View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Afe Babalola University Repository

DNA SEQUENCING ANALYSIS OF AFRICAN *XANTHOMONAS ORYZAE* PV. *ORYZAE* VIRULENCE GENE (*AXAVRG*) DNA MARKER

A. Onasanya[1], M.M. Ekperigin[4], R.O. Onasanya[3], T.O. Obafemi[1], A.T. Ogundipe[2], A.A. Ojo[1], I. Ingelbrecht[5]

[1]Afe Babalola University, Department of Biochemistry, Ado-Ekiti, Nigeria
[2]Afe Babalola University, Department of Mathematical and Physical Sciences, Ado-Ekiti, Nigeria
[3]Federal College of Agriculture, Moor Plantation, Department of Crop Protection, Ibadan, Nigeria
[4]Federal University of Technology Akure, Department of Biochemistry, Akure, Nigeria
[5]Ghent University, Institute of Plant Biotechnology for Developing Countries, Ghent, Belgium

Global rice production is constrained by bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). BLB disease incidence in West Africa was between 70–85% and yield loss in farmers' fields was in the range of 50–90% from 2005 to 2010. In the present study, African *Xoo* virulence gene OPP-17_{2000} DNA marker was identified and purified using randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) products from 50 *Xoo* isolates. Genomic DNA of 50 *Xoo* isolates were analyzed using OPP-17 primer in RAPD-PCR during which African *Xoo* virulence gene OPP-17_{2000} DNA marker was identified, purified, cloned, and sequenced. Cloning and DNA sequencing of African *Xoo* virulence gene OPP-17_{2000} DNA generated a 1953 bp nucleotide sequence consequently tagged as *AXaVrg*-1953. BLAST homologous analysis of the *AXaVrg*-1953 sequence provides comprehensive identification of the type II secretion genes and secreted proteins, type III secretion genes and secreted proteins in African *Xoo* virulence gene. Phylogenetic unweighted pair-group method arithmetic (UPGMA) analysis revealed the African *AXaVrg*-1953 sequence was distinct from the other *Xoo* virulence gene sequences from China, Japan, Korea, Germany, and the United States. This information is potentially useful for effective management of BLB disease in West Africa.

Bacterial leaf blight, Operon primer, RAPD-PCR products, *Xoo* virulence gene DNA marker, cloning, Secreted proteins, BLAST, West Africa

DE GRUYTER OPEN

doi: 10.2478/sab-2018-0012
Received for publication on April 23, 2016
Accepted for publication on September 28, 2017

INTRODUCTION

Global rice production is constrained by bacterial leaf blight (BLB) disease. This BLB disease is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) pathogen. The disease has been reported in Asia, northern Australia, Africa, and the United States (Adhikari et al., 1995; Sere et al., 2005; Jiang et al., 2006). In West Africa, BLB disease incidence and yield loss in farmers' fields was 70–85% and 50–90%, respectively (Sere et al., 2005). In West Africa, known *Xoo* isolates attack rice varieties with high level of pathogenicity and virulence (Sere et al., 2005; Onasanya et al., 2009; Dewa et al., 2011).

In order to gain better knowledge of *Xoo* pathogens virulence, various virulence characters and molecu-

Table 1. Identity of *Xanthomonas oryzae* pv. *oryzae* isolates used for the study

S/N	Isolates code*	Host plant	Country	Pathotype**	Genotype***
1	XN-1	D52-37	Niger	Vr	Vr
2	XN-2	D52-37	Niger	Vr	Vr
3	XN-3	IR15296829	Niger	Vr	MVr
4	XN-4	IR15296829	Niger	MVr	MVr
5	XN-5	WITA 8	Niger	MVr	Vr
6	XN-6	WITA 8	Niger	MVr	Vr
7	XB-7	local	Benin	MVr	Vr
8	XB-8	local	Benin	MVr	Vr
9	XB-9	local	Benin	MVr	MVr
10	XB-10	local	Benin	Vr	MVr
11	XB-11	local	Benin	Vr	Vr
12	XNG-12	WITA9	Nigeria	MVr	Vr
13	XNG-13	WITA9	Nigeria	Vr	MVr
14	XNG-14	WITA 4	Nigeria	Vr	Vr
15	XNG-15	WITA 4	Nigeria	Vr	MVr
16	XNG-16	WITA 8	Nigeria	MVr	Vr
17	XBF-17	TS2	Burkina Faso	Vr	Vr
18	XBF-18	TS2	Burkina Faso	Vr	Vr
19	XBF-19	FKR14	Burkina Faso	Vr	Vr
20	XBF-20	FKR19	Burkina Faso	Vr	Vr
21	XBF-21	FKR14	Burkina Faso	Vr	MVr
22	XBF-22	Chinese	Burkina Faso	Vr	Vr
23	XM-23	Adventices	Mali	Vr	Vr
24	XM-24	Kogoni	Mali	MVr	Vr
25	XM-25	Kogoni	Mali	Vr	Vr
26	XM-26	Kogoni	Mali	MVr	Vr
27	XM-27	Kogoni	Mali	MVr	Vr
28	XM-28	Kogoni	Mali	MVr	Vr
29	XM-29	Jamajigi	Mali	MVr	Vr
30	XM-30	Nionoka	Mali	Vr	Vr
31	XG-31	weed	Guinea	Vr	Vr
32	XG-32	weed	Guinea	Vr	Vr
33	XG-33	weed	Guinea	Vr	Vr
34	XG-34	local	Guinea	MVr	Vr
35	XG-35	local	Guinea	Vr	Vr
36	XG-36	local	Guinea	Vr	Vr
37	XG-37	local	Guinea	MVr	Vr
38	XG-38	local	Guinea	Vr	Vr
39	XG-39	local	Guinea	Vr	Vr
40	XG-40	local	Guinea	Vr	MVr
41	XTG-41	local	The Gambia	Vr	Vr
42	XTG-42	local	The Gambia	MVr	Vr
43	XTG-43	local	The Gambia	MVr	Vr
44	XTG-44	local	The Gambia	MVr	Vr
45	XTG-45	local	The Gambia	MVr	Vr
46	XTG-46	local	The Gambia	Vr	Vr
47	XTG-47	local	The Gambia	Vr	Vr
48	XTG-48	local	The Gambia	Vr	Vr
49	XTG-49	weed	The Gambia	MVr	Vr
50	XTG-50	weed	The Gambia	MVr	Vr

