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yggS Encoding Pyridoxal 5′-Phosphate Binding Protein Is Required for *Acidovorax citrulli* Virulence

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Bacterial fruit blotch, caused by seed-borne pathogen *Acidovorax citrulli*, poses a serious threat to the production of cucurbits globally. Although the disease can cause substantial economic losses, limited information is available about the molecular mechanisms of virulence. This study identified that, a random transposon insertion mutant impaired in the ability to elicit a hypersensitive response on tobacco. The disrupted gene in this mutant was determined to be *Aave_0638*, which is predicted to encode a YggS family pyridoxal phosphate-dependent enzyme. YggS is a highly conserved protein among multiple organisms, and is responsible for maintaining the homeostasis of pyridoxal 5′-phosphate and amino acids in cells. *yggS* deletion mutant of *A. citrulli* strain XjL12 displayed attenuated virulence, delayed hypersensitive response, less tolerance to H_2O_2 and pyridoxine, increased sensitivity to antibiotic β-chloro-D-alanine, and reduced swimming. In addition, RNA-Seq analysis demonstrated that *yggS* was involved in regulating the expression of certain pathogenicity-associated genes related to secretion, motility, quorum sensing and oxidative stress response. Importantly, YggS significantly affected type III secretion system and its effectors *in vitro*. Collectively, our results suggest that YggS is indispensable for *A.citrulli* virulence and expands the role of YggS in the biological processes.

Keywords: *Aicdovorax citrulli*, virulence, YggS, type III secretion (T3S), biological processes

INTRODUCTION

Bacterial fruit blotch (BFB), a seed-borne disease caused by *Acidovorax citrulli*, has caused substantial economic losses to the global cucurbit industry (especially melon and watermelon) (Schaad et al., 1978, 2008; Willems et al., 1992). Since the first report in the United States (Webb and Goth, 1965), BFB has spread worldwide mainly through the international movement of contaminated seeds, which are the main inoculum sources for BFB outbreak (Hopkins and Thompson, 2002; Burdman and Walcott, 2012). However, to date, effective management strategies including watermelon cultivars with significant level resistance to BFB are not commercially

available (Bahar et al., 2009a; Burdman and Walcott, 2012; Ge et al., 2021). Despite the fact that BFB is a serious threat to cucurbit crops production, the mechanism of pathogenicity of *A. citrulli* is largely unknown. Therefore, it is critical to elucidate the pathogenicity factors of *A. citrulli* at the molecular level in order to develop effective BFB management strategies.

The availability of a complete genome sequence of *A. citrulli* strain AAC00-1 (GenBank NC_008752) greatly enhances the investigation of pathogenesis. So far, several diverse virulence-related factors have been characterized for this phytobacterium. Protein secretion systems and motility mediated by pili and flagella are indispensable for phytobacterial pathogenicity (Mattick, 2002; Chaban et al., 2015; Pfeilmeier et al., 2016) and *A. citrulli* is no exception. Previous studies have confirmed that disruption of the T3SS abolished the pathogenicity of *A. citrulli* (Ren et al., 2009; Johnson et al., 2011; Liu et al., 2012; Zhang et al., 2018). Type III-secreted effectors (T3Es) delivered into host cells *via* T3SS promote the invasion of pathogens through interference of the cell metabolisms and/or suppression of the host immune responses (Feng and Zhou, 2012; Macho and Zipfel, 2015). The annotation of genome of AAC00-1 indicates that there are at least 11 T3Es genes in this phytobacterium (Eckshtain-Levi et al., 2014). Recently, the discovery of a wide arsenal of T3Es placed *A. citrulli* among the "richest" bacteria in terms of T3E cargo (Jiménez-Guerrero et al., 2020). More than 50 T3Es sharing similarity with known T3Es from other pathogenic bacteria were revealed in *A. citrulli* strain M6 by computational approach, and seven new putative T3Es were further validated as real effectors through T3SS-dependent translocation assay (Jiménez-Guerrero et al., 2020). Two effectors, AopN and AopP, were confirmed to inhibit plant immunity by interacting with ClHIPP, ClLTP and ClWRKY6 in watermelon, respectively (Zhang et al., 2020a,b). In addition, type II and VI secretion systems were reported to play key roles in *A. citrulli* virulence (Johnson et al., 2009; Tian et al., 2015). Polar flagellum and type IV pili (TFP) of *A. citrulli* are required for motility, colonization ability and virulence, and the lack of ability to synthesize TFP causes phenotypic variation in *A. citrulli* (Bahar et al., 2009b, 2011; Rosenberg et al., 2018). In addition to the above-mentioned classical apparatus related to bacterial pathogenicity, other factors such as ferric uptake regulator (FurA) (Liu et al., 2019), quorum sensing (QS) (Wang et al., 2016), and bifunctional chorismate mutase/prephenate dehydratase (Cmp) (Kim et al., 2020), have been reported to contribute to *A. citrulli* virulence. These advances have improved our understanding of the complex pathogenic mechanisms.

