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Three New Loci of Insertion Element IS*1112* in Chinese Strains of *Xanthomonas oryzae* pv. *oryzae*

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Insertion sequence IS*1112* is a repetitive element with a relatively high number of copies in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of bacterial blight of rice (*Oryza sativa* L.). Three new loci of IS*1112* were identified in seven Chinese strains of *Xoo* using a single oligonucleotide primer J3; 5'-GCTCA GGTCAGGTGGCCTGG-3' by insertion-sequence-based polymerase chain reaction (IS-PCR). Among the three new loci of IS*1112*, two were located in the open-reading frame region of genes *fhu*A and *cir*A, which encode TonB-dependent receptors, and the third in IS*Xo2*, another type of insertion sequence in *Xoo* genome. Three variants of IS*1112* were identified in those three loci based on their sequence similarities: two were identical to IS*1112a* and IS*1112b*, reported in strain PXO86 from the Philippines, while the third was a new member of IS*1112*, defined as IS*1112d*. Inserting IS*1112* in gene *fhu*A caused three bases, GGT, to be duplicated at the target site, but inserting it in gene *cir*A did not cause any duplication in the target site. The diversity of IS*1112* sequence and insertion loci in *Xoo* genome and their potential effects are discussed.

Keywords: *Xanthomonas oryzae* pv. *oryzae*, insertion locus, IS*1112*, IS-PCR

Xanthomonas oryzae pv. *oryzae* (*Xoo*) causes bacterial blight of rice (*Oryza sativa* L.), one of the major rice diseases all over the world, especially in both irrigated and rainfed regions in Asia (Ou, 1985; Mew, 1987). In severely infected fields, the disease could cause yield loss as high as 30% to 50% (Reddy, 1989). In addition to its importance as a pathogen, the bacterium is known to be an ideal model for studying plant-microbe interactions, race differentiation, and evolution of plant pathogens because both the rice genome (Yu *et al.*, 2002) and the *Xoo* genome (Lee *et al.*, 2005; Ochiai *et al.*, 2005) have been sequenced completely.

The disease is conventionally controlled by introducing resistant genes that mediate strain-specific initiation of defense responses due to gene-for-gene interaction of the gene for resistance with *avr* or effector genes in the pathogen (Keen, 1990; Leach and White, 1996). However, widespread and long-term use of a very limited number of resistance genes might accelerate the selection of new pathogenic races of the pathogen and result in the loss of disease resistance in rice (Leach and White, 1996). On the other hand, knowledge of the pathogen's population structure could aid in screening for new sources of disease resistance in a regional crop breeding program (Leach *et al.*, 2001). The diversity of races in *Xoo* is remarkable; so far, more than 30 races of different virulence have been reported worldwide (Noda *et al.*, 1996). In China, *Xoo* populations have been characterized on the basis of their pathogenicity patterns on five *indica* and *japonica* varieties of rice, and seven races or pathogenic types of *Xoo* were recognized among 835 isolates collected from different regions of China (Fang *et al.*, 1990). However, such grouping of race provides little insight into the genetic structure of the bacterial population because most isolates share many traits.

Great strides have been made in the last decade in understanding the molecular basis of interactions between rice and *Xoo* (Shen and Ronald, 2002). These data have helped to resolve difficulties in studying the genetic diversity of *Xoo*. Molecular probes, restriction fragment length polymorphism (RFLP), and PCR-based techniques have been used to study the structure and genetic diversity of *Xoo*. Insertion sequence (IS) elements and transposons are prominent features of bacterial genomes, generally considered to play an important role in adaptation to the environment (Galas and Chandler, 1989). A total of 611 IS elements of 25 types were found in the genome of Japanese strain MAFF 311018 of *Xoo*, which has been sequenced completely (Ochiai *et al.*, 2005). These elements are useful markers for characterizing the structure or composition of bacterial populations. Leach *et al.* (1992) used a probe (pJEL101) carrying the insertion sequence IS*1112*, isolated from *Xoo*, for RFLP analysis of a collection of strains of *Xoo* from the Philippines. They evaluated a number of sets of strains collected over defined time periods and from different regions and determined the relationship between pathogenic races and phylogeny of the pathogen. Nelson *et al.* (1994) analyzed a similar set of strains with four mobile, repetitive elements and an avirulence gene, *avr*Xa10, isolated from *Xoo*. Of the five probes tested, probe IS*1112* led to the most robust phylogeny.

