In planta gene expression analysis of Xanthomonas oryzae pathovar oryzae, African strain MAI1

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Abstract
Background: Bacterial leaf blight causes significant yield losses in rice crops throughout Asia and Africa. Although both the Asian and African strains of the pathogen, Xanthomonas oryzae pv. oryzae (Xoo), induce similar symptoms, they are nevertheless genetically different, with the African strains being more closely related to the Asian X. oryzae pv. oryzicola (Xoc).

Results: Changes in gene expression of the African Xoo strain MAI1 in the susceptible rice cultivar Nipponbare were profiled, using an SSH Xoo DNA microarray. Microarray hybridization was performed comparing bacteria recovered from plant tissues at 1, 3, and 6 days after inoculation (dai) with bacteria grown in vitro. A total of 710 bacterial genes were found to be differentially expressed, with 407 up-regulated and 303 down-regulated. Expression profiling indicated that less than 20% of the 710 bacterial transcripts were induced in the first 24 h after inoculation, whereas 63% were differentially expressed at 6 dai. The 710 differentially expressed genes were one-end sequenced. 535 sequences were obtained from which 147 non-redundant sequences were identified. Differentially expressed genes were related to metabolism, secretion and transport, pathogen adherence to plant tissues, plant cell-wall degradation, IS elements, and virulence. In addition, various other genes encoding proteins with unknown function or showing no similarity to other proteins were also induced. The Xoo MAI1 non-redundant set of sequences was compared against several X. oryzae genomes, revealing a specific group of genes that was present only in MAI1. Numerous IS elements were also found to be differentially expressed. Quantitative real-time PCR confirmed 86% of the identified profile on a set of 14 genes selected according to the microarray analysis.

Conclusions: This is the first report to compare the expression of Xoo genes in planta across different time points during infection. This work shows that as-yet-unidentified and potentially new virulence factors are appearing in an emerging African pathogen. It also confirms that African Xoo strains do differ from their Asian counterparts, even at the transcriptional level.

Background
Xanthomonas oryzae pv. oryzae (Xoo) is the causal agent of bacterial leaf blight in rice. Bacterial cells on leaf surfaces enter the rice leaf by either swimming passively through the fluid oozing from hydathodes in the morning and spreading systemically in the plant through the xylem, or it enters directly into the xylem through wounds [1]. In Asia, this disease is the most economically important within the irrigated environment. It appeared in Africa in the 1980s, and has since been growing in importance [2]. The use of varietal resistance is a highly efficient way of controlling the disease in Asia, but, in Africa, adequate control methods and deployment of resistant varieties are still lacking. Among the prerequisites for finding adequate control strategies are an understanding of the biology of the host-pathogen interaction and the characterization of those genes involved in pathogenicity.

Numerous studies [1] have been carried out on the interaction between both host (rice) and pathogen (Asian Xoo strains). In Asia, Xoo shows important variations, as revealed by virulence and DNA fingerprinting analyses [3-5]. A race is a group of strains sharing common pheno-
type of virulence to a set of host cultivars. In the case of 
Xoo near isogenic lines (IRBB lines) are being used and 
more than 30 Xoo races have been reported in Asia so far. 
New ones are emerging that overcome deployed resis-
tance [6]. Identification of the genes used by the bacteria 
to colonize plants may give new insights into the plant 
defence pathways that are vulnerable to pathogen attack 
and provide better understanding of the processes in 
both bacterial pathogenesis and plant immunity. 

Microarray technology has been widely used to explore 
threats in plant pathogenic bacteria such as 
*Pseudomonas syringae*, *Ralstonia solanacearum*, *Xan-
thomonas axonopodis*, *X. campestris*, and *Xylella fastid-
iosa* [7-15]. These analyses were conducted to study 
responses to environmental factors such as heat shock, 
changes in iron bioavailability or carbon sources [7-9], 
expression changes related to pathogenesis [10-13], and 
biofilm formation [13]. Another significant field of 
microarray analysis is that of genome diversity [14] and 
horizontal gene transfer events [15], using comparative 
genome hybridization. One example was the recent 
development of an *Xanthomonas oryzae* 5K oligoarray, 
with oligos designed according to the sequences of the 
genomes of Asian strains of *Xoo* and *X. oryzae* pv. *oryzi-
cola* (Xoc) [16]. Xoc is the causal agent of bacterial leaf 
streak, a non-vascular counterpart of *Xoo* [1]. *Xoo* and 
*Xoc* showed differentially expressed genes when grown in 
enriched versus minimal media [16]. For example, the 
minimal medium XOM2 induces the *in vitro* expression of 
the *hrp* genes in *Xoo* but not in *Xoc*, presumably by 
mimicking the pH and nutrient content in the apoplast 
[17]. The great potential of microarray technology was 
also demonstrated by several other studies that used the 
technique based on whole or partial plant-bacterial 
genomes [18-20]. Most analyses addressing bacterial gene 
expression were conducted under *in vitro* conditions. 

Strain variations were recently documented in whole-
genome analyses of three *Xoo* strains: the Korean *Xoo*
strain KACC10331 [21], the Japanese *Xoo* strain 
MAFF311018 [22], and the Philippine *Xoo* strain PXO99A 
[23]. A whole-genome sequence is also available for one 
Asian *Xoc* strain BLS256. Several characteristics different-
te the *Xoo* genome from those of other xanthomonads: 
a higher abundance of IS elements, and prevalence of 
TAL effector genes of the *avrBs3*/*pthA* family [1,22]. TAL 
genes are widespread among *Xanthomonas* spp., but this 
family of effectors has expanded specifically in the 
genomes of *X. oryzae* pathovars. Recent studies 
identified African *Xoo* strains as a significantly different 
genetic group that appears more closely related to the 
Asian *Xoc* than to Asian *Xoo* [24]. In contrast to Asian 
*Xoo* strains, African *Xoo* strains show a reduced number 
of both TAL genes and IS elements in their genomes [24]. 
African *Xoo* strains induce a non-host hypersensitive 
response (HR) in tobacco leaves suggesting that these 
strains display one or several specific non-host HR elicitors, 
such as type III effectors or harpins. Finally, three 
new races have been determined among the African 
strains [24]. However, except for the role of one TAL 
effector, almost nothing is known about the specific 
genetic determinants of pathogenicity in *Xoo* African 
strains (Yu Y., Szurek B., Mathieu T, Feng X., Verdier V. 
2009, unpublished data). Much remains to be learned 
about the genes involved in the pathogenicity and viru-
ulence of this African pathogen. Identification of such 
genres can improve understanding of how *Xoo* causes dis-
ease.

