MPMI Vol. 9, No. 7, 1996, pp. 664-666. Publication no. M-1996-0730-02O. © 1996 The American Phytopathological Society

For the Record

Cloning and Characterization of the *rpfC* Gene of *Xanthomonas oryzae* pv. *oryzae*: Involvement in Exopolysaccharide Production and Virulence to Rice

Ji-Liang Tang,[1,2] Jia-Xun Feng,[1,2] Qi-Qin Li,[1] Hai-Xiang Wen,[1] Dong-Lin Zhou,[1] T. J. Greer Wilson,[2] J. Maxwell Dow,[2] Qing-Sheng Ma,[1] and Michael J. Daniels[2]

[1]Laboratory of Molecular Genetics, Guangxi Agricultural University, Nanning, Guangxi 530005, P. R. China; [2]The Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, U. K.

Received 5 March 1996. Accepted 31 May 1996.

***rpfC* is one of a cluster of genes which coordinately regulate the synthesis of extracellular enzymes and exopolysaccharide and pathogenicity in *Xanthomonas campestris* pv. *campestris*, the black rot pathogen of brassicas. An *rpfC* homolog which could functionally complement an *rpfC* mutant of *X. campestris* pv. *campestris* was identified in *Xanthomonas oryzae* pv. *oryzae* and the gene was characterized. Mutation of this gene in *X. oryzae* pv. *oryzae* had no effect on extracellular enzymes, but exopolysaccharide synthesis and virulence to rice were substantially reduced.**

Additional keywords: bacterial blight of rice, two-component regulation.

Xanthomonas oryzae pv. *oryzae* causes bacterial blight of rice, one of the most destructive diseases in rice, particularly in Asia (Mew et al. 1993). Although several genes involved in the interaction of *X. oryzae* pv. *oryzae* and its host plant have been identified (Kelemu and Leach 1990; Hopkins et al. 1992; Kamdar et al. 1993), the molecular mechanisms underlying pathogenesis are poorly understood. In our previous studies on *Xanthomonas campestris* pv. *campestris*, the black rot pathogen of cruciferous plants, we have described a nonpathogenic mutant strain 8237 which is deficient in the synthesis of extracellular enzymes which include protease (PRT), polygalacturonate lyase (PGL), endoglucanase (EGL), and amylase (AML), and extracellular polysaccharide (EPS). This mutant could be complemented *in trans* by a recombinant plasmid (pIJ3020) isolated from a *X. campestris* pv. *campestris* genomic library, which concomitantly restores both the synthesis of the enzymes and EPS and the virulence (Daniels et al. 1984). It was subsequently found that pIJ3020 carries a cluster of positive global regulatory genes spanning 23 kb designated *rpfA-H*; mutation in any of these genes results in significant reduction of both the synthesis of enzymes and EPS and virulence (Tang et al. 1991; Dow and Daniels 1994).

Corresponding author: M. J. Daniels;
E-mail: DANIELS@BBSRC.AC.UK

DNA sequence: GenBank accession number X97865.

One of these genes (*rpfC*) encodes a fused two-component sensor-regulator (Tang et al. 1991) of the ITRO class (Parkinson and Kofoid 1992). DNA sequences cross-hybridizing to the insert DNA of pIJ3020 have been found in Southern hybridizations of genomic DNA of a number of pathovars of *Xanthomonas campestris* and *X. oryzae* pv. *oryzae* (Sawczyc et al. 1989; Todd et al. 1990). Although recombinant plasmids containing these homologous DNA fragments were able to restore pathogenicity and protease production to the mutant 8237 (Sawczyc et al. 1989; Todd et al. 1990), the function of the homologous DNA in the parent organism was not investigated. In this paper we address this question for *X. oryzae* pv. *oryzae*. We describe the cloning and characterization of the *rpfC* homolog from *X. oryzae* pv. *oryzae* and show that it is involved in EPS production and virulence to rice.

Southern hybridizations of genomic DNA from 10 different Chinese *X. oryzae* pv. *oryzae* strains digested with *Eco*RI using the *X. campestris* pv. *campestris* insert DNA of pIJ3020 as a probe revealed that each contained cross-hybridizing DNA. The patterns of hybridizing bands were similar to each other and to those found in the Philippine *X. oryzae* pv. *oryzae* strains by Todd et al. (1990). To clone the homologous sequences a genomic library of the *X. oryzae* pv. *oryzae* wild-type strain T3000 was constructed in pLAFR1 as described by Daniels et al. (1984) and 2,000 individual *Escherichia coli* library colonies were screened by colony hybridization with pIJ3020 insert DNA as a probe. One colony showed strong hybridization with the probe and the recombinant plasmid designated pGXN3000 was isolated from the colony, digested with *Eco*RI (to release whole insert DNA from the vector) and analyzed further by Southern blotting with pIJ3020 insert DNA as a probe. All the insert fragments hybridized with the probe and the pattern was the same as that obtained in hybridizations with the total genomic DNA (data not shown). A restriction map of pGXN3000 is shown in Figure 1.

