

Global Transcriptome Profiling of *Xanthomonas oryzae* pv. *oryzae* under *in planta* Growth and *in vitro* Culture Conditions

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Xanthomonas oryzae pv. *oryzae* (*Xoo*), the causative agent of bacterial blight, is a major threat to rice productivity. Here, we performed RNA-Seq based transcriptomic analysis of *Xoo* transcripts isolated under *in planta* growth (on both susceptible and resistant hosts) and *in vitro* culture conditions. Our *in planta* extraction method resulted in successful enrichment of *Xoo* cells and provided RNA samples of high quality. A total of 4,619 differentially expressed genes were identified between *in planta* and *in vitro* growth conditions. The majority of the differentially expressed genes identified under *in planta* growth conditions were related to the nutrient transport, protease activity, stress tolerance, and pathogenicity. Among them, over 1,300 differentially expressed genes were determined to be secretory, including 184 putative type III effectors that may be involved in *Xoo* pathogenicity. Expression pattern of some of these identified genes were further validated by semi-quantitative RT-PCR. Taken together, these results provide a transcriptome overview of *Xoo* under *in planta* and *in vitro* growth conditions with a focus on its pathogenic processes, deepening our understanding of the behavior and pathogenicity of *Xoo*.

Keywords : bacterial blight, *in planta* transcriptome analysis, pathogenicity, plant-pathogen interaction, secretory proteins, *Xanthomonas oryzae* pv. *oryzae*

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Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a Gram-negative bacteria that causes bacterial blight disease in rice (Niño-Liu et al., 2006). *Xoo* infection initiates from the leaf sheath and eventually spreads to mature leaves through the water flow under optimum temperature and high humidity conditions (Niño-Liu et al., 2006). Bacterial leaf blight disease caused by *Xoo* can result in a 20-50% yield loss in rice (Zhang and Wang, 2013). Therefore, analyzing the interaction between rice and *Xoo* could provide avenues for the genetic modification of crops against bacterial leaf blight disease (Gupta et al., 2015; Wang et al., 2017).

In the past decade, efforts have been made to elucidate the lifecycle, pathogenicity, and adaptation mechanisms of *Xoo* using various omics tools, including genomics, transcriptomics, and proteomics (Kim et al., 2016; Soto-Suarez et al., 2010; Wang et al., 2017; Zhang et al., 2013). Comparison of differentially expressed proteins among phyto-bacteria cultured under different conditions has been carried out by several research groups (Mehta and Yoko, 2001; Tahara et al., 2003; Vileth et al., 2009; Wang et al., 2017; Zhang and Wang, 2013). *Xoo* secretome analysis led to the identification of various hydrolytic enzymes and proteins involved in multiple functional categories, such as protein degradation and cell wall modification (Kazemi-Pour et al., 2004; Watt et al., 2005). Differentially modulated proteins were previously identified from the culture

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medium of *X. axonopodis* pv. *citri*, supplemented with the leaf extract of the host plant (Mehta and Yoko, 2001) or *X. axonopodis* pv. *passiflorae* (Tahara et al., 2003). A similar method was employed to investigate differentially modulated proteins of *X. campestris* pv. *campestris* (Villette et al., 2009). Proteins of *X. campestris* pv. *campestris* cells were extracted and identified from susceptible plants and compared with those from a resistant plant (Villette et al., 2009). Among these studies, enrichment of Calvin cycle-related proteins was detected, suggesting that regulation of energy metabolism and carbon fixation processes is essential for the interaction between host and pathogen.

Compared to a proteomic approach, a microarray-based high throughput approach is more widely used for genome-wide investigation of bacterial transcripts under *in vitro* and *in planta* growth conditions (Dugé de Bernonville et al., 2014; Zhou et al., 2011). However, microarray technology has limitations in the detection of weakly expressed genes. This is a major bottleneck, especially during *in planta* growth analysis of bacterial cells since the percentage of bacterial infected host cells is always less than the total host and microbe cell numbers. Therefore, to detect the transcriptional changes in *Xoo* under *in planta* growth conditions, two methods can be employed. Firstly, next-generation sequencing technology such as RNA-Seq has been developed to reduce inherent defects in microarray methods (Wang et al., 2009). RNA-Seq technology is a powerful method for high-throughput transcriptome analysis based on deliberate measurement of gene expression levels under given sample conditions. Especially, Illumina Genome Analyzer has been successfully employed to examine differential gene expression in pathosystems, including *Xanthomonas axonopodis* pv. *glycines* in soybean (Orshinsky et al., 2012), *Sclerotinia homoeocarpa* in bentgrass (Adhikari et al., 2012), and *Pseudoperonos poracubensis* in cucumber (Hinton et al., 2004). Secondly, enrichment of bacterial cells under *in planta* growth conditions could aid detection as well as purity of pathogen transcription (Tokuda et al., 2008). Thus, a combination of RNA-Seq and bacterial enrichment methods could increase our understanding of *Xoo* behavior under *in planta* infection conditions.

