The **Pid** Family Has Been Diverged into **Xian** and **Geng** Type Resistance Genes against Rice Blast Disease

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Abstract: Rice blast (the causative agent the fungus *Magnaporthe oryzae*) represents a major constraint on the productivity of one of the world’s most important staple food crops. Genes encoding resistance have been identified in both the *Xian* and *Geng* subspecies genepools, and combining these within new cultivars represents a rational means of combating the pathogen. In this research, deeper allele mining was carried out on *Pid2*, *Pid3*, and *Pid4* via each comprehensive FNP marker set in three panels consisting of 70 *Xian* and 58 *Geng* cultivars. Within *Pid2*, three functional and one non-functional alleles were identified; the former were only identified in *Xian* type entries. At *Pid3*, four functional and one non-functional alleles were identified; once again, all of the former were present in *Xian* type entries. However, the pattern of variation at *Pid4* was rather different: here, the five functional alleles uncovered were dispersed across the *Geng* type germplasm. Among all the twelve 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. Profiting from the merits of three comprehensive FNP marker sets, the study has validated all three members of the *Pid* family as having been strictly diverged into *Xian* and *Geng* subspecies: *Pid2* and *Pid3* were defined as *Xian* type resistance genes, and *Pid4* as *Geng* type. Rather limited genotypes of the *Pid* family have been effective in both *Xian* and *Geng* rice groups, of which *Pid2-ZS, Pid3-ZS* has been central to the Chinese rice population.

Keywords: *Oryza sativa; Magnaporthe oryzae; Xian* and *Geng* type resistance gene; resistance function confirmation

1. Introduction

Rice (*Oryza sativa* L.), a crop domesticated in Asia and now cultivated worldwide, is used as a staple food for half of mankind [1–5]. As a result of two major and independent domestication events two different subspecies have been recognized, namely, ssps. *Xian* (*indica*) and ssps. *Geng* (*japonica*) [1,2,4,6]. The two genepools have differentiated over time through their been grown in distinct eco-geographical environments, and have diverged with respect to both the structure of the genome and their gene content [1–4,7]. 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 rice crop is the fungus *Magnaporthe oryzae* Couch (syn. *Pyricularia oryzae* Cavara), the causative agent of the damaging disease rice blast [8–13].
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 [10,11,14–16]. The genetics of resistance largely follow the gene-for-gene principle, involving an interaction between a host’s resistance gene and a matching avirulence gene in the pathogen [17–20]. As a result, following the mutation of matching avirulence genes, major gene-based resistances are prone to rapid breakdown. This in turn enables the creation of a new genotype of resistance gene to overcome the emerged new race with its new resistance specificity [10–13,19–22]. New resistance specificities can be generated by mutations to a resistance gene’s coding sequence (CDS) as well as its regulating region, either in the form of single nucleotide polymorphic (SNP) or even multiple-nucleotide polymorphic mutations (called insertion/deletion, or InDel) [12,15,23–28]; where such changes result in an altered reaction to the pathogen, the mutation is referred to as a functional nucleotide polymorphism (FNP) [15,27,28]. As per the gene-for-gene principal, it is envisaged that a stronger arms race will lead to more FNPs emerging in any particular resistance gene [10–13,19,20,22]. Searching for FNPs in established host cultivars is considered an efficient way of identifying the novel resistances required for crop improvement [29–33].

At least 100 major genes encoding resistance to *M. oryzae* are known, an increasing number of which have been isolated [9,12]. Among the latter are the three genes *Pid2*, -3, and -4, present as a cluster on chromosome 6 (hereafter the *Pid* family), of which *Pid2* encodes for a B-lectin receptor kinase and the other two for nucleotide-binding site (NBS) and leucine-rich repeat (LRR) proteins [34–36]. 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 *Xian* and *Geng* subspecies.

### 2. Materials and Methods

#### 2.1. Development of a Comprehensive FNP Marker System

The DNA sequences of *Pid2* (FJ915121.1), *Pid3* (FJ745364.1), and *Pid4* (MG839283.1) present in Digu as well as in a number of reference cultivars were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/, accessed on 12 September 2020), and each set of alleles was aligned using Multalin (http://multalin.toulouse.inra.fr/multalin/, accessed on 13 September 2020). A comprehensive FNP marker system consisting of two sets of FNP markers, one for functional/non-functional haplotypes and another for individual alleles, was developed for 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, accessed on 28 September 2020). The necessary primer sequences were generated using Primer3 software (https://primer3.ut.ee, accessed on 28 September 2020). For sequencing largely diverged intervals, PCR was driven by triple and/or degenerate primers (Table S1) [37,38].