Efficient methods for recovering bacterial cells directly 
from plant tissues permit analyses of *in vivo* expression in 
plant-pathogen interactions [25,26]. Conducting gene 
expression analyses of bacterial pathogens *in planta* may 
prove the understanding of the mechanisms underly-
ing plant-pathogen interactions and may help in the early 
detection of genes involved in pathogenicity [25,27]. 
Because whole genome is not yet available for African 
*Xoo* strains, we used SSH libraries of *Xoo* strain MAI1 
[28] that were then spotted onto a microarray and used to 
analyse *in planta* gene expression at different time points 
deriving infection. Combining the SSH method, *in vivo* 
analysis, and microarrays to study the *Xoo* MAI1-rice 
interaction offers considerable advantages, particularly as 
in vitro approaches are frequently limited in their ability 
to mimic all aspects of the *in vivo* state. Additionally, 
constructing an *Xoo* MAI1 microarray, based on SSH DNA 
libraries, allows the enrichment of *Xoo* MAI1 sequences. 
Hence, the likelihood is higher that the microarray will 
reveal novel genes involved in *Xoo*-rice infection. 
Although the *Xoo* MAI1 SSH-microarray does not allow 
analyses of genome-wide gene expression profiles, spe-
cific biological questions can be answered more effi-
ciently, for example, identification of virulence determinants in 
African *Xoo* strains. In contrast with 
other large-scale approaches to the study of gene expres-
sion in the *Xanthomonas* genus [10,16,25,29,30], this is 
the first report to compare bacterial gene expression *in planta* 
and at different time points during infection.

**Results and discussion**

**Bacterial recovery from plant tissues, and RNA isolation**

We determined *Xoo* MAI1 multiplication *in planta* at 
seven time points after infection into five 2-cm leaf sect-
ions (A-E, Figure 1). The *Xoo* strain MAI1 multiplied to 
a population size of almost $10^4$ colony-forming units (cfu) 
in section A within 12 h after inoculation (hai). There-
ter, the population continued increasing until it reached a 
size of more than $10^{12}$ cfu within 15 days after inocula-
tion (dai; Figure 1). That is, colonization along the leaf 
was fast. Initially, *Xoo* bacterial cells were concentrated in
the first 2 cm behind the inoculation point but, within 3 dai, they were found in section B. By day 6, the bacterium had colonized more than 8 cm, reaching section D. Levels of Xoo MAI1 populations increased gradually from sections A to D, reaching $10^{-9}$ to $10^{-13}$ cfu per section of leaf by 15 dai. By that time, visible lesions were about 10 cm long. We selected three time points (1, 3, and 6 dai) and the first 2-cm lesion to perform bacterial RNA extractions from leaf tissues for subsequent microarray experiments. Possible genomic DNA contamination was tested by PCR, using primers corresponding to the genomic region flanking the $hrpX$ (hypersensitive reaction and pathogenesis) gene and purified RNA as PCR template. No DNA contamination was found (data not shown).

Differentially expressed genes were identified at late stages of infection

The DNA microarray constructed consists of about 4708 randomly selected clones. The quality of PCR amplification was verified for 20% of the amplified genes (1330 clones), with sizes ranging from 600 to 900 bp. The arrays were hybridized with Cy labelled cDNA probes prepared from total RNA from plant-grown bacteria at 1, 3, and 6 dai, or from bacteria cultured in media and re suspended in water.

We used bootstrap analysis with SAM to identify differentially expressed genes. Significance Analysis of Microarrays (SAM) calculates the fold change and significance of differences in expression. The delta-delta Ct values ranged from 1.21 to 2.37 for each time point. The false significant number (FSN) ranged between 0.80 and 4.99, while the false discovery rate (FDR) ranged from 0.25 to 3.80. Of the 4708 Xoo strain MAI1 clones analyzed, 710 genes were found to be differentially expressed with 407 up- and 303 down-regulated. The proportions of differentially expressed genes (up- or down-regulated) remained relatively constant over the first 3 days after inoculation but had changed markedly by day 6, with the proportions reversing (Table 1).

Table 1: Statistical summary of Significance Analyses of Microarrays (SAM)

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>Days after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Delta-delta Ct value</td>
<td>1.21</td>
</tr>
<tr>
<td>False significant number (FSN)</td>
<td>4.99</td>
</tr>
<tr>
<td>False discovery rate (FDR)</td>
<td>3.80</td>
</tr>
<tr>
<td>Up-regulated</td>
<td>58 (47%)</td>
</tr>
<tr>
<td>Down-regulated</td>
<td>66 (53%)</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
</tr>
</tbody>
</table>

The number of up- and down-regulated genes that are differentially expressed at different time points during infection by Xanthomonas oryzae pv. oryzae, African strain MAI1.
showed that gene FI978197 was present only in Xoo strain MAI1 and absent in the other, both African and Asian, Xoo and Xoc strains (data not shown). Those genes corresponding to ‘unknown function’ may therefore represent interesting candidates for further functional analyses.

**Cluster analysis of microarray data**

A k-means clustering analysis was performed to obtain an overview of the performance of each differentially expressed gene, compared with the others during infection. Seven clusters were defined (Figure 3). Genes that were up-regulated were represented by clusters 1 (at 3 and 6 dai), 2 (1 and 3 dai), 3 (at 3 dai), and cluster 4 (at 1 and 6 dai). Down-regulated genes were represented by clusters 5, 6, and 7 at 1, 3, and 6 dai, respectively. Those differentially expressed genes in Xoo strain MAI1, which are discussed below as related to pathogenicity fell into these clusters.

**Activation of genes related to adhesion to plant system and plant cell-wall degradation during infection**

*Xanthomonas oryzae* pv. *oryzae* is a vascular pathogen. A critical step in infection is adherence to the host’s vascular surfaces [32]. Electron microscopy analysis during interaction between rice and Xoo showed bacterial cells within xylem vessels in both compatible and incompatible interactions after 1 dai [32]. Recently, the use of green fluorescent protein (GFP) technology showed that Xoo strain PXO99<sub>GFP</sub> proliferated in susceptible rice lines but not in resistant lines at 12 dai [33].

