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Genetics and Genomics of Resistance

Characterization of Blast Resistance in a Diverse Rice Panel from Sub-Saharan Africa

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Abstract

There is a recent unparalleled increase in demand for rice in sub-Saharan Africa, yet its production is affected by blast disease. Characterization of blast resistance in adapted African rice cultivars can provide important information to guide growers and rice breeders. We used molecular markers for known blast resistance genes (*Pi* genes; $n = 21$) to group African rice genotypes ($n = 240$) into similarity clusters. We then used greenhouse-based assays to challenge representative rice genotypes ($n = 56$) with African isolates ($n = 8$) of *Magnaporthe oryzae* which varied in virulence and genetic lineage. The markers grouped rice cultivars into five blast resistance clusters (BRC) which differed in foliar disease severity. Using stepwise regression, we found that the *Pi* genes associated with reduced blast severity were *Pi50* and *Pi65,* whereas *Pik-p, Piz-t,* and *Pik* were associated with increased susceptibility. All rice genotypes in the most resistant cluster, BRC 4, possessed *Pi50* and *Pi65*, the only genes that were significantly associated with reduced foliar blast severity. Cultivar IRAT109, which contains *Piz-t*, was resistant against seven African *M. oryzae* isolates, whereas ARICA 17 was susceptible to eight isolates. The popular Basmati 217 and Basmati 370 were among the most susceptible genotypes. These findings indicate that most tested genes were not effective against African blast pathogen collections. Pyramiding genes in the *Pi2/9* multifamily blast resistance cluster on chromosome 6 and *Pi65* on chromosome 11 could confer broad-spectrum resistance capabilities. To gain further insights into genomic regions associated with blast resistance, gene mapping could be conducted with resident blast pathogen collections.

Keywords: Africa, characterization, *Magnaporthe oryzae*, rice blast resistance, rice diversity

Rice is a staple food for more than half of the world's human population (Muthayya et al. 2014). With a per capita consumption that has doubled since 1970, rice is now a major staple food in sub-Saharan Africa (SSA). Rice blast disease is one of the major biotic constraints to attaining self-sufficiency in rice production in SSA, causing significant damage at all stages of crop development. It has been estimated that rice blast disease destroys enough rice to feed 60 million people annually (Dean et al. 2005). In Africa, blast has been reported in all rice growing areas, and causes up to 100% grain yield losses across different countries (Mutiga et al. 2021). Adoption of sustainable blast management strategies, such as the use of durably resistant cultivars would enhance the expansion of rice cultivation and productivity, hence, boosting food security in SSA.

Blast is caused by *Magnaporthe oryzae* (synonym of *Pyricularia oryzae*) (Zhang et al. 2016), a devastating hemibiotrophic pathogen characterized by a wide distribution in rice growing areas, rapid spread by crop debris, pathogen-infected seed, storms, water, and wind. Blast disease is also particularly serious when fields are over-fertilized with nitrogen (Asibi et al. 2019; Osuna-Canizalez et al. 1991; Sharma et al. 2012). The disease can be controlled using fungicides, but the associated cost is prohibitive for small-scale rice growers in SSA. Growing blast-resistant rice cultivars has been used as a sustainable disease control strategy (Ning et al. 2020). Blast resistance is based on the gene-for-gene model (Flor 1971) with many dominant disease resistance loci having been identified. Extensive blast resistance characterization and gene mapping have been conducted in other continents, but there are limited efforts to characterize and/or map blast resistance in SSA (Séré et al. 2013; Singh et al. 2000). Only in the last decade have there been efforts to characterize blast resistance in native African rice (*Oryza glaberrima*) and in inter-specific hybrid backgrounds such as the New Rice

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for Africa (NERICA) cultivars (Mutiga et al. 2017; Odjo et al. 2017). As a consequence, there is very little information regarding genes for blast resistance based on studies that utilize African blast pathogen populations (Mgonja et al. 2016, 2017).

Approximately 100 blast resistance genes have been mapped in different rice cultivars globally using *M. oryzae* pathogen from different parts of the world (Miah et al. 2013). Of the currently identified blast resistance genes (*Pi* genes), one-third ($n = 31$) have been cloned to date, and the majority ($n = 28$) encode proteins with classical nucleotide binding site–leucine-rich repeat (NBS–LRR) domains (Bai et al. 2002; DeYoung and Innes 2006; Ning et al. 2020). Pathotyping analysis shows that some of the genes confer broad-spectrum resistance (e.g., *Pi-1*(*t*), *Pi2*, *Pi9*, *Pi20*(*t*), *Pi27*(*t*), *Pi39*(*t*), *Pi40*(*t*), and *Pikh*) (Jeung et al. 2007, Li et al. 2008, Liu et al. 2002, 2007; Zhu et al. 2004), whereas others show narrower race-specific forms of resistance (e.g., *Pia*, *Pib*, *Pii*, *Pi-km*, *Pi-t*, *Pi12*(*t*), and *Pi19*(*t*)) (Ashikawa et al. 2008; Hayashi and Yoshida 2009; Yang et al. 2008). Except for *pi21*, which is a recessive gene, the rest of the characterized genes are dominant loci (Fukuoka and Okuno 2001). Pathotyping has shown that some cultivars (the *Pi9* donor 75-1-127, for example), which carry some of the major blast resistance genes, are resistant to a wide collection of blast pathogens from SSA (Mutiga et al. 2017). It remains unclear, however, whether genes conferring blast resistance in rice cultivars from SSA utilize the same resistance mechanisms or are the same as those reported in other parts of the world.

Knowledge of the most effective rice blast resistance genes can enable plant breeders to design more robust strategies to overcome the virulence spectra of prevailing populations of *M. oryzae* in SSA. Additionally, knowledge of cognate effectors among the pathogen population would enable breeders to determine which *Pi* genes to pyramid or deploy in locally adapted rice cultivars (Leach et al. 2001; Pietravalle et al. 2006). Because the evolving pathogen population will develop mechanisms to overcome the resistance spectra of existing resistant cultivars, there is also a need for breeders to continuously introduce new resistance alleles and characterize them in parallel with pathogen surveillance programs (Nelson et al. 2018). To characterize the virulence spectra of the resident pathogen population, rice differential lines (hereinafter referred to as international rice blast lines or IRBLs), which carry known individual blast resistance genes were developed by the International Rice Research Institute (IRRI) (Kobayashi et al. 2007; Telebanco-Yanoria et al. 2010). Resistance profiles of available rice germplasm can be characterized by inoculating with isolates of known pathogen races (Zhang et al. 2017). Notwithstanding the inability to identify functional genes, PCR tests could be conducted to identify the presence of known resistance genes within a rice germplasm collection (Imam et al. 2014). With information on the genes being publicly available, molecular markers can, therefore, be used to characterize resistance profiles of any available rice germplasm for use in subsequent breeding programs.

In a case where resistance has not been characterized, new genes could be mapped using diverse rice populations in a genome-wide association analysis, followed by the identification of novel resistance loci (Nelson et al. 2018). We reasoned that there is considerable value in the identification of resistance using resident blast pathogen collections in SSA, and this was the main goal for establishing a biobank at the Biosciences facility of the International Livestock Research Institute with collections of isolates of *M. oryzae* isolates from across Africa to aid in the identification of rice resistance using resident blast pathogen (Mutiga et al. 2021). Assessments of disease reactions when the local germplasm is inoculated with blast pathogen isolates representative of the prevailing pathogen population, therefore, provide a reliable measure of the resistance spectra of contemporary rice genotypes and the corresponding virulence spectrum of the *M. oryzae* populations. A breeding pipeline that involves the characterization of new collections of *M. oryzae* and rice germplasm to enhance the tracking of pathogen virulence spectra and to develop durably resistant rice cultivars for SSA was also established based on this information (Mutiga et al. 2021).

