Virulence analysis and gene expression profiling of the pigment-deficient mutant of Xanthomonas oryzae pathovar oryzae

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Abstract

Xanthomonas oryzae pathovar oryzae (Xoo) causes bacterial blight disease in rice (Oryza sativa L.). For a study of function, we constructed a random insertion mutant library of Xoo using a Tn5 transposon and isolated the mutant strain (M11; aroK::Tn5) that had extremely low pigment production. In addition, M11 had decreased virulence against the susceptible rice cultivar IR24. Thermal asymmetric interlaced-PCR and sequence analysis of M11 revealed that the transposon was inserted into the aroK gene (which encodes a shikimate kinase). To investigate the expression patterns of the pigment- and virulence-deficient mutant, DNA microarray analysis was performed. In addition, reverse transcriptase-PCR was performed to confirm the expression levels of several genes, including the aro genes of the aroK mutant. Our findings reveal that several crucial genes for virulence, including cellulase and hypersensitive response and pathogenicity (hrp) genes, were regulated by mutations in the aroK gene.

Introduction

Bacterial blight (BB) is one of the major rice diseases in Asian countries (Ezuka & Kaku, 2000). To overcome the serious damage from BB disease, we previously determined the whole genome sequence of Xanthomonas oryzae pathovar oryzae (Xoo) KACC10331 (Lee et al., 2005). Furthermore, we generated a random insertion mutant library and also constructed the Xoo DNA chip. Based on these preliminary resources, we analysed the functional role of genes that affected the virulence of Xoo.

The shikimate pathway is responsible for the biosynthesis of aromatic compounds (phenylalanine, tyrosine and tryptophan) in bacteria, yeasts and other fungi, plants and apicomplexan parasites such as Plasmodium falciparum (Roberts et al., 1988; Herrmann & Weaver, 1999). In general, bacteria spend > 90% of their total metabolic energy on protein biosynthesis. Likewise, the bacterial shikimate pathway serves almost exclusively to synthesize aromatic amino acids (Herrmann, 1983; Pittard, 1987). The biosynthesis of these three aromatic amino acids (phenylalanine, tyrosine and tryptophan) is considered to occur in two parts: the shikimate pathway from phosphoenolpyruvate and erythrose-4-phosphate to chorismate and the three specific terminal pathways that use chorismate as a substrate (Herrmann & Weaver, 1999). Although these aromatic amino acids found in proteins are synthesized via the shikimate pathway, not all natural aromatic products originate through such a metabolic sequence (Bentley, 1990).

Most Xanthomonas bacteria produce yellow, membrane-bound, brominated aryl-polyene pigments referred to as xanthomonadins (Starr, 1981). Poplawsky & Chun (1997) reported that pathogenicity, symptomatology and in planta growth are unaffected in the xanthomonadin-deficient strains of Xanthomonas campestris pv. campestris. Goel et al. (2001) reported that the pigment-deficient mutant of Xoo is also virulence deficient and auxotrophic for aromatic amino acids. However, little is known regarding the correlation between the metabolic pathway and virulence genes of Xoo.

Eight ORFs associated with the shikimate pathway have been identified from the Xoo KACC10331 genome sequence (Lee et al., 2005). In general, the shikimate pathway consists of seven enzymes, including 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHPS), 3-dehydroquinate...
synthase (DHQS), 3-dehydroquinate dehydratase (DHQD), shikimate-5-dehydrogenase (SDH), shikimate kinase (SK), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and chorismate synthase (CS) (Campbell et al., 2004). Shikimate kinase (encoded by the aroK gene) catalyses the phosphorylation of shikimate to yield shikimate-3-phosphate in the fifth step of the shikimate pathway (Herrmann & Weaver, 1999).

In this study, we verified that the mutation in the aroK gene affects pigment production and virulence in Xoo as well as the expression of other genes that are known to play a crucial role in its pathogenicity.

