Fructose-Bisphosphate Aldolase Exhibits Functional Roles between Carbon Metabolism and the hrp System in Rice Pathogen Xanthomonas oryzae pv. oryzicola

Wei Guo1,2, Li-fang Zou1,9, Yu-rong Li1, Yi-ping Cui2, Zhi-yuan Ji1, Lu-lu Cai1, Hua-song Zou1, William C. Hutchins3, Ching-hong Yang2,3, Gong-you Chen1,2*

1 Key Laboratory of Urban (South) by Ministry of Agriculture, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China, 2 Key Laboratory of Integrated Management of Crop Diseases and Pests, Ministry of Education of China, College of Plant Protection, Nanjing Agricultural University, Nanjing, China, 3 Department of Biological Sciences, University of Wisconsin, Milwaukie, Wisconsin, United States of America

Abstract

Fructose-bisphosphate aldolase (FbaB), is an enzyme in glycolysis and gluconeogenesis in living organisms. The mutagenesis in a unique fbaB gene of Xanthomonas oryzae pv. oryzicola, the causal agent of rice bacterial leaf streak, led the pathogen not only unable to use pyruvate and malate for growth and delayed its growth when fructose was used as the sole carbon source, but also reduced extracellular polysaccharide (EPS) production and impaired bacterial virulence and growth in rice. Intriguingly, the fbaB promoter contains an imperfect PIP-box (plant-inducible promoter) (TTTCGT-N9-TCGT). The expression of fbaB was negatively regulated by a key hrp regulatory HrpG and HrpX cascade. Base substitution in the PIP-box altered the regulation of fbaB with the cascade. Furthermore, the expression of fbaB in X. oryzae pv. oryzicola RS105 strain was inducible in planta rather than in a nutrient-rich medium. Except other hrp-hrc-hpa genes, the expression of hrpG and hrpX was repressed and the transcripts of hrcC, hrpE and hpa3 were enhanced when fbaB was deleted. The mutation in hrcC, hrpE or hpa3 reduced the ability of the pathogen to acquire pyruvate and malate. In addition, bacterial virulence and growth in planta and EPS production in RAfbaB mutant were completely restored to the wild-type level by the presence of fbaB in trans. This is the first report to demonstrate that carbohydrates, assimilated by X. oryzae pv. oryzicola, play critical roles in coordinating hrp gene expression through a yet unknown regulator.

Introduction

Carbohydrate nutrient acquisition is essential for bacterial pathogen growth to establish successful infections in host plants [1,2,3]. As in other living organisms, plant pathogenic bacteria carry out the catabolic process via the Emden-Meyerhof-Parnas (EMP) pathway of glycolysis, Entner-Doudoroff (ED), pentose phosphate pathway (PPP) and terminal oxidation mediated by the tricarboxylic acid (TCA) cycle to break down hexoses, like glucose, outside of their cells for energy and carbon molecules. Bacteria may also use gluconeogenesis to synthesize glucose from non-sugar C2 or C3 compounds or the intermediates of the TCA cycle when there is not sufficient hexoses in their immediate environment [4]. In Xanthomonas, ED, in conjunction with TCA, has been confirmed to be the predominant pathway for glucose catabolism, and a small portion (8 to 16%) of substrate glucose is routed into PPP [5], whereas the EMP pathway of glycolysis does not play a significant role in glucose catabolism, since Xanthomonas species, including rice bacterial leaf streak X. oryzae pv. oryzicola, lack an essential phosphofructokinase activity which converts fructose 6-phosphate to fructose-1,6-bisphosphate [5,6,7]. Moreover, little is known about the relationship of carbon metabolism to virulence. The genome data of X. oryzae pv. oryzicola (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=Xoc), X. oryzae pv. oryzae [8,9], X. campestris pv. campestris [10], X. axonopodis pv. citri [11] and X. campestris pv. vesicatoria [12] show that xanthomonads possess essential genes for the EMP pathway of glycolysis, ED, PPP, gluconeogenesis and TCA cycle. Currently, great interests have been focused on whether or not and how the carbon metabolic pathways are involved in the virulence of plant pathogenic bacteria. For example, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), converting glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, is required for ED, extracellular polysaccharide (EPS) production and full virulence of X. campestris pv. campestris [13]. The phosphogluconate dehydratase gene (edd) in ED is necessary for xanthan biosynthesis and the 6-phosphogluconate dehydrogenase gene (gndA) in PPP does not influence xanthan biosynthesis in X. oryzae pv. oryzicola [6]. The malate: quinone oxidoreductase gene (mpo) in TCA cycle is required for the wild-type growth, disease symptom development and full
virulence of *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis thaliana* [14]. The phosphoenolpyruvate synthase gene (ppsA), converting pyruvate to phosphoenolpyruvate, is essential for gluconeogenesis, in *planta* growth, and full virulence of *X. campestris* pv. *campestris* [4]. However, little is known about other carbon metabolic factors.

Previous reports have confirmed that some carbohydrates and sulfur-containing amino acids have the ability to induce the expression of *hrp* genes in Gram-negative phytopathogenic bacteria [15,16,17,18]. The *hrp* genes, normally within a 25–27 kb gene cluster in *Xanthomonas* species, encoding a type-III secretion system (T3SS), enable bacterial pathogens to trigger a rapid, localized, programmed hypersensitive response (HR) in nonhost plants and become pathogenic in hosts [18,19,20]. Expression of *hrp* is actually suppressed in nutrient-rich media but induced in *planta* and in apoplast-mimicking media, XVMM containing sucrose and fructose for *X. campestris* pathogens or species [17,18,21,22,23]; XOM3 only containing xylose for *X. oryzae* pathogens [20,24,25], except inorganic salt(s), implying that some nutrients released from plant tissues, which are degraded for bacterial growth, may have effects on induction of *hrp* gene expression. For instance, the *hrp* expression in *Ralstonia solanacearum* is activated possibly by ubiquitous and non-diffusible molecules in the presence of pathogen-plant cell contact [15,16,26]. The above prompts us to assume that there are unknown correlations between carbon metabolism and the *hrp* system for bacterial pathogenesis in plants.

