The Rice Blast Resistance Gene *Pid* Family Has Been Strictly Diverged Into *Indica* And *Japonica* Subspecies

Ruipeng Chai
South China Agricultural University
Jinyan Wang
South China Agricultural University
Xing Wang
South China Agricultural University
Jianqiang Wen
South China Agricultural University
Zhijian Liang
South China Agricultural University
Xuemei Ye
South China Agricultural University
Yaling Zhang
South China Agricultural University
Yongxiang Yao
South China Agricultural University
Jianfu Zhang
Fujian Academy of Agricultural Sciences
Yihua Zhang
Fujian Academy of Agricultural Sciences
Ling Wang
South China Agricultural University
Qinghua Pan (panqh@scau.edu.cn)
South China Agricultural University

Research Article

Keywords: Oryza sativa, Pyricularia oryzae, indica type resistance gene, japonica type resistance gene, resistance function confirmation

Posted Date: March 2nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-430464/v2

Abstract

Background: Rice blast (causative agent the fungus *Pyricularia oryzae*) represents a major constraint over the productivity of one of the world's most important staple foods. Genes encoding resistance have been identified in both the *indica* and *japonica* subspecies genepools and combining these within new cultivars represents a rational means of combating the pathogen.

Results: In this research, a deeper allele mining was carried out on *Pid-2*, *Pid-3*, and *Pid-4* by each of comprehensive FNP marker set in the three panels consisting of 70 *indica* and 58 *japonica* cultivars. Within *Pid-2,* three functional and one non-functional alleles were identified; the former were only identified in *indica* type entries. At *Pid-3,* four functional and one non-functional alleles were identified, and once again, all of the formers were present in *indica* type entries. However, the pattern of variation at *Pid-4* was rather different: here, the five functional alleles uncovered were dispersed across the *japonica* type germplasm. Among all the 12 candidate functional alleles, both *Pid2*-ZS and *Pid3*-ZS were predominant. Furthermore, the resistance functions of both *Pid2*-ZS and *Pid3*-ZS were assured by transformation test.

Conclusions: Profiting from merits of three comprehensive FNP marker sets, the study has validated that all three members of the *Pid* family have been strictly diverged into *indica* and *japonica* subspecies: *Pid-2* and *Pid-3* were defined as *indica* type resistance genes, and *Pid-4* as *japonica* one. Rather limited genotypes of the *Pid* family have been effective in both *indica* and *japonica* rice groups, of which *Pid2*-ZS+*Pid3*-ZS has been central to the Chinese rice population.

Background

Rice (*Oryza sativa*), a crop domesticated in Asia but now cultivated worldwide, is used as a staple food for half of mankind (Huang et al. 2012; Zhang et al. 2016; Du et al. 2017; Wang et al. 2018; Lv et al. 2020). As a result of two major and independent domestication events, two subspecies have been recognized, namely ssp. *indica* and ssp. *japonica* (Huang et al. 2012; Zhang et al. 2016; Choi & Purugganan 2018; Wang et al. 2018). The two genepools have differentiated over time through them been grown in distinct eco-geographical environments, and have diverged with respect to both the structure of the genome and their gene content (Huang et al. 2012; Ouyang & Zhang 2013; Zhang et al. 2016; Du et al. 2017; Wang et al. 2018). Introgression from one gene pool to the other is seen as a useful strategy for increasing the crop's genetic diversity.

One of the major pathogens of the crop is the fungus *Pyricularia oryzae* Cavara (syn. *Magnaporthe oryzae* Couch), the causative agent of the damaging disease rice blast (Dean et al. 2012; Liu and Wang 2016; Zhang et al. 2017, 2019; Zhao et al. 2018; Huang et al. 2021). A wealth of genes determining resistance to this pathogen has supported the success of using breeding to provide a sustainable means of mitigating the damage caused by blast (He et al. 2012; Zhai et al. 2014; Deng et al. 2017; Zhang et al. 2017, 2019). The genetics of resistance largely follows the gene-for-gene principle, involving an

interaction between a host's resistance gene and a matching avirulence gene in the pathogen (Flor 1971; Ebbole 2007; Wu et al. 2014; Zhang et al. 2015). As a result, following the mutation of matching avirulence genes, major gene-based resistances are prone to rapid breakdown. That in turn enable it to create a new genotype of resistance gene to overcome the emerged new race with its new resistance specificity (Zeigler et al. 1995; Wu et al. 2014; Zhang et al. 2015; Wang et al. 2017; Zhang et al. 2017, 2019; Zhao et al. 2018; Huang et al. 2021). New resistance specificities can be generated by mutations to a resistance gene's coding sequence, either in the form of a single nucleotide polymorphic (SNP) and even multiple nucleotide polymorphic mutations (so-called insertion/deletion, InDel) (Bryan et al. 2000; Lin et al. 2007; Liu et al. 2007; Fukuoka et al. 2009; Hua et al. 2012; Zhai et al. 2011, 2014;Zhao et al. 2018); where such changes result in an altered reaction to the pathogen, the mutation is referred to as a functional nucleotide polymorphism (FNP) (Hua et al. 2012; Zhai et al. 2011, 2014). As per the gene-for-gene principal, it is envisaged that the stronger arms race led to more FNPs emerged in any given resistance gene (Wu et al. 2014; Zhang et al. 2015; Wang et al. 2017; Zhang et al. 2017, 2019; Zhao et al. 2018; Huang et al. 2021). Searching for FNPs in established host cultivars is considered as an efficient way of identifying the novel resistances required for crop improvement (Li et al. 2019; Teerasan et al. 2019; Tian et al. 2020; Xiao et al. 2020; Zhou et al. 2020).

At least 100 major genes encoding resistance to *P. oryzae* are known, an increasing number of which have been isolated (Liu and Wang 2016; Zhao et al. 2018). Among the latter are the three genes *Pid-2*, *-3* and *−4*, present as a cluster on chromosome 6 (here after the *Pid* family) (Chen et al. 2006, 2018; Shang et al. 2009). The objective of the present study was to devise a set of reliable FNP markers based on variations in genomic sequences of the *Pid* family, and to use these to exploit the extent of allelic variation available in rice germplasm. A particular focus was to reveal the genetic basis underlying resistance gene divergence between *indica* and *japonica* subspecies.

Methods

Development of a comprehensive FNP marker system

The DNA sequences of *Pid-2* (FJ915121.1), *Pid-3* (FJ745364.1), and *Pid-4* (MG839283.1) present in Digu as well as in a number of reference cultivars were retrieved from GenBank (http://www.ncbi.nim.nih.gov/), and each set of alleles was aligned using Multalin (http://multalin.toulouse.inra.fr/multalin/). A comprehensive FNP marker system, which consists of two sets of FNP markers, one for functional /non-functional haplotypes and another for individual alleles, was developed for a deeper allele mining of each member of the *Pid* family. An interval sequence of each candidate FNP was subjected to various marker designations including CAPS (cleaved amplified polymorphism sequences), and dCAPS (derived CAPS) using dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html). The necessary primer sequences were generated using Primer3 software (https://primer3.ut.ee). As for sequence largely diverged intervals, PCR was drove by triple and/or degenerate primers (Table S1; Zeng 2020; Zhang 2020).

Marker Verification

The FNP assays were validated by testing a larger set of control cvs (so-called CKs), namely Digu (DIG), Tetep (TTP), CO39, Zhenshan 97 (ZS97), Tadukan (TDK), Nipponbare (NPB), Koshihikari (KSH) and Shennong 265 (SN265). Each 20 μL PCR contained 0.1 μL 5 U/μL rTaqase (TaKaRa, Dalian, China), 2.0 μL 10× rTaq Mg^{2+} plus buffer, 0.5 μL 10 mM dNTP (TaKaRa), 1.0 μL 2.5 μM primers (Sangon Biotech, Guangzhou, China), 1.0 μL 10–20 ng/μL template DNA and 14.4 μL ddH_2O. The PCR regime was initiated with a denaturing step (94°C/3 min), which was followed by 35 cycles of 94°C/30 s, 50–62°C/30 s, 72°C/25–30 s and completed with a 72°C/5 min final extension. The resulting amplicons were digested for 3 h with the appropriate restriction enzyme (NEB Inc., Ipswich, MA, USA) at the recommended temperature in a 10 μL reaction containing 1.5 μL PCR product, 0.2 μL 3 U/μL enzyme, 1.0 μL 10× digestion buffer, 8.3 μL ddH_2O. The digested amplicons were electrophoretically separated through 10–12% polyacrylamide gels in the presence of Tris-boric acid-EDTA buffer run at 250 V for 20–50 min depended on the sizes of PCR products.

