Characterization of New Races of *Xanthomonas oryzae* pv. *oryzae* in Mali Informs Resistance Gene Deployment

C. Tekete,¹ S. Cunnac,² H. Doucouré,¹ M. Dembele,³ I. Keita,¹ S. Sarra,³ K. Dagno,³ O. Koita,¹ and V. Verdier^{2,†}

¹ Applied Molecular Biology Laboratory, Faculty of Science and Technology, University of Sciences, Techniques and Technologies of Bamako, Bamako, Mali

² IRD, CIRAD, University of Montpellier, IPME, Montpellier, France

³ Institute of Rural Economy, Regional Center for Agronomic Research, Niono and Bamako, Mali

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ABSTRACT

Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* represents a severe threat to rice cultivation in Mali. Characterizing the pathotypic diversity of bacterial populations is key to the management of pathogen-resistant varieties. Forty-one *X. oryzae* pv. *oryzae* isolates were collected between 2010 and 2013 in the major rice growing regions in Mali. All isolates were virulent on the susceptible rice variety Azucena; evaluation of the isolates on 12 near isogenic rice lines, each carrying a single resistance gene, identified six new races (A4 to A9) and confirmed race A3 that was previously reported in Mali. Races A5 and A6, isolated in Office du Niger and Sélingué, were the most prevalent races in Mali. Race A9 was the most virulent, circumventing all of the resistance genes tested. Xa3 controlled six of seven races (i.e., 89% of the isolates tested). The expansion of race A9 represents a major risk to rice cultivation and highlights the urgent need to identify a local source of resistance. We selected 14 isolates of *X. oryzae* pv. *oryzae* representative of the most prevalent races to evaluate 29 rice varieties grown by farmers in Mali. Six

isolates showed a high level of resistance to *X. oryzae* pv. *oryzae* and were then screened with a larger collection of isolates. Based on the interactions among the six varieties and the *X. oryzae* pv. *oryzae* isolates, we characterized eight different pathotypes (P1 to P8). Two rice varieties, SK20-28 and Gigante, effectively controlled all of the isolates tested. The low association observed among races and pathotypes of *X. oryzae* pv. *oryzae* suggests that the resistance observed in the local rice varieties does not simply rely on single known *Xa* genes. *X. oryzae* pv. *oryzae* is pathogenically and geographically diverse. Both the races of *X. oryzae* pv. *oryzae* characterized in this study and the identification of sources of resistance in local rice varieties provide useful information to inform the design of effective breeding programs for resistance to bacterial leaf blight in Mali.

Keywords: bacterial leaf blight, Mali, pathotype, race, resistance, rice, *Xanthomonas oryzae* pv. *oryzae*

More than half of the world's population relies on rice as a staple source of nourishment (Sahu et al. 2018). Rice occupies a very important place in agriculture and in food security in Mali. Because of the extensive deployment of nitrogen-responsive modern rice varieties, bacterial leaf blight (BLB) caused by the vascular pathogen *Xanthomonas oryzae* pv. *oryzae* has become a major rice disease in the last decade worldwide (Busungu et al. 2018; Khoshkdamani et al. 2012; Tilman et al. 2017). In Mali, BLB was first reported in the late 1980s and thereafter in various African countries (Buddenhagen et al. 1979; European and Mediterranean Plant Protection Organization 2017). Since 2002, plant health monitoring has reported increasing epidemics of BLB in Mali (Sarra et al. 2010). Variations in yield losses depend on the rice growing stage, geographical location, and seasonal conditions (Ou 1985). Annual yield losses can vary between 50 and 80% in the case of severe infections (Mishra et al. 2013; Verdier et al. 2012). Wounds and hydathodes are the main entry sites for *X. oryzae* pv. *oryzae*, where it multiplies in the epitheme, subsequently enters into

the xylem vessels, and starts active multiplication, leading to leaf wilting (Ray et al. 2000; Sahu et al. 2018).

To cause disease in rice, *X. oryzae* pv. *oryzae* injects transcription activator-like effector (TALE) virulence proteins into the host cell through the molecular syringe of the type III secretion system in order to upregulate the expression of target nuclear genes similar to eukaryotic transcription factors (Boch and Bonas 2010; Moore et al. 2014). TALE repertoire inventories based on genomic sequences revealed that individual African strains of *X. oryzae* pv. *oryzae* contain nine TALEs that are classified in nine groups (Doucouré et al. 2018; Tran et al. 2018). The involvement of three of them (TalB, TalC, and TalF) in the virulence of *X. oryzae* pv. *oryzae* strains has been described (Tran et al. 2018; Yu et al. 2011). However, no clear relationship between the contribution of TALEs to virulence and their evolutionary conservation has yet been observed in the *Oryza sativa*–*X. oryzae* pv. *oryzae* pathosystem (Tran et al. 2018).

An understanding of the genetic and pathogenic diversity of the pathogen population is an absolute prerequisite for developing efficient breeding strategies adapted to local rice cultivation systems. Extensive studies on *X. oryzae* pv. *oryzae* have been conducted in different rice producing countries such as China, India, Bangladesh, Japan, Nepal, Korea, Philippines, and Mali (Adhikari et al. 1999; Chen et al. 2012; Doucouré et al. 2018; Li et al. 2009; Ochiai et al. 2000). Populations of *X. oryzae* pv. *oryzae* are variable (Gautam et al. 2015; Khaeruni and Wijayanto 2013) with a high degree of genetic and pathotypic diversity, as reported in the major rice growing countries of Asia (Adhikari et al. 1995; Islam et al. 2016; Ochiai et al. 2000).