When *Xanthomonas* species interact with plants, some of the *hrp* gene products generate a pedestal-like T3S structure that traverses the two bacterial membranes [27,28]. For example, a pilus-like secretion channel (HrpE), which is outside of HrcC [29], and also a translocon protein (HrpF) in the plant membrane [27,30,31,32,33]. As a whole, the T3S apparatus injects a number of effectors into the apoplast and cytosol of plant cells leading to disease in hosts or HR in non-hosts. Conceptually, expression of the *hrp* genes is controlled by two key regulatory genes, *hrpG* and *hrpX*, which are located outside of the *hrp* gene cluster [19]. *HrpG* is predicted to be an OmpR-type response regulator of a two-component signal transduction system and presumably perceives an environmental signal via an unknown sensor kinase [34,35]. *HrpX* is an AraC-type of transcriptional activator [36] which forms a homodimer containing a helix-turn-helix domain which interacts with each TTCGC motif of the PIP-box (plant-inducible promoter) in *X. campestris* pathovars or species [17,18,21,22,23]; XOM3 only containing xylose for *X. oryzae* pathovars [20,24,25]. Except inorganic salt(s), implying that some nutrients released from plant tissues, which are degraded for bacterial growth, may have effects on induction of *hrp* gene expression. For instance, the *hrp* expression in *Ralstonia solanacearum* is activated possibly by ubiquitous and non-diffusible molecules in the presence of pathogen-plant cell contact [15,16,26]. The above prompts us to assume that there are unknown correlations between carbon metabolism and the *hrp* system for bacterial pathogenesis in plants.

**Bacterial strains, cultures media and growth conditions**

Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in LB (Luria-Bertani) medium at 37°C [30]. *X. oryzae* pv. *oryzicola* strains were performed at 28°C in NA (1 g/L yeast extract, 3 g/L beef extract, 5 g/L polypeptone, 10 g/L sucrose, 15 g/L agar), NB (NA without agar), NAN (NA without sucrose) or NAS (NA with 100 g/L sucrose), NY (NB without beef extract and sucrose), the non-carbohydrate minimal medium (NCM) (2 g/L (NH4)2SO4, 4 g/L KH2PO4, 6 g/L KH2PO4, 0.2 g/L MgSO4 · 7H2O) [4] or rice suspension cells [25] when required. Antibiotics were used when required at the following concentrations: kanamycin (Kan), 25 µg/ml; rifampicin (Rif), 50 µg/ml; ampicillin (Amp), 100 µg/ml and spectinomycin (Sp), 50 µg/ml.

**DNA manipulation**

DNA manipulation was performed following the standard procedures described by Sambrook [51]. The transconjugation between the *X. oryzae* pv. *oryzicola* and plasmids was performed as described by Turner [52]. Restriction enzymes and DNA ligases were performed in accordance with the manufacturer’s instructions (Takara, Dalian, China). The PCR primers (Table S1) for gene targets in this report were purchased from Jinsite Biotechnology (http://www.croasia.net/company/jinsite_biotecnology_.co.html). The genes cloned or amplified in this study were referred to the *hrp* clusters of *X. oryzae* pv. *oryzicola* RS105 strain (AF272885, AY875714) and the genome sequence of *X. oryzae* pv. *oryzicola* BLS256 strain (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage. cgi?org=Xoc).

**Rice suspension cell cultures**

*X. oryzae* pv. *oryzicola* RS105 strain, was used for callus induction. Seeds were dehulled and sterilized in 70% ethanol for 10 min and then in 50% commercial bleach with a few drops of Tween-20 for 30 min and then in 1% HgCl2 for 15 min. The sterilized seeds were washed 5 times with sterile distilled water and placed on N6 medium (10) with 2, 4-D (3 mg/L) for induction of rice callus at 28°C in the dark. The actively growing calli were selected and transferred to liquid N6 medium containing 5 mg/L 2, 4-D and 1 mg/L kinetin (KT). The cells were maintained in the dark on a 7 day subculture schedule at a dilution of 1:5 (inoculum: fresh medium). Generally, large amounts of rice suspension cells can be obtained after 4–5 week subculture and then dispersed or single round rice cells could be observed under the microscope.

**Construction of a non-polar mutant in fbaB of *X. oryzae* pv. *oryzicola***

The non-polar mutant of *fbaB* in *X. oryzae* pv. *oryzicola* RS105 strain was constructed by using homologous recombination as described by Jiang [53], using pKMS1 as a suicide vector. Two flanking fragments, left and right to *fbaB* (Figure S1), were amplified using the genomic DNA of strain RS105 as the template and the primers *fbaB*I-F/*fbaB*I-R and *fbaB*I-F/*fbaB*I-R (Table S1), respectively, and then cloned into pMD18-T vectors (Takara, Dalian, China), respectively. After confirmed by sequencing, the two fragments were digested and cloned into the vector pKMS1 at BamHI and PstI sites, resulting in pKΔfbaB (Table 1). The plasmid tagged mutant library of *X. oryzae* pv. *oryzicola* [49] and got a mutant Mxoc0504 where the Tn5 was inserted in a unique gene *fbaB*. In this report, we present genetic evidence demonstrating that *fbaB* is required for gluconeogenesis, EPS production and the expression of *hrp* genes, as well as the full virulence of *X. oryzae* pv. *oryzicola* in rice.
pKΔfbaB was introduced into RS105 by electrotransformation, and then the electrotransformants were plated on N4 plates supplemented with kanamycin. The emerged colonies suggested that the first homologous crossover event occurred in the electron transformants in which the DNA of the deletion vector was integrated into either the left or the right border of fbaB (Figure S1). The single colonies of the mutant produced by single homologous crossover event were then transferred to NBN broth to culture for 12 h at 28°C. Then the bacterial cell was plated on NAS plates. The single colonies emerged within 3–4 day were then picked up into NA and NA bacterial cell was plated on NAS plates. The single colonies integrated into either the left or the right border of transformants in which the DNA of the deletion vector was supplemented with kanamycin. The emerged colonies suggested transferred to NBN broth to culture for 12 h at 28°C. mutant produced by single homologous crossover event were then recipient chromosome (Figure S1). The single colonies of the mutant harboring fbaBdGUS, Rifr, Spr This work
RS105(pfbaBcGUS) The wild-type RS105 harboring pfbaBcGUS, Rifr, Spr This work
RS105(pfbaBbGUS) The wild-type RS105 harboring pfbaBbGUS, Rifr, Spr This work
RS105(pfbaBaGUS) The wild-type RS105 harboring pfbaBaGUS, Rifr, Spr This work
RS105(pfbaBgGUS) The wild-type RS105 harboring pfbaBgGUS, Rifr, Sp' This work
RS105(pfbaBdGUS) The wild-type RS105 harboring pfbaBdGUS, Rifr, Sp' This work