Materials and methods

**Bacterial strains and culture conditions**

The Xoo KACC10859 strain was obtained from the Korean Agricultural Culture Collection (KACC) in Suwon, Korea, and was cultured on YDC medium [2.0% d-(+)-glucose, 2.0% CaCO3, 1.0% yeast extract and 1.5% agar] at 28 °C for 48 h.

**Transposon mutagenesis and virulence assay**

We achieved insertional mutagenesis using a transposon<sup>Tm</sup> (20 ng µL<sup>-1</sup>; Epicentre Technologies) and analysed the insertion site by thermal asymmetric interlaced PCR (TAIL-PCR) by a previously described method (Park et al., 2007).

The virulence of the Xoo strains was assayed with 40-day-old susceptible rice plants of cultivar IR24 by a previously described method (Goel et al., 2007). The length of the lesions was measured at 2 weeks after inoculation. The virulence assay was performed three times for both the wild-type (WT) and mutant strains with calculation of the average and SD of the lesion lengths.

**Quantification of pigment production and complementation analysis**

Pigment extraction from Xoo was performed by a previously described method (Goel et al., 2001) by mixing with chloroform/methanol (2:1) in a vortex mixer (Vortexer Genie-2) for 3 h at room temperature. The pigment extracted from 0.5 mg (dry weight) of each strain was quantified using a Genesys-20 spectrophotometer (Thermo-Spectronic) and was expressed as the absorbance (OD<sub>445 nm</sub>) of the crude pigment extract (Poplawsky & Chun, 1997). The assay was performed three times with calculation of the average and SD of the experiment.

The genomic region (2418 bp), including the aroK gene and the aroB gene, was amplified from the WT genomic DNA by PCR using primers aroKBF (5'-TTATCCGCCGAGGATCCTCA-3') and aroKBFR (5'-TCACGGATACACGATCCGCT-3'). The PCR-amplified DNA was cloned into the pHM1 broad-host-range vector at the EcoRI site. The clone (pHMaroKB) was introduced into M11 by electroporation (12 kV cm<sup>-1</sup>) to obtain the complemented strain (M11c).

**DNA microarray analysis**

**Oligonucleotide and target preparation**

DNA microarrays with 50-mer oligo spots representing 3382 ORFs, i.e. approximately three-quarters of the Xoo KACC10331 genome, were synthesized and spotted on addressable electrodes of CustomArray<sup>Tm</sup> 12K microarrays. Microarray analysis was performed according to the CustomArray 12K microarray protocols provided by Combimatrix (CombiMatrix Corp.; http://www.combimatrix.com).

RNA was isolated from a stationary phase culture (OD = 0.8) by the using an RNeasy mini kit in accordance with the manufacturer’s instructions (Qiagen, Germany). Total RNA was finally eluted with 50 µL of RNase-free water and quantified using a UV spectrophotometer (ND-1000). Each sample was run on a 1.2% agarose gel to check for the purity and integrity of RNA. cDNA was generated using the MessageAmp<sup>Tm</sup> II-Bacteria Kit (Ambion). Polyadenylation of the bacterial RNA molecules was achieved using Escherichia coli poly (A) polymerase. Subsequently, the tailed RNA was reverse transcribed in a reaction mixture primed with an oligo (dT) primer and ArrayScript<sup>Tm</sup> reverse transcriptase. Second-strand cDNA was synthesized and purified for in vitro transcription in a reaction mixture containing biotin-modified UTP and T7 RNA polymerase.

**Microarray hybridization**

Hybridization was performed using 5 µg of a labelled target sample per CustomArray<sup>Tm</sup> 12K microarray designated for the Xoo genome for 16 h at 45 °C with gentle rotation. Thereafter, the arrays were washed with (1) 6 × SSPE (3 M sodium chloride, 0.2 M sodium hydrogen phosphate, 0.02 M EDTA, pH7.4; 20 × SSPE), 0.05% Tween 20 for 5 min, (2) 3 × SSPE, 0.05% Tween 20 for 1 min, (3) 0.5 × SSPE, 0.05% Tween 20 for 1 min and (4) 2 × phosphate-buffered saline, 0.1% Tween 20 for 1 min.