#### 2.2. Marker Verification

The FNP assays were validated by testing a larger set of control cvs (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 mixture contained 0.1 µL 5 U/µL Taqase (TaKaRa, Dalian, China), 2.0 µL 10 x Taq Mg²⁺ plus buffer, 0.5 µL 10 mM dNTP (TaKaRa), 1.0 µL 2.5 µM primers (Sangon Biotech, Guangzhou, China), 1.0 µL 100 ng/µL template DNA, and 14.4 µL ddH₂O. 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 x digestion buffer, and 8.3 µL ddH₂O. The
digested amplicons were electrophoretically separated through 10–12% polyacrylamide gels in the presence of Tris-boric acid–EDTA buffer and run at 250 V for 20–50 min, depending on the sizes of the PCR products.

### 2.3. 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 first determined with two haplotype-specific FNP markers, then candidate functional alleles were determined with a set of allele-specific FNP markers by testing a regular panel consisting of 30 representative Xian and 30 Geng type cultivars. To confirm the genetic divergence of alleles between Xian and Geng rice groups (if any), allele mining was then extended to two additional germplasm panels, one consisting of 40 Xian type cultivars used as parents in rice breeding programs based in the southern province of Guangdong and the other of 28 Geng type cultivars used similarly in the north-eastern province of Heilongjiang (Table S2). An $\chi^2$ test was used to determine whether the two genepools had or had not experienced divergence. The test was based on the formula $\chi^2 = \frac{N[(ad-bc)-\frac{1}{2}N]^2}{(a+b)(c+d)(a+c)(b+d)}$, where $a$ and $b$ represent the number of Xian type entries scored as respectively harbouring or not harbouring a given allele or genotype, while $c$ and $d$ represent the same for the Geng type entries. $N$ denotes the total number of alleles or genotypes detected for each Pid gene or genotype [11,13,39]. If all alleles derived from a given resistance gene, which was extremely diverged into Xian group, then the resistance gene was defined as Xian type one, and that in turn called as Geng type one.

### 2.4. Validation of Candidate Functional Allele

The full length of each genomic sequence of two paired alleles, Pid2-ZS vs. Pid2-DIG and Pid3-ZS vs. Pid3-DIG, 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 [40] to form a construct carrying an individual allele. Each construct was transformed into the blast-susceptible cv. Nipponbare following Hiei et al. [41]. Phenotypes of the T$_1$ transgenic plants were determined via challenge with the recipient cultivar-virulent isolate CHL346, and then the viable plants were further inoculated with the reference allele-avirulent isolates (ZB15 for Pid2-DIG [34] and Zhong-10-8-14 for Pid3-DIG [36]) according to Pan et al. [42]. The transgenic plants were confirmed by PCR-based genotyping with a set of three vector-related markers, namely, the selective marker (HYG) plus two directional vector-gene across markers (Table S1).

### 3. Results

#### 3.1. Pid2 Alleles

An alignment of Pid2 CDSs of the fifteen reference cultivars revealed the presence of seven SNPs (Figure S1). A pair of FNP’s, 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 (Figure 1). Pid2-DIG$^{T2058C}$ was informative for Pid2-DIG alleles (DIG) and Pid2-ZS$^{A355C}$ for Pid2-ZS alleles (TTP, CO39, ZS97, and TDK). When the regular panel was screened, all 30 Xian type entries were found to carry a functional Pid2 allele, while this was the case for only four of the 30 Geng types (Figure 2; Table S2). Of the 34 Pid2 carriers, 14 belonged to Pid2-DIG and 18 to Pid2-ZS, while two carried a distinct allele (hereafter referred to as Pid2-New). The screen of the additional 40 Xian rice panel revealed that of the 39 carrying a functional copy of Pid2, 32 had the Pid2-ZS allele, six the Pid2-DIG allele, and one the Pid2-New allele. None of the additional Geng germplasm panel carried a functional copy of Pid2 (Figure S2). A homogeneity test suggested that divergence of Pid2 was specific to the Xian genepool (Table 1). It was, therefore, defined as a Xian type resistance gene.
was the case for only four of the 30 Geng types (Figure 2; Table S2). InDel alleles of Pid2 were both effective for distinguishing 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, Nipponbare (Pid2-Null); CK7, Koshihikari (Pid2-Null); CK8, Shennong 265 (Pid2-Null). C1022T in superscript represents the FNP at the 1022 position of Pid2, C represents target, T non-target, and M the DL-500 size marker.