YggS is a member of a conserved COG0325 protein family of PLP (pyridoxal 5′-phosphate)-binding proteins and widely present and highly conversed in various organisms (Ito et al., 2013; Darin et al., 2016; Labella et al., 2017). Although this protein family is similar to bacterial alanine racemase and eukaryotic ornithine decarboxylase in structure, no enzymatic activity was detected (Eswaramoorthy et al., 2003; Ito et al., 2013; Tremino et al., 2017). Several studies have shown that YggS is responsible for maintaining the homeostasis of PLP, a biologically active form of vitamin B_6 and an essential cofactor in various kinds of enzymes (Prunetti et al., 2016; Ito et al., 2019). The lack of YggS or its orthologs in this protein family exhibit pleiotropic phenotypes in multiple organisms by unknown mechanisms. In *E. coli*, the absence of *yggS* leads to perturbations in levels of amino acid metabolic and α-ketobutyrate (Ito et al., 2013, 2016, 2019), the accumulation of the PLP precursor pyridoxine 5′-phosphate (PNP) and the sensitivity to pyridoxine (PN), which can be suppressed by pyridoxal (PL) (Prunetti et al., 2016). PipY, a homolog of YggS, was studied in *Synechococcus elongates*, and the *pipY* mutant was more susceptible to PN, but also to the antibiotics D-cycloserine (DCS) and β-chloro-D-alanine (BCDA), both targeting key PLP-holoenzymes. The addition of D-alanine or L-alanine was shown to rescue the susceptibility to both antibiotics (Labella et al., 2017; Tremino et al., 2017). Recently, a study on *Salmonella enterica* lacking YggS suggested that other than the accumulation of endogenous PNP, approximately 10-fold more PLP were detected in growth medium as compared to the wild-type strain (Vu et al., 2020). In addition, in humans, vitamin B_6-dependent epilepsy is attributed to the variation in *PLBP* (formerly called *PROSC*), a homolog of *yggS*. This mutant alters the level of vitamin B6 and neurotransmitters (Darin et al., 2016; Johnstone et al., 2019). Despite the important biological function of YggS in organisms, the role of YggS associated with biochemistry or virulence has not been investigated in *A. citrulli*.

The current study was initiated to identify novel factors related to *A. citrulli* virulence. By screening transposon (Tn5)-insertion library, we obtained a mutant strain of *A. citrulli* with an altered HR phenotype in tobacco and reduced virulence to melon compared to the wild-type strain. The gene disrupted by transposon insertion mutation was identified as *Aave_0638* that encoded a YggS family pyridoxal phosphate-dependent enzyme (YggS). *Aave_0638* was homologous with YggS in *E. coli* by BLASTp analysis in National Center for Biotechnology Information (NCBI). The data indicates that YggS was involved in oxidative stress response, motility, the sensitivity to PN and BCDA and the regulation of T3SS in *A. citrulli*. The RNA-Seq revealed that the absence of YggS had a greater impact on T3SS and its effectors *in vitro*. In the current study, we uncovered the first insights into the role of *yggS* in virulence of *A. citrulli*.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Plant Material

Bacterial strains and plasmids used in this study are listed in **Supplementary Table 1**. Bacteria were cultured in Luria-Bertani (LB) agar or broth medium (Sambrook et al., 1989). *Acidovorax citrulli* and *Escherichia coli* were cultured at 28 and 37°C, respectively. When required, the appropriate dose of antibiotics were added to media at the following final concentrations: rifamycin (Rif) 100 μg/mL, kanamycin (Km) 50 μg/mL, gentamicin (Gm) 50 μg/mL, chloramphenicol (Cm) 20 μg/mL and ampicillin (Amp), 100 μg/mL. The turbidity of the cell suspensions was measured by optical density at 600 nm using a spectrophotometer (Thermo Scientific, Waltham, MA, United States). For pathogenicity

assays, melon (cv. Huanghou) seeds were planted in soil mixed with 50% vermiculite and grown under standard greenhouse conditions including 24°C and 12 h of natural light until inoculated. The inoculated seedlings were incubated in growth chamber with 28°C, 85% relative humidity (RH) and 12 h of fluorescent light.