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IS*1112* is a repetitive element, and its copies are dispersed differently in *Xoo* genome in relatively high numbers: the Japanese strain MAFF 311018 has 20 full-length copies and 12 truncated copies (Ochiai *et al.*, 2005). Studies using insertion-sequence-based polymerase chain reaction (IS-PCR) based on outwardly directed primers of IS*1112* also reveal the diversity among *Xoo* isolates (George *et al.*, 1997; Adhikari *et al.*, 1999; Adhikari and Vera Cruz, 1999; Sukhwinder-Singh *et al.*, 2003). Three types of IS*1112*, namely IS*1112a*, IS*1112b*, and IS*1112c*, were identified based on their sequences in the Philippine strain PXO86 (Ryba-White *et al.*, 2005). Although widely used for characterizing strains and populations, the insertion loci of IS*1112* in different strains have not been reported completely. This study sought to analyze the genetic structure of seven model strains of *Xoo* in China using a single oligonucleotide primer, J3, by IS*1112*-based IS-PCR and to determine the differences among insertion loci based on the differential bands sequenced in the fingerprinting of IS-PCR.

Materials and Methods

Bacterial isolates

Seven Chinese strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), namely C1 (strain no. HLJ72), C2 (strain no. HB17), C3 (strain no. NX42), C4 (strain no. Z173), C5 (strain no. GD1358), C6 (strain no. LN57), and C7 (strain no. JS49-6), were provided by Dr. Kaijun Zhao (Institute of Crop Sciences, Chinese Academy of Agricultural Sciences) and selected for comparison. Each strain represented a typical pathotype (pathotypes I to VII) in China based on the pattern of its pathogenicity to five basic differentials, which were collected during 1985 to 1990 (Fang *et al.*, 1990).

DNA preparation

Xanthomonas oryzae pv. *oryzae* strains were prepared for DNA extraction by growing them at 28°C on peptone sucrose agar containing 20 g sucrose, 5 g peptone, 500 mg K_2HPO_4, 250 mg $MgSO_4 \cdot 7H_2O$, and 15 g agar in 1 L distilled water. The bacteria were scraped from the plates and genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Ochiai *et al.*, 2000).

PCR primers and reaction

PCR primers used in this study are listed in Table 1. The reaction mixtures for PCR contained 10× PCR buffer, 5 μl; 10 mmol/L dNTP mix, 4 μl; 25 μmol/L primer, 2 μl; rTaq, 1.25 U (TaKaRa, China); and genomic DNA, 2 μl (10 ng/μl) in a total volume of 50 μl. The primer, J3, was used in IS-PCR, which is located at 46-65 nucleotide of the insertion sequence IS*1112* in the reverse direction (Adhikari and Vera Cruz, 1999). The IS-PCR was amplified as described by Adhikari (Adhikari *et al.*, 1999), with some modifications. The PCR reaction was carried out using a PCR system (PTC-100, USA) involving initial incubation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, annealing at 60°C for 1 min, and extension at 68°C for 1 min, and final incubation at 68°C for 10 min. A 10 μl portion of each amplified PCR product was confirmed by electrophoresis in 2.5% (w/v) agarose gels. The experiments were repeated three times to confirm the identities and the differences in the DNA bands.

Three pairs of primers, namely 2F1-P1/2F1-P2, 5F2-P1/5F2-P2, and 5F3-P1/5F3-P2 were designed based on genome sequences of the three new insertion loci identified in seven Chinese strains by IS-PCR. The amplification program consisted of denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min, and the final extension cycle at 72°C for 10 min. A 10-μl portion of each amplified PCR product was confirmed by electrophoresis in 1.0% (w/v) agarose gel.

Molecular cloning and sequencing

The fragments were cut from the gel, purified by using a DNA gel extraction kit (TaKaRa, China), and cloned into the pGEM-T easy vector (Promega, USA) following the manufacturer's protocols. The ligated vectors were transformed into *Escherichia coli* strain DH5α and plasmid DNA was isolated from overnight cultures by alkaline lysis. The cloned fragments were sequenced using the dideoxynucleotide chain termination method using an automated sequencer (ABI).

Data analysis

The sequences were determined using the Vector NTI 10.0 sequence analysis package (Invitrogen) and the homology sequences searched using the BlastN program in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Multiple alignments were done using the CLUSTAL W algorithm in DNAMAN (Lynnon Corp, Canada). Phylogenetic trees were constructed using the neigh-

Table 1. Primers and their sequences used in this study

Primer	Sequence	Location
J3	5'-GCTCAGGTCAGGTGGCCTGG-3'	IS*1112a* [46-65(r)]
2F1-P1	5'-TTGACCTCGGGTGCATTCC-3'	1246681-1246699[a]
2F1-P2	5'-CGCGTTGCCTTTCACCTGTAC-3'	1247006-1247026 (r)[a]
5F3-P1	5'-GTCGAAGGCGGAGGTGGAT-3'	2648540-2648558[a]
5F3-P2	5'-GGCGAAGGCCGCTTTGTTG-3'	2648992-2648940 (r)[a]
5F2-P1	5'-CTTTCGCCGTCTGGAGGAG-3'	IS*Xo2* (743-761)
5F2-P2	5'-TGATCGGGCCGATACTTCG-3'	IS*1112b* [237-255(r)]

[a]location in Korean strain KACC10331 of *Xanthomonas oryzae* pv. *oryzae*; (r), reverse direction.

