pib* was 60.9%, whereas the remaining 143 isolates were virulent to the *Pib* gene (Table 1). The percentage of avirulent isolate was 100, 75.9, 75.0, 57.6, 53.6, and 48.2% in northwestern, central, northeastern, southeastern, southwestern, western Yunnan Province, respectively. These results suggested that *Pib* loci had different efficacy against blast infections in most rice-growing regions of Yunnan Province. Among 366 isolates, three genotypes (L1 with 1231bp, L2 with 3100bp, L3 with both of 1231bp and 3100bp) of *AVR-Pib* alleles in 162 isolates were amplified by *AVR-Pib*-specific primers (AVR-Pib F1/AVR-Pib R1) (Table 1; Additional file 4: Fig. S1), and the average percentage of the *AVR-Pib* allele was 44.3%. The highest percentage of amplification of *AVR-Pib* was 73.3% in the rice-blast isolates collected from northwestern Yunnan Province, whereas the lowest percentage was 36.1% from northeastern Yunnan Province (Table 1). The percentage of *AVR-Pib* was 46.3, 36.1, 73.3, 51.5, 67.9, and 39.0% in central, northeastern, northwestern, southeastern, southwestern and western Yunnan Province, respectively. The percentage of *AVR-Pib* was 47.0 and 42.4% in *Xian/ Indica* (*XI*) rice- and *Geng/ Japonica* (*GJ*) rice-production areas in Yunnan. The genotype of L1, L2 and L3 alleles of *AVR-Pib* was detected in 104, 53 and 5 isolates, with percentages of 28.4%, 14.5% and 1.3%, respectively (Table 1). The genotype of L1, L2 and L3 alleles of *AVR-Pib* was detected in the *XI* rice-production area, whereas L3 was absent in the *GJ* rice-production area (Table 1). These results suggested that the genome structure of *AVR-Pib* loci was complicated considerably in *M. oryzae*.

Table 1
Frequency of *AVR-Pib* genotypes and avirulent isolates of *M. oryzae* collected from Yunnan, China to IRBLb-B

Locations	No. of isolates	PCR detection				Pathogenicity assay[b]
		Genotype and No. of isolates and frequency (%)				No. of avirulence isolates and Frequency (%)
		L1	L2	L3	Total isolates and frequency (%)	
Central	54	22 (40.7)	3 (5.6))	0	25 (46.3)^{B}	41 (75.9)^{AB}
Northeastern	72	23 (31.9)	3 (4.2)	0	26 (36.1)^{B}	54 (75.0)^{B}
Northwestern	15	11 (73.3)	0	0	11 (73.3)^{A}	15 (100)^{A}
Southeastern	33	10 (30.3)	6 (18.2)	1 (3.0)	17 (51.5)^{B}	19 (57.6)^{C}
Southwestern	28	9 (32.1)	8 (28.6)	2 (7.1)	19 (67.9^{A}	15 (53.6)^{C}
Western	164	29 (17.7)	33 (20.1)	2 (1.2)	64 (39.0)^{B}	79 (48.2)^{C}
Total	366	104 (28.4)	53 (14.5)	5 (1.4)	162 (44.3)	223 (60.9)
XI	149	31 (20.8)	34 (22.8)	5 (3.4)	70 (47.0)*	69 (46.3)**
GJ	217	73 (33.6)	19 (8.8)	0	92 (42.4)*	154 (71.0)**
Total	366	104 (28.4)	53 (14.5)	5 (1.4)	162 (44.3)	223 (60.9)

[a] L1 indicates the *AVR-Pib* genotype with the expected size (1231bp), L2 and L3 indicates the *AVR-Pib* genotype with TE insertion (L2 with 3100bp, L3 with both of 1231bp and 3100bp). The superscript of A and B indicates the significant difference at 0.01 level, and * indicates non significant.

[b] Indicates pathogenicity assay on monogenic line IRBLb-B containing *Pib*. *XI* and *GJ* indicates *Xian/ Indica* and *Geng/ Japonic*, respectively. The superscript of A, B and C indicates the significant difference at 0.01 level, ** indicates significant difference at 0.01 level.

Virulence function of AVR-Pib **variations against the** Pib **gene**

Twelve *AVR-Pib* haplotypes (H01 to H12) (Table 2), excluding the original *AVR-Pib* allele (GenBank accession number, KM887844), were detected on the nucleotide sequence assemblies of 90 isolates of L1 alleles containing a 719-bp 5'-region, 225-bp coding DNA sequence (CDS), and 302-bp 3'-region of *AVR-Pib* (Table 2; Additional file 5: Fig. S2). Also, insertion of Pot2 (at position -275) and Pot3 (at position -240) was identified based on the DNA sequence assemblies of six and 30 isolates (Table 2; Fig. 1), respectively, and the amplicon size difference between L1 and L2 (Additional file 4: Fig. S1). The 12 novel *AVR-Pib* haplotypes (H01-H12) were identified compared to previous published alleles [3, 27]. Alignment of DNA sequence assemblies of the *AVR-Pib* gene from 90 isolates revealed 18 mutation sites, including six mutant sites in the CDS region which weren't in the signal-peptide region (Table 2; Additional file 5 and 6: Fig. S2 and S3). Six mutant sites in the CDS region led to changes in amino acids (Table 3). The CDS sequence assemblies of the *AVR-Pib* allele among the 126 isolates (including L1, L2 and L3) were predicted to produce four AVR-Pib proteins (Table 3). Among them, amino-acid variations were predicted to occur at six positions (Table 3). Amino-acid variations at F54L in H05 and H06, E46V, F53S, and F54V in H07 were found, these isolates of the corresponding haplotypes were avirulent on the monogenic line IRBLb-B (with *Pib*), whileas, the amino-acid variations at F47L, I49T, and R50G were found in one isolate with H08, which was virulent on the monogenic line IRBLb-B (with *Pib*) (Table 3), the amino-acid variations at F47, I49, and R50 in H01, H02, H03, H04, H05, H06, H07, H09, H10, and H12, and these isolates were avirulent on the monogenic line IRBLb-B (with *Pib*), whileas the amino-acid variations at 47L, 49T, and 50G in H08, and the isolate was virulent on IRBLB-b (Table 3). This finding suggested that the amino acids F47, I49, and R50 were crucial for the avirulence function of *AVR-Pib*.