This study aimed to validate the utility of the *M. oryzae* SSA biobank at the ILRI Biosciences as a means of screening for blast resistance to the SSA blast population under controlled conditions. The objectives of this study were to assess the frequency of known blast resistance genes and their association with the disease reaction phenotype in a diverse collection of African rice germplasm, assess the potential of known individual or combinations of blast resistance genes, and then evaluate blast resistance profiles in popular rice cultivars of Africa. In this way, we set out to assess the utility of genetically distinct isolates of *M. oryzae* for the evaluation of blast resistance in rice germplasm of SSA and to use this information to guide future rice blast resistance breeding programs.

Materials and Methods

Rice germplasm

The rice panel ($n = 240$) consisted of collections of landraces from East Africa ($n = 42$) and West Africa ($n = 16$), improved African cultivars of Advanced Rice for Africa (ARICA, $n = 16$), hybrid varieties of New Rice for Africa (NERICA, $n = 6$), Alliance fora Green Revolution in Africa (AGRA, $n = 6$), Bayer Crop Science ($n = 2$), Korea-Africa Food and Agriculture Cooperation Initiative (KAFACI, $n = 121$), and monogenic lines carrying rice blast resistance genes ($n = 25$), as well as some international cultivars ($n = 6$) (Supplementary Table S1). Except for ARICA seeds, which were kindly provided by AfricaRice Center, Bouaké, Côte d'Ivoire, the rest of the germplasm was provided by the Kenya Agricultural and Livestock Research Organization (KALRO) and IRRI within a collaboration in the Durable Rice Blast Resistance for sub-Saharan Africa project, coordinated by The Sainsbury Laboratory, the University of East Anglia in the United Kingdom.

Genomic DNA extraction

Genomic DNA was extracted from approximately 100 mg of leaf tissue of 10-day-old rice seedlings using the CTAB method (Saghai-Maroof et al. 1984). DNA quality and quantity were analyzed using 0.8% agarose gel electrophoresis and a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Genotyping of the rice blast *R* genes

Purified genomic DNA was used in the amplification of 21 PCR markers linked to, or representing, functional alleles for rice blast resistance loci (Supplementary Table S2). PCR amplification was performed in a total volume of 10 μl containing AccuPower PCR Master Mix with dye (Bioneer, Korea), 0.1 μM each primer, and 25 ng of template DNA. Amplification was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, U.S.A.) using the following PCR program: an initial denaturation step at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at a specific temperature for 1 min (Table 2), and extension at 72°C for 1 min; and then a final extension step at 72°C for 10 min. The PCR amplicons were size fractionated in a 2% agarose gel stained with 0.25× GelRed (Biotium, U.S.A.) run at 7 V/cm for 45 min in 1× Tris TBE buffer. Gels were visualized under UV light using the UVP GelDoc-It Imaging System (Analytik Jena GmbH, Upland, CA, U.S.A.). Alleles were scored as presence (1) or absence (0). The lines containing *Pi50* were subsequently retested using *Pi2/9F3/R4* primer pair, which distinguishes among haplotypes of the genes within the the *Pi2/9* cluster of chromosome 6 (Olukayode et al. 2019; Xiao et al. 2020). Amplicons were purified according to the Qiagen QIAquick PCR Purification Kit (Hilden, Germany) protocol and DNA sequenced at Macrogen, Netherlands. Sequenced DNA fragments were subsequently used for bioinformatics analyses to identify the individual *R* genes.

TABLE 1. Description of the rice panel ($n = 56$) that was inoculated with *Magnaporthe oryzae* in this study

Lab name	Cultivar name	Pedigree	BRC	Origin	Description
EA 33	08FAN10	GSR-CAAS	2	KALRO	Early maturing and high yielding
I 38	75-1-127	WHD-1S-75-1-127	4	IRRI	Broad spectrum resistance to blast conferred by *Pi9* gene
A 7	ARICA 01	WAB 2094-WAC2-TGR 2-B	3	Africa Rice Center	High yielding; recommended for rainfed lowlands in Mali
A 8	ARICA 02	WAB-2056-2-FKR 2-5-TGR1-B	1	Africa Rice Center	High yielding; recommended for rainfed lowlands in Mali and Nigeria
A 9	ARICA 03	WAB 2076-WAC1-TGR 1-B	1	Africa Rice Center	High yielding; recommended for rainfed lowlands in Mali and Nigeria
A 10	ARICA 04	ART3-11-L1P1-B-B-2	1	Africa Rice Center	Drought tolerant; recommended for uplands in Uganda
A 11	ARICA 05	WAB 95-B-B-40-HB	1	Africa Rice Center	Drought tolerant; recommended for uplands in Uganda
A 12	ARICA 06	IR75887-1-3-WAB1	2	Africa Rice Center	Tolerant to iron toxicity; recommended for lowlands in Côte d'Ivoire and Ghana
A 13	ARICA 07	WAS 21-B-B-20-4-3-3	1	Africa Rice Center	Tolerant to iron toxicity; recommended for irrigated lowlands in Burkina Faso, Côte d'Ivoire and Ghana
A 14	ARICA 08	WAT 1046-B-43-2-2-2	2	Africa Rice Center	Tolerant to iron toxicity; recommended for irrigated lowlands in Burkina Faso and Côte d'Ivoire
A 15	ARICA 09	SIM2 SUMADEL	1	Africa Rice Center	Cold tolerant; recommended for irrigated lowlands of Mali and Senegal
A 16	ARICA 10	WAS 200-B-B-1-1-1	2	Africa Rice Center	Cold tolerant; recommended for irrigated lowlands of Mali and Senegal
A 17	ARICA 11	IR 63275-B-1-1-1-3-3-2	1	Africa Rice Center	Salinity tolerant; recommended for mangrove ecology of Gambia
A 18	ARICA 14	ART15-11-8-5-2-B-1	1	Africa Rice Center	Drought tolerant; recommended for uplands in Côte d'Ivoire
A 19	ARICA 15	WAB 881-SG-12	1	Africa Rice Center	Drought tolerant; recommended for uplands in Côte d'Ivoire
A 20	ARICA 16	CNAX 3031-78-2-1-1	2	Africa Rice Center	Drought tolerant; recommended for uplands in Benin, Burkina Faso, and Côte d'Ivoire
A 21	ARICA 17	Scrid017-1-4-4-4-1	3	Africa Rice Center	Cold tolerant; recommended for irrigated highlands of Ethiopia
A 22	ARICA 18	FAROX 521-83-H1	1	Africa Rice Center	High yielding; recommended for rainfed lowlands of Côte d'Ivoire
H 35	ARIZE 6444	KEH10004	3	KALRO	High yielding hybrid
H 36	ARIZE TEJ Gold	KEH10005	3	KALRO	High yielding hybrid
EA 24	Basmati 217	NIBAM 11	3	KALRO	Aromatic indica; highly preferred by consumers in East Africa; susceptible to blast
EA 26	Basmati 370	NIBAM 10	3	KALRO	Aromatic indica; highly preferred by consumers in East Africa; susceptible to blast
EA 23	BW196	NIBAM 109	5	KALRO	Tolerant to multiple stresses; field evaluation showed moderate resistance to blast in Kenya
WA 207	IRAT 109	IRAT 109	4	KALRO	Tropical japonica; tolerant to drought
WA 195	ITA310	NIBAM 110	5	KALRO	High yielding; highly susceptible to blast
K 157	K2-9	K2-9	3	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 128	KAFAGRA 8	KAFAGRA 8	2	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
WA 206	Kamenoo	NSFTV83	3	INERA	Tolerant to stress
AGRA 3	MWUR-3	MWUR-3	1	KALRO	Drought tolerant, early, and good grain quality
N 194	NERICA 1	WAB 56-104 / CG 14//2*WAB 56-104	3	KALRO	Interspecific cultivar; early maturing and tolerant to stress
N 191	NERICA 10	WAB 56-104 / CG 14//2*WAB 56-104	4	KALRO	Interspecific cultivar; early maturing and tolerant to stress
N 192	NERICA 11	WAB 56-104 / CG 14//2*WAB 56-104	4	KALRO	Interspecific cultivar; early maturing and tolerant to stress
N 193	NERICA 12	WAB 56-50 / CG 14//2*WAB 56-50	3	KALRO	Interspecific cultivar; early maturing and tolerant to stress
N 190	NERICA 2	WAB 56-104 / CG 14//2*WAB 56-104	3	KALRO	Interspecific cultivar; early maturing and tolerant to stress
AGRA 1	PAN 84	PAN 84	1	KALRO	High yielding, drought-blast-cold tolerant
K 74	PBR1000922-3	PBR1000922-3	1	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
AGRA 4	R-1081	R-1081	2	KALRO	Good grain quality, early and high yielding, developed using TGMS
EA 30	R-3054	R-3054	3	KALRO	Good grain quality, early and high yielding, developed using TGMS
WA 209	SAHEL 210	SAHEL 210	3	KALRO	An indica with good grain quality, high yielding
EA 25	SARO 5	SARO 5	3	KALRO	High yielding aromatic rice variety
EA 34	Silewah	Silewah	2	KALRO	Tolerant to cold weather conditions
K 131	SR33705F2-67-1-1-HV-1-1	SR33705F2-67-1-1-HV-1-1	4	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 133	SR34053(#5-52)-1-4-2-10-1-2	SR34053(#5-52)-1-4-2-10-1-2	2	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 97	SR34054-1-21-4-1-3-3	SR34054-1-21-4-1-3-3	2	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 82	SR34598-HB-8-HV-1	SR34598-HB-8-HV-1	2	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 138	SR35230-1-12-1-3	SR35230-1-12-1-3	1	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 104	SR35230-2-9-2-2	SR35230-2-9-2-2	3	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 84	SR35250-1-23-2-1	SR35250-1-23-2-1	5	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 141	SR35250-2-19-3-1	SR35250-2-19-3-1	5	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 109	SR35266-2-4-4-1	SR35266-2-4-4-1	5	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 188	SR35266-2-6-1-1	SR35266-2-6-1-1	3	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 120	SR35266-3-3-5-1	SR35266-3-3-5-1	5	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 152	SR35278-1-9-1-2	SR35278-1-9-1-2	4	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
K 155	SR35300-1-HV-1-2	SR35300-1-HV-1-2	4	KALRO	Breeding line; segregating for blast tolerance, cold, salinity, yield, maturity
WA 197	WAB96-1-1	ITA 257/YS 121	4	INERA	Improved Japonica cultivar; earlier studies showed resistance to blast
WA 199	WITA 3	11975/IR 13146-45-2-3	4	INERA	Improved indica cultivar; tolerant to iron toxicity; earlier studies showed resistance to blast