Construction of a Transposon-Insertion Library of *A. citrulli* XjL12 and Identification of Disrupted Genes

To obtain a high efficiency, random insertion mutant library, triparental mating was conducted. In brief, cells of wild-type *A. citrulli* strain XjL12, and *E. coli* containing pUTKm and pRK600, respectively, were mixed and cultured on nitrocellulose membrane (NCM) on an LB agar plate. After 48 h, the lawn was harvested, washed and plated on LB agar supplemented with Rif and Km. The resulting mutants were confirmed by *A. citrulli*-specific primers WFB1/ WFB2 (Walcott and Gitaitis, 2000) and KMF/KMR for the Tn5 transposon. Mutants were grown overnight and bacterial cell suspensions were adjusted to OD_{600} = 0.3 (3×10^8 CFU/mL). Approximately 10 μL of each cell suspension was infiltrated into tobacco leaves to test for HR induction. The inoculated tobacco leaves were grown at 28°C and observed per 8 h.

To identify the disrupted gene of the Tn5 insertion mutant of *A. citrulli*, a plasmid rescue method was employed as described previously (Bahar et al., 2009b) with some modifications. Briefly, the genomic DNA of the *A. citrulli* mutant was extracted and digested with *Pst*I restriction enzyme that is unable to digest the Tn5 region, but is able to excise the genome into fragments. The digestion products were ligated into pUC19 digested with the same restriction enzyme, and the recombinant vector was introduced into DH5α. Due to the existence of a kanamycin (KM) cassette in Tn5, the flanking regions of Tn5 were identified by backward sequencing using the specific primers Tn5-F/Tn5-R for KM cassette. The sequence was then blast searched against the *A. citrulli* AAC00-1 genome in NCBI using Blastn. All primer sequences used in this study are listed in **Supplementary Table 2**.

Construction of a Δ*yggS* Mutant and Complemented Strains of *Acidovorax citrulli*

The Δ*yggS* mutant of *A. citrulli* was generated through homologous recombination as described previously (Johnson et al., 2011). The *yggS* gene (locus tag: *Aave_0638*) is located in region 688,056 to 688,781 in the *A. citrulli* AAC00-1 genome (GenBank NC_008752). The upstream and downstream fragments of *yggS* were amplified using primer pairs (*yggS*-upF/*yggS*-upR, *yggS*-downF/*yggS*-downR) designed using Primer3 online[1], and then digested with restriction enzymes (*Hin*dIII and *Xba*I, *Bam*HI and *Kpn*I, respectively).The Km fragment was cloned from pET30 using Km cassette primer pair Km-F/Km-R and digested with *Xba*I and *Bam*HI simultaneously. Three fragments were ligated with suicide vector pEX18 (digested with *Hin*dIII and *Kpn*I), and the recombinant vector pEX18*yggS*Km was introduced into *E. coli* BW20676 for biparental mating with wild-type XjL12. The resulting *A. citrulli* mutant Δ*yggS* was confirmed by PCR assay using primers *yggS*-upF/*yggS*-downR.

To construct complemented strains, the *yggS* expression vector was first constructed using pBBR1-MCS-5. The *yggS* promoter was predicted using the online promoter prediction program[2]. The sequence containing the promoter and open reading frame of *yggS*, was generated using primers *yggS*-F/*yggS*-R and digested with *Kpn*I and *BamH*I and then ligated with pBBR-MCS-5 digested with the same enzymes. Afterwards, the recombinant vector pBBR-*yggS* verified by Sanger sequencing was introduced into *E. coli* BW20676 for biparental mating with the *A. citrulli* mutant Δ*yggS*. All primer sequences used in this assay are listed in **Supplementary Table 2**.