Four genes fimbrial assembly protein (FI978267), pilin (FI978178), type IV pilin (FI978319), and the pilY1 gene (FI978318) that are associated with bacterial adhesion and biofilm formation were found as up-regulated in Xoo MAI1 in planta at 6 dai. These genes belong to cluster 1. Type IV pili are bacterial major virulence factors supporting adhesion, surface motility, and gene transfer [34-36].

The role of type IV pili genes in biofilm formation and virulence of phytopathogenic bacteria has been largely studied in the vascular pathogens *Ralstonia solanacearum* and *Xylella fastidiosa* [37-39] and, most recently, in Xoo, *Xac*, and *Xcc* [35,40,41]. Type IV in Xoo virulence increased with the presence of two *pilY1* insertion mutants [42]. In *Xylella fastidiosa*, disruption of *pilY1* reduced the number of type IV pili and the bacterium’s capacity for twitching motility [43]. In Xoo and Xoc, grown on enriched medium, microarray analysis revealed the differential expression of several fimbrial assembly proteins [16]. Unlike the findings of previous studies which showed the presence of bacterial cells in xylem vessels after 12 hai [33], adherence-related genes were found to be induced later (cluster 1) in Xoo MAI1.

Biofilm formation and adherence capacities have been associated with virulence of pathogenic bacteria in Xoo,
Table 2: Differentially expressed genes that are specific to the African strain MAI1 of *Xanthomonas oryzae pv. oryzae* (Xoo)

| GenBank accession | Library origin† | Seq. no. ‡ | Putative function | Organism § | E-value | Size | Time point|| | Xanthomonas oryzae genome¶ |
|------------------|----------------|------------|------------------|-----------|---------|------|---------| | | |
| FJ978294         | 1              | 1          | No protein match (NPM) | -         | -       | 1203 | -       | - | - | - | - |
| FJ978293         | 1              | 1          | NPM               | -         | -       | 974  | +       | - | - | - | - |
| FJ978295         | 1              | 1          | NPM               | -         | -       | 1233 | +       | - | - | - | - |
| FJ978297         | 1              | 1          | NPM               | -         | -       | 906  | +       | - | - | - | - |
| FJ978298         | 1              | 1          | NPM               | -         | -       | 975  | +       | - | - | - | - |
| FJ978299         | 1              | 1          | NPM               | -         | -       | 1499 | +       | - | - | - | - |
| FJ978300         | 1              | 1          | NPM               | -         | -       | 1122 | -       | - | - | - | - |
| FJ978301         | 1              | 1          | NPM               | -         | -       | 1659 | +       | - | - | - | - |
| FJ978302         | 1              | 1          | NPM               | -         | -       | 674  | -       | - | - | - | - |
| FJ978303         | 1              | 1          | NPM               | -         | -       | 1232 | +       | - | - | - | - |
| FJ978101         | 1              | 1          | NPM               | -         | -       | 409  | +       | - | - | - | - |
| FJ978177         | 1              | 1          | NPM               | -         | -       | 399  | +       | - | - | - | - |
| FJ978197         | 1              | 1          | NPM               | -         | -       | 248  | -       | - | - | - | - |
| FJ978310         | 1              | 1          | NPM               | -         | -       | 942  | +       | - | - | - | - |
| FJ978308         | 1              | 1          | NPM               | -         | -       | 931  | +       | - | - | - | - |
| FJ978317         | 1              | 1          | NPM               | -         | -       | 1175 | +       | - | - | - | - |
| FJ978273         | 1              | 1          | NPM               | -         | -       | 897  | +       | - | - | - | - |
| FJ978320         | 1              | 1          | NPM               | -         | -       | 1471 | -       | - | - | - | - |
| FJ978321         | 1              | 1          | NPM               | -         | -       | 1902 | -       | - | - | - | - |
| FJ978086         | 1              | 1          | NPM               | -         | -       | 544  | -       | - | - | - | - |
| FJ978068         | 1              | 1          | NPM               | -         | -       | 638  | -       | + | + | - | - |
| FJ978327         | 2              | 1          | NPM               | -         | -       | 876  | -       | - | - | - | - |
| FJ978316         | 2              | 1          | NPM               | -         | -       | 1157 | +       | - | - | - | - |
| FJ978296         | 2              | 1          | NPM               | -         | -       | 1529 | +       | - | - | - | - |
| FJ978323         | 1              | 1          | NPM               | -         | -       | 933  | -       | - | - | - | - |
| FJ978322         | 2              | 1          | NPM               | -         | -       | 861  | +       | - | - | - | - |

† Library origin: MAFF 311018, KACC 10331, PXO 99A, BLS 256, BAI3
‡ Seq. no.
§ Organism: *Xanthomonas oryzae*
¶ Xanthomonas oryzae genome

**Biological Process Unknown**
Table 2: Differentially expressed genes that are specific to the African strain MAI1 of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Continued)

<table>
<thead>
<tr>
<th>Hypothetical protein</th>
<th>FI978307</th>
<th>2 and 1</th>
<th>Hypothetical protein XCC2965</th>
<th>Xcc strain ATCC 33913</th>
<th>3.0E 12</th>
<th>835</th>
<th>-</th>
<th>-</th>
<th>-</th>
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</thead>
<tbody>
<tr>
<td>Phage-related and IS elements</td>
<td>FI978239</td>
<td>1 and 2</td>
<td>Hypothetical protein XCC2966</td>
<td>Xcc strain ATCC 33913</td>
<td>7.0E 11</td>
<td>244</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Metabolism</td>
<td>FI978271</td>
<td>1 and 7</td>
<td>Gene transfer agent (GTA) like protein</td>
<td><em>Parvibaculum lavamentivorans</em> strain DS 1</td>
<td>8.0E 50</td>
<td>788</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>FI978324</td>
<td>1 and 1</td>
<td>Haemolysin III</td>
<td>Xcc</td>
<td>5.0E 17</td>
<td>853</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

†SSH library and/or libraries in which the clone was identified, where 1 corresponds to SSH library *Xoo* strain M1/PXO86, and 2 to SSH library *Xoo* strain M1/Xoc BLS256.
‡Number of sequences by contig, where 1 indicates singleton.
§*Xcc* is *Xanthomonas campestris* pv. *campestris*; *Xoo* is *Xanthomonas oryzae* pv. *oryzae*.
||Time point, in days after inoculation, where + indicates up-regulated, and - indicates down-regulated.
¶*Xanthomonas oryzae* genomes, where + indicates presence of gene homologues to Xoo MAI1 in the genome analysed, and - indicates absence.
Differentially expressed transcripts were clustered, using the k-means method. The mean expression levels of genes in each cluster are shown as a centroid graph. Error bars represent standard deviations of expression within the cluster. Seven clusters were created, with clusters 1, 2, 3, and 4 comprising up-regulated genes and clusters 5, 6, and 7 comprising down-regulated genes at 1, 3, and 6 dai, respectively. The x axis represents time-points during infection (1, 3, and 6 dai) and the y axis the expression level.