To date, there is no report on the transcriptome analysis of *Xoo* genes, obtained from *in planta* growth conditions, probably because of the difficulties in obtaining pure *Xoo* RNAs from *in planta* grown bacteria. Moreover, there is no information on the comparative transcriptome analysis of the *Xoo* genes grown under *in vitro* and *in planta* growth conditions. Alternatively, prior studies have attempted to monitor differentially expressed genes and proteins from bacteria in medium incorporated with host plant leaf ex-

tract in order to omit the RNA and protein extraction steps from *in planta* grown bacteria contaminated by host RNA and proteins, respectively (Mehta and Yoko, 2001; Tahara et al., 2003). However, few reports have provided suitable techniques to purely isolate RNA and proteins from *in planta* cultured bacteria cells for transcriptomic or proteomic analysis, respectively, during interactions between bacteria and the host plant (La et al., 2008; Wang et al., 2013). Recently, a highly efficient method for the isolation of *in planta* grown bacteria cells was reported in rice leaves using CaCl_2 extraction buffer to purify bacterial samples from the debris of an infected host plant (Wang et al., 2017).

In this study, bacterial cells were harvested in adequate amounts from susceptible (Dongjin cv.) and resistant (Samgwang cv.) hosts using a CaCl_2 method and subjected to RNA-Seq based transcriptomic analysis in order to gain deeper insights into *in vitro* and *in planta* expressed genes of *Xoo* during pathogenic interactions with rice. Illumina Genome Analyzer sequencer was employed to identify differentially expressed genes of *Xoo* grown under *in vitro* and *in planta* growth conditions.

Materials and Methods

Bacteria and plant growth conditions. *Xoo* strain K3 (KACC 10385/JH89011) was used in this study. Two rice cultivars (*Oryza sativa* L. cultivar Dongjin and Samgwang), which are compatible and incompatible with *Xoo* K3 strain, respectively, were used. Rice seeds were imbibed in sterilized water at 4°C for 2 days, germinated on moist tissue paper for 7 days, and transferred to soil in a growth chamber at 25°C (16/8 h day/light cycle, 70% relative humidity). Rice at the tillering stage was used for *Xoo* inoculation.

***Xoo* culture and infection of rice.** *Xoo* strain was grown on Peptone sucrose agar (PSA) plate (1% w/v peptone, 1% w/v sucrose, 0.1% w/v glutamic acid, and 1.5% agar) at 28°C. For *in vitro* culture, *Xoo* cells were incubated in Peptone sucrose broth (PSB) medium and shaken at 200 rpm in the dark at 28°C for 3 days. Cultured cells were washed twice with distilled water and diluted to 10^8 cells mL^{-1} ($\text{OD}_{600} = 0.1$) with water containing 0.01% tween-20. Leaves from 50-day-old (at the tillering stage) rice cultivars were artificially infected with *Xoo* strains (10^8 cells mL^{-1}) by clipping the leaf tips with sterile scissors. Infected rice plants were grown under normal light conditions (16 h light/ 8 h dark) for 5 or 10 days.

Xoo cell collection from *in vitro* culture medium and rice leaves. Cultured *Xoo* cells on PSB medium were collected by centrifugation at 12,000 g for 15 min and washed twice with distilled water. The *in planta* grown *Xoo* cells were collected using the CA extraction method (Wang et al., 2017). Briefly, infected rice leaves were detached, divided into 3 cm segments, immediately immersed in CA extraction buffer (200 mM CaCl₂, 5 mM Na-acetate, pH 4.3) on ice, and shaken at 170 rpm for 1 h. *Xoo* cells, collected by filtering extraction buffer through filter paper, were harvested by centrifugation at 12,000 g for 15 min at 4°C. Cell pellet was frozen in liquid nitrogen and then used for RNA extraction.

RNA extraction and library preparation. Total RNA was extracted using a GeneAll Hybrid R™ Kit (GeneAll Biotechnology, Korea) according to the manufacturer's instructions. RNA purity and concentration were assessed using an RNA Nano 6000 Assay Kit (Agilent Technologies, CA, USA) with the Bioanalyzer 2100 system (Agilent Technologies). For mRNA library construction and deep sequencing, RNA samples were prepared using a TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Finally, the two libraries were sequenced using the Illumina HiSeq2000 platform in accordance with the manufacturer's protocol (Illumina, Inc., San Diego, CA, USA). Construction of libraries and RNA sequencing were performed by the National Instrumentation Center for Environmental Management (NICEM) at Seoul National University (<http://nicem.snu.ac.kr/>). The quality control process was conducted to filter high-quality RNA reads using an NIPGR, Delhi, India (v2.3.3) (Zhao et al., 2011). Data normalization for differentially expressed analysis was calculated by RPKM (Reads Per Kilobase per Million reads) as described previously (Wagner et al., 2012).