The variation in the Pid3 CDSs identified in the fifteen reference cultivars comprised 29 SNPs and a single InDel (Figure 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 (Figure 3; Table S2). Three pairs, Pid3-DIG^C775A vs. Pid3-DIG^G2695A, Pid3-TTP^C1136T vs. Pid3-TTP^C1623C, and Pid3-ZS^G2477A vs. Pid3-ZS^C225T, were confirmed as allele-specific FNP markers responsible for Pid3-DIG, Pid3-TTP, and Pid3-ZS, respectively (Figure 3). As was the case for Pid2, all 30 members of the Xian panel carried a functional Pid3 haplotype, whereas only four of the Geng panel did (Figure 4; Table S2). The distribution of effective alleles was highly uneven: 29 of the Pid3 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 Xian panel, where 28 of the Pid3-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 Geng panel.

![Figure 1](image1.png)

**Figure 1.** Development of a comprehensive FNP marker system able to distinguish both haplotypes and alleles of Pid2: (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, Nipponbare (Pid2-Null); CK7, Koshihikari (Pid2-Null); CK8, Shennong 265 (Pid2-Null). C1022T in superscript represents the FNP at the 1022 position of Pid2, C represents target, T non-target, and M the DL-500 size marker.

![Figure 2](image2.png)

**Figure 2.** Alleles of Pid2 represented in the regular panel consisting of both Xian (CV1-30) and Geng (CV31-60) types: (A) Functional and non-functional haplotypes; (B) Pid2-DIG allele; and (C) the Pid2-ZS allele. CK1, Digu (Pid2-DIG; red); CK2, Tetep (Pid2-ZS; blue); CK3, Zhenshan 97 (Pid2-ZS; red); CK4, Nipponbare (Pid2-Null; black); CK5, Shennong 265 (Pid2-Null; black); CK6, CO39 (Pid2-ZS; blue); the undefined alleles carried by CV6 and CV14 are marked in purple. Detailed information on each entry is shown in Table S2. M represents the DL-500 size marker.

3.2. Pid3 Alleles

The variation in the Pid3 CDSs identified in the fifteen reference cultivars comprised 29 SNPs and a single InDel (Figure 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 (Figure 3; Table S2). Three pairs, Pid3-DIG^C775A vs. Pid3-DIG^G2695A, Pid3-TTP^C1136T vs. Pid3-TTP^C1623C, and Pid3-ZS^G2477A vs. Pid3-ZS^C225T, were confirmed as allele-specific FNP markers responsible for Pid3-DIG, Pid3-TTP, and Pid3-ZS, respectively (Figure 3). As was the case for Pid2, all 30 members of the Xian panel carried a functional Pid3 haplotype, whereas only four of the Geng panel did (Figure 4; Table S2). The distribution of effective alleles was highly uneven: 29 of the Pid3 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 Xian panel, where 28 of the Pid3-positive entries carried the Pid3-ZS allele, three the Pid3-DIG allele, one the Pid3-TTP and one Pid3-New; none of the members of the additional Geng panel.
carried an effective allele (Figure S4). A homogeneity test implied that divergence at Pid3 has occurred in the Xian gene pool (Table 1); thus, it was termed a Xian type resistance gene.