Major virulence genes are up-regulated in planta
Five classes of virulence genes were found regulated during infection. They corresponded to three genes related to the avrBs3/pth family (F1978282, M1P3115, and AF275267), a leucin-rich protein (BAE68417), a virulence regulator (F1978260), and a xopX (ACD57163) and hrpF gene (F1978263). Most of these major virulence genes fell into cluster 1, corresponding to genes that are activated after 3 dai. Xoo pathogenicity is highly dependent on the type III secretion system (TTSS) injecting effector proteins into the eukaryotic host cell [48]. Most knowledge on TTSS in Xoo is based on studies of the large AvrBs3/ pthA family of Xanthomonas effector proteins [49]. This family includes proteins with avirulence activities, virulence functions, or both [48]. It includes the well-characterized AvrXa7 protein, which plays a role in bacterial growth and lesion development in rice [50,51]. Genes avrXa7 (AF275267) and xopX (ACD57163) are up-regulated at both 3 and 6 dai. The xopX gene encodes a TTSS effector protein and contributes to the virulence of X. campestris pv. vesicatoria on hosts pepper and tomato [52]. XopX targets the innate immune response, resulting in enhanced plant disease susceptibility [52]. The XopX protein from Xcc is required for full virulence, as shown by the XccN mutant that produced weaker disease symptoms than the wild-type strain [53].

The HrpF protein is probably inserted into the plant-cell membrane and may be required for the bacterium's type III effector proteins to enter host cells [54]. As a bacterial translocon, HrpF would therefore be in direct contact with the plant-cell membrane and even possibly subjected to the plant's surveillance mechanisms while it mediates effector protein delivery across the host-cell membrane. To demonstrate that HrpF is required for pathogenicity, Sugio et al. [55] used Xoo hrpF mutants, which had a reduced ability to either grow within rice plants or cause lesions. For the Xoo MA11 strain, we found a hrpF gene that was differentially expressed at 3 dai during infection. The activation of different genes encoding proteins secreted by TTSS (hrpF, avrXa7, and

X. axonopodis pv. citri (Xac), X. campestris pv. campestris (Xcc), and others [35,36,40,44]. Inside plant tissues, biofilms are thought to contribute to virulence by blocking sap flow in the xylem vessels and promoting plant wilt [39]. The up-regulated genes involved in biofilm formation and pathogenicity were identified in Xylella fastidiosa through microarray analysis, which compared cells growing in a biofilm with planktonic cells [45]. In Xoo MA11, we identified several of these genes as corresponding to type IV pili genes (e.g. F1978319) and the fimbrial assembly protein (e.g. F1978267) (Additional file 1, Table S1). Given that Xoo, like Xylella fastidiosa, is a restricted vascular pathogen, the induction of genes related to adhesion and motility suggests a role in biofilm formation and vascular colonization. The Xoo MA11 strain regulates the expression of a group of genes for adherence and biofilm formation in the nutrient-limited environment of xylem in rice. This group's role in pathogenicity should be investigated.

Among the up-regulated genes in the Xoo MA11 strain, we found one cellulase (F1978181) and one xylanase (F1978325) gene activated at 3 dai (cluster 1). Using an SSH approach, Qi et al. [46] identified the unique Fibrobacter intestinalis genes coding for plant cell-wall hydrolytic enzymes. More than 40 cellulases play a major role in F. intestinalis plant cell-wall degradation. An xylanase of Xoo was differentially expressed in planta [47]. Both enzymes (cellulase and xylanase) may play a similar role in Xoo MA11 in degrading rice cell walls, thus facilitating pathogen multiplication.
xopX genes) during Xoo MAI1-rice interaction was consistent with TTSS being essential for Xoo pathogenicity.

Expression of IS elements in Xoo MAI1 during infection

Insertion sequence (IS) elements have recently been shown to play a role in plant pathogenicity [56-59]. These elements may inactivate genes on insertion or activate and/or enhance the expression of nearby genes [57,60,61]. One characteristic of the Xoo genomes sequenced to date is the accumulation of many IS elements, representing as much as 10% of the Xoo genome size [23]. In Xanthomonas spp., virulence and pathogenicity islands are commonly associated with mobile genetic elements such as phages and transposons [56,58]. By comparing gene expression of both Xoo and Xoc grown in enriched versus minimal medium, Seo et al. [16] determined that IS elements are differentially expressed in minimal medium.

In our study, we identified 27 IS elements in Xoo MAI1 that are up- or down-regulated in planta. Most of these IS elements belong to cluster 1, corresponding to genes that are activated after 3 dai. Twelve elements were classified into the following IS families: IS30 (4 elements), IS5 (7), and IS3 (1), with 15 IS elements unclassified. Members of the IS5 family have been reported previously in bacterial pathogens and it has been speculated that expression of some pathogenicity genes might be controlled by the expression/insertion of IS5 family elements [58,62,63]. Expression of IS5 members in the neighboring region of their hrp gene cluster was observed in Pseudomonas syringae [64] It has been also demonstrated that IS elements (among them some IS5 family members) can act as a mobile switch for the downstream genes, creating new transcriptional promoters and increasing the expression levels of downstream genes [65]. Members of the IS3 and IS30 families have also been reported in bacterial pathogens, some of them controlling the expression of other genetic elements [60,66]. The expression of IS elements in Xoo MAI1 in planta suggests that these elements may play a significant role in bacterial pathogenicity or may be associated with genes related to pathogenicity.