Table 2. IS*1112* sequences from the genomes of Japanese strain MAFF 311018 and Korean strain KACC10331

Name[a]	Direction[b]	Start	End	Length (bp)	Copy	5' Border	3' Border
JXO1	F	134540	135594	1055	full	GCT	GGT
JXO2	F	281611	282663	1053	full	CGG	CCC
JXO3	F	312807	313860	1054	full	CGG	CCC
JXO4	F	495918	496443	526	truncated	CGG	
JXO5	F	835768	836822	1055	full	CTC	CTC
JXO6	F	993020	993960	941	truncated		CAA
JXO7	R	1226893	1227777	885	truncated	GGC	
JXO8	R	1517771	1518825	1055	full	GGC	GGC
JXO9	F	1543026	1544080	1055	full	GAG	ATT
JXO10	F	1599821	1600881	1061	full	AGC	GCT
JXO11	F	2321379	2322433	1055	full	CTT	CTT
JXO12	F	2393659	2394333	675	truncated	GGC	
JXO13	R	2394964	2396018	1055	full	GGC	GGC
JXO14	F	2404079	2404948	870	truncated		CAG
JXO15	R	2448080	2448751	672	truncated	CTG	
JXO16	R	2474602	2475121	520	truncated	GGG	
JXO17	F	2796970	2798024	1055	full	GGG	CCC
JXO18	R	2835589	2836640	1052	full	TCG	AAG
JXO19	F	3028724	3029777	1054	full	CAC	ACA
JXO20	R	3085308	3086362	1055	full	CAC	CAC
JXO21	F	3108634	3109597	964	truncated	TAG	
JXO22	F	3186762	3187816	1055	full	CGA	CGA
JXO23	R	3509612	3510261	650	truncated	GTT	
JXO24	R	3518288	3518991	704	truncated		GCA
JXO25	F	3718818	3719872	1055	full	CCC	CCC
JXO26	R	3824664	3825632	969	full	AGT	AGT
JXO27	F	3902098	3903152	1055	full	GAC	GAC
JXO28	R	4158840	4159895	1056	full	GAC	GAC
JXO29	R	4189320	4190374	1055	full	CCC	GAC
JXO30	F	4450951	4451866	916	truncated		GAA
JXO31	F	4737495	4738478	984	truncated		CAG
KXO1	R	41511	40457	1055	full	CGT	CGT
KXO2	R	151499	150445	1055	full	ATC	ATC
KXO3	F	152089	153143	1055	full	GGT	GGT
KXO4	F	294864	295916	1053	full	CGG	CCC
KXO5	F	324914	325967	1054	full	CGG	CCC
KXO6	F	508061	508586	526	truncated	CGG	
KXO7	F	1026688	1027628	941	truncated		CAA
KXO8	R	1261682	1260798	885	truncated	GGC	
KXO9	R	1535377	1534323	1055	full	GGC	GGC
KXO10	F	1559570	1560623	1054	full	GAG	ATT
KXO11	F	1617657	1618711	1055	full	AGT	GCT
KXO12	F	2274563	2275617	1055	full	GAC	GAC
KXO13	F	2346528	2347582	1055	full	CTT	CTT
KXO14	F	2412571	2413245	675	truncated	GGC	

Name[a]	Direction[b]	Start	End	Length (bp)	Copy	5' Border	3' Border
KXO15	R	2414930	2413876	1055	full	GGC	GGC
KXO16	F	2422991	2423860	870	truncated		CAG
KXO17	R	2469056	2468385	672	truncated	CTG	
KXO18	R	2495428	2494909	520	truncated	GGG	
KXO19	R	2821554	2820500	1055	full	AGG	CCC
KXO20	R	2858494	2857443	1052	full	TCG	AAG
KXO21	F	2977592	2978646	1055	full	CGG	CGG
KXO22	F	3035921	3036974	1054	full	CAC	ACA
KXO23	R	3093586	3092532	1055	full	CAC	CAC
KXO24	F	3115939	3116902	964	truncated	TAG	
KXO25	R	3506016	3505367	650	truncated	GGC	
KXO26	R	3514510	3513807	704	truncated		GCA
KXO27	R	3826387	3825333	1055	full	AGT	AGT
KXO28	R	4164120	4163053	1068	full	GAC	GAC
KXO29	R	4193797	4192743	1055	full	CCC	GAC
KXO30	F	4453519	4454434	916	truncated		AGT
KXO31	F	4741501	4742484	984	truncated		CAG