Phylogeny of the rice genotypes and identification of representative lines for inoculation

Genetic similarity of the rice genotypes was established based on the data which revealed the presence (1) or absence (0) of alleles for individual markers for blast resistance. Binary data for all genotypes was used to generate a phylogenetic tree based on hierarchical clustering using the Adegenet package in R (Jombart 2008). The main genetic lineages of the rice genotypes (clades) were assigned to five identifiers called "rice blast gene cluster groups or RBCs" from which representative rice genotypes were randomly selected for greenhouse-based inoculation with a genetically diverse set of blast pathogen isolates.

Inoculation of rice using a diverse panel of *magnaporthe oryzae*

A subset of rice genotypes ($n = 56$) was inoculated with eight isolates of *M. oryzae* which represented the diversity of virulence from pathogen collections from nine countries in SSA (Mutiga et al. 2017). The eight isolates represented members of the seven genetically distinct lineages which differed in virulence within the tested panel (Mutiga et al. 2017). Isolates included those from Kenya ($n = 4$), Tanzania ($n = 2$), Benin ($n = 1$), and Nigeria ($n = 1$). The isolates are stored at –20°C in dry filter papers in a Biobank at the ILRI Biosciences Facility (Mutiga et al. 2021).

Preparation of fungal inoculum

Fungal cultures were grown in rice bran agar (RBA) media and stored at 20°C on dry filter papers in 2-ml plastic vials within the ILRI Biobank (Mutiga et al. 2021). Inoculum was multiplied by inducing sporulation in RBA under direct white light for 18 days. Conidia were then washed from the surface of plate cultures using 0.2% Tween 20 solution and suspensions normalized to a concentration of 2×10^5 conidia/ml using a hemocytometer (Paul Marienfeld GmbH & Co. KG, Am Wöllerspfad 497922 Lauda-Königshofen, Germany).

Fungal inoculation and evaluation of the rice panel

We assessed the disease reaction using eight genetically diverse isolates of *M. oryzae* against rice genotypes ($n = 56$) which represented lineages of the entire rice panel that had been previously genotyped for the presence of blast resistance genes, ARICA cultivars ($n = 16$), and checks (resistant: BW96 and 75-1-127; susceptible: NIBAM110, NIBAM111, and ITA310; based on the disease reactions previously observed during field trials in Kenya and also published data for inoculations with some of the isolates from SSA) as shown in Table 1 and also reported earlier (Mutiga et al. 2017). We chose to test the disease responses of all the ARICA lines because the panel has been identified as a very promising and superior group of cultivars based on nominations for performance in specific rice-growing countries of Africa. Rice lines were inoculated with the blast pathogen under controlled temperature and humidity conditions, as described earlier, with some modifications (Mutiga et al. 2017). Briefly, the rice panel ($n = 56$) was grown in rectangular vented nursery trays; each of which was planted with 28 genotypes in a greenhouse (temperature range 19 to 26°C) for 21 days before being inoculated with individual *M. oryzae* isolates. Each rice genotype was represented by 10 to 15 seedlings, which were sown in a tray containing sterile forest soil mixed with animal manure. The conditions of growth (with variations in light, temperature, and soil types) for rice were first optimized in pilot studies within the ILRI greenhouse environment. Seedlings were inoculated with fungal conidial suspensions at a concentration of 2×10^5 conidia/ml^{-1} using a handheld plastic sprayer. Inoculated seedlings were incubated in a dew chamber (relative humidity ≈100%; temperature 21 to 25°C) for 48 h prior to turning off humidifiers and resumption of ambient conditions of the greenhouse. Foliar blast symptoms were evaluated at 7 days after inoculation on a 0 to 9 visual score scale, where 0 = no visible damage, 1 to 3 = varying degrees of hypersensitive reaction, and 4 to 9 = varying degrees of blast severity (IRRI 2013). Test results with contrasting disease reactions were identified in the first two tests, and the rice genotype-isolate sets were retested in subsequent experiments.