To establish a correlation between the presence of IS elements and adjacent genes differentially expressed in MAI1, we used the draft genome of Xoo African strain BAI3 (Genoscope project 154/AP 2006-2007 and our laboratory, 2009, unpublished data) and the published genome of Xoo strain MAFF311018 [22]. We compared the location of the 147 Xoo MAI1 differentially expressed genes with the presence of adjacent IS elements in the Xoo BAI3 and MAFF311018 genomes. For this, homologous sequences of IS elements, found as differentially expressed in the Xoo strain MAI1, were first identified in the BAI3 draft genome. We then extracted 10 kb from each of up- and downstream flanking regions of IS elements. BLAST searches were performed against these flanking regions, using the Xoo MAI1 non-redundant set of sequences. For the sequences located within 20 kb of sequences flanking the IS elements, we compared the relative distance of each sequence to the IS element in BAI3 with the relative distance of their respective homologues in the Xoo MAFF311018 genome (Table 3).

Results showed that homologues of the 11 selected Xoo MAI1 differentially expressed genes are located in the vicinity of IS elements in BAI3 genome, within the same 20-kb region (Table 3). In the Xoo MAFF311018 genome, Xoo MAI1 differentially expressed genes are not located in a vicinity of 20 kb of the IS elements. Given that the African Xoo strain BAI3 is more closely related to Xoo MAI1 than Xoo MAFF311018, a similar organization of IS elements and presence of neighbour genes is expected for MAI1. Correlation between differential expression of IS elements, genome location, and role played in the control of expression of nearby genes in African Xoo strains need further study.

Validation of differentially expressed genes, using QRT-PCR

To validate the Xoo MAI1 microarray results, QRT-PCR was performed on a set of 14 genes of different functions and which were up- or down-regulated during infection. Table 4 lists the primers, putative function, and average fold-change expression of genes used for QRT-PCR validation. The genes selected for QRT-PCR correspond to four hypothetical proteins (FI978067, FI978252, FI978305, and FI978328), one gene showing no similarity to known proteins (FI978310), two putative transposases (FI978288 and FI978099), two genes related to transport and motility (FI978259 and FI978319), one hrpF gene (FI978263), and one avirulence protein from the AvrBs3/ pthA family (FI978282), the avr/ pth14 gene (M1P3I15), the xopX gene (ACD57163), and the avrXa7 gene (AF275267). Figure 4 shows five genes out of the 14 tested that were up-regulated by QRT-PCR and having a larger than 4-fold change. Of the 14 genes selected according to the microarray data (Table 4), 13 were up-regulated and 1 (FI978067) was down-regulated. The QRT-PCR results supported these data, and also showed that the gene expression pattern was identical for all genes tested, except two (FI978259 and FI978319). Gene expression values, however, differed between microarrays and QRT-PCR. As shown in Figure 4, the expression values for the five genes FI978252, FI978263, FI978328, AF275267, and ACD57163 were higher in QRT-PCR than for microarray, indicating that QRT-PCR may be more sensitive than microarray analysis. The xopX gene (ACD57163) was highly up-regulated in Xoo strain MAI1 in planta, indicating that induction of this gene is important during interaction between Xoo strain MAI1 and rice. These five genes belonged to cluster 1.
Conclusions
Sustainable control measures for bacterial blight in Africa will depend on understanding and characterizing those of the microbe's genes involved in the rice-\(Xoo\) interaction. We therefore focused our study on analysing and characterizing \(Xoo\) MAI1 at the transcriptional level. For this we constructed a \(Xoo\) MAI1 SSH array, performed in planta gene expression analysis and selected and validated by QRT-PCR various gene expressions to generate robust and reliable data. Although the SSH microarray may not be as sensitive as QRT-PCR for some genes, results included several candidate genes whose regulation and function will need to be elucidated to better understand the \(Xoo\)-rice interactions.

Our study shows that the regulation of gene expression in the \(Xoo\) strain MAI1 is controlled at different time points during pathogen infection. We identified conserved mechanisms for which some were reported in other \(Xoo\)-plant interactions but not yet described for African strains. We also identified differentially regulated genes specific to the \(Xoo\) strain MAI1. Several homologues of \(Xoo\) MAI1 differentially expressed genes were located in the vicinity of \(Xoo\) elements in the \(Xoo\) BAI3 genome. The role played by these IS elements in controlling neighbouring-gene expression needs to be elucidated. More data on African \(Xoo\) strains also need to be generated. Recently, the sequencing of various African \(Xoo\) and \(Xoc\) strains has been initiated at our laboratory and others. With this information, the full-length cDNA of desired genes can be easily obtained and their specific functions in pathogenicity studied, using available gene knockout technology. Functional characterization of the proteins/genes related to virulence will be of particular importance in understanding the complex interaction between \(Xoo\) MAI1 and rice. Our work constitutes a significant contribution towards the biology of an emerging

<table>
<thead>
<tr>
<th>Flanking sequence of IS element</th>
<th>Genes in vicinity</th>
<th>Putative function</th>
<th>Relative distance (kb) between differentially expressed genes and IS elements in genome:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BAI3</td>
</tr>
</tbody>
</table>
| F1978233  
ISXo8 transposase (ISS family) | F1978262  
ISXo8 transposase (ISS family) |                | 10001+10132          | 920135..920004           |
|                               | F1978083  
Putative transposase |                | -2.0                 | -3723                   |
|                               | M1P482  
No protein match |                | +8.2                 | +621                    |
|                               | F1978246  
Transposase |                | +1.2                 | -3796                   |
|                               | F1978279  
ribonucleoside-diphosphate reductase, beta subunit | | -0.9                 | +153                    |
|                               | F1978268  
No protein match |                | 7.8                  | +761                    |
|                               | F1978290  
dTDP-glucose 4,6-dehydratase | | -10.1                | +144                    |
|                               | F1978285  
Hypothetical protein XOO1934 | | -1.2                 | +150                    |
|                               | F1978270  
Putative transposase | | -3.8                 | +757                    |
| F1978246  
transposase | F1978181  
Cellulase | | 10001+10299          | 2009657..2009789       |
|                               | F1978274  
ISXo15 transposase (ISS0 family) | | 13384+14161          | 14199973..1420912      |