**Data acquisition and data analysis**

The hybridized microarrays were scanned (PMT, photomultiplier tube), 500–700; pixel size, 5; focus position, 130) using a GenePix 4000B microarray scanner (Axon Instruments). After data extraction (https://webapps.combimatrix.com), the background was calculated for individual samples. For background calculation, factory-built controls...
with low intensities (lowest intensity, 5–30%) were used, and their median signal intensities were then calculated for subtraction. The microarray data of individual samples were normalized by global normalization using probes with signal values greater than zero, < 60,000 (the saturation value) and greater than the lowest 5% of the signal value of each sample. A total of 747 probes were used for the final analysis. The local pooled error (LPE) test (http://bioinformatics.oxfordjournals.org/cgi/reprint/19/15/1945) and fold change were applied to determine the differentially expressed sets of genes using AVADIS PROPHETIC version 3.3 software (Strand Genomics).

Reverse transcriptase (RT)-PCR assay

First-strand cDNA was synthesized using an Omniscript RT KIT (Qiagen) with 200 ng of total RNA in a total volume of 20 μL containing 20 pmol of the reverse primer. The reaction mixtures were incubated at 37 °C for 60 min. After cDNA synthesis, 2 μL of the cDNA was used for PCR in 50-μL reaction mixtures [containing 10 pmol of the gene-specific primer, 10 mM dNTPs, 1 U Taq DNA polymerase (Toyobo), and 10 × Taq buffer supplied by the manufacturer]. Each reaction included an initial 5-min denaturation at 94°C, followed by 25 cycles of PCR (94°C, 15 s; 50 to ~60°C, 15 s; 72°C, 30 s), and a final extension for 10 min at 72°C. Subsequently, 10 μL of each reaction mixture was separated on a 1.0% agarose gel.

In vivo assay of cellulase activity

The bacterial strains were grown at 28°C for 24 h in 5 mL nutrient broth. One millilitre of each bacterial culture was sonicated and centrifuged at 9100g for 10 min (4°C). The culture supernatant was transferred to a new tube and used as the extracellular enzyme. The bacterial pellet was suspended in 1 mL of 100 mM sodium phosphate (pH 6.5), sonicated and centrifuged at 9100 g for 10 min (4°C). The prepared enzyme was incubated with 1.0% carboxymethyl-cellulose (Sigma), and the reducing sugar content was measured by the dinitrosalicylic acid (DNS) method (Miller et al., 1960). The cellulase activity assay was performed three times with calculation of the average and SD for each repetition of the experiment.

Results

Molecular characterization of the mutant M11 strain

The insertion site of the transposon was determined by TAIL-PCR and sequence analysis. A 1221-bp transposon including a kanamycin-resistant gene was inserted at nucleotide 174 of the aroK gene (Fig. 1a). The aroK gene (encoding a 180-amino-acid shikimate kinase) harbours a 543-bp ORF. The aroB gene harbours a 1113-bp ORF and encodes a 370-amino-acid DHQS. Transposon insertion in the aroK gene was further reaffirmed by PCR and Southern hybridization analysis (Fig. 1b and c).

Virulence and pigment production

In the virulence assay, M11 showed a severe decrease in its virulence against the susceptible rice plant. Fifteen days after inoculation, the average length of the lesion caused by M11 was 1.7 cm, whereas that of the lesion caused by the WT strain was 14.2 cm (Fig. 2a). Pigment production by M11 was drastically reduced (Fig. 2b). The average OD value of the pigment from M11 was 0.08 (approximately 10% that of the WT strain).

In the complementation test, the length of the lesion caused and pigment production by the M11c strain was 14.3 cm and 0.76 OD445 nm, respectively, values similar to those of the WT strain (Fig. 2a and b).