Plasmids

| Strains or plasmids | Relevant characteristics* | Reference or source |
|---------------------|---------------------------|---------------------|
| pMD18-T             | pUC ori, cloning vector, Ap' | Takara             |
| pKMS1               | Suicide vector derivative from pK18mobsGII, sacB', Km' | This lab |
| pHM1                | Sp' or Sm' IncW, MobIp, Mob, LacIP', PK2 replication, cosmid | This lab |
| pKMBaB              | A 522 bp fusion cloned in pKMS1 for a 439 bp deletion in fbaB, Km' | This work |
| pCfbaB              | pHM1 expressing fbaB under its own promoter, Sp' | This work |
| pBaBaGUS            | pHM1 expressing gusA under the fbaB promoter, Sp' | This work |
| pBaBcGUS            | pHM1 expressing gusA under the site (c)-mutated promoter of fbaB, Sp' | This work |
| pBaBdGUS            | pHM1 expressing gusA under the site (d)-mutated promoter of fbaB, Sp' | This work |

*Ap' = ampicillin resistance, Km' = kanamycin resistance, Rif' = rifampicin resistance, Sp' = spectinomycin.
PCR using the total DNA of *X. oryzae* pv. *oryzicola* RS105 as the template and the primer pair fbaB-F/fbaB-R (Table S1). After being confirmed by sequencing, the amplified DNA fragment was cloned into pH1M vector at *Hind*III and *Kpn* sites to create a recombinant plasmid pCfbaB (Table 1). Plasmid pCfbaB was then transferred into the RAfbaB strain by electroporation. The transconjugants carrying pCfbaB were screened on NA plates with rifampicin and spectinomycin. A confirmed representative was verified by colony-PCR amplification for further study and named CRAfbaB (Table 1).

**HR and pathogenicity assays**

HR and pathogenicity assays were performed as described [19]. *Xanthomonas* bacteria were grown in NB liquid with appropriate antibiotics at 28°C with shaking at 200 rpm for 16 h. The bacterial inocula were washed twice and resuspended in sterile water to $1 \times 10^8$ cfu/ml and used to infiltrate into tobacco leaves (*Xanthii*) for HR detection and into rice seedlings (cv. Shanyou63, susceptible to *X. oryzae* pv. *oryzicola* infection, two-week old) for water-soaking formation with needleless syringes, respectively, and to inoculate in adult rice plants (cv. Shanyou63, two-month old) by leaf-needling for lesion length measurement. All plants were grown and maintained in a greenhouse with 12-h day-night cycle and relative humidity at 75 to 80% [54]. Plant responses were scored at 24 h for HR, in 3 days for water-soaking symptoms, and in 14 days for lesion lengths after inoculation. Five leaves were inoculated for each independent experiment, and each treatment was repeated three times.

**Determination of bacterial growth ability in planta**

and in minimal medium supplemented with different carbohydrates

*Xanthomonas* bacterial suspensions at $1 \times 10^8$ cfu/ml were infiltrated into the intercellular spaces of fully expanded leaves of rice (cv. Shanyou63, two-week old) with needleless syringes at three spots on each leaf. Three 0.8 cm diameter leaf discs were harvested with a cork borer from each infiltration area after infiltration. After being sterilized in 70% ethanol and 30% hypochlorite, the leaf discs were homogenized in 1 ml of distilled water. Diluted homogenates were plated on NA agar plates supplemented with appropriate antibiotics. The number of bacterial colonies on these plates was counted after incubation at 28°C until single colonies could be counted after 3 to 4 days. The number of bacterial CFU per square centimeter of leaf area was then estimated, and the standard deviation was calculated using colony counts from the three triplicate spots from each of the three samples per time point per inoculum. Experiments were repeated at least three times.

As to the detection of bacterial growth influenced by different carbohydrates, *Xanthomonas* bacteria were preincubated in 5 ml NB medium for 16–20 h at 28°C with shaking at 200 rpm until the $OD_{600}$ value reached 0.6, and 2% of this culture was subcultured into 20 ml of the fresh NB for 16–18 h incubation. The bacterial cells were collected and washed twice, and resuspended to an optical density of 600 nm of 0.1 in 100 ml of the minimal medium NCM supplemented with different carbon source at 0.5%. For each time point, 200 µl of each culture was removed and determined by measuring $OD_{600}$ against the medium blank. Data presented were from a representative experiment; the experiment was repeated independently three times.

**Site-directed mutagenesis in the PIP-box motifs of fbaB by PCR amplification**

There is a PIP-box sequence, TTCGT-N9-TTCGT, which is not typical to TTCGG-N15-TTCCG, following 30 base pair interval space before a -10 box-like motif CAGCAT in the fbaB promoter region. Base-substitution in the PIP-box sequence of the fbaB promoter region was performed via a PCR amplification strategy. Briefly, three substituted sequences, TTCGG-N9-TTCCG (fbaBB), TTCGG-N9-TTCCG (fbaBc) and TGATA-N9-TTCCG (fbaBd) within the fbaB promoter region were generated by using three primer pairs, fbaBa-F/fbaBb-R, fbaBa-F/fbaBc-R and fbaBa-F/fbaBd-R (Table S1), respectively, for PCR amplification with the genomic DNA of *X. oryzae* pv. *oryzicola* RS105 as the template. Then, these PCR products were cloned into pMD18-T vector and confirmed by sequencing for further study.

**Construction of the fbaB reporter plasmids**

To investigate whether the expression of fbaB is or not regulated by HrpG and HrpX, four fbaB reporter plasmids, pFbaBaGUS, pFbaBbGUS, pFbaBcGUS and pFbaBdGUS, were constructed by cloning the PIP-box promoter region and three mutated PIP-box promoters of the fbaB gene which were fused with the promoterless β-glucuronidase (gusA) gene into the broad-host-range cloning vector pHM1 (Table 1) at MCS (multiple cloning site). A 366 bp region upstream of the fbaB was amplified by PCR using the total DNA of the wild-type RS105 strain as the template and the primer pair FbafBPF/FbafBPR (Table S1). The amplified fragment of the wild-type type of promoter of fbaB, confirmed by sequencing, was fused with the promoterless gusA in the vector pHM1 at *Hind*III and EcoRI sites to create the recombinant plasmid pFbaBaGUS (Table 1). In contrast, the wild-type promoter was replaced by three site-directed substitutes in the PIP-box of the fbaB promoter, as mentioned above, and fused with the gusA gene in the vector pHM1 at *Hind*III and EcoRI sites, generating the recombinant plasmids pFbaBbGUS, pFbaBcGUS and pFbaBdGUS (Table 1). The plasmids obtained were further confirmed by restriction analysis and sequencing.