Homologues of IS elements, found as differentially expressed in the African strain MAI1 of \(Xanthomonas oryzae\) pv. \(oryzae\) (\(Xoo\)) were identified in the \(Xoo\) BAI3 draft genome. Extractions (10 kb) were made from up- and downstream flanking regions of IS elements. BLAST searches were performed locally, using the MAI1 differentially expressed genes. For the sequences located within the 20-kb sequence flanking the IS elements, the relative distance of each sequence to the IS element in BAI3 was compared with the relative distance of their respective homologues in the \(Xoo\) MAFF311018 genome. The designation + indicates upstream location of the sequence relative to the IS element, and the designation - indicates downstream location. For IS elements, gene locations within the 20-kb sequence flanking the IS element in BAI3 and within the genome of \(Xoo\) MAFF311018 are presented.
### Table 4: Validation by QRT-PCR of differentially expressed genes

| Gene          | Putative function                          | Primer sequence                  | Size, bp | AT, °C | 1 dpi | 3 dpi | 6 dpi | Array | QRT-PCR |
|---------------|--------------------------------------------|-----------------------------------|----------|--------|-------|-------|-------|-------|---------|---------|
| FI978319      | Type IV pilin                              | 5' CTACCGGCTAGCTATTCG
3' CAGCAGAGCTGGAAAGAGGCTG | 166      | 60     | 0,0   | 0,0   | 1,3   | 2,7   | 2,9   | 2,0   |
| FI978328      | probable TonB-dependent receptor           | 5' CGCCTAATCGACTTCTCA
3' AACGGGCGATAGTGAAGACAG | 167      | 60     | 0,0   | 29,0  | 11,7  | 29,9  | 69,0  | 22,5  |
| FI978288      | putative transposase                        | 5' CGAAGACCTGGGAACACTT
3' CAGTTCAGAGCGAATAA | 156      | 60     | 0,0   | 1,7   | 0,8   | 0,5   | 0,4   | 1,0   |
| FI978282      | avirulence protein AvrBs3/pth family        | 5' AGAGGAATCGCAATGGTGA
3' TTGAAACCCATGTGCAACTG | 167      | 60     | 0,0   | 0,6   | 1,3   | 0,7   | 0,6   | 1,6   |
| FI978099      | putative transposase                        | 5' TGTTTTGTAGCCGCTCTTT
3' GAGCCGACTGGCATTTGAT | 188      | 60     | 0,0   | 0,9   | 1,4   | 0,0   | 0,8   | 1,6   |
| M1P3115       | Aavr/Pth14 (avr/pth14) gene                 | 5' AGGTACGAGGCCTCACTGAA
3' CAATTCCCTATCCCGAGGAG | 157      | 60     | 0,0   | 5,0   | 9,8   | 8,3   | 26,7  | 12,5  |
| FI978263      | HrpF protein                               | 5' GGGCTAACAATCCAGAGGAG
3' CAGTTCATTCAGCAGGAGCATC | 150      | 60     | 0,0   | 20,0  | 12,3  | 4,3   | 47,5  | 24,9  |
| FI978252      | hypothetical protein Xoo0776               | 5' AGAAGTTCAGGCTGGAAAGAG
3' CGCAAGTGCAAAAAGAG | 150      | 60     | 0,0   | 0,0   | 1,5   | 0,0   | 1,2   | 1,1   |
| FI978310      | -                                         | 5' AATGATCACTGGGTTGGAG
3' GAGGCAGCCTGTCGTTCAGG | 224      | 60     | 0,0   | 0,0   | 1,5   | 0,0   | 1,2   | 1,1   |
| FI978259      | ATP-binding protein of ABC transporter     | 5' TCACCTACAGTGGTCAGAG
3' CAGACGGGTGGATGTAAGCA | 215      | 60     | 0,0   | 0,0   | 1,6   | 2,5   | 1,7   | 1,6   |
| FI978067      | conserved hypothetical protein             | 5' GCCATATGGTCTCTCAAC
3' GGTTCATCCTTGCGATATT | 160      | 60     | 0,0   | -2,2  | 0,0   | -0,8  | -2,8  | -0,2  |
| FI978305      | hypothetical protein xccb100_3708          | 5' AGAAGGCAACGCCAATTAACA
3' TGAGGAGTGGGGGAAAGTGG | 170      | 60     | 0,0   | 0,0   | 0,5   | 0,5   | 0,6   | 1,2   |
| ACD57163      | XopX effector protein                      | 5' TTGTTCCTGGCCATTTGGAAT
3' GATGTGCTGCAGAAAGAAGG | 150      | 60     | 10,0  | 14,7  | 11,0  | 198,5 | 49,0   | 43,3  |
| AF275267      | avirulence protein gene (avrXa7)           | 5' GCACGCAACATTTCCAGAGGT
3' CACCTTGTCCGCCACACTAC | 172      | 60     | 0,0   | 7,2   | 3,0   | 9,8   | 12,3  | 4,8   |

List of DNA fragments used to validate the *Xanthomonas oryzae* pv. *oryzae* (Xoo) MAI1 strain expression changes as determined by microarray analysis. Sequences of forward and reverse primers, putative function; average of fold-change expression, gene product sizes, and annealing temperatures (AT) are indicated.
and devastating pathogen under a specific, but insufficiently studied, environment in West Africa.

**Methods**

**DNA microarray construction**

Two subtracted DNA libraries (SSH) were previously constructed in our laboratory and partially characterized [28]. For the first library (MAI1-PXO86), the tester was the African *Xoo* MAI1 (race A3) and the driver the Philippine *Xoo* PXO86 (Phil race 2). For the second library (MAI1-BLS256), the same tester was used, with *Xoc* BLS256 being the driver. We randomly selected 2112 clones from MAI1-PXO86 library and 2304 from MAI1-BLS256. From the MAI1-PXO86 SSH library, we selected another 88 clones that represented a non-redundant set of sequences selected from a previous analysis of 265 sequences from that library [28]. Several controls (clones from genes of other bacteria, fungi, rice, and humans) were also included. Inserts from each DNA clone were PCR-amplified directly from bacteria. Amplification reactions were performed in 96-well plates, with each well carrying a 50-μl volume containing 0.2 μM of each primer (T7 and SP6), 200 μM of each dNTP, 1× PCR buffer, and 1.25 units of *Taq* polymerase (AmpliTaq DNA polymerase, Promega Corporation). An MJ Research thermal cycler was used for 35 PCR cycles, as follows: 95°C for 45 s, 56°C for 45 s, and 72°C for 1 min. We also amplified a selected set of conserved effector and *hrp* genes (e.g. *XopX*, *avrXa7*, *XopD*, *avrRxv*, *avrXv3*, *hpaF*, and *hrpx*), housekeeping genes, and other conserved bacterial genes from genomic DNA of *Xoo* MAI1. Random PCR samples were visualized on agarose gels. All PCR products were transferred to a 384-well plate and a volume of 2× betaine solution was added. The PCR products were arrayed once on poly-L-lysine slides (TeleChem International, Inc., Sunnyvale, CA, USA), using an SPBIO™ Microarray Spotting Station (MiraiBio, Inc., Alameda, CA, USA). The microarray contained 4708 elements.