Xanthomonas oryzae* pv. *oryzae* isolates obtained from Onasanya et al. (2009),*Xanthomonas oryzae* pv. *oryzae* isolates pathotypes from Onasanya et al. (2009), ****Xanthomonas oryzae* pv. *oryzae* isolates genotypes from Onasanya et al. (2013).

Vr=Virulent; MVr=Mildly Virulent.

lar markers have been studied (Jiang et al., 2006). Transcription activator-like and other effectors of *Xoo* are delivered into rice cells through the type III secretion system (T3SS). The T3SS is encoded by hypersensitive response and pathogenicity (*hrp*) genes (Gurlebeck et al., 2006). T3SS mediated secretion and delivery of *AvrXa10*, *hrpF*, *hrpA*, *Hpa1*, *hpaB*, *hpa3,* and *hpa4* into rice cells by *Xoo* have been demonstrated (Zhu et al., 2000; Noel et al., 2002; Furutani et al., 2003; Oku et al., 2004; Roden et al., 2004; Makino et al., 2006). In *Xoo,* six open reading frames are present between *hpaB* and *hrpF* in which four encode transposases or transposase derivatives while the other two designated as *hpa3* and *hpa4* appear to encode a chaperone and effector pair (Zhu et al., 2000; Roden et al., 2004).

Besides the *hrp* encoded genes secreted via T3SS, there are extracellular polysaccharide and proteins secreted through the general type II secretion system which includes degradative enzymes such as pectate, lyases, cellulases, xylanases, proteases and toxin production that contributes to the virulence of phytopathogenic bacteria in host plant cells (Alfano, Collmer, 1996). Type II secretion genes and secreted proteins also play important roles in the interaction of *Xoo* with its host (Ray et al., 2000; Chatterjee et al., 2003; Sun et al., 2005). Many studies of *Xoo* have led to the discovery of new types of genes required for virulence (Goel et al., 2002; Chatterjee et al., 2003). In West Africa, *Xoo* virulence pathotypes based on cultivars reactions have been determined (Onasanya et al., 2009; Basso et al., 2011; Dewa et al., 2011), while little information is available on *Xoo* virulence genotypes, sequencing and analysis of DNA marker tagged to virulence genes in *Xoo*. The main goal of this study is to carry out molecular DNA sequencing and analysis of the African *Xoo* virulence gene DNA marker using the characterized *Xoo* isolates virulence pathotypes and genotypes previously identified by Onasanya et al. (2009) and Onasanya et al. (2013), respectively.

MATERIAL AND METHODS

Bacterial isolates

Fifty *Xoo* isolates (Table 1) used in this study were from the studies by Onasanya et al. (2009) and Onasanya et al. (2013). The identity of all the fifty *Xoo* isolates was confirmed by oxidative biochemical test, as well as their virulence pathotypes and genotypes (Onasanya et al., 2009, 2013).

Isolates propagation

Xoo isolates were first propagated using a modified procedure developed by Akanji et al. (2011). Nutrient broth (75 ml, pH 7.5; Sigma-Aldrich, St. Louis, Missouri, USA) was prepared inside a 100 ml conical flask. Each stored *Xoo* isolate (100 µl) was transferred into 50 ml of nutrient broth and kept under constant shaking at 30°C for 24 h for bacterial growth. The bacterial cell was removed by centrifugation, washed with 0.1 mM Tris-EDTA (pH 8.0), and kept at –20°C for DNA extraction.

Genomic DNA extraction

The DNA extraction was performed according to Onasanya et al. (2013) with some modification. Totally 0.3 g of washed bacterial cell was suspended in 200 µl of Cetyl trimethylammonium bromide (CTAB) buffer (50 mM Tris, pH 8.0; 0.7 mM NaCl; 10 mM EDTA; 2% hexadecyltrimethylammonium bromide; 0.1% 2-mercaptoethanol – all Sigma-Aldrich), followed by 100 µl of 20% sodium dodecyl sulfate, and incubated at 65°C for 20 min. DNA was purified by two extractions with chloroform and precipitated with absolute ethanol at –20°C. Purified DNA was washed with 70% ethanol, then was dried and resuspended in 200 µl of sterile distilled water. DNA concentration was measured using a DU-65UV spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA) at 260 nm. DNA quality was checked on a 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.0; Sigma-Aldrich) after electrophoresis.