TABLE 2. Frequency of *R* genes in the genotyped African rice population ($n = 240$)

Cluster Gene/cultivars (*n*)	Frequency of *R* genes[y] 1 (32)	2 (64)	3 (78)	4 (42)	5 (24)	Overall (240)	PIC[z]
Pi50	21.9	0.0	14.1	100.0	37.5	28.8	0.92
Piz	53.1	73.4	91.0	90.5	95.8	81.7	0.33
Pita	43.8	6.3	93.6	50.0	50.0	51.7	0.73
Pii	50.0	45.3	46.2	50.0	54.2	47.9	0.77
Pik	12.5	100.0	96.2	97.6	87.5	85.4	0.27
Pik-p	31.3	20.3	35.9	14.3	0.0	23.8	0.94
Pi65	84.4	93.8	97.4	100.0	95.8	95.0	0.10
pi21	87.5	100.0	98.7	97.6	91.7	96.7	0.07
Pi2	81.3	92.2	98.7	100.0	95.8	94.6	0.11
Pikm	100.0	93.8	85.9	95.2	50.0	87.9	0.23
Pi5	100.0	100.0	100.0	100.0	87.5	98.8	0.02
Pi1	96.9	100.0	100.0	95.2	91.7	97.9	0.04
Pi37	96.9	98.4	100.0	100.0	87.5	97.9	0.04
Pi56	93.8	100.0	98.7	100.0	87.5	97.5	0.05
Pish	100.0	98.4	98.7	92.9	79.2	95.8	0.08
Pi33	96.9	92.2	97.4	97.6	87.5	95.0	0.10
Pia	93.8	82.8	100.0	97.6	91.7	93.3	0.13
Pi25	100.0	98.4	97.4	100.0	0.0	88.8	0.21
Pib	84.4	84.4	88.5	76.2	79.2	83.8	0.30
Pid2	100.0	98.4	100.0	97.6	95.8	98.8	0.02
Pikh	100.0	100.0	94.9	100.0	91.7	97.5	0.05

[y] *R* genes is the blast resistance genes or *Pi* genes that were tested using specific markers in a conventional polymeric chain reaction.
[z] PIC refers to polymorphic information content.

Data analysis

Phylogenetic analysis was conducted using binary responses based on the amplification of *R* genes. The resulting dendrogram provided genetic lineages or major clades which were used in the identification of representative rice genotypes for subsequent inoculation with eight isolates of *M. oryzae*. Frequencies of amplification of the 21 *R* gene markers within the entire rice panel were computed in Microsoft Excel. Tests for statistical association between markers, marker groups, isolates, and rice cultivars and the disease reactions were implemented in JMP Pro ver. 16 (SAS Institute Inc., Cary, NC, 1989–2021). Means of disease severity were compared using Tukey's HSD ($\alpha = 0.05$). Mosaic plots of binary disease reactions were based on binning disease severity into resistance category (ds = 0 to 3 were scored as resistant and coded as 0) and susceptible reaction (ds = 4 to 9 coded as 1).

Results

A panel of rice cultivars from SSA ($n = 240$) was grouped into five blast resistance clusters (BRC) using PCR markers for known *Pi* genes ($n = 21$) (Fig. 1). The markers' polymorphic information content (PIC) ranged from 0.02 to 0.94, with the most distinctive being *Pik-P* (0.94), *Pi50* (0.92), *Pii* (0.77), *Pita* (0.73), and *Piz* (0.33) (Table 2). The number of rice genotypes within each BRC ranged from 24 to 78. The lowest *Pi* gene frequencies were observed for *Pik-P* (24%), *Pi50* (29%), and *Pii* (48%). The rest of the *Pi* genes had frequencies ranging from 52% (*Pita*) to 99% (*Pid-2* and (*Pi5*) (Table 2). All members of BRC 1 possessed *Pik-m*, *Pi5*, *Pish*, *Pi25*, *Pid2*, and *Pik-h* resistance loci. BRC 2 included all known R genes being present in some members, except for *Pi50* which was not detected in any member. BRC 3 contained 14% genotypes with *Pi50,* and all members had *Pi5*, *Pi1*, *Pi37*, *Pia*, and *Pid-2*. All members of BRC 4 had *Pi50*, *Pia*, *Pi65*, and *Pikh*. BRC 5 lacked *Pik-p* and *Pi25* and no individual gene was common to all members

(Table 2). We conclude that there is considerable variability in *Pi* gene profile among the most grown rice cultivars in SSA.

A subset ($n = 56$) of rice cultivars representing the five BRCs were then challenged with eight isolates of *M. oryzae* from SSA that are representative of the virulence spectrum of the prevailing pathogen population (Tables 2 and 3). The percentages of rice cultivars from individual blast resistance clusters (BRCs) that were inoculated with the eight isolates were as follows: BRC 1 (44%), BRC 2 (17%), BRC 3 (21%), BRC 4 (19%), and BRC 5 (25%). The inoculated rice cultivars did not include the IRBL set as these had been tested in our earlier study. All ARICA cultivars were inoculated because they represented novel germplasm of interest in regional rice development, and their resistance spectra had not been previously evaluated using the set of *M. oryzae* isolates used in our panel. We tested for the effects of isolate, cultivar, and BRC on blast severity in a standard least square regression model (Table 4). This revealed that isolates, BRC, and cultivars within a BRC, all had significant effects on disease severity ($P < 0.0001$). The isolates attacked at least 40% and at most 84% of all tested rice cultivars (Table 3). Overall, the mean blast severity caused by individual isolates on the inoculated rice panel ranged from 1.8 to 4.2. Half the isolates showed a moderate degree of foliar blast severity against the cultivars with a mean disease severity score higher than 3. The Kenyan isolate KE0215 was the most aggressive (foliar blast severity score 4.2 ± 0.25) followed by a Nigerian isolate NG0026 (3.3 ± 0.26) and a Tanzanian isolate TZ0077 (3.3 ± 0.26). The