pGXN3000 was transferred by conjugation into *X. campestris* pv. *campestris* strains 8237 and 8473, an *rpfC* mutant with the chromosomal copy of the gene inactivated by Tn*5* insertion (Tang et al. 1991). The transconjugant strains

8237/pGXN3000 and 8473/pGXN3000 were tested for pathogenicity and production of the enzymes and EPS. The results showed that pGXN3000 could complement both mutants for pathogenicity and production of extracellular enzymes and EPS suggesting that it carries a functional *rpfC* homolog. The precise location of this homolog within the insert DNA of pGXN3000 was established by Southern hybridization with a 3-kb *Bam*HI fragment from pIJ3020 which carries the *rpfC* gene (Tang et al. 1991). pGXN3000 DNA was digested with *Eco*RI or *Bam*HI and the DNA fragments were separated by agarose gel electrophoresis. On Southern analysis with the 3-kb *X. campestris* pv. *campestris rpfC* probe, one *Bam*HI fragment (3 kb) and three *Eco*RI fragments (1.8, 1.3, 1.3 kb) were seen to hybridize. This located the *rpfC* homolog to the middle of the pGXN3000 insert DNA (Fig. 1). The 3-kb *Bam*HI fragment was deleted from pGXN3000 by partial digestion with *Bam*HI and religation of the fragments bigger than 27 kb. This deletion derivative plasmid could no longer complement 8473 for pathogenicity, production of the enzymes, and production of EPS.

The nucleotide sequence of the 3-kb *Bam*HI fragment comprises 3,018 base pairs. Analysis of this sequence with the FRAME program (Bibb et al. 1984) revealed a similar open reading frame (ORF) to the *X. campestris* pv. *campestris rpfC* ORF (Tang et al. 1991), extending from an ATG start codon at position 189 to a TAG stop codon at position 2219. Potential –10 and -35 consensus promoter sequences and a ribosome binding site upstream of the ATG start codon were similar to those of the *rpfC* gene from *X. campestris* pv. *campestris*. The nucleotide sequences of the two ORFs showed 83.3% identity. The predicted protein in *X. oryzae* pv. *oryzae* has 676 amino acids, one less than RpfC of *X. campestris* pv. *campestris*, and the amino acid sequences of the two proteins show 85.2% identity and 91.7% similarity. The greatest variation between the sequences appears mainly in two domains; from amino acids 365 to 388 and from amino acids 526 to 546. As expected, RpfC from *X. oryzae* pv. *oryzae* shows extensive homology to conserved domains of both sensor and regulator proteins of two-component regulatory systems (reviewed by Parkinson and Kofoid 1992) where the N-terminal region of the protein is related to the sensors and is separated by a linker segment from a region related to the regulators (data not shown).

As a first step to creating an *rpfC* mutant of *X. oryzae* pv. *oryzae*, pGXN3000 was mutagenized with the transposon Tn*5* by the method described by Turner et al. (1985). Five Tn*5* insertions located in different positions within the 3-kb *Bam*HI fragment were obtained (insertions 1,22,39,47 and 57 in Fig. 1). The plasmids were transferred individually by mating into the *X. campestris* pv. *campestris rpfC* mutant 8473 with appropriate antibiotic selection. When the transconjugants were tested for pathogenicity on turnip leaves and for production of extracellular enzymes and EPS, all of them had reduced pathogenicity and produced similar levels of enzymes (AML, EGL, PGL, and PRT) and EPS to 8473. As controls, two other plasmids with Tn*5* insertions located outside the 3-kb *Bam*HI fragment (insertions 34 and 19 in Fig. 1) were also transferred into 8473. These plasmids were still able to restore pathogenicity and production of the enzymes and EPS to the *X. campestris* pv. *campestris* mutant.