Cultural, chemical, and biological control approaches have been developed to control BLB, but currently none are fully effective in the case of severe disease outbreaks (Islam et al. 2016; Mundt 2014). The selection of disease-resistant rice varieties is the most

[†]Corresponding author: V. Verdier; valerie.verdier@ird.fr

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effective, economical, and least environmentally damaging method for BLB control (Mew 1987; Verdier et al. 2012). Varietal resistance is thus considered a top-priority approach for controlling BLB in Mali, although major resistance genes are not durable in some cases because of the high degree of variation of the pathogen population (Vera Cruz et al. 2000). Breeding programs can also unintentionally alter the pathogen population structure by favoring the emergence of underrepresented virulent clones (McDonald and Linde 2002). Thus, before any breeding program for varietal resistance begins, the genetic and pathotypic diversity of the pathogen must be studied in detail. Different rice genes conferring resistance to *X. oryzae* pv. *oryzae* have been identified and designated in a series from *Xa1* to *Xa44* (Busungu et al. 2016, 2018; Vikal and Bhatia 2017). Major resistance genes have been incorporated into rice to develop BLB resistant varieties and to construct near isogenic lines (NILs) (Kim et al. 2011; Ogawa et al. 1991). These NILs and the recurrent parent IR24 are commonly used to characterize the race structure of the pathogen (Triplett et al. 2014). An *X. oryzae* pv. *oryzae* race is a group of strains sharing the same virulence profile on a panel of 12 rice NILs. Several dozen *X. oryzae* pv. *oryzae* races have been reported in Asia, and new races, virulent on the deployed breeding materials, are frequently emerging (Liu et al. 2007; Triplett et al. 2014). In West Africa, three races have been identified, with race A3 reported in Mali, races A1 and A2 reported in Burkina Faso, and race A1 also reported in Niger (Gonzalez et al. 2007).

The efficiency and sustainability of the available resistance genes for BLB control in West Africa and in Mali in particular are poorly characterized. This study primarily aimed to expand our knowledge on the presence of *X. oryzae* pv. *oryzae* surveying fields in the main rice growing centers in Mali and the race structure of *X. oryzae* pv. *oryzae* isolates using a rice panel of NILs deployed by the International Rice Research Institute (IRRI) in the Philippines. We also evaluated the known resistance genes available to control BLB and the resistance of cultivated rice varieties to *X. oryzae* pv. *oryzae* in Mali.

MATERIALS AND METHODS

Surveys and isolation of *X. oryzae* pv. *oryzae*. Simple random sampling was used to collect rice leaves with BLB symptoms between 2010 and 2013 in three rice production centers in Mali: Office du Niger, Baguinéda, and Sélingué regions (Fig. 1). Samples were tagged to record the collection date, host plant, site, and GPS data and were stored at -20°C in the laboratory before processing. A total of 60 samples were analyzed. Bacteria were isolated from infected leaves by adapting the method described by Adhikari et al. (1994). After a brief surface disinfection of the infected tissue, a 5-cm fragment of the leaf sample was macerated with a homogenizer in a plastic bag (Bioreba) containing sterilized water. The macerate was incubated at 4°C for 30 min to 1 h, diluted (in a series of 1:10, 1:100, and 1:1,000), and then spread on a semiselective PSA medium (10 g/liter of peptone, 10 g/liter of sucrose, 1 g/liter of glutamic acid, 16 g/liter of Bacto agar, 50 mg/liter of actidione, 40 mg/liter of cephalaxin, and 20 mg/liter of kasugamycin). Cultures were incubated for up to 3 days at 28°C . Simple colonies (pale yellow) were isolated and purified twice on PSA medium. A single purified bacterial colony was then selected per sample. Nomenclature was adopted in 2003 (Gonzalez et al. 2007) for the codification of *X. oryzae* pv. *oryzae* isolates in Mali. The MAI term for Mali Institut de Recherche pour le Développement followed by a number as an index ranging from 1 to n was assigned to each isolate. Four reference strains of *X. oryzae* pv. *oryzae* originated from Mali (Gonzalez et al. 2007) were used as controls. Colony-multiplex PCR was used to confirm that each isolate belonged to the *X. oryzae* species and *oryzae* pathovar (Lang et al. 2010). Amplification was carried out using a MJ Research PTC-200 thermocycler according to the conditions described by Lang et al. (2010). Visualization of the PCR product after electrophoresis on a 1.5% agarose gel was performed using a Kodak device with EDAS software.

Pathogenicity assays. The pathogenicity of the *X. oryzae* pv. *oryzae* isolates was evaluated on the susceptible variety Azucena. Characterization of the *X. oryzae* pv. *oryzae* isolates into virulence groups (races) was conducted on a set of 12 NILs each carrying a specific resistance gene and the recurrent parent IR24 using a simplified scale developed by IRRI (Quibod et al. 2016). Seeds were obtained from IRRI. As part of our objective to search for a broad-spectrum source of resistance against *X. oryzae* pv. *oryzae* isolates, we evaluated the resistance of 29 rice varieties grown in Mali (Supplementary Table S1). The seeds were obtained from the breeding program at the Institute of Rural Economy (IER) in Mali.

