Efficient Discrimination of Race Mutants of *Xanthomonas campestris* pv. *oryzae* by RFLP Analysis Using Plasmid pJEL101 Containing a Highly Repetitive DNA Element

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Xanthomonas campestris pv. *oryzae* (XCO) is a causal agent of bacterial leaf blight of rice. So far XCO has been divided into 7 to 9 races in Japan⁹⁾ and 6 races in the Philippines⁷⁾, based on the pathogenicity to differential rice cultivars. When genomic DNA of XCO was probed with pJEL101, a pUC18 plasmid containing a 2.4 kb *EcoRI*-*HindIII* fragment with one highly repetitive DNA sequence derived from XCO, restriction fragment

length polymorphism (RFLP) analysis was efficient to differentiate strains of races from Japan⁹⁾ and the Philippines⁴⁾. In the present study, we compared RFLP patterns of various XCO pathogenicity mutants induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment with those of wild type strains, using 4 kinds of restriction enzymes (*EcoRI*, *HindIII*, *EcoRV* and *Clal*) and pJEL101 as a probe.

Fig. 1. Southern blot analysis of total DNA from strains of *Xanthomonas campestris* pv. *oryzae* wild types and pathogenicity mutants. The probe was photobiotin-labeled pJEL101. Lanes 1, 8 and 16, λ DNA digested by *HindIII*; lane 2, wild type, race 1, PXO61; lane 3, race 1, PXO61PT; lane 4, PXO61NT3; lane 5, PXO61NT3RV1; lane 6, race 1 $\rightarrow$ 2, PXO61NT3RV2; lane 7, race 1 $\rightarrow$ 2, PXO61NT3RV3; lane 9, wild type, race 2, PXO63-6; lane 10, race 2, PXO63-6PT1; lane 11, race 2, PXO63-6PT2; lane 12, PXO63-6NT2; lane 13, PXO63-6NT3; lane 14, PXO63-6NT5; lane 15, PXO63-6NT8; lane 17, PXO63-6NT8I-1; lane 18, PXO63-6NT8I-2; lane 19, PXO63-6NT8Lh; lane 20, PXO63-6NT11; lane 21, PXO63-6NT12; lane 22, PXO63-6NT15 and lane 23, PXO63-6NT17. DNA samples were digested with *EcoRI* before electrophoresis and transfer.

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Bacterial strains. The wild type strains used in this study were obtained from the International Rice Research Institute (IRRI). Pathogenicity mutants listed in Table 1 were derived from Tsuchiya *et al.*¹⁰⁾ All strains have been verified by extensive screening with pathogenicity tests to fully-grown rice plants^{10,11)}.

Preparation of bacterial DNA. Bacteria were streaked on peptone sucrose agar plates (PSA: peptone 10 g, sucrose 10 g, sodium glutamate 1 g, agar 15 g, distilled water 1000 ml, pH 7.0)¹⁰⁾ and kept at 28°C for 48

Table 1. Wild type strains and pathogenicity mutants of *Xanthomonas campestris* pv. *oryzae* used in RFLP analyses

Strain	Race	Characteristics ^{a)}
Wild type (Philippine isolate)		
PXO61	1	SM ^s ^{b)}
PXO23	1	SM ^s
PXO63-6	2	SM ^s
PXO86	2	SM ^s
PXO138	2	SM ^s
PXO158	2	SM ^s
PXO159	2	SM ^s
PXO171	2	SM ^s
PXO172	2	SM ^s
PXO175	2	SM ^s
PXO22	3	SM ^s
PXO71	4	SM ^s
PXO80	5	SM ^s
Mutant		
PXO61PT	1	SM ^r , parent strain
PXO61NT3	—	SM ^r , avirulent, non-pigmented
PXO61NT3RV1	—	SM ^r , avirulent, revertant for pigmentation
PXO61NT3RV2	1 → 2	SM ^r , reverted from PXO61NT3, race mutant
PXO61NT3RV3	1 → 2	SM ^r , reverted from PXO61NT3, race mutant
PXO63-6PT1	2	SM ^r , parent strain 1
PXO63-6PT2	2	SM ^r , parent strain 2
PXO63-6NT2	2	SM ^r , non-pigmented, resistant to OP ₂ phage
PXO63-6NT3	2	SM ^r , resistant to OP ₂ phage
PXO63-6NT5	—	SM ^r , weakly virulent
PXO63-6NT8	2	SM ^r , non-pigmented
PXO63-6NT8I-1	2	SM ^r , reisolated from inoculated plant
PXO63-6NT8I-2	2	SM ^r , reisolated from inoculated plant
PXO63-6NT8Lh	2	SM ^r , non-pigmented, colony type variant ^{c)}
PXO63-6NT11	2	SM ^r , non-pigmented
PXO63-6NT12	2	SM ^r , non-pigmented
PXO63-6NT15	—	SM ^r , weakly virulent
PXO63-6NT17	—	SM ^r , weakly virulent

a) Details were described in Refs. 10) and 11).