Table 1. Distribution of alleles and genotypes of the Pid family in the Xian and Geng rice gene pools.

| Alleles vs. Genotypes | Xian Group (a, Presence/b, Absence) | Geng Group (c, Presence/d, Absence) | $\chi^2$ for Homogeneity $^a$ |
|-----------------------|-------------------------------------|-------------------------------------|---------------------------|
| Pid2 alleles          | 69/1                                | 4/54                                | 105.07 ***                |
| Pid2-ZS               | 49                                  | 1                                   |                          |
| Pid2-DIG              | 17                                  | 3                                   |                          |
| Pid2-new              | 3                                   | 0                                   |                          |
| Pid2-null             | 1                                   | 54                                  |                          |
| Pid3 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                                  |                          |
| Pid4 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             |                                     |                                     |                          |
| $d_4$                 | 1/69                                | 54/4                                | 105.07 ***                |
| $d_2$-$d_3$           | 51/19                               | 2/56                                | 60.16 ***                |
| $d_2$-$d_3$-$d_4$     | 18/52                               | 2/56                                | 10.30 **                 |

$^a$ $\chi^2$ homogeneity test to determine whether the paired Pid alleles varied between the two gene pools. Calculations based on the formula, $\chi^2 = \frac{N(ad - bc)^2}{(a+b)(c+d)(a+c)(b+d)}$, where ** and *** represent the paired Pid alleles and genotypes differing significantly between the Xian and Geng gene pools ($p < 0.01$ and $p < 0.001$, respectively; df = 1). The frequency of the presence and absence of each allele/genotype in the Xian gene pool is provided by a and b, respectively, and in the Geng gene pool by c and d, respectively. N denotes the total number of alleles/genotypes detected for each Pid gene/genotype.
Figure 3. Development of a comprehensive FNP marker system able to distinguish both haplotypes and alleles of Pid3: (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 represents the DL-500 size marker.

Figure 4. Alleles of Pid3 represented in the regular panel consisting of both Xian (CV1-30) and Geng (CV31-60) types: (A) Functional and non-functional haplotypes; (B) the Pid3-DIG allele; (C) the Pid3-TTP allele; and (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); the undefined allele carried by CV14 is marked in purple. Detailed information on each entry is shown in Table S2. M represents the DL-500 size marker.

3.3. Pid4 Alleles

Pid4 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 thirteen reference cultivars (Figure S5): a sample of these (seventeen 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 (Figure 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/COT1841A coupled with Pid4-SN/COC2250G as responsible for both Pid4-SN and Pid4-CO (Figure 5). Unlike the situation in Pid2 and Pid3, functional Pid4 alleles were present in many (28/30) of the Geng type entries, while the frequency of functional alleles was only moderate (12/30) in the Xian germplasm (Figure 6; Table S2). The distribution of the various alleles was even more than was the case for Pid2 and Pid3, with fourteen entries carrying the Pid4-SN allele, eleven the Pid4-NPB allele, eight Pid4-New, and six the Pid4-CO allele (Figure 6). Extending the screen to the two additional panels revealed that 28/40 Geng type cultivars harboured a functional allele, while only 7/40 Xian type cultivars did. Of the 35 functional haplotypes, twelve were present in entries carrying the Pid4-CO allele, eleven in those carrying the Pid4-NPB allele, seven in those carrying Pid4-New, and five in those carrying the Pid4-SN allele, while a single entry carried the Pid4-DIG allele (Figure S6). A homogeneity test confirmed that significant divergence at Pid4 has occurred in the Geng genepool (Table 1). Thus, it was termed a Geng type resistance gene.

![Figure 5](image-url) Development of a comprehensive FNP marker system able to distinguish both haplotypes and alleles of Pid4: (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 represents the DL-500 size marker.
Figure 6. Alleles of Pid4 represented in the regular panel consisting of both Xian (CV1-30) and Geng (CV31-60) types: (A) Functional and non-functional haplotypes; (B) the Pid4-DIG allele; (C) the Pid4-NPB allele; and (D) the Pid4-SN and Pid4-CO alleles. CK1, Digu (Pid4-DIG; red); CK2, Tetep (Pid4-Null; black); CK3, ZS97 (Pid4-Null; black); CK4, Nipponbare (Pid4-NPB; blue); CK5, Shennong 265 (Pid4-SN; green); CK6, CO39 (Pid4-CO; rose-red); the undefined alleles carried by CVs 10, 20, 21, 29, 30, 42, 50, and 55, are marked in purple. Detailed information on each entry is shown in Table S2. M represents the DL-500 size marker.