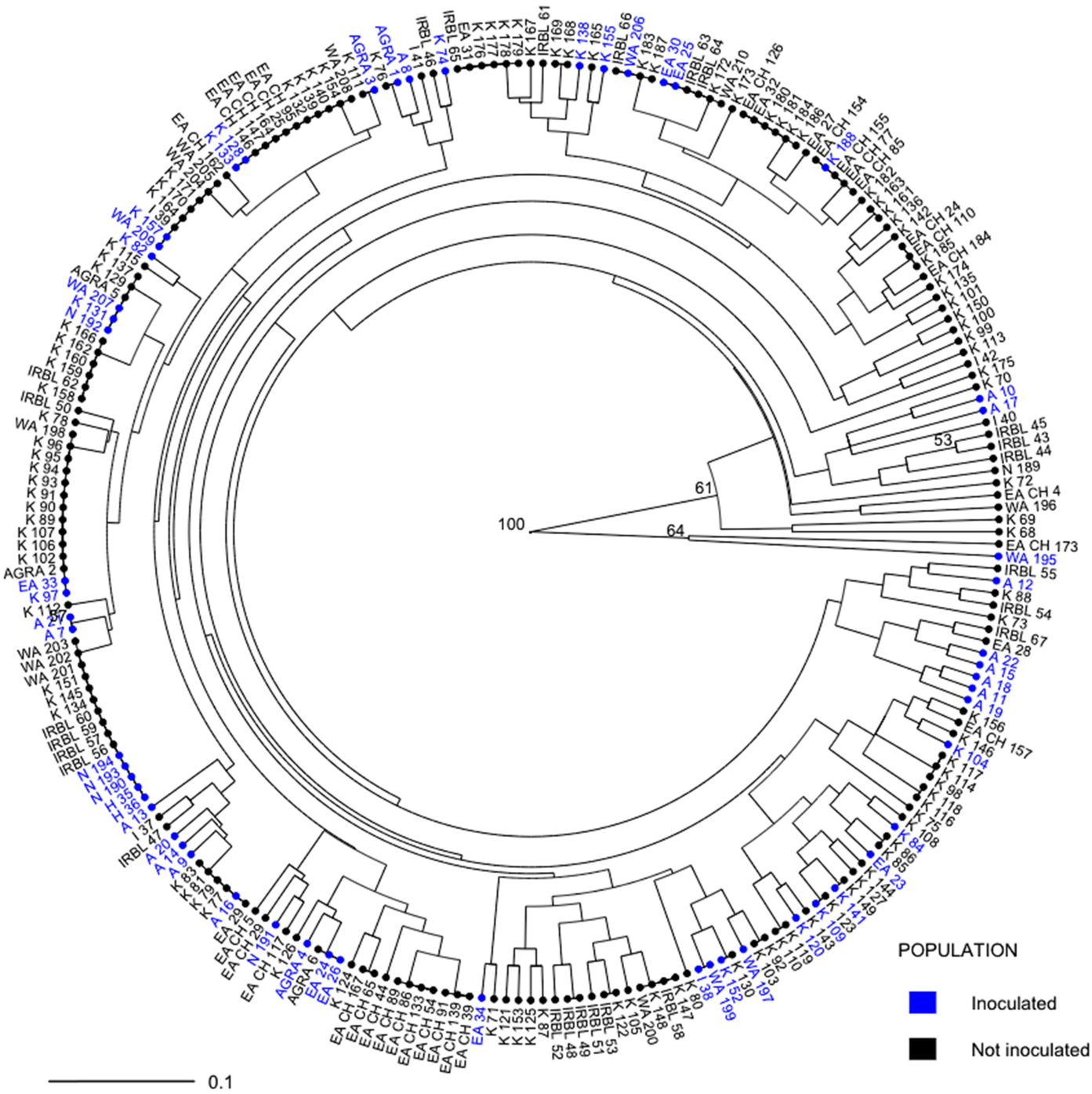

Fig. 1. Phylogenetic tree based on the presence of blast resistance genes in rice cultivars ($n = 240$) used in this study. Rice genotypes with blue highlights were inoculated with *Magnaporthe oryzae*, whereas those in black were not inoculated. Bootstrap values above 50% are indicated above the branches.

overall mean disease severity score for the remaining isolates was below 3, and these isolates could not attack most of the rice cultivars tested (Table 3). The proportions of *M. oryzae* isolates that attacked rice cultivars in different BRCs were as follows: BRC 4 had the least proportion of isolates (53%), followed by BRC 1 (65%), then BRC 5 (71%) and BRC 2 and 3 (72%) (Table 5).

The inoculated panel rice genotypes were susceptible to 0 to 8 isolates with mean disease severity ranging from 1.8 to 7.2 (Figs. 2 and 3). Based on foliar blast score means, nine cultivars were resistant to all the eight isolates tested (Fig. 3). When the data was transformed using a binary method (where maximum severity scores were used to code disease reactions as 0 for <4, and 1 for scores ≥4, cultivar 75-1-127 was found to be resistant to eight *M. oryzae* isolates, whereas cultivar IRAT109 was susceptible to only one pathogen isolate, KE0401. Among other most resistant cultivars were those that were attacked by at most three isolates, including NERICA 10, ARICA 3, NERICA 2, and NERICA 12 (Fig. 2). Four cultivars, including NSFTV83 and ARICA 17 were susceptible to all *M. oryzae* isolates tested (Fig. 2). Some of the most popular and widely grown rice cultivars, including Basmati 217, Basmati 370, ITA 310, ARICA 15, NERICA 1, WITA 3, ARIZE 6444 Hybrid, and SARO 5 in the region were susceptible to at least five *M. oryzae* isolates (Fig. 2).

By using stepwise regression analysis, we identified five *Pi* genes that were significantly associated with blast severity (Table 6; Fig. 4). Markers for genes *Pi50* and *Pi65* were associated with reduced blast severity, whereas *Pik-p*, *Piz*, and *Pik* were associated with increased disease severity (Table 6; Fig. 4). Cultivars with *Pi65* had a disease severity of 2.8, whereas those without this resistance specificity had a severity of 3.5. Similarly, lines containing *Pi50* had a mean severity of 2.5, whereas those without the gene had 2.8. Although the presence of *Pik-m* had a notable reduction in disease severity, the mean severities for lines with and without the gene were not statistically significant ($P = 0.73$) (Table 6; Fig. 4). No statistically significant gene interactions affected foliar blast severity ($P > 0.05$). However, the identified blast resistance clusters did differ in blast severity (Table 4). Blast severity was, for example, least in BRC 4, BRC 1, and BRC 2, and highest in BRC 3 and BRC 5. Confidence intervals for blast severity showed that BRC 4 mainly consisted of incompatible disease reactions, whereas BRCs 2, 3, and 5 had notable proportions of genotypes with compatible disease reactions (Table 4).

As *Pi50* was significantly associated with reduced blast severity and is also linked with *Pi9*, a gene that was found to confer resistance against all tested African blast pathogen isolates in our ongoing blast resistance breeding program, we conducted an additional molecular analysis by re-amplifying and sequencing with a primer that targeted the *Pi2/9* locus (see Materials and Methods section). The *Pi2/9* multigene family cluster carries multiple rice blast resistance genes (which have been cloned) on chromosome 6 of rice. Out of

TABLE 5. Resistance profile of clusters of rice genotypes

BRC[x]	Rice (*n*)	Attacked by isolates (%)[y]	Blast severity (score scale 0–9): Mean and confidence interval (95%)		
			(X ± SE)[z]	Lower	Upper
3	16	72	3.7 ± 0.2 a	3.3	4.0
2	11	72	2.9 ± 0.2 b	2.5	3.3
5	6	71	2.9 ± 0.3 ab	2.4	3.4
1	14	65	2.7 ± 0.2 b	2.4	3.1
4	8	53	2.4 ± 0.2 b	1.9	2.8

[x] BRC = blast resistance gene cluster.
[y] Proportions do not differ, $X^2 = 8.032$, $P = 0.09$.
[z] Means separated by the same letters do not differ significantly (Tukey's HSD $\alpha = 0.05$).

TABLE 6. Estimates of gene effects on susceptibility of rice genotypes to blast disease based on inoculation with eight African isolates

Rice gene	Estimate	Standard error	*t* Ratio	$P > \|t\|$	LogWorth
Pik-p	0.38447665	0.106186	3.62	0.0003	3.325
Piz-t	0.33733916	0.116442	2.9	0.0039	2.223
Pi65	−0.3784536	0.130993	−2.89	0.004	2.187
Pik	0.27908152	0.112214	2.49	0.0131	1.912
Pi50	−0.4700297	0.186135	−2.53	0.0117	1.198
Pikm	−0.064492	0.185674	−0.35	0.7284	1.19

TABLE 3. Virulence spectrum and aggressiveness of *Magnaporthe oryzae* isolates used in this study

Isolate code	Lineage and virulence in past study		Aggressiveness in current study		Reference
	Clade[x]	Virulence category[y]	Blast severity (0–9) ± SE[z]	Attacked rice (%)	
KE0215	7	Moderately virulent	4.2 ± 0.25 a	84	Mutiga et al. 2017
NG0026	1	Moderately virulent	3.3 ± 0.26 ab	78	Mutiga et al. 2017
KE0401		Moderately virulent	3.1 ± 0.25 b	78	This paper
KE0002		Virulent	2.9 ± 0.25 b	73	Mutiga et al. 2021
TZ0077	4	Virulent	3.3 ± 0.26 ab	69	Mutiga et al. 2017
KE0218		Virulent	2.7 ± 0.26 bc	58	This paper
BN0125	1	Moderately virulent	2.4 ± 0.26 bc	58	Mutiga et al. 2017
TZ0001		Moderately virulent	1.8 ± 0.25 c	40	Huang et al. 2019

[x] Based on genetic analysis using single-nucleotide polymorphisms derived from a genotyping-by-sequencing study reported earlier (Mutiga et al. 2017). Blank cells in the clade column indicate that the isolate was not genotyped in the past study (reported as Mutiga et al. 2017), but was tested in the current study.
[y] Based on pathotyping with rice differentials in a previous study.
[z] Means separated by the same letters do not differ significantly (Tukey's HSD $\alpha = 0.05$).

