Reciprocal adaptation of rice and *Xanthomonas oryzae pv. oryzae*: cross-species 2D GWAS reveals the underlying genetics

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Abstract

A 1D/2D genome-wide association study strategy was adopted to investigate the genetic systems underlying the reciprocal adaptation of rice (*Oryza sativa*) and its bacterial pathogen, *Xanthomonas oryzae pv. oryzae* (*Xoo*) using the whole-genome sequencing and large-scale phenotyping data of 701 rice accessions and 23 diverse *Xoo* strains. Forty-seven *Xoo* virulence-related genes and 318 rice quantitative resistance genes (QR-genes) mainly located in 41 genomic regions, and genome-wide interactions between the detected virulence-related genes and QR genes were identified, including well-known resistance genes/virulence genes plus many previously uncharacterized ones. The relationship between rice and *Xoo* was characterized by strong differentiation among *Xoo* races corresponding to the subspecific differentiation of rice, by strong shifts toward increased resistance/virulence of rice/*Xoo* populations and by rich genetic diversity at the detected rice QR-genes and *Xoo* virulence genes, and by genome-wide interactions between many rice QR-genes and *Xoo* virulence genes in a multiple-to-multiple manner, presumably resulting either from direct protein–protein interactions or from genetic epistasis. The observed complex genetic interaction system between rice and *Xoo* likely exists in other crop–pathogen systems that would maintain high levels of diversity at their QR-loci/virulence-loci, resulting in dynamic coevolutionary consequences during their reciprocal adaptation.
Introduction

The coevolution between host plants and their pathogens can be best defined as a dynamic process involving reciprocal and adaptive genetic changes in the interacting species (Woolhouse et al., 2002). In the modern agriculture system, the relationships between crop plants and many of their pathogens are often represented by more extreme cases of arms-race, which are genetically governed by the classical gene-for-gene theory (Flor, 1971) and well explained by the benchmarked “zigzag model” of plant immune systems (Jones and Dangl, 2006). This model has since guided both basic and applied research on plant disease resistance. Considerable advances have been made in understanding the dynamics of direct or indirect interactions between plant resistance genes (R-genes) and pathogen virulence effector proteins (Hammond-Kosack and Jones, 1997; De Wit et al., 2009; Kapos et al., 2019). However, recent findings in diverse pathosystems are becoming increasingly difficult to fit into the simplified binary view of plant–pathogen interactions (Wu et al., 2017, 2018). An alternative and more inclusive “invasion model” (Cook et al., 2015) and a further refined “spatial invasion model” (Kanyuka and Rudd, 2019) of plant immunity have been proposed to be more general in defining the host–pathogen systems.

Over the past several years, rapid development of sequencing technologies and computational methods, and genome-wide association studies (GWAS) have been demonstrated to be a powerful and feasible strategy to detect natural variation underlying complex quantitative traits in crop plants (Huang and Han, 2014). Since then, GWAS has been applied to gene/QTL discovery in both host plants and pathogens (Genissel et al., 2017). The application of GWAS in identifying genomic regions associated with pathogenicity has been reported in bacterial and fungal pathogens (Bartoli and Roux, 2017; Sánchez-Vallet et al., 2018), including Pseudomonas syringae (Monteil et al., 2016), Parastagonospora nodorum (Gao et al., 2016), and Zymoseptoria tritici (Hartmann et al., 2017). Characterizing the molecular landscape of plant–pathogen intergenic interactions can considerably enrich our knowledge on the coevolutionary processes and adaptive dynamics of microbial species in plant communities (Bartoli and Roux, 2017). Wang et al. (2018a) reported the first case of applying a joint GWAS to quantifying the interaction between Arabidopsis thaliana and its bacterial pathogen, Xanthomonas arboricola. However, the genetic architecture underlying complex genome-wide interactions between crop plants and their pathogens have rarely been characterized at a population level.

Rice bacterial blight (BB), caused by Xanthomonas oryzae pv. oryzae (Xoo), is the most devastating rice bacterial disease worldwide (Niño-Liu et al., 2006). As an excellent model for understanding host–pathogen interactions, the rice-Xoo pathosystem has been intensively investigated in past decades and is well-known for the high degree of differential interactions between rice varieties and Xoo races (Ji et al., 2018). Xoo attacks rice mainly by suppressing host immunity with its type-III secretion system (T3SS), which contains transcription activator-like (TAL) effectors and non-TAL effectors. During the Xoo invasion, TAL effectors are injected into rice cells through the T3SS, trigger specific defense reactions, and transcriptionally activate corresponding host genes by recognizing and binding to specific sequences in their promoters (Boch and Bonas, 2010). Non-TAL effectors such as Xanthomonas outer proteins (Xops) also play important roles in the modulation of signaling in rice defense responses through peptidoglycan-triggered mitogen-activated protein kinase (MAPK) activation (Mudgett, 2005). The same Xops may contribute differently to virulence in different genetic backgrounds of Xoo strains or rice varieties primarily in a quantitative manner (Ji et al., 2018).

The past decades also witnessed tremendous progress in developing and deploying resistant varieties for effective BB management in rice production. Major efforts have been taken to develop BB-resistant rice varieties in breeding programs worldwide (Zhai et al., 2002; Zhou et al., 2011), but rapid losses of resistance of rice varieties carrying single R-genes have been frequently reported (Vera Cruz et al., 2000). Thus, there are well-documented cases of the coadaptation and arms-race between rice and Xoo, particularly since the Green Revolution (Mew et al., 1992). Continuous searching in rice gene pools have identified 46 major Xa/xa genes and many quantitative resistance (QR) loci, each conferring high-level resistance or partial resistance to a specific set of Xoo races (Liu et al., 2014; Hutin et al., 2015; Kim et al., 2015; Zhang et al., 2015; Busungu et al., 2016; Kim, 2018; Kim and Reinke, 2019; Neelam et al., 2020; Chen et al., 2020). Genetically, different rice Xa-genes and QR-loci tend to interact strongly with one another against specific Xoo races (Li et al., 2001, 2006). At least 11 Xa/xa genes of diverse functions have been cloned, including eight Xa/xa genes (Xa1, xa5, Xa10, xa13, Xa23, xa25, Xa27, and xa41) each of which mediates resistance through their interactions with corresponding TAL effectors of Xoo. Three dominant Xa-genes (Xa3/Xa26, Xa4, and Xa21) each encoding a kinase protein are known to mediate high-level resistance to a specific set of Xoo races (Ji et al., 2018). The relationship between rice and Xoo appears to be largely governed by the gene-for-gene system, as well demonstrated by several studied interactions between Xoo TAL effectors and rice Xa-genes, including Xa10/avrXa10 (Tian et al., 2014), Xa27/avrXa27 (Gu et al., 2005), Xa23/avrXa23 (Wang et al., 2015a), Xa21/RaxX (Pruitt et al., 2015; Luu et al., 2019), and PthXo2/xax25 or OsSWEET13 (Zhou et al., 2015). While the presence of complex epistasis among rice Xa-genes and QR-loci provided insights into complex genetic networks underlying the rice defensive system to Xoo (Li et al., 2006), many important questions regarding the interactions between rice and Xoo remain unanswered. In particular, how rice defensive system (Xa/xa genes and QR-loci) interact with Xoo virulence genes at the whole genomic and population levels.
remains poorly understood. Answers to this question are important for a deep understanding of the coadaptation of crop plants and their bacterial pathogens and for efficient deployment of the host defensive system for effective disease control.

Here, we performed whole-genome sequencing of 23 diverse Xoo strains and two large sets of rice accessions and evaluated the reactions of these rice accessions to the Xoo strains. Comprehensive analyses of this big data led us to reveal the complex genome-wide interactions that underlie the reciprocal adaptation between rice and Xoo, which shed important light on the coadaptation patterns and mechanistic relationships between crop plants and their bacterial pathogens.

Results

Phenotypic and genomic diversity of the Xoo strains

For the first set rice materials, analysis of variance (ANOVA) using the mean lesion lengths indicated that resistance differences among the 73 rice accessions, virulence differences among the 23 Xoo strains, and the interactions between rice accessions and Xoo strains were all highly significant, and explained 52.2%, 18.1%, and 27.5% of the variance, respectively (Supplemental Data Set S1). The difference between the two experimental replications was insignificant ($P = 0.855$). The broad-sense heritability estimates were very high for all Xoo races, ranging from 0.871 for P6 to 0.979 for P9a (Supplemental Data Set S1).

Based on the lesion lengths of 73 diverse rice accessions (Supplemental Data Sets S1 and S2), the 23 Xoo strains were classified into four virulence groups (Figure 1A). Group I included six weak virulence (WV) races (C1, C2, C3, C4, C6, and C7) from China. Group II contained seven moderate virulence (MV) races (GIV from China, and P1, P4, P5, P7, P8, and P10 from Philippines). Group III included nine strong virulence (SV) races (C5, GV from China, and P3b, P3c, P6, P6d, P9b, P9c, P9d from Philippines). Group IV contained a single race, P9a from Philippines, which showed a unique pathogenicity pattern in the tested rice accessions.

Whole-genome sequencing of the 23 Xoo strains using next-generation sequencing technology produced a total of 19.5 GB high-quality bases with an average sequencing depth of $172.9 \pm 4.3x$ for each strain (Supplemental Data Set S3). Comparing sequenced genomes with the PXO99A reference genome (Salzberg et al., 2008) identified 33,006 single-nucleotide polymorphisms (SNPs), including 13,053 nonsynonymous SNPs, 11,113 synonymous SNPs, and 8,840 SNPs in intergenic regions. Remarkably, 50% of genic SNPs occurred in approximately 300 highly variable genes. Principal component analysis (Figure 1B) and population structure analysis (Figure 1C) resolved all 23 Xoo strains into four major clusters. Six Chinese races (C1–C4, C6, and C7) and two Philippine races (P1 and P9d) formed a single cluster. Four Philippine races (P3c, P9a, P9b, and P9c) plus two Chinese races (GV and C5) formed the second one. Six Philippine races (P3b, P5, P7, P8, P6, and P6d) constituted the third cluster. The remaining three races (Philippine races P4 and P10, and Chinese race GIV) were admixtures (Figure 1C). We noted that the Xoo race structure (Figure 1, B and C) depicted by the SNP data was very similar to the grouping by the phenotypic data (Figure 1A) except for the three tropical Xoo race groups each containing several highly related MV (II) and SV (III) races. A relatively high number (22) of multi-locus genotypes (MLGs) were found out of the 23 strains, indicating a low clonality of the sampled Xoo population.