Table 2
Haplotypes of *AVR-Pib* loci in *M. oryza*e field populations of Yunnan, China

Haplotype	No. of isolates	% of total	Variant locus[a]														
			5' UTR							CDS regions							3
			-338	Between -325 and -326	Between -239 and -240	Between -216 and -217	-192	-175	Between -210 and -211	-93	137	141	146	148	158	160	+
KM887844			T	-	-	-	C	C	-	T	A	T	T	C	A	T	C
H01	33	26.2	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
H02	4	3.2	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.
H03	4	3.2	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.
H04	1	0.8	.	ACTTA	.	C	.	.	.	.	.	.	.	.	.	.	T
H05	8	6.3	C	.	.	C	.	.	.	.	.	.	.	.	.	C	.
H06	1	0.8	C	.	ACGTTA	C	.	.	.	.	.	.	.	.	.	C	.
H07	3	2.4	.	.	.	C	.	T	.	.	T	.	.	.	C	G	.
H08	1	0.8	.	.	.	C	.	.	ACA	.	.	A	C	G	.	.	.
H09	13	10.3	.	ACTTA	.	C	.	.	.	.	.	.	.	.	.	.	.
H10	10	7.9	.	AGTTA	.	C	.	.	.	.	.	.	.	.	.	.	.
H11	2	1.6	.	ATTA	.	C	.	.	.	.	.	.	.	.	.	.	.
H12	10	7.9	.	.	.	C	.	.	ACA	.	.	.	.	.	.	.	.
Pot2	6	4.8	-275 insert Pot2		.	C	.	.	.	.	.	.	.	.	.	.	.
Pot3 rev-A	1	0.8	-240 insert Pot3		.	C	.	.	.	.	.	.	.	.	.	.	.
Pot3 rev-B	2	1.6	-240 insert Pot3		.	C	.	.	.	.	.	.	.	.	.	C	.
Pot3-A	24	19.1	-240 insert Pot3		.	C	.	.	.	.	.	.	.	.	.	.	.
Pot3-B	2	1.6	-240 insert Pot3		.	C	.	.	.	.	.	.	.	.	.	C	.
Pot3-C	1	0.8	-240 insert Pot3		.	C	.	.	.	.	.	.	.	.	.	.	.

[a] . Indicates the same with KM887844 (GenBank Accession No.). The KM887844 of *AVR-Pib* was obtained from GenBank. rev: indicates reverse insertion of P

Table 3
Variation of the *AVR-Pib* loci proteins in *M. oryza*e populations of Yunnan, China

Haplotype	No. of isolates	% of total	Variant locus[a]						Disease reaction[b]
			46	47	49	50	53	54	
KM887844			E	F	I	R	Y	F	
H01	33	26.2	.	.	.	.	.	.	24R+5M+4?
H02	4	3.2	.	.	.	.	.	.	3R+1M
H03	4	3.2	.	.	.	.	.	.	4R
H04	1	0.8	.	.	.	.	.	.	1R
H05	8	6.3	.	.	.	.	.	L	7R+1?
H06	1	0.8	.	.	.	.	.	L	1R
H07	3	2.4	V	.	.	.	S	V	3R
H08	1	0.8	.	L	T	G	.	.	1S
H09	13	10.3	.	.	.	.	.	.	11R+2M
H10	10	7.9	.	.	.	.	.	.	9R+1M
H11	2	1.6	.	.	.	.	.	.	2S
H12	10	7.9	.	.	.	.	.	.	9R+1M
Pot2	6	4.8	.	.	.	.	.	.	7S
Pot3 rev-A[c]	1	0.8	.	.	.	.	.	.	1S
Pot3 rev-B	2	1.6	.	.	.	.	.	L	2S
Pot3-A	24	19.1	.	.	.	.	.	.	22S+2M
Pot3-B	2	1.6	.	.	.	.	.	.	2S
Pot3-C	1	0.8	.	.	.	.	.	L	1S

[a]. Indicates the same with KM887844.

[b] Indicates pathogenicity assay on the monogenic lines IRBLb-B containing the resistant gene of *Pib*. R, M and S indicate disease reaction was resistant, moderate resistant and susceptible, respectively; Ex. 24R indicated 24 isolates were avirulent to IRBLb-B; and ? indicates unknown.

[c] rev: indicates reverse insertion of Pot3 in *AVR-Pib*.