Genotyping And Data Analysis

A smaller set of control cultivars, i.e., DIG, TTP, ZS97, NPB, SN265, and CO39, were involved in each genotyping experiment (Table S2). The functional and nonfunctional haplotypes of each member of the *Pid* family were firstly determined with a set of haplotype specific FNP markers, and candidate functional alleles were then determined with a set of allele specific FNP markers by testing a regular panel consisting of each 30 representative *indica* and *japonica* type cultivars. For confirming genetic divergence of alleles between *indica* and *japonica* rice groups (if any), allele mining was then extended to two additional germplasm panels, one consisting of 40 *indica* type cultivars used as parents in rice breeding programs based in the southern province of Guangdong and the other of 28 *japonica* type cultivars used - similarly in the north-eastern province of Heilongjiang (Table S2). A χ^2 test was used to determine whether the two genepools had or had not experienced divergence. The test was based on the formula $^2=\frac{N\left[|ad-bc|-\left(\frac{1}{2}\right)N\right]^2}{(a+b)(c+d)(a+c)(b+d)}$, where *a* and *b* represent the number of *indica* type entries scored as, respectively, harbouring or not harbouring a given allele or genotype, while *c* and *d* represent the same for the *japonica* type entries. *N* denotes the total number of alleles or genotypes detected for each *Pid* gene or genotype (Fernando et al. 2018; Zhang et al. 2019; Huang et al. 2021). If all alleles derived from a given resistance gene, which was extremely diverged into *indica* group, then the resistance gene was defined as *indica* type one, and that in turn called as *japonica* type one.

Validation Of Candidate Functional Allele

The full-length of each genomic sequence of two paired alleles of *Pid2*-ZS vs *Pid2*-DG, and *Pid3*-ZS vs *Pid3*-DG was amplified with Q5® High-Fidelity 2X Master Mix (NEB Inc., Ipswich, MA, USA) and fused within a pGEM®-T Easy Vector (Promega Inc., Madison, WI, USA). Then, the correct fragment was digested with the common restriction enzyme *Asc* I and fused into the binary vectors, pYLTAC380H (Zhu et al. 2017) to form a construct carrying individual allele. Each construct was transformed into the blast-susceptible cv. Nipponbare, following Hiei et al. (1994). Transgenic plants were confirmed by PCR-based genotyping with a set of three vector-related markers, the selective marker (HYG) plus two directional vector-gene across markers (Table S1). Then, Phenotypes of transgenic plants were determined by challenging with the reference allele-avirulant isolates (ZB15 for *Pid2*-DG, Chen et al. 2006; and Zhong-10-8-14 for *Pid3*-DG, Shang et al. 2009), according to Pan et al. (1996).

Results

Pid-2 **alleles**

An alignment of *Pid-2* coding sequences (CDSs) of the 15 reference cultivars revealed the presence of seven single nucleotide polymorphisms (Fig. S1). A pair of FNPs, Pid2-F/N^{C1022T} and Pid2-F/N^{A1383G} effectively distinguished between the functional and the non-functional alleles. DIG, TTP, CO39, ZS97 and TDK each carried a functional allele, while NPB, KSH and SN265 carried a non-functional one (Fig. 1). Pid2-DIG^{T2058C} was informative for *Pid2*-DIG allele (DIG), and Pid2-ZS^{A555G} for *Pid2*-ZS allele (TTP, CO39, ZS97, and TDK). When the regular panel was screened, all 30 *indica* type entries were found to carry a functional *Pid-2* allele, but this was the case for only four of the 30 *japonica* types (Fig. 2; Table S2). Of the 34 *Pid-2* carriers, 14 belonged to *Pid2*-DIG, and 18 to *Pid2*-ZS, and two carried a distinct allele (hereafter referred to as *Pid2*-New). The screen of the additional 40 *indica* rice panel revealed that of the 39 carrying a functional copy of *Pid-2*, 32 had the *Pid2*-ZS allele, six the *Pid2*-DIG allele and one the *Pid2*-New allele. None of the additional *japonica* germplasm panel carried a functional copy of *Pid-2* (Fig. S2). A homogeneity test suggested that divergence of *Pid-2* was specific to the *indica* genepool (Table 1). It was, therefore, defined as an *indica* type resistance gene.

Table 1
Distribution of alleles and genotypes of the *Pid* family in the *indica* and *japonica* rice gene pools

Alleles/ Genotypes	***indica* group (*a*, presence / *b*, absence)**	***japonica* group (*c*, presence / *d*, absence)**	**χ^2 for homogeneity [a]**
Alleles			
Pid-2 **alleles**	**69/1**	**4/54**	**105.07*** **
Pid2-ZS	49	1	
Pid2-DIG	17	3	
Pid2-new	3	0	
Pid2-null	1	54	
Pid-3 **alleles**	**69/1**	**4/54**	**105.07*** **
Pid3-ZS	60	3	
Pid3-DIG	5	1	
Pid3-TTP	2	0	
Pid3-new	2	0	
Pid3-null	1	54	
Pid-4 **alleles**	**19/51**	**56/2**	**60.16*** **
Pid4-NPB	1	21	
Pid4-SN	3	16	
Pid4-CO	4	14	
Pid4-new	9	5	
Pid4-DIG	2	0	
Pid4-null	51	2	
Genotypes			
d4	**1/69**	**54/4**	**105.07*** **

[a] χ^2 homogeneity test to determine whether the paired *Pid* alleles varied between the two genepools. Calculations based on the formula, $^2=\frac{N\left[|ad-bc|-\left(\frac{1}{2}\right)N\right]^2}{(a+b)(c+d)(a+c)(b+d)}$, where ** and *** represent the paired *Pid* alleles and genotypes differed between *indica* and *japonica* genepools, significantly (P<0.01 and P<0.001, respectively; df=1). The frequency of the presence and absence of each allele/genotype in the *indica* genepool is given by, respectively, *a* and *b*, and in the *japonica* genepool by, respectively, *c* and *d*. *N* denotes the total number of alleles/genotypes detected for each *Pid* gene/genotype.

Alleles/ Genotypes	*indica* group (*a*, presence / *b*, absence)	*japonica* group (*c*, presence / *d*, absence)	χ^2 for homogeneity [a]
d2-d3	**51/19**	**2/56**	**60.16*** **
d2-d3-d4	**18/52**	**2/56**	**10.30** **

[a] χ^2 homogeneity test to determine whether the paired *Pid* alleles varied between the two genepools. Calculations based on the formula, $^2 = \frac{N\left[|ad-bc| - \left(\frac{1}{2}\right)N\right]^2}{(a+b)(c+d)(a+c)(b+d)}$, where ** and *** represent the paired *Pid* alleles and genotypes differed between *indica* and *japonica* genepools, significantly (P<0.01 and P<0.001, respectively; df=1). The frequency of the presence and absence of each allele/genotype in the *indica* genepool is given by, respectively, *a* and *b*, and in the *japonica* genepool by, respectively, *c* and *d*. *N* denotes the total number of alleles/genotypes detected for each *Pid* gene/genotype.

