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Molecular and Pathotypic Characterization of New *Xanthomonas oryzae* Strains from West Africa

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DNA polymorphism analysis and pathogenicity assays were used to characterize strains of *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola* collected from rice leaves in West Africa. Restriction fragment length polymorphism (RFLP), repetitive sequence–based polymerase chain reaction, fluorescent amplified fragment-length polymorphism (FAFLP) analyses were assessed for molecular characterization, while pathogenicity was tested by leaf clipping and leaf infiltration. Dendrograms were generated for the data sets obtained from RFLP analysis and repetitive polymerase chain reaction suggesting that the interrelationships between strains were dependent on the technique used. In all cases, data showed that African strains of *X. oryzae* pv. *oryzae* form a group genetically distant from Asian strains. FAFLP analyses separated the *X. oryzae* strains into three groups with significant bootstrap values. A specific and intriguing feature of African strains of *X. oryzae* pv. *oryzae* is a reduction in the number of insertion sequence elements and transcription activator-like (*avrBs3/pthA*) effector genes, based on the molecular markers employed in the study. In addition, pathogenicity assays conducted with African strains of *X. oryzae* pv. *oryzae* on a series of nearly isogenic lines (NILs) identified three new races. Finally, leaf infiltration assays revealed the capacity of African strains of *X. oryzae* pv. *oryzae* to induce a nonhost hypersensitive response in *Nicotiana benthamiana*, in contrast with Asian *X. oryzae* pv. *oryzae* and *X. oryzae* pv. oryzicola strains. Our results reveal substantial differences between genomic characteristics of Asian and African strains of *X. oryzae* pv. *oryzae*.

Two important diseases of rice are due to *Xanthomonas oryzae* pathovars, bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* and bacterial leaf streak (BLS) caused by *Xanthomonas oryzae* pv. *oryzicola*. These two phytopathogenic bacteria are highly related, showing more than 90% similarity by the DNA:DNA hybridization and are difficult to differentiate genetically and phenotypically (Rademaker et al. 2000; Swings et al. 1990). Indeed, the symptoms provoked by *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* are different at early but not later stages of infection (Niño-Liu et al. 2006). They infect their host in distinct ways; *X. oryzae* pv. *oryzae* enters through hydathodes and spreads systemically through the xylem, while *X. oryzae* pv. *oryzicola*, which is a nonvascular pathogen, enters through stomata and colonizes the mesophyll parenchyma (Niño-Liu et al. 2006). Bacterial blight is a major disease in rice-irrigated environments, causing a substantial yield loss of up to 50% in severe epidemics (Ou et al. 1985). BB has been reported in Asia, Australia, and Latin America (Mew et al. 1993) and is emerging in importance in Africa, as briefly reported by Ryba-White and associates (1995). BLS is increasing in importance in areas of Asia where hybrid rice is widely grown (Raymundo et al. 1999). The disease was also reported in Madagascar, Nigeria, and Senegal (Buddenhagen 1985). Although both diseases were reported earlier in Africa, a detailed molecular and pathotypic analysis of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains was still lacking until this study. Sustainable control measures for BB and BLS in Africa, however, will depend on parallel improvements in the understanding of the pathogen population structure and evolution and on the analysis of microbial genes involved in the interaction.

Numerous studies have been carried out on the rice–*X. oryzae* pv. *oryzae* pathosystem, resulting in powerful advancement in the understanding of the molecular basis of the interaction (Shen and Ronald 2002). The rice–*X. oryzae* pv. *oryzae* interaction follows the classical gene-for-gene model. As a prerequisite for designing breeding strategies to control bacterial blight disease, a comprehensive study of *X. oryzae* pv. *oryzae* pathogen diversity was conducted in different Asian countries. Pathogen populations of *X. oryzae* pv. *oryzae* are highly variable, as revealed by virulence and DNA fingerprinting analysis (Adhikari et al. 1995, 1999; Leach et al. 1992; Nelson et al.1994). A race or pathotype is a group of strains sharing common phenotype of virulence to a set of host cultivars. Over thirty *X. oryzae* pv. *oryzae* races have been reported in Asia, and new ones have emerged to overcome deployed resistance (Adhikari 1994; Jeung et al. 2006; Nelson et al. 1994; Niño-Liu et al. 2006; Noda et al. 1996; Vera Cruz et al. 2000). Adaptation to rice varieties containing a single dominant resistance gene often results from the loss of function of the corresponding pathogen *avr* gene. Consequently, the durability and quality of a resistance gene is a function of the amount of fitness penalty imposed on the pathogen (Vera Cruz et al. 2000).

Host resistance is considered to be a sound approach to control BB, although major resistance genes are not durable because of the high degree of pathogenic variation exhibited by *X. oryzae* pv. *oryzae* (Vera Cruz et al. 2000). Durable resistance is more likely to be achieved through pyramiding different types of resistance (*R*) genes (Li et al. 2006). Currently, various rice genes conferring resistance to *X. oryzae* pv. *oryzae* have been identified genetically and designated in a

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series from *Xa1* to *Xa29*, of which nine were identified as recessively inherited. So far, six *R* genes (*Xa1*, *xa5*, *xa13*, *Xa21*, *Xa26*, and *Xa27*) have been characterized as encoding different types of proteins, suggesting multiple mechanisms of *R* gene–mediated *X. oryzae* pv. *oryzae* resistance (Chu et al. 2006; Gu et al. 2005; Iyer and Couch 2004; Song et al. 1995; Sun et al. 2004; Yoshimura et al. 1998). Various polymerase chain reaction (PCR)-based markers were developed for *Xa* genes, providing powerful tools for marker-assisted selection of *R* genes in breeding programs. Major genes of resistance have been incorporated into rice varieties to develop BB-resistant varieties and to build near-isogenic lines (NILs) (Ogawa et al. 1991).

The population structure of the BLS pathogen *X. oryzae* pv. *oryzicola* has been little studied, except in the Philippines where a high diversity was observed among strains (Raymundo et al. 1999). Haplotypes were characterized allowing further selection of a set of strains for resistance analysis. Resistance to BLS is believed to be quantitative; however, no resistance genes against *X. oryzae* pv. *oryzicola* have been characterized in rice so far (Zhao et al. 2004a). Recently, maize single dominant gene *Rxo1* was identified for its property to mediate a nonhost defense response to *X. oryzae* pv. *oryzicola* (Zhao et al. 2004b). *Rxo1* also prevents the development of BLS when it is expressed as a transgene in rice (Zhao et al. 2005).

X. oryzae pv. *oryzae* pathogenicity is highly dependent on a type III secretion system (T3SS) injecting effector proteins into the eukaryotic host cell (Buttner and Bonas 2002). Most of our knowledge on T3SS effectors in *X. oryzae* pv. *oryzae* is based on studies of the large AvrBs3/PthA family of *Xanthomonas* effector proteins, the abundance of which in individual strains is a unique feature of *Xanthomonas oryzae* pathovars as compared with other *Xanthomonas* spp. (Ochiai et al. 2005). Also referred to as transcription activator-like (TAL) effectors, this family includes proteins with avirulence activities, virulence functions, or both (Gurlebeck et al. 2006). Among them are *avrXa7*, *avrXa10*, *avrxa5*, and *avrXa27*, which have been cloned from *X. oryzae* pv. *oryzae* Philippine strains and which all exhibit resistance gene–specific avirulence function (Gu et al. 2005; Hopkins et al. 1992). In addition to the well-characterized AvrXa7 protein (Yang et al. 2000), new TAL effectors were recently reported to be major virulence determinants (Yang and White 2004). Analysis of the genome sequences of the *X. oryzae* pv. *oryzae* Korean KACC10331 and Japanese MAFF311018 strains enabled the identification of a few other T3SS effector candidates, including *avrBs2*, *hpaF*, and *popC*-like genes (Lee et al. 2005; Ochiai et al. 2005). Isolated from *X. oryzae* pv. *oryzicola* BLS256, *avrRxo1* encodes the corresponding avirulence protein that was reported to be associated with the plasma membrane and to interact with *Rxo1* inside maize cells to elicit cell death (Zhao et al. 2004a). This novel effector gene, the action of which is T3SS-dependent, is not related to TAL effector genes but is conserved in all *X. oryzae* pv. *oryzicola* strains tested so far, suggesting that it may act as a critical fitness or virulence factor, or both.

Well-developed genetic tools together with reproducible inoculation methods and the availability of rice NILs provide good support for analyses of *Xanthomonas oryzae* pathovar strains. We report here on the characterization of *Xanthomonas oryzae* strains in West Africa.

RESULTS

Identification of three new *X. oryzae* pv. *oryzae* races among African strains.

A total of 96 severely blighted leaves of different African cultivars were collected in three West African countries (Mali, Niger, and Burkina Faso) and were processed for bacterial isolation. A total of 26 *X. oryzae* strains were isolated and are referred to here as field isolates. These 26 field isolates together with selected strains of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* obtained from international collections were then evaluated for virulence by leaf-clip inoculation on the rice variety Nipponbare. Typical BB symptoms were visible for 16 field isolates (MAI1, MAI2, and MAI9, BAI1 to BAI4, and NAI1 to NAI9) and were similar to those observed upon inoculation of *X. oryzae* pv. *oryzae* Asian strains PXO339, PXO71, and CFBP1949, i.e., chlorotic regions in leaf tissue spreading from the leaf tip toward the leaf base and forming lesions >15 cm. Ten field isolates (MAI3 to MAI8 and MAI10 to MAI13) did not induce any symptoms, nor did *X. oryzae* pv. *oryzicola* strains BLS256, UPB497, or CFBP2286 (Table 1). Syringe infiltration of all these strains also resulted in two different types of reaction, as exemplified for field isolates BAI4 and MAI3 in Figure 1. Localized lesions at the inoculation point were observed for the above-mentioned 16 isolates (MAI1, MAI2, and MAI9, BAI1 to BAI4. NAI1 to NAI9) and reference strains *X. oryzae* pv. *oryzae* PXO339, PXO71, and CFBP1949 (Fig. 1 and Table 1). In contrast, the ten other isolates (MAI3 to MAI8 and MAI10 to MAI13) together with reference *X. oryzae* pv. *oryzicola* strains induced typical BLS water-soaking lesions visible by day 5, developing into yellow translucent lesions with droplets of bacterial exudates by day 10 (Fig. 1). The average size of the yellow translucent lesions varied among the isolates. One-way analysis of variance (ANOVA) revealed significant effects of isolates ($F = 82.74$; $P < 10^{-4}$) that were grouped in four classes (A to D). Therefore and for simplicity, we referred to the field isolates inducing typical BB symptoms as *X. oryzae* pv. *oryzae* (strains MAI1, MAI2, and MAI9, BAI1 to BAI4, NAI1 to NAI9) and the ones inducing typical BLS symptoms as *X. oryzae* pv. *oryzicola* (strains MAI3 to MAI8 and MAI10 to MAI13; Table 1).