[a]JXO1 to JXO31 were from Japanese strain MAFF 311018; KXO1 to KXO31 were from Korean strain KACC10331
[b]F, forward; R, reverse direction

bor-joining algorithm in DNAMAN. Complete genome sequences of the Japanese strain MAFF 311018 (GenBank accession number NC_007705) and the Korean strain KACC 10331 (GenBank accession number AE013598) were downloaded from NCBI for analysis. Three IS*1112* elements, namely IS*1112a* (GenBank accession number AY 691649), IS*1112b* (GenBank accession number AY691647), and IS*1112c* (GenBank accession number AY691648) were used for analysis and for locating copies of IS*1112* in the two genome sequences. The full-length copy of IS*1112* used for analysis in *Xoo* genome was defined as one that had imperfect inverted repeats at both terminals and showed similarity over 95% with IS*1112*. The truncated copy of IS*1112* used for analysis was defined as one that had one imperfect inverted repeat and over 500-bp long homology sequence with IS*1112*. Nineteen full-length copies and twelve truncated copies of IS*1112* were identified from the Japanese and the Korean strains, respectively (Table 2).

Results and Discussions

Three patterns of IS-PCR fingerprints were obtained in the seven Chinese strains of Xoo based on IS1112

IS-PCR technology has already proved useful in molecular screening of different strains of microorganisms. In *Xoo*, primer J3, located in the reverse direction in the 46-65 nucleotides of insertion sequence IS*1112a*, has been widely used to analyze diversity and genetic relationships (George *et al.*, 1997; Adhikari *et al.*, 1999; Adhikari and Vera Cruz, 1999; Sukhwinder-Singh *et al.*, 2003). Four to five strongly amplified fragments less than 2 kb were obtained from seven strains of *Xoo* collected from different regions of China using IS-PCR and primer J3 (Fig. 1). Based on the amplification patterns by IS-PCR, the seven Chinese strains of *Xoo* were visually classified into three groups: Group A (comprising strains C1 and C4) lacked a fragment about 420 bp long present in Group B (comprising strains C2, C3, C6, and C7) whereas Group C (comprising strain C5) had three unique fragments (about 250 bp, 550 bp, and 1100 bp in length) but lacked two fragments (about 840 bp and 870 bp in length) present in both Group A and Group B.

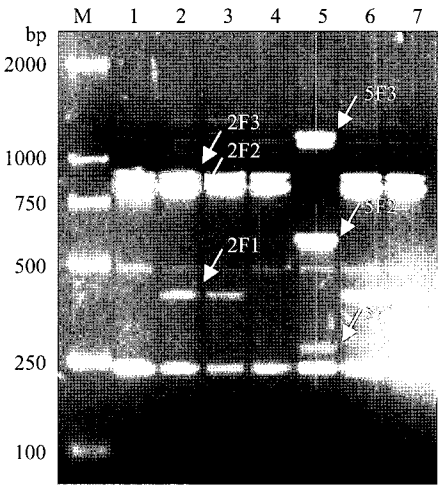

Fig. 1. Fingerprints of *Xanthononas oryzae* pv. *oryzae* strains using IS-PCR. Lane 1 to lane 7, seven strains C1 to C7. The molecular size marker (Lane M) is DL2000 (TaKaRa).

Six strongly amplified fragments, namely 2F1-2F3 and 5F1-5F3, were found to be different in the seven Chinese strains of *Xoo* in IS-PCR fingerprinting (Fig. 1). When all the six

fragments were sequenced, four (2F1, 2F2, 5F2, and 5F3) were identified as the insertion loci of IS*1112*, which consisted of a 65-bp-length sequence of 5'-terminal of IS*1112a* and the *Xoo* genomic sequence, and two (2F3 and 5F1), without the sequence of IS*1112* fragment, were identical to the *pilX* gene and cysteine protease gene of *Xoo*, respectively-they might represent a non-specific amplification by the primer J3 because the 3' terminal region of it was a multicopy repeat sequence in *Xoo* genome. In the genome of the Japanese strain MAFF 311018, 96 copies of repeats were found of a sequence of ten nucleotides in the 3' terminal region of primer J3: if searched with a sequence of the last nine nucleotides, the number of copies of the repeats goes up to 184. Therefore, using the IS-PCR based primer J3 could fingerprint the product just as rep-PCR, and also some bands obtained reflected the location of IS*1112* in the *Xoo* genome.