b) SM^s: streptomycin susceptible, SM^r: streptomycin resistant.

c) See Refs 2) and 11).

hr. From these plates, single colonies were picked and cultured on PSA slants. Then the bacteria were grown in 10 ml of PS broth on a reciprocal shaker (120 rpm) at 28°C for 24 hr. Genomic DNA was extracted by a modification of the procedure of Wilson¹²⁾. Bacterial cells were pelleted by centrifugation at 5000 × *g* for 10 min and washed with STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM Na₂EDTA, pH 8.0). The washed bacteria were resuspended with a mixture of 2.8 ml of TE buffer (10 mM Tris-HCl, 1 mM Na₂EDTA, pH 8.0), 150 μl of 10% sodium dodecyl sulfate (SDS), 30 μl of proteinase K (10 mg/ml) and incubated at 37°C for 2 hr. Then 5 M NaCl solution was added to adjust the final concentration to 0.7 M and 400 μl of hexadecyltrimethylammonium bromide (CTAB) solution in 0.7 M NaCl was added. After incubation at 65°C for 15 min, an equal volume of chloroform was mixed. The mixture was centrifuged at 6000 × *g* for 10 min. After the upper layer was transferred to a fresh tube, the DNA was precipitated by adding two volumes of ethanol and kept at 4°C for 10 min. The DNA was collected by centrifugation at 6000 × *g* for 10 min at 4°C, dried under vacuum and suspended in TE buffer. CsCl (2.1 g) was added to the DNA solution (2 ml) and completely dissolved. One hundred μl of ethidium bromide (EtBr) solution (10 mg/ml) was then added. The mixture was centrifuged at

Fig. 2. Southern blot analysis of total DNA from strains of *Xanthomonas campestris* pv. *oryzae* race mutants and representing 5 different races. The probe was photobiotin-labeled pJEL101. Lane 1, race 1, PXO61; lane 2, race 1 → 2, PXO61NT3RV2; lane 3, race 1 → 2, PXO61NT3RV3; lane 4, race 1, PXO23; lane 5, race 2, PXO86; lane 6, race 3, PXO22; lane 7, race 4, PXO71 and lane 8, race 5, PXO80. DNA samples were digested with *Eco*RI before electrophoresis and transfer.

70,000 rpm in the Hitachi RP100 AT4 rotor for 24 hr at 20°C. The DNA band was collected by side puncture of the tube with a 18-gauge needle. An equal volume of *n*-butanol was added to the DNA solution and the elimination of EtBr was repeated several times. After the final elimination, the DNA was subsequently precipitated by adding two volumes of ethanol, collected by centrifugation at 6000×*g* for 10 min at 4°C, dried under vacuum and suspended in TE buffer.

Agarose gel electrophoresis. Approximately 1 µg of DNA was digested to completion with restriction enzymes (*Eco*RI, *Hind*III, *Eco*RV or *Cla*I, TOYOBO, 2–10 unit/µg DNA). Digested DNA samples (1 µg/well) were run in 0.8% agarose gel immersed in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM Na₂EDTA) with electrophoresis at 50 V for 4 hr. Restriction fragment sizes were estimated by comparison to λ DNA digested with *Hind*III.