The different haplotypes of H01, H02, H03, H04, H09, H10, H11 and H12 had no change on amino acids sequence (Table 3). The three-dimensional protein structures built by homology modeling (SWISS-MODEL; https://swissmodel.expasy.org/) showed the different protein structures of these four AVR-Pib variants (Additional file 7: Fig. S4). Isolates of H11 with insertion of ATTA in the 5' UTR may change *AVR-Pib* expression and cause loss of the avirulent function (Tables 2 and 3; Additional file 6: Fig. S3). Isolates of H01 (amino acids same as that with a GenBank accession number of KM887844), H02, H03, H04, H05, H06, H07, H09, H10 and H12 haplotypes harbored *AVR-Pib* because these isolates were avirulent to the *Pib*-containing monogenic line IRBLb-B (Table 3). Isolate of H08 defeated the resistance of *Pib* because this isolate was virulent to the *Pib*-containing monogenic line IRBLb-B (Table 3). Furthermore, Pot2 and Pot3 inserted in the 5' UTR of the *Pib* gene were identified in six isolates and 30 isolates (Table 3; Fig. 1), respectively, and these isolates were virulent to the *Pib*-containing monogenic line IRBLb-B (Table 3). These findings suggested that insertion of TEs (Pot2 and Pot3) and small segments of the nucleotide in the promoter region, and nuclear substitution in the ORF region, resulted in variation of *AVR-Pib* from avirulence to virulence, and that the diverse mutations of the *AVR-Pib* allele of *M. oryzae* were involved.

Haplotype diversity of AVR-Pib **of** M. oryzae

Among 12 *AVR-Pib* haplotypes, none were identical to the original *AVR-Pib* (GenBank accession number, KM887844) (Table 2). Eight haplotypes, as well as Pot2 and Pot3 insertion were detected in 50 *M. oryzae* isolates from western Yunnan Province (Table 4). Five haplotypes, as well as Pot3 and Pot3 reverse-insertion were identified in 23 *M. oryzae* isolates from central Yunnan Province. Three haplotypes, as well as Pot2 and Pot3 reverse-insertion were identified in 15 isolates of *M. oryzae* from northeastern Yunnan Province. Three haplotypes and Pot2 insertion were detected in 19 isolates from northeastern Yunnan Province. Three haplotypes and Pot3 insertion were identified in six *M. oryzae* isolates from southeastern Yunnan Province. Three haplotypes were identified in 13 isolates of *M. oryzae* from northwestern Yunnan Province (Table 4). Eleven and nine haplotypes were detected in *GJ* rice- and *XI* rice-production areas, and the diversity index (DI) of haplotypes was 0.84 and 0.79 for these areas, respectively. The DI of *AVR-Pib* was 0.72, 0.71, 0.70, 0.63, 0.59, and 0.54 for southeastern, central, western, southwestern, northeastern, and northwestern Yunnan Province, respectively (Table 4). In brief, the DI of *AVR-Pib* alleles in Yunnan Province was in the order southeastern > central > western > southwestern > northeastern > northwestern. The DI of *AVR-Pib* alleles in the *GJ*-rice production area was higher than that of in the *XI* rice-production area. These results indicate that the genetic divergence of *AVR-Pib* of *M. oryzae* in each rice growing region was occurred under fields condition.

Table 4
Haplotype distribution of *AVR-Pib* in different Yunnan rice growing regions

Haplotype	No. isolates	Percent (%)	Regions						Production[c]	
			Central	Northeastern	Northwestern	Southwestern	Southeastern	Western	*XI*	*GJ*
H01	33	26.2	11(47.8)[a]	9(60.0)	2(15.4)	10(52.6)	0	1(2.0)	10(20.8)	23(29.5)
H02	4	3.2	0	0	0	4(21.1)	0	0	4(8.3)	0
H03	4	3.2	4(17.4)	0	0	0	0	0	0	4(5.1)
H04	1	0.8	0	0	0	0	0	1(2.0)	0	1(1.3)
H05	8	6.3	1(4.3)	1(6.7)	3(23.1)	0	2(33.3)	1(2.0)	2(4.2)	6(7.7)
H06	1	0.8	0	0	0	0	1(16.7)	0	1(2.1)	0
H07	3	2.4	0	0	0	0	0	3(6.0)	0	3(3.8)
H08	1	0.8	0	0	0	0	0	1(2.0)	1(2.1)	0
H09	13	10.3	2(8.7)	0	0	0	0	11(22.0)	0	13(16.7)
H10	10	7.9	2(8.7)	0	8(61.5)	0	0	0	0	10(12.8)
H11	2	1.6	0	0	0	0	1(16.7)	1(2.0)	1(2.1)	1(1.3)
H12	10	7.9	0	3(20.0)	0	1(5.3)	0	6(12.0)	6(12.5)	4(5.1)
Pot2	6	4.8	0	1(6.7)	0	4(21.1)	0	1(2.0)	6(12.5)	0
Pot3 rev	3	2.4	2(8.7)	1(6.7)	0	0	0	0	0	3(3.8)
Pot3	27	21.4	1(4.3)	0	0	0	2(33.3)	24(48.0)	17	10(12.8)
Total	126	100	23	15	13	19	6	50	48	78
No. of haplotypes			7	5	3	4	4	10	9	11
Index of diversity[b]			0.71	0.59	0.54	0.63	0.72	0.70	0.79	0.84

[a] Number and frequency (in bracket) of isolates of each haplotype.

[b] Diversity index was calculated as the frequency of haplotypes types in the *M. oryzae* population following Fontaine's method [43]. Diversity index = $(1-\sum^{n}_{i=1}p_i^2)$ (where pi is the frequency of the haplotype i in a population).

[c] *XI* and *GJ* indicates *Xian/ Indica* and *Geng/ Japonic*, respectively.