Three new loci of IS1112 were found in Chinese Xoo strains, which are located in gene fhuA, cirA, and insertion sequence ISXo2

Three of the four IS*1112* insertion loci identified in the Chinese strains of *Xoo* using IS-PCR were new: they had not been reported before and were not found in the full genome of the Japanese strain MAFF 311018 and the Korean strain KACC10331 either.

Fragment 2F1, found in four Chinese strains C2, C3, C6, and C7, was composed of a 353-bp length of gene *fhu*A (XOO1221, Korean strain KACC10331, putative TonB-dependent receptor) and a 65-bp length of 5'-terminal sequence of IS*1112a* but there were no insertion sequences in the gene *fhu*A region in both the Korean strain and the Japanese strain. To confirm the assumption that the insertion sequence IS*1112* had been inserted in gene *fhu*A, two primers, 2F1-P1 and 2F1-P2, were designed according to the sequence of gene *fhu*A flanking the inserted target site of band 2F1. When seven Chinese strains of *Xoo* were identified using these primers by PCR, a fragment about 1400 bp long could be obtained from four strains (C2, C3, C6, and C7), but another fragment, about 350 bp long, was also obtained from the other three strains, namely C1, C4, and C5 (Fig. 2A). When all the amplified fragments were sequenced, a full-length IS*1112* was found in strains C2, C3, C6, and C7, located just after 450 nucleotides of the open-reading frames

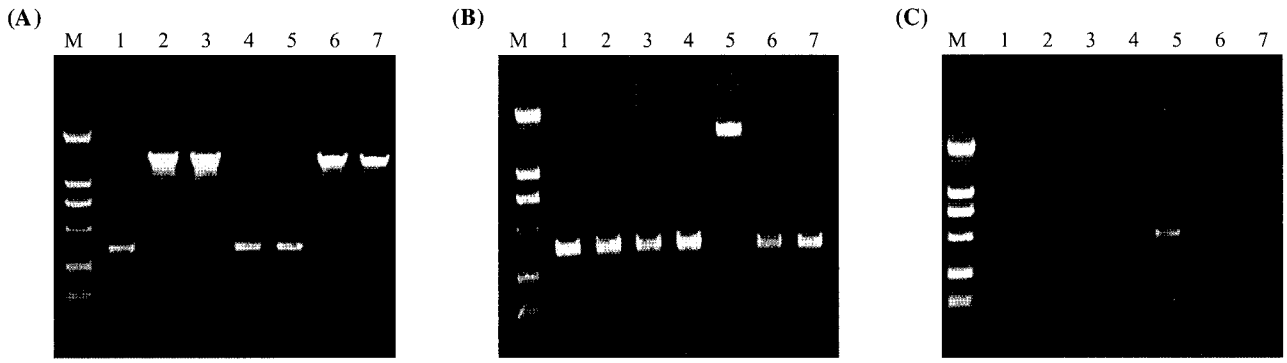

Fig. 2. Identification of three new insertion loci of IS*1112* in *Xanthomonas oryzae* pv. *oryzae* strains by PCR. (A) Pair of primers 2F1-P1/2F1-P2, (B) Pair of primers 5F3-P1/5F3-P2, (C) Pair of primers 5F2-P1/5F2-P2. Lanes 1-7, *Xoo* strains C1-C7; the molecular size marker (lane M) is DL2000.