TABLE 4. Analysis of variance for blast disease components associated with blast severity

Source	df[z]	Sum of squares	Mean square	*F* ratio	$P > F$
Blast resistance cluster (BRC)	4	158.2269	39.55672	7.0812	<0.0001
Pathogen isolate	7	377.0242	53.86059	9.6418	<0.0001
Rice cultivar (BRC)	50	815.9689	16.31938	2.9214	<0.0001
Replicate	1	1.92683	1.92683	0.3449	0.5572
Model	62	1,379.782	22.2545	3.9839	<0.0001
Error	821	4,586.214	5.5861	–	–
Corrected total	883	5,965.996	–	–	–

[z] df is degrees of freedom.

the 47 cultivars that were sequenced in this way, the majority (64%) sequences identified *Piz-t*, whereas 15% showed *Pi9*, another 6 and 4% showed *Pi2* and *Pigm*, respectively. Alignment of the sequences for cultivars that showed similar genes in this region revealed that the sequence for the *Pi9* donor did not differ from that of other lines carrying the gene, such as Wita 3 and NSFTV83. Additionally, a sequence alignment and comparison of cultivar IRAT109 with those of NERICA 1, NERICA 10, and the other cultivars carrying *Piz-t* did not reveal any polymorphism in the region. Interestingly, there were differences in disease reactions for cultivars carrying similar genes. Cultivar 75-1-127 was resistant to all isolates, for example, whereas the other cultivars were attacked by at least five isolates.

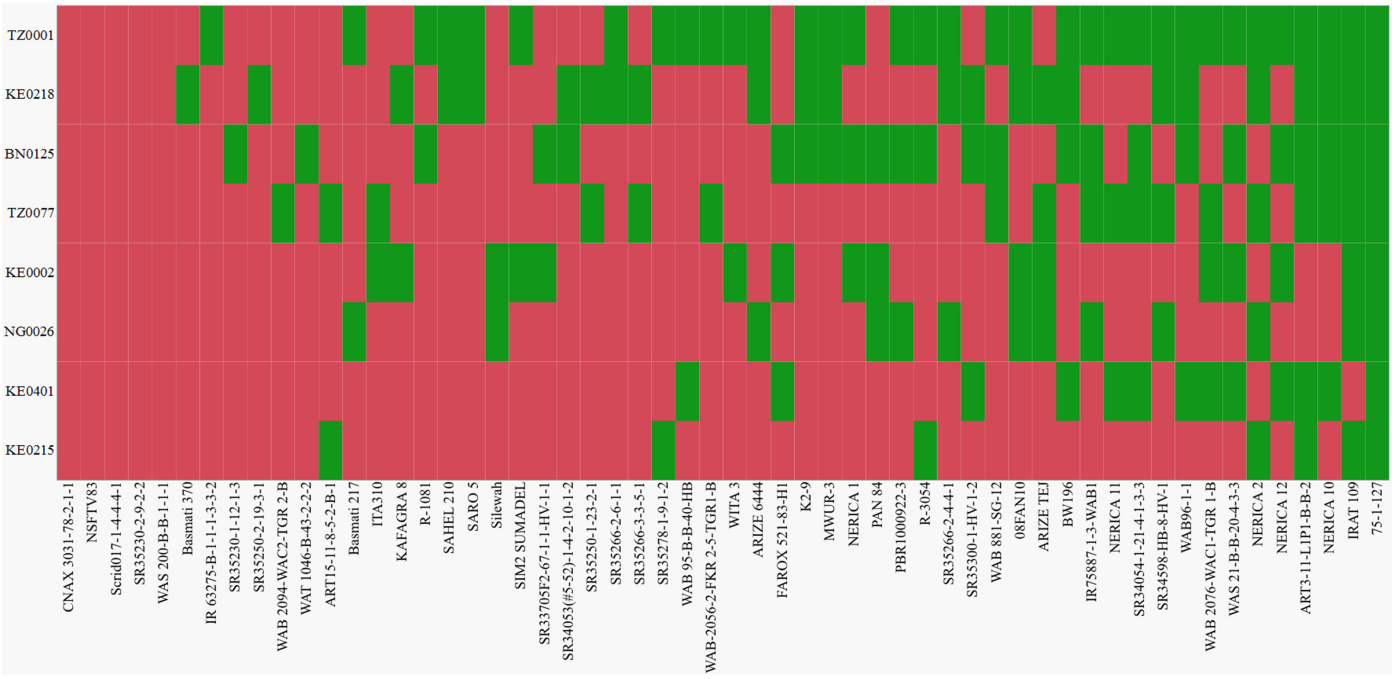

Fig. 2. Disease reactions for a subset of rice genotypes (*x*-axis) inoculated with isolates (*y*-axis) of *Magnaporthe oryzae* from sub-Saharan Africa. Colors denote disease reactions; green for resistant (foliar blast severity score ≤3) and red for susceptible (severity score ≥4). The binary transformation of the disease reaction is based on mean disease score severity; where 0 is for a mean <4 and 1 is for ≥4 in at least two tests, with a repeat test being conducted there were contradictions in whether the foliar blast score was below or above 4. Sorting on the *x*-axis is based on the proportion of isolates which attacked the rice cultivars. Sorting on the *y*-axis is based on the proportion of cultivars that were attacked by individual isolates.

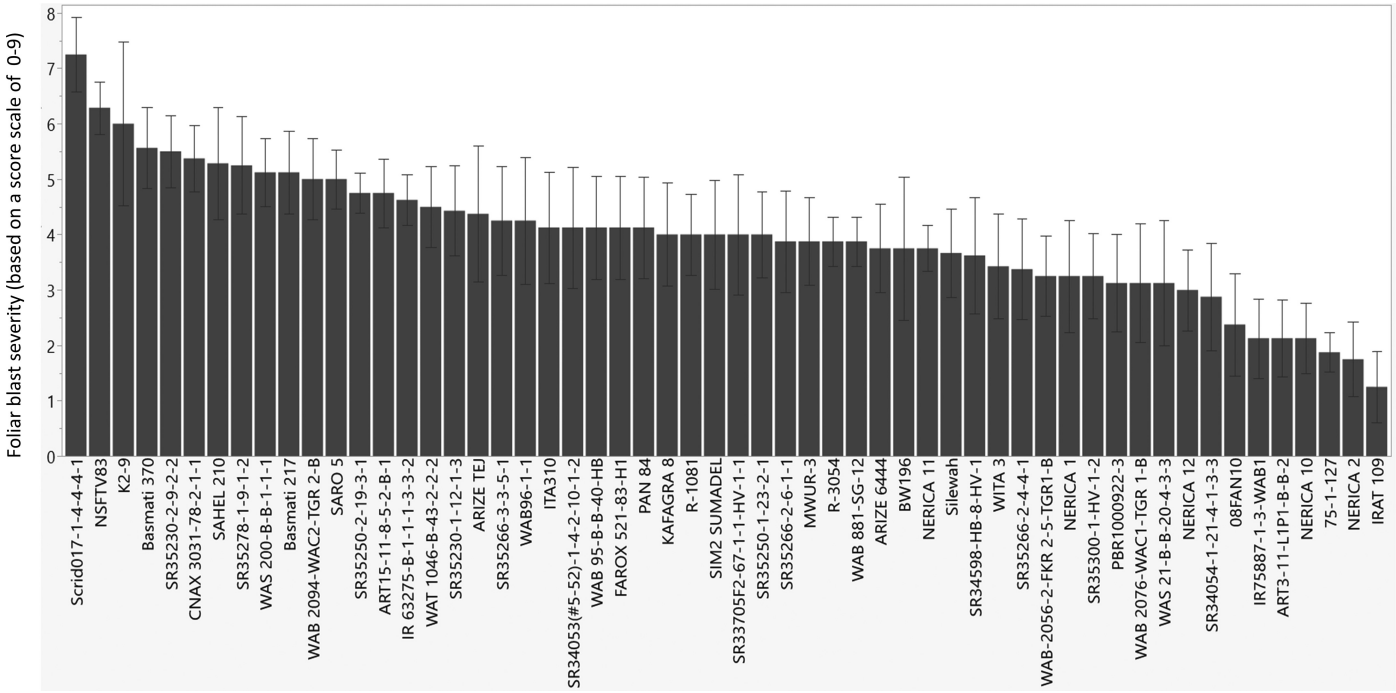

Fig. 3. Foliar blast severity (on a scale of 0 to 9) for a subset of rice genotypes (*x*-axis) that were inoculated with isolates of *Magnaporthe oryzae* from sub-Saharan Africa. Error bars are standard errors for individual rice genotypes based on at least two tests. Cultivars were considered resistant if the mean disease score is below 4.