Eighteen nucleotide variable sites in *AVR-Pib* alleles were identified (Table 2; Additional file 5 and 6: Figs. S2 and S3). A haplotype network based on sequence variations of 90 isolates of L1 alleles was developed (Fig. 2). Four main lineage branches (A to D) of *AVR-Pib* were divided among 90 field isolates (Fig. 2), and different evolution of *AVR-Pib* among them was noted. Isolates of B and D lineage branches of *AVR-Pib* were avirulent to IRBLb-B (with *Pib*) (Fig. 2; Table 3). Isolates of H11 of the A-evolved branch and H08 of the C-evolved branch were virulent to the rice blast-resistant gene *Pib*, respectively (Fig. 2; Table 3). These data suggested that the A and C branches of *AVR-Pib* had evolved to virulence from avirulent origins *via* base substitution and insertion, and evaded the recognition of the rice blast-resistance gene *Pib* in field isolates. The virulence of H08 and H11 was identified in southeastern and western Yunnan Province (Table 4). Also, TE insertion in rice samples in all regions except northwestern Yunnan Province (Table 4) suggested that virulent evolution of *AVR-Pib* occurred in most rice-production areas of Yunnan Province.

Selection pressure on AVR-Pib in M. oryzae

The natural-selection pressure on *AVR-Pib* was calculated by Tajima's neutrality test on 126 *AVR-Pib* CDS sequences: the Tajima's *D* value was not significantly different from zero ($D=-1.61687$; $0.10>P>0.05$) (Additional file 1: Table S1). This result suggested that *AVR-Pib* may suffer neutral selection and evolving neutrally in the population of *M. oryzae*. Furthermore, the results of three positive-selection models kept higher similatity (Additional file 8: Fig. S5). The "sliding window" under M8, M8a, and M7 models showed values of Ka/Ks (Ka, rate of nonsynonymous substitutions; Ks, rate of synonymous substitutions) across all 74 amino acids (Additional file 8: Fig. S5). The Ka/Ks value of all sites was >1 under M8 and M8a model, and the value was 1 under M7 model for entire residues, those results implying that these sites may have suffered neutral selection. These findings suggest that the *AVR-Pib* maybe under neutral evolution.

Adaption of TE insertion in AVR-Pib

We wished to confirm the host (rice and non rice) selection pressure on TE insertion in *AVR-Pib*. A total of 27 isolates from *O. rufipogon* (with *Pib* homologs) [20], *Digitaria sanguinalis*, *Eleusine indica, E. coracana*, and *Musa nana* Lour, which were stored in our lab, and 5 isolates genome sequence from *Lolium perenne* Linn (2 isolates), *Setaria viridis* (Linn.) Beauv. (1 isolate), and *Triticum aestivum* Linn (2 isolates) from Genbank were selected and analyzed

(Additional file 2: Table S2). The *AVR-Pib* allele was not detected in the isolate from *D. sanguinalis*, *M. nana* Lour, *Lolium perenne* Linn, and *Setaria viridis* (Linn.) Beauv. (Additional file 2: Table S2). Only the L1 genotypes (with the expected size) of *AVR-Pib* were detected in isolates from *E. indica*, *E. coracana*, and *Triticum aestivum* Linn, suggesting that these isolates did not have a TE insertion in the *AVR-Pib* allele. Three genotypes of L1, L2 (with TE insertion), and L3 (with TE insertion) of *AVR-Pib* were detected in 18 isolates from the *Pib* homolog-containing *O. rufipogon*, and the isolate of YN441 (with the H9 haplotype of *AVR-Pib* and identical with the original haplotype of KM887844) was virulent to *O. rufipogon* (Fig. 3). These findings showed that the diversification of *AVR-Pib* of *M. oryzae* was dependent upon *Pib* homolog-containing *O. rufipogon*, and that variation in TE insertion in *AVR-Pib* could be selected and adapted to rice and other Gramineae species.

Phylogeny of Pib allele partial to CDS regions

Fifty-seven sequences of *Pib* were obtained from GenBank (Additional file 3: Table S3). Eleven of them were from five wild-rice species (seven from *O. rufipogon*, one from *O. meyeriana*, one from *O. officinalis*, one from *O. longistaminata*, one from *O. nivara*), and 46 accessions from *O. sativa*, including the original *Pib* (GenBank accession number, AB013448.1) (Additional file 3: Table S3). These sequences were aligned. A minimum-evolution phylogenetic tree was constructed based on the nucleotide sequences of exon 1 of *Pib* in 34 accessions and partial regions of exon-3 nucleotide sequences (from 7633 to 8484 of AB013448.1) of *Pib* in 49 accessions, respectively (Fig. 4). Exon 1 of *Pib* in wild-rice species (*O. rufipogon*, *O. meyeriana*, *O. officinalis*, *O. longistaminata*) was close to that in *O. sativa* (Fig. 4B). The DQ317978.1 group of the wild rice *O. rufipogon* shared >90% identity with the nucleotide sequences of the JN564624.1 group of *Indica*. Two major clades emerged in one part of exon 3 of *Pib* (Fig. 4C). One clade contained two wild-rice species (*O. rufipogon* and *O. nivara*) and *O. sativa*. The EF642422.1 group of the wild-rice species *O. nivara* shared >90% identity in nucleotide sequences with the EF642423.1 group of *O. sativa*. The EF642442.1 group of *O. rufipogon* shared >75% identity of nucleotide sequences with the EF642423.1 group of *O. sativa*. The other clade contained *O. rufipogon* and *O. sativa*. The EF642440.1 group of the wild-rice species *O. rufipogon* shared >75% identity of nucleotide sequences with the EF642433.1 group of *O. sativa*. The isolate of YN441 (with the H9 haplotype of *AVR-Pib*) was virulent to *O. rufipogon*, *O. meyeriana*, and *O. officinalis* (Fig. 3). These results suggested that different regions of the *Pib* gene may have suffered different selection pressures in the host rather than domestication.

Discussion

We identified 12 new haplotypes, as well as Pot2 and Pot3 insertion in the *AVR-Pib* DNA sequences among rice-blast isolates from different rice-growing areas in Yunnan Province. The many virulent isolates to *Pib*-containing rice varieties implied that *Pib* was overcome in these rice-growing regions because of massive exploitation of *Pib* in China.