We found that recombination along the Xoo genomes occurred in a nonuniform manner with a recombination hotspot in the region of 1.57–1.86 Mb of the Xoo genome where genes related to chemotaxis and two-component systems are highly enriched (Supplemental Figure S1A). Notably, recombination occurred more frequently in genomes of SV or MV races P6, P6d, P5, P7, P8, and P3b with approximately 900 recombination events. In contrast, recombination occurred much less frequently in genomes of WV races C3, C4, C2, C1, C7, and C6. Thus, elevated recombination in the SV and MV races may have directly contributed to virulence shifts of SV and MV Xoo races in the tropics.

To examine the effect of the recombination hotspot on the genome-wide linkage disequilibrium (LD) decay distance, we compared the LD decay curves across the Xoo genomes with and without the recombination hotspot (Supplemental Figure S1B). Clearly, similar LD decay curves were observed, but the observed average LD block ($r^2$ decays to 0.25) with the recombination hotspot (1.57–1.86 Mb) was ∼2.1 kb, smaller than the average LD (∼2.4 kb) without the recombination hotspot. Because higher recombination rates cause faster LD decay which ultimately results in higher mapping resolution, the high level of genomic diversity and small LD decay values indicated that the Xoo population was suitable for further identification of virulence genes by GWAS.

Identification of genes related to Xoo virulence

We first examined SNPs in 48 known virulence-related genes (Supplemental Table S1) and found that ten TAL effectors and two T3SS genes (hrpF and hpaF) showed significantly higher SNP densities than the genome-wide background of 0.0058, indicating that these genes had undergone fast functional diversification. Three TAL effectors (talC8b, talC8a, and talC7A) and two T3SS genes (hrpA and hrcC) showed significantly lower SNP densities, implying that these genes are essential for the fitness of Xoo and thus had gone through strong purifying selection.

To identify additional virulence-related genes, we associated genic variants of the 23 Xoo strains with the mean lesion lengths caused by inoculation with these strains on 73 diverse rice accessions. To overcome the small sample size and take advantage of multiple phenotypes, we utilized a combined association score (CAS) that combined independent association results (see “Methods” section for details). As a result, 86 significant SNPs in 47 Xoo genes were detected (Supplemental Table S2), 5 of which (PXO_00274,
PXO_00272, pthXo1, PXO_00124, and PXO_00502) are located within the 290-kb recombination hotspot. We noted that the small difference in LD block sizes within and outside the recombination hotspot had little impact on the number and resolution of the detected virulence-related genes (Fisher’s exact test, \( P = 0.119 \)), though we indeed observed a higher power (more significant SNPs in the recombination hotspot, Fisher’s exact test \( P = 0.009 \)). Permutation analysis suggested that the probability of more than 86 significant SNPs in a random GWAS is less than 0.0001. This suggested a low false discovery rate of 0.17, or on average, 71 out of the 86 SNPs were true signals.

The 47 genes contained 5 of the 48 known virulence-related genes previously reported in Xoo (a T3SS gene hrpF and four TALs, pthXo1, pthXo7, talCSa, and talC9b), and 18 highly likely virulence-related genes based on the literature on other related bacterial species, including four type-VI secretion system (T6SS) genes, two TonB-dependent receptors

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**Figure 1** The origin of 23 diverse Xanthomonas oryzae pv. oryzae (Xoo) strains and their virulence. A, Reaction type of 73 rice accessions caused by 23 Xoo strains from China and the Philippines and classification of the 23 Xoo strains into four major groups based on their virulence levels (mean lesion length [LL]): resistant (LL < 3 cm), moderately resistant (3 cm \( \leq \) LL < 5 cm), moderately susceptible (5 cm \( \leq \) LL < 10 cm), susceptible (10 cm \( \leq \) LL < 15 cm), and highly susceptible (LL \( \geq \) 15 cm). B, Principal component analysis plots for the first two principal components of the 23 Xoo genomes. C, The population structure and geographic distribution of the 23 Xoo strains. The neighbor-joining tree was constructed from LD pruned SNPs. Fraction ancestry was calculated with STRUCTURE software using an ancestry number of 3, which is shown in different colors.
(TBDRs), and a chorismate mutase (Supplemental Table S3). This report thus implicates four T6SS genes (PXO_03644, PXO_04700, PXO_04712, and PXO_00502) and TBDRs (PXO_01644 and PXO_03467) in the virulence of Xoo to rice. T6SS functions as a contractile nanomachine to puncture target cells and deliver effectors highly associated with the virulence of plant and animal pathogens (Alteri and Mobley, 2016). TBDRs were reported to play important roles in Xoo infection by obtaining the required iron from host iron-carrying proteins (Grinter et al., 2016). The chorismate mutase was experimentally validated to affect the pathogenicity of Xoo XKK.12 (Degrassi et al., 2010). The remaining 11 significantly associated genes are linked to known virulence-related genes (Supplemental Table S3).

Selective signatures on virulence-related genes in Xoo
To determine which of these Xoo virulence-related genes were the targets of selection from rice, we computed nucleotide diversity \((\pi)\) across the Xoo genome (Supplemental Figure S1C). Divergent patterns of nucleotide diversity across the Xoo genome differentiated the WV races, MV races, and SV races, indicating that the evolution of these Xoo races from WV to MV and SV resulted from genome-wide positive selections for functional diversification of many virulence-related genes. Twenty-three of the 47 detected virulence-related genes had significantly higher SNP densities than the genome-wide background genes in Xoo (Supplemental Table S2). Of these, six important ones were noted. The first was hrpF (PXO_03417), a T3SS gene with a high SNP density of 0.0203 (23 nonsynonymous SNPs). hrpF was under strong positive selection for functional diversification in all Xoo races except for races P1 and P9d (Supplemental Figure S2A). There are two major haplotypes at hrpF among the Xoo races (Supplemental Figure S2B). Hap1 was strongly associated with increased virulence and mainly present in one branch of the SV Xoo races (Supplemental Figure S2C). The second gene was a TBDR (PXO_01644), which showed an extremely high SNP density of 0.116 (96 nonsynonymous SNPs) and exhibited distinct haplotypes among the four Xoo virulence groups (Supplemental Figure S3, B and C). Interestingly, one of the two nonsynonymous SNPs causing a substitution from Glu to Lys in the domain loop of this protein showed the strongest associations with strong virulence (Supplemental Figure S3, D–F). In WV races, this particular SNP was very likely to cause reduced ability of the TBDR in binding specific iron-carrying rice proteins required for Xoo infection (Grinter et al., 2016; Supplemental Figure S3G). This TBDR gene (PXO_01644) was under very strong positive selection for functional diversification in all WV races and some of the SV and MV races (Supplemental Figure S3A). The remaining four were T6SS genes showing high SNP densities (Supplemental Table S2), implying that they were the key virulence genes responding to selection from the host.

Differentiated responses of 701 rice accessions to four representative Xoo races
To better characterize the interaction between rice and Xoo, we selected four races representing the four major Xoo virulence groups, based on the results shown in Figure 1: C3 (WV or I), P1 (MV or II), C5 (SV or III), and P9a (IV), and used these to inoculate 701 second-set rice accessions of diverse origins (Figure 2A; Supplemental Data Set S4). ANOVA indicated that resistance differences among the 701 rice accessions, virulence differences among the four Xoo races, the interactions between rice accessions and Xoo races were all highly significant and explained 46.1%, 17.4%, and 30.2% of the total phenotypic variation in mean lesion lengths of the second set rice materials, respectively (Supplemental Data Set S4). The difference between the two experimental replications was insignificant \((P=0.236)\). The broad-sense heritability estimates were 0.797, 0.811, 0.857, and 0.880 for Xoo races C5, C3, P1, and P9a, respectively. Population structure analyses from LD pruned SNPs classified the 701 rice accessions into two main well-known subspecies, Xian (indica) and Geng (japonica; Supplemental Figure S4, A, B, and D). The LD \((r^2)\) dropped to half of its maximum value at ~300 kb in the whole population (Supplemental Figure S4C).

To evaluate the specific impact of population structure, geographical origins, and variety types of rice accessions, the 701 accessions were divided into six panel subpopulations, including Xian (419 accessions), Geng (219 accessions), Chinese (451 accessions), overseas (241 accessions), and two subpopulations of Chinese accessions, including 135 Chinese landraces (CHN-LAN) and 316 Chinese modern varieties (CHN-MV), with 158 accessions shared between subpopulations Chinese and Geng, 266 accessions shared between subpopulations Chinese and Xian, 60 accessions shared between subpopulations Overseas and Geng, 145 accessions shared between subpopulations Overseas and Xian (Supplemental Data Set S4). The proportion of the 701 accessions showing high level (lesion length <3 cm) resistance was highest \((42.9\%)\) against WV race C3, followed by P9a (19.5%), P1 (17.1%), and C5 (4.3%; Figure 2). Rice accessions of the whole and specific panel populations showed considerable variation and race-specificities in their resistances to the four Xoo races (Figure 2B). In general, more Geng accessions were resistant to C5 and P9a than Xian accessions, and the opposite was true when against P1, indicating a major difference between the two rice subspecies in their resistance to Xoo races. The proportion of resistant accessions in CHN-LAN was 19.3%, 2.2%, 9.6%, and 1.5% against Xoo races C3 (WV or I), P1 (MV or II), P9a (IV), and C5 (SV or III), which was much lower than those \((43.7\%, 19.6\%, 19.9\%, and 5.1\%)\) of CHN-MV (Figure 2B). Clearly, CHN-MV had much stronger resistance against all four Xoo races, as a result of modern breeding for improving BB resistance in China.

Identification of rice QR genes and their race specificities
GWAS using data of the whole population and six panel subpopulations (Xian, Geng, Chinese, overseas, CHN-LAN
and CHN-MV) detected 5,432 significant SNPs associated with QR to one or two Xoo races (Supplemental Data Set S5), which are mainly clustered in 41 genomic regions (R1–R41 < 300 kb (Figure 3; Supplemental Figure S5 and Supplemental Table S4). Of the 5,432 SNPs, 2,703 were computationally predicted in 318 QR-genes and the remaining ones were located in 372 intergenic regions based on the Nipponbare reference genome annotation (MSU v7; Supplemental Data Set S5). The detected QR-genes mainly encode proteins with nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4 (NB-ARC) domains and leucine-rich repeats (NLR; Supplemental Figure S6E) and were enriched in biological processes related to plant responses to pathogens, including apoptosis, programmed cell death, defense response, and protein amino acid phosphorylation (Supplemental Figure S6D). Interestingly, 827 (15.2%) of the significant SNPs are located in retrotransposon or transposon genes (Supplemental Data Set S5).