RAPD-PCR analysis

The randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis was performed according to Onasanya et al. (2013). The OPP-17 primer (5'-TGACCCGCCT-3') used was previously identified by Onasanya et al. (2013) and was purchased from Operon Technologies (Alameda, USA). OPP-17 was used in amplifying the genomic DNA from all the 50 *Xoo* isolates. Amplifications were performed in a 25 µl reaction mixture consisting of genomic DNA, reaction buffer (Promega, Madison, Wisconsin, USA), 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.2 µM Operon random primer (OPP-17), 2.5 µM $MgCl_2$, and 1 U of *Taq* polymerase (Sigma-Aldrich). Amplification was performed in a thermowell microtiter plate (ICN Biomedicals, *Costa Mesa, USA*) using a MJ Research programmable Thermal Controller. The cycling program was (*i*) 1 cycle of 94°C for 3 min; (*ii*) 45 cycles of 94°C for 1 min for denaturation, 40°C for 1 min for annealing of primer, and 72°C for 2 min for extension; and (*iii*) a final extension at 72°C for 7 min. Amplification products were maintained at 4°C until electrophoresis.

Electrophoresis of PCR products and purification of the African Xoo virulence gene OPP-17_{2000} DNA marker

The amplification products were resolved by electrophoresis in a 1.4% agarose gel (Sigma-Aldrich) using TAE buffer at 100 V for 2 h. A 1 kb ladder (Sigma-Aldrich) was used as the molecular size marker. Gels were visualized by staining with ethidium bromide solution (0.5 µg/ml; Sigma-Aldrich) and banding patterns were photographed over UV light using a UVP-computerized gel photo documentation system. After an initial electrophoresis of PCR products in a 1.4% agarose gel, a separate and second electrophoresis of the PCR products in a 1.0% low melting agarose gel was conducted in TAE buffer at 100 V for 2.5 h. A 1 kb ladder was used as the size marker. Gels were visualized by staining with ethidium bromide solution (0.5 µg/ml; Sigma-Aldrich). Under a UV light, a specific DNA fragment of 2 kb size (OPP-17_{2000} DNA marker tagged to African *Xoo* virulence gene, *AXaVrg*) (Fig. 1) was excised from low melting agarose gel and purified using a Qiagen DNA gel extraction kit (Q i a g e n , 2001).

Cloning and sequencing of AXaVrg OPP-17_{2000} DNA marker

The purified PCR 2 kb DNA fragment (OPP-17_{2000} DNA marker tagged to African *Xoo* virulence gene, *AXaVrg*) (Table 2) was cloned and sequenced according to the procedure outlined in J u n g et al. (1999).

Sequence analysis

Using the obtained nucleotide sequence and translated amino acid sequence, computer-based homology search was performed with the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST+) software program while nucleotides to amino acid translation and phylogenetic unweighted pair-group method arithmetic (UPGMA) analysis were carried out using the CLC Main Workbench (Version 7.6) software (A l t s c h u l et al., 1990).

RESULTS

Cloning and DNA sequencing of the African *Xoo* virulence gene OPP-17_{2000} DNA marker generated a 1953 bp nucleotide sequence, and consequently the African *Xoo* virulence gene DNA marker size was tagged as *AXaVrg*-1953 (Table 2). BLAST homology analysis using nucleotide sequence of *AXaVrg*-1953 gave significant alignments, at 100% nucleotide identity with *Xanthomonas oryzae* pv. *oryzae* PXO99A complete genome (CP000967.1), *Xanthomonas oryzae* pv. *oryzae* MAFF 311018 DNA complete genome (AP008229.1), and *Xanthomonas oryzae* pv. *oryzae* KACC10331 complete genome (AE013598.1) (Table 2). Significant alignments at 96% nucleotide identity were also obtained with *Xanthomonas oryzae* 16S-23S rDNA spacer region (AB026287.1), at 88% nucleotide identity with *Xanthomonas oryzae* pv. *oryzae* strain JXOIII *HrpG* gene (AY048682.1), and *Xanthomonas oryzae* pv. *oryzicola* regulatory protein *HrpG* and regulatory protein *HrpX* genes (AF272885.2), and at 82% nucleotide identity with *Xanthomonas oryzae* 16S rRNA gene (X95921.1) (Table 2). Lineage and taxonomy nucleotide sequence alignment analyses revealed that *AXaVrg*-1953 was primarily of *Xanthomonas oryzae* pv. *oryzae* genome origin rather than that of *Xanthomonas oryzae* pv. *oryzicola* (Table 2). BLAST homology analysis using translated amino acid sequence from the nucleotide sequence of *AXaVrg*-1953 produced significant alignments with known virulence proteins. The *AXaVrg*-1953 translated amino acid sequence has 10 virulence gene proteins which were penicillin acylase II, gamma-glutamyl phosphate reductase, putative outer membrane protein, TAL effector PthXo6 protein, TAL effector *AvrBs3*/PthA protein, *HrpD1* protein, *HrpD5* protein, *HrcC* protein, putative IS1113 transposase, and glycosyltransferase (Table 3).