Pib alleles have been used widely and have shown strong resistance to disease in China [18]. Complete deletions have occurred in *AVR-Pib* sequences among field isolates of *M. oryzae* from various rice-growing regions of Guangdong, Hunan, and Liaoning Provinces [3]. Also, TE insertion has occurred in *AVR-Pib* in *M. oryzae* isolates from south and northeast China [3] and the Philippines [13]. Those data are consistent with our results. The L1 genotype of *AVR-Pib* identified in rice-blast isolates collected from rice fields implied that *Pib* has been effective in preventing rice blast. Li and colleagues showed that rice cultivars with *Pib* were resistant to 74.9% of isolates (282 isolates) from Yunnan Province [26]. The corresponding value was 2.1% in Guangdong Province (146 isolates were tested) [28], and the percentage resistance was <31% in Hunan Province [29]. These results show that *Pib* alleles had poor effects in these rice-production areas. Further inspection of variation in *AVR-Pib* DNA sequences in these isolates could reveal the molecular evolutionary patterns of *AVR-Pib* and predict the durability and effectiveness of *Pib* allele-mediated resistance under field conditions in rice-production regions.

AVR-Pia, *AVR-Pii* and *AVR-Pita1* located on telomere regions tend to be unstable, and effective mutants in these genes have been identified [6, 30–31]. The retrotransposon (MINE) insertion in the *ACE1* gene [8] and Pot3 insertion in *AVR-Pita1* [32–33] and *AVR-Pizt* [7] have caused new virulent alleles. TE (Pot2 and Pot3) insertion, complete absence, segmental deletion, and a point mutation have been found in *AVR-Pib* alleles, all of which lead to gain of virulence [3]. Three expression patterns have been identified among different haplotypes of *AVR-Pib* [3]. Recently, insertion of a Pot3 transposon in *AVR-Pib* was shown to mediate the loss of function of *AVR-Pib* in all 248 isolates collected from the Philippines [13]. These findings showed that rice blasts can use transposons to suppress expression of *AVR* genes to defeat the rice-blast resistance gene. The *AVR-Pib* gene was identified in nearly half of rice-blast isolates (44.3%) in Yunnan Province (Table 1). This percentage was higher than that in rice-blast isolates in Jilin and Heilongjiang Provinces, but lower than those in Guangdong, Hainan and Liaoning Provinces [3]. Meanwhile, 28.6% of isolates contained a TE insertion in *AVR-Pib*. Among them, 21.4% isolates contained a Pot3-element insertion and 2.4% of isolates contained a reverse Pot3-element insertion, and 4.8% isolates contained a Pot2-element insertion in *AVR-Pib* of *M. oryze* from Yunnan Province (Table 4; Fig. 1). These insertions resulted in variation from avirulence to virulence to the corresponding *R* gene. Several nucleotide variations in *AVR-Pib* alleles were identified, which led to variations in amino acids and implied that *AVR-Pib* alleles suffer strong selection pressure in rice-production regions of Yunnan Province.

We observed no TE insertions in *AVR-Pib* of isolates from *E. indica* or *E. coracana*, and TE insertion in *AVR-Pib* was selected by the host, data that are consistent with the results of Zhang et al. [3]. Pot2 and Pot3 insertions were identified in the *XI*-rice growing areas, whereas only Pot3 insertion was identified in *GJ*-rice growing areas of Yunnan Province. TE insertion of *AVR-Pib* was noted in all rice-growing regions except northwestern Yunnan Province, and Pot3 insertion was distributed mainly in western Yunnan Province. These results showed that the virulent *AVR-Pib* alleles had been involved in most of the rice-growing regions of Yunnan Province. Hence, monitoring of these virulent alleles in field populations is important for employing *Pib*-containing rice varieties.

Various mutations were identified in CDS regions of *AVR-Pib*, and 12 *AVR-Pib* haplotypes were found based on 18 variant nucleotides among 90 isolates of L1 alleles collected from Yunnan Province (Table 2). Six new variant amino acids of the *AVR-Pib* loci variants were identified in the 90 *M. oryze* isolates in the

present study, and resulted in identification of four novel haplotypes. A more holonomic network was constructed based on the new variations among different alleles of *AVR-Pib*. The putative secreted proteins of AVR-Pib in 126 isolates were identified (Table 3), and they were in accordance with the results of Zhang et al. [3]. Nine isolates had variations at the amino-acid position F54L; three isolates had variations at the amino-acid positions of E46V, Y53S, and F54V; one isolate had variations at the amino-acid positions of F47L, I49T, and R50G (Table 3). These isolates were virulent to the monogenic line IRBLb-B (with *Pib*), suggesting that these amino acids are crucial for avirulent function.

In the course of interactions and co-evolution between pathogens and plants, the *R* genes of plants can discern the cognate *AVR* genes of pathogens and inspire immunity. The genetic variation of the *AVR* genes of the pathogen is dependent upon the *R* genes of the host and changeable environmental conditions. The DI of *AVR-Pib* was higher in the *GJ* rice-production areas than that of *XI* rice-production areas (Table 4). Different variations were observed in *AVR-Pib* between *XI* rice- and *GJ* rice-production areas (Table 4). These findings imply that the adaptive mutations of *AVR-Pib* occurred in Yunnan Province under natural conditions.