The 16 *X. oryzae* pv. *oryzae* African strains, together with control *X. oryzae* pv. *oryzae* strains corresponding to different Phillipine races (from the International Rice Research Institute [IRRI] collection) and African *X. oryzae* pv. *oryzae* strains isolated in 1980 (Collection Française de Bactéries Phytopathogènes [CFBP]) were next evaluated for virulence on a series of 10 NILs, each carrying a single (*Xa*) resistance gene. As shown in Table 2, three races (A1, A2, and A3) were thereby characterized. Race A1 strains isolated in Cameroon, Niger, and Burkina are incompatible on IRBB4, IRBB5, and IRBB7 lines and showed a moderately susceptible reaction on IRBB8 and IRBB21 lines. Race A2 consists of one strain (BAI4), which was isolated in Burkina on *Oryza glaberrima* and is incompatible on almost all IRBB lines tested, except for line IR24. Race A3 strains, which were exclusively isolated in Mali in 1980 and 2003, are incompatible on all IRBB lines tested, including IR24. The *X. oryzae* pv. *oryzae* Phillipine races tested showed different reactions on the IRBB lines (Table 1 and data not shown) and confirmed previous results (Ogawa et al. 1991; Nelson et al. 1994).

X. oryzae pv. *oryzae* African strains induce a nonhost hypersensitive response (HR) in *Nicotiana benthamiana*.

Next, we addressed the question of whether high-titer inoculation of *N. benthamiana* leaves with African and reference Asian *Xanthomonas oryzae* strains of both pathovars would help in discriminating our strains, upon scoring of potential nonhost HR induction. All *X. oryzae* pv. *oryzae* African strains induced a typical nonhost cell-death response 48 h postinoculation in *N. benthamiana*, while none of the *X. oryzae* pv. *oryzae* Asian ones did (Fig. 2 and Table 1). In contrast, no

Table 1. Characteristics of the *Xanthomonas oryzae* strains used in this study

Strains[c]	Origin[d]	Pathogenicity assays[a]				RFLP[b]				Rep-PCR		AFLP[e]
		A	B	C	D	PJEL101	*avrXa10*	Tnx1	*Xoo*1762	Rep	Eric	
Xanthomonas oryzae* pv. *oryzae												
A3842	India	nt	nt	nt	nt	1^{fi}	1^{av}	1^{te}	nt	1^{r}	1^{er}	nt
A3857	India	nt	nt	nt	nt	2^{fi}	2^{av}	2^{te}	1^{so}	2^{r}	1^{er}	nt
HN35	Chine	nt	nt	nt	nt	3^{fi}	3^{av}	3^{te}	nt	3^{r}	2^{er}	1
IXO57	Indonesia	nt	nt	nt	nt	4^{fi}	4^{av}	4^{te}	2^{so}	4^{r}	2^{er}	nt
IXO60	Indonesia	nt	nt	nt	nt	5^{fi}	4^{av}	5^{te}	nt	5^{r}	2^{er}	1
MB18	Malaysia	nt	nt	nt	nt	6^{fi}	5^{av}	3^{te}	3^{so}	6^{r}	2^{er}	1
MXO92	Malaysia	nt	nt	nt	nt	7^{fi}	6^{av}	5^{te}	nt	7^{r}	3^{er}	nt
NXO537	Nepal	nt	nt	nt	nt	8^{fi}	7^{av}	6^{te}	nt	8^{r}	4^{er}	1
NXO622	Nepal	nt	nt	nt	nt	9^{fi}	8^{av}	2^{te}	nt	9^{r}	2^{er}	nt
XOO212	Korea	nt	nt	nt	nt	10^{fi}	4^{av}	6^{te}	1^{so}	10^{r}	4^{er}	1
PXO61	Philippines	+	nt	1	nt	11^{fi}	9^{av}	7^{te}	nt	11^{r}	5^{er}	nt
PXO71 (CFBP1946)	Philippines	+	a	4	–	12^{fi}	10^{av}	7^{te}	nt	11^{r}	6^{er}	nt
PXO79 (CFBP1981)	Philippines	+	nt	3B	nt	nt	nt	nt	nt	nt	nt	nt
PXO86	Philippines	+	nt	2	nt	13^{fi}	11^{av}	8^{te}	nt	12^{r}	7^{er}	nt
PXO99	Philippines	+	nt	6	nt	14^{fi}	12^{av}	9^{te}	nt	13^{r}	8^{er}	nt
PXO112	Philippines	+	nt	5	nt	15^{fi}	13^{av}	10^{te}	nt	14^{r}	8^{er}	nt
PXO145	Philippines	+	nt	7	nt	16^{fi}	14^{av}	11^{te}	nt	12^{r}	7^{er}	nt
PXO280	Philippines	+	nt	8	nt	16^{fi}	9^{av}	12^{te}	nt	15^{r}	7^{er}	nt
PXO339	Philippines	+	a	9	–	17^{fi}	15^{av}	13^{te}	1^{so}	16^{r}	5^{er}	1
PXO340	Philippines	+	nt	3C	nt	17^{fi}	16^{av}	13^{te}	nt	16^{r}	5^{er}	nt
PXO341	Philippines	+	nt	10	nt	17^{fi}	17^{av}	13^{te}	nt	16^{r}	5^{er}	nt
PXO345	Philippines	+	nt	9c	nt	18^{fi}	18^{av}	13^{te}	nt	nt	nt	nt
PXO448	Philippines	+	nt	9b	nt	19^{fi}	18^{av}	14^{te}	nt	nt	nt	nt
CFBP 2532* (NCPPB3002)	India	+	nt	nt	nt	20^{fi}	19^{av}	9^{te}	4^{so}	13^{r}	9^{er}	1
CIAT 1185 (LMG634)	Colombia	+	nt	nt	nt	21^{fi}	20^{av}	15^{te}	nt	14^{r}	7^{er}	nt
CIAT 1186 (LMG635)	Colombia	+	nt	nt	nt	22^{fi}	20^{av}	16^{te}	nt	11^{r}	5^{er}	nt
CFBP 1948 (LMG12465)	Cameroon	+	nt	A1	nt	23^{fi}	21^{av}	nh	nt	17^{r}	10^{er}	nt
CFBP1947 (LMG12464)	Cameroon	+	a	A1	nt	23^{fi}	21^{av}	nh	nt	17^{r}	10^{er}	nt
CFBP1949 (LMG12466)	Mali	+	a	A3	nt	23^{fi}	21^{av}	nh	nt	17^{r}	11^{er}	2
CFBP1951 (LMG12467)	Mali	+	a	A3	nt	23^{fi}	21^{av}	nh	nt	17^{r}	12^{er}	nt
CFBP1952 (LMG12468)	Mali	+	a	A3	nt	23^{fi}	21^{av}	nh	nt	17^{r}	12^{er}	nt
BAI1	Burkina, Bagre	+	a	A1	+	23^{fi}	22^{av}	nh	nt	18^{r}	13^{er}	nt
BAI2	Burkina, Bagre	+	a	A1	+	23^{fi}	23^{av}	nh	nt	18^{r}	13^{er}	nt
BAI3	Burkina, Bagre	+	a	A1	+	23^{fi}	23^{av}	nh	5^{so}	18^{r}	13^{er}	2
BAI4	Burkina, Bagre	+	a	A2	+	23^{fi}	21^{av}	nh	5^{so}	18^{r}	13^{er}	nt
NAI1	Niger, Saga	+	a	A1	+	23^{fi}	24^{av}	nh	nt	18^{r}	13^{er}	nt
NAI2	Niger, Toula	+	a	A1	+	23^{fi}	21^{av}	nh	5^{so}	18^{r}	13^{er}	2
NAI3	Niger, Bonfeba	+	a	A1	+	23^{fi}	21^{av}	nh	nt	18^{r}	13^{er}	nt
NAI4	Niger, Bonfeba	+	a	A1	+	23^{fi}	21^{av}	nh	nt	18^{r}	13^{er}	nt
NAI5	Niger, Toula	+	a	A1	+	23^{fi}	21^{av}	nh	nt	18^{r}	13^{er}	nt
NAI6	Niger, Toula	+	a	A1	+	23^{fi}	21^{av}	nh	nt	18^{r}	13^{er}	nt
NAI7	Niger, Toula	+	a	A1	+	23^{fi}	21^{av}	nh	nt	18^{r}	13^{er}	nt
NAI8	Niger, Bonfeba	+	a	A1	+	23^{fi}	21^{av}	nh	nt	18^{r}	13^{er}	nt
NAI9	Niger, Bonfeba	+	a	A1	+	23^{fi}	23^{av}	nh	nt	18^{r}	13^{er}	nt
MAI1	Mali, Niono	+	a	A3	+	24^{fi}	21^{av}	nh	nt	18^{r}	13^{er}	nt
MAI2	Mali, Niono	+	a	A3	+	24^{fi}	21^{av}	nh	5^{so}	18^{r}	13^{er}	2
MAI9	Mali, Molodo	+	a	A3	+	24^{fi}	22^{av}	nh	nt	18^{r}	13^{er}	2
Xanthomonas oryzae* pv. *oryzicola												
CFBP2286	Malaysia	–	b	–	–	25^{fi}	25^{av}	17^{te}	6^{so}	19^{r}	14^{er}	3
CFBP 2287	Malaysia	–	b	–	–	26^{fi}	26^{av}	17^{te}	6^{so}	20^{r}	14^{er}	nt
UPB497	Malaysia	–	b	–	–	nt	26^{av}	nt	nt	26^{r}	20^{er}	3
BLS256	Philippines	–	b	–	–	nt	nt	nt	nt	nt	nt	nt
MAI3	Mali, Niono	–	b	–	–	27^{fi}	27^{av}	18^{te}	7^{so}	21^{r}	15^{er}	3
MAI4	Mali, Niono	–	b	–	–	28^{fi}	28^{av}	19^{te}	8^{so}	22^{r}	16^{er}	nt
MAI5	Mali, Niono	–	b	–	–	29^{fi}	28^{av}	19^{te}	9^{so}	23^{r}	16^{er}	3
MAI6	Mali, Niono	–	b	–	–	29^{fi}	28^{av}	19^{te}	nt	22^{r}	16^{er}	nt
MAI7	Mali, Niono	–	b	–	–	30^{fi}	29^{av}	17^{te}	10^{so}	24^{r}	16^{er}	3
MAI8	Mali, Niono	–	b	–	–	31^{fi}	28^{av}	17^{te}	8^{so}	22^{r}	17^{er}	3
MAI10	Mali, Niono	–	b	–	-	27^{fi}	30^{av}	18^{te}	8^{so}	21^{r}	18^{er}	3
MAI11	Mali, Niono	–	b	–	–	27^{fi}	30^{av}	17^{te}	11^{so}	25^{r}	19^{er}	3
MAI12	Mali, Niono	–	b	–		27^{fi}	30^{av}	17^{te}	nt	25^{r}	19^{er}	nt
MAI13	Mali, Niono	–	b	–	–	27^{fi}	30^{av}	17^{te}	nt	25^{r}	19^{er}	nt