The identified SNPs (genes) for resistance showed strong race-specificity with 4,847 (89.2%) of the detected SNPs and

Figure 2 Summary of the resistance reactions of 701 rice accessions to four representative Xoo races (C3, C5, P1, and P9a). A, The heatmap of reactions of 701 rice accessions to four representative Xoo races (C3, C5, P1, and P9a). XI, Xian/indica; GJ, Geng/japonica B, Proportions of different reactions in rice subpopulations to the four Xoo races, in which Pearson’s Chi-squared tests were performed with “***” representing $P < 0.001$ and “NS” representing no significant difference.
272 (85.5%) of the detected genes associated with resistance to a single Xoo race and almost all the remaining SNPs and genes associated with resistance to both C5 and P1 (Supplemental Figure S6, A–C). The strong race specificity of the detected SNPs was primarily reflected in the Xian-Geng differentiation as a result of the adaptation of Xoo to rice. Specifically, of the 46 SNPs (10 genes) for resistance to WV race C3 detected in Xian or Geng subpopulations, 16 SNPs
(3 genes) and 30 (7 genes, respectively) were detectable only in Geng and Xian accessions, respectively (Supplemental Data Set S5). Similarly, of the 1,471 SNPs (44 genes) for resistance to P9a in Xian or Geng subpopulations, 1,454 were detectable only in Xian accessions and the remaining 17 only in Geng accessions. Of the 1,983 SNPs (167 genes) for resistance to MV race P1 in Xian or Geng subpopulations, 1,878 were detected only in Xian accessions and 105 only in Geng accessions. Of the 259 SNPs (24 genes) for resistance to SV race C5 in Xian or Geng subpopulations, 49 were detected only in Xian accessions, 159 only in Geng accessions, and the remaining 51 in both Xian and Geng.

We also performed two-way ANOVA to examine the interaction effects between SNP and subspecific populations using 3,656 subpopulation-specific SNPs. Of these, 414 (11.3%) SNPs were fixed in one of the subpopulations. For the remaining 3,242 SNPs, 2,482 (76.6%) SNPs were detected with significant interaction effects between SNPs and subpopulations, indicating that these SNPs have different effects between the two subpopulations (Supplemental Data Set S5). Taking these results together, of the 228 detected rice genes for resistance to the four Xoo races in Xian or Geng subpopulations, 213 (93.4%) were associated either in Xian or Geng with only 15 (6.6%) shared with both. We noted that 5, 257, 965, and 572 SNPs for resistance to C3, P9a, P1, and C5 detectable only in the whole population but neither in Xian nor in Geng also resulted from the Xian-Geng differentiation (Supplemental Data Set S5).

The high resolution of our GWAS resolved several of the detected QR-genes into a few or single candidate genes with clear resistance haplotypes. The first was xa25 (LOC_Os12g29220) on chromosome 12, which harbors a group of SNPs with the strongest signals for resistance to P9a. As a cloned recessive R-gene encoding a sucrose transporter (OsSWEET13; Liu et al., 2011), three major haplotypes were detected at xa25 (Supplemental Figure S7A). Hap3 was associated with susceptibility to P9a and had a frequency of 13.8% in Xian but absent in Geng, while Hap1 and Hap2 were associated with resistance. Hap2 was associated with significantly higher frequencies in Geng (58.4% and 0.5%) than in Xian (7.4% and 11.9%). This explained the different responses of Xian and Geng accessions to P9a (Supplemental Figure S7, B and C). The second candidate was Xa26 (LOC_Os11g47210), which encodes a receptor kinase-like protein (Sun et al., 2004). In the intragenic region of Xa26, we detected five significant signals for resistance to C5 and four significant signals for resistance to P1. However, we did not observe any resistance-related haplotypes. Alternatively, much stronger signals with 38 highly significant SNPs were detected for resistance to P1 in its neighboring locus within the Xa26 gene family cluster (Li et al., 2012a), which (LOC_Os11g47240) encodes a leucine-rich-repeat receptor kinase EXS precursor (Supplemental Data Set S5). Sixteen significant nonsynonymous SNPs together with a SNP that produced a stop codon revealed three major haplotypes at LOC_Os11g47240 (Supplemental Figure S7D). Hap1 and Hap2 were associated with susceptibility to P1 and had significantly lower frequencies (23.2% and 19.1%) in Xian than in Geng (50.2% and 25.1%), while Hap3 was associated with resistance with a frequency of 15.5% in Xian but absent in Geng (Supplemental Figure S7, D and E). Meanwhile, Hap3 was more abundant in CHN-MV than in CHN-LAN, showing strong Xian-Geng differentiation and strong selective signature during breeding (Li et al., 2012a). Specifically, the kinase domains in exons 2 and 3 of LOC_Os11g47240 appeared to be more important for its resistance function since two nonsynonymous SNPs and the SNP that produced a stop codon in its kinase domains showed the strongest associations with resistance. These results indicated that LOC_Os11g47240 is an important member of the Xa26 gene family cluster conferring BB resistance.

Another example is Xa40, a reported dominant R-gene conferring high-level resistance to all Korean Xoo races, which was fine-mapped within a ~80-kb region on chromosome 11 (Kim et al., 2015). A total of eight candidate genes were functionally predicted in the target region, including a WAK3 gene (LOC_Os11g46900) and a protein-coding gene of unknown function (LOC_Os11g46890) with gradually increased levels of expression over time after Xoo inoculation. We detected five haplotypes for LOC_Os11g46900 (P = 5.3 × 10^{-11} of the lead SNP; Figure 4A). Hap3 and Hap5 were associated with resistance to P1 and had significantly higher frequencies (23.6% and 8.1%) in Xian than in Geng (0.9% and 3.2%), while Hap1, Hap2, and Hap4 were associated with susceptibility with differentiated frequencies of in Xian (11.9%, 10.5%, and 11.9%), and Geng (14.6%, 42.0%, and 0%; Figure 4, A and C). Interestingly, its neighboring gene, LOC_Os11g46890 contains more significant SNPs (P = 4.0 × 10^{-18} of the lead SNP) for resistance to C5 and P1 with four haplotypes (Figure 4B). Hap3 and Hap4 were associated with resistance to P1 and had significantly higher frequencies (37.0% and 0.7%) in Xian than in Geng (0.9% and 12.8%), while Hap1 and Hap2 were associated with susceptibility with high frequencies in both Xian (9.1% and 41.1%) and Geng (9.1% and 47.0%; Figure 4, B and D).

We further validated the function of LOC_Os11g46890 on BB resistance by knockout in a Geng variety Wuyugeng20, which carries the major resistance haplotype Hap4. The mutants of Wuyugeng20 were generated from two CRISPR/Cas9 targeting sites of LOC_Os11g46890 using the pYLCRISPR/Cas9Pubi-H system (Figure 4, E and F). Three independent homoygous T1 mutants with different types of mutation in the coding region were obtained. Among them, line JK143 (1-bp insertion in target site 1) caused a stop codon and produced a truncated protein, while the other two lines (JK150 and JK151) caused frame shifts and encoded putative new proteins (Supplemental Figure S8). Analysis of these T1 plants containing homozygous mutations within LOC_Os11g46890 indicated that resistance of the mutants to C5 was significantly lower compared to the wild-type (Figures 4, G and H), suggesting that LOC_Os11g46890 is more likely to be the true candidate for Xa40, though the

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possibility that LOC_Os11g46900 might also contribute to resistance jointly with LOC_Os11g46890 could not be excluded.

We identified a protein-coding gene with unknown function (LOC_Os11g46250) as the candidate for Xa22 (Wang et al., 2003), within which 152 significant SNPs cluster in its promoter and genic region showing the strongest associations with resistance to races P1 \((P = 2.0 \times 10^{-14})\) and C5 \((P = 6.7 \times 10^{-23})\). Interestingly, we observed differentiated resistance of different alleles at LOC_Os11g46250 against different Xoo races. We discovered three major haplotypes at this locus based on 58 significant SNPs in its 1-kb upstream promoter region, 5′-untranslated region (UTR), and nonsynonymous SNPs in the coding region (Supplemental Figure S9A). Hap2 was associated with resistance against MV race P1 (Supplemental Figure S9B), but with susceptibility to SV race C5 (Supplemental Figure S9C). Hap2 had frequencies of 18.4% and 0% in populations Xian and Geng, respectively. These results suggest that LOC_Os11g46250 encoding an expressed protein of unknown function is the most likely candidate for Xa22.
To identify which loci contribute to high-level resistance to the SV race (C5), we examined the graphical genotypes of the 30 accessions resistant to C5 for all loci associated with resistance to C5 (Supplemental Figure S10). Interestingly, we could not attribute the high level of resistance of the 30 accessions to C5 to any single locus. However, the coexistence of the resistance alleles at eight loci appeared to be responsible for the resistance of 19 accessions to C5, suggesting the eight loci jointly determined the resistance to C5, while the resistance of the remaining 11 accessions to C5 appeared to under different genetic control.

Selective signatures at the detected QR-loci in rice from modern breeding

To determine the overall impact of modern breeding on QR-loci across the rice genome, we examined the genome-wide frequency shifts of resistance alleles (defined as the alleles at detected SNPs with the shortest mean lesion length compared with the other alleles) at all significant SNPs for resistance to the four Xoo races both in the whole and in the six rice panel populations. Xian and Geng apparently achieved resistance by using different alleles at most detected QR-loci, though no global resistance allele frequency shifts were observed between Xian and Geng (Figure 5A; Supplemental Figure S11A). When comparing CHN-MV with CHN-LAN, we observed an apparent genome-wide frequency increase in resistance alleles by 0.15 (Figure 5B; Supplemental Figure S11B). Among them, 3,735 (68.8%) of the 5,432 significant SNPs showed significant frequency shifts for resistance alleles, resulting presumably from the artificial selection for improving resistance via modern breeding. Notably, 55% of the significant SNPs and 44% of nontransposon genes locate in the ~3.3 Mb region of R30–R37 on chromosome 11, where seven fine-mapped (Xa22, Xa32, Xa35, Xa36, and Xa40) cloned (Xa4 and Xa26) Xa-genes reported reside. R40–R41 (~0.5 Mb) on chromosome 12 contains an additional 30.8% (11%) of significant SNPs (genes) detected.

Compared with CHN-LAN, CHN-MV displayed greatly decreased genome-wide nucleotide diversity (Figure 6, A and B; Supplemental Figure S11C). However, R40–R41 for resistance to P9a were apparently under strong directional selection in the Geng population, while regions R30–R37 were characterized with high-level diversity (Figure 6, C and D). Using the RPAN database (Sun et al., 2017), we found that 67.5% of the genes detected in regions R30–R37 were distributed/dispenesable. These identified genes showed 2.2-fold enrichment in comparison to the genome background with a distributed mean frequency of 0.30 (the hypergeometric test, \(P = 1.7 \times 10^{-23}\)). In particular, genes in R30–R37 tended to have significantly higher gene copy number variations (CNVs) when the Xian Minghua63/Zhenshan97 genomes (Zhang et al., 2016) were compared with the Geng Nippobare genome (Supplemental Figure S11D). This is consistent with the reported presence of many tandemly arrayed R-genes in this region (Rizzon et al., 2006). An additional peak of large deletions (>100 bp) was detected in R30–R37 among the 701 genomes (Figure 5C), suggesting high frequencies of homologous recombination in this region. All these results indicate that R-genes in this region evolved quickly, resulting primarily from the unique nature of this region harboring high numbers of SNPs and CNVs.