Relationships between the African *Xoo* virulence gene (*AXaVrg*-1953) and the other known 17 *Xoo* virulence genes have been revealed by a BLAST homology nucleotide sequence alignment UPGMA analysis

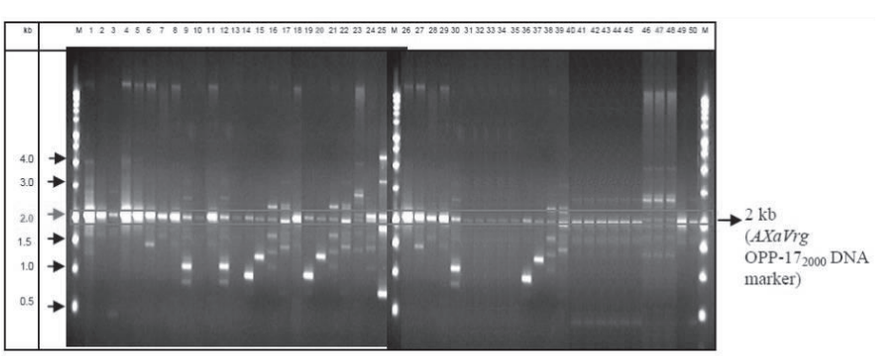

Fig. 1. *Xoo* OPP-17 DNA fingerprint revealed African *Xoo* virulence gene *AXaVrg* OPP-17_{2000} DNA marker

Xanthomonas oryzae pv. *oryzae* isolate: 1–6 = XN-1 to XN-6; 7–11 = XB-7 to XB-11; 12–16 = XNG-12 to XNG-16; 17–22 = XBF-17 to XBF-22; 23–30 = XM-23 to XM-30; 31–40 = XG-31 to XG-40; 41–50 = XTG-41 to XTG-50; M = 1 kb DNA ladder, kb = kilobase pair

Table 2. African *Xoo* virulence gene (*AXaVrg*-1953) DNA marker sequence, alignment, lineage, and taxonomy details

African *Xoo* virulence gene DNA marker (*AXaVrg*-1953)	
Sequence (1953 bp)	tgacccgcct accacaccca gccccctccc cgaaggaaga agaacccgtg cagcggttcg tggtccaagg accggcccgt accaggcacc ccaccaaccg gtggccggtg acctgaggac ccaacagtgt gctcgacaag ccccccaacc tcacccgtga tgttccacca ccacccgcac aaacccccga agagacacgt gcgagccggc aggtactgac accagcaaga ccgaagaagc tctttcgtca atgttccacc catgagcaac cacccaccac acggacggcg atggcgtggc acctgaacca cccacccacc agcaaacacc ggcaagatga gggttggtag ctccttagaa aggaggtgat ccagccgcac cttccggtac ggctaccttg ttacgactta gtcccaatcg ccagtcccac cttcgaccac tccccccgcg aacggttggg ccatgggctt cgggtgttac cgactttcgt gacttgacgg gcggtgtgta caaggcccgg gaacgtattc accgcagcgt tgctgatctg cgattactag cgactccgac ttcatggggt cgagttgcag accccaatcc gaactgagac cggctttttg ggattcgctc caccttacgg tatcgcagcc ctttgtaccg gccattgtag catgcgtgaa gcccaagaca taaggggcat gatgatttga cgtcatcccc accttcctcc gagttgaccc cggcagtctc ccatgagtcc ccggcataac ccgctggcaa catgggacga gggttgcgct cgttgcggga cttaacccaa catctcacga cacgagctga cgacaaccat gcaccacctg tgcacgagtg tccaaagaga ccaccatctc tggtggcttc tcgtgcatgt caagccttgg taaggttctt cgcgttgcat cgaattaatc cgcatgctcc gccgcttgtg cgggcccccg tcaattcctt tgagttttag ccttgcggcc gtactcccca tgacccgcct accacaccca gccccctccc cgaaggaaga agaacccgtg cagcggttcg tggtccaagg accggcccgt accaggcacc ccaccaaccg gtggccggtg acctgaggac ccaacagtgt gctcgacaag ccccccaacc tcacccgtga tgttccacca ccacccgcac aaacccccga agagacacgt gcgagccggc aggtactgac accagcaaga ccgaagaagc tctttcgtca atgttccacc catgagcaac cacccaccac acggacggcg atggcgtggc acctgaacca cccacccacc agcaaacacc ggcaagatga gggttggtag ctccttagaa aggaggtgat ccagccgcac cttccggtac ggctaccttg ttacgactta gtcccaatcg ccagtcccac cttcgaccac tccccccgcg aacggttggg ccatgggctt cgggtgttac cgactttcgt gacttgacgg gcggtgtgta caaggcccgg gaacgtattc accgcagcgt tgctgatctg cgattactag cgactccgac ttcatggggt cgagttgcag accccaatcc gaactgagac cggctttttg ggattcgctc caccttacgg tatcgcagcc ctttgtaccg gccattgtag catgcgtgaa gcccaagaca taaggggcat gatgatttga cgtcatcccc accttcctcc gagttgaccc cggcagtctc ccatgagtcc ccggcataac ccgctggcaa catgggacga gggttgcgct cgttgcggga cttaacccaa catctcacga cacgagctga cgacaaccat gcaccacctg tgcacgagtg tccaaagaga ccaccatctc tggtggcttc tcgtgcatgt caagccttgg taagggcggg tca
Sequences alignments	(i) *Xanthomonas oryzae* pv. *oryzae* PXO99A, complete genome; 100% identity; Accession No: CP000967.1 (ii) *Xanthomonas oryzae* pv. *oryzae* MAFF 311018 DNA, complete genome; 100% identity; Accession No: AP008229.1 (iii) *Xanthomonas oryzae* pv. *oryzae* KACC10331, complete genome; 100% identity; Accession No: AE013598.1 (iv) *Xanthomonas oryzae* 16S-23S rDNA spacer region; 96% identity; Accession No: AB026287.1 (v) *Xanthomonas oryzae* pv. *oryzae* strain JXOIII *HrpG* (*hrpG*) gene, complete proteincoding sequences (cds); 88% identity; Accession No: AY048682.1 (vi) *Xanthomonas oryzae* pv. *oryzicola* regulatory protein *HrpG* (*hrpG*) and regulatory protein *HrpX* (*hrpX*) genes, complete cds; 88% identity; Accession No: AF272885.2 (vii) *X.oryzae* 16S rRNA gene; 82% identity; Accession No: X95921.1