The pathogenicity assays were carried out in pots under the agroecological conditions of the IER Sotuba agricultural experiment station in 2014 and 2015. Two-week-old plants were transplanted into 10-kg pots filled with natural potting soil following the experimental split plot designs with three replicates. The soil used for the experimentations results from the natural decomposition of the organic matter of the virgin forest of the station. The experiments were carried out during wintering (rainy season) periods in order to benefit from the effect of the high relative humidity content, with a mean relative humidity of 74% (minimum, 61%; maximum, 88%) and a mean temperature of 28.05°C (minimum, 22.96°C ; maximum, 33.15°C). Approximately 3 g of diammonium phosphate (18-46-0) fertilizer was used three times per pot on days 15, 25, and 35 after sowing. Strain MAI1 (Gonzalez et al. 2007) was used as a control and sterilized distilled water was used as a negative control for leaf inoculations in all assays. The inoculum was prepared with sterilized distilled water and bacterial colonies from a 24-h culture on PSA medium at 28°C . Bacterial suspensions were adjusted at an optical density (measured at a wavelength of 600 nm) of 0.2 (10^8 CFU/ml) using a Biophotometer 6131 spectrophotometer (Eppendorf). The 45-day-old plants were inoculated by leaf clipping following the procedure proposed by Kauffman et al. (1973). A total of six plants (12 leaves per rice line) were inoculated with each isolate. After inoculation, a 5-cm-thick layer of water was maintained under the plants to maximize relative humidity. Observations were done 15 days after inoculation. Lesion lengths was measured in centimeters using a graduated ruler. Disease reactions were classified as follows: interactions with an average lesion length ≤ 5 cm were classified as resistant and those > 5 cm were classified as susceptible (Adhikari et al. 1999; Quibod et al. 2016).

Statistical analysis. Mean lesion lengths associated with each isolate were used to study the responses of rice lines to BLB. Isolates were grouped into races or into pathotypes when presenting the same virulence profile on NILs or on the six Malian varieties, respectively. To achieve this, a data matrix associated with the virulence of isolates was generated by noting avirulence as “0” when the lesion length was ≤ 5 and virulence as “1” when the lesion length was > 5 . Lesion length data for each rice line-isolate combination were subjected to two-factor analysis of variance (ANOVA) following the Fisher least significant difference (LSD). The LSD significance level was estimated at 5%. Comparative analyses of lesion means were performed using the Bonferroni test with Genstat software. These data were analyzed with R statistical software to perform hierarchical clustering. Hierarchical clustering on principal components was conducted using the FactoMineR package (Cornillon et al. 2012). Distance chosen was the Euclidean distance and the Ward aggregation method was used. Races (virulence profiles) were described with variables using the desc function. The ComplexHeatmap package was used to build and visualize the various heatmaps generated in this study.

RESULTS

Isolation and Identification of *X. oryzae* pv. *oryzae* in Mali. Field surveys were conducted during the rainy season in three major rice growing areas in Mali (Baguinéda, Office du Niger, and Sélingué). BLB symptoms were observed in almost all visited areas except in Baguinéda, where the disease has not yet been reported.

Between 2010 and 2013, 60 rice leaf samples showing BLB symptoms were collected (Fig. 1). One leaf per sample was processed for bacterial isolation. In total, 41 *X. oryzae* pv. *oryzae* isolates forming pale yellow colonies on PSA medium were isolated and confirmed by multiplex PCR (Table 1). We isolated *X. oryzae* pv. *oryzae* from many pilot rice varieties grown in Mali and from wild species, *O. longistaminata* and *O. barthii* (Table 1).

To confirm their pathogenicity, all isolates were tested on the susceptible rice variety Azucena using a leaf clipping assay. Typical BLB symptoms were visible for all isolates 15 days after inoculation. Lesion lengths caused by the isolates varied from 6 to 50 cm (Fig. 2). Many of the isolates caused complete leaf blight 3 to 4 weeks after inoculation and plant death after 2 months. One-way ANOVA on lesion length revealed a significant difference

Fig. 1. Map of Mali showing the different rice regions visited and the geographical distribution of *Xanthomonas oryzae* pv. *oryzae* races. Diseased leaf samples were collected in the rice growing perimeters of Niono (Office du Niger [ON]) and Sélingué and their location is represented by dots on the map. The race composition of *X. oryzae* pv. *oryzae* populations in these perimeters is reported using shape symbols as described on the map.

among isolates ($P < 0.001$). Overall, symptoms were comparable to those observed with the *X. oryzae* pv. *oryzae* reference strains. Altogether, our data (PCR and pathogenicity testing) confirmed the 41 Malian isolates as *X. oryzae* pv. *oryzae* (Table 1).

Evaluation of the NILs carrying resistance genes. To evaluate the effectiveness of available *Xa* resistance genes, 45 *X. oryzae* pv. *oryzae* isolates were inoculated on 12 NILs (also referred to as IRBB lines), each containing a single BLB resistance gene. The IRBB lines showed differential reactions against the 45 *X. oryzae* pv. *oryzae* isolates (Fig. 3). Lesion length varied from 0 to 35 cm (Fig. 3B). The Bonferroni test, according to which the averages bearing the same letter were not significantly different, confirmed that IRBB lines showed different levels of resistance (Fig. 3B). Similarly, a significant difference was observed among the IRBBs and between the IRBBs and the isolates tested ($P < 0.001$). IR24, the recurrent parent of the NILs, was susceptible to 40 of 45 isolates tested. Most NILs were susceptible to the majority of the isolates (Fig. 3). The hierarchical clustering analysis showed four clusters, with IRBB1 and IRBB3 forming one cluster with high resistance to *X. oryzae* pv. *oryzae* in Mali (Fig. 3A; Table 2). The four most susceptible NILs formed another cluster. The two other clusters consisted of the most differentials NILs (Fig. 3A). None of the IRBB lines showed complete resistance to all of the *X. oryzae* pv. *oryzae* isolates (Fig. 3B and C; Table 2).