**Measurement of EPS production**

EPS production was measured as previously described by Tang [55]. In brief, *Xanthomonas* bacteria were grown in 100 ml of NY medium supplemented with 2% (w/v) various sugars at 28°C with constant shaking at 200 rpm for 3 days. EPS was precipitated from the culture supernatant with ethanol, and dried to constant weight at 55°C, and weighed. Every experiment was repeated at least three times.

**Semi-quantitative RT-PCR and Real-time quantitative RT-PCR**

The expression of tested genes, including the reporter gusA, was assayed by semi-quantitative RT-PCR or real-time quantitative RT-PCR with corresponding primer pairs (Table S1). *Xanthomonas* bacteria were preincubated in 20 ml NB medium for 16–20 h, until the $OD_{600}$ value reached 0.6, and 2% of this culture was subcultured into 20 ml of the fresh NB for 16–18 h incubation. The bacterial cells were collected and washed twice, and resuspended to an optical density of 600 nm of 2.0 by sterilized water. Then, 40 µl of bacterial suspension was inoculated into 1.5 ml of NB, NY medium or rice suspension cells incubating for 16 h at 25°C. As a template, total RNAs were extracted using the Trizol reagent (Takara, Dalian, China) according to the manufacturer’s protocol. cDNA synthesis was conducted with
AMV random primers (order no. D3801) provided by the manufacturer (Takara, Dalian, China). Before synthesis of the first-strand cDNA, total RNAs were digested with RNase-free DNase I (TaKaRa, Dalian, China) to remove potential traces of genomic DNAs. Semi-quantitative RT-PCR was performed on the ordinary PCR apparatus and the PCR program was as follows: step 1, 95°C for 3 min; step 2, 95°C for 20 s; step 3, 55°C for 30 s; step 4, 72°C for 40 s; 35 cycles from steps 2 to 4; and step 5, 72°C for 10 min. Real-time quantitative RT-PCR was performed on the Applied Biosystems 7500 real-time PCR System using SYBR Premix EX Taq™ (Takara, Dalian, China), and the PCR thermal cycle condition was as following: denature at 95°C for 30 s and 41 cycles for 95°C, 5 s; 60°C, 34 s. The expression level of the 16S rRNA gene was used as an internal standard. The comparative-threshold method was used to calculate the relative mRNA level with respect to the corresponding transcript in cells cultured in NB or NY medium or rice suspension cells. All RT-PCRs were performed in triplicate.

Results

\textit{fbaB} is required for full virulence and growth of \textit{X. oryzae pv. oryzicola} in planta

The discovery of the \textit{fbaB} gene as a virulence factor came from work aimed at the identification of genes involved in virulence alteration of \textit{X. oryzae pv. oryzicola} RS105 strain in rice. The approach was to screen a Tn5-tagged mutant library of RS105 [20] for mutants that could impair virulence of the bacterium in rice. One mutant Mxoc0504 (Table 1), where the Tn5 transposon was inserted in an open-reading frame (ORF) of \textit{Xoryp\_17640} at the 185 bp site (Figure S1), reduced virulence of \textit{X. oryzae pv. oryzicola} RS105 in rice (data not shown). The genome location and genetic organization of the Tn5-insertion in \textit{Xoryp\_17640} of \textit{X. oryzae pv. oryzicola} RS105 suggests the presence of a transcriptionally active gene \textit{Xoryp\_17640} (Figure S1). Fructose-bisphosphate aldolase is an enzyme encoded by just one gene, which is highly conserved in \textit{Xanthomonas} species (data not shown). It performs the reversible action of converting fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, which are involved in functional glycolytic and gluconeogenic pathways [1]. Thus, \textit{Xoryp\_17640} is hereafter named as \textit{fbaB}.

To facilitate the functional study of \textit{fbaB}, a nonpolar \textit{fbaB} mutant, named RA\textit{fbaB} (Table 1), was constructed by homologous suicide plasmid integration (Figure S1) (see Materials and Methods for detail). A complemented strain named CRA\textit{fbaB} was also constructed by introducing the recombinant plasmid pCfbaB, which carries the entire ORF \textit{fbaB} with a 297 bp promoter region upstream of the start codon (Table 1), into the mutant RA\textit{fbaB}.

The virulence of the \textit{fbaB} mutant RA\textit{fbaB}, the complemented strain CRA\textit{fbaB} and the wild-type strain RS105 was tested on the hybrid rice cultivar Shanyou63 by the leaf-needling inoculation method [4]. Although the mutant strain RA\textit{fbaB} still caused obvious bacterial leaf streak (BLS) symptoms, the symptoms were significantly less severe than that caused by the wild-type RS105 (Figure 1A). The mean lesion length caused by RA\textit{fbaB} was significantly reduced (P = 0.01, t test) by approximately 1.5 cm compared to the wild-type RS105, while the T3SS mutant RA\textit{hrcV}, used as a negative control, did not cause any BLS symptoms in rice (Figure 1A). The BLS lesion length caused by the complemented strain CRA\textit{fbaB} were however obviously the same as those caused by the wild-type RS105 (Figure 1B). These results demonstrated that \textit{fbaB} is required for full virulence of \textit{X. oryzae pv. oryzicola} in rice.

In order to determine whether \textit{fbaB} results in a decrease in the proliferation of \textit{X. oryzae pv. oryzicola} in the host rice, we investigated the growth capacity of the \textit{fbaB} mutant RA\textit{fbaB}, the complemented strain CRA\textit{fbaB} and the wild-type strain RS105 in planta. During the observation days, the bacterial number of the RA\textit{fbaB} mutant recovered from the infected rice leaves was significantly lower than that of the wild-type RS105 at each of the test points. The growth capacity of the RA\textit{fbaB} strain in planta was completely restored to the wild-type level by \textit{fbaB} in trans (Figure 1C), whereas the T3SS mutant RA\textit{hrcV} did not grow more in inoculated rice tissues. These results indicated that the \textit{fbaB} is required for growth of \textit{X. oryzae pv. oryzicola} in planta.