Yunnan Province is abundant in genetic resources of rice. The wild species of *O. officinalis*, *O. meyeriana* and *O. rufipogon* also coexist in this Province [34]. More than 5000 rice accessions germplasms have been conserved in Yunnan Province. Among them, 12 out of 227 accessions carried the *Pib* resistance gene screened by resistance gene identification using different isolates [34]. *Pib* gene homologs have been identified in wild rice *O. rufipogon* from Yuanjiang County [20], and four genotypes (L0 to L3) of *AVR-Pib* have been detected in *M. oryzae* and *O. rufipogon* in Yuanjiang County. TE insertion of L2 and L3 genotypes of *AVR-Pib* was absent in the isolates from *D. sanguinalis* and *M. nana* Lour. These results suggest that adaptive variation of *AVR-Pib* is involved during interactions and co-evolution between *AVR-Pib* of *M. oryzae* and *Pib* of *O. rufipogon*. The Tajima's *D* value of −1.61687 (Additional file 1: Table S1) indicates that *AVR-Pib* loci may suffer purifying selection by the corresponding *R* gene in Yunnan rice-production areas.

Massive variations and stepwise mutations in *AVR-Pib* of rice-blast isolates were observed in Yunnan Province (Table 2; Fig. 2), which suggests that there is abundant diversity of rice accessions and *M. oryzae* isolates in Yunnan Province. Pot2 insertion in *AVR-Pib* was found in western, southwestern, and northeastern Yunnan Province. Pot3 reversed-insertion was found in central and northeastern Yunnan Province. Also, Pot3 insertion occurred mainly in western Yunnan Province. Pot3 insertion of *AVR-Pib* was found in *GJ* rice- and *XI* rice-production areas, whereas Pot2- and Pot3-reverse insertion was found only in the *XI* rice-production area and *GJ* rice-production area, respectively (Table 4). The virulent haplotype of H11 was detected in the *XI* rice-production area and *GJ* rice-production area, and H08 was detected in the *XI* rice-production area. These data showed high variation of *AVR-Pib* in different rice-growing regions, which may be due to rice variety and the environment.

The stepwise mutations that result in loss of avirulence function have been identified in *AVRL567* [35] and *AVR-Pik* [36–38]. Based on the *Pib* homologs identified by Yang et al. [20], and our result for *AVR-Pib* in the present study, the potential interactions and co-evolution of *AVR-Pib* alleles in *M. oryzae* and *Pib* alleles of rice were constructed (Additional file 9: Fig. S6). The *AVR-Pib* homolog L1 (H01) originated from an ancestral *M. oryzae* gene. The *Pib* allele (87-bp deletion in exon 1 of *Pib*) in *O. rufipogon* could not recognize the L1 alleles of *AVR-Pib*. Thus, the other *Pib* allele (gained 87 bp in exon 1 of *Pib*) in cultivated rice evolved to recognize the L1 alleles (H01) of *AVR-Pib*, whereas the altered alleles L2 and L3 evolved to virulence from avirulent origins by TE insertion, base substitution (H08) and segment insertion (H11) to avoid recognition by *Pib* (Table 2; Additional file 9: Fig. S6). These actions indicated stepwise evolution of *AVR-Pib* as well as *Pib* interaction and co-evolution. Intriguingly, the *AVR-Pib* alleles H08 and H11 were derived from H01, and could escape recognition by *Pib* (Table 2; Additional file 9: Fig. S6), but several extinct or missing haplotypes were not identified in the sample (Fig. 2). These findings imply that: (i) the *AVR-Pib* loci of *M. oryzae* evolved gradually during the interaction and coevolution between the *Pib* loci of *M. oryzae* in field conditions; (ii) the genome organization of the *AVR-Pib* locus is much more intricate than anticipated.

Conclusions

We detected twelve novel haplotypes in the field population by using 90 isolates, transposable element (TE) insertion in 36 of 126 isolates, constructed a complex network of *AVR-Pib* alleles, and assessed the efficacy of *Pib* alleles in rice production areas of Yunnan, analyzed the adaption of TE insertion of *AVR-Pib* in the isolates from different host. Our findings support the hypothesis that functional *AVR-Pib* possesses varied sequence structures and can escape surveillance by hosts via multiple variation manners. Haplotype H08 and H11 can overcome all detected *Pib* alleles to date, and Pot insertion can changed the avirulent function of *AVR-Pib*. Despite the H08, H11 haplotypes and TEs insertions have low frequencies, monitoring of these alleles in field populations is critical because of their high risk for *Pib*-holding rice varieties. The TE insertion was not detected in *AVR-Pib* allele in the isolates from *E. indica*, *E. coracana*, and *Triticum aestivum* Linn, while three genotypes of *AVR-Pib* were detected in isolates from *O. rufipogon*, the selected and adapted variation of TE insertion in *AVR-Pib* is occurrence in the long-term co-evolution between *M. oryzae* and hosts (rice and other Gramineae species).

Abbreviations

AVR
Avirulence gene
DI
Diversity index
GJ
Geng/Japonica
Ka
The rate of nonsynonymous substitution
Ks
The rate of synonymous substitution

LTH
Lijiangxintuanheigu
R
Resistance
M
Moderate resistant
S
Susceptible
TE
Transposable element
XI
Xian/Indica

Methods

Blast isolates, rice accessions, culture, and pathogenicity identification

The seedlings of the rice monogenic line IRBLb-B (which contains *Pib*) and a susceptible cultivar Lijiangxintuanheigu (LTH; does not contain *Pib*) were used for pathogenicity assays (the seeds were acquired originally from Cailin Lei). The total of 366 isolates of *M. oryzae* in the present study were same with the published paper [38]. The storing and culturing methods of the isolates were described in the published paper [38]. Disease reactions were referred as the method of Jia et al. [39]. In a few words, when rice seedlings were at the 3- to 4-leaf stage, they were inoculated with a spore suspension ($1–5\times10^5$ spores/mL containing 0.05% Tween 20). After innoculation, rice seedlings were put into a plastic bag and sealed securely for keeping high relative humidity of 90 -100% at 25°C for 24 h in the dark. Then, the plants were shifted into a greenhouse for 6 days to develop disease lesion extension fully.