In vitro Bacterial Growth Assays

Acidovorax citrulli growth was measured in LB broth. After culturing overnight, the strains were harvested and adjusted to an OD_{600} of 0.1 with sterilized water. The bacterial suspensions were added to fresh LB broth at 1:100 (vol/vol) and then incubated at 28°C with shaking at 220 rpm. A growth curve was investigated by measuring bacterial cell turbidity using a spectrophotometer (BioPhotometer, Eppendorf) at OD_{600} at 2 h intervals until the cultures reached the plateau phase. In this assay, three biological replicates were performed and the experiment was repeated three times independently.

Biofilm Formation

The ability of biofilm formation of *A. citrulli* was measured as described previously (Bahar et al., 2009b). Briefly, all strains cultured overnight were harvested and washed twice with sterilized water. Forty microliters of each bacterial suspension at OD_{600} of 1.0 were added to 4 mL LB broth, in 12-well polyvinyl chloride (PVC) plates and incubated at 28°C. After 48 h, the cell suspension in each well was removed and the plate was dried at 80°C for 20 min. The biofilms attached to the plate well walls were stained with 1% methyl violet for 50 min and then solubilized in absolute ethyl alcohol. The biofilms were quantified by measuring solutions at OD_{590} with a Microplate Reader (Synergy H1, Biotek). Three replicates for each strain were performed per experiment and the experiment was repeated three times.

Bacterial Motility Assay

Swimming assay was conducted as a previous protocol (Wang et al., 2016) with some modifications. For the swimming assay, 5 μL of cell suspensions at OD_{600} of 0.3 (3×10^8 CFU/mL) were deposited in the center of oligotrophic medium containing 0.3% agar and incubated at 28°C. The diameters of three colonies of each strain were measured after 48 h. Each strain was tested three times per experiment and this experiment was conducted three times.

[1]https://bioinfo.ut.ee/primer3-0.4.0/

[2]http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb

Hypersensitive Response Assay

To determine the ability of *A. citrulli* to induce hypersensitive response (HR), cell suspensions were injected into the leaves of *Nicotiana tabacum* as described previously (Liu et al., 2019). In brief, *A. citrulli* strains grown in LB overnight were washed with sterilized water and adjusted to OD_{600} = 0.3. About 10 μL of cell suspensions were syringe-infiltrated into the leaves of *N. tabacum* grown at 28°C and HR was observed after 12 h. Three leaves at the same leaf position on the different stem were inoculated by tested strains. The experiment was conducted three times.

H_2O_2, Antibiotic and Pyridoxine (PN) Susceptibility Assays

To evaluate *A. citrulli* sensitivity to some chemical compounds, the inhibition zone method was used. For sensitivity to H_2O_2 and antibiotics, 1 mL of cell suspensions at OD_{600} of 0.3 were added to 50 mL LB agar medium and poured into sterilized petri dishes. A sterilized paper disk, approximately 4 mm in diameter, was placed in the center of each plate containing bacteria. Ten microliters of H_2O_2 (5% and 10%) or antibiotics were dropped on the disk. After incubating at 28°C for 48 h, the diameters of inhibition zones were measured. Similarly, sensitivity to 1 M PN was tested as described above except for the concentration of cell suspension. To make the inhibition zone more easily visible, cell suspension added to LB agar medium was at an OD_{600} = 0.1. In this assay, the final concentrations of tested antibiotics were as follows (antibiotic/concentration in mg/mL): ampicillin/10, β-chloro-D-alanine (BCDA)/100, chloramphenicol/4, D-cycloserine (DCS)/10, gentamicin/12.5, spectinomycin /50, tetracycline/5. Each strain was tested three times per experiment and the experiment was conducted three times.

Virulence Assays

In order to evaluate the effect of *yggS* on *A. citrulli* virulence, three inoculation methods were performed as described previously (Liu et al., 2019).