Identification of new Malian *X. oryzae* pv. *oryzae* races. The same data set was analyzed for the identification of *X. oryzae* pv. *oryzae* races based on the interactions among isolates and NILs. Forty-five *X. oryzae* pv. *oryzae* isolates were classified into seven

races, named A3 to A9, following a categorical analysis of the lesion length associated with each combination of NIL and *X. oryzae* pv. *oryzae* isolates (Fig. 3A; Table 2). The proportion of different races among the isolates varied from 4 to 29% (for races A7 and A6, respectively) (Table 2). Using an additional NIL carrying *Xa23*, A6 and A5 subdivided into two distinct races, respectively (Table 2). Race A3 was avirulent on all NILs and the recurrent parent IR24. Importantly, strains belonging to race A9 were virulent on all IRBB lines, including IRBB23 (*Xa23*). None of the resistance genes tested could effectively control all of the different races of *X. oryzae* pv. *oryzae* (Table 2). The most effective resistance gene, *Xa3*, controlled six of the seven races. *Xa1* and *Xa4* each controlled four of the seven races. The recessive *xa5* gene controlled the A4 race (Table 2).

The races are described according to the variables that characterize them (Supplementary Table S2). Races A4 and A5 showed significantly lower virulence than the overall mean. Race A5 also showed a significantly higher virulence on IRBB5 and race A7 was significantly less virulent on IRBB1.

To obtain a quantitative representation of the virulence of the isolates belonging to the different races, we plotted side by side the virulence profiles with the race classification of Malian *X. oryzae* pv. *oryzae* isolates (Fig. 3A). Except for race A3, it appeared that there was a low correlation between virulence profile and race classification.

The geographical distribution of the different races of *X. oryzae* pv. *oryzae* in Mali is shown in Table 3. Isolates from Office du Niger were the most diverse, with all of the races present in this region (Table 3). Two predominant races, A5 and A6, were also reported in Sélingué (Table 3).

TABLE 1. List of *Xanthomonas oryzae* pv. *oryzae* strains isolated in Mali between 2010 and 2013 and used in this study

Strain	Region	Site	Year	Host variety	Race	Pathotype
MAI55	Office du Niger	Niono	2010	Adny11	A4	P1
MAI64	Office du Niger	Segou Coura	2010	<i>Oryza longistaminata</i>	A4	P1
MAI65	Office du Niger	Dioro Coura	2010	Adny11	A4	P3
MAI68	Office du Niger	Niono	2010	Huang Huazhon	A4	P3
MAI70	Office du Niger	Niono	2012	Adny11	A6	P1
MAI71	Office du Niger	Niono	2012	Adny11	A6	P8
MAI72	Office du Niger	Niono	2012	Adny11	A5	P3
MAI73	Office du Niger	Niono	2012	Adny11	A5	P4
MAI74	Office du Niger	Niono	2012	Adny11	A5	P1
MAI75	Office du Niger	Niono	2012	Adny11	A5	P1
MAI81	Sélingué	Sélingué	2012	Wassa	A6	P3
MAI83	Sélingué	Sélingué	2012	Wassa	A5	P3
MAI92	Office du Niger	Niono	2012	Adny11	A5	P3
MAI93	Office du Niger	Niono	2012	Adny11	A7	P3
MAI94	Office du Niger	Niono	2012	Adny11	A5	P1
MAI95	Office du Niger	Niono	2012	Adny11	A6	P1
MAI96	Office du Niger	Niono	2012	Adny11	A7	P3
MAI98	Office du Niger	Niono	2012	Adny11	A8	P1
MAI99	Office du Niger	Niono	2012	Adny11	A8	P1
MAI100	Office du Niger	Niono	2012	Adny11	A5	P1
MAI101	Office du Niger	Niono	2012	Adny11	A4	P1
MAI102	Office du Niger	Niono	2012	Adny11	A6	P1
MAI103	Office du Niger	Niono	2012	Adny11	A5	P1
MAI104	Office du Niger	Niono	2012	Adny11	A6	P4
MAI105	Office du Niger	Niono	2012	Adny11	A4	P1
MAI106	Office du Niger	Niono	2012	Adny11	A6	P2
MAI107	Sélingué	Sélingué	2012	Wassa	A6	P4
MAI108	Sélingué	Sélingué	2012	Wassa	A6	P4
MAI127	Office du Niger	Macina 7	2013	Adny11	A9	P6
MAI128	Office du Niger	Macina Niaro	2013	Gambiaka Kokoum	A6	P5
MAI129	Office du Niger	Bewani 1	2013	Adny11	A8	P7
MAI130	Office du Niger	Bewani 2	2013	Adny11	A9	P2
MAI131	Office du Niger	Bewani 3	2013	Adny11	A9	P2
MAI132	Office du Niger	Bewani 3	2013	<i>O. barthii</i>	A9	P7
MAI133	Office du Niger	Bewani 10	2013	Kogoni91-1	A8	P4
MAI134	Office du Niger	Kala 3	2013	<i>O. longistaminata</i>	A3	P1
MAI135	Office du Niger	Kala 3	2013	<i>O. longistaminata</i>	A3	P1
MAI136	Office du Niger	Kala 9	2013	Adny11	A8	P6
MAI145	Office du Niger	Kouroumari	2013	Kogoni91-1	A6	P3
MAI146	Office du Niger	Kouroumari	2013	Kogoni91-1	A6	P3
MAI147	Office du Niger	Kalana	2013	Nionoka	A6	P3

Screening local rice varieties for resistance to BLB.