GO and KEGG enrichment analysis. To analyze the functional properties of the identified *Xoo* genes, gene ontology (GO) enrichment analysis was carried out in the categories of biological processes and molecular function using the Universal Protein Resource online web-based toolkit (<http://www.uniprot.org/>) as described previously (Kim et al., 2011). Hierarchical clustering and Heatmap were generated using MA, USA (Orshinsky et al., 2012). Genome information resource was observed from KEGG (<http://www.genome.jp/kegg/>) and identified *Xoo* transcripts from susceptible and resistant hosts were mapped into the KEGG pathways using BLAST KOALA program (<http://www.kegg.jp/blastkoala/>) (Nakaya et al., 2013).

Prediction of putative secreted proteins. Different web-based prediction tools were employed to check the secretory gene products. Classical secreted proteins, derived from N-terminal signal peptides, and non-classical secreted proteins were predicted using SignalP (version 4.1) (<http://www.cbs.dtu.dk/services/SignalP/>) (Petersen et al., 2011) and SecretomeP (version 2.0) (<http://www.cbs.dtu.dk/services/SecretomeP/>), respectively (Bendtsen et al., 2005a). Bacterial specific twin-arginine signal peptide-containing proteins were predicted by TatP (version 1.0) (<http://www.cbs.dtu.dk/services/TatP/>) (Bendtsen et al., 2005b). Type III secretion effectors were predicted by the Effectors 4.0 online program (<http://www.effectors.org/>) (Jehl et al., 2011).

Semi-quantitative RT-PCR. To analyze expression levels of *in planta* *Xoo* genes, semi-quantitative real-time PCR was conducted using a Maxime RT PreMix Kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. Equal amount of purified RNA was used to synthesize cDNA, using a first strand cDNA synthesis kit (Sigma-Aldrich, Germany). PCR was performed with 30 cycles using gene-specific primers. The primer sequences used in this study are listed in Supplementary Table 1.

Results and Discussion

Differentially expressed genes during *in planta* growth. A total of 92,620,466 raw reads were identified from *in vitro* cultured *Xoo*; 94,542,634 and 104,550,144 raw reads were identified from *in planta* grown *Xoo* in susceptible and resistant hosts, respectively (Supplementary Table 2). Among raw reads from *Xoo* under *in vitro* conditions, more than 97.45% of bases had a q-value ≥ 20 , whereas under *in planta* conditions, 97.92% (susceptible) and 97.71% (resistant) satisfied this criterion. GC contents were 53.64%, 53.75%, and 53.82% under *in vitro* as well as *in planta* susceptible and resistant conditions, respectively. There were 4,468,510,187 clean reads for *Xoo* under *in vitro* conditions as well as 5,235,181,135 and 4,697,020,133 clean reads under *in planta* conditions (susceptible and resistant), respectively (Supplementary Table 2, 3). Expression levels of genes were determined in accordance with the RPKM method (Wagner et al., 2012). Differentially expressed *Xoo* genes (DEGs) under *in planta* and *in vitro* conditions were selected based on criteria [q-value ≥ 0.001 and log₂ (≥ 2 fold change)] using the MeV program (Supplementary Fig. 1). A total of 4,619 genes, including gene clusters with high (2784) and low (548) expression levels, in given sample

sets, were identified (Supplementary Table 4). Among all DEGs, 81 *Xoo* genes were differentially expressed under susceptible and resistant interactions. Interestingly, over 10% of total genes were identified as transposable elements with approximately 25 kinds of transposase genes found in both the samples. Transposable elements (TEs) lead to chromosomal rearrangements such as deletion, inversion, duplication, and translocation (Hacker and Kaper, 2000). *Xoo* TEs were classified into six TE families: TE3, TE4, TE5, TE30, ISNCY, and TE630. In the *Xoo* genome, the most abundant TE was the TE5 family for which 117 cop-

ies were detected out of 207 total identified TEs. Many of these elements are located adjacent to strain-specific genes in regions with altered codon usage and G+C content. Of these, TEXo5 transposase was most abundant, followed by TEXo8 and TEXo3. However, expression of TEXo5 was higher in S than R cultivars. Several TEs and transposon sequences can be found in virulence-associated genes. Therefore, these elements might play important roles in horizontal gene transfer and internal modification of the genome. Furthermore, these TEs can convert pathogenic genes into non-pathogenic ones (Hacker and Kaper, 2000).