i. Cotyledon infiltration assay: Each *A. citrulli* suspension (approximately 1×10^4 CFU/mL) was injected into cotyledons of five one-week-old melon (cv. Huanghou) seedlings and the plants were incubated in a growth chamber at 28°C, 85% RH and exposed to 12 h of fluorescent light daily. BFB symptoms were observed at 3, 5, and 7 days postinoculation (dpi).
ii. Seed-to-seedling transmission assay: Germinating melon (cv. Huanghou) seeds were immersed in 2 mL of each cell suspension diluted to 1×10^8 CFU/mL until the seeds were air-dried at room temperature. Twenty seeds inoculated with each strain were planted in one cup and incubated in growth chamber with 28°C, 85% RH and 12 h of fluorescent light. After one week, seedlings were visually observed.
iii. Seedling spray inoculation: When the second euphylla of melon (cv. Huanghou) seedlings fully emerged (about 3 weeks old), seedlings were inoculated with cell suspension at OD_{600} of 0.3 by spraying. About 50 mL cell suspension of each strain was spray-inoculated evenly onto twenty seedlings per experiment. Seedlings were incubated at 100% RH for two days and then at 85% RH. After one week, the euphylla were observed for BFB symptoms. Each seedling was evaluated for BFB severity based on disease index (DI) as described previously (Araújo et al., 2005), with modifications. Briefly, disease severity scale ranged from 0 to 5: 0 for no symptoms; 1, 2, 3, 4 for necrotic lesions on approximately 25, 50, 75, 100% of the leaves, respectively; 5 for complete death of seedling. The DI was calculated based on the formula: $DI = \Sigma(A \times B) \times 100/\Sigma B \times 5$ (where A: disease class (0, 1, 2, 3, 4, 5); B: the number of seedlings in the corresponding disease class). This experiment was conducted three times.

Bacterial Colonization of Melon Cotyledons and Seeds Assay

A previous established protocol (Tian et al., 2015) was used to assess the role of *yggS* in *A. citrulli* colonization of melon seedlings with slight modification. For cotyledon colonization assay, cell suspensions of each *A. citrulli* strain (approximately 1×10^3 CFU/mL) were injected into at least twenty-five cotyledons of melon (cv. Huanghou) seedlings per experiment. Five 5-mm disks were collected from cotyledons injected with each strain at 0, 1, 2, 3, 4 dpi and triturated in 1 mL of buffer in the sterilized 1.5-mL centrifuge tubes. Homogenate was 10-fold serially diluted with sterile water and 100 μL of homogenate was spread on LB plates with appropriate antibiotics. Resulting *A. citrulli* colonies were counted after two days. For seed colonization assays, the seeds (cv. Huanghou) in the assay were disinfected with 5% H_2O_2 for 20 min before germination to prevent microbe contamination. The front end of the germinating seeds were opened gently and five microliters of cell suspensions (approximately 1×10^5 CFU/mL) were inoculated into the melon seeds. Each strain was inoculated into at least thirty-five melon seeds per experiment. The inoculated seeds were placed on moist filter paper. Five seeds inoculated with each strain were collected at 0, 1, 2, 3, 4, 5, 6, 7 dpi, and each seed was shaken for 10 min in a sterilized 2-mL centrifuge tube containing 1 mL sterilized water. Seed homogenate was 10-fold serially diluted with sterilized water and 100 μL was spread on LB plates with appropriate antibiotics.

Bacterial colonization was quantified by calculating the area under population dynamics curve (AUPDC) as follows: $AUPDC = \sum_{i=1}^{n} [(Y_i + Y_{i+1})/2] \times (X_{i+1} - X_i)$ (Bjarko and Line, 1988). (X_i: the value of horizontal coordinates at *i*th observation; Y_i : the value of vertical coordinates at the *i*th observation; n: the total number of observation). This assay was repeated three times.

Transcriptome Sequencing and Data Analysis

To determine the regulatory mechanism of YggS in *A. citrulli*, RNA-Seq was conducted commercially by Beijing Allwegene Technology Company Limited (Beijing, China). Briefly, total RNA were extracted from *A. citrulli* strains that were cultured

in LB broth to OD_{600} = 1.0 using the TRIzol method (TIANGEN BIOTECH, Bejing). RNA was quantified by Agilent 2100 (Agilent Technologies, CA, USA), and the quality and integrity were detected by NanoDrop spectrophotometer (IMPLEN, CA, United States). Ribosomal RNA (rRNA) was removed from qualified RNA sample using Vazyme Ribo-off rRNA depletion kit (Bacteria) (Vazyme biotech, United States). Subsequently, the sequencing libraries were generated using NEBNext ULtraTM RNA library Prep Kit (NEB, United States). Library quality was assessed on the Agilent Bioanalyzer 2100 system. The qualified library was sequenced by Illumina Hiseq 4000 platform. The reads cleaned by Trimmomatic V0.33 were mapped to *A. citrulli* AAC00-1 genome (GenBank NC_008752) by Bowtie2 V2.2.6. Resulting data were subjected to DESeq R package (1.10.1) for analyzing differential expression genes between mutant and wild-type strain. Genes with an adjusted *P*-value < 0.05 found by DESeq were assigned as differentially expressed. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed by GOSeq and KOBAS, respectively. GO term and KEGG pathway with corrected *P* value < 0.05 were defined as significantly