To gain insights into the question of whether deployment of single major Xa/xa genes were the driving force shaping the Xoo race structure, we examined the frequency distribution of major functional haplotypes at four large-effect QR-genes located in the two genomic regions of chromosomes 11 and 12 where the highest frequency shifts from breeding were observed. At xa25, differential shifts were observed for the resistance haplotypes Hap1 and Hap2. When CHN-MV was compared with CHN-LAN, Hap1 decreased by 1.9% and Hap2 (predominant in Xian but rare in Geng) decreased by 2.3% (Supplemental Figure S7A). At Xa26 homolog (LOC_Os11g47240), the resistance Hap3 had a significantly higher frequency in CHN-MV (7.0%) than in CHN-LAN (1.5%; Supplemental Figure S7D). For the Xa40 candidate (LOC_Os11g68890), the resistance Hap3 and Hap4 had significantly higher frequency (26.9% and 4.7%) in CHN-MV than in CHN-LAN (4.4% and 3.0%; Figure 4B). For the Xa22 candidate (LOC_Os11g46250), the resistance Hap2 against MV race P1 had a significantly higher frequency (16.1%) in CHN-MV than in CHN-LAN (1.5%; Supplemental Figure S9, A and B). When against SV race C5, resistance Hap3 at Xa22 was associated with moderate resistance with a significantly higher frequency (3.2%) in CHN-MV than in CHN-LAN (1.5%; Supplemental Figure S9, A and C). These results plus those in the previous section led us to the conclusion that artificial selection of modern breeding acting on the major Xa genes locate in the ~3.3 Mb region of R30–R37 were the primary driving force shaping the observed Xoo race structure (Figure 1), consistent with the extensive deployment of Xa4 in breeding programs during 1970s–1990s at IRRI and China (Mew et al., 1992; Zhang, 2009).

Cross-species genome-genome interactions between Xoo and rice

We adopted a cross-species 2D GWAS strategy to identify the genetic interactions between Xoo and rice using the whole-genome sequencing data of 49 rice accessions and the 23 Xoo strains plus the lesion length data \((n = 49 \times 23 = 1,127)\) of the 49 rice accessions inoculated by all 23 Xoo strains (see “Methods” for details). Practically, to limit the number of SNP pairs in the same LD blocks in the rice–Xoo interaction analysis, 75 rice LD blocks containing all significant SNPs detected in the rice GWAS panels were constructed and then their 172 tag SNPs were selected for rice–Xoo interaction analyses (Supplemental Data Set S6). Then, we examined interactions between 1,909 SNPs in Xoo virulence-related genes (known virulence genes and novel
candidate genes detected from GWAS) and the 172 rice tag SNPs (Supplemental Data Set S7). Based on a Bonferroni corrected threshold of \( P \leq 1.52 \times 10^{-7} \) [0.05/(1,909 \times 172)], we detected 18,142 significant SNP-SNP interactions between 625 SNPs within 51 Xoo genes and 59 SNPs within 35 rice LD blocks (Figure 7; Supplemental Data Set S8). The 51 virulence-related Xoo genes included 13 TAL effectors (avrBs2, pthXo1, pthXo6, pthXo7, talC3a, talC3b, talC5a, talC6a, talC6b, talC9a, talC9b, talC9d, and avrXa27), 2 non-TAL effectors (xopF1 and xopX), 4 T6SS genes (PXO_03404, PXO_04712, PXO_04700, and PXO_00502), 7 T3SS genes (hrpA, hrcJ, hrcN, hrcP, hrcR, hrcX, and hpaA), plus 24 other genes detected in the 1D GWAS. Most of these interactions showed a general “multiple-to-multiple” pattern, where a group of Xoo genes each interacted with a group of rice LD blocks distributed throughout the genome, and vice versa (Figure 7; Supplemental Data Set S8).

Based on the strengths (levels of statistical significance) and number of interactions, the 51 involved Xoo virulence genes and the 35 involved rice LD blocks could be roughly classified into four major interacting groups, i.e. Xoo gene groups I–IV versus rice QR-gene groups 1–4 (Figure 7B). Xoo gene group I was the most important group containing seven genes that interacted very strongly with rice QR-gene groups 2 and 4, strongly with rice QR-gene group 3 and weakly with rice QR-gene group 1. This group contains two T6SS genes (PXO_00502 and PXO_04700), a non-TAL effector (xopX), a TAL effector (talC3b), a T6SS gene (PXO_01644), and two others (PXO_02058 and PXO_03643). Also important was Xoo gene group IV containing 22 genes that interacted strongly with rice QR-gene groups 2–4. This group included five TAL effectors (pthXo1, talC6a, pthXo6, talC9a, and talC9b), five T3SS genes (hrpF, hrcG, hrcJ, hrcP, and hrcR), a T6SS gene (PXO_03644), a NADHR (PXO_00908), a glk (PXO_01645), and nine others (Figure 7B). Xoo gene group III has 13 genes, which interacted relatively weakly with rice QR-gene group 2 and most of the QR-gene group 4. This group includes a T6SS gene (PXO_04712), three TAL effectors (talC5a, talC6b and pthX07), two T3SS genes (hrpA and hrcP), an hrpA (PXO_03408), a lipoprotein (PXO_04702), plus five others. Xoo gene group II includes only nine genes, six of which (talC9d, xopF1, avrBs2, dnaE, PXO_03021, and PXO_03270) interacted weakly with most blocks in rice QR-gene groups 2 and 3. The remaining three genes (talC3a, avrXa27, and glmU) each interacted with 1–4 rice QR-gene blocks. One T6SS gene (PXO_00502) was the most important one that showed the strongest interactions with 33 rice LD blocks containing 174 rice genes. Other important Xoo genes

Figure 5 Selection and evolution of the rice QR-genes. A, Comparison of resistance allele usage between Xian accessions (n = 419) and Geng accessions (n = 219) for each SNP associated with resistance to Xoo. XI, Xian/indica; GJ, Geng/japonica. B, Comparison of resistance allele usage between CHN-MV (n = 316) and CHN-LAN (n = 135) for each SNP associated with resistance to Xoo. The dotted red lines in (A) and (B) indicate the mean frequency difference of all significant SNPs detected in rice GWAS panel. The resistance allele was determined by its corresponding phenotypes in the whole population. C, Density of large-sized deletions (>100 bp) calculated by a sliding window of 500 kb with each moving step of 100 kb. The density was further adjusted to deletion numbers per 100 kb. The two important regions (R30–R37 and R40–R41) were highlighted. The dotted red line indicates the genome-wide mean value.
included another T6SS gene (PXO_04700), a non-TAL effector (xopX), a TAL effector (talC3b), a TBDR gene (PXO_01644), and three other genes (PXO_01443, PXO_02058, and PXO_03643), which interacted more strongly and with more rice genes (Supplemental Data Set S8).

Similarly, rice QR-gene groups 2 and 4 were most important and interacted very strongly with Xoo gene group I and strongly with Xoo gene group IV (Figure 7B). The strongest interactions occurred between Xoo gene group I and QR-gene block L71 of 1.948 Mb on rice chromosome 11 containing seven NBS-LRR genes and 17 receptor-like protein genes. Moreover, most of the interacting rice genes within rice LD block L71 are enriched in pathways related to programmed cell death, protein amino acid phosphorylation, ATP binding, protein serine/threonine kinase activity, etc. (Supplemental Table S5). Furthermore, most rice genes that interacted with more Xoo genes are typical plant R-genes encoding signal-peptides, LRR, and tyrosine–protein kinase domains (Liu et al., 2014). Within rice LD block L71, Xa3/Xa26 (LOC_Os11g47210) was the most important one that interacted strongly with four T6SS genes (PXO_00502, PXO_04700, PXO_03644, and PXO_04712), six TAL effectors (talC3b, talC6a, pthXo1, pthXo6, talC9a, and talC9b), five T3SS genes (hrcN, hrpF, hrcJ, hrpG, and hrpX), a non-TAL effector xopX, and 17 other Xoo genes. Rice QR-gene group 4 was also important and contains 15 LD blocks on nine rice chromosomes (1–4, 6–9, and 11) that interacted strongly with Xoo gene groups I and IV.

**Discussion**

Most rice landraces in tropic Asia and China were known to lack resistance to Xoo (Ou and Jennings, 1969; Zhang, 2009) and no major epidemics of rice BB were documented until the early 1970s in Philippines and slightly later in China when the semi-dwarf varieties from the Green Revolution were spreading (Zhang, 1991; Mew et al., 1992). The Xoo strains used in this study represented 14 well-characterized races that emerged sequentially in the Philippines since late 1960s, while the Chinese strains represented nine major Xoo races collected from six provinces of China (Vera Cruz et al., 2000; Zhou et al., 2011; Quibod et al., 2016). Consistent with the previously reported Philippine race structure (Quibod et al., 2016), we found four Philippine races (P3c, P9a, P9b, and...
Figure 7 Genome-wide interactions between Xoo and rice detected by a cross-species 2D GWAS. A, 18,142 significant genome-wide SNP–SNP interactions between 625 SNPs within 51 Xoo genes and 59 SNPs within 35 rice LD blocks associated with resistance to Xoo detected by 2D GWAS. The P-value threshold for Bonferroni-corrected significance with the number of tests is set at $1.52 \times 10^{-6}$ [0.05/(1,909 Xoo variants in virulence-related genes $\times$ 172 tag SNPs)]. Xoo genomic region highlighted with a cyan background is the recombination hotspot. Coordinates of the detected Xoo genes are displayed with red dotted arrows. B, A heatmap with two-way hierarchical clustering of genome-wide interactions between Xoo and rice shown in (A), in which the left side included all involved 51 Xoo virulence-related genes and the top side included all involved 35 rice LD blocks. The heatmap colors indicate the interaction intensity using the negative logarithmic values of the minimum P-values of the interaction signals if multiple significantly interacted SNPs were located in the same Xoo gene and the same rice LD block, respectively. The color scale for interaction intensity is shown on the right.
the host was the driving force shaping the data (Figure 1A), suggesting that the selection pressure from specific differentiation of rice.

Xoo and arms-race between rice and Xoo among races from South China with those tropical Xoo races suggest they were more likely to have been introduced from the Philippines. The Xoo race structure depicted by the SNP data (Figure 1, B and C) was very similar to the grouping by the phenotypic data (Figure 1A), suggesting that the selection pressure from the host was the driving force shaping the Xoo race structure, which is in agreement with the previous study on Philippine races (Quibod et al., 2016).