**Bacterial inoculation and quantification**

The *Xoo* strain MAI1 was grown on PSA medium (10 g l⁻¹ peptone, 10 g l⁻¹ sucrose, 1 g l⁻¹ glutamic acid, 16 g l⁻¹ agar, and pH 7.0) for 2 days at 30°C. The bacterial cells were suspended in sterilized water at an optical density of 600 nm (OD₆₀₀) (about 10⁻⁹ cfu ml⁻¹). Bacterial blight inoculation was carried out on the two youngest, fully expanded leaves on each tiller of 6-week-old rice plants (var. Nipponbare), using the leaf-clipping method [67]. Experiments were conducted under greenhouse conditions at 26°C and 80% relative humidity.

We determined *Xoo* MAI1 multiplication *in planta* at seven time points after infection by leaf clipping (0 and 12 h, and 1, 3, 6, 10, and 15 days after inoculation) in 8-week-old plants of the susceptible rice cultivar Nipponbare. The number of cells in the leaves was determined at the top 10 cm of each leaf which was cut into five 2-cm sections, and labelled A, B, C, D, and E, with A being the inoculation point. The leaf pieces were then ground in 1 ml of sterilized water. Serial dilutions were made and spread onto PSA agar plates. The plates were incubated at 28°C until single colonies could be counted. The number of colony-forming units (cfu) per leaf (equivalent to about 2 cm²) was counted and standard deviations calculated. The experiment was repeated independently three times.

**RNA extraction**

To obtain RNA from cells growing *in planta*, 30 rice leaves were inoculated by the leaf-clipping method. At each time point, leaves extending 2 cm from the tip were collected and, to facilitate exudation of bacterial cells, vortexed for 30 s with RNAprotect Bacteria Reagent (QIAGEN, Inc., Courtaboeuf, France). The leaves were removed and bacterial cells were collected in a 15-ml tube by centrifuging at 4000 rpm for 30 min at 4°C. Total RNA extraction and DNase treatment were conducted, using the RNaseasy Mini Kit according to the manufacturer’s recommendations (QIAGEN, Inc.). To obtain RNA from bacterial cells, bacterial cultures were grown on PSA medium at 28°C until the early stationary phase. They were then re-suspended in 15 ml sterilized Milli-Q water, adjusted to OD₆₀₀ of 0.2 (about 10⁻⁸ cfu ml⁻¹), pelleted by centrifuging, and transferred to 1.5-ml tubes. Total RNA and DNase I treatments were performed as described above. The RNA quality was verified both by agarose-gel electrophoresis and by PCR (for presence of...
genomic DNA), using the genomic region flanking the hrpX gene as control and purified RNA as the PCR template. About 1 μg of Xoo MAI1 total RNA, obtained from cells grown in culture medium or in planta and treated with DNase I, were used individually to synthesize single-stranded cDNA. The SMART™ PCR cDNA Synthesis Kit (BD Biosciences Clontech) was used, following the manufacturer’s instructions. The cDNA was then quantified, using the PicoGreen® reagent (Invitrogen, Ltd., Paisley, UK), an ultra-sensitive, fluorescent, and nucleic dye.

**DNA microarray hybridization**

Fluorescent-labelled probes were prepared, following the Klenow labelling method (indirect labelling). Briefly, 500 ng of cDNA were labelled, using 1 μl of either Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech, Little Chalfont, UK), 10 U Exonuclease-Free Klenow (USB Corporation, Cleveland, OH, USA), and 3 μg random primers (Invitrogen Life Technologies, Carlsbad, CA, USA), and incubated 2 h at 37°C. Unincorporated nucleotides were removed, using a QIAquick PCR Purification Kit (QIAGEN, Inc.). Cleaned probes were concentrated in a speedvac (Eppendorf® Vacufuge Concentrator 5301, Hamburg, Germany).

Before hybridization, glass slides were snap-dried on a 95°C heating block for 10 s. DNA was crosslinked to the slides, using 65 mJ of 254-nm UV-C radiation from a 95°C heating block for 10 s. DNA was crosslinked to the Hamburg, Germany).

About 1 μg of 2× hybridization buffer (1× formamide, 1× SSC, and 0.04× SDS). The mixture was denatured at 95°C for 2 min and transferred to ice. The hybridization mixture was applied to a microarray slide, transferred immediately to a hybridization chamber (Corning, Inc., Lowell, MA, USA), and incubated overnight (15-17 h) at 42°C. The slide was then washed for 5 min successively in each of 2× SSC, 0.1% (w/v) SDS at 54°C, 1× SSC, and 0.1× SSC at room temperature. Slides were immediately dried by centrifuging at 1000 rpm for 4 min. At each time point, cDNA, obtained from bacteria used as inoculum and re-suspended in water (time 0), was compared with bacteria recovered from inoculated plants at 1, 3, and 6 dai. The comparisons made were 0 - 1, 0 - 3, and 0 - 6 dai. To maximize the statistical reliability of the data, three biological replicates were carried out. In addition, for each time point comparison and each biological replicate, three technical replicates (cDNA obtained from the same mRNA extraction) were used for hybridization. For one of the three technical replicates, the labelling of the two cDNA samples with either Cy5 or Cy3 fluorescent dye was reversed to prevent potential dye-related differences in labelling efficiency. Overall, 27 images were analysed, 9 for each time point during Xoo infection. The nine data points obtained for each gene were used in the analyses.