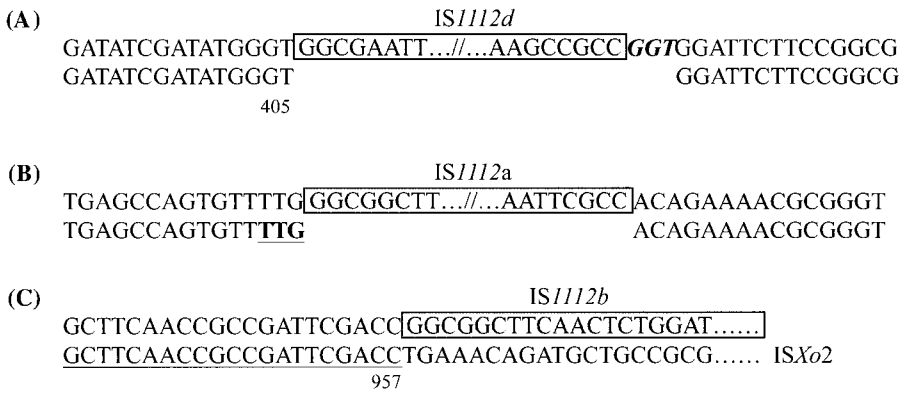

Fig. 3. Structures of three new loci of IS*1112* in *Xanthomonas oryzae* pv. *oryzae* in China. (A) Locus of IS*1112d* in gene *fhu*A (XOO1221). The first line shows the sequence from strain C2 and the second line, that from strain C5. Three bases in italics are duplicated at the target site. The number below is that of the nucleotide of coding region of gene *fhu*A. (B) Locus of IS*1112a* in gene *cir*A (XOO2500). The first line shows the sequence from strain C5 and the second line, that from strain C2. Bases underlined are the start codon of gene *cir*A. (C) Locus of IS*1112b* in insertion sequence IS*Xo2*. The first line shows the sequence from strain C5 and the second line, that from IS*Xo2*.

(ORF) of gene *fhu*A. However, no sequences were found inserted in gene *fhu*A in strains C1, C4, and C5 (Fig. 3A).

Fragment 5F3, found in Chinese strain C5, was composed of a 1114-bp length of gene *cir*A gene (XOO2500, Korean strain KACC10331, putative TonB-dependent receptor) and a 65-bp length of IS*1112*. However, this fragment too did not show any insertion sequence in gene *cir*A region either in the Korean strain or in the Japanese strain. A pair of primers 5F3-P1/5F3-P2 was designed based on the sequences of gene *cir*A flanking the inserted target site of band 5F3. A 1450-bp fragment was obtained only from strain C5, but another 400-bp fragment was obtained from the other six strains (Fig. 2B). A full-length IS*1112* was found only in strain C5, inserted just after the start codon of gene *cir*A in the reverse direction (Fig. 3B).

Fragment 5F2, also found in strain C5, was composed of a 531-bp long insertion sequence IS*Xo*2 and a 65-bp length of IS*1112*. Primers 5F2-P1 and 5F2-P2 were designed based on insertion sequence IS*Xo*2 and IS*1112*, respectively. When the seven strains were amplified with these primers, a 470-bp long fragment was obtained only in strain C5 (Fig. 2C), and no fragment was obtained in any of the other six strains. The fragment amplified was sequenced, a 255-bp length of 5' terminal of IS*1112* linked after the 957th nucleotide of IS*Xo*2 (Fig. 3C). Insertion IS*1112* in IS*Xo*2 resulted in a new complex of ISs found in *Xoo*, but structural details of this complex were not completely clear due to the multiple copies of IS*Xo2* in *Xoo* genome, and need further study.

The insertion structure of IS*1112* in gene *cir*A in strain C5 was confirmed by RFLP in an earlier study (Adhikari *et al.*, 1995). When the region of gene *cir*A in strain C5 is cleaved by *Eco*RI, it is theoretically possible to obtain a 2864 bp band containing a full length of IS*1112* . Among 308 strains from different Asian countries, only the Chinese strain GD 1358 (strain C5 of this study) exhibited a strong and clear band about 2.9-kb long in the RFLP map using *Eco*RI-cleaved *Xoo* genomic DNA hybridized with pJEL101 (containing a full length of IS*1112*). This result also indicated that the insertion of IS*1112* in gene *cir*A might be limited specifically to the Chinese strain C5. Another new locus of IS*1112* in gene *fhu*A identified in four Chinese strains was also analyzed again, none of the Asian strains exhibited the one 1301-bp hybrid band containing a full length of IS*1112* obtainable in theory when genomic DNA is digested with *Eco*RI (Adhikari *et al.*, 1995), indicating that the insertion loci of IS*1112* in gene *fhu*A might be unique to Chinese strains.

Nineteen full-length copies and twelve truncated copies of IS*1112* were found in the genomes of both the Korean strain and the Japanese strain of *Xoo* (Table 2), which have been sequenced completely (Lee *et al.*, 2005; Ochiai *et al.*, 2005). Seven of the nineteen full-length IS*1112* dispersed differently, but all loci of the 12 truncated copies of IS*1112* were identical in the two strains. This suggests active movement of full-length IS*1112* among the Korean, Japanese, and Chinese strains. DNA fingerprinting of *Xoo* strains from Asia using IS*1112* and *avrXa*10 as probes often groups strains from a single country or geographic region in Asia into separate independent groups (Adhikari *et al.*, 1995). This supports the assumption that different loci of IS*1112* in *Xoo* strains could be specific to the strains collected from