Considering that none of the resistance genes present in the NILs tested were able to control the different races of *X. oryzae* pv. *oryzae* reported in Mali, we next aimed to identify sources of broad resistance against *X. oryzae* pv. *oryzae* in a panel of 29 local rice varieties (Fig. 4). These varieties were first screened with 14 isolates representative of the most prevalent races (Fig. 4A).

Our data showed a significant difference in virulence among the 14 isolates ($P < 0.001$) (Fig. 4; Supplementary Fig. S1). Different responses were also observed among the varieties (Fig. 4). Overall lesion length data by variety revealed large lesion length variations ranging from 0 to 45 cm (Fig. 4B). Thirteen varieties were susceptible to all *X. oryzae* pv. *oryzae* isolates tested (Fig. 4C). Three (Gigante, Kogoni 91-1, and SK-20-28) were resistant to all 14 isolates (Fig. 4A and C). Thirteen other varieties showed different reactions (resistant or susceptible) to *X. oryzae* pv. *oryzae* (Fig. 4A and C). Among these, three (SK-19-34, SK-95-4, and WAS-203-B) controlled at least 50% of the isolates (Fig. 4C).

Overall lesion length data by isolate highlighted their virulence diversity on the panel of rice varieties tested, with the exception of three isolates that presented the same virulence (Supplementary Fig. S1). The hierarchical clustering dendrogram of this data set containing the 14 isolates was arbitrarily cut into four clusters (C1 to C4) (Fig. 4A). *X. oryzae* pv. *oryzae* MAI100 and MAI105 belonging to cluster (C1) were incompatible on the variety Gambiaka Kokoum. Similarly, *X. oryzae* pv. *oryzae* belonging to cluster C4

was weakly virulent on SK-7-8. Two varieties, Gigante and SK20-28, controlled all of the isolates.

Figure 4A shows the low association among virulence clusters and *X. oryzae* pv. *oryzae* races. The hierarchical clustering tree of rice varieties based on lesion length data was arbitrarily cut into four clusters (R1 to R4) (Fig. 4A). The R2 cluster represented the most susceptible varieties (Gambiaka Kokoum and DM16). Rice variety SK-7-8 alone formed cluster R3 and effectively controlled 5 of 14 isolates. Cluster R4 encompassed 12 varieties mainly susceptible to *X. oryzae* pv. *oryzae* isolates.

The R1 cluster consisted of three subgroups, with one encompassing six varieties with a high level of resistance to *X. oryzae* pv. *oryzae*. These six resistant varieties were subsequently tested with a larger number of *X. oryzae* pv. *oryzae* isolates (Fig. 5). A two-way ANOVA showed a significant difference among the isolates ($P < 0.001$). A significant difference ($P < 0.001$) was also observed between varieties and in the interaction between varieties and isolates. The Bonferroni test confirmed these results. Gigante and SK-20-28 displayed a broad spectrum of resistance (lesion length = 0) against the seven races reported in Mali (Fig. 5B and C; Table 4). The response of both varieties to *X. oryzae* pv. *oryzae* infection was characterized by a rapid necrosis reminiscent of a hypersensitive response. Four varieties showed a high level of resistance by controlling 56 to 91% of the *X. oryzae* pv. *oryzae* isolates in this extended panel, respectively.

Identification of *X. oryzae* pv. *oryzae* pathotypes. Lesion length data aggregated by isolate and variety (45 isolates $\times$ 6

Fig. 2. Virulence of 45 isolates of *Xanthomonas oryzae* pv. *oryzae* on the rice variety Azucena. Overall lesion length data by isolate. The x-axis indicates the *X. oryzae* pv. *oryzae* isolates tested. The y-axis indicates the lesion length (in centimeters) per isolate 14 days after inoculation using the leaf clipping method. Boxes are the box plots of lesion lengths, and lines indicate the median. Data from at least three independent replicates of the experiment with six replicate lesion length values each per treatment (Azucena $\times$ isolate combination) were used to generate the graph. Dots represent the lesion lengths that are outside of the whiskers.

Fig. 3. Efficiency of rice resistance genes (Xa) in near isogenic lines (NILs; IRBB lines) to 45 isolates of *Xanthomonas oryzae* pv. *oryzae*. **A**, Virulence profile clustering of *X. oryzae* pv. *oryzae* isolates on a set of 12 IRBB lines. On the heatmap, each interaction (IRBB lines $\times$ *X. oryzae* pv. *oryzae*; i.e., individual cell colors) codes for the corresponding mean lesion length (LL) across three independent replicates of the experiment with six values each. Races are color coded in the colored bar on the right. Isolates were also clustered according to their quantitative virulence profiles using a hierarchical clustering approach based on pairwise Euclidian distances as displayed in the tree on the left. Susceptibility profiles of the NILs were also clustered using the same method as displayed on the hierarchical tree above the heatmap. **B**, Overall LL data by IRBB line. The x-axis indicates the IRBB lines. The y-axis indicates the LLs (in centimeters) observed 14 days after inoculation by leaf clipping. The displayed data (box plots) correspond to LLs of three repetitions per treatment (combination IRBBs $\times$ isolates). Mean LLs with the same letter (a to l) are not significantly different (Bonferroni test). **C**, Relative frequency of resistance (R, in blue) or susceptibility (S, in pink) reactions against Malian isolates for individual IRBB lines derived from the data set in B. Reactions with an average LL $\leq$ 5 cm are considered resistant and those $>$ 5 cm are considered susceptible.