[a] Pathogenicity tests were performed as follows. Column A: leaf clipping inoculation on *O. sativa* var. Nipponbare (susceptible check). + indicates strains inducing typical BB symptoms and – indicates those not inducing symptoms. Column B: leaf infiltration on *O. sativa* var. Nipponbare. a indicates localized lesions and b indicates spreading translucent and yellow lesions. Column C: leaf clipping inoculation on a series of near isogenic lines carrying different *Xa* genes here and in previous studies (Ogawa et al. 1991). Column D: leaf infiltration of *Nicotiana benthamiana* leaves; reaction is observed 48 h after infiltration. + indicates for hypersensitive reaction (HR) and – indicates no HR.

[b] Restriction fragment length polymorphism (RFLP) groups with the different probes tested and as defined in the text. nt: not tested, nh: no hybridation.

[c] PXO = Philippines *Xanthomonas oryzae* pv. *oryzae* strains collection, The International Rice Research Institute (IRRI), Los Baños, Philippines; CIAT = Lab Rice Pathology collection, Centro Internacional de Agricultura Tropical, Cali, Colombia; CFBP = Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; NCPPB = National Collection of Plant Pathogenic Bacteria, United Kingdom; BLS = strains of bacterial leaf streak of cereals; MAI NAI and BAI = *Xanthomonas oryzae* WARDA-IRD (Africa Rice Center- Institut de la Recherche pour le Développement) collection from Mali, Niger, and Burkina, respectively; UPB = Unité de Phytopathologie Bacterial Collection, Louvain La Neuve, Belgium. Strains A, HN, IX, MB, MXO, NXO and XOO were provided by J. Leach as DNA. An asterisk (*) indicates a pathovar reference strain.

[d] Country and locality where the isolates were collected.

[e] Amplification fragment length polymorphism (AFLP) groups with significant bootstrap values.

differences could be observed upon inoculation of African and Asian *X. oryzae* pv. *oryzicola* strains, all leading to a mild chlorosis response (Fig. 2).

RFLP analyses distinguish African *X. oryzae* pv. *oryzae* strains from Asian ones.

Restriction fragment length polymorphism (RFLP)-based DNA polymorphism analyses were then performed to characterize *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* African strains, comparing them with several reference *Xanthomonas* pathovars. The RFLP probes used in this study enabled us to differentiate the *X. oryzae* strains at the pathovar and infrapathovar level. Probes pJEL101 and pBS*avrXa10* were the most discriminative, resolving a high number of haplotypes for both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains (Table 1). Probe pJEL101 resolved 31 unique RFLP patterns (haplotypes), seven among *X. oryzae* pv. *oryzicola* and 24 among *X. oryzae* pv. *oryzae* strains (Table 1). Haplotypes were clearly distinct when comparing African and Asian *X. oryzae* pv. *oryzae* strains, African ones exhibiting a lower number of DNA fragments (15 to 16) per strain than did the Asian ones (18 to 25) (Fig. 3). Only two haplotypes could be differentiated within *X. oryzae* pv. *oryzae* African strains, and this enabled us to distinguish strains isolated in Mali in 1980 from those isolated in 2003. Strains originating from Burkina and Niger showed the same pattern as those isolated in Mali and Cameroon in the 1980s. Five haplotypes were observed among the African *X. oryzae* pv. *oryzicola* strains that are clearly distinct from those exhibited by *X. oryzae* pv. *oryzicola* Asian strains. Finally, none of the *Xanthomonas translucens* strains hybridized with the pJEL101 probe. Hybridization with probe pBSTnX1 revealed 15 different-sized bands ranging between 15 and 2 kb (data not shown). *X. oryzae* pv. *oryzae* African strains did not hybridize with pBSTnX1, confirming previous results by Ryba-White and associates (1995). Three haplotypes were observed among *X. oryzae* pv. *oryzicola* strains with a pattern showing only 1 or 2 bands (data not shown).

Probe pBS*avrXa10* resolved 24 haplotypes among *X. oryzae* pv. *oryzae* and six among *X. oryzae* pv. *oryzicola* strains (Table 1). Again, haplotypes clearly differentiated *X. oryzae* pv. *oryzae* African and Asian strains (Fig. 4A). African *X. oryzae* pv. *oryzae* strains exhibited four specific and different haplotypes (Fig. 4B), with a lower number of DNA fragments (7 to 8) per strain than Asian *X. oryzae* pv. *oryzae* ones (10 to 13). Finally, four other conserved T3SS effector (*avrBs2* and *XOO1762*) and associated (*hpa*) *X. oryzae* pv. *oryzae* genes (*hpaF* and *hpa1*), as well as *avrRxo1*, which encodes a novel type III effector protein recently characterized in *X. oryzae* pv. *oryzicola* BLS256, were used as probes. *hpa1* and *XOO1762* differentiated *X. oryzae* strains at the pathovar level. The most discriminative probe was *XOO1762*, which enabled us to set apart African from Asian *X. oryzae* pv. *oryzae* strains. Five *X. oryzae* pv. *oryzae* African strains were tested and were found to exhibit the same haplotype, with four bands ranging from 4 to 12 kb (data not shown).

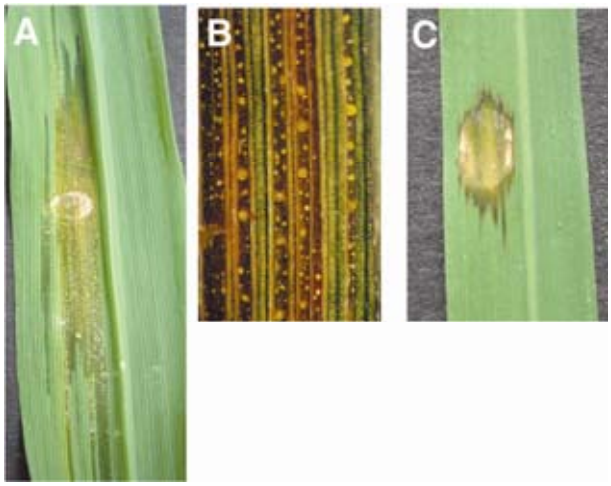

Fig. 1. Disease symptoms observed upon leaf infiltration of the rice susceptible variety Nipponbare ten days after inoculation with **A,** *Xanthomonas oryzae* pv. *oryzicola* MAI3. **B,** Bacterial leaf streak (BLS) symptoms with droplets of bacterial exudates (close-up of A). **C,** Symptoms observed after inoculation with *X. oryzae* pv. *oryzae* BAI4.

Table 2. Reactions of near isogenic lines with single bacterial-resistance genes (*Xa*) against *Xanthomonas oryzae* pv. *oryzae* African strains[a]

NILS	*Xa*-gene	Race A1[b]	Race A2[c]	Race A3[d]
IR24		S	S	R
IRBB3	*Xa3*	S	R	R
IRBB4	*Xa4*	R	R	R
IRBB5	*xa5*	R	R	R
IRBB7	*Xa7*	R	R	R
IRBB8	*xa8*	MS	R	R
IRBB10	*Xa10*	S	MR	R
IRBB11	*Xa11*	S	R	R
IRBB13	*xa13*	S	MR	R
IRBB14	*Xa14*	S	MR	R
IRBB21	*Xa21*	MS	MR	R

[a] Resistance or susceptibility of rice plants to *X. oryzae* pv. *oryzae* is expressed in lesion lengths measured 14 days after inoculation. Resistant (R) < 5 cm, moderately resistant (MR) = 5 to 10 cm, moderately susceptible (MS) = 10 to 15 cm, and susceptible (S) > 15 cm.
[b] Strains NAI1 to NAI9, BAI1, BAI2, BAI3, CFBP1947, CFBP1948.
[c] Strain BAI4.
[d] Strains MAI1, MAI2, MAI9, CFBP1949, CFBP1951, CBFP1952.