Pid-3 **alleles**

The variation in the *Pid-3* CDSs identified in the 15 reference cultivars comprised 29 SNPs and one InDel (Fig. S3); 18 of the SNPs and the indel were targeted for marker development (data not shown). The Pid3-F/N^{G2009A} and Pid3-F/N^{C2209T} were both effective for distinguishing between functional and non-functional alleles: the five cultivars DIG, TTP, CO39, ZS97 and TDK each carried a functional allele, while NPB, KSH and SN265 each carried a non-functional one (Fig. 3; Table S2). Three pairs, Pid3-DIG^{G775A} vs Pid3-DIG^{G2695A}, Pid3-TTP^{C1136T} vs Pid3-TTP^{C1623G}, and Pid3-ZS^{G477A} vs Pid3-ZS^{C525T}, were ensured as allele-specific FNP markers responsible for *Pid3*-DIG, *Pid3*-TTP, and *Pid3*-ZS, respectively (Fig. 3). As was the case for *Pid-2*, all 30 members of the *indica* panel carried a functional *Pid-3* haplotype, whereas only four of the *japonica* panel did so (Fig. 4; Table S2). The distribution of effective alleles was highly uneven: 29 of the *Pid-3* carriers harboured the *Pid3*-ZS allele, three the *Pid3*-DIG allele, one the *Pid3*-TTP and one a novel allele (*Pid3*-New). The distribution was similarly uneven in the additional *indica* panel, where 28 of the *Pid-3* positive entries carried the *Pid3*-ZS allele, three the *Pid3*-DIG allele, one the *Pid3*-TTP allele and one *Pid3*-New; none of the members of the additional *japonica* panel carried an effective allele (Fig. S4). A homogeneity test implied that divergence for *Pid-3* has only occurred in the *indica* genepool (Table 1). It was, also, referred to as an *indica* type resistance gene.

Pid-4 **alleles**

Pid-4 was by far the most diverse of the three members, with 149 SNPs and six InDels identified in the CDSs plus one intron of the 13 reference cultivars (Fig. S5), a sample of these (17 SNPs and two InDels) were targeted for marker development (data not shown). Both Pid4-F/N^{C1217G} and Pid4-F/N^{A1452G} were informative with respect to functionality: five cultivars, DIG, NPB, KSH, CO39, and SN265, were recognized as carriers of functional alleles, while TDK, TTP and ZS97 harboured non-functional alleles (Fig. 5; Table S2). Two pairs, Pid4-DIG^{A1149T} *vs* Pid4-DIG^{A1898G}, and Pid4-NPB^{G1362A} *vs* Pid4-NPB^{C1554A}, were confirmed as allele-specific FNP markers responsible for *Pid4*-DIG and *Pid4*-NPB, respectively; and Pid4-

SN/CO^{T1841A} coupled with Pid4-SN/CO^{G2250C} responsible for both *Pid4*-SN and *Pid4*-CO (Fig. 5). Unlike the situation in both *Pid-2* and *Pid-3*, functional *Pid-4* alleles were present in many (28/30) of the *japonica* type entries, while the frequency of functional alleles was only moderate (12/30) in the *indica* germplasm (Fig. 6; Table S2). The distribution of the various alleles was more even than was the case for *Pid-2* and *Pid-3*, with 14 entries carrying the *Pid4*-SN allele, 11 the *Pid4*-NPB allele, eight *Pid4*-New and six the *Pid4*-CO allele (Fig. 6). Extending the screen to the two additional panels revealed that 28/40 *japonica* type cultivars harboured a functional allele, while only 7/40 *indica* type cultivars did so. Of the 35 functional haplotypes, 12 were present in entries carrying the *Pid4*-CO allele, 11 in those carrying the *Pid4*-NPB allele, seven in those carrying *Pid4*-New, five in those carrying the *Pid4*-SN allele, while just a single entry carried the *Pid4*-DIG allele (Fig. S6). A homogeneity test confirmed that significant divergence at *Pid-4* has occurred in the *japonica* genepool (Table 1). Thus, it was termed '*japonica* type resistance gene'.

Performance Of Candidate Functional Allele

The transgenic progenies derived from two paired alleles of *Pid2*-ZS vs *Pid2*-DG, and *Pid3*-ZS vs *Pid3*-DG revealed that both new functional alleles (*Pid2*-ZS and *Pid3*-ZS) expressed slightly higher resistance than their reference alleles (*Pid2*-DG and *Pid3*-DG; Fig. 7). It was, therefore, demonstrated that the candidate functional alleles being explored by the comprehensive FNP marker systems were promising ones for conveying their resistance.

Discussion

The comprehensive FNP marker systems were largely improved the marker works

In the present study, the comprehensive FNP marker systems consisting of two set of FNP markers was carried out on the *Pid* family in the three panels consisting of 70 *indica* and 58 *japonica* cultivars, which were selected from various regions across landrace and modern rice eras (Tables 1; S2). Each comprehensive FNP marker system for a deeper allele mining of the *Pid* family was devised based on several criterions such as representative of FNPs over a given CDS, clear genotyping, and easy-accession to users (Zeng 2020; Zhang 2020). As almost resistance genes have been initially diverged into functional and nonfunctional haplotypes (Figs. S1, S3, S5; Bryan et al. 2000; Lin et al. 2007; Liu et al. 2007; Fukuoka et al. 2009; Hua et al. 2012; Yuan et al. 2011; Zhai et al. 2011, 2014; Zhao et al. 2018; Zeng 2020), allele mining of each member of the *Pid* family was, therefore, initiated from haplotype differentiation with a set of haplotype specific FNP markers, which enabled us to identify any new allele in a given cultivar belonging to the functional haplotype. It means that allele mining could be stopped when the panel where was not any functional haplotype (Figs. S2, S4). Then allele mining was pursued to individual alleles with a set of allele-specific FNP markers, which helped us finding out more certain alleles within the functional haplotypes. Even so, there were still 3, 2, and 14 cultivars in *Pid-2*, *Pid-3*, and *Pid-4* categories, respectively, which were presumed to carry new types of alleles, compared to the defined

alleles (Table 1). That, in turn, indicated that each comprehensive FNP marker system was inclusive for finding unlimited new alleles, as it has included the major FNPs at each locus. Collectively, the comprehensive FNP marker systems used in the present study were largely improved from those used in the previous investigations, as almost of those were in a working model, as called “the specific marker(s)-for-the specific allele” model, for allele mining. In contrast, the working model (the deeper allele mining model) used in the current study could be defined as “a comprehensive marker system-for-the whole locus/cluster”, which makes an inclusive and comparable approach for finding out a series of new alleles as were the three cases shown in the current study (Shang et al. 2009; Lv et al. 2017; Promchuay and Nilthong 2017; Teerasan et al. 2019; Tian et al. 2020; Zhou et al. 2020). The outstanding merit of the comprehensive FNP marker system was not only for mining new alleles for breeding, but also for revealing molecular mechanisms underlying genetic divergence of the whole locus/cluster (Table 1).

The Pid **family has been strictly diverged into** indica **and** japonica **subspecies**

Four alleles were detected at *Pid-2*, of which the three functional ones were almost entirely restricted to *indica* type cultivars, while the null allele was only present in *japonica* type germplasm (Table 1). The distribution of alleles at *Pid-3* was very similar: the four functional ones were harboured for the most part by *indica* type entries and the null allele was common in the *japonica* genepool (Table 1). The latter result echoes a prior finding that the alleles of *Pid-3* present in *japonica* type cultivars are pseudogenes (Shang et al. 2009; Lv et al. 2017). In contrast, the distribution of alleles at *Pid-4* featured five functional alleles which were shared evenly among the *japonica* type entries, with the null allele found only in *indica* type ones (Table 1). It is notable that the well-known resistance alleles at *Pi2/ 9*, *Pia* and *Pita* loci/cluster have evenly diverged into both subspecies (Zeng et al. 2020; Zhang et al. 2020). It might be the first time to find out and define both *indica* and *japonica* type resistance genes within individual cultivars through the deeper allele mining with the comprehensive FNP marker system (Table S2). The data revealed by the FNP screen suggest a plausible genetic basis for the stable and broad blast resistance exhibited by the modern cultivars, Digu, R207, Lu28S, Tianfeng B, R217, Zhonghua 11, Gumeizao 4, Moliruanzhan, Yuehesimiao and Yuejingsimiao 2, in that all these cultivars harbour a functional allele at each of the three *Pid* genes (Table S2). It might be truly expected that integration of both *indica* and *japonica* resistance genes into upcoming cultivars would be one of the most promising ways to enlarge their genetic diversities of resistance genic resources thereby withstanding ever-growing pressure from the pathogen across *indica* and *japonica* rice areas (Shang et al. 2009; Zhang et al. 2016; Lv et al. 2017, 2020; Zhang et al. 12017, 2019; Huang et al. 2021).