**Microarray data analysis**

The slides were scanned, using a chip reader/scanner (Virtek Vision International, Inc., Waterloo, ON, Canada). The signal was initially normalized during image scanning to adjust the average ratio between the two channels, using control spots. Spot intensities from scanned slides were quantified, using the Array-Pro 4.0 software (Media Cybernetics, Inc., Silver Spring, MD, USA). With this program, local corner background correction was carried out. Array-Pro 4.0 output data files (in Excel) were used to perform the lowest intensity normalization, standard deviation regularization, low intensity filtering, and dye-swap analysis, using the MIDAS computer program [68]. Normalization between different slides was carried out by centring [69]. MIDAS [68] was also used for replicate analysis and dye-swap filtering.

Bootstrap analyses with SAM enabled us to identify the differentially expressed genes, using a cut-off of two and adjusting the delta-delta Ct value, FDR, and FSN to minimize the number of false positives genes [70]. We conducted k-means clustering analysis to group the cDNA clones according to the similarity of their expression patterns, using MeV software available from TIGR and the default options [68].

**Sequence data analysis**

The 710 genes identified as differentially expressed were one-end sequenced. Sequence data were processed, using a PerlScript pipeline, to remove vector and low-quality sequences and to assemble sequences into a non-redundant set of sequences [71]. The Xoo MAI1 non-redundant set of sequences was deposited at GenBank’s GSS Database [72], under accession numbers F1978231-F1978329.

Processed sequences were initially searched against the NCBI database with BLASTN and TBLASTX [73], setting BLAST parameters to search against the complete non-redundant database and the genomes of Xoo strains KACC10331, MAFF311018, and PXO99A, and Xoc strain BLS526. A BLAST search was also performed with the partial genome of the African Xoo strain BAI3, which is currently being sequenced (Genoscope project 154/AP 2006-2007 and our laboratory, 2009, unpublished data). Results of these comparisons are summarized in the Additional file 1, Table S1. The Xoo MAI1 non-redundant set of sequences was grouped into functional categories.

To establish if differentially expressed genes are located in the vicinity of the IS elements in the genomes of Xoo African strain BAI3 and Xoo Asian strain MAFF311018, we selected a region of 20 kb that flanked the IS elements in both the MAI1 and BAI3 genomes. BLAST searches were performed against these flanking sequences, using the Xoo MAI1 non-redundant set of sequences. For the sequences located within the 20-kb sequence flanking the IS elements, the relative distance of each sequence to the IS element was calculated and compared between the two genomes.

**Southern blot analysis of differentially expressed genes**

Southern blot analysis was used to confirm that the DNA fragments derived from individual clones were present in the initial tester (Xoo MAI1 strain) and absent in the driver DNA (Xoo PXO86 or Xoc BLS256 strain). Eight genes (F1978263, F1978169, F1978079, F1978093, F1978109, F1978168, F1978197 and F1978322) were selected according to sequence similarities and library origin. Additionally, the gene F1978197 was selected to screen genomic DNA from different Xoo Asian strains (HN35, PXO339, PXO341, and PXO86), Xoo African strains (MAI1, BAI3, NAI8, and BAI4), Xoc African strains (MAI1 and MAI3), and the Xoc Asian strain BLS256 (Figure 2).

Briefly, for each strain, 5 μg of genomic DNA was digested with 10 units of Rsal and run on 0.8% agarose gels. The DNA was transferred to Hybond-N1 nylon membranes (Amersham Pharmacia Biotech) by capillary transfer. The insert DNA was amplified by PCR, using the nested primers provided with the PCR-Select™ Bacterial Genome Subtraction Kit (Clontech Laboratories, Inc.). The amplified DNA fragment was gel purified, using the QIAquick Gel Extraction Kit (QIAGEN, Inc.), as recommended by the manufacturer. The DNA fragments were labelled with [α-32P]dCTP by random priming (MegaPrime labelling kit, Amersham Biosciences). Conditions of hybridization and washes were done at 65°C. Filters were washed with three solutions: the first of 2× SSC and 0.1% SDS for 20 min, followed by two washings with 1× SSC and 0.1% SDS for 10 min each, and a final wash with 0.1× SSC and 0.1% SDS for 20 min. Blots were exposed on a PhosphorImager (model Storm 860, Amersham Pharmacia Biotech Inc.-Molecular Dynamics Division, Piscataway, NJ, USA).

**Validation by quantitative QRT-PCR**

We selected 14 genes that had been differentially expressed at various time points during infection by Xoo MAI1 for confirmation by QRT-PCR. The primers for quantitative detection were designed, using the Beacon Designer™ software (PREMIER Biosoft International, Palo Alto, CA, USA) (Table 4). All experiments were performed in triplicate. PCR mixtures were prepared, using FullVelocity® SYBR® Green QPCR Master Mix (Stratagene). PCR was performed on an Mx3005P thermal cycler (Stratagene), with the following cycling program: 95°C for 5 min, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C.

Analysis was performed, using the delta-delta Ct method. The gene expression levels obtained by QRT-PCR were normalized, using the 16S ribosomal gene, which showed similar expression levels at different time points after infection. The gene expression level was compared between microarray and QRT-PCR. Similarly, the microarray ratio for each gene analysed was normalised against the microarray ratio obtained for 16S ribosomal gene. This allowed direct comparison between the 16S ribosomal gene-normalized QRT-PCR ratio and the 16S ribosomal gene microarray ratio for each transcript investigated.

**Additional material**

**Additional file 1.** Xoo strain MAI1 genes identified as differentially expressed in planta by microarray analysis. The non-redundant set of sequences, composed of 147 Xoo strain MAI1 genes differentially expressed during infection, was searched against the genomes of all available sequenced strains of X. oryzae (Xoo strains KACC10331, MAFF311018, and PX0999, and Xoc strain BLS256), and against the draft genome of the African Xoo strain BA13. Changes in gene expression across different time points during infection are also presented.

**Authors’ contributions**

MS, JJ and VV designed the research project. MS DB and CG constructed the SSH, prepared samples for microarray studies and performed the microarray experiments. MS and DB analyzed microarray data. MS and RG carried out sequence analysis, MS and BS designed QRT-PCR experiments. MS and VV drafted the manuscript. All authors read and approved the final manuscript.

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66. BLAST (Basic Local Alignment Search Tool), BLAST Assembled Genomes [http://blast.ncbi.nlm.nih.gov/Blast.cgi]

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