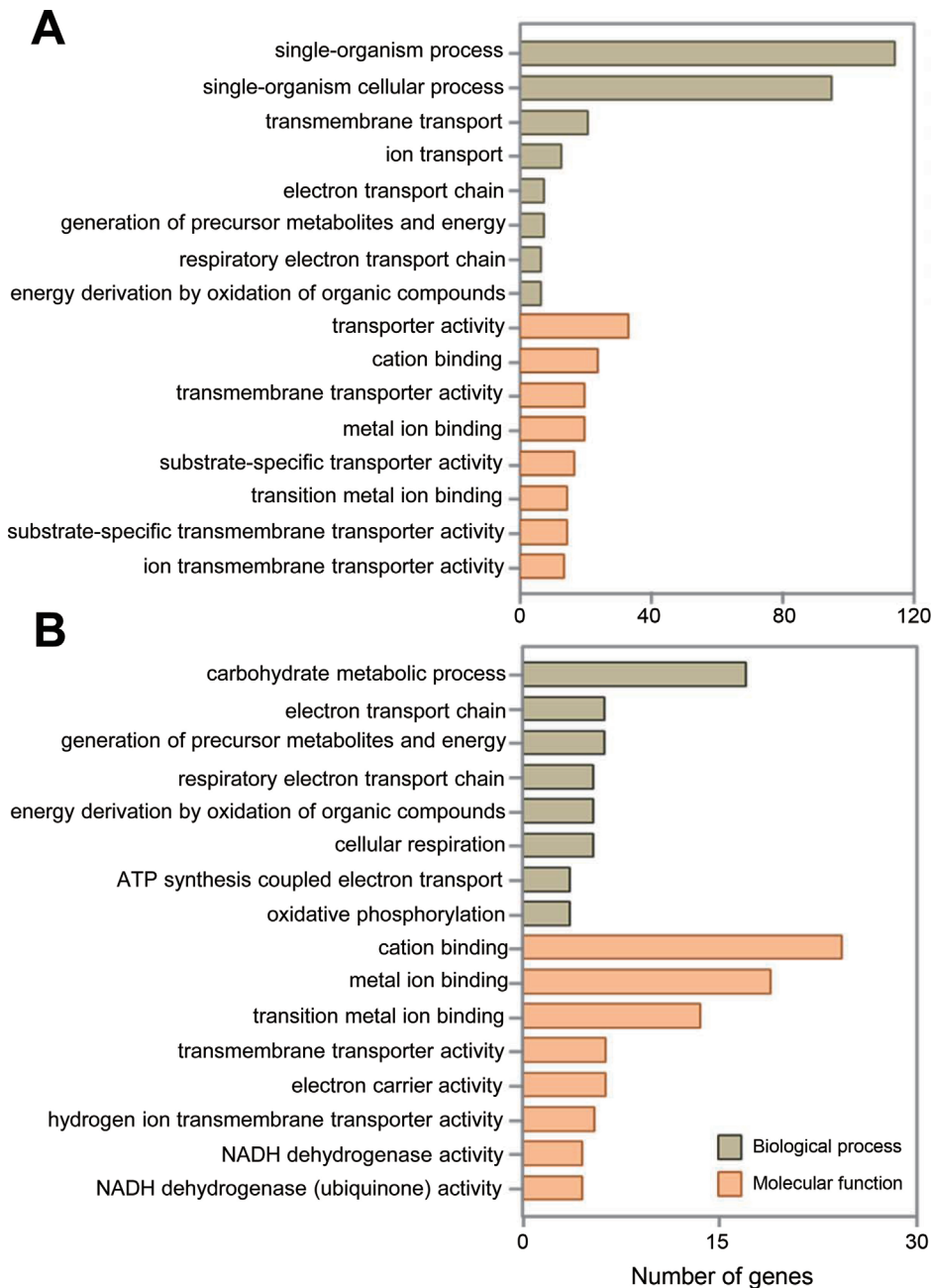


Fig. 1. Gene ontology analysis of enriched up-regulated genes of *Xoo* during *in planta* growth on [susceptible (S) and resistant (R)] hosts. Data were analyzed in biological process and molecular function categories.

These observations indicate that higher expression of TEs under *in planta* conditions could be a means of adaptation in the host plant.

Functional annotation and classification of identified genes. GO term enrichment analysis was carried out to determine the functional roles of DEGs between *in planta* and *in vitro* conditions (Fig. 1, Supplementary Table 5). A total of 1,619 *Xoo* genes from the susceptible host and 1,127 genes from the R host were functionally annotated into the molecular function and biological process categories (Fig. 1). In the molecular function category, up-regulated genes from the susceptible host were clustered into 27 categories. The largest category was ‘transporter activity’ (12.7%), followed by ‘cation binding activity’ (9.26%). In the case of biological processes, genes were divided into 14 categories of which the most abundant was ‘single-organism process’ (39.5%). Up-regulated *Xoo* genes from the resistant host were clustered into 13 groups, and most of these were related to ‘cation binding’ (23.68%) and ‘metal ion binding’ (18.42%) activities. For biological processes, the largest