Disease reactions were monitored externally on the second youngest leaf based on the number and degree of lesions using a 0-to-5 disease scale (Additional file 10: Fig. S7). The disease scale method was described in the published paper [38] Five seedlings at each time and twice repeats were arranged in the experiment. And the average value of disease scales was used to discrinate resistance *versus* susceptibility. The disease reaction of wild rice *O. rufipogon*, *O. meyeriana*, and *O. officinalis* was determined using a detached-leaf method, as described by Jia et al. [39]. One virulent blast isolate was used for inoculation. Disease reactions were evaluated 5–7 days after inoculation.

DNA extraction, PCR amplification, and DNA sequencing

Culturing method of vegetative mycelia of *M. oryzae* isolates were described in the published paper [38]. The genomic DNA of each isolate was extracted from vegetative mycelia by CTAB method [40]. The primers AvrPibF1 (5'-GGACAAGGGAGGCAAATCTAAC-3') and AvrPibR1 (5'-ATGCCGACAATGCGAGGTAT-3') were used to amplify the *AVR-Pib* allele and for sequencing according to the method of Zhang et al. [3]. Each PCR reaction was amplified in a total reaction volume of 50 μL containing the following components: 25 μL of 2× Taq PCR MasterMix (Tiangen Biotech, Beijing, China), 1 μL (10 μM) of each primer, 2 μL of template DNA, and 21 μL of ddH_2O (provided in the Tiangen kit). PCRs procedure were done in a C1000 Touch™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) as the following steps: initial denaturation at 94°C for 3 min, followed by 29 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 2.5 min, and a final extension at 72°C for 5 min. Each reaction was repeated twice. The size of the PCR products was valued by a DNA marker (DL2000, Tiangen Biotech). Amplicons were sequenced twice by Life Technologies Biotechnology (Shanghai, China).

Data analyses

DNA sequences of *AVR-Pib* were assembled and aligned using DNASTAR v7.1.0 (www.dnastar.com/). DnaSP v5.10.01 [41] was used for calculation of polymorphic sites (π), the number of DNA haplotypes, and the sliding window. TCS1.21 (http://darwin.uvigo.es/) [42] was used for analyses of the haplotype network of *AVR-Pib*. The DI (haplotype diversity index) was counted in *M. oryzae* populations following the method of Fontaine et al. [43]:

$$DI = (1 - \sum^{n}_{i=1} p_i^2)$$

where p_i is the frequency of haplotype *i* in a population. Tajima's neutrality test was done using MEGA X (www.megasoftware.net) [44]. The Selection Server program (http://selecton.tau.ac.il) was used for analyses of purifying selection. The purifying selected sites of AVR-Pib was identified by used three models: M8 (positive selection enabled, beta + w ≥ 1), M7 (beta, null model) and M8a (beta + w = 1, null model). Then, the sliding window of purifying selected sites of AVR-Pib was draw under the M8, M7, and M8a models by Excel™ (Microsoft, Redmond, WA, USA). MEGA X [44] was used for construction of phylogenetic trees by the minimum-evolution method [45]. The SWISS-MODEL (http://swissmodel.expasy.org) with ProMod v3.7.0 was used to built the protein homology model. The significant difference of distribution of *AVR-Pib* genes and avirulence isolates of *M. oryzae* in each region was analyzed by Excel software with CHITEST.

Declarations

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JBL was the leading investigators of this research programme. JBL planned and designed the research; JBL, QW and LL performed the majority of experiments with the help of CYL; LL, ZFS and CYL contributed reagents, materials, and analysis tools; JBL and QW analyzed the data; JBL and QW wrote the paper with suggestions from LL, CYL and ZFS. All authors commented on the article before submission. The author(s) read and approved the final manuscript.

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Figures

Figure 1

Characterization of allelic variation at *AVR-Pib*. The functional nucleotide polymorphic maps of the six natural alleles in 126 isolates of *M. oryzae* in Yunnan Province. **Dis.** indicates disease reaction on monogenic line IRBLb-B (containing *Pib*), V indicate the isolates were virulent to IRBLb-B.