For *Piz-t*, the most resistant cultivar was IRAT109, but this was attacked by KE0401, whereas the rest of the cultivars were susceptible to at least three isolates. For example, the hybrid NERICA 10 cultivar was attacked by three isolates (Fig. 5). An inoculation of NERICA 12 (*Pi2*) showed that it was susceptible to four isolates (Fig. 5).

Discussion

Breeding for blast resistance requires a good understanding of the genetic diversity within the available rice germplasm, the mechanisms of resistance gene action, and the virulence spectrum of the prevailing pathogen population. Recently, there have been efforts to map genes conditioning blast resistance based on rice germplasm characterization using African collections of *M. oryzae* (Mgonja et al. 2016, 2017). In this paper, we endeavored to characterize blast resistance through molecular analysis and phenotyping by inoculation with genetically distinct isolates of *M. oryzae* showing variation in virulence spectrum. We screened for the presence of known blast resistance genes in diverse populations of rice germplasm from SSA, including the majority of the ARICA (a collection of rice cultivars with superior traits across multiple countries in Africa), some members of the NERICA interspecific hybrid rice, as well as breeding lines from an ongoing KAFACI rice development program, and other popularly grown commercial rice cultivars in SSA (Gridley et al. 2002; Wopereis 2013). We reasoned that grouping rice cultivars based on the presence/absence of known blast resistance genes would enable us to identify representative lines for further characterization of phenotype of blast resistance profiles among the evaluated germplasm. The utility of this study approach is that it reduces the time and cost of phenotyping and could be harnessed in identifying cultivars rapidly for genetic improvement for blast resistance in rice.

Based on the polymorphic information content, we identified five distinctive markers, and those for the rest of the genes showed a high frequency (52 to 99%) in the rice panel. This implies that most of the *Pi* genes or their allele versions existed in the tested rice panel. Most of the rice cultivated in SSA was introduced from Asia by

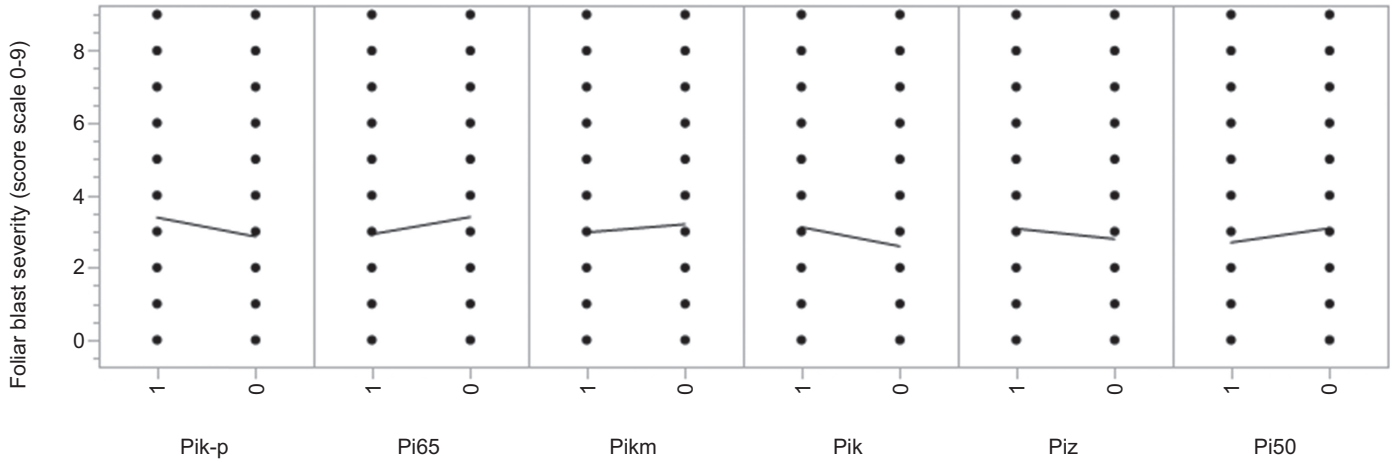

Fig. 4. Effect of the presence of individual genes on the blast phenotype based on inoculation of rice genotypes with eight isolates of *Magnaporthe oryzae.* Genes coded as 1 = present; 0 = absent on the *x*-axis. *y*-axis is the disease severity.

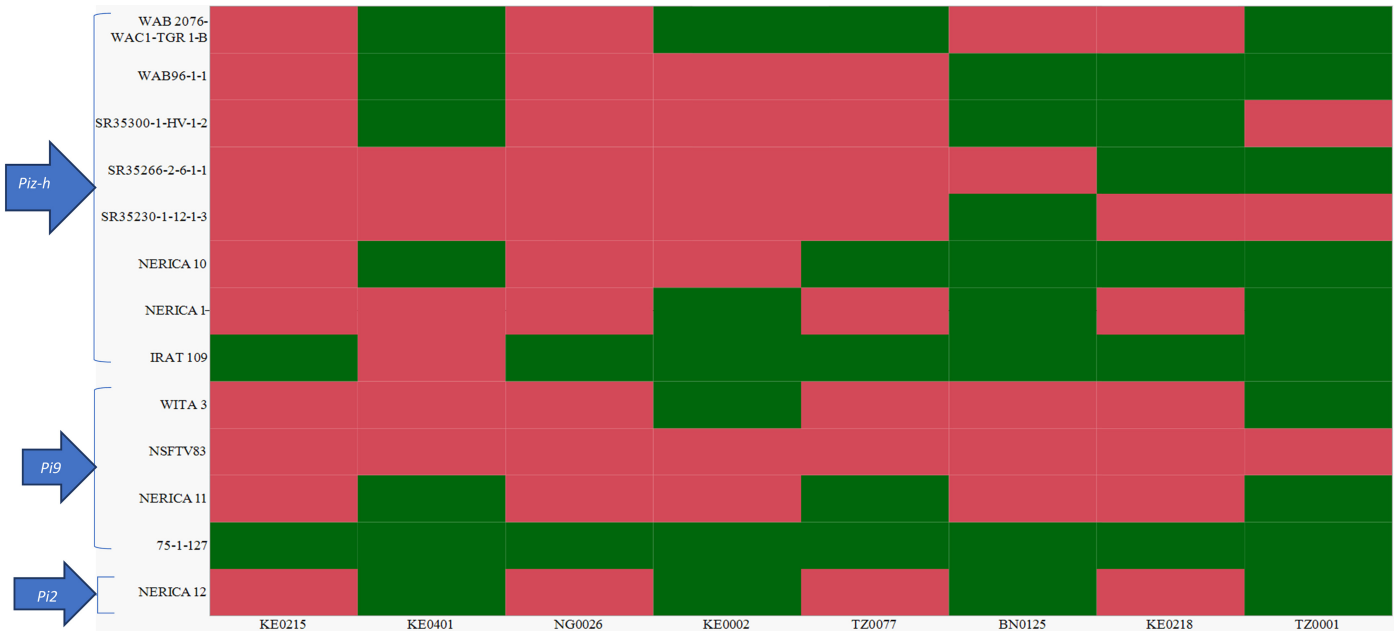

Fig. 5. Disease reaction for rice lines that contained *Pi2/9* gene family (*y*-axis) based on inoculation with eight African isolates of *Magnaporthe oryzae* (*x*-axis). Disease reactions are color coded as green for resistant (foliar blast severity score ≤3) and red for susceptible (foliar blast severity score ≥4).