category was ‘carbohydrate metabolic process’ (32.2%). Organism processes, including cell proliferation, utilization of nutrition sources, and membrane organization, were highly detected in the susceptible host, whereas most of these were related to the carbohydrate metabolic process in the resistant host. In the molecular function category, genes related to cation binding activity were predominant in both susceptible and resistant interactions. Cation binding proteins play crucial roles in reactive oxygen species (ROS) stress as well as uptake of ammonium and metallic cations (such as copper, iron, and nickel) (Tam and Saier, 1993). It is well known that essential nutrients, such as iron, are required for the infection processes of pathogenic bacteria (Messenger and Barclay, 1983). In addition, the ability to scavenge these nutrients is required for survival and multiplication of pathogens (Hacker and Kaper, 2000). KEGG analysis revealed no major differences between susceptible and resistant hosts. In both the cases, ‘metabolic pathways’ and ‘biosynthesis of secondary metabolites’ were major pathways, and other parts such as ‘oxidative phosphorylation’ and ‘bacteria secretion system’ were detected (Fig.

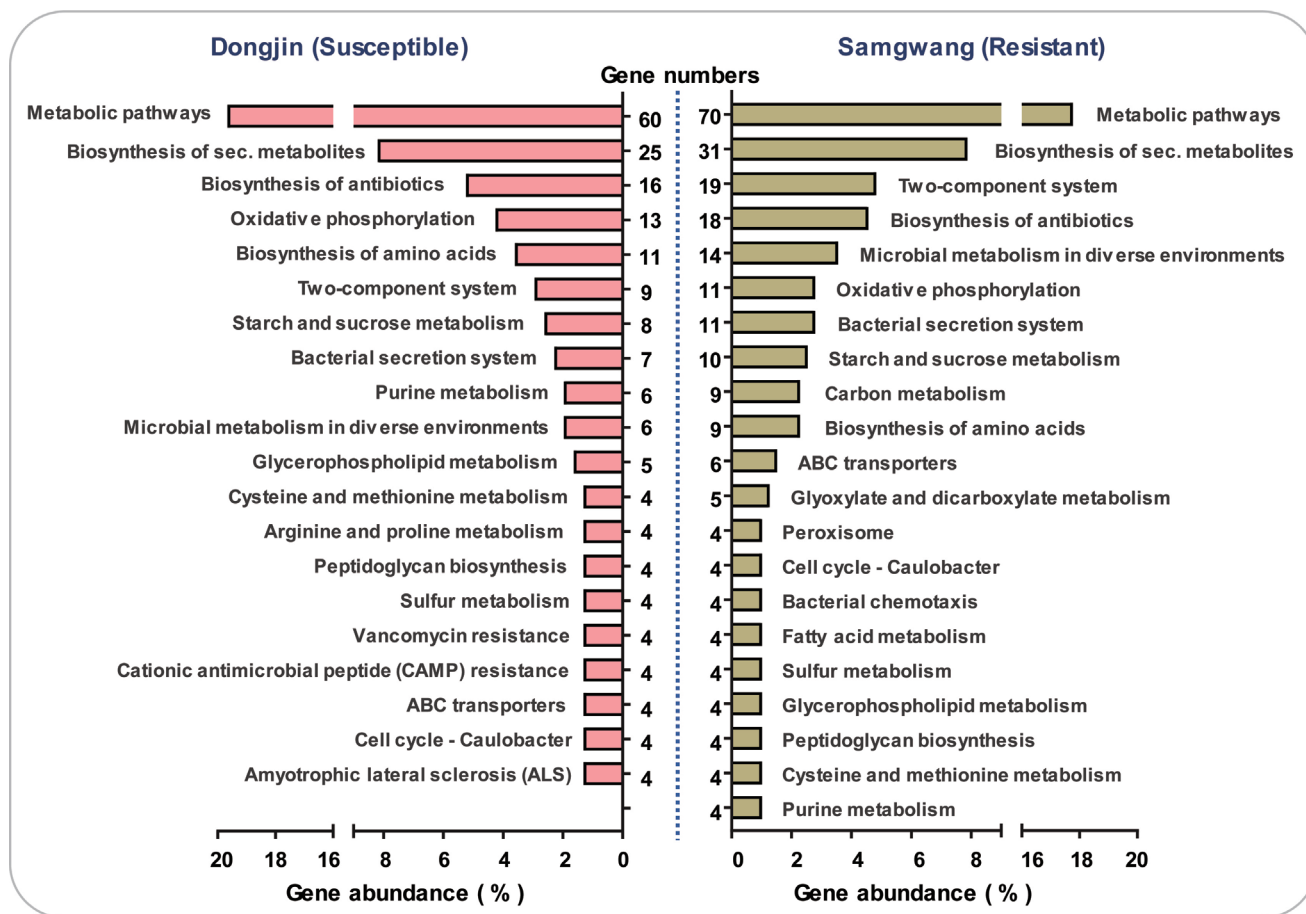


Fig. 2. Enriched KEGG pathways of up-regulated *Xoo* genes during *in planta* growth on [susceptible (S) and resistant (R)] hosts.