3.4. Performance of Candidate Functional Allele

The transgenic T1 plants derived from the two paired alleles Pid2-ZS vs. Pid2-DIG and Pid3-ZS vs. Pid3-DIG revealed that both new functional alleles (Pid2-ZS and Pid3-ZS) expressed slightly higher resistance than their reference alleles (Pid2-DIG and Pid3-DIG; Figure S7). It has thus been demonstrated that the candidate functional alleles being explored by the comprehensive FNP marker systems are promising ones for conveying resistance.

4. Discussion

4.1. The Comprehensive FNP Marker Systems Have Largely Improved the Marker Works

In the present study, analysis via comprehensive FNP marker systems consisting of two sets of FNP markers was carried out on the Pid family in the three panels consisting of 70 Xian and 58 Geng cultivars selected from various regions across landrace and modern rice eras (Tables 1 and S2). Each comprehensive FNP marker system for deeper allele mining of the Pid family was devised based on several criteria, including representative FNPs over a particular CDS, clear genotyping, and easy access for users [37,38]. As almost all resistance genes have diverged into functional and nonfunctional haplotypes (Figures S1, S3 and S5) [12,15,23–28,37,43], allele mining of each member of the Pid family was initiated with haplotype differentiation using a set of haplotype-specific FNP markers, enabling us to identify any new allele in each cultivar belonging to the functional haplotype. This means that allele mining could be stopped when there was not any functional haplotype in the panel (Figures S2 and S4). Then allele mining was pursued to individual alleles with a set of allele-specific FNP markers, which helped to discover more specific alleles within the functional haplotypes. Despite this, there were three, two, and fourteen cultivars in the Pid2, Pid3, and Pid4 categories, respectively, that were presumed to carry new types of alleles compared to the defined alleles (Table 1). This, in turn, indicated that each comprehensive FNP marker system was inclusive for finding unlimited new alleles, as it 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 previous investigations, as almost of those were in the context of a working model (such as is the ‘specific marker(s) for specific alleles’ model) for allele mining. In contrast, the working model (the deeper allele mining model) used in the current study can be defined as a ‘comprehensive marker system for the whole locus/cluster’, which makes for an inclusive
and comparable approach for discovering a series of new alleles, as in the three cases shown in the current study [30,31,33,36,44,45]. The outstanding merit of the comprehensive FNP marker system is advantageous for mining new alleles for breeding as well as for revealing the molecular mechanisms underlying the genetic divergence of the whole locus/cluster (Table 1).

4.2. The Pid Family Has Strictly Diverged into Xian and Geng Subspecies

Four alleles were detected at Pid2, of which the three functional ones were almost entirely restricted to Xian type cultivars while the null allele was only present in Geng type germplasm (Table 1). The distribution of alleles at Pid3 was very similar: the four functional ones were harboured for the most part by Xian type entries and the null allele was common in the Geng genepool (Table 1). The latter result echoes a prior finding that the alleles of Pid3 present in Geng type cultivars are pseudogenes [36,44]. In contrast, the distribution of alleles at Pid4 featured five functional alleles which were shared evenly among the Geng type entries, with the null allele found only in Xian type ones (Table 1). It is notable that the well-known resistance alleles at the Pi2/9, Pia, and Pita loci/clusters have evenly diverged into both subspecies [37,38]. This may represent the first time that both Xian and Geng type resistance genes have been discovered and defined within individual cultivars through 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 of these cultivars harbour a functional allele at each of the three Pid genes (Table S2). It might be truly expected that integration of both Xian and Geng resistance genes into upcoming cultivars would be one of the most promising ways to enlarge the genetic diversities of resistance genic resources, thereby withstanding ever-growing pressure from the pathogen across the Xian and Geng rice areas [2,5,10,11,13,36,44].