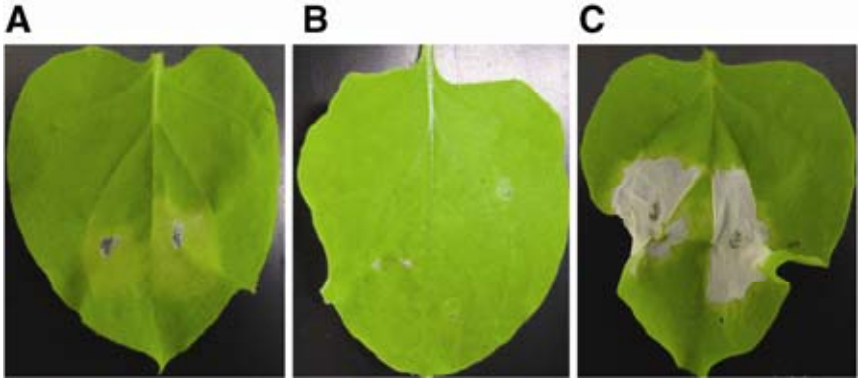

Fig. 2. *Xanthomonas oryzae* pv. *oryzae* nonhost hypersensitive response induction assay in *Nicotiana benthamiana*. Leaves were infiltrated with **A,** water, **B,** *X. oryzae* pv. *oryzae* Asian strain PXO339, and **C,** *X. oryzae* pv. *oryzae* African strain BAI3, at approximately 1×10^9 CFU/ml. Photographed 48 h after infiltration.

Seven haplotypes were obtained among the nine *X. oryzae* pv. *oryzicola* strains analyzed, which clearly differentiated African and Asian strains. Finally, while *avrRxo1* probe hybridized with Asian *X. oryzae* pv. *oryzicola* strains, no band could be detected in *X. oryzae* pv. *oryzicola* African strain, except for MAI3. No homologs could either be detected in *X. oryzae* pv. *oryzae* Asian or African tested strains (data not shown).

Rep-PCR analyses confirm RFLP results.

Repetitive sequence–based (Rep)-PCR genomic fingerprints generated with the Rep and enterobacterial repetitive intergenic consensus (ERIC) primers from the entire set of *Xanthomonas* strains enabled the amplification of 35 and 49 bands, respectively, with sizes ranging from 4,500 to 200 bp (Fig. 5). Rep- and ERIC-PCR–derived DNA fingerprints enabled us to set apart *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *X. translucens* strains (Fig. 5 and data not shown). For both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, they also clearly distinguished African from Asian strains, confirming thereby our RFLP analysis (Fig. 5). ERIC and Rep-PCR defined 18 and 13 haplotypes among *X. oryzae* pv. *oryzae* strains, respectively (Table 1). ERIC primers allowed the amplification of 7 to 16 different-sized bands per *X. oryzae* pv. *oryzicola* strain, and seven distinct haplotypes were inferred (Table 1). Amplification with the Rep primers yielded eight haplotypes among *X. oryzae* pv. *oryzicola* strains with 9 to 19 bands per strain (Fig. 5). Finally, Rep- and ERIC-PCR analysis of *X. translucens* strains defined seven haplotypes (data not shown) clearly distinct from the ones exhibited by *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains.

***X. oryzae* pv. *oryzae* African strains form a separate group distant from *X. oryzae* pv. *oryzae* Asian strains.**

To study the genetic relationships between *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains, RFLP and Rep-PCR) haplotypes were first analyzed separately by cluster analysis. Dendrograms were generated for the data sets obtained from RFLP analysis (probes pBS*avrXa10*, pJEL101, and pBSTnX1) and Rep- and ERIC-PCR (data not shown). Visual inspection of these dendrograms suggested that the interrelationships between strains were dependent on the technique used. However, in all cases, obtained groups of strains clearly separated *X. oryzae* pv. *oryzae* African strains, *X. oryzae* pv. *oryzae* Asian strains, and *X. oryzae* pv. *oryzicola* strains. Identical distribution was inferred from the RFLP data obtained with pBS*avrXa10*, pJEL101, and pBSTnX1 probes. While *X. oryzae* pv. *oryzae* Asian strains grouped into several clusters (four with pJEL101, seven with pBS*avrXa10*, and nine with pBSTnX1), *X. oryzae* pv. *oryzae* African ones grouped into a single cluster at 70% similarity (data not shown). *X. oryzae* pv. *oryzicola* strains grouped into different clusters (three with pBS*avrXa10* and pBSTnX1, five with pJEL101) at 70% similarity (data not shown), highlighting the high diversity observed among *X. oryzae* pv. *oryzicola* African strains. The dendrogram generated by ERIC-PCR data defined 16 clusters at 70% similarity (Fig. 6A), with *X. oryzae* pv. *oryzae* strains grouping into five clusters, one of them comprising all *X. oryzae* pv. *oryzae* African strains. Very similarly, Rep-PCR inferred data defined seven *X. oryzae* pv. *oryzae* strain clusters, one of which grouped all the African strains (data not shown). ERIC- and Rep-PCR dendrograms grouped *X. oryzae* pv. *oryzicola* strains into four and five clusters at 70% similarity, respectively (Fig. 6A). African and Asian strains of *X. oryzae* pv. *oryzicola* were grouped in distinct clusters. *X. translucens* strains each grouped in separate clusters, different from *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* ones (Fig. 6A). Finally, the dendrogram derived from all the combined RFLP and Rep-PCR data grouped the strains in 20 clusters at 70% similarity (Fig. 6B). *X. oryzae* pv. *oryzae* strains clustered into nine groups, with one regrouping all African *X. oryzae* pv. *oryzae* strains. African and Asian *X. oryzae* pv. *oryzicola* strains were clustered into five groups at 70% similarity (Fig. 6B). Altogether our data demonstrate that *X. oryzae* pv. *oryzae* African strains form a separate group genetically distinct from *X. oryzae* pv. *oryzae* Asian strains.

Fluorescent amplified fragment-length polymorphism (FAFLP) identifies *X. oryzae* pv. *oryzae* African strains as a separate group.

To confirm their relatedness, a set of selected strains of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* together with

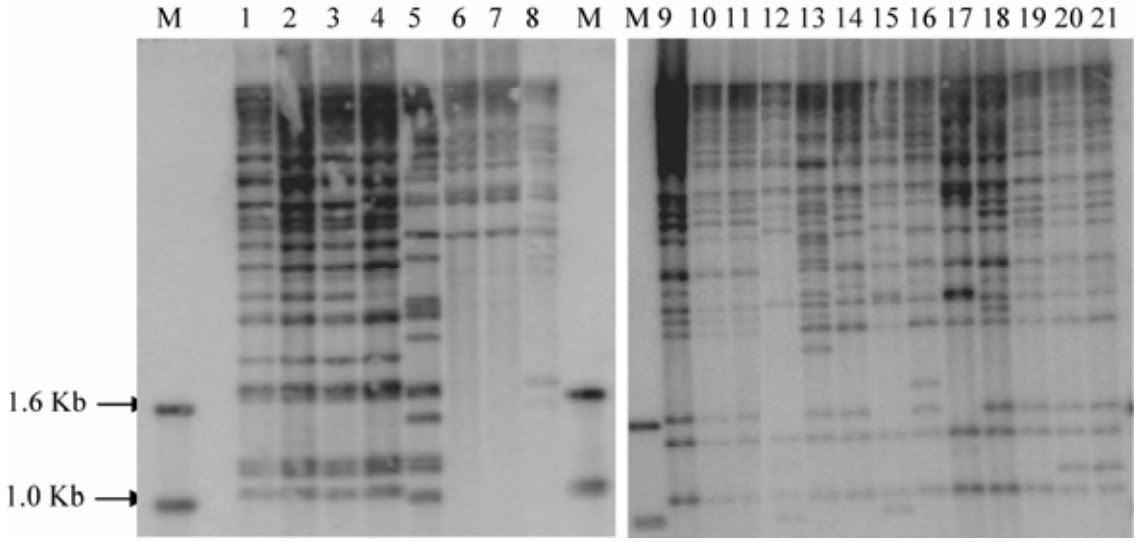

Fig. 3. Hybridization patterns obtained with probe pJEL101 on *Eco*RI-digested DNA of *Xanthomonas oryzae* strains, 1, *X. oryzae* pv. *oryzicola* CFBP2286; 2, *X. oryzae* pv. *oryzicola* MAI13; 3, *X. oryzae* pv. *oryzicola* MAI12; 4, *X. oryzae* pv. *oryzicola* MAI11; 5, *X. oryzae* pv. *oryzicola* MAI10; 6, *X. oryzae* pv. *oryzae* BAI4; 7, *X. oryzae* pv. *oryzae* NAI8; 8, *X. oryzae* pv. *oryzae* BAI1; 9, *X. oryzae* pv. *oryzae* PXO448; 10, *X. oryzae* pv. *oryzae* PXO345; 11, *X. oryzae* pv. *oryzae* PXO339; 12, *X. oryzae* pv. *oryzae* A3857; 13, *X. oryzae* pv. *oryzae* A3842; 14, *X. oryzae* pv. *oryzae* XOO212; 15, *X. oryzae* pv. *oryzae* NXO622; 16, *X. oryzae* pv. *oryzae* NXO537; 17, *X. oryzae* pv. *oryzae* MXO92; 18, *X. oryzae* pv. *oryzae* IXO60; 19, *X. oryzae* pv. *oryzae* IXO57; 20, *X. oryzae* pv. *oryzae* HN35; and 21, *X. oryzae* pv. *oryzae* HB18. M = 1-kb$^+$ ladder.