(Fig. 2). The analysis revealed *AXaVrg*-1953 was distinct from the other 17 *Xoo* virulence genes originating from China, Japan, Korea, Germany, and the United States (Fig. 2). However, only *Xoc-HrpG*-China and *Xo*16SrRNA-Germany virulence genes were slightly related to the African *Xoo* virulence gene (*AXaVrg*-1953) at 37% similarity coefficient (Fig. 2).

DISCUSSION

Molecular based PCR techniques have created opportunities for the application of DNA markers both in the identification of bacterial virulence genes and characterization to identify proteins involved in virulence and their functions in bacterial pathogenicity

(Nelson et al., 1994; Vera Cruz et al., 1996). DNA marker linked to *Xoo* virulence genes could circumvent many traditional problems associated with phenotypic selection for *Xoo* virulence and offers a unique opportunity for identification of durable resistance cultivars to BLB disease (Adhikari et al., 1995; Adhikari et al., 1999). In the present study the African *Xoo* virulence gene (*AXaVrg*) DNA marker has been identified, sequenced, and characterized. Molecular sequencing has identified the *AXaVrg*-1953 DNA marker present in each of the 50 *Xoo* isolates analyzed in this study. This *AXaVrg*-1953 DNA marker was known to contain penicillin acylase II, gamma-glutamyl phosphate reductase, putative outer membrane protein, TAL effector PthXo6 protein, TAL effector *AvrBs3*/PthA protein, *HrpD1* protein, *HrpD5* protein, *HrcC* protein, putative IS1113 transposase, and glycosyltransferase in its translated amino acid sequence. The sequence analysis of *AXaVrg*-1953 DNA marker revealed that every *Xoo* isolate and strain in West Africa are virulent and possibly carry multiple virulence gene proteins to elicit hypersensitive reactions leading to BLB disease in host cells. This possibly suggests the need for pyramiding multiple resistance genes into rice cultivars would provide high performance and durability of genetic resistance to BLB disease in West Africa (Narayanan et al., 2002; Sere et al., 2005).

It has been reported that penicillin acylase II, gamma-glutamyl phosphate reductase, putative outer membrane protein, TAL effector PthXo6 protein, TAL effector *AvrBs3*/PthA protein, *HrpD1* protein, *HrpD5* protein, *HrcC* protein, putative IS1113 transposase, and glycosyltransferase obtained in this study are involved in pathogenicity and virulence of *Xanthomonas oryzae* pv. *oryzae* (Buttner et al., 2006; Gurlebeck et al., 2006; Makino et al., 2006). The *hrp* genes (*HrpD1*, *HrpD5*), and TAL effector *AvrBs3* protein encode the type III secretion system (T3SS) for their secretion pathway in *Xanthomonas oryzae* pv. *oryzae* (Buttner et al., 2006; Gurlebeck et al., 2006; Makino et al., 2006). Besides, *HrcC* protein, penicillin acylase II, gamma-glutamyl phosphate reductase, and glycosyltransferase encode the type II secretion system (T2SS) for their secretion pathway in *Xanthomonas oryzae* pv. *oryzae* (Ray et al., 2000; Chatterjee et al., 2003; Sun et al., 2005). The type III secretion system is well-known to be present among Gram-negative plants and animal pathogens, and made up of a supermolecular structure called *Hrp* mutants pilus that deliver TAL and other effectors into plant cells (Yang, White, 2004; Makino et al., 2006). The environmental conditions are known to regulate the expression of *hrp* genes in *Xoo* and can be induced only in plants or specific synthetic media (Tsuge et al., 2005). The type II secretion genes and secreted proteins play important roles in the host–*Xoo* interaction (Sun et al., 2005).

Molecular phylogeny and cluster analyses of nucleotide sequence revealed the African *Xoo* virulence gene (*AXaVrg*-1953) DNA marker was distinct from the other 17 *Xoo* virulence genes originating from China, Japan, Korea, Germany, and the United States. Similarly, previous DNA fingerprinting studies revealed that South American *Xoo* strains are closely related to Asian strains while the African strains form two clearly distinct groups (Gonzales et al., 2005). Because the *Xoo* virulence gene (*AXaVrg*-1953) DNA