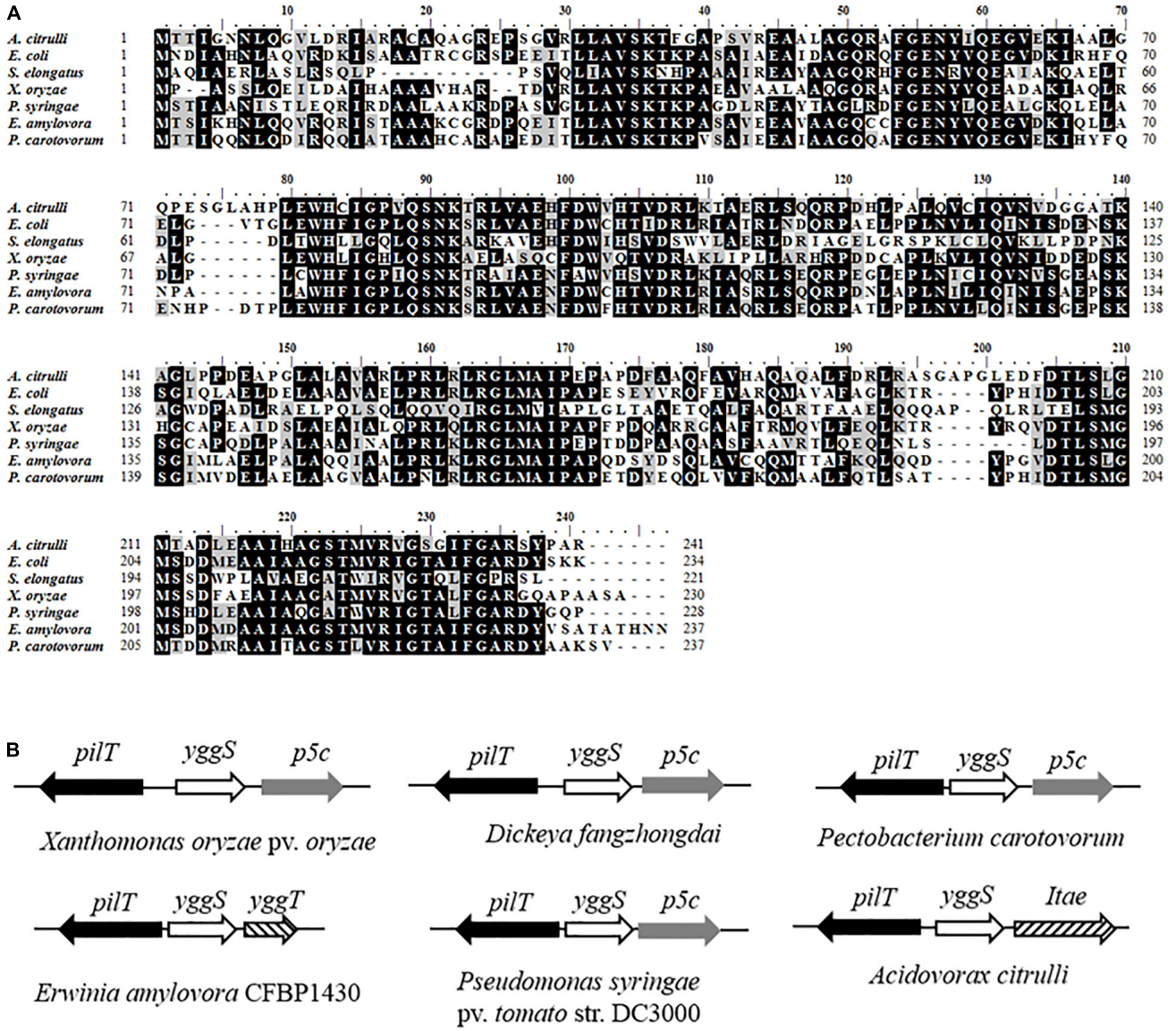

FIGURE 1 | Amino acid sequences of YggS and physical clustering of *yggS* genes. **(A)** Multiple alignments of YggS amino acid sequences for *Acidovorax citrulli* and other important bacteria including *Escherichia coli* str. K-12 substr. MG1655, *Synechococcus elongates* PCC7942, *Pseudomonas syringae* pv. *tomato* str. DC3000, *Xanthomonas oryzae* pv. *oryzae* PXO99A, *Dickeya fangzhongdai* strain DSM 101947, *Erwinia amylovora* CFBP1430 and *Pectobacterium carotovorum* PC1. The analysis was performed by BioEdite V7.0.5. Among these sequences, amino acids marked with black are conserved, and marked with gray are highly homologous. **(B)** Physical clustering of *yggS* genes in phytopathogenic bacteria. The direction of the arrow is consistent with the direction of the gene transcription.

enriched by the DEGs. Each strain was analyzed in three biological repetitions.

Quantitative Real-Time PCR Analysis

To validate the result of RNA-Seq, quantitative real-time PCR analysis (qPCR) was conducted. The culture condition for bacteria growth was the same as described in the transcriptome sequencing and data analysis. Total *A. citrulli* RNA was extracted from bacteria using the bacterial RNA kit (OMEGA), and the concentration of RNA was measured by spectrophotometer (Nanodrop One, Thermo Scientific). cDNA was synthesized and purified using HiScript III RT SuperMix kit (Vazyme, Nanjing, China) and diluted to 100 ng/μL for qPCR with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The qPCR assay was carried out in a real-time PCR machine (7,500, Applied Biosystems) as the following program: 95°C for 30 s (1 cycle); 95°C for 10 s, 60°C for 30 s (40 cycles); melting curve profiled from 60°C to 95°C to check the specialty of reaction. The primers of selected genes used in the assay are listed in **Supplementary Table 2**. 