Thus, we observed two consequences of the coadaptation and arms-race between rice and Xoo populations in tropic Asia and China. First, there is the strong differentiation among Xoo races corresponding to the well-known subspecific differentiation of rice. Xoo races from Geng accessions in the temperate areas of China all had weak virulence, while those on Xian accessions in the subtropic and tropical areas had moderate and strong virulence. Correspondingly, most QR-genes detected in Xian accessions are different from those in Geng accessions, suggesting a long history of reciprocal adaptation of rice and Xoo that may have started long before domestication. Second, the CHN-LAN population showed low levels of resistance to Xoo and had few detectable genes for BB resistance, which was accompanied by the weak virulence of the Chinese Xoo races. In contrast, modern Xian rice varieties had significantly improved resistance to Xoo. At the genomic level, we observed a high level of diversity at 3,735 (68.8%) of 5,432 significant SNPs across the rice genome at which significant frequency shifts for resistance alleles occurred during modern breeding, which was accompanied by a very high level of diversity at many virulence-loci (23 of 47 virulence-related genes with significantly higher SNP densities than other genome-wide background genes) in the Xoo populations. This correspondence in gene number and diversity between QR-loci in rice and virulence loci in Xoo has been the foundation of their reciprocal adaptation. Thus, our results revealed several important genomic features and genetic mechanisms of rice and Xoo that have been responsible for maintaining the observed genetic diversities at the resistance/virulence loci in the host plant and pathogen populations and for continuously generating “novel/new” resistance/virulence during the reciprocal adaptation between crops and their pathogens.

On the host side, as well demonstrated in lettuce (Lactuca sativa; Meyers et al., 1998) and in the case of Xa21 of rice (Song et al., 1997), the most important mechanism for generating new and novel resistance genes/alleles is recombination in R-gene clusters present in plant genomes. For example, the Xa21 locus is a receptor kinase gene cluster at ~5 Mb upstream of regions R30–R37 on rice chromosome 11, in which recombination events in the highly conserved regions of the Xa21 family members could result in precise swapping of promoter regions and generating new genes/alleles for novel resistance to Xoo. In this study, approximately half of the large-effect QR-genes were detected in the ~3.3 Mb region of R30–R37 on rice chromosome 11 that contains many receptor-like kinases and NB-ARC gene clusters where most previously reported dominant Xa-genes (Xa4, Xa22, Xa26, Xa32, Xa35, Xa36, and Xa40) reside (Figure 3). We also observed a peak of large deletions in the R30–R37 region among the 701 rice genomes, implicating that recombination occurred frequently within this region (Figure 5C). Moreover, this region is characterized by high levels of SNPs, CNVs, and showed the most dramatic frequency shifts toward resistance alleles in modern varieties. Clearly, this region has been the primary targets of selection for high-level resistance to Xoo during modern breeding and recombination events in the R30–R37 region during breeding could easily generate “new” R-genes and/or R-gene combinations for novel resistance to new virulent races of Xoo (Zhang et al., 2015). One interesting observation was that most of the detected QR-genes against the SV race (C5) were of “small effect”, and the high-level resistance of 30 rice accessions could not be attributed to any single QR-genes but to associations of a set of “small-effect” genes (Supplemental Figure S10). This suggested novel high-level resistance could result from accumulation of many “small-effect” QR-genes from the CHN-LAN population. Experimental determination of the genetic relationships among these “small-effect” QR-genes leading jointly to high-level resistance to SV Xoo races are of great interest for future investigation.

On the pathogen side, mutations of large effect on traits associated with virulence and host adaptation are expected to evolve more rapidly and under strong positive selection in the modern crop–pathogen interaction systems. Also, rice responses to Xoo measured by lesion lengths using the leaf-clipping artificial inoculation method are known to be a highly heritable trait that, unlike most quantitative traits, shows minimum G × E (years and locations) interactions (Nayak et al., 1987; Li et al., 2001). Thus, these large-effect causal mutations in a pathogen population can be relatively easily detected even with small bacterial samples (Farhat et al., 2014). In this study, the problem of Xoo population stratification was controlled by using the first two principal components as covariates in GWAS using a linear mixed model (LMM). Thus, with the actual population size of 23 × 73 = 1,679 phenotypic observations for each of the 33,006 SNPs and the observed LD decay of 2.4 kb (~3 genes) in the Xoo genome, we expected a reasonably high overall mapping resolution of the virulence-related loci identified in the Xoo population, similar to a previously reported case of phytopathogenic fungi (Gao et al., 2016). The false discovery rate of 0.17 of our GWAS estimated by permutation analysis was reasonable. Thus, most of the 47 detected virulence-related genes in Xoo should be real. We noted that

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23 (~49%) of these virulence loci showed significantly higher SNP diversity than the genome-wide background genes and more dramatic functional diversification. Of these, four TAL effectors, one T3SS gene (hrpf) and four T6SS genes showed extremely high levels of diversity and functional diversification among different Xoo races, indicating they had gone through a rapid evolution during their adaptation to rice host.

Most importantly, we discovered three groups of virulence-related genes. The first group included several T6SS genes involved in virulence of Xoo. T6SS effectors were reported to be important in fitness, virulence, and competence of pathogenic bacteria of several plant species (Ryu, 2015), including rice bacterial pathogen Acidovorax avenae subsp. Avenae, which causes rice bacterial brown stripe through the T6SS-mediated protein (toxic effectors) translocation (Basler and Mekalanos, 2012; Masum et al., 2017). The second group was the TBDRs. TBDRs in phytopathogenic bacteria play important roles in bacterial infection by obtaining iron through specifically targeting host iron-containing proteins (Parker Siburt et al., 2009; Grinter et al., 2016), by transporting specific carbohydrates across the host outer membrane (Blanvillain et al., 2007; Xu et al., 2013), or by mediating antibiotic resistance (Zhao et al., 1998). We found that many nonsynonymous SNPs in several TBDRs could clearly differentiate the 23 Xoo strains (Supplemental Figure S3, B and C). These results strongly suggested that TBDRs in Xoo had similar functions to acquire essential iron(s) and/or to transport carbohydrate(s) as required for Xoo infection and growth in rice (Supplemental Figure S3, D to G). The third group included three genes, NADH dehydrogenase (PXO_00908), ATP-dependent RNA helicase DbpA (PXO_00394), and ATP-dependent RNA helicase (PXO_03270), involved in energy metabolism. The SV Xoo races are differentiated strongly from the WV/MV races at the three loci, suggesting the adaptation of Geng and Xian types races to the rice host might have involved in energy use differences. Taking these findings together, recombination and mutation are expected to have played an important role in generating new and novel virulence to rice resistance and maintaining high levels of genetic diversity at many virulence-related genes in the tropical Xoo races even under the strong selection from the host populations during the coadaptation between rice and Xoo. This was because Xoo had been evolving at a much greater rate than rice, particularly in the tropic areas (Midha et al., 2017), and because various Xoo races of diverse virulence, including incompatible ones, are expected to be present in very different frequencies in the field populations of single modern rice varieties with high-level resistance.

Given the complex genetic systems underlying resistance/virulence in each case of plant–pathogen interactions regarding the numbers of loci involved, it has been a great challenge to characterize experimentally genome-wide interactions between defensive genes of a plant species and virulence genes of its pathogen because of the technical difficulty in phenotyping large numbers of plant accessions with many pathogen strains of diverse origins/virulence, particularly for crop plants. Here, we demonstrated that even with relatively small numbers of well-sampled rice accessions and Xoo races, it was possible to identify and shortlist causal rice QR-genes and Xoo virulence-genes involved in interactions underlying the reciprocal adaptation process between rice and Xoo. Statistically, the sample size of our data set (49 × 23 = 1,127) was expected to have sufficient power in detecting significant interactions between rice QR-gene blocks and Xoo virulence genes, though the rice sample size was too small to resolve causal QR-genes involved in specific interactions in most cases. While we adopted the tag SNPs of LD blocks each covering multiple significant SNPs in a single LD block as the input rice genotypic data to control the number of significant interactions detected in our 2D GWAS, the high mapping resolution of most QR-genes detected in our 1D GWAS allowed shortlisting of candidate QR-genes in many LD gene blocks involved in important interactions.

Based on current knowledge, the detected multiple-to-multiple interactions between the rice and Xoo genes were expected to be of two types in nature. The first type would result from direct interactions between rice R-genes and Xoo genes at the molecular level (protein–protein, protein–DNA, protein–RNA, DNA–DNA interactions, etc.) according to their functional correspondences, leading to high-level resistance/immunity or susceptibility (Nino-Liu et al., 2006; White and Yang, 2009) and the second type, described below, result from indirect genetic interactions. For the first type, previously reported cases of direct gene-for-gene interactions between Xoo TAL effectors and dominant rice Xa-genes are of this type, including Xa10/avrXa10 (Tian et al., 2014), Xa27/avrXa27 (Gu et al., 2005), Xa23/avrXa23 (Wang et al., 2015a), and Xa21/RaxX (Pruitt et al., 2015). In this study, the strongest interactions between the seven genes of Xoo gene group I and genes of rice QR-gene groups 2 and 4 (particularly those in block L71) are most likely of this type, which may have played the most important role during the observed arms-race between rice and Xoo during modern breeding. These included the strongest interactions between Xa26 (LOC_Os11g47210) and two T6SS genes (PXO_00502 and PXO_04700), hrpf and seven Xoo genes (xopX, talC3b, pthXa1, talC6a, pthX6, talC9a, and talC9b), and the interaction between LOC_Os11g46890 (the Xa40 candidate) and hrpf.

The direct interactions may also have occurred between some Xoo genes and their target rice susceptibility genes that function to acquire energy, essential nutrients, etc., for successful infection and growth of Xoo. Also, bacteria need resistance to plant antibiotics such as the phenazine-1-carboxylic acid in order to grow and reproduce normally (Wang et al., 2011). Thus, the (recessive) nonfunctional alleles at the involved rice susceptibility loci would act as recessive “r-genes”. Correspondingly, Xoo genes encoding enzymes involved in energy/nutrient/antibiotics metabolism
and relevant transport systems may potentially be involved in this type of interaction, as demonstrated in the cases of PthXo1/OsSWEET11 (Yuan et al., 2011), PthXo2/OsSWEET13 (Zhou et al., 2015), and (AvrXa7, PthXo3, TalC and Tal5)/OsSWEET14 (Blanvillain-Baufume et al., 2017) interactions. We believe that the strong interactions between Xoo TBDR (PXO_01644) and rice “r-genes” (iron-carrying proteins, such as a 2OG-Fe oxygenase family protein gene LOC_Os11g43610) may be of this type.