2, Supplementary Table 6). However, a high percentage of genes involved in ‘microbial metabolism in diverse environments’ and ‘carbon metabolism’ pathways were observed in the resistant host. These results indicate that *Xoo* cells in the resistant host were under stress as compared to the susceptible host. Compared with *in vitro* cultured *Xoo*, *in planta* grown *Xoo* were more exposed to unfamiliar and nutrient-deficient conditions. During *in planta* growth, especially in the resistant host, genes related to stress and carbohydrate consumption were highly expressed. However, a considerable number of metabolic processes involved in reproduction and development were detected under susceptible conditions. Taken together, these results constitute detailed information on differentially up- and down-regulated *Xoo* genes between susceptible and resistant host-*Xoo* interactions.

Prediction of putative effectors from *Xoo* under *in planta* conditions. To determine putative secretory proteins and effectors from the identified genes, the SecretomeP, SignalP, and TatP programs were used. Of the identified genes, a total of 1,334 genes were found to have an N-terminal signal peptide and thus encode for the secretory proteins (Fig. 3). Of these secretory proteins, 184 putative

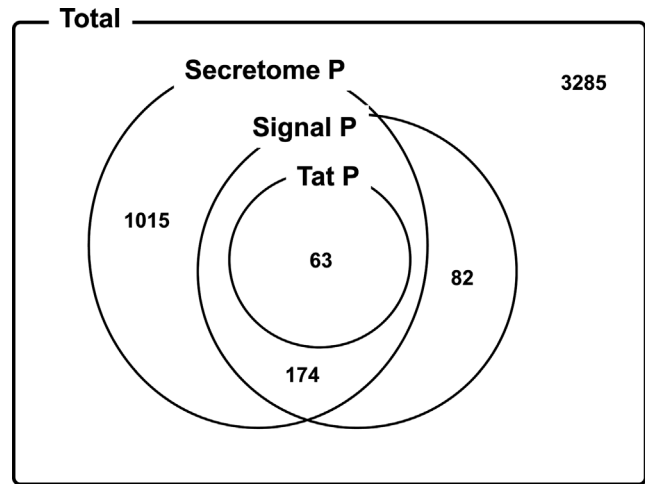


Fig. 3. Analysis of putative secretory proteins using bioinformatic tools such as SecretomeP, SignalP, and TatP. A total of 1,334 candidate proteins was detected.

type III effectors were predicted by a web-based analysis tool (Supplementary Table 7). The Hypersensitive reaction and pathogenicity (*hrp*), and avirulence genes (*avr*) accounted for a high percentage among others. Particularly, the AvrBs3 family, which is well known for its TAL (transcription activator-like) effector activity, was also detected

