

Marker-Exchange Mutagenesis and Complementation Strategies for the Gram-Negative Bacteria *Xanthomonas oryzae* pv. *oryzae*

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Summary

This chapter describes methods for targeted knockouts using marker exchange mutagenesis and complementation of the Gram-negative bacteria *Xanthomonas oryzae* pv. *oryzae*. We have used these methods to demonstrate that type I secretion and modification systems are involved in avrXa21 activity of *X. oryzae* pv. *oryzae*.

Key Words: Marker-exchange mutagenesis; overexpression; Gram-negative bacteria; *Xanthomonas oryzae* pv. *oryzae*.

1. Introduction

Innate immunity provides a first line of defense against pathogen attack and is activated rapidly after infection. In contrast to the adaptive immune system that depends on somatic gene rearrangements for the generation of antigen receptors with random specificities, the innate immune system uses a set of defined receptors for pathogen recognition (**1**). Although it is now widely appreciated that pathogen recognition receptors play a key role in innate immunity in plants and animals, very little is known about the bacterial molecules recognized by such receptors.

Components of innate immune systems in both plants and animals share many conserved features. Most notably, they sense the presence of pathogen-associated molecular patterns (PAMPs), which represent conserved molecular structures, and avirulence (Avr) factors that are strain-specific molecules produced by phytopathogens. Recognition by the host is via cell surface or cytoplasmic receptors (**2,3**). These receptors share common protein domains such as leucine-rich repeats (LRRs), which act as ligand recognition domains, and

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conserved signaling domains, such as Toll-interleukin 1 and serine threonine kinase domains (4). Naturally occurring mutations of LRR residues that interfere with ligand binding are correlated with several human diseases, including Bernard-Soulier syndrome and Chron's disease (5,6).

Intracellular recognition of both PAMPs and Avr factors is largely carried out by the cytoplasmic nucleotide-binding oligomerization domain (NOD) protein family. The NOD family contains a large number of proteins from animals, plants, fungi, and bacteria (7). Genetic variation in three human NOD family members has been implicated in the development of disease (7). Similarly, variations in plant NOD family members determine levels of resistance to bacterial, fungal, insect, and viral pathogens underscoring the essential role of the NOD-mediated innate immune response in plant and animal biology.

In animals, recognition of PAMPs in extracellular compartments or at the cell surface is largely carried out by the Toll-like receptor (TLR) family that contain LRRs in the extracellular domain and a Toll-interleukin 1 intracellular domain (8). Although TLRs recognize diverse molecules, they activate a common signaling pathway to induce a core set of defense responses (9). Several bacterial PAMPs have been identified to date, including flagellin (recognized by TLR5 [10]), lipopolysaccharide (recognized by TLR4 [11]), and a modified peptide (muramyl dipeptide, recognized by Nod1 [12]).

Surprisingly, little is known about how plant hosts sense and respond to PAMPs or Avr factors at the cell surface. The best characterized examples are the tomato CF receptors that detect *Cladosporium fulvum* Avr peptides (13), the *Arabidopsis* FLS2 receptor kinase (RK) that detects flagellin, a proteinaceous component of bacterial polar flagella, and the rice Xa21 RK that mediates recognition of *Xanthomonas oryzae* pv. *oryzae* (Xoo) strains expressing AvrXa21 activity. In this chapter, the term AvrXA21 pathogen-associated molecule(s) (PAM) will be used to designate the molecule(s) produced by Xoo that triggers the Xa21-mediated innate immune response. Resistance conferred by the Xa21 gene is quite broad spectrum, with resistance to 29 of 32 strains tested, suggesting that all 29 strains carry AvrXa21 activity (14). Whereas plants lacking XA21 are susceptible to most races of the pathogen Xoo, *Arabidopsis* plants lacking FLS2 display no disease phenotype (15), confounding the precise role of FLS2 in disease resistance.

Despite these distinctions, both FLS2 and XA21 carry LRRs in the presumed extracellular domain, are members of large polymorphic gene families (in the case of Xa21, at least 40), and fall into a distinct phylogenetic subclass, the LRRXII class ([16]; CD and PR, unpublished), suggesting that FLS2 and XA21 mediate recognition of PAMs in a conserved manner. Recently, a rice RK named XA26 that is closely related to XA21 and FLS2 was cloned and demonstrated to confer resistance to Xoo (Q. Zhang, personal communication). This

result suggests that many of the approx 1100 largely uncharacterized rice RKs may be involved in PAM perception. Interestingly, like FLS2, at least two other plant LRR–RKs serve as receptors for small peptides, including the presumed receptor for phytosulfokine (a sulfated peptide that plays a key role in cellular dedifferentiation and proliferation in plants), and systemin (a plant signalling molecule [17–19]). As is the case with RKs in animals, most plant RK ligands identified so far are secreted peptides (20).

In summary, there is increasing evidence that TLRs, NODs, and plant RKs share conserved recognition and signaling domains, that their signaling pathways are conserved, and that they recognize diverse PAMs from plant and animal pathogens (15,21). Given the importance of these proteins in innate immune recognition and host defense, there is great interest in identifying the PAMs that they detect, elucidating the secretion and modification of these molecules, and determining their role in the biology of the pathogen.

