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Insertional Transposon Mutagenesis of *Xanthomonas oryzae* pv. *oryzae* KXO85 by Electroporation

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The bacterial leaf blight, which is caused by *Xanthomonas oryzae* pv. *oryzae*, is the most damaging and intractable disease of rice. To identify the genes involved in the virulence mechanism of transposon Tn5 complex, which possesses a linearized transposon and transposase, was successfully introduced into *X. oryzae* pv. *oryzae* by electroporation. The transposon mutants were selected and confirm the presence of transposition in *X. oryzae* pv. *oryzae* by the PCR amplification of transposon fragments and the Southern hybridization using these mutants. Furthermore, transposon insertion sites in the mutant bacterial chromosome were determined by direct genomic DNA sequencing using transposon-specific primers with ABI 3100 Genetic Analyzer. Efficiency of transposition was influenced mostly by the competence status of *X. oryzae* pv. *oryzae* cells and the conditions of electroporation. These results indicated that the insertion mutagenesis strategy could be applied to define function of uncharacterized genes in *X. oryzae* pv. *oryzae*.

Keywords **:** Mutagenesis, Transposon, Tn5, *Xanthomonas oryzae* pv. *oryzae*

Xanthomonas oryzae pv. *oryzae*, the causal agent of bacterial leaf blight (BLB), is the most destructive bacterial disease of rice (*Oryza sativa* L.) which occurs in tropical, subtropical, and warm temperate regions of the world (Swing et al., 1990). Rice plants infected with BLB show various changes in their physiology, such as increase or decrease of component substances, change in enzyme activities, increase in respiratory rate, accumulation of photosynthetic assimilates, decrease in photosynthetic efficiency, and increase in water permeability of leaf cell (Misawa and Miyazaki, 1972; 1973).

The transposable elements have become valuable mutagenic tools for genetic and molecular analyses of different bacteria for creating insertional knockout mutations (Voelker and Dybvig, 1998). The Tn5 is the most widely used transposon in Gram-negative bacteria, which transposes at high frequency, has relatively little target sequence specificity and low homology with genomic sequences of most bacterial species (Reznikoff, 1993). However, transposon mutagenesis has the following technical limitations: (i) the transposase gene must be expressed in the target host, (ii) the transposon must be introduced into the host using suicide vector and (iii) the transposase gene on the transposon should be expressed in subsequent generations, otherwise the insertion may result in genetic instability (Akerley et al., 1998; Devine and Boeke, 1994; Goryshin and Reznikoff, 1998; Gwinn et al., 1997; Haapa et al., 1999).

Electroporation is a method of transformation that allows the introduction of foreign DNA into host cells (prokaryotic or eukaryotic) via the application of high-voltage electric pulses. The electric field induces pore formation in the cell wall and increases the permeability of the host cells to macromolecules, which allows for uptake of DNA (Lurquin, 1997). Previously, triparental mating was commonly used for the introduction of foreign DNA into plant-pathogenic bacteria (Ditta et al., 1980). Triparental mating requires a helper strain, carrying the genes that code for conjugation and DNA transfer, and a donor strain, carrying the plasmid to be introduced into the new bacterial strain. At least five to seven days are required in order to determine if the plasmid was successfully introduced into the new bacterial strain and conform that there is no carryover of the helper or donor strain. Electroporation does not require a helper or a donor strain and helps to avoid possible contamination with other strains. In addition, introduction of the foreign DNA can be verified in the recipient strain within two days, making electroporation the faster and more efficient method of transformation. Electroporation is being used for transformation of both Gram-positive and Gram-negative species of plant-pathogenic and plant-associated bacteria (Dennis and Sokol, 1995; Grewal et al., 1993; Smith and Iglewski, 1989). The electroporation conditions can differ not only for bacterial species but also strains within a species.

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In this study, the genome sequence of the *X. oryzae* pv. *oryzae* KACC 10331 (KXO85) was determined for functional analysis of uncharacterized genes. A technique for *in vivo* transposition that involves the *in vitro* formation of released Tn5 transposition complexes (Transposome™) was used and followed by introduction of the complexes into the KXO85 of choice by electroporation. The Transposome™ is an association between a hyperactive Tn5-derived transposase and linearized transposon that contains a selectable marker (kanamycin resisatance gene) flanked at each end by modified 19-bp transposase recognition sequences of Tn5 (Goryshin and Reznikoff, 1998). In addition, we report that simple and robust technology can generate high-efficient transposition in *X. oryzae* pv. *oryzae*.