16s ribosomal RNA gene was used as a reference gene. Each sample was tested four times per experiment and experiments were conducted three times independently. Relative gene expression was calculated in the method of $2^{-\Delta\Delta ct}$ as described previously (Livak and Schmittgen, 2001).

Statistical Analyses

All data were analyzed by SPSS Statistics 26. The one-way analysis of variance (ANOVA) and least Significant Difference (LSD) test were used to determine the significant difference in disease index, biofilm assay, motility assay and AUPDC. Differences with $p < 0.05$ were considered significant.

RESULTS

YggS Is Conserved and Clusters Strongly With PilT in Multiple Organisms

Through library screening, an *A. citrulli* mutant named sk24 that was unable to induce a HR on tobacco at 12 h after inoculation was obtained (**Supplementary Figure 1**). By subcloning and sequence analysis, we identified the gene disrupted by transposon as *Aave_0638* (**Supplementary File 1**). This gene putatively encodes YggS family pyridoxal phosphate-dependent enzyme, which exhibits structual similarity to the *N*-terminal domain of alanine racemase (EC 5.1.1.1). Multiple sequence alignment shows that the amino acid sequence of YggS from *A. citrulli* has high identity among the tested bacteria including *E. coli* (**Figure 1A**). By BLASTP sequence homology analysis, YggS displays 53.94, 38.97, 49.57, 53.78, 55.46, and 55.14% sequence identity with homologs from *E. coil* MG1655, *S. elongates* PCC7942, *X. oryzae* PXO99A, *P. syringae* DC3000, *E. amylovora* CFBP1430 and *P. carotovorum* PC1, respectively. Furthermore, previous studies have shown that *yggS* clusters strongly with genes such as those related to metabolism, including cell division and cell wall (Prunetti et al., 2016). We observed the position of *yggS* on genome and found that *yggS* and *pilT*, encoding