In our laboratory, efforts are underway to identify new genes required for AvrXa21 PAM activity and to determine the product and function of the genes with various molecular techniques. Among them, inactivation of a gene via marker exchange mutagenesis and recovery of the gene via complementation of a mutant are invaluable tools for understanding the physiology and the significance of specific genes in the virulence of pathogens. For the last a few years, we have applied marker exchange mutagenesis using double crossover (DCO) and complementation strategies to understand the function of the *rax* (required for AvrXa21 activity) genes. We have cloned eight *rax*-genes from *Xoo*, which causes bacterial blight disease in rice. We generated nonpolar mutants using the cloned genes and pUC18, and complement mutants with the cloned genes and the pUFR027 or pML122 vector (22–25). Through analysis of phenotype changes of the mutants in inoculation experiments, we confirmed that the genes are required for AvrXa21 activity.

2. Materials

1. pUC18 vector.
2. pUC-4K vector (Pharmacia).
3. Restriction enzymes and reaction buffers (NEB).
4. T4 DNA ligase with reaction buffer (NEB).
5. NB medium.
6. PSB medium: 10 g of peptone, 10 g of sucrose, 1 g of sodium glutamate for 1 L, pH 7.0).
7. Antibiotics (kanamycin, cephalixin, gentamycin, ampicillin).
8. Spectrophotometer.
9. Cell-Porator™ (BRL).
10. *Escherichia coli* strain DH10B.
11. *Xoo* strain, PX099.

12. pET15-b vector.
13. pUFR027 or pML122 vector.
14. TEN buffer: 200 mM Tris-HCl, pH 7.5, 1 mM ethylene diamine tetraacetic acid, 1 M NaCl.
15. TMN buffer: 50 mM Tris-HCl, pH 7.5, 50 mM MgSO₄.

3. Methods

The following protocols are described based on our work for *Xoo*.

3.1. Marker-Exchange Mutagenesis

3.1.1. Vector Construction for DCO Event

1. Construct a plasmid coding the target gene by using multiple cloning sites in a suicide plasmid (*see Note 1*), which are not able to replicate in *Xoo* (conditional replicons). The conditional replicon must have a gene encoding a selectable marker for antibiotics resistance. General *E. coli* vector such as a pUC18 has been used for generation of *Xoo* knockout mutants in our laboratory.
2. Disrupt the coding sequences of the target gene with restriction enzyme(s) available for insertion or substitution of a marker gene. An antibiotic resistance marker such as the Kanamycin-resistant gene (*Kan^r*) or another gene for which there is an easily selected phenotype are generally used as the marker gene (*see Note 2*). The marker must be different from the plasmid marker (Ampicillin resistant gene [*Amp^r*] in pUC18). In this step, homologous fragments for DCO event of your target gene disrupted by the inserted marker would be better to be longer than 400 bp (*see Note 3*).
3. Ligate the linearized plasmids and marker genes with T4 DNA ligase at 4°C overnight.

3.1.2. Preparation of *Xoo*-Competent Cells

1. Grow an overnight culture (OD₆₀₀ = 0.8-1.0) of *Xoo* cells in 40 mL of NB containing cephalixin (25 µg/mL) on a rotary shaker at 28°C.
2. Harvest by centrifugation at 2500g at 4°C for 10 min.
3. Suspend the cell pellet with 15 mL of cold TEN buffer by pipetting.
4. Repeat **steps 2 and 3** three times.
5. Centrifuge at 2500g at 4°C for 10 min.
6. Resuspend with 15 mL of TMN buffer by pipetting.
7. Chill on ice for 2 h.
8. Repeat **steps 5 and 6**.
9. Suspend with 15 mL of cold DDW by pipetting.
10. Harvest the cell with centrifugation at 2500g at 4°C for 10 min.
11. Suspend with 15 mL of cold 15% glycerol–water solution.
12. Transfer 20 µL of cells to 0.5-mL tubes on ice.
13. Stock in –80°C freezer.

3.1.3. Electroporation of the Construct Into Xoo-Competent Cells

1. Mix 20 μL of *Xoo*-competent cells and 1 to 2 μL (10 ng) of recombinant plasmids.
2. Transform by using electroporation (Cell-Porator™: 700 V, 4K Ω).
3. Transfer the cell to 1 mL of liquid PSB medium and culture for 2 to 3 h at 28°C.
4. Plate the cells onto PSA medium plates that contain the appropriate antibiotics (Kanamycine: 50 $\mu\text{g}/\text{mL}$) for selection of mutants, and incubate at 28°C.