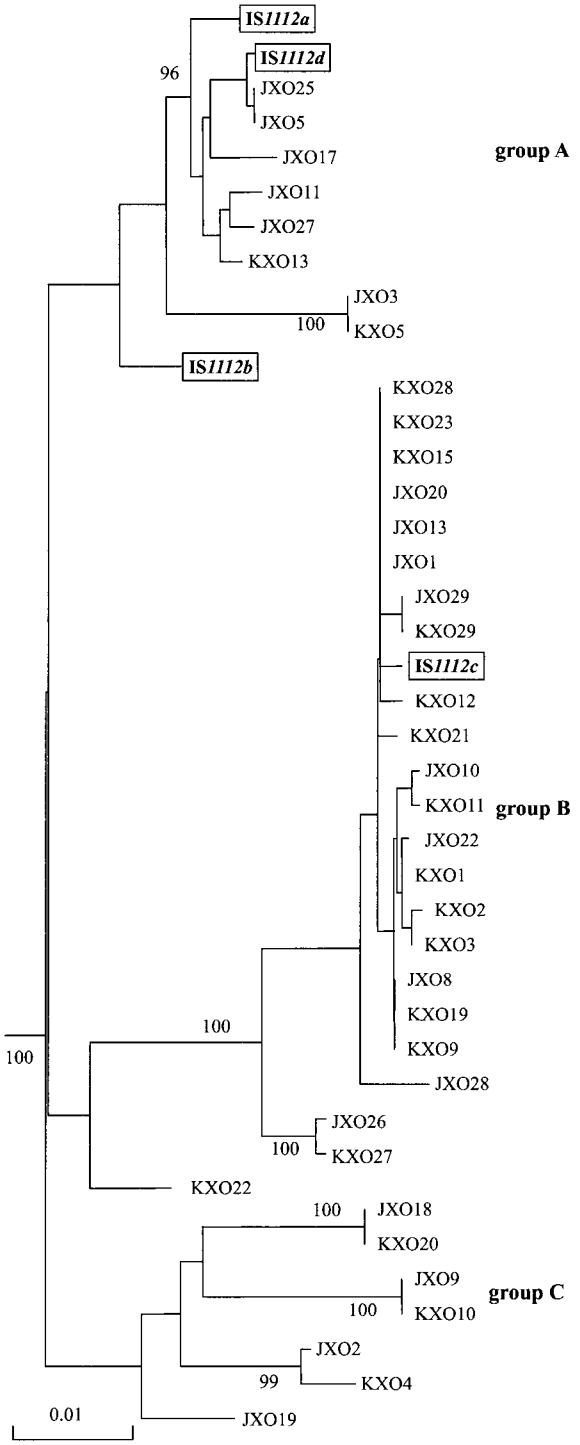

Fig. 4. Phylogenetic analysis of IS*1112a*, IS*1112b*, IS*1112c*, IS*1112d*, and 38 full-length copies of IS*1112* from *Xoo* Japanese strain MAFF 311018 and Korean strain KACC10331. Thirty-one loci of IS*1112* in Japanese strain numbered JXO1 to JXO31 and those in Korean strain numbered KXO1 to KXO31, according to their location in genome. Nineteen from each selected for analysis and retain their number. Multiple-sequence alignments generated by using DNAMAN and phylogenetic tree constructed by the neighbor-joining algorithm. Lengths of horizontal branches proportional to the genetic distance, and the number at each point indicates bootstrap values. The data set was subjected to 1000 bootstrap replicates.

the same region or country, and that the pattern of movement of IS*1112* in *Xoo* might be different among different ecological regions.

The diversity of the sequences of IS1112 found in the three new loci in Chinese strains

The sequences of IS*1112* in the three new loci also varied: the sequence in gene *cir*A of strain C5 was 100% identical with the full-length sequence of IS*1112a* in strain PXO86 from the Philippines and that in IS*Xo*2 100% identical with the corresponding region of IS*1112b*, also identified in strain PXO86. However, the sequence in gene *fhu*A of strains C2, C3, C6, and C7 did not show more than 99% similarity to any of the IS*1112* variants reported earlier. This novel IS*1112* element, designated as IS*1112d* (GenBank accession number EF140722), was 1055 bp in length, with a 25-bp imperfect inverted repeats, and showed 98.6%, 98.5%, and 95.4% similarities with IS*1112a*, IS*1112b*, and IS*1112c*, respectively. The putative protein encoded by the ORF of IS*1112d* consisted of 318 amino acids and showed 97.5%, 97.2%, and 95.0% similarities with IS*1112a*, IS*1112b*, and IS*1112c*, respectively.