strains belonging to other pathovars of *Xanthomonas* were analyzed by FAFLP. The reproducibility of FAFLP patterns was first evaluated by performing several independent DNA extractions, amplifications, and runs with strains CFBP2532, CFBP2286, and CFBP1121. These strains were then incorporated in the global analysis, and only runs with reproducible patterns were analyzed. Totals of 13 to 42 bands and 23 to 39 bands per strain were obtained with the selective primers *Msp*I+TA and *Msp*I+TG, respectively. Size of the bands varied between 50 and 494 bp. A total of 453 fragments were analyzed in the global study, each strain producing a unique pattern. FAFLP analysis generated 184 fragments for *X. oryzae* strains, 92% of these fragments being polymorphic. The number of polymorphic bands among *X. oryzae* pv. *oryzae* strains (55%) was similar to that of *X. oryzae* pv. *oryzicola* strains (49%). Level of polymorphism was similar among African and Asian *X. oryzae* pv. *oryzae* strains (53 and 50%, respectively). The analysis generated 188 fragments for the *X. transluscens* strains, 98% of them being polymorphic. The intraspecies diversity was not assessed for *X. axonopodis*, *X. campestris*, or *X. hiacinthi* because of a too-low number of strains used in the analysis. All strains of each bacterial species formed separate groups with high bootstrap values (89% to 100%) (Fig. 7). Strains of *X. oryzae* separated into three groups with significant bootstrap values (100). *X. oryzae* pv. *oryzae* Asian strains clustered in group 1, *X. oryzae* pv. *oryzae* African strains formed group 2, and *X. oryzae* pv. *oryzicola* African strains clustered in group 3 together with *X. oryzae* pv. *oryzicola* Asian strains (Fig. 7 and Table 1). The *X. oryzae* strains were clearly differentiated from the *X. translucens* group, confirming results obtained by RFLP and Rep-PCR.

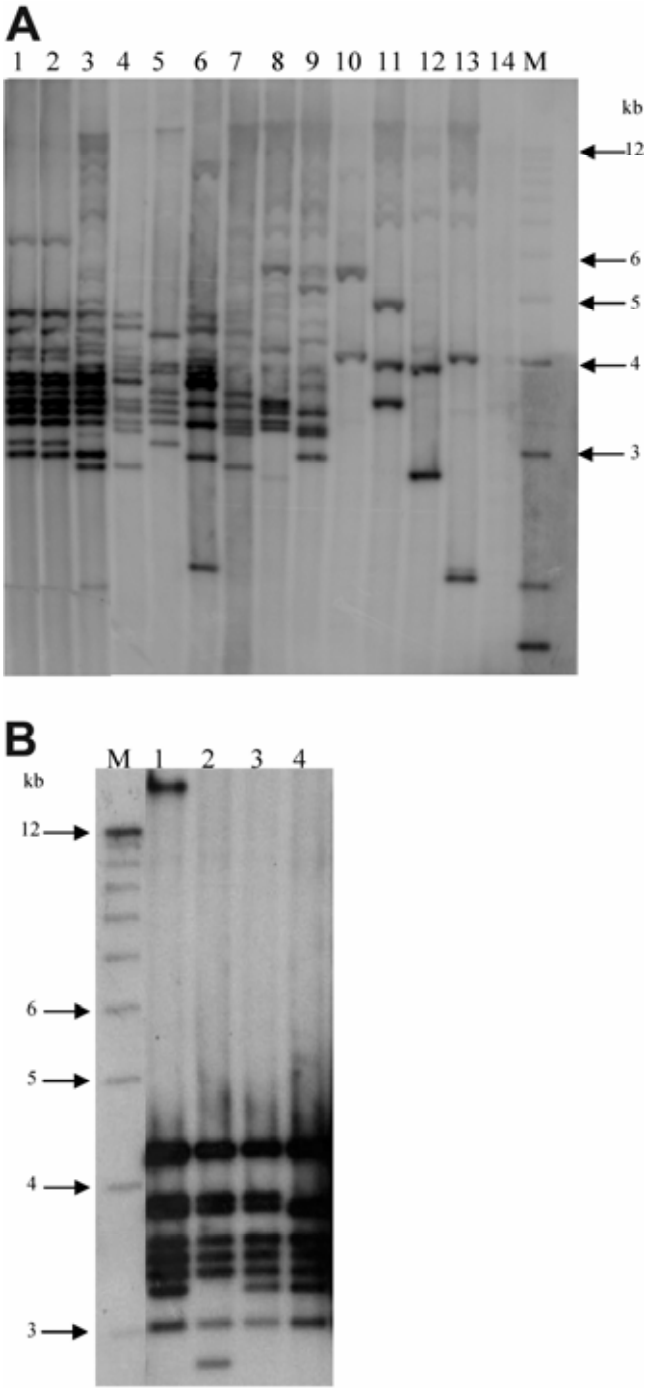

Fig. 4. A, Hybridization patterns obtained with probe pBS*avrXa10* on *Bam*HI-digested DNA obtained from the following strains. 1, *Xanthomonas oryzae* pv. *oryzicola* CFBP2286; 2, *X. oryzae* pv. *oryzicola* UPB497; 3, *X. oryzae* pv. *oryzicola* MAI11; 4, *X. oryzae* pv. *oryzae* NXO537; 5, *X. oryzae* pv. *oryzae* CFBP1949; 6, *X. oryzae* pv. *oryzae* PXO339; 7, *X. translucens* pv. *undulosa* UPB513; 8, *X. translucens* pv. *secalis* UPB469; 9, *X. translucens* pv. *hordei* UPB458; 10, *X. translucens* pv. *arrhenatheri* UPB455; 11, *X. translucens* pv. *poae* UPB454; 12, *X. translucens* pv. *cerealis* UPB453; 13, *X. translucens* pv. *phleipratensis* UPB441; and 14, *X. translucens* pv. *graminis* UPB437. M = 1-kb$^+$ ladder. **B,** Close-up of the *avrXa10* groups obtained with *X. oryzae* pv. *oryzae* African strains. 1. strain MAI9, group 22^{avr}, 2. strain BAI3, group 23^{avr}, 3. strain MAI1, group 21^{avr}, and 4. strain NAI1, group 24^{avr}, M = 1-kb$^+$ ladder.

DISCUSSION

X. oryzae isolates were collected on rice from different countries in West Africa and were characterized in detail by DNA and pathotypic analysis. Our work led to the identification and characterization of new *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains in Africa. Interestingly, molecular analyses showed that *X. oryzae* pv. *oryzae* African strains form a group genetically distant from *X. oryzae* pv. *oryzae* Asian

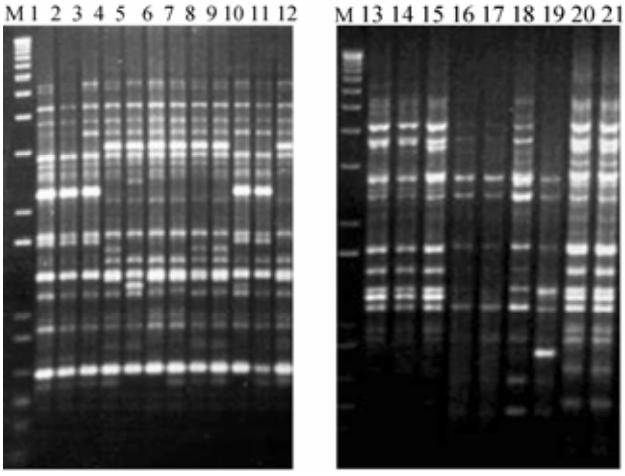

Fig. 5. Polymerase chain reaction (PCR) fingerprint patterns from genomic DNA of *Xanthomonas oryzae* strains obtained by repetitive-PCR. 1, *X. oryzae* pv. *oryzae* PXO61; 2, *X. oryzae* pv. *oryzae* PXO71; 3, *X. oryzae* pv. *oryzae* CIAT1186; 4, *X. oryzae* pv. *oryzae* PXO86; 5, *X. oryzae* pv. *oryzae* PXO99; 6, *X. oryzae* pv. *oryzae* CIAT1185, 7, *X. oryzae* pv. *oryzae* PXO112; 8, *X. oryzae* pv. *oryzae* PXO145; 9, *X. oryzae* pv. *oryzae* PXO280; 10, *X. oryzae* pv. *oryzae* PXO339; 11, *X. oryzae* pv. *oryzae* PXO341; 12, *X. oryzae* pv. *oryzae* CFBP1951; 13, *X. oryzae* pv. *oryzae* CFBP 1947; 14, *X. oryzae* pv. *oryzae* CFBP1948; 15, *X. oryzae* pv. *oryzae* CFBP1949; 16, *X. oryzae* pv. *oryzicola* MAI5; 17, *X. oryzae* pv. *oryzicola* MAI6; 18, *X. oryzae* pv. *oryzicola* MAI4; 19, *X. oryzae* pv. *oryzicola* MAI3; 20, *X. oryzae* pv. *oryzicola* MAI1; and 21 *X. oryzae* pv. *oryzicola* MAI2. M, 1-kb$^+$ ladder.