\textit{fbaB} is important in acquisition of fructose, pyruvate and malate for \textit{X. oryzae pv. oryzicola} growth

\textit{FbaB} reversibly converts fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. This prompted us to investigate whether \textit{fbaB} affects \textit{X. oryzae pv. oryzicola} growth in a non-sugar NY medium (see Materials and Methods for detail). The result showed that the \textit{fbaB} mutant RA\textit{fbaB} grew identically as the wild-type RS105 (Figure 2A),
indicating that the RAfbaB mutant was not auxotrophic. To further examine the effect of the fbaB gene on the ability of X. oryzae pv. oryzicola to utilize various carbon sources, the growth of the fbaB mutant RAfbaB, the complemented strain CRAfbaB and the wild-type RS105 were tested by using the liquid NCM (non-carbohydrate minimal medium) supplemented with glucose, sucrose, fructose, mannose, galactose, pyruvate and malate, respectively, as the sole carbon source. The growth of the RAfbaB strain grew in a similar fashion to that of the wild-type strain RS105 when supplemented with glucose, sucrose, mannose or galactose (data not shown). However, the growth of RAfbaB was significantly slower than that of the wild-type strain RS105 in liquid NCM supplemented with fructose as the sole carbon source, while the complemented strain CRAfbaB with the fbaB gene restored the growth to the wild-type level (Figure 2B), suggesting that the mutation in fbaB diminishes the capability of X. oryzae pv. oryzicola to utilize fructose.

Since pyruvate is the final product in glycolysis, and the initial carbohydrate for gluconeogenesis [1], the mutation in fbaB may presumably lead no gluconeogenesis. To seek this, we then investigated whether the mutagenesis in fbaB causes X. oryzae pv. oryzicola unable to utilize pyruvate or not. Indeed, the fbaB mutant RAfbaB was unable to grow in NCM medium supplemented with pyruvate as the sole carbon source, whereas the complemented strain CRAfbaB harboring the fbaB gene restored the ability to acquire pyruvate to the wild-type level (Figure 2C).

Pyruvate may be catalyzed by pyruvate carboxylase into oxaloacetate, or by the pyruvate dehydrogenase complex into acetyl-CoA which essentially flows into the TCA cycle. Malate is reversibly converted by malic enzyme into pyruvate for gluconeogenesis [1]. This prompted us to investigate whether or not the mutation in fbaB impairs the ability of X. oryzae pv. oryzicola to utilize malate for growth. The results showed that the fbaB mutant RAfbaB was unable to grow in NCM supplemented with malate as the sole carbon source, while the complemented strain CRAfbaB with fbaB was recovered to the wild-type level to use malate for growth (Figure 2D), implying that the conversion of malate into pyruvate can not flow through gluconeogenesis because of the mutation in fbaB.

Briefly, the above data indicate that fbaB of X. oryzae pv. oryzicola has limited influence on fructose utilization due to the presence of ED and PPP pathways when the downstream glycolysis is blocked, but plays important roles in gluconeogenesis when malate from TCA cycle is converted into pyruvate.

fbaB influences EPS production of X. oryzae pv. oryzicola

It has been demonstrated that EPS as a virulence factor plays an important role during bacterial infection [56,57] and ED is necessary for xanthan biosynthesis and PPP does not influence xanthan biosynthesis in X. oryzae pv. oryzae [6]. In order to determine whether the mutation in fbaB has any effect on EPS production of X. oryzae pv. oryzicola, the EPS yields of the fbaB mutant RAfbaB, the complemented strain CRAfbaB and the wild-type strain were quantitatively measured after the strains grew in NY liquid medium supplemented with 2% of fructose, pyruvate and malate, respectively, for 3 days. Meanwhile, NY medium and NY medium added with 2% of glucose were used as the control. After the EPS of the tested strains was extracted from the cultures (see Materials and Methods for detail), we found that there were no significant (P = 0.01, t test) difference in EPS production among

![Figure 2. Growth curves of X. oryzae pv. oryzicola in sole carbon media.](https://example.com/figure2.png)

RS105, the wild-type strain; RAfbaB, the fbaB deletion mutant; CRAfbaB, the complemented strain of RAfbaB with the fbaB gene. The initial concentration of the tested strains was adjusted to OD_{600} of 0.1 with NCM supplemented with fructose, pyruvate or malate as the sole carbon source. Aliquots were taken in triplicate at intervals of 120 h after incubation at 28°C, and bacterial growth was determined by measuring OD_{600} against the medium blank. Values given are the means ± SD of triplicate measurements from a representative experiment; similar results were obtained in two other independent experiments.

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fbaB is negatively regulated by HrpX and HrpG

Previous reports have demonstrated that the PIP-box of HrpX regulons serves as a cis-regulated element in a HrpX-dependent manner [37,38,44]. Analysis of the promoter region of fbaB of X. oryzae pv. oryzae BL256 (Figure S1) by searching the existence of similar PIP-box sequence and by using a promoter-prediction software (http://www.fruitfly.org/seq_tools/promoter.html) revealed an imperfect PIP-box (TTCGT-N_9-TTCGT) interval by 50 bp sequence with a -10 box-like motif (CAGCAT) upstream of the fbaB start codon (Figure 3A), suggesting that the expression of fbaB may be regulated by HrpX and HrpG, the latter controls the expression of gusA [34,35]. To investigate this, a real-time quantitative RT-PCR was employed to assay the action of the fbaB transcript with gusA and gusG. The fbaB relative transcript level displayed a significant increase (P = 0.01, t test) in the hrpG mutant R. oryzae pv. oryzae when fed with different carbohydrates. The expression level of fbaB was measured by real-time PCR (Figure 3C). Compared to pFbaBaGUS, pFbaBbGUS and pFbaBcGUS in the hrpG mutant R. oryzae pv. oryzae was significantly lower than that in the hrpG mutant or in the wild-type (Figure 3C). Intriguingly, the base substitution of the fifth residue in the right motif of the PIP-box (Figure 3A) significantly increased the gusA transcript of pFbaBeGUS in either R. oryzae pv. oryzae or R. oryzae pv. oryzae, compared with the wild-type (Figure 3C). The gusA expression of pFbaBeGUS in R. oryzae pv. oryzae was significantly higher (approximately 20-fold higher than that in the wild-type) than that in R. oryzae pv. oryzae (Figure 3C). The GUS activity assay also demonstrated the same results above (data not shown). The above evidence suggests that expression of fbaB is negatively regulated by HrpG and HrpX via the PIP-box promoter where a yet unknown factor might bind for the involved regulation.