The 42 full-length copies of IS*1112* used for analyzing their diversity comprised 38 full-length copies from the Japanese strain and the Korean strain (Table 2), 3 copies (IS*1112a*, IS*1112b*, and IS*1112c*) from strain PXO86 from the Philippines, and IS*1112d*, identified from the Chinese strain in this study. IS*1112* was 1052-1061 bp long, and similarities among its copies ranged from 94.1% to 100%. The copies were classified into three groups: IS*1112a*, IS*1112b*, IS*1112d* and 8 copies of IS*1112* from the Japanese and the Korean strains formed Group A; IS*1112c* and 23 copies of IS*1112* from the Japanese and the Korean strains formed Group B; and the remaining 7 IS*1112* from the Japanese and the Korean strains formed Group C (Fig. 4).

The sequences of IS*1112* at the same position between two strains are conservative. Of 12 copies of IS*1112* at identical positions in the genomes of the Japanese and the Korean strains, 10 were classified into the same subgroups, for example JXO2/KXO4, JXO9/KXO10, and JXO18/KXO20. The similarities in these pairs of IS*1112* exceeded 99.5% except two pairs, JXO19/KXO22 and JXO28/KXO28, which could not be assigned either to the same subgroup or to any other subgroup at other loci (Fig. 4). IS*1112a* and IS*1112d*, identified at new loci in the Chinese strains in this study, and IS*1112b*, identified from the Philippine strain PXO86, could not be placed in the same subgroup with IS*1112* from the Japanese and the Korean strains; presumably, each locus of IS*1112* in the *Xoo* genome might be conservative and inherited independently.

Twelve truncated copies of IS*1112* in genomes of both the Japanese and the Korean strains were compared pair by pair according to their location. To our surprise, the location and length of all twelve pairs were identical. The similarities in pairs of IS*1112* at the same locations were almost 99.0%, indicating that the two strains are very closely related, and also supported the idea that each location of IS*1112* in *Xoo* genome might be conservative and inherited independently.

Three bases duplicated in the target site were often found with insertion of IS1112

Three bases of the target sites were often found duplicated at the insertion locus of IS*1112*. In this study, IS*1112d* caused three bases, GGT, to be duplicated at the target site of gene *fhu*A whereas IS*1112a* did not cause any duplication at the target site of gene *cir*A (Fig. 3). Among 19 full-length copies of IS*1112*, 10 caused three bases to be duplicated at the target site in the Japanese strain and 11 did so in the Korean strain (Table 2). Three IS*1112* elements, namely IS*1112a*, IS*1112b*, and IS*1112c*, in the Philippine strain caused three bases - CGG, CAC, and GGC - to be duplicated respectively also at the target site (Ryba-White *et al.*, 2005).

It could be concluded that the IS*1112* element could cause three bases to be duplicated at about half the insertion sites in *Xoo*. However, other insertion sequences, such as IS*Xo1* and IS*Xo2*, could respectively cause four and eight bases to be duplicated in *Xoo* (Rajeshwari and Sonti, 2000).

No sequence preference was found in the insertion site of IS*1112*. In this study, the three bases of the target sequences of these three new insertion sites of IS*1112* were different. Three bases to the right and three to the left of the borders of the insertion site of IS*1112* were used for further analysis (Table 2). In the Japanese strain, 19 insertion target sequences to the right and left of the borders were both classified into 13 types. In the Korean strain, the 19 sequences on either side of the border were classified into 13 types in the case of those to the right of the border and 14 in the case of those to the left. In the Philippine strain, target sequences of three insertions were classified into three types (Ryba-White *et al.*, 2005).

Phenotype could be changed due to the insertion of IS1112 in Chinese strains

Changes in phenotype due to transposition of insertion sequences under natural conditions have been reported in *Xoo*. Some mutants deficient in virulence or with lower production of extracellular polysaccharides were reported following the insertion of IS*Xo*1 or IS*Xo*2 into *gumM* gene (Rajeshwari and Sonti, 2000). In this study, two of the three new loci of IS*1112* in the Chinese strains were located in an open-reading frame (ORF) of functional genes. One was found in strains C2, C3, C6, and C7, located just after 450 nucleotides of gene *fhu*A and another, in strain C5, was located after the start codon of gene *cir*A in the reverse direction. Although the functions of *fhu*A and *cir*A genes in *Xoo* are not known so far, the phenotype could be changed following the insertion because both belong to putative TonB-dependent receptors, which are involved in the uptake of macromolecules, play an important role in perceiving environmental signals, and are associated with pathogenicity of plant pathogens (Koebnik, 2005).

In this study, three new loci of IS*1112* were found in Chinese *Xoo* strains using IS-PCR. Among the three new loci of IS*1112*, two located in the open reading frame region of genes *fhu*A and *cir*A, both belong to putative TonB-dependent receptors, which might relate to the pathogenicity of *Xoo* strains. The diversity of IS*1112* sequence and insertion loci in *Xoo* genome was analyzed to discuss the movement and inheritance of IS*1112*.

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