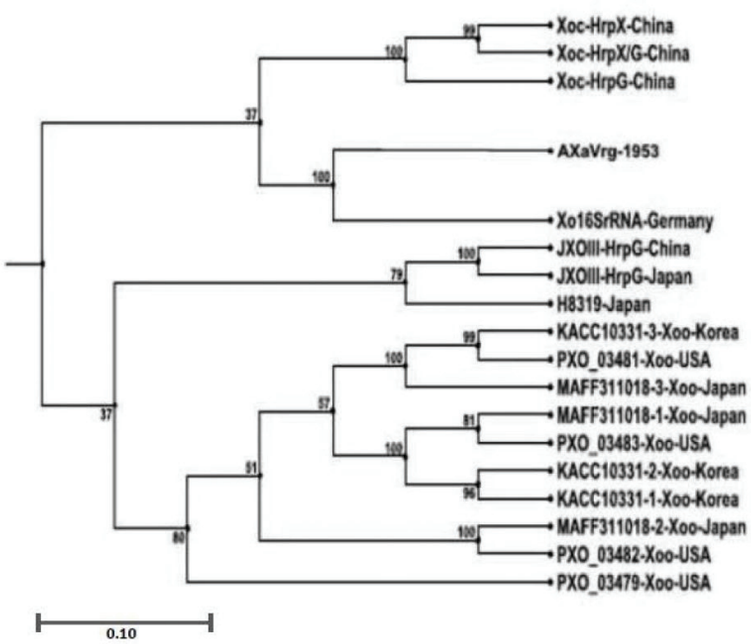

Fig. 2. Relationships between African *Xoo* virulence gene DNA marker (*AXaVrg-1953*) obtained and other known 17 *Xoo* virulence genes as revealed by nucleotide sequence alignment unweighted pair-group method arithmetic (UPGMA) analysis using CLC Main Workbench 7.6 software program. The tree branch annotations are % bootstrap values. Nucleotide distance scale used was Jukes-Cantor.

Table 3. *AXaVrg*-1953 translation protein sequence and sequence alignment details

African *Xoo* virulence gene DNA marker (*AXaVrg*-1953)	
Translation protein sequence (650 bp)	1 TRLPHPAPSP KEEEPVQRFV VQGPARTRHP TNRWPVT*GP NSVLDKPPNL TRDVPPPPAQ 61 TPEETRASRQ VLTPARPKKL FRQCSTHEQP PTTRTAMAWH LNHPPTSKHR QDEGW*LLRK 121 EVIQPHLPVR LPCYDLVPIA SPTFDHSPRE RLGHGLRVLP TFVT*RAVCT RPGNVFTAAL 181 LICDY*RLRL HGVELQTPIR TETGFLGFAP PYGIAALCTG HCSMREAQDI RGMMI*RHPH 241 LPPS*PRQSP MSPRHNPLAT WDEGCARCGT *PNISRHELT TTMHHLCTSV QRDHHLWWLL 301 VHVKPW*GSS RCIELIRMLR RLCGPPSIPL SFSLAAVLPM TRLPHPAPSP KEEEPVQRFV 361 VQGPARTRHP TNRWPVT*GP NSVLDKPPNL TRDVPPPPAQ TPEETRASRQ VLTPARPKKL 421 FRQCSTHEQP PTTRTAMAWH LNHPPTSKHR QDEGW*LLRK EVIQPHLPVR LPCYDLVPIA 481 SPTFDHSPRE RLGHGLRVLP TFVT*RAVCT RPGNVFTAAL LICDY*RLRL HGVELQTPIR 541 TETGFLGFAP PYGIAALCTG HCSMREAQDI RGMMI*RHPH LPPS*PRQSP MSPRHNPLAT 601 WDEGCARCGT *PNISRHELT TTMHHLCTSV QRDHHLWWLL VHVKPW*GRV
Sequence alignments	(i) Penicillin acylase II sequence: 322 RLCGPPSIPLSFSLAAVLPMTRLPHPAPSPKEEEPVQRFVVQGPART 367 sequence: 322 LCGPPSIPLSFSLAAVLPMTRLPHPAPSPKEEEPV 356 (ii) Gamma glutamyl phosphate reductase sequence: 338 LPMTRLPHPAPSPKEEEPVQRFVVQGPARTRH 369 (iii) Putative outer membrane protein sequence: 255 HNPLATWDEGC 265 sequence: 595 HNPLATWDEGC 605 (iv) TAL effector PthXo6 sequence: 330 LSFSLAAVLPMTRLPHPAPSPKEEEPVQ 357 (v) TAL effector *AvrBs3*/PthA sequence: 47 PPNLTRDV-PPPPAQTPEETRASR 69 sequence: 387 PPNLTRDV-PPPPAQTPEETRASR 409 (vi) *HrpD1* protein sequence: 67 ASRQVLTPARPKKLFRQCSTHEQPPTTRTAMAWHLNHPPTSKHRQ, DEGWXLLRKEVIQPHLPVRLPCYDLVPIASPTFDHSPRE 150 sequence: 407 ASRQVLTPARPKKLFRQCSTHEQPPTTRTAMAWHLNHPPTSKHRQ, DEGWXLLRKEVIQPHLPVRLPCYDLVPIASPTFDHSPRE 490 (vii) *HrpD5* protein sequence: 202 ETGFLGFAPP-YGIAALCTGHCS 223 sequence: 542 ETGFLGFAPP-YGIAALCTGHCS 563 (viii) *HrcC* protein sequence: 108 KHRQDEGWXLLRKEVIQPHL 127 sequence: 448 KHRQDEGWXLLRKEVIQPHL 467 (ix) Putative insertion sequence (IS) IS1113 transposase sequence: 73 TPARPKKLFRQCSTHEQPPTTRTAMAWHLNHPP 105 sequence: 413 TPARPKKLFRQCSTHEQPPTTRTAMAWHLNHPP 445 (x) Glycosyltransferase sequence: 124 QPHLPVRLPCYDL 136 sequence: 464 QPHLPVRLPCYDL 476

marker from Africa has been confirmed different from those from China, Japan, Korea, Germany, and the United States by molecular sequencing and phylogeny in this study, with resistance genes against *Xoo* from China, Japan, Korea, Germany, and the United States possibly would not provide high performance and durable genetic resistance to BLB disease in West Africa (A d h i k a r i et al., 1999; N a r a y a n a n et al., 2002; S e r e et al., 2005).