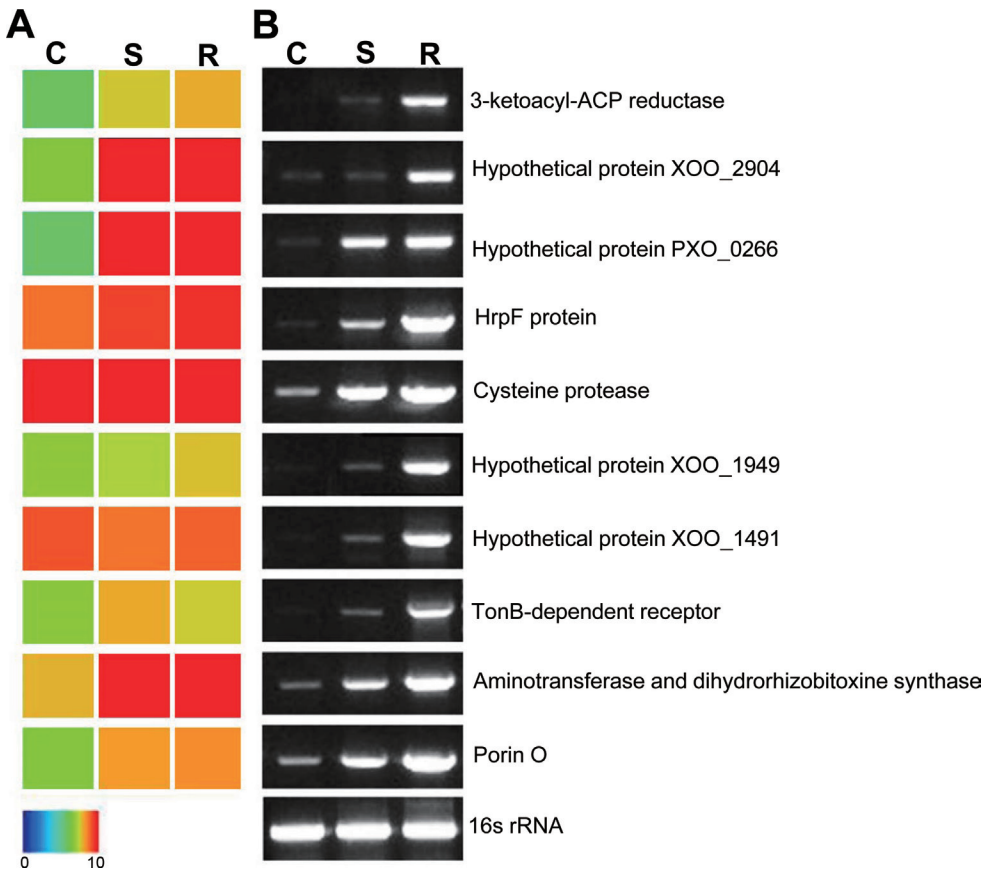


Fig. 4. Heat map of *Xoo* genes specifically expressed during *in planta* growth (A). Interestingly, 10 candidate genes were differentially up-regulated in *Xoo* cultured *in planta* by RNA-Seq analysis. Expression patterns were confirmed by semi-quantitative RT-PCR (B). C-R: C; *in vitro*, S; susceptible, R; resistant host conditions of RNA-Seq analysis.

in this study. TAL effectors act as transcription factors and influence host gene expression (Kay et al., 2007). Among these, candidate genes were sorted into their functional categories and combined together. Most of the genes related to nutrient uptake or transport showed similar expression patterns under *in vitro* and *in planta* growth conditions while genes related to protein degradation and modification and bacterial virulence were more highly accumulated *in planta* as compared to *in vitro*. Taken together, our results provide a deeper understanding of *Xoo* behavior during *in vitro* and *in planta* growth on resistant and susceptible hosts.

Validation of *in planta* expressed *Xoo* genes. Transcriptome analysis showed that a total of 81 genes were highly up-regulated under *in planta* infection conditions as compared to *in vitro* culture conditions. To validate our transcriptome results, we randomly selected differentially expressed *Xoo* genes related to the pathogenicity (rtxA and HrpF), nutrition uptake (Porin-O and TonB), and fatty acid synthesis (FabG) and validated them by semi-quantitative RT-PCR (Fig. 4). ThertxA was reported to be involved in bacterial pathogenicity (Cirillo et al., 2001, 2002). HrpF is

essential for the bacterial type III secretion system and is involved in pathogenicity (Rossier et al., 2000). Porin-O- and TonB- dependent receptors are related to the nutrition uptake for *Xoo* growth under *in planta* infection conditions (Blanvillain et al., 2007; Wang et al., 2002). FabG protein is known to be involved in bacterial type II fatty acid synthesis (Zhang et al., 2006). 16s rRNA primers were used as an internal control for normalization of cDNA concentration under each condition. Most genes were expressed under *in planta* infection conditions. In addition, genes related to motility such as flagella biosynthesis and chemotaxis were expressed. For several kinds of phyto-bacteria species, motility is an important factor for virulence (Josenhans and Suerbaum, 2002). *Xoo* secretes many extracellular enzymes during culture. In *Xoo*, cellulase and protease have been proposed to play important roles in bacterial virulence and nutrition (Ryan et al., 2011). Based on the results obtained here, an overview of *Xoo* candidate genes, which were differentially regulated under *in planta* and *in vitro* growth conditions, is provided (Fig. 5). A variety of genes involved in cell wall degradation were highly expressed under both *in planta* and *in vitro* growth conditions, includ-