varieties) are displayed in the heatmap presented in Figure 5A. A clustering analysis was performed on lesion lengths coded as resistant (average lesion length ≤ 5 cm) or susceptible (average lesion length > 5 cm), revealing the existence of eight virulence clusters called pathotypes, noted from P1 to P8 (Fig. 5A; Table 1; Supplementary Table S1). Pathotype P1 was the most abundant (including 20 of 45 isolates) and was controlled by the six varieties tested (Fig. 5A). The other seven pathotypes (P2 to P8) regrouped 25 of 45 isolates and were each virulent on one, two, or three varieties (Fig. 5A). Gigante and SK-20-28 were highly resistant to all races and pathotypes of *X. oryzae* pv. *oryzae* (Fig. 5A; Table 4). Figure 5A illustrates the low level of association among races and pathotypes.

DISCUSSION

Isolates of *X. oryzae* pv. *oryzae* were collected from rice in different regions in Mali and characterized. Our study revealed the existence of seven races among 41 *X. oryzae* pv. *oryzae* isolates. Although *Xa1* and *Xa3* are the most effective resistance genes to control *X. oryzae* pv. *oryzae* in Mali, no single resistance gene is effective against the different races. A new race (A9) is overcoming all of the known resistance genes tested. Two varieties grown in Mali, SK20-28 and Gigante, are broadly resistant to the Malian *X. oryzae* pv. *oryzae* strains.

Virulence of *X. oryzae* pv. *oryzae* is not related to the host plant. Bacteria were predominantly isolated from the high-yielding rice variety, Adny. Adny is the most cultivated variety in Mali and is highly susceptible to BLB. We did not detect any distinct virulence pattern among *X. oryzae* pv. *oryzae* isolates collected from cultivated varieties or from wild rice (*O. barthii* and *O. longistaminata*). Similar results have been observed on the *X. oryzae* pv. *oryzae* population in Asia (Adhikari et al. 1999; Ardales et al. 1996), suggesting that the genetic background of the rice varieties does not significantly affect the virulence of *X. oryzae* pv. *oryzae* strains.

Six new races of *X. oryzae* pv. *oryzae* have been characterized, with *Xa3* being the most efficient resistance gene. Based on their reaction on 12 IRBB lines including IR24, seven races of *X. oryzae* pv. *oryzae* were identified among the isolates collected between 2010 and 2013 in Mali. Race A3 was previously described in two distinct regions in Mali: Kayes and Office du Niger (Gonzalez et al. 2007). Our study confirmed its presence in Office du Niger. Race A3 is totally incompatible with

the NILs and its founder IR24 carrying the resistance gene *Xa18* (Noda et al. 1996). Race A3 might carry an *avr* gene in its genome that would be specifically recognized by *Xa18* and therefore by all of the derivative NILs (Gonzalez et al. 2007). The absence of race A3 in Asia may be attributable to the fact that IR24, the founder of the NILs (IRBB lines), was selected based on its susceptibility to all *X. oryzae* pv. *oryzae* isolates in Japan and the Philippines (Ogawa 1993). Races A1 and A2, previously described in Africa (Gonzalez et al. 2007), were not found in this study. In addition, 10 *X. oryzae* pv. *oryzae* races were described in the Philippines during a 40-year collection period (Quibod et al. 2016; Triplett et al. 2014) and none of these were characterized in Mali. The burst of *X. oryzae* pv. *oryzae* races in Mali, from the single race A3 to six new races in less than 30 years, could be explained by a bias attributable to the very limited number of previously collected and characterized *X. oryzae* pv. *oryzae* strains (Gonzalez et al. 2007). However, the bacterial races tend to evolve continually, influenced by the artificial and natural selection of BLB resistance genes in rice (Vikal and Bhatia 2017). The emergence of novel pathogen populations is a common phenomenon in the agricultural landscape that can be associated with events of selection (Burdon and Thrall 2008). In the Philippines, a major race shift has been observed associated with the introduction of a resistance gene (*Xa4*) in rice (Mew et al. 1992; Quibod et al. 2016). A gene transfer event or a single mutation could also provoke the emergence of new *X. oryzae* pv. *oryzae* races (Ochiai et al. 2005). However, in Mali, no known resistance gene has yet been deployed for the control of BLB and we do not yet know which resistance genes are present in the Malian varieties. Therefore, it is currently difficult to draw conclusions on the evolution of races in Mali in relation to the presence of the resistance gene in cultivated or wild rice.

Race A9 collected on cultivated and wild rice species in three locations of Office du Niger is the most virulent, as it circumvents

TABLE 3. Geographical distribution of races of *Xanthomonas oryzae* pv. *oryzae* in two regions in Mali

Region in Mali	<i>X. oryzae</i> pv. <i>oryzae</i> race (n) ^z							Strains (n)
	A3	A4	A5	A6	A7	A8	A9	
Sélingué	0	0	2	2	0	0	0	4
Office du Niger	5	6	9	10	2	5	4	41

^z The number of strains belonging to each race is indicated.