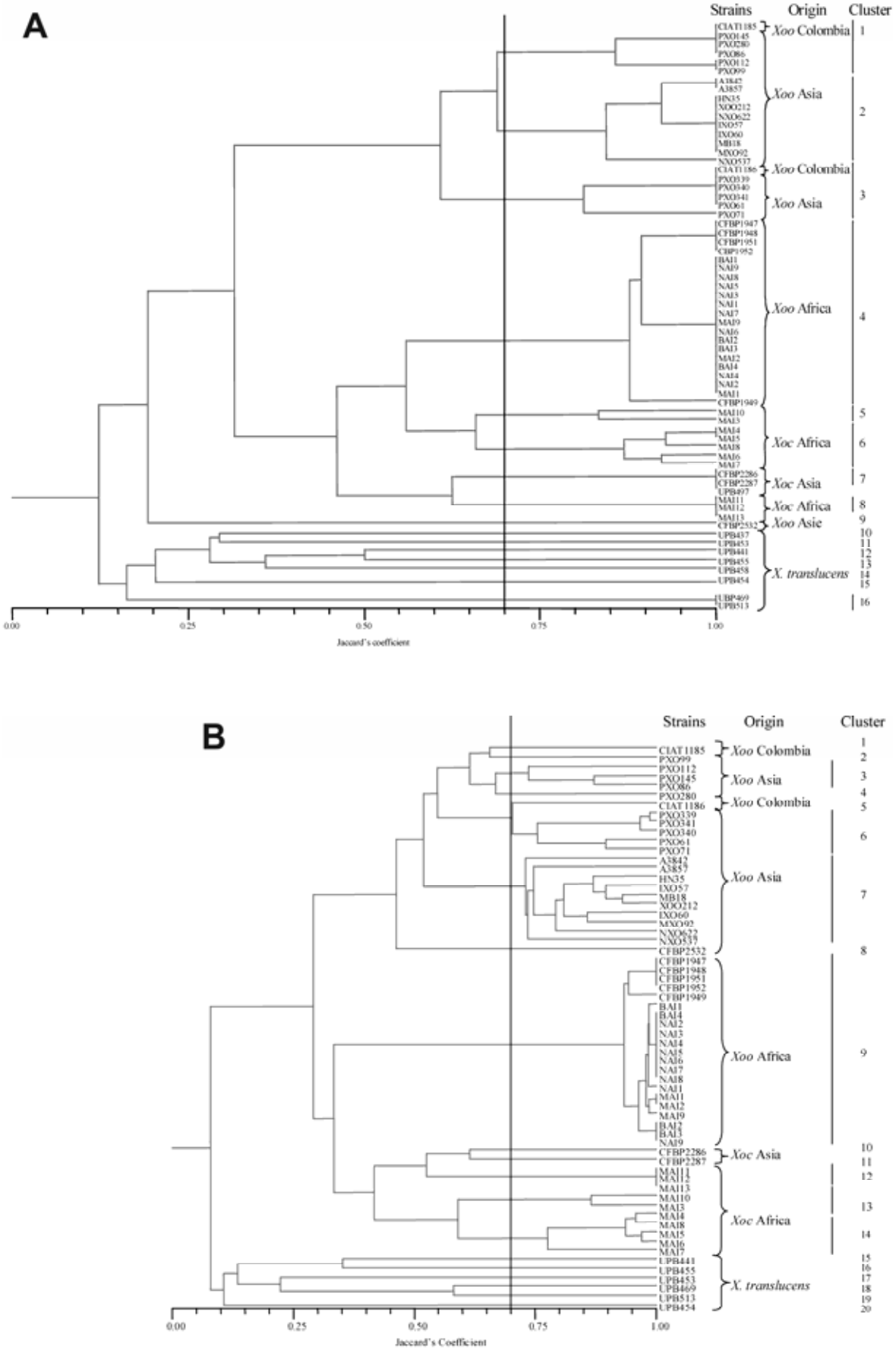

Fig. 6. Dendrograms obtained by cluster analysis using the unweighted pair-group arithmetic mean method by the CLUSTER statistical procedure of SAS, clusters were delineated at an arbitrary level of similarity =0.70. **A,** Enterobacterial repetitive intergenic consensus polymerase chain reaciton (ERIC-PCR). **B,** Combination of restriction fragment length polymorphism (RFLP) and PCR data.

strains, while pathotypic analysis led to the identification of three new *X. oryzae* pv. *oryzae* races. As compared with their Asian relatives, specific features of *X. oryzae* pv. *oryzae* African strains include i) a reduction of several molecular markers analyzed in this study, i.e., insertion sequence (IS) element IS*1112* and TAL (*avrBs3*/*pthA*) effector genes, ii) the absence of *TnX1* (IS*1113*), an IS found to be conserved in all Asian *X. oryzae* pv. *oryzae* strains tested so far, and iii) the capacity to induce nonhost HR in *N. benthamiana* leaves. In addition and to our knowledge, our study constitutes the first report on the characterization of *X. oryzae* pv. *oryzicola* strains in Africa.

X. oryzae pv. *oryzae* African strains cluster in a genetic group distinguishable from Asian ones.

To gain insight into the genetic diversity and evolutionary history of *X. oryzae* isolates in West Africa, DNA polymorphism characterization based on RFLP, Rep-PCR, and FAFLP analyses were performed. RFLP analyses were achieved using IS and T3SS effector (or associated) genes as probes, since they are presumably phenotype-neutral repetitive elements and involved in host-pathogen interaction, respectively. IS element IS*1112* and IS*1113* probes were previously found by Nelson and associates (1994) to be the most discriminative for phylogenetic analysis of a collection of *X. oryzae* pv. *oryzae* strains from the Philippines and, therefore, were used in this study. *avrXa10* is an avirulence gene of the *avrBs3*/*pthA* multiple gene family, some members of which are essential for *X. oryzae* pv. *oryzae* full pathogenicity (Hopkins et al. 1992, Niño-Liu et al. 2006). Both IS*1112* and *avrXa10* probes effectively enabled to differentiate African and Asian strains within *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* pathovars, respectively. In addition, new markers were developed using

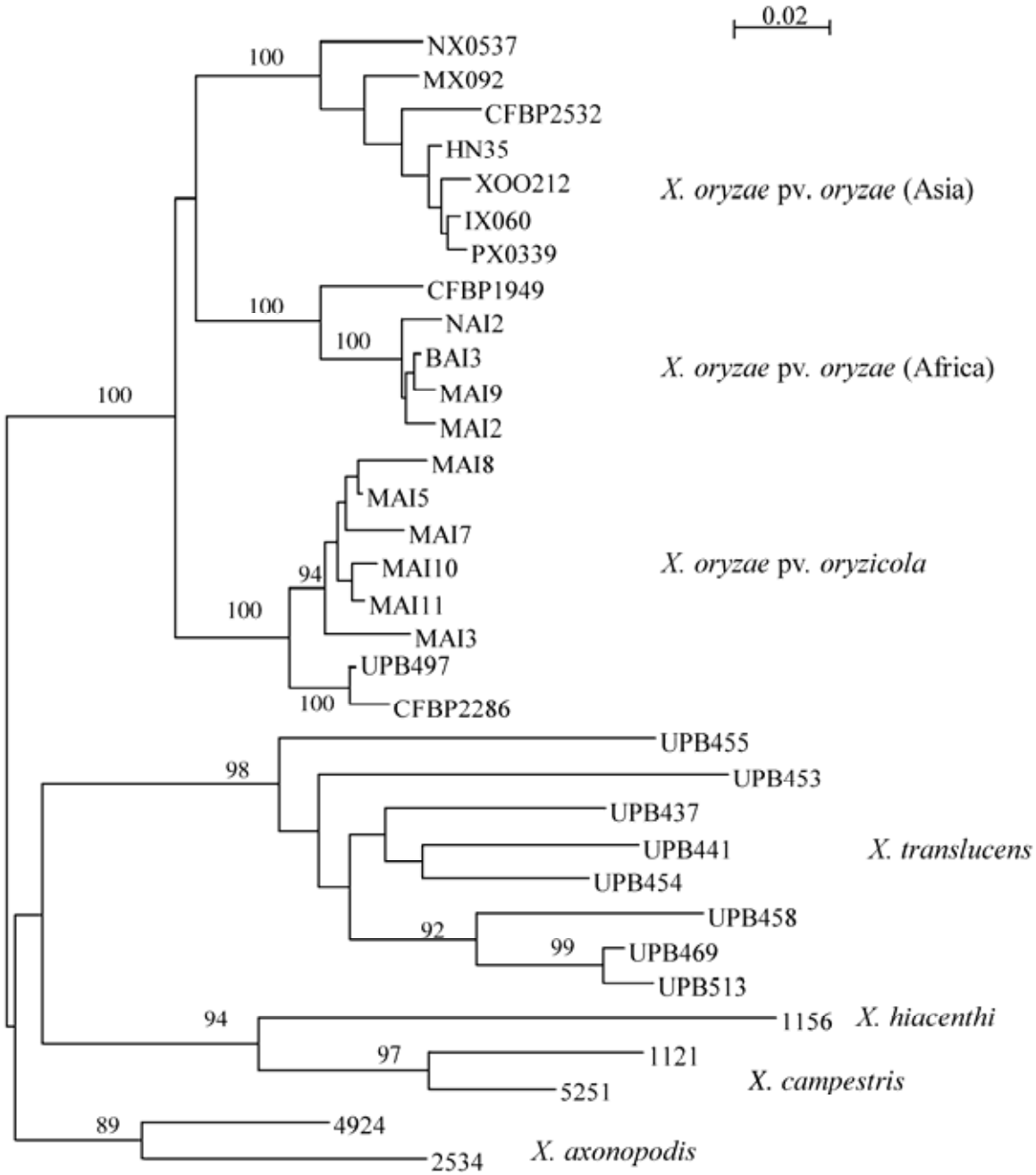

Fig. 7. Dendrogram obtained by fluorescent amplified fragment-length polymorphism (FAFLP) analysis of *Xanthomonas* strains. Bootstrap values are indicated on the root branching.

type III effector (*avrBs2*, *XOO1762*, and *avrRxo1*) and *hrp*-associated (*hpa1* and *hpaF*) genes. *XOO1762*, which encodes a protein with leucine-rich repeat motifs (Lee et al. 2005), appeared to be the most discriminative at the pathovar level and, relatively, to the geographical origin (Africa or Asia), while the harpin-like protein–encoding gene *hpa1* (Zhu et al. 2000) enabled us to set apart African strains from Asian ones in *X. oryzae* pv. *oryzicola*. Data were successfully confirmed by Rep-PCR analysis but also by FAFLP. Cluster analysis of the combined data deriving from our DNA polymorphism study revealed *X. oryzae* pv. *oryzae* African strains as a clear separate genetic group, as compared with reference strains representative of the high degree of genetic diversity among *X. oryzae* pv. *oryzae* in Asia (Adhikari et al. 1995). Visual inspection of each RFLP data–inferred dendrogram indicated that African *X. oryzae* pv. *oryzae* strains and *X. oryzae* pv. *oryzicola* ones always tend to group together on the same branch, while Asian *X. oryzae* pv. *oryzae* strains all clustered apart. This suggests that African *X. oryzae* pv. *oryzae* strains are more related to *X. oryzae* pv. *oryzicola* strains than to *X. oryzae* pv. *oryzae* Asian ones, although pathogenicity assays undoubtedly discriminated *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* African strains as such.