Rather limited genotypes of the Pid **family have been effective in both** indica **and** japonica **rice groups**

By owning three members of the *Pid* family, there would be seven all possible genotypes (combinations) (2^3-1 = 7), shortly as: *d2*, *d3*, *d4*; *d2-d3*, *d2-d4*, *d3-d4*; and *d2-d3-d4*, irrespective of specific alleles. However, only three genotypes, *d4*, *d2-d3*, and *d2-d3-d4*, were detected in the three panels consisting of 128 diversified rice germplasms (Tables 1, S2). The indication is therefore that rather limited genotypes of the *Pid* family have been integrated into both *indica* and *japonica* rice cultivars in China. Since all the three

members have been strictly diverged into the two subspecies across landrace and modern rice eras, *d2-d3* was centralized in *indica* group and *d4* in *japonica* one both reached at overwhelming proportions; and *d2-d3-d4* also in *indica* group but with a rather moderate rate (Table 1). The genomic structure of the region harbouring the *Pid* family does not suggest any obvious barrier to local recombination (Fig. S7). That is, there were four types of such barriers in the target genomic region, the key subspecies hybrid sterile gene cluster *S5* (Chen et al. 2008), the heading date gene *Hd1* (Yano et al. 2000), the photonasty gene *Se5* (Izawa et al. 2000), and the centromere of rice chromosome 6 (Zhao et al. 2019), all of which were enough far from the genic positions of the *Pid* family. In addition, genomic intervals among the three members were also enough long for independently segregation each other in a given genetic cross (Fig. S7). Again, the *P2/ 9* cluster near the *Pid* family did not show any subspecific divergence in the same rice population tested in the current investigation (Fig. S7; Zeng et al. 2020; Zhang et al. 2020). That, in turn, indicates that the subspecific divergence in the genomic region was specific to the *Pid* family.

One of the most possible genetic determinants leading to establish such specific allelic and genotypic structures of the *Pid* family was, therefore, due to the specific lineage(s) of the Chinese rice population. That is, the Chinese rice population has, indeed, been derived from rather limited founder parents for an age. By reviewing the pedigrees of Top-10 of several cultivar types including the general cultivars and F_1 hybrid crosses, Liu (2021) pointed out that the specific lineage, 'Zhenzhuai 11-ZS97', both were recognized as *Pid2*-ZS_*Pid3*-ZS, has been central to Chinese *indica* type rice breeding programs since the 1960s (also see www.ricedata.cn/variety). The specific lineage perfectly addressed to both questions: why there were two *indica* type alleles with much higher rates in the respective allelic structures, *Pid2*-ZS with 71.4% (50/73), and *Pid3*-ZS with 86.3% (63/73); and why there was not any single gene genotype for *d2* or *d3*, but *d2-d3* for being predominant among the three effective genotypes for a long time (Tables 1, S2). That is, the unique allelic structures of the three members of the *Pid* family have been mainly constructed by the genotype, *d2-d3*, exactly, *Pid2*-ZS_ *Pid3*-ZS, carried by the lineage in rice breeding programs in China since the 1960s. It was, again, concerned that updating the lineage would be the key to enlarge genetic diversities of rice cultivars in the next generation in China (Shang et al. 2009; Zhang et al. 2016; Lv et al. 2017, 2020; Liu 2021; www.ricedata.cn/variety).

A further priority will be to address an intriguing question whether and why rather limited genotypes of the *Pid* family have also been predominant in other rice germplasm populations via plant genetic resources and functional genomics approaches.

Conclusions

The study has demonstrated that all the three members of the *Pid* family have been strictly diverged into *indica* and *japonica* subspecies: *Pid-2* and *Pid-3* were defined as *indica* type resistance genes, and *Pid-4* as *japonica* one. Rather limited genotypes of the *Pid* family have been effective in both *indica* and *japonica* rice groups, of which *Pid2*-ZS + *Pid3*-ZS has been central to the Chinese rice population. The resistance functions of both *Pid2*-ZS and *Pid3*-ZS were demonstrated by their transgenic progenies.

Declarations

Acknowledgements

The authors appreciate the team-works contributed from the Pan's laboratory members and thank Dr. Qinlong Zhu at South China Agricultural University for providing the vector, pYLTAC380H, for genetic transformation; and Dr. Xuewei Chen at Sichuan Agricultural University for providing *P. oryzae* isolates for inoculation.

Author's contributions

Project conception (QHP), marker development (JYW, JQW, XW, QHP), material composition (YZ, YXY, LW, XW, JFZ, YHZ, QHP), allele mining (RPC, JQW, XW, XMY), transformation test (XW, ZJL), data analysis (JYW, RPC, QHP), manuscript preparation (JYW, QHP). All authors read and approved the final manuscript.

Funding

This study was supported by grants from the National Key R&D Project (2016YDF0100601), the National Transgenic Research Project (2015ZX08001-002), and the National Natural Science Foundation of China (U1131003).

Availability of Data and Materials

The data sets supporting the results of this article are included within the article and its supporting files.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bryan G, Wu K, Farrall L, Jia Y, Hershey H, McAdams S, Faulk K, Donaldson G, Tarchini R, Valent B (2000) A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene *Pita*. Plant Cell 12: 2033–2045
2. Chen J, Ding J, Ouyang Y, Du H, Yang J, Cheng K, Zhao J, Qiu S, Zhang X, Yao J, Liu K, Wang L, Xu C, Li X, Xue Y, Xia M, Ji Q, Lu J, Xu M, Zhang Q (2008) A triallelic system of S5 is a major regulator of

the reproductive barrier and compatibility of indica-japonica hybrids in rice. Proc Natl Acad USA 195: 11436–11441

3. Chen X, Shang J, Chen D, Lei C, Zou Y, Zhai W, Liu G, Xu J, Ling Z, Cao G, Ma B, Wang Y, Zhao X, Li S, Zhu L (2006) A B-lectin receptor kinase gene conferring rice blast resistance. Plant J 46: 794–804
4. Chen Z, Zhao W, Zhu X, Zou C, Yin J, Chen M, Zhou X, Ying H, Jiang X, Li Y, Liao H, Cheng M, Li W, He M, Wang J, Wang J, Ma B, Wang J, Li S, Zhu L, Chen X (2018) Identification and characterization of rice blast resistance gene *Pid4* by a combination of transcriptomic profiling and genome analysis. J. Genet Genomics 45: 665–672
5. Choi J, Purugganan M (2018) Multiple origin but single domestication led to *Oryza sativa*. G3 8: 797–803
6. Dean R, Van Kan J, Pretorius Z, Hammond-Kosack K, Pietro A, Spanu P, Rudd J, Dickman M, Kahmann R, Ellis J, Foster G (2012) The Top 10 fungal pathogens in molecular plant pathology. Mol Plan Pathol 13: 414–430
7. Deng Y, Zhai K, Xie Z, Yang D, Zhu X, Liu J, Wang X, Qin P, Yang Y, Zhang G, Li Q, Zhang J, Wu S, Milazzo J, Mao B, Wang E, Xie H, Tharreau D, He Z (2017) Epigenetic regulation of antagonistic receptors confers rice blast resistance with yield balance. Science 355: 962–965
8. Du H, Yu Y, Ma Y, Gao Q, Cao Y, Zhen Z, Ma B, Qi M, Li Y, Zhao X, Wang J, Liu K, Qin P, Yang X, Zhu L, Li S, Liang C (2017) Sequencing and *de novo* assembly of a near complete *indica* rice genome. Nature Commun 8: 15324
9. Ebbole D (2007) Magnaporthe as a model for understanding host-pathogen interactions. Annu Rev Phytopathol 45: 437–456
10. Fernando D, Zhang X, Selin C, Zou Z, Liban S, McLaren D, Kubinec A, Parks P, RashodH, Pamathilake R, Rong L, Yang C, Gnanesh B, Huang S (2018) A six-year investigation of the dynamics of avirulence allele profiles, blackleg incidence, and mating type alleles of *Leptosphaeria maculans* populations associated with canola crops in Manitoba, Canada. Plant Dis 102: 790–798
11. Flor H (1971) Current status of the gene for gene concept.Ann Rev Phytopathol 9:275–296
12. Fukuoka S, Saka N, Koga H, Ono K, Shimizu T, Ebana K, Hayashi N, Takahashi A, Hirochika H, Okuno K, Yano M (2009) Loss of function of a proline-containing protein confers durable disease resistance in rice. Science 325: 998–1001
13. He X, Liu X, Wang L, Lin F, Cheng Y, Chen Z, Liao Y, Pan Q (2012) Identification of the novel recessive gene *pi55*(t) conferring resistance to *Magnaporthe oryzae*. Sci China Life Sci 55:141–149
14. Hiei Y, Ohta S, Komari T, Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6: 271–282
15. Hua L, Wu J, Chen C, Wu W, He X, Lin F, Wang L, Ashikawa I, Matsumoto T, Wang L, Pan Q (2012) The isolation of *Pi1*, an allele at *Pik* locus which confers broad spectrum resistance to rice blast. Theor Appl Genet 125:1047–1055