The finding that Xoo NADH may have played an important role in determining Xoo virulence was a big surprise because NADH dehydrogenase is well-known for its key role in energy metabolism for all bacteria (Heikal et al., 2014). In contrast to TBDRs, the Xoo NADH dehydrogenase gene, PXO_00908, is highly conserved harboring only two nonsynonymous SNPs (Supplemental Figure S12). Nevertheless, the two nonsynonymous SNPs distinguish seven SV races from the WV/MV races. Furthermore, the Xoo NADH interacts strongly with the same set of the rice genes as hrpF does (Figure 7B), suggesting that shifts between MV and SV of the closely related Philippine Xoo races caused by hrpF might be accompanied with a change or cost in their energy use associated with the corresponding functional changes in the NADH dehydrogenase protein.

Taking these results together, we have demonstrated an efficient strategy to identify and shortlist the numbers of important QR-genes/virulence-genes underlying the rice/Xoo coadaptation, which would greatly facilitate future efforts to elucidate the molecular mechanism(s) of the QR-gene–virulence-gene interactions underlying the coadaptation of rice and Xoo. Clearly, the power and mapping resolution of the 2D GWAS can be further improved by increasing the representativeness and sizes of sampled host genotypes such as well-designed mapping populations or near-isogenic line sets of QR-genes, and/or by increasing the pathogen population size. Nevertheless, our results support the concept that any specific plant–pathogen interaction involves complex immune receptor networks (Wu et al., 2018), within which the gene-for-gene theory would largely hold true in most cases of these direct interactions between plant R-genes and virulence genes in pathogens, though these direct interactions may not have to be one-for-one.

The second type of interactions between rice genes and Xoo genes are genetic and inferred to be indirect, resulting primarily from the functional complementarity between or among different types of Xoo virulence genes or rice defensive genes (genetic epistasis; Li et al., 2006), and most QR-gene blocks/virulence genes interactions detected in this study were expected to be of this type. Obviously, the observed multiple-to-multiple pattern of the interactions between rice genes and Xoo genes would arise in any of the following scenarios: (1) genetic epistasis arising from genes acting in the same defensive pathways of the host; (2) the co-existence of different allelic combinations of defensive genes in specific rice accessions and/or those of virulence genes in specific Xoo races (the small population size); and (3) the complex structure of the rice population used in the 2D GWAS. This is because a susceptible (compatible) phenotype would be expected only when none of the defensive genes in the host genome is effective or the pathogen genome contains the virulent alleles at all its virulence loci. Otherwise, a resistance phenotype would arise when a host plant carries one or more effective Xa/xa genes. Thus, it would be much more difficult genetically for a pathogen to adapt to its host genetic system containing large numbers of QR-genes. Fortunately, the short generation time (Grewal et al., 2012) and fast evolution rate of pathogens would allow them to generate sufficient new virulence alleles by recombination and/or by mutation to overcome host resistance, and strong selection from host populations with new R-gene(s) would quickly pick one or more virulent races from existing pathogen populations consisting of diverse races.

In summary, we demonstrated the power and efficiency to apply both 1D and 2D GWAS for genome-wide search for QR-genes/virulence-genes in rice and Xoo and their interactions underlying their coadaptation, which led to the identification of 47 Xoo virulence-related genes and 318 rice gene loci of both the detected virulence genes and QR-genes. The discovered QR-genes included previously reported Xa26, xa25, Xa40 (experimentally validated) and Xa22 plus many new ones. The new and novel virulence genes included several T6SS genes, TBDRs and many others. Recombination in the QR-gene clusters was suggested as the most important mechanism for generating novel resistance in rice to new virulent Xoo races, while recombination and mutation may have played an important role in generating new and novel virulence to rice resistance and maintaining high levels of genetic diversity at many virulence genes in Xoo populations. The relationship between rice and Xoo was characterized by strong differentiation among Xoo races corresponding to the subspecific differentiation of rice, by corresponding strong shifts toward increased resistance/virulence of rice/Xoo populations, by corresponding rich genetic diversity at the detected rice QR-genes and Xoo virulence genes, and by genome-wide interactions between many rice QR-genes and Xoo virulence genes in a multiple-to-multiple manner, presumably either from direct protein–protein interactions or from genetic epistasis. The observed complex genetic interaction system between rice and Xoo is expectedly to exist in other crop–pathogen systems, which would maintain high levels of diversity at their QLoci/virulence-loci under strong directional selection from host populations, resulting in the dynamic coevolutionary consequences during their reciprocal adaptation. This relationship would last when a balance in evolutionary potential is reached, in which pathogens are depending primarily on their extraordinary abilities to evolve, while plants are dependent on their tremendous within-species genetic diversity and large complex genomes (more genes and more complex genetic systems). The arms-races between crop
plants and their pathogens represent more extreme cases of the dynamic coevolutionary consequences of the complex genetic interaction system between pathogens and plants under strong artificial selection for high-level resistance/immunity that tends to break the balance between host plants and pathogens. In the end, the relationships between pathogens and their hosts would persist only if neither side wins.

**Materials and methods**

**Xoo strains and artificial inoculation**

Based on the previous determination of race grouping (Vera Cruz et al., 2000; Zhou et al., 2011; Quibod et al., 2016), the 23 Xoo strains representing 14 well-characterized Xoo races from tropical Asia (IRRI at the Philippines) and nine races from China collected during 1973–2012 (Supplemental Data Set S3) were used in this study. For the phenotyping experiments, seeds of individual rice (Oryza sativa) accessions were sown in the seedling nursery in the summers of 2015 (73 accessions for inoculation with all 23 strains) and 2016 (701 accessions for inoculation with four representative races C3 [JS158-2], C5 [GD1358], P1 [PXO61], and P9a [PXO339]), and 30-day-old seedlings of each accession were transplanted in paddy fields of experiment farms in the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China. Each of the rice accessions was planted into a 23-row plot for the first set of 73 rice accessions and a four-row plot for the second set of 701 rice accessions (the number of rows within a plot depended on the number of Xoo strains used for inoculation) with nine plants in each row at a spacing of 20 × 17 cm. The field planting followed a randomized complete block design with two experimental replications. All plants were managed under standard cultural practices. Each Xoo strain was incubated on peptone sucrose agar at 30°C for 2 days, and inoculum was prepared by suspending the bacterial mass in sterile water at a concentration of ∼10⁶ cells mL⁻¹.

At the tillering stage 30 days after rice seedlings were transplanted, plants were inoculated by the leaf-clipping method (Kauffman et al., 1973). For the accuracy of inoculation, only a single Xoo strain was used to inoculate rice plants in the same row. Briefly, four to five top fully expanded leaves from each of five and three central plants per row of each (accession) plot were clipped using scissors and then dipped into the Xoo inoculum for the first and second set of rice materials. The lesion lengths (cm) were measured 21 days after inoculation on two (three) leaves per plant of five (three) inoculated plants per experimental replication per Xoo strain for the first (second) set of rice materials. Thus, these combinations resulted in a total of 73 rice accessions × 23 Xoo strains × 5 plants × 2 replications for the first set of rice materials and 701 rice accessions × 4 Xoo strains × 3 plants × 2 replications for the second set of rice materials. Phenotypic data for each accession were defined as the average of the two experimental replications in each set of rice materials.

**Plant materials**

Two nonoverlapping sets of plant materials were used in this study. The first set consisted of 73 rice accessions from 21 countries, including 49 accessions from the 3,000 Rice Genomes Project (3KRGP, 3KRGP, 2014) plus 24 additional accessions (Supplemental Data Set S2). The second set included 701 rice accessions from 40 countries (Supplemental Data Set S4), 515 of which were from 3KRGP plus 186 additional accessions composed mainly of modern Chinese varieties. According to the population structure, geographical origins, and variety types of accessions, we divided the 701 second-set accessions into different subpopulations, consisting of 419 Xian accessions, 219 Geng accessions, 451 Chinese accessions (135 landraces and 316 modern varieties), and 241 overseas accessions for specific comparisons of results.

**Heritability estimates**

Broad-sense heritability was estimated using the conventional method of ANOVA with two experimental replications. The linear model for estimation of heritability is

\[ y_{jk} = \mu + a_j + b_k + e_{jk} \]

where \( y_{jk} \) is the lesion length of \( j \)th accession caused by one Xoo strain from \( k \)th replication, \( \mu \) is the population mean, \( a_j \) is the genetic effect of the \( j \)th accession as a random effect following a \( N(0, \sigma^2_G) \) distribution, \( b_k \) is the effect of the \( k \)th replication, and \( e_{jk} \) is the residual error with an assumed \( N(0, \sigma^2_e) \) distribution. The broad-sense heritability is defined as

\[ H^2 = \frac{\sigma^2_G}{\sigma^2_G + \sigma^2_e} \]

The two variance components were estimated from the variance component analysis using the lme4 package (Bates et al., 2015) in R.

**Whole-genome sequencing and SNP calling of the 23 Xoo strains**

Genomic DNA of each of the 23 Xoo strains was isolated using a modified cetyltrimethylammonium bromide (CTAB) protocol. Specifically, 2-mL cultures were grown in NYGB (nutrient yeast extract glycerol broth) for two days at 28°C under constant shaking. Cells were pelleted and re-suspended in Tris/ethylenediaminetraacetic acid. Cells were lysed in sodium dodecyl sulfate/CTAB extraction buffer before phenol: chloroform: isoamyl alcohol extraction. DNA was precipitated in isopropanol and washed with 70% (vol/vol) ethanol. Quantity and quality of extracted DNA were assessed by a combination of nanodrop and gel electrophoresis. Next, the genomic DNA was fragmented by nebulization with compressed nitrogen gas. The overhangs of the fragments were converted to blunt ends using T4 DNA polymerase and Klenow polymerase. After adding an “A” base to the blunt ends of the double-stranded DNA fragments, adaptors with “T” base overhangs were ligated to the genomic DNA fragments. These fragments were separated on an
agrose gel and excised from the gel at the DNA band around 200 bp. Finally, the DNA fragments were enriched by a ten-cycle polymerase chain reaction (PCR) process. The sequencing libraries with insert sizes of ~500 bp and ~6,000 bp were generated using a Covaris S-series instrument. We sequenced 100 bp at each end by Illumina Genome Analyzer Ixl or Illumina HiSeq 2000. In total, each Xoo strain was sequenced with a high sequencing depth of ~100×. After removal of sequencing adapters and cleaning of duplicated reads of PCR amplification, the clean reads were mapped to the PX099X genome (Salzberg et al., 2008) by BWA (V0.6.2; Li and Durbin, 2009) with default parameters. Reads realignment around indels were performed by Realigner Target Creator and Indel Realigner in GATK (V2.5-2; McKenna et al., 2010). SNPs were called by HaplotypeCaller in GATK (V2.5-2; McKenna et al., 2010) with default options, and SNPs were further filtered by SNP quality (QUAL > 30) and depth (DP > 10).