Various carbohydrates have different effects on expression of hrgp, hrx, and fbaB in X. oryzae pv. oryzae

Environmental signals like carbon sources presented in plants may serve as inducers or inhibitors of virulence-associated gene expression in plant bacteria [17,21]. We sought to investigate transcript production of hrgp, hrx and fbaB when X. oryzae pv. oryzae is fed with different carbohydrates. The expression level of hrgp, hrx, and fbaB was measured by real-time PCR after the wild-type RS105 strain grew for 16 h in NY medium supplemented with 0.5% of sucrose, galactose, glucose, mannose, fructose, pyruvate and malate, respectively. Using NY medium as the control, we found that, besides malate, the other six carbon sources enhanced the expression of hrgp. Fructose, mannose, galactose, pyruvate and malate had little effect on the expression of hrx, while sucrose and glucose increased the transcript level of hrx (Figure 4). The transcript level of fbaB was increased by sucrose, galactose, glucose and fructose rather than pyruvate and malate (Figure 4). Noticeably, fructose, mannose and malate repressed the expression of hrgp, pyruvate and malate inhibited the expression of fbaB, and malate suppressed the expression of imperfect PIP-box (TTCGT-N_9-TTCGT) of fbaB are affected by Hrgp and Hrx, the fifth base of the first or second TTTG motif was substituted with a C to create TTTGC by site-direct substitutions, which generated pFbaBeGUS and pFbaBeGUS (Figure 3A), respectively. In addition, the first TTTG motif was completely changed to a TGATA motif to produce pFbaBeGUS (Figure 3A) by using site-mutagenesis primers (Table S1). The GUS reporter strains (Table 1) were incubated in rice suspension cells for 16 h. The gusA transcript level was measured by real-time PCR (Figure 3C). Compared to pFbaBaGUS, pFbaBbGUS and pFbaBcGUS in the hrpG mutant R. oryzae pv. oryzae was significantly lower than that in the hrpG mutant or in the wild-type (Figure 3C). The GUS activity assay also demonstrated the same results above (data not shown). The above evidence suggests that expression of fbaB is negatively regulated by Hrgp and Hrx via the PIP-box promoter where a yet unknown factor might bind for the involved regulation.

Table 2. EPS products in X. oryzae pv. oryzae strains.

| Strainsa | EPS yield (g/100 ml)b | NY | NY plus 2% glucose | NY plus 2% fructose | NY plus 2% pyruvate | NY plus 2% malate |
|----------|----------------------|----|--------------------|---------------------|---------------------|------------------|
| R. oryzae pv. oryzae RS105 | 0.005 ± 0.009b | 0.03 ± 0.02b | 0.01 ± 0.02b | 0.01 ± 0.02b | 0.01 ± 0.02b |
| CR. fbaB | 0.006 ± 0.006c | 0.06 ± 0.02c | 0.08 ± 0.02c | 0.10 ± 0.02c | 0.12 ± 0.02c |
| RS105/pPHM1 | 0.05 ± 0.007c | 0.005 ± 0.003c | 0.01 ± 0.003c | 0.01 ± 0.003c | 0.01 ± 0.003c |

aStrains were cultured in NY medium alone and supplemented with 2% various carbon sources. 
bData presented are the means ± standard deviations of triplicate measurements from a representative experiment, and similar results were obtained in two other independent experiments. Different letters in each data column indicate significant differences (P ≤ 0.01; t test). 
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Figure 3. The expression of fabB in X. oryzae pv. oryzicola is negatively by hrpX and hrpG in rice suspension cells. (A) Schematic map of the promoter region containing PIP-box and -10 box-like motif of fabB fused with a promoterless gusA gene. * stands for base substitutions. The constructs are listed on the right. (B) Expression analysis of fabB by real-time quantitative RT-PCR. RNAs were isolated from cultures of the wild-type RS105, the hrpG deletion mutant RΔhrpG and the hrpX deletion mutant RΔhrpX strains which were grown in NB medium and rice suspension cells for 16 h, respectively. The relative mRNAs level of fabB was calculated with respect to the level of the corresponding transcript in the wild-type RS105. (C) Effects of the mutated PIP-box on gusA transcript. The gusA transcript level by the wild-type PIP-box promoter (a) and three base-substituted PIP-box promoter (b, c, d) in the wild-type RS105, the hrpX mutant RΔhrpX and the hrpG mutant RΔhrpG were investigated, respectively. All the reporter strains above were cultured in rice suspension cells for 16 h and gusA transcript levels were then determined by real-time PCR. The transcript of gusA in the wild-type was taken as one unit. Data are the mean ± SD of triplicate measurements from a representative experiment; and similar results were obtained in two other independent experiments. The asterisks in each horizontal data column indicate significant differences at P = 0.01 by t test.

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Figure 4. Effects of different carbohydrates on the expression of hrpX, hrpG and fabB in X. oryzae pv. oryzicola. RNAs were isolated from cultures of the wild-type RS105 strain and the fabB deletion mutant RΔfabB grown in NY medium alone and NY supplemented with 0.5% of various carbohydrates for 16 h. The relative mRNAs levels of hrpX, hrpG and fabB genes were calculated by real-time quantitative RT-PCR with respect to the level of the corresponding transcript in the wild-type RS105 cultured in NY medium alone. Data presented are the means ± SD of triplicate measurements from a representative experiment; similar results were obtained in two other independent experiments.

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hrpX (Figure 4). The results above demonstrate that expression of certain genes involving the carbon metabolic pathways may be regulated by the two key hrp regulatory genes hrpG and hrpX, and in turn the expression of hrpG and hrpX may also be enhanced or repressed by the carbon sources or intermediates in the metabolic pathways.

The negative regulation of fabB with HrpG and HrpX prompted us to determine whether it is influenced by the carbohydrates tested in the previous section when fabB is mutated. Using the same real-time PCR assay, we found that the relative mRNA level of hrpX was significantly reduced in the fabB mutant than that in the wild-type RS105 strain when sucrose, mannose, galactose, pyruvate and malate were complemented in NY medium as the sole carbon sources, but increased when fructose was used. The expression of hrpG was significantly enhanced by glucose, fructose, sucrose, mannose and galactose, respectively, and repressed by pyruvate and malate (Figure 4). These results demonstrated that the mutation in fabB may alter the expression of
fbaB positively affects the expression of hrgG and hrgX, but negatively influences the transcripts of hrcC, hrgE and hpa3