Materials and Methods

Bacterial strain and culture condition. *X. oryzae* pv. *oryzae* KACC10331 (KXO85) was obtained from Korean Agricultural Culture Collection (KACC) of Genetic Resource Division in National Institute of Agricultural Biotechnology, Suwon, Korea and cultured on YGC medium [2.0% D-(+)-glucose, 2.0% $CaCO_3$, 1.0% Yeast extract, 1.5% agar].

Preparation of electrocompetent *X. oryzae* pv. *oryzae* cells. To prepare competent cells for electroporation, *X. oryzae* pv. *oryzae* KXO85 cells were inoculated into 40 ml of nutrient broth (NA, Difco) after incubating for three days at 28°C on YGC agar plate. The cells were further cultured for two days at 28°C on a rotary shaker (OD_{600}=0.8-1.0) and harvested by centrifugation (6,000 × g) at 4°C for 10 min. The cells were washed with 15 ml of cold, sterile ETN buffer [1 mM EDTA, 200 mM Tris-HCl (pH 7.5), 1 M NaCl], concentrated by centrifugation and washed in 15 ml of cold, sterile MTN buffer [50 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl (pH 7.5), 80 mM NaCl]. The suspension was centrifuged at 6000 × g for 10 min at 4°C, resuspended in 1 ml of cold, sterile 15% glycerol and kept on ice until electroporated with the transposon.

***In vivo* transposition mutagenesis by electroporation.** One microliter of a premade Transposome™ (20 ng/μl; Epicentre Technologies, Madison, WI) was mixed with 50 μl of electrocompetent KXO85 cells and placed in a 0.2 cm gap electroporation cuvette (Bio-Rad). A single high-voltage pulse (12.5 kV/cm for 5 ms with a resistance value of 200 Ω and a capacitance of 25 μF) was applied across the chilled suspension with the Gene Pulser II system (Bio-Rad). After the pulse delivery, the cells were immediately removed from the electroporation curvette and inoculated into 1 ml of SOC medium (2.0% tryptone, 0.5% yeast extract, 0.05% NaCl, 20 mM glucose) without antibiotics. The cells were incubated for 18 h at 28°C with constant shaking (120 rpm) to allow expression of antibiotics resistance. After incubation, the putative transformants were plated on nutrient agar plates containing 10 μg/ml of kanamycin. The 1 ml of SOC medium containing the electroporated sample was entirely plated on 10 plates of selective NA medium and incubated at 28°C for three days.

Genomic DNA isolation. After incubation, colonies were inoculated into 1.5 ml of NB medium containing 10 μg/ml of kanamycin and incubated at 28°C for 24 h. Genomic DNAs from KXO85 mutants were extracted using the genomic DNA extraction kit (Genomic-tips) supplied by Qiagen (Hilden, Germany).

PCR amplification of insertion mutants. PCRs were carried out with a PTC-200™ thermocycler (MJ research, Watertown, mass.) using the primer set (KANF: 5'-CAATCAGGTGCGACAATC-3', KANR: 5'-TCACCGAGGCAGTTCCAT-3') for confirming the insertion of transposon. PCR reactions were performed in a 50 μl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM of each dNTP, 10 pM of each primer, 2 units of *Taq* polymerase (Promega, Madison, Wis.). The total amount of genomic DNA from KXO85 insertion mutants added to the PCR mixture was approximately 50 ng. Reactions were run for 25 cycles, each consisting of 15 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C, with initial denaturation of 10 min at 94°C and final extension of 7 min at 72°C. An eight μl aliquot of each amplified PCR product was electrophoresed on a 1.0% agarose gel, stained with ethidium bromide and visualized under a UV transilluminator.

Southern hybridization. To confirm whether the mutants obtained were derived from true transposition or not, genomic DNA from KXO85 mutants was digested with *Eco*RI and separated by electrophoresis in 0.8% agarose gel. Transposon was labeled as probe with [α-^{32}P] dCTP using the random primed method according to the manufacturer's instructions (Ladderman™ Labeling kit, Takara, Japan). Southern hybridization analysis was carried out by standard methods (Sambrook et al., 1989).