CONCLUSION

The gene sequence of *AXaVrg*-1953 provides a comprehensive identification of the type II secretion genes, specific type II-secreted proteins, type III secretion genes, and specific type III-secreted proteins in the African *Xoo* virulence gene, and a distinct differentiation from the other *Xoo* virulence genes from China, Japan, Korea, Germany, and the USA. This information is potentially useful for identifying rice cultivars with high performance and durable genetic resistance to BLB disease in West Africa.

ACKNOWLEDGEMENT

We are very grateful to Dr. Ivan Ingelbrecht, Head, Central Biotechnology Laboratory, International Institute of Tropical Agriculture (IITA) Nigeria, and

to Dr. Stephan Winter, Head, Virology and Diagnostics, German Collection of Microorganisms and Cell Cultures, Germany for support in molecular cloning and sequencing part of A. Onasanya's Ph.D. research reported in this article.

REFERENCES

Adhikari TB, Vera Cruz CM, Zhang Q, Nelson RJ, Skinner DZ, Mew TW, Leach JE (1995): Genetic diversity of *Xanthomonas oryzae* pv. *oryzae* in Asia. Applied and Environmental Microbiology, 61, 966–971.

Adhikari TB, Basnyat RC, Mew TW (1999): Virulence of *Xanthomonas oryzae* pv. *oryzae* on rice lines containing single resistance genes and gene combinations. Plant Disease, 83, 46–50. doi: 10.1094/PDIS.1999.83.1.46.

Akanji BO, Ajele JO, Onasanya A, Oyelakin O (2011): Genetic fingerprinting of *Pseudomonas aeruginosa* involved in nosocomial infection as revealed by RAPD-PCR markers. Biotechnology, 10, 70–77. doi: 10.3923/biotech.2011.70.77.

Alfano JR, Collmer A (1996): Bacterial pathogens in plants: life up against the wall. Plant Cell, 8, 1683–1698. doi: 10.1105/tpc.8.10.1683.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990): Basic local alignment search tool. Journal of Molecular Biology, 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2.

Basso A, Onasanya A, Issaka S, Sido AY, Haougui A, Adam T, Sere Y, Saadou M (2011): Bacterial leaf blight of rice in Niger: Pathological diversity of isolates collected on irrigated lands. Journal of Applied Biosciences, 38, 2551–2563.

Buttner D, Lorenz C, Weber E, Bonas U (2006): Targeting of two effector protein classes to the type III secretion system by a *HpaC*- and *HpaB*-dependent protein complex from *Xanthomonas campestris* pv. *vesicatoria*. Molecular Microbiology, 59, 513–527. doi: 10.1111/j.1365-2958.2005.04924.x.

Chatterjee S, Sankaranarayanan R, Sonti RV (2003): PhyA, a secreted protein of *Xanthomonas oryzae* pv. *oryzae,* is required for optimum virulence and growth on phytic acid as a sole phosphate source. Molecular Plant–Microbe Interactions, 16, 973–982. doi: 10.1094/MPMI.2003.16.11.973.

Dewa KMK, Banito A, Onasanya A, Kpemoua KE, Sere Y (2011): Rice bacterial blight in Togo: importance of the disease and virulence of the pathogen. Current Research in Bacteriology, 4, 94–100. doi: 10.3923/crb.2011.94.100.

Furutani A, Tsuge S, Oku T, Tsuno K, Inoue Y, Ochiai H, Kaku H, Kubo Y (2003): *Hpa1* secretion via type III secretion system in *Xanthomonas oryzae* pv. *oryzae*. Journal of General Plant Pathology, 69, 271–275. doi: 10.1007/s10327-003-0042-2.

Goel AK, Rajagopal L, Nagesh N, Sonti RV (2002): Genetic locus encoding functions involved in biosynthesis and outer membrane localization of xanthomonadin in *Xanthomonas oryzae* pv. *oryzae*. Journal of Bacteriology, 184, 3539–3548. doi: 10.1128/JB.184.13.3539-3548.2002.

Gonzalez C, Szurek B, Manceau C, Mathieu T, Sere Y, Verdier V (2007): Molecular and pathotypic characterization of new *Xanthomonas oryzae* strains from West Africa. Molecular Plant–Microbe Interactions, 20, 534–546. doi: 10.1094/MPMI-20-5-0534.

Gurlebeck D, Thieme F, Bonas U (2006): Type III effector proteins from the plant pathogen Xanthomonas and their role in the interaction with the host plant. Journal of Plant Physiology, 163, 233–255. doi: 10.1016/j.jplph.2005.11.011.

Jiang GH, Xia ZH, Zhou YL, Wan J, Li DY, Chen RS, Zhai WX, Zhu LH (2006): Testifying the rice bacterial blight resistance gene *xa5* by genetic complementation and further analyzing *xa5* (*Xa5*) in comparison with its homolog *TFIIAγ1*. Molecular Genetics and Genomics, 275, 354–366. doi: 10.1007/s00438-005-0091-7.

Jung G, Skroch PW, Nienhuis J, Coyne DP, Arnaud-Santana E, Ariyarathne HM, Marita JM (1999): Confirmation of QTL associated with bacterial blight resistance in four different genetic backgrounds in common bean. Crop Science, 39, 1448–1455. doi: 10.2135/cropsci1999.3951448x.