Whole-genome sequencing of 186 Chinese accessions and SNP calling of 701 rice accessions

Total genomic DNA was isolated from leaf tissues of each of the 186 Chinese accessions. A sequencing library with insert sizes of ~500 bp was constructed for each accession according to Illumina’s standard instructions. Paired-end 90-bp reads were sequenced on Illumina HiSeq 2000 platform, and the raw sequences were further processed to remove adapter sequences and low-quality reads, yielding approximately 5-Gb sequencing bases with an average sequencing depth of ~15.2×, similar to sequencing data of the 3KRGP (3K RGP, 2014). All library construction, sequencing, and sequence cleaning were carried out by BGI-Shenzhen, China.

We further collected sequencing data of the 515 rice accessions from the 3KRGP (3K RGP, 2014). SNPs were called for all these 701 rice accessions. Specifically, high-quality reads were aligned to the Nipponbare reference genome (Kawahara et al., 2013) using the BWA software (V0.6.2; Li and Durbin, 2009) with the parameter “-m 10000 -o 1 -e 10 -t 4”. Aligned duplicated reads were then removed using Picard tools (V1.171; http://broadinstitutegithub.io/picard/), reads around indels were realigned using GATK (V 2.0-35; McKenna et al., 2010) IndelRealigner, alignments were recalibrated by BaseRecalibrator in GATK. SNP calling was performed using UnifiedGenotyper in GATK with a minimum phred-scaled confidence threshold of 50, a minimum phred-scaled confidence threshold for emitting variants at 30, a minimum mapping quality at 20, and a minimum sequencing depth at 2. Finally, 8,661,876 high-quality SNPs were obtained for further analysis.

Population analysis

We used the SNPPhylo (V20160204; Lee et al., 2014) to construct the unrooted and unweighted neighbor-joining tree for the 701 rice accessions and the 23 Xoo strains by the Neighbor program. The genetic ancestry was inferred with STRUCTURE (V2.2.3; Pritchard et al., 2000). Principal component analysis was performed by EIGENSOFT(V6.0.1; Patterson et al., 2006) using pruned SNPs selected by PLINK (V1.90; Purcell et al., 2007) with the parameter “--indep-pairwise 50 10 0.2”. To estimate clonality of the Xoo population, MLGs were assigned by the poppr package (Kamvar et al., 2015) in R. By running the software HRefinder (Wang et al., 2013), with the information of SNP sites as input, we characterized the recombination events during the evolution and divergence of the 23 Xoo strains. The patterns of LD decay were calculated by PopLDdecay (V3.40; Zhang et al., 2019) in the whole rice population and PLINK in the whole Xoo population. Nucleotide diversity (π) for each 10-kb window across the genome with overlapping 5-kb step size was calculated using Variscan (V2.0.3; Vilella et al., 2005). Weir and Cockerham’s FST statistics at all significantly associated SNPs between rice subpopulations were calculated by VCFTools (V0.1.15; Danecek et al., 2011).

Identification of virulence-related SNPs in Xoo

We integrated phenotypes of 73 rice accessions to detect virulence-related genes in Xoo. Existing multi-phenotype GWAS methods can only handle less than 10 phenotypes. Here, we used a customized strategy to integrate the GWAS results, which was demonstrated correct by revealing many known and highly possible virulence-related genes (see “Results” section for details). Specifically, we first carried out independent GWAS for Xoo SNPs with phenotyping data of lesion lengths from the first set of 73 rice accessions. Only 16,607 binary genic variants (SNPs within nonprotein-coding genes, transposases excluded) with MAF ≥ 0.05 were used for the association studies. We used both linear (LM) and LMMs, which took the population structure into consideration. Association tests based on LM were carried out using PLINK (V1.90; Purcell et al., 2007) with parameters “--linear”. Association tests based on LMM were carried out using EMMAX (Kang et al., 2010) with default parameters. An identical-by-state matrix based on all SNP data was used to create the kinship matrix that measures the genetic similarity between individual Xoo strains. The first two principal components were used as covariates to control for population structure. Finally, a CAS for each SNP (SNP i) defined as $C_{Ai} = LM_i + LMM_i$. LM and LMM are the integrated scores for LM and LMM and were calculated similarly as a weighted sum of 73 independent GWAS results: $LM_i or LMM_i = \sum_{j=1}^{73} D_{ij}$, where $\sum D_i$ is the sum of weighted scores of SNPs in the 73 independent LM/ LMM GWAS

$$D_{ij} = \begin{cases} 0, & p_{ij} > 10^{-3} \\ \frac{1}{K_j}, & p_{ij} \leq 10^{-3} \end{cases}$$

in which, $K_j$ is the total number of significant SNPs ($P < 10^{-3}$) in the jth LM/LMM GWAS. Those SNPs with detected times less than 2 under both LM and LMM models, or with its minimum P-value among all tests higher than $10^{-4}$ were not considered as significantly associated. Finally, those SNPs with a CAS ≥ 0.3 were considered as significant associations. This threshold was determined by considering the ranks of the known and candidate virulence-related
genes. Specifically, we first utilized three different P-value cutoffs (1e−3, 1e−4, and 1e−5) to calculate the CAS scores; next, by manually exploring literature in PubMed database (to see if a gene is reported as a virulence-related gene in other bacteria), we manually annotated 113 genes with CAS over 0.1 at each P-value cutoff; we then assessed the performance of combinations of the P-value and CAS score cutoffs by assuming that all the candidate pathogen genes were true positives, the combination of the P-value cut-off at 0.001 and CAS cutoff at 0.3 gave the highest overall accuracy (the average value of sensitivity and specificity at the genic level). We carried out genotype permutation analysis to estimate the false discovery rate. Specifically, we shuffled the genotypes (exchange “whole” genotypes among the 23 level). We carried out genotype permutation analysis to estimate the false discovery rate. Specifically, we shuffled the genotypes (exchange “whole” genotypes among the 23 Xoo strains) and performed the CAS-based GWAS. We did 10,000 permutations and for each permutation, 15.0 ± 8.0 (mean ± SD of the mean) SNPs could be flagged as significant [as described previously in the method section: CAS ≥ 0.3 (calculated at 0.001), detected times ≥2 and minimum P < 0.0001], which suggested a low SNP-level false discovery rate of 15/86 (the significant SNP number detected in this article) = 0.17.

Phylogeny and positive selection analysis of Xoo virulence genes
In order to obtain evidence for positive selection of two virulence-related genes (PXO_03417 and PXO_01644) during the evolution and divergence of Xoo, the CDSs of all genes from the 23 Xoo strains were built artificially by replacing SNPs to their corresponding CDS in the PXO99A reference genome and aligned by using PHYML (V3.0; Guindon et al., 2010), and then phylogenetic trees were inferred using the maximum likelihood method accordingly. The software was set to use a BIONJ start tree, the JTT substitution model, the default NNI tree searching method, gamma-distributed rates of evolution with four discrete categories and empirical nucleotide frequencies. Nonparametric bootstrap repetitions were used to evaluate statistical support to branches. Rooting was performed using the midpoint method. The resulting phylogenetic trees were drawn with iTOL (V4; Letunic and Bork, 2019). Sequence alignments and phylogenetic tree files can be found in Supplemental File S1. By comparing a free parameter model (dn/ds is free) and a restricted parameter model (dn/ds is restricted to be 1 on individual tree branches) to detect positive selection genes in the Xoo genome, and positively selected amino acid residues in proteins (NSites model) were inferred using the codeml program from the PAML software package (Yang, 2007). The dN and dS values were estimated using the nonsynonymous and synonymous nucleotide substitutions along lineages on the phylogenetic trees.

Identification of rice resistance genes by GWAS
A total of 5,379,674, 5,359,202, 5,003,609, 5,828,337, 5,168,652, and 4,784,025 SNPs with minor allele frequencies >5% and the number of accesses with minor alleles ≥6 were filtered for the association analyses of the whole population, Xian, Geng, Chinese, overseas, Chinese landrace, and Chinese modern variety panel populations, respectively. All GWAS were completed using a LMM in EMMAX (Kang et al., 2010) to determine the associations between each SNP and phenotyping data of lesion lengths from the second set of 701 rice accessions for resistance to four representative Xoo races (C3, C5, P1, and P9a). We used the Balding–Nichols matrix based on LD pruned SNPs across the whole rice genome (with parameter “indep-pairwise 50 10 0.2” in PLINK) to develop the kinship matrix, which measured the genetic similarity between individuals in each GWAS panel. The first three principal components were used as covariates to control for population structure. The effective number of independent markers (N) was calculated using the GEC software (Li et al., 2012b), and significant thresholds (0.05/N) were calculated in each GWAS panel. To account for the effects of replication, the best linear unbiased predictions (BLUPs) for the lesion lengths of the two replications were calculated with the Imer function of the R package lme4 (Bates et al., 2015), using rice accession as a random effect and replication as a fixed effect (Supplemental Data Set S4). Mean lesion lengths and BLUPs of the two replications were both used as the phenotypic input for the association analysis. Because both approaches produced very consistent results, only the results based on the phenotypic means across two replications are presented. Adjacent lead SNPs separated by <300 kb were defined as a single association region based on the LD decay in the whole population and the method described by Chen et al. (2014) and Wang et al. (2015b). Gene symbols of functionally characterized rice genes were obtained from funRiceGenes database (Yao et al., 2018). The Manhattan and quantile–quantile plots for the GWAS results were created using R. Enriched GO terms (FDR < 0.05) were identified using the agrigo v2.0 with the Singular Enrichment Analysis method (Tian et al., 2017).

Vector construction and rice transformation
To analyze the functions of the predicted candidate genes, the CRISPR/Cas9 binary vector of LOC_Os11g46890 in a resistant haplotype variety, Wuyugeng20, was constructed according to the multiplex editing method of Ma et al. (2015). Specifically, two guide RNAs were designed to target exon of LOC_Os11g46890 using the web-based software CRISPR-GE (http://skl.scau.edu.cn/; Xie et al., 2017). To construct the guide RNA constructs for target site 1, the first PCR was carried out in two separate reactions with U-F/U6a-LOC_Os11g46890 and gRT1-LOC_Os11g46890/gR-R using pYLsgRNA-OsU6a as a template. Then the second PCR was performed to generate a guide RNA construct containing target site 1 by overlapping PCR with the first PCR products. Likewise, to construct the guide RNA constructs for target site 2, the first PCR was carried out in two separate reactions with U-F/U6b-LOC_Os11g46890 and gRT2-LOC_Os11g46890/gR-R using pYLsgRNA-OsU6b as a template. Then the second PCR was performed to generate a guide RNA construct containing target site 2 by overlapping PCR with the first PCR products. Finally, these two guide
RNA constructs were inserted into the pYLCRISPR/Cas9Pubi-H vector through restriction–ligation reaction. The transgenic plants were created by the Agrobacterium-mediated transformation method (Hiei et al., 1994). To confirm the mutations, we designed primer LOC_Os11g46890-TF and LOC_Os11g46890-TR to amplify the fragment spanning the two target sites in the T0 transgenic lines. Through Sanger sequencing with gene-specific sequencing primer LOC_Os11g46890-SP, the homozygous mutants in target sites were identified. See Supplemental Table S6 for the primer sequences used for vector construction and mutation detection. The resistance levels of the mutants and Wuyugeng20 were determined by inoculation with Xoo race C5 at the tillering stage 30 days after rice seedling transplanting using the leaf-clipping method (Kauffman et al., 1973). Briefly, five fully expanded leaves from each of three plants of each T1 mutant and Wuyugeng20 were clipped using scissors dipped with the Xoo inoculum. The water-soaked lesion lengths (cm) of nine leaves (three leaves per plant) of three inoculated plants from each mutant and Wuyugeng20 were measured 21 days after inoculation.