Direct genomic DNA sequencing and sequence analysis. Transposon insertion sites were sequenced bidirectionally using sequencing primers specific for the ends of the inserted transposon (TF: 5'-AATCACCAACTGGTCCACCT-3', TR: 5'-CGATAGATTGTCGCACCTGA-3'). Two micrograms of genomic DNA and 10 pmoles of primer were used in "2X" Big Dye Terminator sequencing reactions according to the manufacturer's protocols (PE Applied Biosystems). Samples were cycled by PTC-200™ thermocycler (MJ research, Watertown, mass.) for 5 min at 95°C, then 55 cycles of 30 sec at 95°C, 20 sec at 50°C and 4 min at 60°C followed by 4°C indefinitely. Samples were purified and analyzed with ABI 3100 Genetic Analyzer (PE Applied Biosystems).

Results

Efficiency of random mutagenesis system. The transposon mutagenesis was performed using different conditions of electroporation since a transformation system can be highly strain-dependent. It was important to define the optimal parameters of electroporation to ensure that each bacterial strain of interest is transformed efficiently. These parameters include the time constant and the field strength applied to the sample (Lurquin, 1997). The time constant is dependent on the total resistance of the sample and the

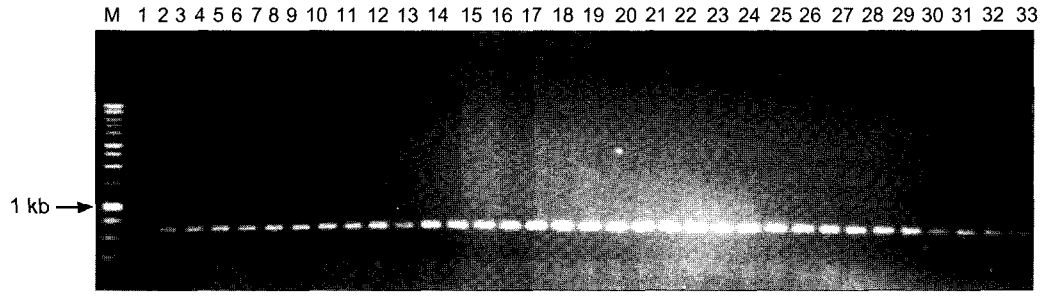

Fig. 1. PCR amplification of Tn5 fragment using kanamycin resistant gene-specific primers from KXO85 mutants. 1, *Xanthomonas oryzae* pv. *oryzae* (control); 2-33, random insertion mutants (*X. o.* pv. *oryzae*); M, Size marker (1-kb ladder).

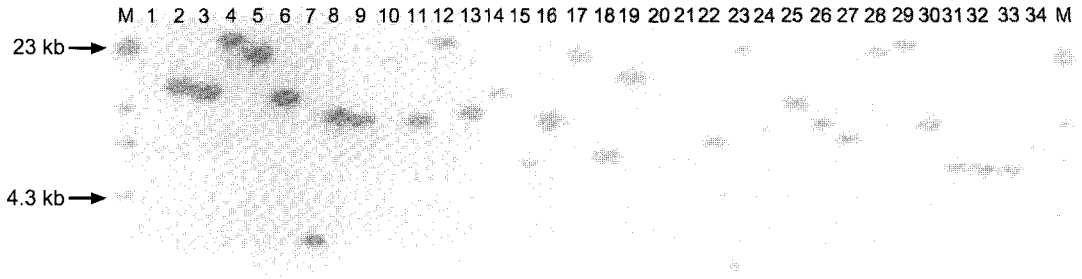

Fig. 2. Southern hybridization of *X. o.* pv. *oryzae* KXO85 and its Tn5 insertion mutants. 1, *Xanthomonas oryzae* pv. *oryzae* (control); 2-34, random insertion mutants (*X. o.* pv. *oryzae*); M, Size marker (lambda/*Hin*dIII). Genomic DNA from 33 mutants was digested with *Eco*RI and hybridized with [α-^{32}P] dCTP labeled transposon.

capacitance of the pulse circuit for the electroporation device. The field strength is dependent on the initial voltage delivered by the electroporator and the distance between the electrodes of the cuvette. The optimal time constant for prokaryotic cells should range from 5 to 10 ms and the field strength should be 16 to 19 kV/cm (Lurquin, 1997). We defined the optimal parameters of electroporation for transposon mutagenesis of KXO85. The optimal time constant for KXO85 should be 5 ms and the field strength should be 12.5 kV/cm with a resistance value of 200 Ω and a capacitance of 25 μF. When the 20 ng of transposome was electroporated into *X. o.* pv. *oryzae* Kxo85 competent cells, 664 mutant clones were obtained in the first experiment. However, in subsequent experiments with optimized competent cells and electroporation conditions, about 30,000 clones per reaction were obtained from KXO85 using 20 ng transposome.