3.1.4. Selection of the Mutant by DCO Event

1. Plate the putative mutants from PSA plate containing 50 $\mu\text{g}/\text{mL}$ of kanamycin on PSA containing kanamycin and kanamycin (50 $\mu\text{g}/\text{mL}$) / ampicillin (100 $\mu\text{g}/\text{mL}$), respectively.
2. Incubate at 28°C for 2 or 3 d.
3. Select mutants grown on PSA plate containing kanamycin (50 $\mu\text{g}/\text{mL}$), not on PSA plate containing kanamycin (50 $\mu\text{g}/\text{mL}$) / ampicillin (100 $\mu\text{g}/\text{mL}$; *see* **Notes 4 and 5**).
4. After selection on replica plates, the marker exchange event can be confirmed by Southern blot analysis (*see* **Note 6**) or colony polymerase chain reaction.

3.2. Complement and Overexpression Mutant

3.2.1. Vector Construction

1. Clone your favorite gene into the pET-15b vector by using available cloning site. Using this cloning step, six sequential copies of Histidine are fused to N-terminus of the coding sequences. This His-tag from pET-15b will be feasible for confirmation of the gene expression in the target cells with Western blot analysis (*see* **Note 7**).
2. Excise the fused fragment from the construct by using available restriction enzyme(s) for cloning to expression vector. We have used pML122 or pUFR027 (*see* **Note 1**).
3. Ligate the gene fused by six histidines and vectors (pML122 or pUFR027) with T4 DNA ligase at 4°C overnight.

3.2.2. Introduction of the Construct Into Xoo-Competent Cells

1. Introduce pML122 carrying the His-tag fused gene into *Xoo*-competent cells (*see* **Subheading 3.1.2.**) in which target gene expression was inactivated by marker exchange mutagenesis by using electroporation (Cell-Porator: 700 V, 4K Ω).
2. Transfer the cell to 1 mL of liquid PSB medium and culture for 2 to 3 h at 28°C and then plate onto PSA plates containing kanamycin (50 $\mu\text{g}/\text{mL}$)/gentamycin (15 $\mu\text{g}/\text{mL}$; *see* **Note 8**).
3. Confirm the transformant with isolated plasmids, and Western blot analysis with His-antibody for expression of the gene (*see* **Note 6**).
4. Stock in -80°C freezer (*see* **Note 9**).

4. Notes

1. A narrow host range vector for *E. coli* can be used as a suicide vector for *Xanthomonas* broad host range vectors, such as pML122 and pML123 (26), or pUFR027 and pUFR034 (27), which replicate in *Xanthomonas* and cannot be used. pML122/123 uses pML10 as the template vector and contains two selective marker genes (*Kan^r*, *Gm^r*) and the promoter of the *Nm^r* gene. The vectors (pUFR027 and 034) contain the pSa origin of DNA replication, *parA* from the *Agrobacterium* plasmid pTAR, neomycin-resistant gene as a selection marker, and a *lacZ* cassette with cloning sites.
2. In our laboratory, the *Kan^r* gene from pUC-4K (Pharmacia) or the *Spec^r* gene from the TOPO have both been used for the marker. In the case in which a double gene knockout mutant is being generated, two different selective markers are needed.
3. We recommend using more than 400 bp for the DCO event, but it is not impossible to cause the DCO event with shorter DNA fragments. However, the efficiency of the DCO event is considerably lower with shorter DNA fragments.
4. The putative mutants from kanamycin plates might have both (DCO and single crossover [SCO]) mutants, but the DCO mutants can be selected by replica plating (kanamycin and kanamycin/ampicillin). DCO mutants carry only the *Kan^r* gene used for disruption of the target gene, whereas SCO mutants contain both the *Kan^r* gene and the plasmid marker gene (*Amp^r*) in the *Xoo* genome. This selection step is important because if the homologous regions for recombination include sequences 5'- or 3'- to the coding portion of the target gene, SCO events can recreate a complete gene and DCO mutagenesis will be unsuccessful.
5. In some cases, a direct screen for DCO is not feasible because DCO events that incorporate a gene from a plasmid into the chromosome are infrequent. In this case, a two-step method is used. Although the SCO mutants carry the entire plasmid containing both the mutant and wild-type copies, the wild-type copy can be removed by second recombination event between the flanking direct repeats through succeeding a generation.
6. The standard technique for southern and western blot analyses is used (28).
7. If you have other methods to detect expression of your gene, you don't need to use pET15-b and start from **step 3**. In some case, the six histidines at the N-terminus can change conformational structure of protein and, therefore, the biological function of the protein could be lost.
8. Growth of the transformant could be slow or unsuccessful on selection medium containing two antibiotics (kanamycin and gentamycin) because pML10, the template vector for pML122/123, has different copy numbers in different species (45, 70, 105, 45 copies in *E. coli*, *Pseudomonas putida*, *Rhizobium meliloti*, and *Rhizobium leguminosarum*, respectively) and the copy number is much lower than other *E. coli* vectors. In this case, you can select for transformants using one half the concentration of antibiotics (25 µg/mL of kanamycin and 7.5 µg/mL of gentamycin) or you can use a two-step selection, with kanamycin followed by gentamycin.

9. Safekeeping of the transformants carrying pML122 constructs in -80°C are important, because the vector is not stable and has low copy number. To obtain accurate results with the transformants, it would be better to use the fresh cells from stock.

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