Europeans in the 16th century (Linares 2002). This finding shows that cultivars derived from these ancestral populations originally had effective resistance genes, but these may have been overcome or rendered ineffective because of shifts in the virulence spectrum of the pathogen within the new rice growing ecologies of Africa (McDonald and Linde 2002; Onaga et al. 2020). Our findings imply that *Pi50* and *Piz* members of the *Pi2/9* multigene family were among the least frequent in the tested panel. The *Pi2/9* loci is located in the short arm of chromosome 6 in rice and consists of *Pi2*, *Pi9*, *Pi25*, *Pi26*, *Pi40*, *Pi50*, *Pigm*, *Piz*, *Pi2-2*, *Piz-t*, *Pi-kf2*(*t*), and *Piz-5* broad-spectrum resistance genes (Wei et al. 2019; Zhu et al. 2012). Consistent with the results observed in this study, cultivar 75-1-127, a donor of *Pi9* gene, has been reported to be resistant to the majority of blast pathogen isolates collected from SSA (Mutiga et al. 2017). Here, we show an association between foliar blast resistance and the presence of two members (*Pi50* and *Piz-t*) of the *Pi2/9* cluster and imply that it is not the number of genes, but whether those present are effective in the tested rice background. Therefore, it is likely that pyramiding of several specific genes from functional genetic backgrounds could provide durability in blast resistance in African rice varieties.

We observed that eight blast pathogen isolates were able to discriminate African rice cultivars based on disease reaction, with the most resistant being ART3-11-L1P1-B-B-2 (ARICA 4), IRAT109, and NERICA 10, and the most susceptible being the two Basmati varieties 217 and 370, as well as NSFTV83 and Scrid017-1-4-4-4-1 (ARICA 17). An earlier characterization of a diverse rice panel using African collections of *M. oryzae* had shown that NSFTV83 was resistant, but current findings show otherwise (Mgonja et al. 2016, 2017). It should be noted that the current set of isolates was selected based on virulence and diversity reported in an earlier study and may not be similar to those used in the reports shown above (Mgonja et al. 2016; Mutiga et al. 2017). The results of this comprehensive evaluation of ARICA and KAFACI rice germplasm will be useful in guiding further breeding efforts and for varietal recommendations by the relevant stakeholders. Furthermore, this data confirms the susceptibility of the popular aromatic Basmati 217 and Basmati 370, which are among the most widely grown cultivars in East Africa (Mutiga et al. 2021; Nganga et al. 2022). Although we included IRBLs to enhance confirmation of the presence of specific *R* genes and specificity of lines to cultivars, the PCR screening showed that the lines had alleles of other unexpected *Pi* genes and were, therefore, not monogenic. The presence of multiple genes (at least based on PCR data) made these IRBLs not useful for the characterization of pathogen specificity on *Pi* genes. A recent genome-wide characterization of Lijiangxintuanheigu (LTH)-a Japanese universally susceptible cultivar and the recurrent parent for all IRBLs showed that although the cultivar contained alleles for some *Pi* genes, these genes may have been suppressed or the genes are not functional because of mutations of individual loci which have been disrupted by genomic variations (Yang et al. 2022). Testing the functionality of individual genes was not within the scope of the current study but would provide the means to fully evaluate their efficacy and mode of action.

Markers for the 21 known blast resistance genes grouped SSA rice germplasm into five clusters, which differed in mean blast disease score. Based on greenhouse-based inoculations with the eight isolates of *M. oryzae*, cluster 4 consisted of the most resistant cultivars, and all members of this cluster possessed *Pi50*, *Pi65*, and *Pikm*. *Pi50* is a member of the *Pi2/9* multifamily of genes located in chromosome 6 that confer broad-spectrum resistance against blast pathogen (Zhu et al. 2012). Upon sequencing the rice cultivars which had amplified *Pi50*, we found cultivars with other members of the multigene family, including *Piz-t*, *Pi9*, *Pi2*, and *Pigm*. Out of the cultivars that amplified *Pi50* and were sequenced using the *Pi2/9* primer, IRAT109 (*Piz-t*) and 75-1-127 (*Pi9*) cultivars were the most resistant, whereas the rest of the rice cultivars showed varying levels of susceptibility. This finding shows that part of the resistance observed in lines carrying *Pi50* was because of *Pi9* and *Piz-t*. The *R* gene *Pi65* was statistically associated with reduced blast disease based on stepwise regression of the entire phenotypic data. This implies that part of the resistance in cluster 4 may also have been conferred by *Pi65* (Zheng et al. 2016). Although all members of cluster 4 amplified *Pikm*, the gene had an insignificant marginal negative effect on the magnitude of blast severity. The two genes are located in chromosome 11, but *Pi65* confers broad-spectrum resistance, whereas *Pikm* is a race-specific blast resistance gene (Ashikawa et al. 2008; Zheng et al. 2016). Although the stepwise regression model did not show significant synergy among *Pi* genes, it is possible that pyramiding *Pikm, Pi65,* and the members of *Pi2/9* genes within adapted and popular rice could confer durable blast resistance in SSA, since this combination excludes the majority of the prevailing pathogen population. Our breeding program has initiated a gene pyramiding scheme with *Pi2/9* and *pi21* gene specificities in popular cultivars, consistent with this study performed independently.

Some of the genes detected in this study may not, however, be effective in the rice panel. By using the stepwise regression which tested for the markers associated with foliar blast severity, we found that only five markers (including *Pi50, Pi65*, *Pik-p*, *Piz-t*, and *Pik*) had a significant effect on the phenotype. Here, only *Pi50* and *Pi65* were associated with reduced blast severity, whereas the other three acted more like susceptibility genes. It should be noted that all the *Pi* genes screened in this rice panel were previously mapped based on inoculations with blast pathogen isolates from outside SSA. Therefore, the lack of effectiveness of the *Pi* genes could be because of differences in pathogen virulence and resistance mechanisms against the tested rice panel. Pathogenicity is strongly correlated, for example, with the prevailing effector repertoire which can influence disease reactions in the tested host germplasm (Liao et al. 2016). Although *M. oryzae* may have originated and spread to the rest of the world from South East Asia, studies have shown that African populations may have further evolved as a result of changes in host and environmental factors (Onaga et al. 2020; Saleh et al. 2014). Although defeated *R* genes could easily be evaded by pathogen effectors, we do not have sufficient data to validate whether *Pik-p, Piz-t*, and *Pik* are associated with susceptibility. In fact, one of the most resistant rice cultivars in the current study, IRAT109, carried *Piz-t* (Nelson et al. 2018; Petit-Houdenot and Fudal 2017). Despite carrying a disease resistance gene, cultivars may also be susceptible if they are challenged by new strains that use a novel effector repertoire (Jones and Dangl 2006; Lo Iacono et al. 2013; McDonald and Linde 2002). There is, therefore, a need to map and clone genes associated with blast resistance using adapted rice germplasm and based on inoculations with resident pathogen collections of *M. oryzae* from SSA because this will enhance breeding for durably resistant rice varieties.

Recognizing that contemporary *Pi* genes were identified based on tests that involved blast pathogen collections from outside Africa, we set out in this study to evaluate whether the same genes conferred resistance in African rice germplasm. The findings in this study show that very few of the known genes are effective or associated with foliar blast severity. Therefore, there is a need to utilize available germplasm and blast pathogen collections within current biobanks to map and clone blast resistance that can be deployed in SSA because these will enhance efficiency of rice breeding for resistance by using modern molecular tools. We have identified the resistance profiles of some rice cultivars, which could now be utilized as donor parents in the development of mapping populations and/or susceptible and resistant checks in subsequent blast resistance characterization for SSA. In ongoing rice breeding efforts for blast resistance, we recommend initially pyramiding gene specificities in the *Pi2/Pi9* multigene family in chromosome 6 and those in chromosome 11 (*Pi65* and *Pik-m)* of the rice genome to achieve broad and durable blast resistance for SSA.

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