Expression profiles and RT-PCR

The Xoo DNA-chip was used to compare the expression patterns of M11 with those of the WT strain. The resultant expression profile was refined using a false discovery rate of 5% (P < 0.05; LPE test) and a fold-change minimum of ±2 (resulting in 544 genes for M11). Interestingly, 13 hrp/ hrc genes (XO00066, XO00075, XO00076, XO00077,
XOO0078, XOO0082, XOO0087, XOO0086, XOO0088, XOO0089, XOO0090, XOO0094 and XOO0095) and three cellulase genes (XOO4019, XOO4035 and XOO4036) were upregulated by the aroK gene mutation. In addition, the aroK gene mutation did not affect the expression of the other aro genes (XOO0328, XOO1243, XOO2386, XOO3261 and XOO4284), except the aroB gene (XOO1243; Fig. 3). Supporting Information, Table S1 provides details of the expression profiling, hierarchical clustering (using the Euclidean method) and ontological classification (using the COG database) of M11.

RT-PCR was performed to reaffirm the expression pattern of M11. The RNA extracted from M11 and the primers specific to each of the 21 genes were used (Table 1). The results of RT-PCR were similar to those of DNA microarray analysis, except four genes (XOO0086, XOO0088, XOO2386 and XOO3261; Fig. 4).

**Cellulase activity**

The intra- and extracellular cellulase activity of the WT, M11 and M11c strains was validated by the DNS method. The reducing sugar content in the culture supernatants of the WT, M11 and M11c strains (OD600 nm = 1) was 0.24, 0.31 and 0.12, respectively. In contrast, cellulase activity in the WT, M11 and M11c cell extract was low (0.10, 0.07 and 0.06, respectively; Fig. 5). From the DNA microarray analysis and these results, it is clear that the expression of the cellulase genes (XOO4019, XOO4035 and XOO4036) was affected by the mutation in the aroK gene.

**Discussion**

To identify the relationship between the aroK gene and other genes in Xoo, DNA microarray analysis was performed. DNA microarray analysis showed that two types of genes, known to play a crucial role in the virulence of Xoo, were regulated by the mutation in the aroK gene.

First, the expression of three cellulase genes was shown to have been increased significantly by the mutation in the aroK gene. Plant cell walls consist of various components (lignin, cellulose, hemicellulose and pectin) to enable them to defend attacks by plant pathogens. In general, plant pathogenic bacteria produce and secrete cell wall degradation enzymes to degrade the physical barrier of the plant cell wall. In previous reports, mutations in the cell wall degradation enzyme genes have been shown to result generally in the...
attenuation of virulence (Ray et al., 2000; Subramoni et al., 2006; Hu et al., 2007).

Second, as shown in Figs 4 and 5, the expression of 10 hrp/hrc genes was increased by the mutation in the aroK gene. Many pathogenic bacteria use the type III secretion system (TTSS), encoded by the hrp/hrc genes, to deliver virulence factors into host cells (Staskawicz et al., 2001; Buttner & Bonas, 2002). The bacterial TTSS serves to inject several pathogenicity factors, termed effectors, into the cytosol of eukaryotic cells (Cornelis & Van Gijsegem, 2000). Once inside the plant cell, the effector proteins are believed to modulate host defence-signalling pathways and promote disease by interfering with the host cell surveillance