Cross-species genome–genome interaction analysis
Of the 73 rice accessions of the first set, 49 are sequenced in 3KRG. Using the SNP genotypes at the detected virulence loci of Xoo, resistance loci of rice and the phenotypes (mean lesion lengths of two replications) of the 49 rice accessions inoculated by the 23 Xoo strains, we were able to analyze all possible pairwise interactions between the detected virulence loci of Xoo and resistance loci of rice statistically using a linear model via a custom-made script in R, which is available on Github (https://github.com/dookbrhzq/TwoWayGWAS). Specifically, to test the interaction between the pth rice SNP and the qth Xoo SNP, we fit the below linear model:

\[ L \sim \beta_0 + \beta_1M_p + \beta_1M_{q} + \beta_2N_p + \beta_2M_q + \{\beta_4R_1 + \cdots + \beta_4R_{4a}\} + \{\beta_5X_1 + \cdots + \beta_5X_{22}\} \]

\[ \{L_{1,1}, L_{1,2}, \ldots, L_{1,23}, L_{2,1}, \ldots, L_{2,23}, \ldots, L_{49,23}\} \] is a phenotype vector (with size of 49 \times 23) between 49 rice accessions and 23 Xoo strains. \( L_{ij} \) denotes the lesion length of jth Xoo strain on ith rice accession. \( M_p \{M_{1p}, M_{12p}, \ldots, M_{1p} \} \) (i.e. \( M_{1p} \) was repeated 23 times), \( \ldots, M_{1p}, \ldots, M_{1p} \) is the pth genotypes of the corresponding rice accession, and \( N_q \{N_{1q}, N_{2q}, \ldots, N_{23q}\} \) (i.e. a genotype set of the 23 Xoo strains), \( N_{1q}, \ldots, N_{2q}, \ldots, N_{23q}, \ldots \) (the genotype set was repeated a total of 49 times) \( \ldots \) is the qth genotypes of the corresponding Xoo strain. \( M_{p}N_{q} \) denotes the interaction variable, which was our interest. \( R_1, \ldots, R_{4a} \) are 48 covariates displaying if a phenotype involved a specific rice accession. For example, \( R_1 \) (49 times of 1 followed by 49 \times 22 times of 0) represents if the phenotypes were obtained from the first rice accession. Similarly, \( X_{1}, \ldots, X_{22} \) were 22 covariates displaying if a phenotype involved a specific Xoo strain. Here, to test if there was a significant interaction between \( M_p \) and \( N_q \), the null hypothesis is \( \beta_5 = 0 \).

Practically, to limit the number of SNP pairs in the same LD blocks in rice–Xoo interaction analysis, we firstly constructed local LD structures around 5,432 significant SNPs detected by the 1D GWAS in the second set of rice materials and selected independent representative SNPs (for the four Xoo races) with the most significant hit and/or with the largest contribution to the phenotypic variance for each Xoo race within an LD block. Specifically, rice LD blocks were identified to select independent significant association peaks in rice GWAS panels using PLINK command line: “plink –clump –clump-p1 6.81e-8 –clump-p2 1e-5 –clump-r2 0.5 –clump-kb 300”. A total of 75 LD blocks were identified for the four Xoo races, including 28, 23, 46, and 20 LD blocks associated with C3, C5, P1, and P9a, respectively. Then, we identified two representative SNPs for each Xoo race, one with the lowest P-value in 1D GWAS (i.e. the SNP identified by the clump function in PLINK) and one with the largest contribution to the phenotypic variance estimated with the statistical model described by Zhao et al. (2011) within each LD block. Both SNPs were considered as tag SNPs for this LD block to conduct further cross-species interaction analysis. Consequently, a total of 172 tag SNPs within the 75 LD blocks in the rice genome (Supplemental Data Set S6) were selected for determining significant rice–Xoo interactions. Then, we tested interactions (328,348 possible pairs) between 1,909 SNPs in two types of Xoo genes ((1) known virulence-related genes in Supplemental Table S1 and (2) genes associated with virulence detected by Xoo GWAS) and the 172 rice tag SNPs. Using the Bonferroni correction for multiple testing with the number of tests we conducted here, the P-value threshold for significant interaction was 1.52 \times 10^{-7} (0.05/(1,909 Xoo variants in virulence-related genes \times 172 rice tag SNPs)). Finally, significant interactions between all pairs of SNPs were binned into gene-block interactions between Xoo and rice. For example, when multiple significantly interacted SNPs were located in the same Xoo gene and the same rice LD block, respectively, only the strongest SNP–SNP interaction (with the minimum P-value) was retained as the gene-to-block interaction.

The density of large-sized deletions
Based on structural variations detected in the 3KRG (Wang et al., 2018b), in which deletions with size >100 bp were detected, we calculated the genome-wide deletion densities with the sliding window method. Briefly, the average numbers of deletions were calculated in 500-kb windows that slid forward by 100 kb each time.

Statistical analysis
The whole GWAS panel SNPs within 1 kb of the upstream promoter region, 3′-UTR, 5′-UTR, and nonsynonymous SNPs in the coding region of a rice candidate gene or the GWAS panel SNPs within the genomic region of an Xoo candidate gene were concatenated as the haplotype of resistance or
virulence loci identified by GWAS, respectively. SNP annotation information was obtained from Rice SNP-Seek Database (Mansueto et al., 2017). For multiple group comparison of mean lesion lengths of the major haplotypes of resistance loci (shared by at least 10 accessions), Tukey's HSD post hoc tests followed by one-way ANOVA were carried out with the agricolae package in R. Pearson's Chi-squared tests using the “chisq.test” function in R were performed to determine significant differences in frequencies of different reactions in rice subpopulations to each Xoo race and significant differences in frequencies of QR-gene haplotypes among rice subpopulations. A collection of the data for statistical analyses can be found in Supplemental Data Set S9. Enrichment significance of SNP density was detected with the “phyper” function in R for tests of the hypergeometric distribution. Fisher's exact tests by the “fisher.test” function in R were used to compare differences in frequencies of detected virulence-related genes or SNPs within and outside the recombination hotspot in Xoo genome. ANOVA was performed to partition the variance components for lesion lengths due to the effects of the replications, rice accessions, Xoo strains, and the interactions between rice accessions and Xoo strains using the “aov” function in R.

Accession numbers
The datasets supporting the conclusions of this article are included in the article. Raw sequence data of 23 Xoo strains are available in the Genome Sequence Archive in National Genomics Data Center (https://bigd.big.ac.cn/gsa) under accession number CRA004049. Raw sequence data and the SNP dataset in PLINK format of 186 Chinese accessions are available at Rice Functional Genomics and Breeding Database (http://www.rmbreeding.cn/tool/dl_rice701).

Supplemental data
The following materials are available in the online version of this article.

Supplemental Figure S1. Genomic recombination, LD decay and nucleotide diversity of 23 Xoo strains.

Supplemental Figure S2. Positive selection and haplotypes of the hrpF gene (PXO_03417).

Supplemental Figure S3. SNP distribution and positive selection of the TonB-dependent receptor gene (PXO_01644) across the 23 Xoo strains and the effect of the nonsynonymous mutation (SNP position 3248766) on 3D structure.

Supplemental Figure S4. Population structure and LD decay of the 701 rice accessions.

Supplemental Figure S5. Quantile-quantile plots of genome-wide association studies for P1, P9a, C5, and C3 in different panel populations of rice.

Supplemental Figure S6. Candidate QR-genes to bacterial blight identified in the genome-wide association analyses.

Supplemental Figure S7. Haplotype analysis of xa25 (LOC_Os12g29220) and Xa26 homolog (LOC_Os11g47240).

Supplemental Figure S8. Comparison of the predicted proteins in wild type Wuyugeng20 (WYG20) and three knockout mutants of LOC_Os11g46890.

Supplemental Figure S9. Haplotype analysis of LOC_Os11g46250.

Supplemental Figure S10. The graphical genotype showing coexistence of the resistance alleles at eight QR-loci jointly determining high-level resistance to SV Xoo race CS.

Supplemental Figure S11. Extended and detailed differences in frequencies of resistance alleles, nucleotide diversity, and copy number of the rice QR-genes.

Supplemental Figure S12. The SNP distribution in NADH dehydrogenase gene (PXO_00908) across the 23 Xoo strains.

Supplemental Table S1. Summary of SNPs in the 48 known virulence-related genes previously reported in Xoo.

Supplemental Table S2. Candidate SNPs associated with Xoo virulence (lesion length) detected by GWAS.

Supplemental Table S3. Twenty-three genes highly associated with Xoo virulence (lesion length) detected by GWAS.

Supplemental Table S4. Forty-one genomic regions <300 kb each containing >5 significant SNPs for resistance to 1–4 Xoo races detected in the second set of rice materials.

Supplemental Table S5. Results of a GO enrichment analysis of the detected rice genes that interacted with Xoo virulence-related genes.

Supplemental Table S6. Information for vector constructions and primers.

Supplemental Data Set S1. The reactions of 73 rice accessions in the first set of rice materials with 23 Xoo strains.

Supplemental Data Set S2. List of the 73 accessions of the first set of rice materials.

Supplemental Data Set S3. Summary of whole-genome sequencing information of 23 Xoo strains.

Supplemental Data Set S4. The reactions of 701 accessions in the second set of rice materials with four representative Xoo races.

Supplemental Data Set S5. The list of total 5,432 significant SNPs associated with resistance to 1–4 Xoo races detected in the second set of rice materials.

Supplemental Data Set S6. Summary of the 75 LD blocks around significant SNPs identified in rice GWAS.

Supplemental Data Set S7. Complete list of genome-wide pairwise SNP-SNP interactions of rice genes associated with bacterial blight resistance and virulence-related genes of Xoo based on lesion lengths.

Supplemental Data Set S8. Genome-wide interactions of rice LD blocks associated with bacterial blight resistance and Xoo virulence-related genes identified in Supplemental Data Set S7.

Supplemental Data Set S9. Summary of statistical tests.

Supplemental File S1. Sequence alignments and phylogenetic tree files of two Xoo genes (PXO_03417 and PXO_01644).

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