PCR amplification and Southern hybridization. Total thirty-three KXO85 transposition mutants were randomly selected and amplified using PCR reaction with KANF and KANR primers. Single DNA fragment of 611 bp size was successfully amplified from all 32 mutants, but not from wild type *X. oryzae* pv. *oryzae* KXO85. (Fig. 1). Genomic DNA isolated from 33 transposition mutants was digested with *Eco*RI and subjected to Southern hybridization analysis using transposon as a probe. At result, 32 transposition mutants of KXO85 contained single unique transposon inserts (Fig. 2). Correct hybridization signal could not be detected from one of the mutants by Southern hybridization with transposon as a probe. These results indicated that the insertions were single and independent events in most cases. The stability of the transposon in bacterial cells was confirmed (data not shown). After several times subcultures, all transformants retained the antibiotic resistance (kanamycin) when sub-cultured for several times indicating stable transposon within the genome of the Kxo85.

Direct genomic DNA sequencing of transposon insertion sites. Genomic DNAs from eight randomly selected KXO85 transposon mutants were purified and directly sequenced as above described methods. Two micrograms of genomic DNA were sufficient to obtain sequence reads of approximately 450 bases. The location of the transposon inserts was determined by comparing the sequence of regions flanking the element with the genome sequence of the KXO85 (Table 1). Some of these mutant clones were significantly changed with their phenotype. Especially, "MKxo331_#3" clone was a pigment deficient mutant, because *aroE* gene was disrupted by the transposon (Fig. 3) (Goel et al., 2001). All eight-insertion sites were unique. Sequence analysis also indicated that eight insertion sites carried 9-bp duplication at the boundaries, which is the result of Tn5-transposase-mediated transposition (Goryshin and Reznikoff, 1998).

Table 1. DNA sequences and corresponding genes flanking the Tn5-derived transposon insertion sites in KXO85 mutants

KXO85 mutants	Flanking sequence	Insertion site (gene)	Gene locus	E -value
OxoL173(Kxo85)-PL2	<KANR>GCGCTGTCTTGGTCTGAAACACGACCTG	*metB*	AF337647	e-146
OxoL743(Kxo85)-PL8	<KANR>CCCACTTTTGGCTGTTTTTTACACGAATC	*avrXa10*	XOU50552	1e-09
OxoL903(Kxo85)-PL10	<KANR>GGCAAGGTCAGTGCGCAGGATCTGTGG	*hrpF*	AY205561	e-135
OxoL965(Kxo85)-PL11	<KANR>ACAGACACTGGGCGCTTGCAGCACCGG	*gum*	AE011898	1e-69
MKxo331_#3	<KANR>CGCTGGGGCTGGTCAACAGTCTTACCG	*aroE*	AF258797	3e-91
MKxo331_#49	<KANR>CCGTTGTTCAACACCGGGCTGGGGTT	*acp* (acyl carrier protein)	AY010120	e-135
MKxo331_#11	<KANR>GAGCGCATCTGCGCGCGTGGGTTCGTGG	*aroK*	AE011943	e-111

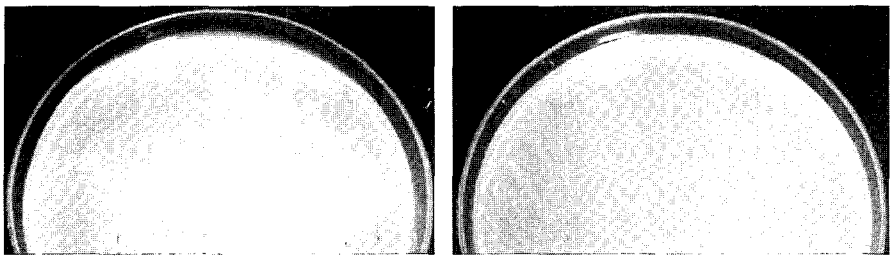

Fig. 3. Phenotypic change of *X. o.* pv. *oryzae* KXO85 by mutation. Left: wild type, right: MKxo331_#3, pigment-deficient mutant (disruption of *aroE* gene).