Five *rpfC* mutants of *X. oryzae* pv. *oryzae* were constructed by introducing the five different Tn*5* insertions located within the *rpfC* gene in pGXN3000 into the corresponding positions of the wild-type strain 13751 genome by marker exchange. This was done essentially as described by Turner et al. (1985), except that the plasmid used to displace pGXN3000 (or pGXN3000::Tn*5*) was pIJ3011 (Sawczyc et al. 1989). The marker exchange was confirmed by Southern analysis of *Eco*RI-digested genomic DNA of the mutants using pGXN3000 as a probe (data not shown). The two other Tn*5* insertions located outside the *rpfC* locus (34 and 19), which could still complement the *X. campestris* pv. *campestris rpfC* mutant, were also exchanged into the 13751 genome. The wild type strain 13751, like the nine other Chinese *X. ooryzae* pv. *oryzae* strains tested, produced large amounts of EPS and EGL. The five *rpfC*::Tn*5* mutants obtained from marker exchange had wild-type levels of EGL, but produced much less EPS than the wild type strain 13751. The levels of EPS and EGL produced by the other two marker exchange products were very similar to the wild type levels.

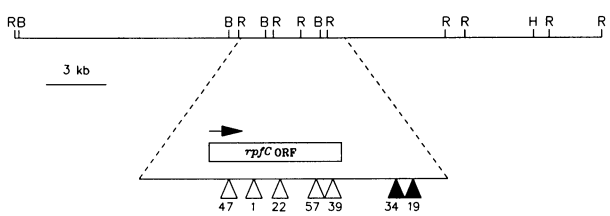

Fig. 1. Physical map of pGXN3000 showing restriction sites for *Bam*HI (B), *Eco*RI (R), and *Hin*dIII (H) and sites of Tn*5* insertions. Open triangles represent Tn*5* insertions that abolish the ability of pGXN3000 to complement the *rpfC* mutant of *Xanthomonas campestris* pv. *campestris* for pathogenicity and production of extracellular enzymes and EPS, closed triangles represent insertions with no effect on complemetation. The position and direction of the *rpfC* open reading frame (ORF) is also shown.

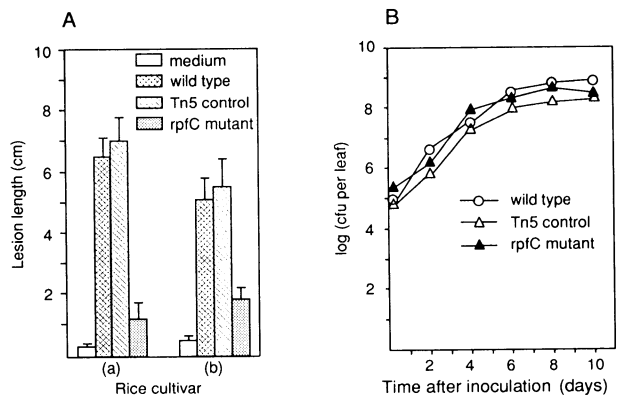

Fig. 2. A, Lesion development on rice cvs. Guanggui 110 **(A)** and Shangyouguang 12 **(B)** at 14 and 16 days, respectively, after inoculation with different strains of *Xanthomonas oryzae* pv. *oryzae*. The *rpfC* mutant and Tn*5* control were created by marker exchange of Tn*5* insertions 57 and 34 (see Fig.1) into the chromosome. Very slight symptoms are seen with the medium alone. The mean and standard deviation of 10 replicate measurements of lesion length are given. **B,** Growth of *X. oryzae* pv. *oryzae* strains in leaves of rice cv. Guanggui 110. The *rpfC* mutant and Tn*5* control strains were created by marker exchange of Tn*5* insertions 57 and 34 (see Fig. 1) into the chromosome. Bacterial numbers are the averages of three replicate measurements which differed from the average by less than 5%.

To study the role of the *rpfC* gene in the pathogenesis of *X. oryzae* pv. *oryzae*, the leaves of 3-week-old seedlings of the rice cultivars Guanggui 110 and Shanyouguang 12 were inoculated as described by Kauffman et al. (1973) by clipping their tips with scissors that had been dipped in bacterial suspensions of 10^9 CFU/ml made in the culture medium of Yuan (1990). The strains tested were the wild type 13751, and marker exchange mutants resulting from exchange of transposon insertions 57 (*rpfC* mutant) and 34 (Tn*5* control). By 5 days after inoculation, no disease symptoms were seen on either cultivar inoculated with the *rpfC* mutant, whereas the wild-type strain 13751 and Tn*5* control strain produced clear symptoms. Although at later time points symptoms did appear on the leaves of both cultivars in response to the *rpfC* mutant, the lesion length was much shorter than that caused by 13751 (Fig. 2A). The Tn*5* control strain showed very similar behaviour to the wild type (Fig. 2A). The growth of the different strains in the leaves of rice seedlings was determined by homogenizing pools of five inoculated leaves for each strain at 2-day intervals after inoculation and plating suitable dilutions of the homogenates on selection plates. The results showed that the *rpfC* mutant and Tn*5* control strain had a similar growth rate and final population size to the wild type strain (Fig. 2B).