Table 1. Primers used for reverse transcription and PCR

<table>
<thead>
<tr>
<th>ORF ID</th>
<th>Gene*</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>XOO0066</td>
<td>hrpF</td>
<td>GGACCCGCGATGTTGTTTGTG</td>
<td>CTTGACCTGCAGGTATGTA</td>
</tr>
<tr>
<td>XOO0075</td>
<td>hpa8</td>
<td>GCTGTTGCTTGGTGGTAGAG</td>
<td>CCCGTAAGCATGCGTAAAT</td>
</tr>
<tr>
<td>XOO0076</td>
<td>hrpE</td>
<td>TAATTGCGCTTCCAAACG</td>
<td>GCCGAGCAACAGAAACCATCT</td>
</tr>
<tr>
<td>XOO0077</td>
<td>hrpD6</td>
<td>AGGGCTGATGCCACTG</td>
<td>CATATGCGGGCAGATGTTG</td>
</tr>
<tr>
<td>XOO0078</td>
<td>hrpD5</td>
<td>CGTCAGCAGCGACATCGT</td>
<td>GCGGCCAGCATCGGACAGAA</td>
</tr>
<tr>
<td>XOO0082</td>
<td>hrcQ</td>
<td>CGCACTGGGGGAGAAAGG</td>
<td>GCAGAGGGGCCCCATGATT</td>
</tr>
<tr>
<td>XOO0086</td>
<td>hrpB2</td>
<td>ACGGCTGATGCCAAGAAAC</td>
<td>GGTCGAGCCAGATGTTG</td>
</tr>
<tr>
<td>XOO0087</td>
<td>hrpB1</td>
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<td>GCGGCCAGCATCGGACAGAA</td>
</tr>
<tr>
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<td>hrpB3</td>
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<td>CTCATCATGCGTGGCTTC</td>
</tr>
<tr>
<td>XOO0089</td>
<td>hrpB4</td>
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<td>GCGGCCAGCATCGGACAGAA</td>
</tr>
<tr>
<td>XOO0090</td>
<td>hrpB5</td>
<td>ATGGTGCTTGGTGAGTGGC</td>
<td>CTCATCATGCGTGGCTTC</td>
</tr>
<tr>
<td>XOO0094</td>
<td>hrcC</td>
<td>TGAGGCGGGGAGGAGGAGG</td>
<td>GCAAGGCCCACCACAGACC</td>
</tr>
<tr>
<td>XOO0095</td>
<td>hpa1</td>
<td>GCAAGGCCCACCACAGACC</td>
<td>GCAAGGCCCACCACAGACC</td>
</tr>
<tr>
<td>XOO0328</td>
<td>aroD</td>
<td>CAGGAGGACGCGACAGGG</td>
<td>AGAAGGCCCACCACAGACC</td>
</tr>
<tr>
<td>XOO1243</td>
<td>aroB</td>
<td>CGCGCTGACGCCAAGACC</td>
<td>CTCGAACCGGCAACAGACC</td>
</tr>
<tr>
<td>XOO1244</td>
<td>aroK</td>
<td>ATGCTGATGCCGCGCGCCGCAGCAG</td>
<td>GCAAGGCCCACCACAGACC</td>
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<tr>
<td>XOO2236</td>
<td>aroA</td>
<td>CGCGCTGACGCCGCGCGCCGCAGCAG</td>
<td>GCAAGGCCCACCACAGACC</td>
</tr>
<tr>
<td>XOO3261</td>
<td>aroC</td>
<td>ATGCTGATGCCGCGCGCGCCGCAGCAG</td>
<td>GCAAGGCCCACCACAGACC</td>
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<tr>
<td>XOO4019</td>
<td>engXCA</td>
<td>GCCCTGATACCTGGCTGCTGCTGCTG</td>
<td>GCAAGGCCCACCACAGACC</td>
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<tr>
<td>XOO4035</td>
<td>cbsA</td>
<td>ATGCTGATGCCGCGCGCGCCGCAGCAG</td>
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<tr>
<td>XOO4036</td>
<td>cellulase</td>
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<tr>
<td>XOO4284</td>
<td>aroG</td>
<td>ATGCTGATGCCGCGCGCGCCGCAGCAG</td>
<td>GCAAGGCCCACCACAGACC</td>
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</tbody>
</table>

All gene sequences were attained from the Xoo KACC10331 genomic sequence in the NCBI GenBank database (accession no. AE013598).