African *X. oryzae pv. oryzae* strains display specific features.

Based on the Asian strains molecular analysis and whole-genome sequencing data, two main characteristics of *X. oryzae* pv. *oryzae* genome, as compared with other xanthomonads, are i) a higher abundance of IS elements and ii) the prevalence of effector genes of the *avrBs3/pthA* family (Ochiai et al. 2005; Niño-Liu et al. 2006). With respect to these two criteria, our data show that African *X. oryzae* pv. *oryzae* strains behave differently, since they carry fewer of both of these two types of genes. Indeed, IS*1112* content is fairly reduced within *X. oryzae* pv. *oryzae* African strains in comparison to Asian ones and there is no IS*1113* (TnX1) copy in their genome, while Asian strains all bear an abundant number of the latter. The role of IS elements in Asian *X. oryzae* pv. *oryzae* race evolution was reported earlier and was recently documented in the genome analysis of two Asian *X. oryzae* pv. *oryzae* strains (Leach et al. 1990, 1992; Lee et al. 2005; Nelson et al. 1994; Ochiai et al. 2005). IS elements are often located near strain-specific genes with distinct GC content, indicating that adjacent genes have been acquired through horizontal gene transfer. The presence of large amounts of transposable elements in a genome may confer adaptive evolution by generating recombination-based rearrangements, as it was hypothesized for *Yersinia* spp. and *Bulkholderia* spp. evolution (Chain et al. 2004; Nierman et al. 2004).

Moreover, *X. oryzae* pv. *oryzae* African strains were all shown to carry up to eight *avrBs3/pthA* members, while up to 16 copies were detected in some Asian strains (Leach et al. 1990; Yang and Gabriel 1995). *avrBs3/pthA*-like genes are widely spread within *Xanthomonas* spp., but this family of effectors seems to have specifically expanded in the *X. oryzae* pv. *oryzae* genome. The function of each of these effector genes is so far unknown, with some exceptions, and the reason why they are so abundant in *X. oryzae* pv. *oryzae* remains unclear. Interestingly, recent data by Yang and White (2004) demonstrated that among all the repertoire of TAL effector genes present in a given strain, only one or two play a crucial role as pathogenicity factors. The function of the other gene members is presumed to be to build up a reservoir of genes in the genome or have moderate or minor function in virulence. Indeed, spontaneous recombination has been proved to take place in *X. oryzae* pv. *oryzae*, albeit with low probability, and to generate new effectors contributing to virulence but not to avirulence (Yang and White 2004). It is hypothesized that the expansion of *avrBs3/pthA* genes throughout the genome reflects a strong adaptive pressure imposed on the pathogen population, due to intensive cultivation and selection of the crop (Yang and Gabriel 1994; Yang and White 2005). Consequently, the differences observed between African and Asian *X. oryzae* pv. *oryzae* strains might be explained by differences with respect to rice cultivation in Africa. The reduced number of *avrBs3/pthA* genes in African *X. oryzae* pv. *oryzae* strains makes them extremely attractive for a functional analysis of this crucial family of effectors in a unique evolutionary context.

Another specific feature of African *X. oryzae* pv. *oryzae* strains is their capacity to induce a nonhost HR when infiltrated with a high-titer inoculum in *N. benthamiana* leaves, while none of the Asian *X. oryzae* pv. *oryzae* (or *X. oryzae pv. oryzicola*) tested ones did. Provided that this phenotype is T3SS-dependent (experiments are in progress in our lab), our data suggest that African *X. oryzae* pv. *oryzae* strains display one or several specific nonhost HR elicitors, such as type III effectors or harpins. Alternatively, *X. oryzae* pv. *oryzae* Asian and *X. oryzae* pv. *oryzicola* strains may harbor specific effectors inhibiting plant defense responses, as recently demonstrated for several *X. oryzae* pv. *oryzae* AvrBs3/PthA effectors that suppress nonhost HR in tobacco (Fujikawa et al. 2006) and so-far-unknown *X. oryzae* pv. *oryzicola* factors inhibiting *R* gene–specific defense responses against *X. oryzae* pv. *oryzae* in rice (Makino et al. 2006). Nonhost HR-inducing ability would offer an easy tool for the isolation of African *X. oryzae* pv. *oryzae*-specific effector genes upon gain-of-function complementation assay of an Asian *X. oryzae* pv. *oryzae* strain.

African *X. oryzae* pv. *oryzicola* strains are deficient for *avrRxo1*.

Until our study, *avrRxo1* effector gene was found to be present in all *X. oryzae* pv. *oryzicola* Asian strains tested, suggesting that it may encode a critical fitness or pathogenicity factor (Zhao et al. 2004a), as is the case, for example, of the *Xanthomonas* spp.–wide conserved *avrBs2* gene (Kearney and Staskawicz 1990). The identification of *X. oryzae* pv. *oryzicola* strains that do not harbor any *avrRxo1* copy (with the exception of strain MAI3) is therefore intriguing and shows how important are population structure and evolution studies prior to the deployment of *R* gene–containing varieties, as it would be the case for the *Rxo1* nonhost matching *R* gene. Did African *X. oryzae* pv. *oryzicola* strains lose *avrRxo1*? Do they harbor another effector that functionally compensates for *avrRxo1* absence? Clues may be obtained from sequence analysis at the *avrRxo1* locus in MAI3 and nonhybridizing strains as well as upon further population structure studies. A high degree of genetic diversity was observed among African strains of *X. oryzae* pv. *oryzicola*, similar to what has been inferred from the analysis of *X. oryzae* pv. *oryzicola* strains isolated in the Philippines (Raymundo et al. 1999). Although visual inspection of RFLP and Rep-PCR data clearly defined specific haplotypes for *X. oryzae* pv. *oryzicola* African strains, we cannot exclude the possibility that they originated in Asia and were introduced to Africa together with contaminated germplasm.

Virulence assays reveal the existence of three new *X. oryzae* pv. *oryzae* races among African strains.

Although *X. oryzae* pv. *oryzae* African and Asian strains were found to provoke similar symptoms on the susceptible cultivar Nipponbare, virulence assays performed on a set of NILs revealed three *X. oryzae* pv. *oryzae* African races that do not represent any of the *X. oryzae* pv. *oryzae* Asian races characterized so far. Race A3 is incompatible on all tested NILs,

suggesting that A3 strains, which all originate in Mali, harbor an *avr* gene that would be specifically recognized by IR24 lines and, consequently, all derived NILs. Indeed, presence of a resistance gene (*Xa18*) in IR24 has been reported in the literature (Noda et al. 1996). The next challenge will be the characterization of *X. oryzae* pv. *oryzae* population structure in Africa, and race screening will provide important information to that end, although the NILs used here seem to be well-adapted only for African *X. oryzae* pv. *oryzae* race A1. The development of appropriate NILs in an African background genotype compatible with races A2 and A3 is therefore an essential prerequisite for such study. Race A1 strains show *avrXa4*, *avrxa5*, and *avrXa7* activities on matching *Xa4*-, *xa5*-, and *Xa7*-containing lines. Such *R* genes could be recommended for use in breeding programs but with great caution. For instance, the large-scale and long-term cultivation of varieties carrying *Xa4* resulted in significant shifts in the race frequency of *X. oryzae* pv. *oryzae* (Mew et al. 1992; Vera Cruz et al. 2000).

What could be the evolutionary history of *X. oryzae* African strains?

In many aspects, the African *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains described in this study differ from their Asian counterparts. Altogether, our data are suggestive of a different evolutionary history for *X. oryzae* pv. *oryzae* strains originating from West Africa, where the rice species *Oryzae glaberrima* originated and was domesticated. An important parameter known to shape pathogen population structure evolution and genome dynamic is the intensification of modern agriculture, which is a relatively recent event in Africa, with respect to rice cultivation, as compared with Asia. Indeed, extensive cultivation of rice started only in the 1960s in West Africa and BB was reported 20 years after. In addition, no breeding program for resistance to BB has ever been conducted in that region. It is therefore likely that, because African *X. oryzae* pv. *oryzae* strains interactions with cultivated rice lines is rather recent, selective pressure for adaptation to the host is only starting. Although only a very small fraction of the *X. oryzae* pv. *oryzae* population has been analyzed in our study, it is appealing to speculate about their origin and evolution. *X. oryzae* pv. *oryzae* African strains were compared with *X. oryzae pv. oryzae* strains from different regions in Asia without finding any common haplotypes or races, but we cannot exclude a possible introduction from another continent through contaminated germplasm. *X. oryzae* pv. *oryzicola* strains isolated in Mali were found to be closely related to *X. oryzae* pv. *oryzicola* strains from Malaysia, suggesting possible transfer between the two continents. Given the striking features of *X. oryzae* pv. *oryzae* African strains and absence of visible relatedness with *X. oryzae* pv. *oryzae* Asian strains, a tempting hypothesis is that they are endemic from Africa. Clues to their specificity may certainly be obtained from further genome analysis such as suppression subtractive hybridization, an approach which has been undertaken in our laboratory to compare *X. oryzae* pv. *oryzae* African and Asian genomes. Sequencing the whole genome of an African *X. oryzae* pv. *oryzae* strain should also reveal precious information about its origin and evolution. Indeed, complete genome sequences are now available for four *X. oryzae* pv. *oryzae* Asian strains and, soon, one Asian strain of *X. oryzae* pv. *oryzicola*, thus facilitating comparative genomic analysis and identification of strain-specific genetic determinants (Niño-Liu et al. 2006).