TABLE 2. Races of *Xanthomonas oryzae* pv. *oryzae* on 12 near isogenic rice lines (NILs) containing a single resistance gene in IR24 genetic background^z

NIL	<i>Xa</i> gene	New <i>X. oryzae</i> pv. <i>oryzae</i> races									Effectiveness of resistance (%)	
		A3	A4	A5		A6		A7	A8	A9	W- <i>Xa23</i>	N- <i>Xa23</i>
				A5-1	A5-2	A6-1	A6-2					
IR 24	<i>Xa18</i>	R	S	S	S	S	S	S	S	S	11	14
IRBB 1	<i>Xa1</i>	R	R	R	R	R	R	S	S	S	67	57
IRBB 3	<i>Xa3</i>	R	R	R	R	R	R	R	R	S	89	86
IRBB 4	<i>Xa4</i>	R	R	R	R	S	S	R	S	S	56	57
IRBB 5	<i>xa5</i>	R	R	S	S	S	S	S	S	S	22	29
IRBB 7	<i>Xa7</i>	R	S	S	S	S	S	S	S	S	11	14
IRBB 8	<i>xa8</i>	R	S	S	S	S	S	S	S	S	11	14
IRBB 10	<i>Xa10</i>	R	S	S	S	S	S	S	S	S	11	14
IRBB 11	<i>Xa11</i>	R	S	S	S	S	S	S	S	S	11	14
IRBB 13	<i>xa13</i>	R	S	S	S	S	S	S	S	S	11	14
IRBB 14	<i>Xa14</i>	R	S	S	S	S	S	S	S	S	11	14
IRBB 21	<i>Xa21</i>	R	S	S	S	S	S	S	S	S	11	14
IRBB 23	<i>Xa23</i>	R	S	S	R	R	S	S	S	S	33	–
Strains with <i>Xa23</i> (n)		5	6	7	3	2	11	2	5	4	–	–
Strains without <i>Xa23</i> (n)		5	6	10		13		2	5	4	–	–

^z Forty-five *X. oryzae* pv. *oryzae* strains (41 strains from this study and four reference strains) cluster into seven races noted A3 to A9. Using *Xa23* in NIL screening, the most dominant and heterogeneous races (A6 and A5) are subdivided into two virulence subclusters. Each race is characterized by its virulence profile on NILs and the number of strains belonging to it is indicated. The ability of *Xa* genes to control different races is displayed. W-*Xa23* = with *Xa23*, N-*Xa23* = without *Xa23*, R = resistant (lesion length ≤ 5 cm), and S = susceptible (lesion length > 5 cm).

Fig. 4. Bacterial leaf blight susceptibility profile of 29 rice varieties cultivated in Mali. **A**, Virulence profile of 14 isolates on the rice varieties tested. Heatmap display of the aggregated lesion length (LL) data by isolate and by variety. For each interaction (rice varieties $\times$ *Xanthomonas oryzae* pv. *oryzae* isolates), individual cell colors code for the corresponding mean LL across three independent replicates of the experiment with six replicates each. Races are color coded in the colored bar on the right. *X. oryzae* pv. *oryzae* strains were also clustered (C1 to C4) according to their quantitative virulence profiles using a hierarchical clustering approach based on pairwise Euclidian distances as displayed in the tree on the left. The susceptibility profiles of the rice varieties were also clustered (R1 to R4) using the same approach as displayed on the hierarchical tree above the heatmap. **B and C**, Efficiency of rice varieties against the 14 isolates tested. The x-axis indicates the rice varieties tested. The y-axis indicates the LLs (in centimeters) observed 15 days after inoculation by leaf clipping (B) or the frequency of resistance reactions (C) per rice variety. B, Overall LL data per rice variety. The displayed data (box plots) correspond to LLs of three repetitions per treatment (rice varieties $\times$ isolates). Mean LLs with the same letter (a to t) are not significantly different (Bonferroni test). C, Frequency of resistance phenotypes among the rice varieties. LLs ≤ 5 cm are considered resistant (R, in blue) and those >5 cm are considered susceptible (S, in pink).

Fig. 5. Virulence profile clustering of Malian *Xanthomonas oryzae* pv. *oryzae* isolates and the resistance level of six rice varieties tested. **A**, Heatmap display of the aggregated lesion length (LL) data by isolate and by variety. For each interaction (rice varieties $\times$ *X. oryzae* pv. *oryzae* isolates), individual cell colors code for the corresponding mean LL across three independent replicates of the experiment with six replicate LL values each. Pathotypes and races, plotted side by side, are color coded in the two colored bars on the right. The LL data on the six rice varieties were clustered as in Figure 4A. Likewise, the six rice varieties are grouped according to their response to infections in the hierarchical tree above the heatmap. **B and C**, The x-axis indicates the rice varieties tested. The y-axis indicates the LLs (in centimeters) observed 15 days after inoculation by leaf clipping (**B**) or frequency of resistance reactions (**C**) per rice variety. **B**, Overall LL data per rice variety. The displayed data (box plots) correspond to aggregated LL data from three independent replicate experiments with six values per treatment (rice varieties $\times$ isolates) and per experiment. Mean LLs with the same letter (a to e) are not significantly different (Bonferroni test). **C**, Percentage of resistance phenotypes by variety. LLs ≤ 5 cm were considered resistant (R, in blue) and those >5 cm were considered susceptible (S, in pink).