The data above demonstrated that expression of the key hrg regulatory genes, hrgG and hrgX, are induced or repressed when X. oryzae pv. oryzicola evidently uses sugars from plants as nutrient sources, implying that the expression of the hrg-hrc-hpa genes, which are regulated by HrgG and HrgX as reported [20], may be altered when fbaB is mutated. To investigate this, we employed a semi-quantitative (Figure 5A) and real-time RT-PCR (Figure 5B) with the specified primer pairs (Table S1) to evaluate the transcript production of representative hrg-hrc-hpa genes in the fbaB mutant RΔfbaB and the wild-type RS105 strain after incubation in rice suspension cells for 16 h, while the nutrient-rich medium NB was used as a synchronous control (data not shown). The results showed that: i) the expression of hrgG and hrgX in the fbaB mutant RΔfbaB was significantly (P = 0.01, t test) lower than that in the wild-type, implying that the dysfunction in glycolysis and gluconeogenesis by the mutation in fbaB represses the transcript of hrgG and hrgX; ii) the mRNA level of the tested genes, hrgD5, hrgD6, hpa1, hpa2, hrgE, and hpa3, in RΔfbaB was similar to that in the wild-type; iii) the transcriptional level of hrcC, hrgE and hpa3 (which was previously reported that their expression was not completely controlled by HrgG and HrgX in X. oryzae [24,31]) in RΔfbaB was significantly higher than that in the wild-type (Figure 5), indicating that certain intermediates in glycolysis and gluconeogenesis derived from the aldol reaction [1] may influence the expression of hrcC, hrgE and hpa3. The above data suggest that the mutation in fbaB may alter the ability of X. oryzae pv. oryzicola to acquire carbon from its living niche which in turn represses the expression of hrgG and hrgX.

hrcC, hrgE and hpa3 are required in utilization of pyruvate and malate for X. oryzae pv. oryzicola

To verify our hypothesis that the hrcC, hrgE and hpa3 genes are involved in acquisition of pyruvate and malate, we tested the growth of the hrcC, hrgE and hpa3 mutants, RAhrcC, RAhrgE, and RAhpa3 (Table 1), respectively, in NCM medium supplemented with sucrose, mannose, galactose, glucose, fructose, pyruvate and malate as the sole carbon sources, while the wild-type strain RS105 was used as the control. Indeed, the mutation in hrcC, hrgE or hpa3 reduced the growth of the pathogen when pyruvate and malate were used as the sole carbon source (Figure 6). By contrast, the growth of the hrgE mutant was affected much more than that of the hrcC or the hpa3 mutants by pyruvate and malate, respectively (Figure 6). These data suggests possible reasons why the expression of hrcC, hrgE and hpa3 was higher than other hrg-hrc-hpa genes when fbaB was mutated (Figure 5).

Discussion

In this study, we identified in X. oryzae pv. oryzicola RS105 strain a novel and unique virulence gene, fbaB, which encodes a fructose-bisphosphate aldolase (FbaB), highly conserved in other Xanthomonads, and converts the intermediate fructose-1,6-bisphosphate to reversible dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, which is essential for glycolysis and gluconeogenesis (Figure 7), and now has been shown to play a role in virulence. Genetic evidence presented here demonstrates that fbaB is required for X. oryzae pv. oryzicola to utilize fructose, pyruvate and malate so that the pathogen produces EPS and expands full virulence and growth in planta for adaptation. The mutation in fbaB does not make the pathogen auxotrophic and lethal. Interestingly, the expression of fbaB is negatively regulated by the HrgG and HrgX cascade via the imperfect PIP-box of fbaB possibly with an unknown regulator. The latter may presumably regulate the expression of hrcC, hrgE, and hpa3 and be influenced by the accumulation of pyruvate for the initiation of gluconeogenesis and malate from the TCA cycle (Figure 7). Intriguingly, the PIP-box spaced, by 30 base pairs with a -10 box-like motif is also highly conserved within the genome sequences of X. oryzae pv. oryzicola PXO99a [9], KACC10331 [8], X. campestris pv. vesicatoria 85-10 strain [12], X. campestris pv. campestris 8004 [10], and X. axonopodis pv. citri 306 [11] (data not shown). This implies that the expression of fbaB homologues in other Xanthomonas species may be regulated by the same manner. However, whether fbaB of other Xanthomonas species plays a similar role as above in host-pathogen interactions needs to be further investigated.

X. oryzae pv. oryzicola is a nonvascular pathogen that enters through leaf stomata or wounds, and propagates and spreads in the intercellular spaces and the parenchyma apoplast to cause BLS in rice [19]. To reach the cell density for pathogenesis in plants, the pathogen has to be able to adapt to intercellular environments and also utilize available nutritional sources, especially carbohy-
given are the means of triplicate measurements from a representative result of other two similar independent experiments.

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Figure 6. The mutation in hrcC, hrpE and hpa3 reduced the ability of X. oryzae pv. oryzicola to acquire pyruvate and malate. RS105, the wild-type strain; RΔhrcC, the hrcC deletion mutant; RΔhrpE, the hrpE deletion mutant; RΔhpa3, the hpa3 deletion mutant. The initial concentration of the tested strains was adjusted to OD600 of 0.05 with NCM supplemented with pyruvate or malate as the sole carbon source. Aliquots were taken in triplicate at intervals of 120 h after incubation at 28 °C, and bacterial growth was determined by measuring OD600 against the medium blank. Values given are the means ± SD of triplicate measurements from a representative result of other two similar independent experiments.

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Figure 7. Working model of FbaB coordinating with hrp genes of X. oryzae pv. oryzicola in carbon metabolic pathways. The lined arrows from the carbohydrates in dashed-line boxes or the double lined arrows from the intermediates indicate carbon flows in glycolysis, gluconeogenesis, pentose phosphate pathway (PPP), entner–doudoroff (ED) and tricarboxylic acid (TCA) cycle pathways, respectively. The grey box displays fbaB encodes a fructose-bisphophate aldolase that converts vertically fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. The mutation in fbaB has no influence on ED and PPP pathways, but impairs glycolysis of the pathogen to use fructose and block gluconeogenesis to use pyruvate and malate. The expression of an unknown regulator in a cycled question mark may be enhanced by galactose, glucose, mannose, sucrose, fructose and pyruvate (as shown by dash-lined arrow) and repressed by malate (a dash-lined arrow with a stop bar). The unknown factor may differentially regulate the expression of hrpG or/and hrpX which down-regulate the expression of fbaB (a dash-lined arrow with a stop bar). The unknown regulator may also control the transcripts of hrcC, hrpE and hpa3 (other than other hrp-hrc-hpa genes) which are not completely regulated by HrpG and HrpX [20]. Being the components of the T3SS apparatus, HrcC, HrpE and Hpa3 may facilitate X. oryzae pv. oryzicola to utilize the intermediates, like pyruvate and malate, of the TCA cycle from plants.