Our study is a first step towards the characterization of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* populations in West Africa. Extensive pathogen-population analyses are needed and will provide clues to better understand the origin, biology, and evolution of *Xanthomonas oryzae* pathovars in Africa. Such studies have important implications in rice breeding programs. Resistance genes urgently need to be identified for developing a breeding strategy for durable BB and BLS rice resistance in Africa.

MATERIALS AND METHODS

Bacterial strains and media.

X. oryzae pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and other *Xanthomonas* strains were obtained from different origin and collections (Table 1). New strains were isolated in 2003 from diseased leaf samples of rice plants collected in West Africa (Mali, Niger, and Burkina-Faso). The field visit was conducted during the rainy season, when symptoms were visible. Samples were collected from farm and experimental fields and were collected from improved *Oryza sativa* cultivars, except strain BAI4 from *O. glaberrima*. The leaves were held at –20°C until bacterial isolations were conducted. Bacteria were isolated from infected leaves as described (Adhikari et al. 1994). Of each sample, a single bacterial colony was purified on PSA (10 g of peptone, 10 g of sucrose, 1 g of glutamic acid, 16 g of agar per liter of H_2O). The isolates were maintained in 15% glycerol at –80°C. For the DNA extraction, the bacteria were grown overnight in nutrient broth, with shaking, at 28°C.

Pathogenicity assays.

All field isolates were tested for virulence on Nipponbare by leaf-clip inoculation. *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains obtained from international collections were included as control. Experiments were conducted under greenhouse conditions at 26°C and 80% relative humidity. Plants were inoculated at 45 days after sowing, using a clipping method (Kauffman et al. 1973). Bacteria for the inoculum were prepared as described previously (Kauffman et al. 1973). Lesion lengths were measured 15 days postinoculation with a ruler. All plant-inoculation experiments contained two replications with 10 plants and two fully expanded leaves inoculated per plant and were repeated at least three times. Disease reactions were classified according to the mean lesion length (LL) as follows: resistant (R), LL < 5 cm; moderately resistant (MR), LL = 5 to 10 cm; moderately susceptible (MS), LL = 10 to 15 cm; and susceptible (S), LL > 15 cm.

All the strains were also inoculated by leaf infiltration. Leaves of 30-day-old plants (rice variety Nipponbare) were infiltrated with a bacterial suspension (approximately 1×10^9 CFU/ml) prepared from 48-h-old PSA plate cultures with a needleless syringe as previously described (Reimers and Leach 1991). Leaves were infiltrated with sterile water and used as controls. The plants were maintained for 2 days after inoculation in a growth chamber with high relative humidity (90%). Disease reaction after inoculation was observed at 2-day intervals starting at 5 days after inoculation, and lesion length was measured 10 days after inoculation. All plant-inoculation experiments contained three replications with one plant, two fully expanded leaves inoculated per plant, and two infiltrations per leaf. All the experiments were repeated twice.

The *X. oryzae* pv. *oryzae* strains were tested on a series of NILs carrying a defined resistance gene backcrossed into the susceptible cultivar IR24 (IR-BB3 [*Xa-3*], IR-BB4 [*Xa-4*], IR-BB5 [*xa-5*], IR-BB7 [*Xa-7*], IR-BB8 [*xa-8*], IR-BB10 [*Xa-10*], IR-BB11 [*Xa-11*], IR-BB13 [*Xa-13*], IR-BB14 [*Xa-14*], IR-BB21 [*Xa-21*]), and with IR24 used as a susceptible check. *X. oryzae* pv. *oryzae* PXO339, PXO341, and PXO99 were used as control. ANOVA statistical analyses were performed for all the assays.

For *N. benthamiana* nonhost HR induction assays, 3 week-old plants were leaf-infiltrated with a bacterial suspension of approximately 1×10^9 CFU/ml and were examined for appearance of nonhost HR-like responses 24 to 48 h after infiltration. These experiments were reproduced three times on independent individuals.

RFLP.

Genomic DNA was extracted by the method of Boucher and associates (1985) and concentration was estimated with a spectrophotometer. For each strain, 5 μg of total DNA was digested to completion with the enzyme *Eco*RI, *Pst*I, or *Bam*HI, as described by the manufacturer (New England Biolabs Inc., Saint Quentin, France). Digested DNA underwent electrophoresis in 0.8% agarose gels in 1× Tris-borate EDTA (TBE) buffer at 35 V for 15 h, except for gels of digested *Bam*HI DNA, which were run for 72 h. Fragments were visualized by UV radiation (302 nm) after staining agarose gels in ethidium bromide (0.5 $\mu g\ ml^{-1}$). A size standard (1-kb^+ ladder) was included in each gel. Fragments were transferred in alkaline solution onto Hybond N^+ membrane (Amersham Biosciences, Saclay, Orsay, France), following manufacturer specifications. Different probes were used in this study. Probe pJEL101 is plasmid pUC18 carrying a 2.4-kb *Eco*RI-*Hin*dIII IS*1112* fragment derived from *X. oryzae* pv. *oryzae* (Leach et al. 1990). Probe pBS*avrXa10* contains an *avrXa10* 3.1-kb *Bam*HI fragment in pBluescript (Hopkins et al. 1992). The DNA probe pBSTnX1 (=IS*1113*) consisted of a 1.05-kb transposable element isolated from *X. oryzae* pv. *oryzae* (Nelson et al. 1994). Probe p*avrRxo1* contains the *avrRxo1* gene from *X. oryzae* pv. *oryzicola* (Zhao et al. 2004b). For *hpaF*, *avrBs2*, *hpa1*, and *XOO1762* analysis, approximately 400-bp amplicons were obtained from *X. oryzae* pv. *oryzae* BAI3, using primers designed according to one or both available PXO99 or KACC10331 sequences and cloned into pGEM-T Easy vector, leading to probes p*hpaF*, p*avrBs2*, p*hpa1*, and p*XOO1762*. Oligonucleotide sequences are available upon request.

The DNA probes were labeled with [α^{32}P] dCTP by random priming (Megaprime, Amersham Biosciences). Conditions of hybridization and washes were of high stringency. Filters were washed once with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) for 20 min, twice with 1× SSC, 0.1% SDS for 10 min, and once with 0.1× SSC, 0.1% SDS for 20 min. Blots were exposed either on the PhosphorImage machine (Molecular Dynamics Storm, Amersham Biosciences, Saclay , Orsay , France) or on X-ray film (Kodak France, Paris), at –80°C with intensifying screens. Each strain was analyzed at least twice, and the RFLP banding patterns were compared in a single blot to confirm that each pattern was unique.

Rep-PCR analysis.

The primers used for Rep and ERIC-PCR amplification were: Rep (REP1R-I: 5′-IIIICGICGICATCIGGC-3′ and REP2-I: 5′-ICGICTTATCIGGCCTAC-3′) and ERIC (ERIC1R: 5′-ATGTA AGCTCCTGGGGATTCAC-3′ and ERIC2: 5′-AAGTAAGTG ACTGGGGTGAGCG-3′). PCR protocols were as described by Vera Cruz and associates (1996), except for the composition of the buffer (100 mM Tris HCl, pH 8.3, 33.5 mM $MgCl_2$, and 500 mM KCl). PCR amplifications were performed in an automated thermal cycler, and the program was as described by Vera Cruz and associates (1996). A sample of 12.5 μl of amplified PCR product was separated by electrophoresis on 1% agarose gels in 1× TBE buffer at 35 V for 15 h, stained with ethidium bromide, and then, photographed on a UV transilluminator. PCR amplifications were done at least two times for each strain for confirmation patterns.

Analysis of RFLP and Rep-PCR data.

Banding patterns of hybridization obtained with the RFLP, Rep-PCR and ERIC-PCR were used to compare the relatedness of strains. The presence of a band was coded as 1 (presence) or 0 (absence). A multiple correspondence analysis, generated by the statistic procedure of SAS (release 8.2 [TS2MO] SunOS 5.8 platform; SAS Software, Cary, NC, U.S.A.), was used to determine the positions of the strains on a three-dimensional graph by use of X^2 distance $[\mathrm{Dist}^2(k,l) = \Sigma^J_j = 1[(n_{kj}/n_{k+}) - (n_{lj}/n_{l+})]^2/(n_{+j}/n)]$. Cluster analysis was done with the unweighted pair-group arithmetic mean method (UPGMA) by the CLUSTER statistical procedure of SAS. The clustering criterion was the average linked method, and the number of clusters was determined by consensus among the two principal clustering statistics, pseudo F^2 and pseudo t^2 and the biological data coherence. Average genetic similarities between and within clusters were calculated by the SAS IML procedure. Jaccard's coefficient of similarity ($S = a/[a + b + c]$) was computed from the binary data for all pairwise combinations of strains. We used NTSYS-PC (version 2.10Y) to calculate a similarity matrix, using Jaccard's coefficient. Dendrogram was constructed using the TREE option.

FAFLP analysis.

The same selected set of *X. oryzae* strains was analyzed by FAFLP. Additionally, few other *X. oryzae* strains were added together with strains representative of the *X. translucens* group, *X. campestris (*CFBP1121 and CFBP5251*)*, *X. hyacinthi* (CFBP1156), and *X. axonopodis* pv. *axonopodis* (CFBP4924, CFBP2534). The FAFLP was performed as described by Boudon and associates (2005). The presence and absence of FAFLP fragments were scored in a binary matrix. Similarity matrices using the Jaccard's coefficient were calculated with the dist AFLP program (Mougel et al. 2001). The distance matrices were used to construct dendrograms with the neighbor-joining method (Saitou and Nei 1987). The strength of the tree was assessed by the bootstrap method with the CONSENSE software of PHYLIP (Felsenstein 1993).

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