TABLE 4. Characterization of eight pathotypes among strains of *Xanthomonas oryzae* pv. *oryzae* in Mali^z

Rice line	Pathotype (n)								Resistance (%)	
	P1	P2	P3	P4	P5	P6	P7	P8	Pathotypes	Strains
SK-20-28	R	R	R	R	R	R	R	R	100	100
Gigante	R	R	R	R	R	R	R	R	100	100
Kogoni 91-1	R	S	R	R	S	S	S	R	50	80
SK-19-34	R	R	R	R	S	R	S	S	62.5	91
SK-95-4	R	R	R	S	R	S	R	S	62.5	84
WAS 203-B	R	R	S	S	R	S	S	S	37.5	56
Azucena	S	S	S	S	S	S	S	S	0	0
Strains (n)	20	4	11	4	1	2	2	1	–	–

^z The pathotypes are noted P1 to P8. The rice variety Azucena was used as a susceptible control. Each pathotype is characterized by its virulence profile on six rice varieties and the number of strains that belongs to it is indicated. The effectiveness of rice varieties to control pathotypes is indicated. R = resistant (lesion length ≤5 cm), and S = susceptible (lesion length >5 cm).

all of the *Xa* genes tested. The dissemination of A9 represents a major risk for rice production in Mali. The ability of *X. oryzae* pv. *oryzae* strains to overcome all *Xa* genes has been reported in different countries by several authors (India, Nepal, and Sri Lanka), arguing for the need to identify new sources of resistance against BLB in targeted areas (Adhikari et al. 1999; Mishra et al. 2013; Triplett et al. 2014). This is also a major challenge for the control of BLB in Mali.

The geographical location of the *X. oryzae* pv. *oryzae* races in Mali shows that Office du Niger has seven races and Sélingué has two. Rice cultivation at Office du Niger began decades before Sélingué and recently intensified. From our discussions with local rice producers during sample collection, the disease appeared with rice cultivation in Sélingué. It is possible that the two predominant races present in Office du Niger (A5 and A6) were disseminated to other regions likely through seed or plant exchanges.

The characterization of races of *X. oryzae* pv. *oryzae* in Mali will inform breeders on the use and development of effective resistance genes in rice varieties (Jeung et al. 2006; Khoshkdamani et al. 2012). *Xa3* and *Xa1* are potentially the most effective resistance genes for controlling the different races described in Mali, although they are not effective for controlling the *X. oryzae* pv. *oryzae* population in the Philippines or in Sri Lanka (Triplett et al. 2014). In Japan, *Xa1* confers a highly specific resistance to race 1 that is widespread in rice fields over the country. In Mali, Sélingué and Office du Niger represent two distinct agroecological regions with an irrigated rice system. Farmers often are supplied with the same seed distributors and, in practice, there is no measure to prevent seed or seedling exchanges between them. Thus, although *Xa1* and *Xa3* each control the two races reported in Sélingué, the deployment of specific resistance genes in one region or the other without taking into account the global structure of the *X. oryzae* pv. *oryzae* population (races) in Mali is not recommended. However, pyramiding resistance genes in a cultivated elite rice line could be an effective solution to control BLB in Mali. Indeed, several authors have reported that the combination of *Xa* genes results in a synergistic effect on the resistance spectrum of rice to BLB (Khoshkdamani et al. 2012; Mishra et al. 2013). Combinations of *Xa1*, *Xa3*, and *Xa4* in elite lines could bring significant results to control BLB in relevant areas of rice production in Mali. Bacterial blight resistance gene *Xa23* has been identified from *O. rufipogon* (Wang et al. 2015). Its transcription is specifically activated by the TALE *AvrXa23* (Zhou et al. 2011). *Xa23* will not be effective alone but could be used in combination with *Xa1* or *Xa3* to control most of the races characterized in Mali thus far.

Varieties SK20-28 and Gigante display broad resistance against *X. oryzae* pv. *oryzae*. Most of the rice varieties widely cultivated by farmers in Mali are susceptible to *X. oryzae* pv. *oryzae*, albeit to variable levels. Six varieties are highly resistant, with SK20-28 and Gigante being the most promising. SK20-28 was

selected from the Gigante parent as part of the national research program against rice yellow mottle virus in Mali. Rice Yellow Mottle Virus 1 (RYMV1) has been identified and characterized in Gigante (Albar et al. 2003; Oludare et al. 2016). SK20-28 does not carry RYMV1, suggesting that resistance to BLB is independent of the RYMV1 gene. SK20-28 and Gigante may possibly carry a new resistance gene(s) against BLB or a natural variation of known *Xa* genes that confer a broad spectrum of resistance against the Malian *X. oryzae* pv. *oryzae* population. Characterization of the gene(s) responsible for SK20-28 and Gigante resistance needs to be further investigated.

In conclusion, the *X. oryzae* pv. *oryzae* population is divided into seven races and eight pathotypes and is geographically diverse. This information should inform breeders on resistance genes to be deployed in Mali. Pyramiding resistance genes should be an effective solution to control the different races characterized thus far in Mali. In addition, two rice varieties displaying broad-spectrum resistance to the *X. oryzae* pv. *oryzae* population were identified. Further characterization, use, and transfer of this resistance in high-yielding varieties should significantly improve rice productivity and BLB control in Mali.

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