Discussion

In this report, we described an *in vivo* mutagenesis system for identifying genes involved in virulence of *X. oryzae* pv. *oryzae* (KXO85). The broad-host-range (bhr) vector pUFR027, pUFR034 (cosmid), pHM1 (cosmid) and pLAFR3 (cosmid) are commonly used cloning vectors for preparing mutant of *X. campestris* pv. *malvacearum* and other xanthomonads which could be introduced into host cell by conjugation or electroporation (DeFeyter et al., 1990). However, mutagenesis using conjugation or electroporation system with bhr vector failed to produce any detectable mutation. In contrast, when the Tn5-derived transposon (Transposome™) containing kanamycin resistance gene was used with electroporation, 664 mutant clones were obtained from a reaction started with 1 μl (20 ng) of transposome mixture. To our knowledge, transposon mutagenesis of Korean *X. oryzae* pv. *oryzae* (KXO85) has not been reported till date. Most Gram-negative bacteria are susceptible to electroporation (Wirth et al., 1989), except those, which contains restriction-modification (R-M) systems. Korean *X. oryzae* pv. *oryzae* KACC10331 (KXO85) used in this study has two R-M (*Xor*I and *Xor*II) systems. The presence of R-M systems in KXO85 influences transformation and conjugation frequencies. Genomic DNA from *X. oryzae* pv. *oryzae* that contains the *Xor*I R-M systems is not digested by the endonuclease *Pst*I(*Xor*I isoschizomer). Genomic DNA from *X. oryzae* pv. *oryzae* that contains the *Xor*II R-M systems is not digested by either *Xor*II or *Pvu*I (isoschizomer of *Xor*II) (Choi and Leach, 1994). Based on these result, it is speculated that the presence of R-M system in *X. oryzae* pv. *oryzae* could be one of the reasons for frequent failure of generating transposon mutants using plasmid delivery based transposition previously. Interestingly, the strain KXO85 was virulent on rice plant carrying the *Xa21* gene, which has been reported to confer broad-spectrum resistance to BLB (Wang et al., 1996). Through mutagenesis approach described here, it would be interesting to analyze functional genes conferring virulence on the strain KXO85 against such rice cultivars carrying such a broad-spectrum resistance gene.

There are many different types of bacterial transposons. Some of the smaller ones are about 1,000 bp long and carry only the genes for the transposases that promote their movement. Larger transposons may also contain one or more other genes, such as those for resistance to an antibiotic (Snyder and Champness, 1997). Tn5 is a bacterial genetic element that transposes *via* a cutand-paste mechanism (Goryshin and Reznikoff, 1998). The only macromolecular components required for this process are the transposase; the transposon, which can presumably be any sequence that is defined by two specific inverted 19 bp sequences; and the target DNA into which the insertions are made (Goryshin and Reznikoff, 1998).

Transposition mutagenesis technique using electroporation with transposon is the simple means of introducing any DNA segment into bacterial chromosome. This technology allows the *in vivo* insertion of transposons into several

bacterial species. In fact, with improvements in the efficiencies of bacterial electroporation, *in vivo* transposition mutagenesis with transposon (Transposome™) could be applied to any bacterium for which a selectable marker exists. Not only is the transposon insertion process easy and fast, but the inserted transposons facilitate rapid analysis of the transposon insertion sites by such means as direct genomic DNA sequencing (Hoffman et al., 2000).

The transposition mutagenesis strategy should allow the identification of KXO85 genes that mediate plant pathogenicity (Rossier et al., 2000). Our KXO85 transposition mutants are currently being inoculated into rice plant in order to identify genes that allows *X. oryzae* pv. *oryzae* cells to colonize, move and cause disease in rice plant. Hopefully, mutagenesis studies, in conjunction with the additional genome sequence of *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri*, will lead to a better understanding of the molecular mechanisms of *X. oryzae* pv. *oryzae* pathogenicity and perhaps suggest novel control measures for BLB.

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