16. Huang X, Zhao Y, Wei X, Li C, Wang A, Zhao Q, Li W, Guo Y, Deng L, Zhu C, Fan D, Lu Y, Weng Q, Liu K, Zhou T, Jing Y, Si L, Dong G, Huang T, Feng Q, Qian Q, Li J, Han B (2012) Genome-wide association study of flowering time and grain yield traits in a worldwide collection of rice germplasm. Nature Genetics 44: 32–39
17. Huang Z, Wang J, Zhang Y, Yao Y, Huang L, Yang X, Wang L, Pan Q (2021) Dynamics of race structures of *Pyricularia oryzae* populations across 18 seasons in Guangdong province, China. Plant Dis 105: 144–148
18. Izawa T, Oikawa T, Tokutomi S, Okuno K, Shimamoto K (20000) Phytochromes confer the photoperiodic control of flowering in rice (a short-day plant). Plant J 22: 391–399
19. Li C, Wang D, Peng S, Chen Y, Su P, Chen J, Zheng L, Tan X, Liu J, Xiao Y, Kang H, Zhang D, Wang G, Liu Y (2019) Genome-wide association mapping of resistance against rice blast strains in South China and identification of a new *Pik* allele. Rice 12: 47
20. Lin F, Chen S, Que Z, Wang L, Liu X, Pan Q (2007) The blast resistance gene *Pi37* encodes an NBS-LRR protein and is a member of a resistance gene cluster on rice chromosome 1. Genetics 177: 1871–1880
21. Liu D (2021) Decoding pedigrees of the top-10 general cultivars and rice F_1 hybrid systems in China. Available via DIALOG. https://mp.weixin.qq.com/s/7L5PcYMxGFXLJlVjfSM3dQ. Accessed 23 Feb, 2021
22. Liu W, Wang G (2016) Plant innate immunity in rice: a defense against pathogen infection. Natl Sci Rev 3: 295–308
23. Liu X, Lin F, Wang L, Pan Q (2007) The *in silico* map-based cloning of *Pi36*, a gene encodes a coiled-coil NBS-LRR protein, conferring race-specific resistance to *Magnaporthe oryzae*. Genetics 176: 2541–2549
24. Lv Q, Huang Z, Xu X, Tang L, Liu H, Wang C, Zhou Z, Xin Y, Xing J, Peng Z, Li X, Zheng T, Zhu L (2017) Allelic variation of the rice blast resistance gene *Pid3* in cultivated rice worldwide. Sci Rep 7: 10362
25. Lv Q, Li W, Sun Z, Ouyang N, Jing X, He Q, Wu J, Zheng J, Zheng J, Tang S, Zhu R, Tian R, Duan M, Tan Y, Yu D, Sheng X, Sun X, Jia G, Gao H, Zeng Q, Li Y, Tang L, Xu Q, Zhao B, Huang Z, Lu H, Li N, Zhao J, Zhu L, Li D, Yuan L, Yuan D (2020) Resequencing of 1,143 indica rice accessions reveals important genetic variations and different heterosis patterns. Nature Commun 11: 4778
26. Ouyang Y, Zhang Q (2013) Understanding reproductive isolation based on the rice model. Annu Rev Plant Biol 64: 111–135
27. Pan Q, Wang L, Ikehashi H, Tanisaka T (1996) Identification of a new blast resistance gene in the *indica* rice cultivar Kasalath using Japanese differential cultivars and isozyme markers. Phytopathology 86: 1071–1075
28. Promchuay A, Nilthong S (2017) Investigation of *Pid3* rice blast resistance gene in northern upland rice varieties (Oryza sativa L.), Thailand using molecular markers. J Advanced Agri Technol 4: 209–214

29. Shang J, Tao Y, Chen X, Zou Y, Lei C, Wang J, Li X, Zhao X, Zhang M, Li Z, Xu J, Cheng Z, Wang J, Zhu L (2009) Identification of a new rice blast resistance gene, *Pid3*, by genome-wide comparison of paired nucleotide-binding site-leucine-rich repeat genes and their pseudogene alleles between the two sequenced rice genomes. Genetics 182: 1303–1311
30. Teerasan W, Srikaew I, Phaitreejit K, Kate-Ngam K, Jantasuriyarat C (2019) Gene-specific marker screening and disease reaction validation of blast resistance genes, *Pid3*, *Pigm* and *Pi54* in Thai landrace rice gerplasm and recommended rice varieties. Plant Genetic Resources: Characterization and Utilization 17: 421–426
31. Tian D, Lin Y, Chen Z, Chen Z, Yang F, Wang F, Wang Z, Wang M (2020) Exploring the distribution of blast resistance alleles at the *Pi2/9* locus in major rice-producing areas of China by a novel Indel marker. Plant Dis 104: 1932–1938
32. Wang X, Jia Y, Wamishe Y, Jia M, Valent B (2017) Dynamic changes in the rice blast population in the USA over six decades. Mol Plant-Microbe Interac 30:803–812
33. Wang W, Mauleon R, Hu Z et al. (2018) Genomic variation in 3,010 diverse accessions of Asian cultivated rice. Nature 557: 43–49
34. Wu W, Wang L, Zhang S, Li Z, Zhang Y, Lin F, Pan Q (2014) Stepwise arms race between *AvrPik* and *Pik* alleles in the rice blast pathosystem. Mol Plant-Microbe Interac 27:759–769
35. Xiao G, Yang J, Zhu X, Wu J, Zhou B (2020) Prevalence of ineffective haplotypes at the rice blast resistance (*R*) gene loci in Chinese elite hybrid varieties revealed by sequence-based molecular diagnosis. Rice 13: 6
36. Yano M, Katayose Y, Ashikari M, Yamanouchi U, Monna L, Fuse T, Baba T, Yamamoto K, Umehara Y, Nagamura Y, Sasaki T (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene *CONSTANS*. Plant Cell 12: 2473–2483
37. Yuan B, Wang L, Zeng X, Xu X, Zhai C, Hu H, Lin F, Pan Q (2011) The *Pik-p* resistance to *Magnaporthe oryzae* in rice is mediated by a pair of closely linked CC-NBS-LRR genes. Theor Appl Genet 122: 1017–1028
38. Zeigler R, Cuoc L, Scott R, Bernardo M, Chen D, Valent B, Nelson R (1995) The relationship between lineage and virulence in *Pyricularia grisea* in the Philippines. Phytopathology 85:443–451
39. Zeng S (2020) Identification of rice blast resistance genes in the key parental cultivars. Dissertation, South China Agri University, Guangzhou, China (In Chinese with English Abstract)
40. Zhai C, Wang L, Dong Z, Zeng X, Lin F, Pan Q (2011) The rice blast resistance gene, *Pik*, is the third allele at the *Pik* cluster that is distinguished by a function-associated SNP. New Phytologist 189: 321–334
41. Zhai C, Zhang Y, Yao N, Lin F, Liu Z, Dong Z, Wang L, Pan Q (2014) Function and interaction of the coupled genes responsible for *Pik-h* encoded rice blast resistance. Plos One 9: e98067
42. Zhang J, Chen L, Xing F, Kudrna D, Yao W, Copetti D, mu T, Li W, Song J, Xie W, Lee S, Talag J, Shao L, An Y, Zhang C, Ouyang Y, Sun S, Jiao W, Lv F, Du B, Luo M, Maldonado C, Goicoechea J, Xiong L, Wu C, Xing Y, Zhou D, Yu S, Zhao Y, Wang G, Yu Y, Luo Y, Zhou Z, Hurtado B, Danowitz A, Wing R, Zhang