4.3. Rather Limited Genotypes of the Pid Family Are Effective in Both the Xian and Geng Rice Groups

By possessing three members of the Pid family, there would be seven total possible genotypes (combinations) \((2^3 - 1 = 7)\): \(d_2, d_3, d_4; d_2-d_3, d_2-d_4, d_3-d_4, \) and \(d_2-d_3-d_4\), irrespective of specific alleles. However, only three genotypes, \(d_4, d_2-d_3, \) and \(d_2-d_3-d_4\), were detected in the three panels consisting of 128 diversified rice germplasms (Tables 1 and S2). The indication is therefore that rather limited genotypes of the Pid family have been integrated into both Xian and Geng rice cultivars in China. As all three members have strictly diverged into the two subspecies across the landrace and modern rice eras, \(d_2-d_3\) was centralized in the Xian group and \(d_4\) in Geng, with both reaching overwhelming proportions, as was \(d_2-d_3-d_4\) in the Xian group at a rather more moderate rate (Table 1). The genomic structure of the region harbouring the Pid family does not suggest any obvious barrier to local recombination (Figure S8); that is, there were four types of such barriers in the target genomic region: the key subspecies hybrid sterile gene cluster S5 [46], the heading date gene Hd1 [47], the photonasty gene Se5 [48], and the centromere of rice chromosome 6 [49], all of which were far enough from the genic positions of the Pid family. In addition, genomic intervals among the three members were long enough to independently segregate each other in any particular genetic cross (Figure S8). 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 (Figure S8) [37,38]. This in turn indicates that the subspecific divergence in the genomic region was specific to the Pid family.

One of the most likely genetic determinants leading to the establishment of such specific allelic and genotypic structures in the Pid family is due to the specific lineage(s) of the Chinese rice population; that is, the Chinese rice population has been derived from rather limited founder parents for its age. In reviewing the pedigrees of several Top-Ten cultivar types, including the general cultivars and F1 hybrid crosses, Liu [50] pointed out
that in the specific lineage ‘Zhenzhui 11-ZS97’ both were recognized as Pid2-ZS_Pid3-ZS, which has been central to Chinese Xian type rice breeding programs since the 1960s (see www.ricedata.cn/variety, accessed on 12 September 2020). The specific lineage perfectly addressed the questions of why there were two Xian 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 (with d2-d3 being predominant among the three effective genotypes for a long time; see Tables 1 and S2). That is, the unique allelic structures of the three members of the Pid family have been mainly constructed by the genotype, d2-d3, specifically Pid2-ZS_Pid3-ZS, carried by the lineage in rice breeding programs in China after the 1960s. Thus, updating the lineage is again found to be the key to enlarging the genetic diversity of next-generation rice cultivars in China [2,5,36,44,50] (www.ricedata.cn/variety, accessed on 12 September 2020).

A further priority is to address the intriguing question of whether and why rather limited genotypes of the Pid family are predominant in other rice germplasm populations using both plant genetic resources and functional genomics approaches.

5. Conclusions

The study has demonstrated that all three members of the Pid family have been strictly diverged into Xian and Geng subspecies: Pid2 and Pid3 can be defined as Xian type resistance genes and Pid4 as Geng type. Rather limited genotypes of the Pid family have been effective in both Xian and Geng rice groups, of which Pid2-ZS_Pid3-ZS is central to the Chinese rice population. This study has demonstrated the resistance functions of both Pid2-ZS and Pid3-ZS via their transgenic progenies.

6. Patents

Qinghua Pan, Xing Wang, Jinyan Wang, Li Wang, Ruipeng Chai, Ying Zhang, Ling Wang. A set of three critical and comprehensive FNP marker systems for deeper allele mining for rice blast resistance genes Pid2, Pid3, and Pid4 (20211102878.7, pending on 3 September 2021).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13050891/s1, Figure S1: The Pid2 CDSs harboured by the 15 reference rice cultivars; Figure S2: Allele mining of the Pid2 alleles in the additional Xian and Geng rice cultivar panels; Figure S3: The Pid3 CDSs harboured by the 15 reference rice cultivars; Figure S4: Allele mining of the Pid3 alleles in the additional Xian and Geng rice cultivar panels; Figure S5: The Pid4 genomic sequences harboured by the 13 reference rice cultivars; Figure S6: Allele mining of the Pid4 alleles in the additional Xian and Geng rice cultivar panels; Figure S7: Validation of resistance function of two paired alleles of the Pid family; Figure S8: The genomic context of the three key genotypes of the Pid family detected in the present study; Table S1: The three comprehensive FNP marker systems for deeper allele mining, and the primers and markers for transformation test of the Pid family; Table S2: Two sets of rice cultivars for control and three panels of rice cultivars for allele mining used in the present study.

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