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photosynthesis induce the expression of virulence factors, which presumably plays a central role in regulation between the plant pathogen and the host. Thus, the disruption in carbon metabolic flux can be balanced by the intermediates from the TCA cycle of the host and the pathogen. In X. oryzae pv. oryzae, the expression of global regulator genes, including HrpG and HrpX which enables the pathogen to respond to environmental factors (such as pH and osmotic strength), plant signals (such as carbon sources, organic nitrogen, and phosphate), and catabolite repression that may be encountered during the infection [17,18,21,25,60]. This regulation is very complex and varies substantially between different Xanthomonas plant pathosystems and in some cases even between closely related bacteria within the same pathosystem [61]. In this study, we found that different carbohydrates have different influences on expression of hppDET and hppX. Sucrose, galactose, mannose, glucose, fructose and pyruvate significantly increase the expression of hppX in X. oryzae pv. oryzae when they used as the sole carbon source, while sucrose and glucose remarkably enhance the expression of hppX (Figure 4). By contrast, the hppX expression goes up while the hppG expression decreases obviously when the pathogen grows on these carbon sources, respectively, and malate represses the expression of hppG and hppX (Figure 4), suggesting that other unknown factors are possibly involved differentially in regulation of the expression of hppG and hppX. This postulation conflicts with the concept that HrpG function as a positive activator upstream of HrpX in regulatory pathways of hpp gene expression. In fact, the expression of hnrT goes up when hppG is mutated and there is no hnrT expression detected when hppX is mutated in X. oryzae pv. oryzae [20], supporting the above hypothesis.

The above findings also theoretically support the development of hpp-inducing media, XCV2 for X. campesiris pv. veicatoria [29], XOM2 for X. oryzae pv. oryzae [61,62] and XOM3 for X. oryzae pv. oryzae [25]. The major carbon source in plant leaf extract is sucrose, followed by glucose and fructose, and the dicarboxylic acid, malate, and the latter induces the secretion of extracellular enzymes and has a negative effect on the expression of the TSSs in X. campesiris pv. campesiris [63]. In P. syringae pv. phaseolicola, the expression of hppAB, hppC, and hppD was reduced when citrate or succinate was added to fructose- or sucrose-containing medium [17]. Taken together, we assumed that hexoses from plant photosynthesis induce the expression of hpp genes and this action can be balanced by the intermediates from the TCA cycle of the plant pathogen. Thus, the disruption in carbon metabolic pathways reduces bacterial virulence in plants through alteration of the expression of global regulator genes, including hppG and hppX, in plant pathogenic bacteria. As we observed, the mutation in fabB of X. oryzae pv. oryzae leads the dysfunction of gluconogenesis and the accumulation of intermediates, like malate, from the TCA cycle represses the expression of hppG and hppX (Figure 4 and 5B), enhances the bacterium to use the TCA intermediates by the help of HrcC, HrpE and Hpa3 (Figure 5B, 6 and 7), and may also affect the ability of the organism to obtain nutrients from the environment.

The interesting finding in this report is that the promoter region of fabB of X. oryzae pv. oryzae assembles the cis-element of PIP-box which is taken as the sequence of HrpX regulons (Figure 3A). The highly conserved PIP-box of the fabB homologue in other typical Xanthomonas species (data not shown) suggests that the expression of fabB may commonly be negatively regulated by HrpG and HrpX. Protein secretion assays demonstrated that FabB is not secreted through the T2SS and T3SS (data not shown). In fact, the expression level of fabB in the hppG mutant is higher than than in the hppX mutant (Figure 3B), suggesting yet unknown factor(s) may strongly regulate the expression of HrpX than HrpG. This is consistent with the following findings. The base substitution in the fifth residue of the left motif TCTCG of the PIP-box significantly reduced the expression of fabB when hppG was mutated rather than in the hppX mutant (Figure 3C). However, the substitution in the right motif TCTCG led the expression of fabB to be 15–20 fold higher than the wild-type promoter when hppG and hppX are disrupted, respectively and the promoter activity in the hppX mutant is significantly higher than that in the hppG mutant (Figure 3C), implying that the alteration of the binding sites of the fabB PIP-box promoter makes the expression of fabB released from the regulation of HrpG and HrpX together with a yet unknown regulator (Figure 7). This unknown regulator may activate the expression of fabB which is also regulated by the HrpG and HrpX cascade (Figure 7), or regulate the expression of HrpG by phosphorylation as speculated in R. solanacearum [64]. Unfortunately, our electrophoretic mobility shift assay (EMSA) showed that HrpX did not bind the fabB promoter (data not shown), suggesting that HrpX, a transcriptional activator, may form a complex with a yet unknown factor to regulate the expression of fabB. The expression of this unknown factor may be inhibited by intermediates, like malate, from the TCA cycle in X. oryzae pv. oryzae, resulting in the lower expression of HrpG and HrpX when the block of gluconogenesis is made by the fabB mutation (Figure 3, 4, 5 and 6).

In addition, the expression of hrcC, hrpE and hpa3, other than other hpp-hrc-hpa genes, of X. oryzae pv. oryzae is still activated when fabB is mutated. Previously, we found that the mutation of hppG and hppX does not abolish the expression of hrcC, hppE and hpa3 and postulated that a yet unknown factor may influence the expression of these genes [20,53]. This speculation is in accordance with our hypothesis in this report that the hexoses from plant photosynthesis induce the expression of virulence-related genes, including hpp and fabB genes, and the intermediates, like malate, from the TCA cycle of X. oryzae pv. oryzae repress the expression of the unknown factor gene that will in turn, directly or indirectly, suppress the expression of HrpG and HrpX and increase the expression of hrcC, hppE and hpa3 which is involved in nutrient acquisition of pyruvate and malate when X. oryzae pv. oryzae contacts the host cells (Figure 5 and 6). Importantly, this report may also provide clues to investigate a yet unknown factor which presumably plays a central role in regulation between carbohydrate metabolism and the hpp system of X. oryzae pv. oryzae mediated by pyruvate and malate (Figure 7).

Supporting Information

Figure S1 Schematic map and molecular analysis of fabB mutation in X. oryzae pv. oryzae. The positions and orientations of Xoryp_17640, encoding FabB, and other adjacent ORFs are shown by using the genome sequence of X. oryzae pv. oryzae BLS256 strain as the reference (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org = Xoc). Arrows indicate the intergenic sequences.

Figure S1

Figure S1 Schematic map and molecular analysis of fabB mutation in X. oryzae pv. oryzae. The positions and orientations of Xoryp_17640, encoding FabB, and other adjacent ORFs are shown by using the genome sequence of X. oryzae pv. oryzae BLS256 strain as the reference (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org = Xoc). Arrows indicate the intergenic sequences.
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Author Contributions

Conceived and designed the experiments: CY GC. Performed the experiments: WG YC ZJYL. Analyzed the data: WH LC HZ. Contributed reagents/materials/analysis tools: LZ. Wrote the paper: WG WH CY GC.
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