Q (2016) Extensive sequence divergence between the reference genomes of two elite indica rice varieties Zhenshan 97 and Minghui 63. Proc Natl Acad Sci USA E5163-5171

43. Zhang R (2020) Resistance gene composition of differential cultivars for rice blast pathosystem. Dissertation, South China Agri University, Guangzhou, China (In Chinese with English Abstract)
44. Zhang S, Wang L, Wu W, He L, Yang X, Pan Q (2015) Function and evolution of *Magnaporthe oryzae* avirulence gene *AvrPib* responding to the rice blast resistance gene *Pib*. Sci Rep 5:11642
45. Zhang Y, Zhu Q, Yao Y, Zhao Z, Correll J, Wang L, Pan Q (2017) The race structure of the rice blast pathogen across southern and northeastern China. Rice 10: 46
46. Zhang Y, Wang J, Yao Y, Jin X, Correll J, Wang L, Pan Q (2019) Dynamics of race structures of the rice blast pathogen populations in Heilongjiang province, China from 2006 through 2015. Plant Dis 103: 2759–2763
47. Zhao H, Wang X, Jia Y, Minkenberg B, Wheatley M, Fan J, Jia M, Famoso A, Edwards J, Wamishe Y, Valent B, Wang G, Yang Y (2018) The rice blast resistance gene *Ptr* encodes an atypical protein required for broad-spectrum disease resistance. Nature Commun 9: 2039
48. Zhao L, Wang S, Cao Z, Ouyang Y, Zhang Q, Xie L, Zheng R, Guo M, Ma M, Hu Z, Sung W, Zhang Q, Li G, Li X (2019) Chromatin loops associated with active genes and heterochromatin shape rice genome architecture for transcriptional regulation. Nature Commun 10: 3640
49. Zhou Y, Lei F, Wang Q, He W, Yuan B, Yuan W (2020) Identification of novel alleles of the rice blast-resistance gene *Pi9* through sequence-based allele mining. Rice 13: 80
50. Zhu Q, Yu S, Zeng D, Liu H, Wang H, Yang Z, Xie X, Shen R, Tan J, Li H, Zhao X, Zhang Q, Chen Y, Guo J, Chen L, Liu Y (2017) Development of “Purple Endosperm Rice” by engineering anthocyanin biosynthesis in the endosperm with a high-efficiency transgene stacking system. Molecular Plant 10: 918–929

Figures

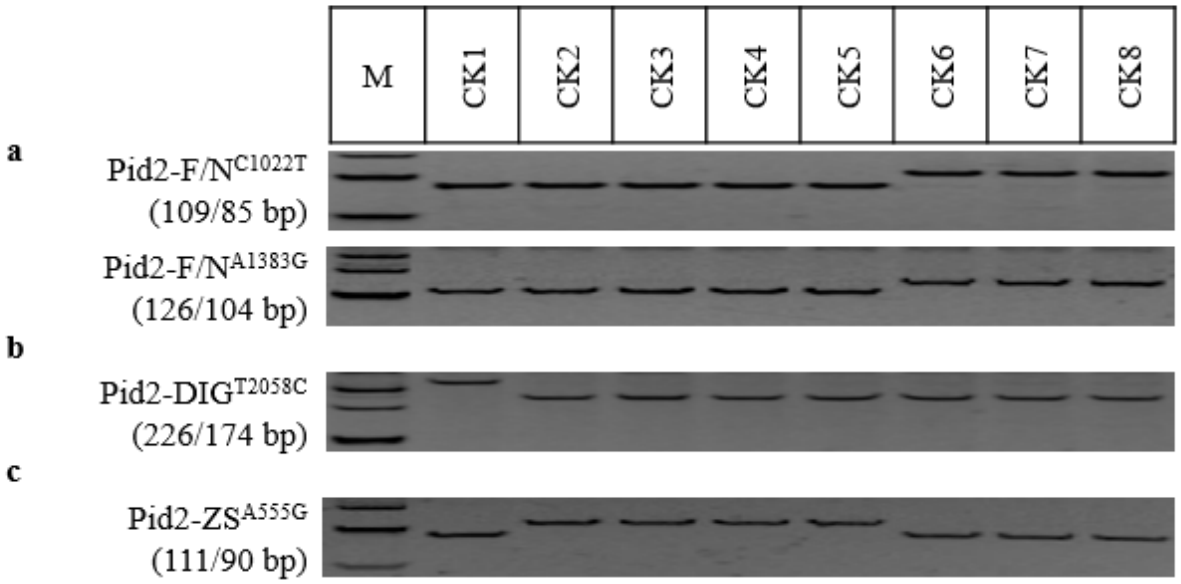

Figure 1

Development of a comprehensive FNP marker system able to distinguish both haplotypes and alleles of *Pid-2*.

(**a**-**c**) Discriminating between functional (F) and non-functional (N) haplotypes and between *Pid2*-DIG and *Pid2*-ZS alleles. CK1, Digu (*Pid2*-DIG); CK2, Tetep (*Pid2*-ZS); CK3, CO39 (*Pid2*-ZS); CK4, Zhenshan 97 (*Pid2*-ZS); CK5, Tadukan (*Pid2*-ZS); CK6, Nippon bare (*Pid2*-Null); CK7, Koshihikari (*Pid2*-Null); CK8, Shennong 265 (*Pid2*-Null). M, DL-500 size marker.

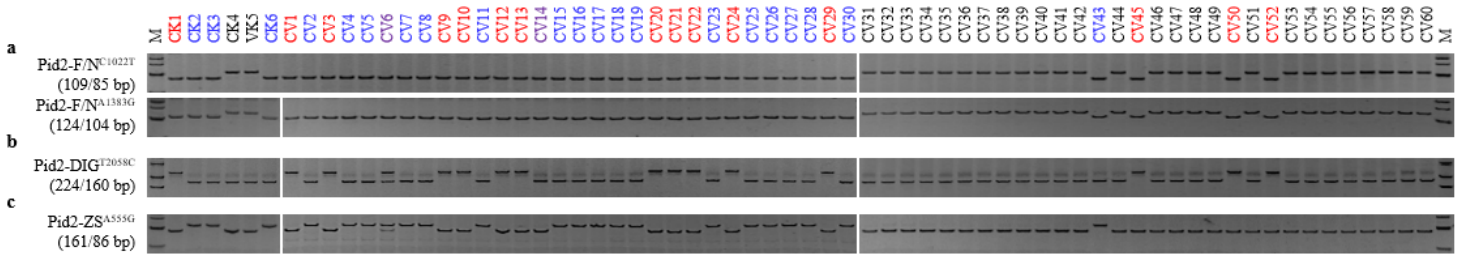

Figure 2

Alleles of *Pid-2* represented in the regular panel consisting of both *indica* (CV1-30) and *japonica* (CV31-60) types.

(**a**) Functional and non-functional haplotypes, (**b**) the *Pid2*-DIG allele, (**c**) the *Pid2*-ZS allele. CK1, Digu (*Pid2*-DIG; red); CK2, Tetep (*Pid2*-ZS; blue); CK3, Zhenshan 97 (*Pid2*-ZS; blue); CK4, Nipponbare (*Pid2*-Null; black); CK5, Shennong 265 (*Pid2*-Null; black); CK6, CO39 (*Pid2*-ZS; blue); and the undefined alleles carried

by CV6 and CV14 was marked in purple. The detailed information on each entry was shown in Table S2. M, DL-500 size marker.