Overall the results suggest that the *rpfC* gene is involved in the pathogenesis of *X. oryzae* pv. *oryzae* to rice where it contributes to symptom production rather than to growth or survival of the bacteria. The effects of mutation of *rpfC* in *X. oryzae* pv. *oryzae* are different in detail from those seen in *X. campestris* pv. *campestris* since the production of extracellular enzymes is not affected in an *rpfC* mutant of *X. oryzae* pv. *oryzae*. We cannot state that the reduced symptoms seen in rice in response to the *rpfC* mutant of *X. oryzae* pv. *oryzae* are solely due to the reduction in EPS biosynthesis, since we do not know how many genes other than those involved in EPS biosynthesis are also positively regulated by *rpfC*. This and other intriguing questions such as what aspects of the bacterial environment or cellular state are "sensed" by RpfC will be the subject of future study.

ACKNOWLEDGMENTS

Work at Guangxi Agricultural University was supported by the 863 Plan, the FEYT of State Education Commission and the International Foundation for Science. The Sainsbury Laboratory is supported by a grant from the Gatsby Charitable Foundation. J-L.T. gratefully acknowledges a Career Fellowship from the Rockefeller Foundation, a Marie Curie Fellowship from the Commission of the European Union and financial support from The Royal Society. We also acknowledge support from the BBSRC (U.K.) (for T.J.G.W.) and from the British Council (for J.-X. F.). This work was carried out according to the provisions of the Ministry of Agriculture, Fisheries and Food licence PHF1185/8(48) issued under the Plant Health (Great Britain) Order 1987 (Statutory Instrument 1758).

LITERATURE CITED

Bibb, M. J., Findlay, P. R., and Johnson, M. W. 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene 30:157-166.

Daniels, M. J., Barber, C. E., Turner, P. C., Sawczyc, M. K., Byrde, R. J. W., and Fielding, A. H. 1984. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. EMBO J. 3:3323-3328.

Dow, J. M., and Daniels, M. J. 1994. Pathogenicity determinants and global regulation of pathogenicity in *Xanthomonas campestris* pv. *campestris*. Pages 29-41 in: Bacterial Pathogenesis of Plants and Animals. J. Dangl, ed. Current Topics in Microbiology and Immunology. Vol. 192. Springer-Verlag, Berlin.

Hopkins, C. M., White, F. F., Choi, S. H., Guo, A., and Leach, J. E. 1992. Identification of a family of avirulence genes from *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interact. 5:451-459.

Kamdar, H. V., Kamoun, S., and Kado, C. I. 1993. Restoration of pathogenicity of avirulent *Xanthomonas oryzae* pv. *oryzae* and *X. campestris* pathovars by reciprocal complementation with the *hrpXo* and *hrpXc* genes and identification of HrpX function by sequence analysis. J. Bacteriol. 175:2017-2025.

Kelemu, S., and Leach, J. E. 1990. Cloning and characterization of an avirulence gene from *Xanthomonas campestris* pv. *oryzae*. Mol. Plant-Microbe Interact. 3:59-65.

Kauffman, H. E., Reddy, A. P. K., Hsieh, S. P. Y., and Merca, S. D. 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis. Rep. 57:537-541.

Mew, T. W., Alvarez, A. M., Leach, J. E., and Swings, J. 1993. Focus on bacterial blight of rice. Plant Dis. 77:5-12.

Osbourn, A. E., Clarke, B. R., Stevens, J. H., and Daniels, M. J. 1990. Use of oligonucleotide probes to identify members of two-component regulatory systems in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 222:145-151.

Parkinson, J. S., and Kofoid, E. C. 1992. Communication modules in bacterial signalling proteins. Annu. Rev. Genet. 26:71-112.

Sawczyc, M. K., Barber, C. E., and Daniels, M. J. 1989. The role in pathogenicity of some related genes in *Xanthomonas campestris* pv. *campestris* and *translucens*: A shuttle strategy for cloning genes required for pathogenicity. Mol. Plant-Microbe Interact. 2:249-255.

Tang, J-L., Liu, Y-N., Barber, C. E., Dow, J. M., Wootton, J. C., and Daniels, M. J. 1991. Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 226:409-417.

Todd, G. A., Daniels, M. J., and Callow, J. A. 1990. *Xanthomonas campestris* pv. *oryzae* has DNA sequences containing genes isofunctional with *X.campestris* pv. *campestris* genes required for pathogenicity. Physiol. Mol. Plant Pathol. 36:73-87.

Turner, P., Barber, C., and Daniels, M. J. 1985. Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 199:338-343.

Yuan, W. 1990. Culture medium for *Xanthomonas campestris* pv. *oryzae*. J. Appl. Bacteriol. 69:798-805.