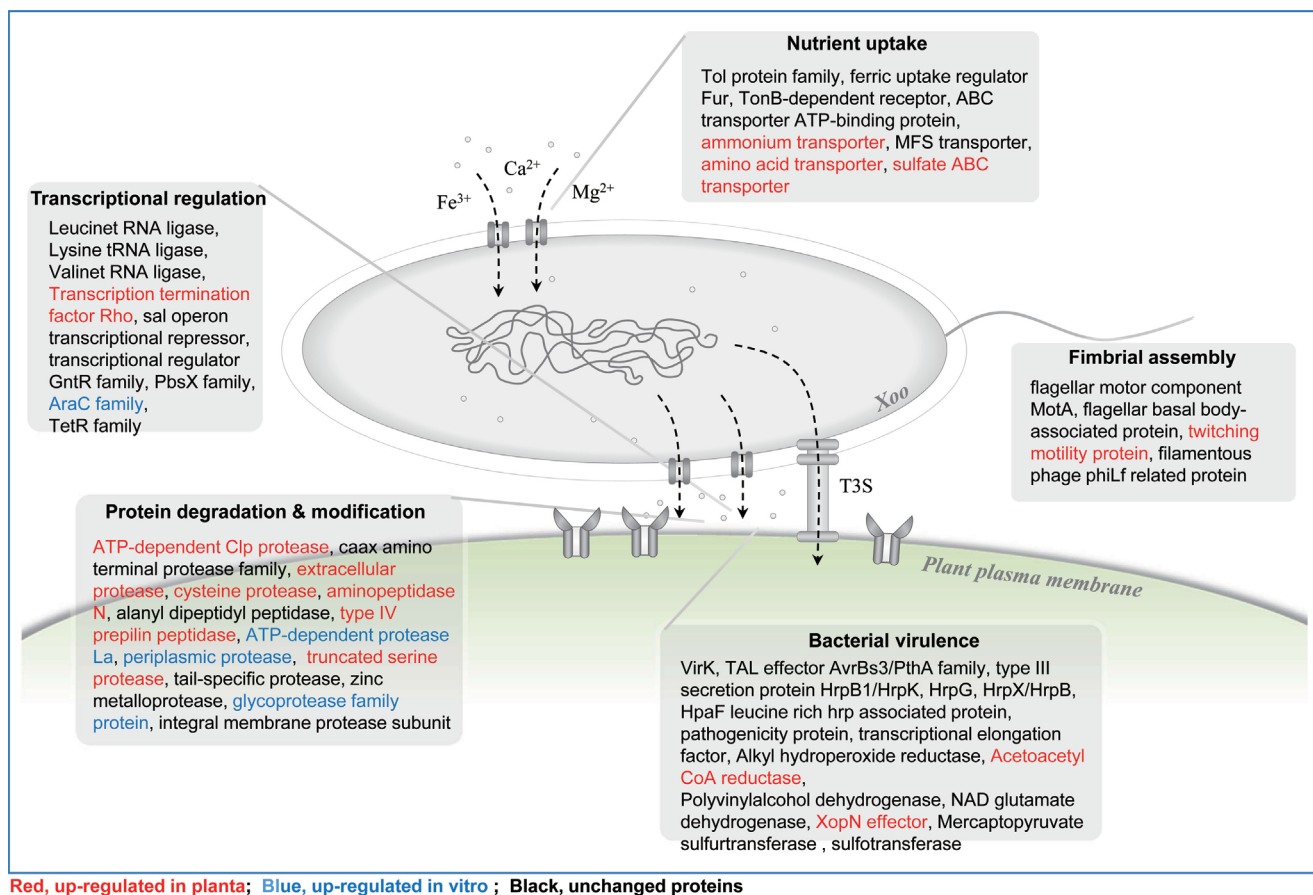


Fig. 5. An overview of *Xoo* candidate genes/proteins differentially regulated under *in planta* and *in vitro* growth conditions.

ing cellulase, xylanases, pectinases, proteases, xylosidases, and polygalacturonases.

Previously, comparative transcriptome analysis was performed on different *Xoo* strains exhibiting differential virulence in rice during *in vitro* culture in minimal medium (Zhang et al., 2013). Genes related to exopolysaccharide (EPS) synthesis, Hrp proteins, and type III effectors, including *Xanthomonas* outer protein (Xop) effectors and transcription activator-like (TAL) effectors, were differentially expressed between *Xoo* strains, suggesting these proteins may affect bacterial pathogenicity in plants. Later, Kim et al. (2016) performed transcriptome analysis of *Xoo* in response to rice leaf extracts under *in vitro* culture conditions. Leaf extracts were shown to induce rapid induction of genes related to ion transport, metabolism, cell motility, pathogenicity, chemotaxis, flagella biosynthesis, and cyclic-di-GMP control. However, the immune related genes or metabolites were induced upon pathogen infection, and these immune outputs play an important role in anti-pathogen processes (Jones and Dangl, 2006; Withers and Dong, 2017). Therefore, investigation of transcriptome or proteome changes during *in planta* infection condition, which could not be monitored via *in vitro* conditions, will provide an in-depth understanding of plant-microbe interaction. Therefore, a microarray-based transcriptome analysis of *Xoo* during *in planta* infection condition was carried out in a susceptible rice species (Soto-Suárez et al., 2010). Differentially expressed genes were related to metabolism, secretion, and transport, pathogen adherence to plant tissues, plant cell-wall degradation, IS elements, and virulence. In a combination of that information, here, a comparative transcriptome analysis of *Xoo* under *in vitro* and *in planta* growth conditions was performed for the first time.

In summary, a total of 4,619 *Xoo* genes were identified under *in planta* and *in vitro* growth conditions. Of these, some of the interesting candidates identified included TAL effector or protein synthesis related genes, EF-Tu, and transposase and these were up-regulated during *in planta* *Xoo* growth conditions. As these proteins are highly expressed during *in planta* growth conditions only, these can be directly linked to the *Xoo* pathogenicity. In addition to these, 184 effector candidate genes were also identified including 10 candidate effector genes that were especially expressed under *in planta* conditions. These genes encode for HrpF, proteases, and TonB. Taken together, list of genes and effectors provided here can serve as resource for further validation and their utilization to genetically modify the *Xoo* genome to reduce its pathogenicity.

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