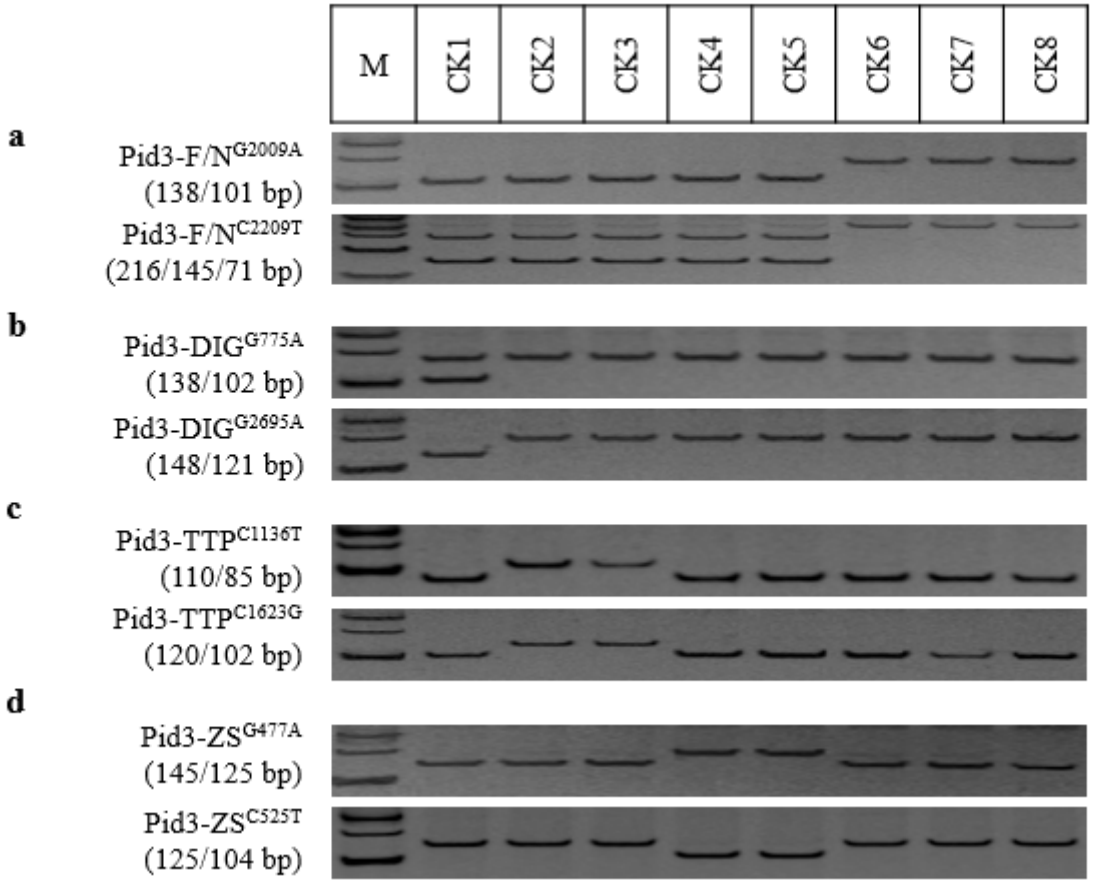

Figure 3

Development of a comprehensive FNP marker system able to distinguish both haplotypes and alleles of *Pid-3*.

(**a**-**d**) Discriminating between functional (F) and non-functional (N) haplotypes and among *Pid3*-DIG, *Pid3*-TTP, and *Pid3*-ZS alleles. CK1, Digu (*Pid3*-DIG); CK2, Tetep (*Pid3*-TTP); CK3, Tadukan (*Pid3*-TTP); CK4, Zhenshan 97 (*Pid3*-ZS); CK5, CO39 (*Pid3*-ZS); CK6, Nipponbare (*Pid3*-Null); CK7, Shennong 265 (*Pid3*-Null); CK8, Koshihikari (*Pid3*-Null). M, DL-500 size marker.

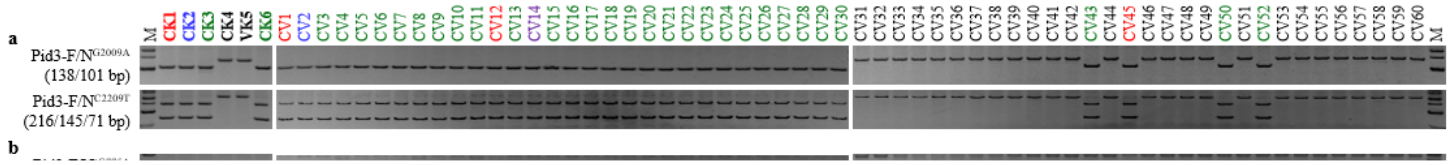

Figure 4

Alleles of *Pid-3* represented in the regular panel consisting of both *indica* (CV1-30) and *japonica* (CV31-60) types.

(**a**) Functional and non-functional haplotypes, (**b**) the *Pid3*-DIG allele, (**c**) the *Pid3*-TTP allele, (**d**) the *Pid3*-ZS allele. CK1, Digu (*Pid3*-DIG; red); CK2, Tetep (*Pid3*-TTP; blue); CK3, Zhenshan 97 (*Pid3*-ZS; green); CK4, Nipponbare (*Pid3*-Null; black); CK5, Shennong 265 (*Pid3*-Null; black); CK6, CO39 (*Pid3*-ZS; green); and the undefined allele carried by CV14 was marked in purple. The detailed information on each entry was shown in Table S2. M, DL-500 size marker.

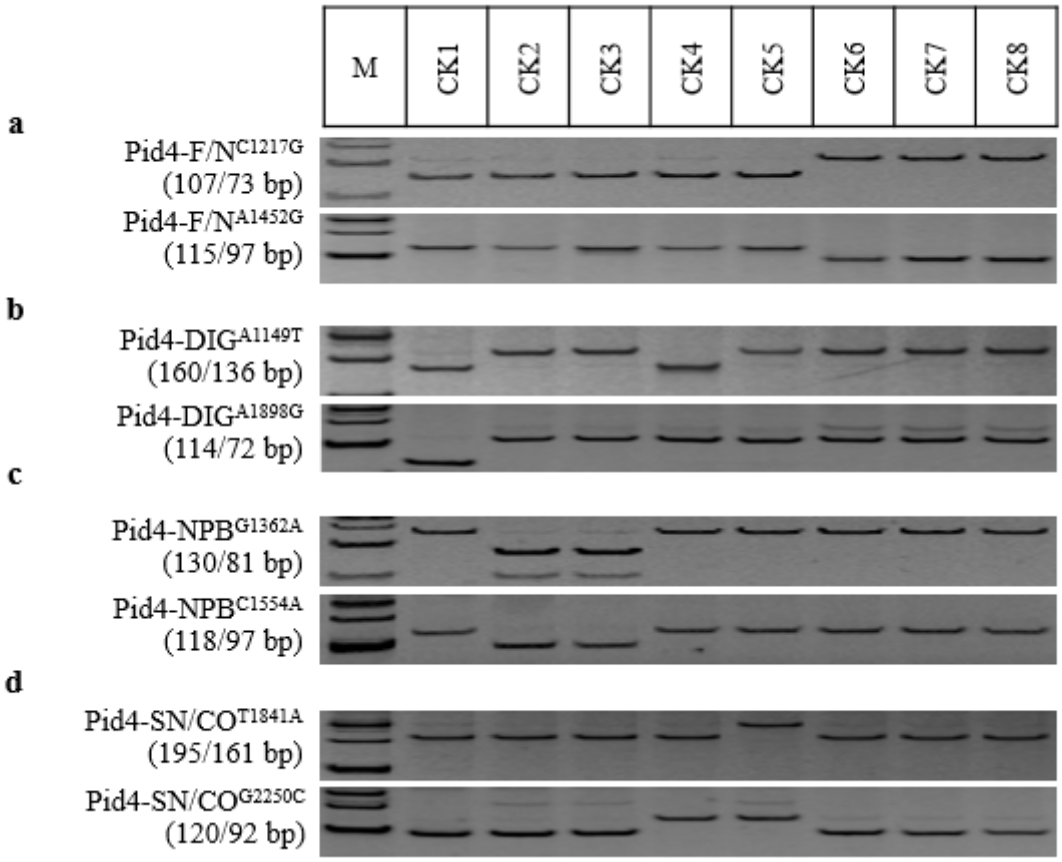

Figure 5

Development of a comprehensive FNP marker system able to distinguish both haplotypes and alleles of *Pid-4*.