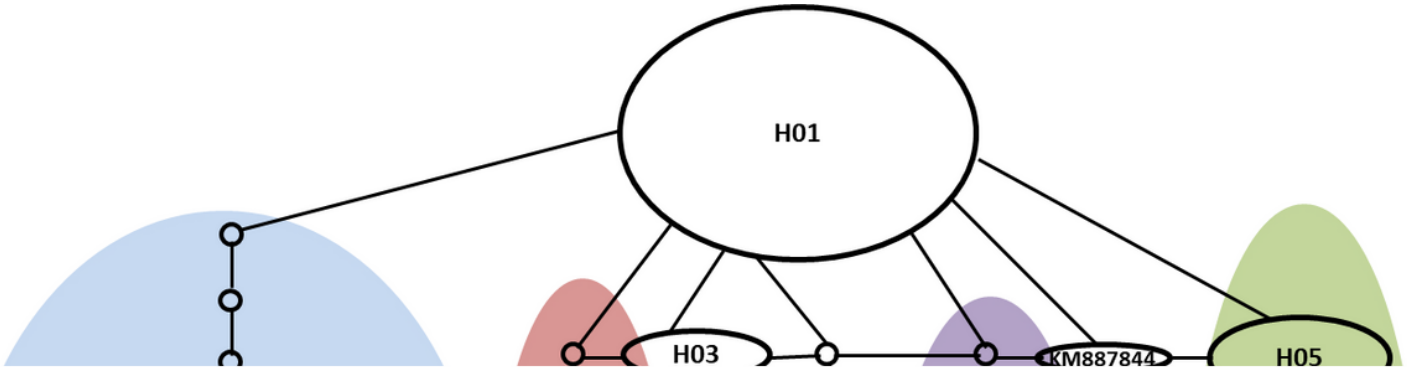

Figure 2

The haplotype network for the 12 *AVR-Pib* alleles. The original *AVR-Pib* allele was designated as the H01 haplotype in the network. Each haplotype was separated by mutational events. The node in the network represents an extinct or a missing haplotype not found among the samples. Each haplotype was separated by mutational events. All haplotypes were displayed as circles. The size of the circles corresponds to the haplotype frequency. The KM887844 (GenBank Accession No.) of *AVR-Pib* was obtained from GenBank. White color indicates avirulent to the *Pib* gene, yellow color indicates virulent to the *Pib* gene. **A to D,** four major haplotypes of *AVR-Pib* in Yunnan Province of China are shaded.

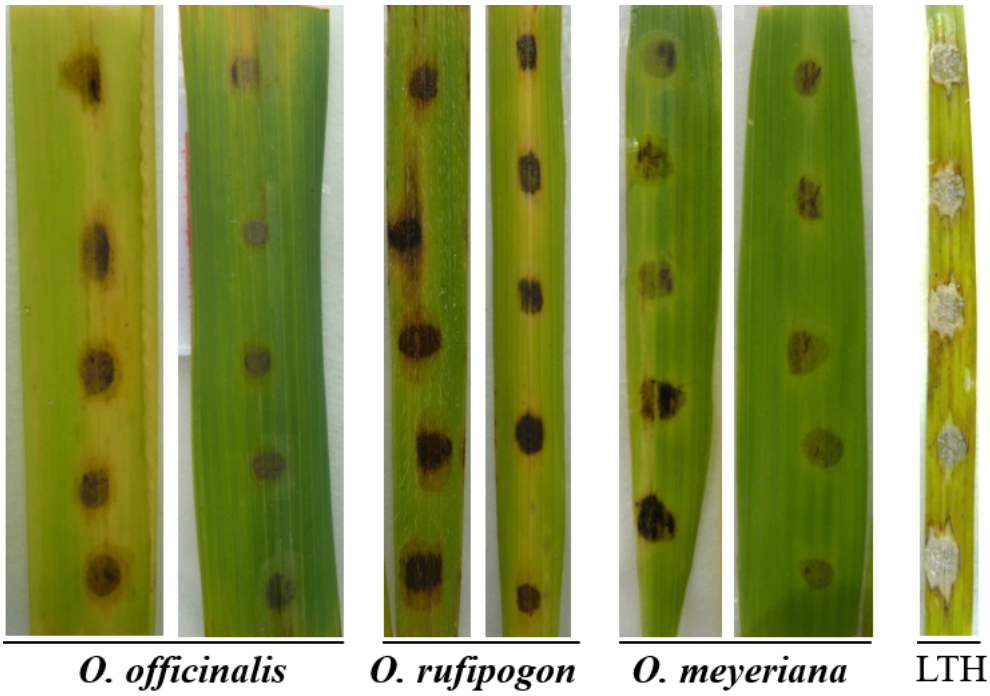

Figure 3

Disease reaction of the identification isolate of YN441 (with the H9 haplotype of *AVR-Pib* which was identical with the original haplotype of KM887844) on *O. officinalis*, *O. rufipogon*, *O. meyeriana*. LTH: Lijiangxintuanheigu.

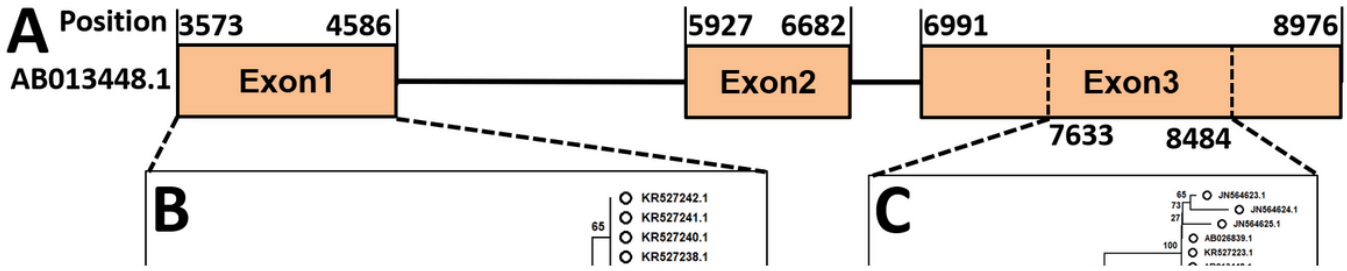

Figure 4

Phylogenetic tree constructed with the nucleotide sequences of *Pib* gene different partial CDS regions from wild rice and *O. sativa* using minimum evolution method of MEGA X. The numbers associated with individual branches indicate confidence levels based on 1000 bootstrap replicates. **A,** structure of *Pib* from AB013448.1 (GenBank ID); **B,** the phylogenetic tree was constructed base on the nucleotide sequences of exon1 of *Pib* regions from 34 accessions. **C,** the phylogenetic tree was constructed base on the nucleotide sequences of partial exon3 (from 7633 to 8484 of AB013448.1) of *Pib* from 49 accessions. All accessions of *Pib* were obtained from GenBank.

Supplementary Files

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