DNA probe. The DNA probe pJEL101 used in this study was derived from a genomic library of XCO PXO86 and constructed into the cloning vector pUC18⁵⁾. Labeling of pJEL101 by photobiotin was done as described by McInnes *et al.*⁶⁾

Southern hybridization. Transfer of DNA fragments from agarose gel onto nylon membrane (Hybond-N) was done according to instructions of the manufacture (Amersham Japan Co.). The blot was prehybridized

at 42°C for 2 hr in a solution composed of 0.5% SDS, 5× Denhardt (100×Denhardt contains 2% bovine serum albumin, 2% Ficoll 400 and 2% polyvinylpyrrolidone), 2×SSC (20×SSC contains 3 M NaCl and 0.3 M Na₃-citrate), 50% formamide and 20 µg/ml of denatured salmon sperm DNA. For hybridization, denatured labeled probe DNA (40 ng/ml) was added directly to the prehybridization solution, and the blot was incubated at 42°C for 24 hr. After hybridization, the blot was washed three times (5 min each) at room temperature in 2×SSC containing 0.1% SDS and then two times (15 min each) at 55°C in 0.1×SSC containing 0.1% SDS. Detection of DNA band was done as described by McInnes *et al.*⁶⁾

When various mutant strains within the same race were compared, such as avirulent, weakly virulent and non-pigmented ones derived from PXO61 (race 1) or from PXO63-6 (race 2), their RFLP patterns were almost the same as those of their respective parent strains. On the other hand, RFLP patterns of two race mutants (race 1→2), PXO61NT3RV2 and PXO61NT3RV3, were significantly different from that of the parent strain (PXO61, race 1) (Fig. 1). RFLP patterns of these two race mutants were similar to that of race 2 strain PXO86, but differed from RFLP patterns of other races (strains PXO23, race 1; PXO22, race 3; PXO71, race 4 and PXO80, race 5) (Fig. 2). Furthermore, the RFLP patterns of the race mutants were almost identical to those of many other isolates belonging to race 2 (Fig. 3). The other enzymes used, *Hind*III, *Eco*RV and *Cla*I, also generated similar restriction patterns as *Eco*RI to discriminate between strains (data not shown).

From these results, RFLP analysis of XCO probed with pJEL101 could be an efficient tool for distinguishing races of wild type strains as well as those induced by mutagenesis. This would avoid the laborious inoculation tests to many rice plants.

Results also suggest that the race 2 strains in the Philippines might contain mutants derived from race 1 strains. Similar race mutants (race 2→3) induced by NTG mutagenesis have been reported earlier¹⁾. Clear changes in RFLP patterns have been shown with race 1→2 mutants. It is difficult to explain this phenomenon only by single point mutations or frame shifts. Nelson *et al.*⁸⁾ reported that XCO have several transposable elements such as TNX1, TNX6, TNX7 and TNX8, in which an element TNX8 hybridized with the repetitive element present in pJEL101 and had been designated IS1112. It is, therefore, possible that NTG mutagenesis induced homologous recombination and/or transposition in connection with IS1112 and arose changes in race specificity. Further research on this is now in progress.

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Fig. 3. Southern blot analysis of total DNA from race mutants and the race 2 strains of *Xanthomonas campestris* pv. *oryzae*. The probe was photobiotin-labeled pJEL101. Lane 1, race 1→2, PXO61NT3RV2; lane 2, race 1→2, PXO61NT3RV3; lane 3, race 2, PXO86; lane 4, race 2, PXO138; lane 5, race 2, PXO158; lane 6, race 2, PXO159; lane 7, race 2, PXO171; lane 8, race 2, PXO172 and lane 9, race 2, PXO175. DNA samples were digested with *Eco*RI before electrophoresis and transfer.

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和 文 摘 要

堀田光生・土屋健一・加来久敏・平八重一之・日比忠明：高頻度反復配列を含むプラスミド pJEL101 をプローブとした RFLP によるイネ白葉枯病菌レース突然変異株の判別

フィリピン産イネ白葉枯病菌 PXO61 株(レース 1)および PXO63-6 株(レース 2)からニトロソグアニジン処理によって誘発された各種病原性変異株について、イネ白葉枯病菌由来の高頻度反復配列を含むプラスミド pJEL101 をプローブとして RFLP 解析を行い、親株および他レース(1~5)との比較を行った。その結果、2つの親株に由来する病原性喪失変異株、病原力低下変異株および色素非産生変異株は、それぞれ親株と概ね相同の RFLP パターンを示した。これに対し、PXO61 株に由来するレース変異株(1→2)の RFLP パターンは親株を含むレース 1 に属する他の菌株とは明らかに異なり、レース 2 に属する多数のフィリピン産菌株の示すパターンとほぼ相同であった。以上の結果から、フィリピン産イネ白葉枯病菌のレース 2 群菌の中には、突然変異によりレース 1 群菌から分化したものが含まれることが推察された。また、同プローブを用いた RFLP 解析は本細菌の野生株のみならず誘発突然変異株のレース判定においても適用可能であることが示唆された。

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