(**a**-**d**) Discriminating between functional (F) and non-functional (N) haplotypes and among *Pid4*-DIG, *Pid4*-NPB, *Pid4*-CO, and *Pid4*-SN alleles. CK1, Digu (*Pid4*-DIG); CK2, Nipponbare (*Pid4*-NPB); CK3, Koshihikari (*Pid4*-NPB); CK4, CO39 (*Pid4*-CO); CK5, Shennong 265 (*Pid4*-SN); CK6, Tadukan (*Pid4*-Null); CK7, Tetep (*Pid4*-Null); CK8, Zhenshan 97 (*Pid4*-Null). M, DL-500 size marker.

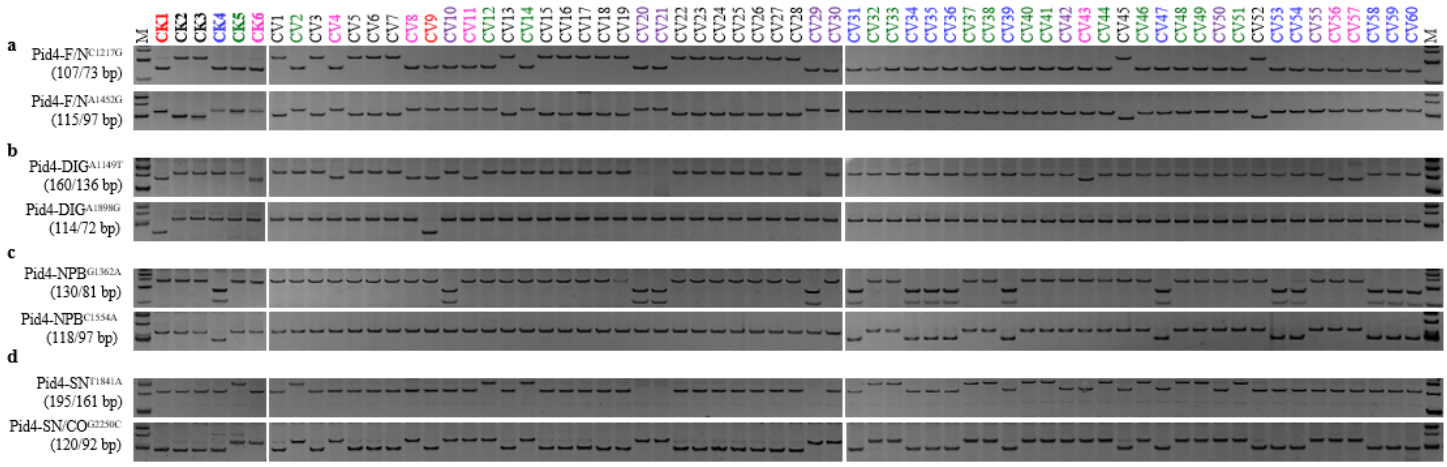

Figure 6

Alleles of *Pid-4* represented in the regular panel consisting of both *indica* (CV1-30) and *japonica* (CV31-60) types.

(**a**) Functional and non-functional haplotypes, (**b**) the *Pid3*-DIG allele, (**c**) the *Pid3*-NPB allele, (**d**) the *Pid4*-SN and *Pid4*-CO alleles. CK1, Digu (*Pid4*-DIG; red); CK2, Tetep (*Pid4*-Null; black); CK3, Zhenshan 97 (*Pid4*-Null; black); CK4, Nipponbare (*Pid4*-NPB; blue); CK5, Shennong 265 (*Pid4*-SN; green); CK6, CO39 (*Pid4*-CO; rose-red); and the undefined alleles carried by CVs 10, 20, 21, 29, 30, 42, 50, and 55, were marked in purple. The detailed information on each entry was shown in Table S2. M, DL-500 size marker.

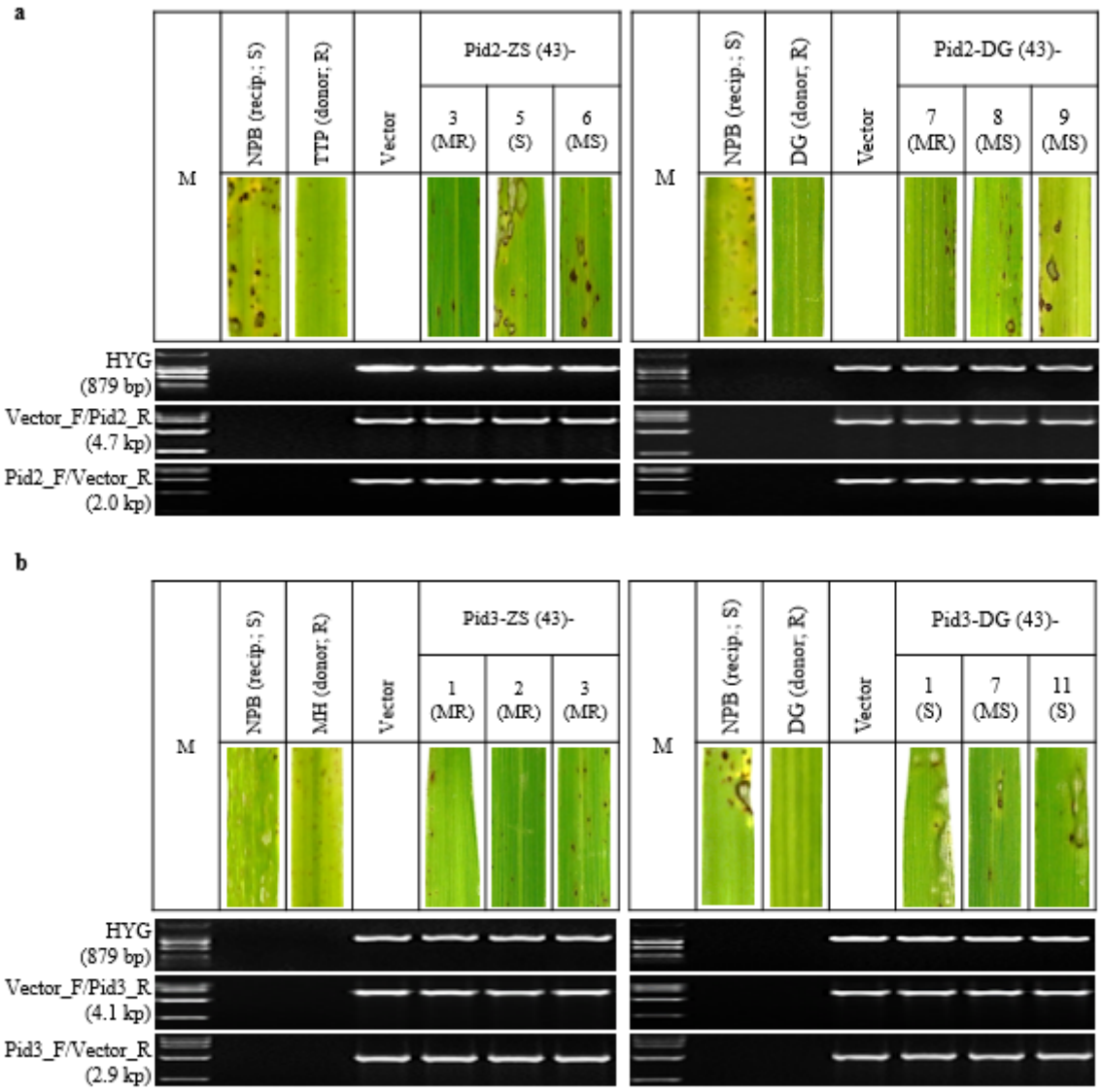

Figure 7

Validation of resistance function of two paired alleles of the *Pid* family. (**a**) Phenotypes and genotypes of the transgenic plants derived from genetic transformation of the new allele *Pid2*-ZS (left) and its reference allele *Pid2*-DG (right). (**b**) Phenotypes and genotypes of the transgenic plants derived from genetic transformation of the new allele *Pid3*-ZS (left) and its reference allele *Pid3*-DG (right). Only survival plants were subjected to phenotyping and genotyping, the former one was determined by inoculating isolates ZB15 (for the *Pid-2* pair) or Zhong 10-8-14 (for the *Pid-3* pair), and the latter one by a set of three vector related markers. NPB, Nipponbare (recipient); TTP, Tetep; DG, Digu; MH, Minghui 63; S, susceptible; R, resistant; MR, moderate resistant; MS, moderate susceptible.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Figs.S17TableS12.Pid.pptx