Makino S, Sugio A, White F, Bogdanove AJ (2006): Inhibition of resistance gene mediated defense in rice by *Xanthomonas oryzae* pv. *oryzicola*. Molecular Plant–Microbe Interactions, 19, 240–249. doi: 10.1094/MPMI-19-0240.

Narayanan NN, Baisakh N, Vera-Cruz CM, Gnanamanickam SS, Datta K, Datta SK (2002): Molecular breeding for the development of blast and bacterial blight resistance in rice cv. IR50. Crop Science, 42, 2072–2079. doi: 10.2135/cropsci2002.2072.

Nelson RJ, Baraoidan MR, Vera Cruz CM, Yap IV, Leach JE, Mew TW, Leung H (1994): Relationship between phylogeny and pathotype for the bacterial blight pathogen of rice. Applied and Environmental Microbiology, 60, 3275–3283.

Noel L, Thieme F, Nennstiel D, Bonas U (2002): Two novel type III-secreted proteins of *Xanthomonas campestris* pv. *vesicatoria* are encoded within the *hrp* pathogenicity island. Journal of Bacteriology, 184, 1340–1348. doi: 10.1128/JB.184.5.1340-1348.2002.

Oku T, Tanaka K, Iwamoto M, Inoue Y, Ochiai H, Kaku H, Tsuge S, Tsuno K (2004): Structural conservation of the *hrp* gene cluster in *Xanthomons oryzae* pv. *oryzae*. Journal of General Plant Pathology, 70, 159–167. doi: 10.1007/s10327-004-0106-y.

Onasanya A, Ekperigin MM, Nwilene FE, Sere Y, Onasanya RO (2009): Two pathotypes of *Xanthomonas oryzae* pv. *oryzae* virulence identified in West Africa. Current Research in Bacteriology, 2, 22–35**. doi:** 10.3923/crb.2009.22.35.

Onasanya A, Ekperigin MM, Afolabi A, Onasanya RO, Ojo AA, Ingelbrecht I (2013): Two genotypes of *Xanthomonas oryzae* pv. *oryzae* virulence identified in West Africa. International Journal of Genetics and Molecular Biology, 5, 28–36. doi: 10.5897/IJGMB2013.0070.

Qiagen (2001): QIAquick® Spin Handbook for QIAquick PCR Purification Kit, QIAquick Nucleotide Removal Kit, and QIAquick Gel Extraction Kit. https://www.calpoly.edu/~bio/ubl/protocols_files/pcrclean.pdf. Accessed 03, 2001.

Ray SK, Rajeshwari R, Sonti RV (2000): Mutants of *Xanthomonas oryzae* pv. *oryzae* deficient in general secretory pathway are virulence deficient and unable to secrete xylanase. Molecular Plant–Microbe Interactions, 13, 394–401. doi: 10.1094/MPMI.2000.13.4.394.

Roden JA, Belt B, Ross JB, Tachibana T, Vargas J, Mudgett MB (2004): A genetic screen to isolate type III effectors translocated into pepper cells during Xanthomonas infection. Proceedings of the National Academy of Sciences of the United States of America, 101, 16624–16629. doi: 10.1073/pnas.0407383101.

Sere Y, Onasanya A, Verdier V, Akator K, Ouedraogo LS, Segda Z, Coulibaly MM, Sido AY, Basso A (2005): Rice bacterial leaf blight in West Africa: preliminary studies on disease in farmers` fields and screening released varieties for resistance to the bacteria. Asian Journal of Plant Sciences, 4, 577–579. doi: 10.3923/ajps.2005.577.579.

Sun QH, Hu J, Huang GX, Ge C, Fang RX, He CZ (2005): Type-II secretion pathway structural gene *xpsE*, xylanase- and cellulase secretion and virulence in *Xanthomonas oryzae* pv. *oryzae*. Plant Pathology, 54, 15–21. doi: 10.1111/j.1365-3059.2004.01101.x.

Tsuge S, Terashima S, Furutani A, Ochiai H, Oku T, Tsuno K, Kaku H, Kubo Y (2005): Effects on promoter activity of base substitutions in the *cis*-acting regulatory element of *HrpXo* regulons in *Xanthomonas oryzae* pv. *oryzae*. Journal of Bacteriology, 187, 2308–2314. doi: 10.1128/JB.187.7.2308-2314.2005.

Vera Cruz CM, Ardales EY, Skinner DZ, Talag J, Nelson RJ, Louws FJ, Leung H, Mew TW, Leach JE (1996): Measurement of haplotypic variation in *Xanthomonas oryzae* pv. *oryzae* within a single field by rep-PCR and RFLP analyses. Phytopathology, 86, 1352–1359.

Yang B, White FF (2004): Diverse members of the *AvrBs3*/PthA family of type III effectors are major virulence determinants in bacterial blight disease of rice. Molecular Plant–Microbe Interactions, 17, 1192–1200. doi: 10.1094/MPMI.2004.17.11.1192.

Zhu W, Magbanua MM, White FF (2000): Identification of two novel *hrp*-associated genes in the *hrp* gene cluster of *Xanthomonas oryzae* pv. *oryzae*. Journal of Bacteriology, 182, 1844–1853. doi: 10.1128/JB.182.7.1844-1853.2000.

Corresponding Author:

Prof. Amos O n a s a n y a , Afe Babalola University, Department of Biochemistry, Ado-Ekiti, Nigeria, phone: +2348 074 289 553, e-mail: onasanyaa@abuad.edu.ng