Fig. 4. RT-PCR assay of 22 genes of the WT and M11 strains. XOO0328, aroD; XOO1243, aroB; XOO2236, aroA; XOO3261, aroC; XOO4019, engXCA (cellulase); XOO4035, cbsA (1,4-β-cellobiosidase); XOO4036, cellulase; XOO4284, aroG. XOO0066, hrpF; XOO0075, hpa8; XOO0076, hrpE; XOO0078, hrpD6; XOO0079, hrpD5; XOO0082, hrcQ; XOO0086, hrpB2; XOO0087, hrpB1; XOO0088, hrpB3; XOO0089, hrpB4; XOO0090, hrpB5; XOO0094, hrcC; XOO0095, hpa1; and 16S rRNA gene.

Fig. 5. In vivo assay of cellulase activity of the WT, M11 and M11c strains. The reducing sugars released from carboxymethylcellulose were measured at OD540 nm. The assay was performed three times, and the averages and SDs of cellulase activity from the intra- and extracellular extracts were calculated for each repetition of the experiment.

gene. Many pathogenic bacteria use the type III secretion system (TTSS), encoded by the hrp/hrc genes, to deliver virulence factors into host cells (Staskawicz et al., 2001; Buttner & Bonas, 2002). The bacterial TTSS serves to inject several pathogenicity factors, termed effectors, into the cytosol of eukaryotic cells (Cornelis & Van Gijsegem, 2000). Once inside the plant cell, the effector proteins are believed to modulate host defence-signalling pathways and promote disease by interfering with the host cell surveillance
mechanisms (Cunnac et al., 2004). In addition, Xoo-secreted proteins such as cellulase, cellobiosidase and xylanase induce very potent host defence responses that are suppressed by proteins secreted through the TTSS. Therefore, plant defence responses can be inhibited by the increased expression of the hrp/hrc proteins, secreted through the Xoo TTSS (Subramoni et al., 2006).

In previous reports, increased expression of the cellulase and hrp/hrc genes has been shown to enhance the ability of pathogens to release host plant nutrients and to inhibit the plant defence responses (Gough et al., 1988; Roberts et al., 1988; Barras et al., 1994; Walker et al., 1994; Walton, 1994; Subramoni et al., 2006). However, M11 failed to develop such pathogenicity against the rice plant. These results suggest that the disruption of a specific gene that plays a crucial role in the metabolic pathway of Xoo attenuates its virulence, despite the high levels of expression of the pathogenicity-related genes (including hrp/hrc and cellulase).

Interestingly, we were able to obtain the complemented strain (M11c) with the clone (pHMaroKB) containing the ORFs of the aroK and aroB (XOO1243) genes. M11 could not recover its virulence and pigment production with the pHMaroK or pHMaroB clone (which contains only the ORF of the aroK or aroB gene as an insert, respectively). These results provide evidence that the mutation in the aroK gene affects pigment production and virulence in Xoo as well as the expression of other genes that are known to play a crucial role in its pathogenicity. DNA microarray and transcription analysis of M11 revealed that the mutation of the aroK gene did not severely affect the expression of the other aro genes, except aroB. This might be due to the polar effect of the mutation in the aroK gene. As shown in Figs 3 and 4, expression of the aroB gene was downregulated by the mutation in the aroK gene. In some cases, the mutation of one gene prevents other genes in the same operon from being transcribed (termed as the polar effect). In addition, M11 was unable to grow in modified Miller's minimal medium M4 (Kelemu & Leach, 1990; data not shown). Goel et al. (2001) reported that one or more aromatic amino acids may be limiting for growth of Xoo in rice plants. Thus, the virulence deficiency of M11 is probably due to a growth defect. These results also suggest that many enzymes involved in the shikimate pathway can be used as a target for antibacterial reagents against Xoo.

Acknowledgements
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Authors' contribution
Y.-J.P. and E.-S.S. are joint first authors.

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Common and contrasting themes of plant and animal diseases. 

Supporting Information

Additional Supporting Information may be found in the 
online version of this article:

Table S1. The complete gene expression profiling data of 
M11 strain.

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for the article.