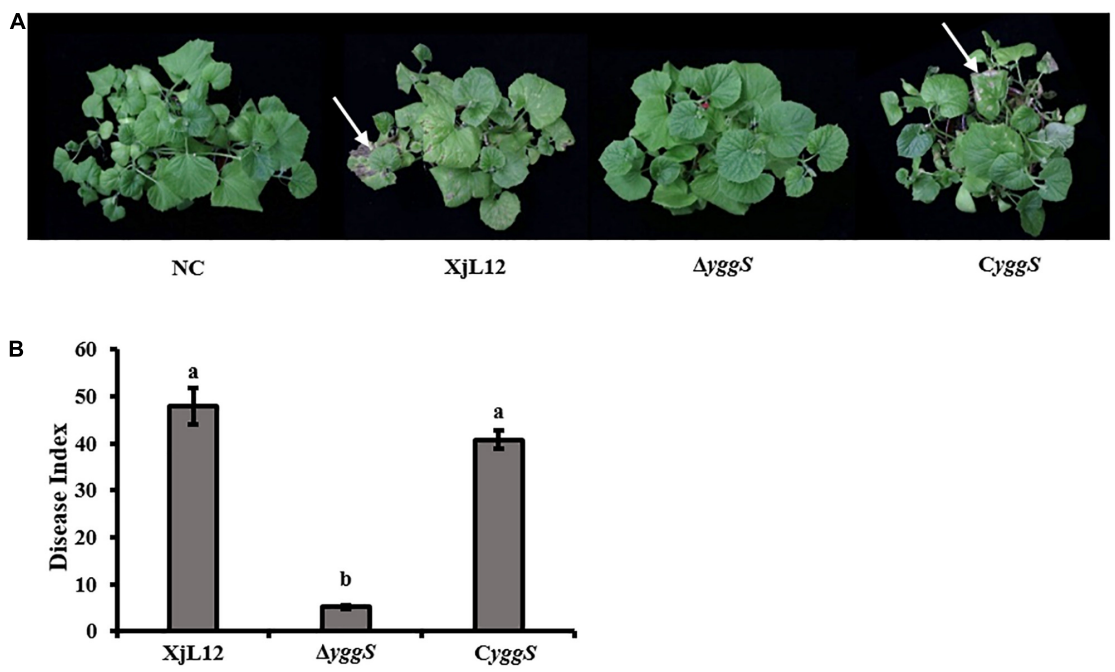

FIGURE 2 | Spray inoculation assay. **(A)** *A. citrulli* suspension (~1 × 10^8 CFU/mL) were inoculated onto the euphylla of melon by spray inoculation. The phenotypes were photographed after the melon seedlings were allowed to grow for 7 days after inoculation. The red arrows indicate the BFB symptoms. **(B)** Disease index for each treatment is represented by a histogram. The bars represent means and lines indicate standard errors of the mean. XjL12: wild-type strain, Δ*yggS*: *yggS* deletion mutant; *CyggS*: complemented strain of Δ*yggS*. Letters above each bar represent significant differences as determined by the LSD test ($P < 0.05$).

ATPase-mediating pilus retraction and disassembly, are clustered together. This genetic organization appears to be conserved for serval phytopathogenic bacteria (**Figure 1B**).

yggS Contributes to *Acidovorax citrulli* Virulence

To investigate the effect of *yggS* on *A. citrulli* virulence, we compared the pathogenicity of the wild-type strain XjL12, the mutant strain Δ*yggS* and complemented strain C*yggS* by conducting cotyledon injection and spray inoculation of melon seedling euphylla. The results of the spray inoculation assay showed that mutation of *yggS* impaired the virulence of *A.citrulli* (**Figure 2A**). Seven days after inoculation, necrotic lesions developed on the euphylla sprayed with *A. cirulli* strains XjL12 and C*yggS*, while the euphylla sprayed with Δ*yggS* showed mild necrotic symptoms. The disease indices (DI) caused by XjL12, Δ*yggs* and C*yggS* were 47.87, 5.15, and 40.78, respectively. The DI of Δ*yggS* was significantly lower than the wild-type and complemented strains ($P < 0.05$) (**Figure 2B**). As shown in **Figure 3A**, the cotyledons injected with Δ*yggS* showed no symptoms, similar to the negative control (NC) at 5 days after inoculation. Meanwhile, melon cotyledons injected with XjL12 and C*yggS* showed typical BFB symptoms, including seedling blight/collapse. By 7 dpi, there were no visible BFB symptoms on melon cotyledons injected with the mutant strain. In agreement with the results of cotyledon injection, Δ*yggS* impaired its ability to colonize melon cotyledons. The bacterial populations of XjL12, Δ*yggS* and C*yggS* were approximately 2.94×10^8, 1.69×10^6, 1.95×10^8 CFU/cm^2 by 4 dpi, respectively (**Figure 3B**). As expected, AUPDC data showed that the population of Δ*yggS*

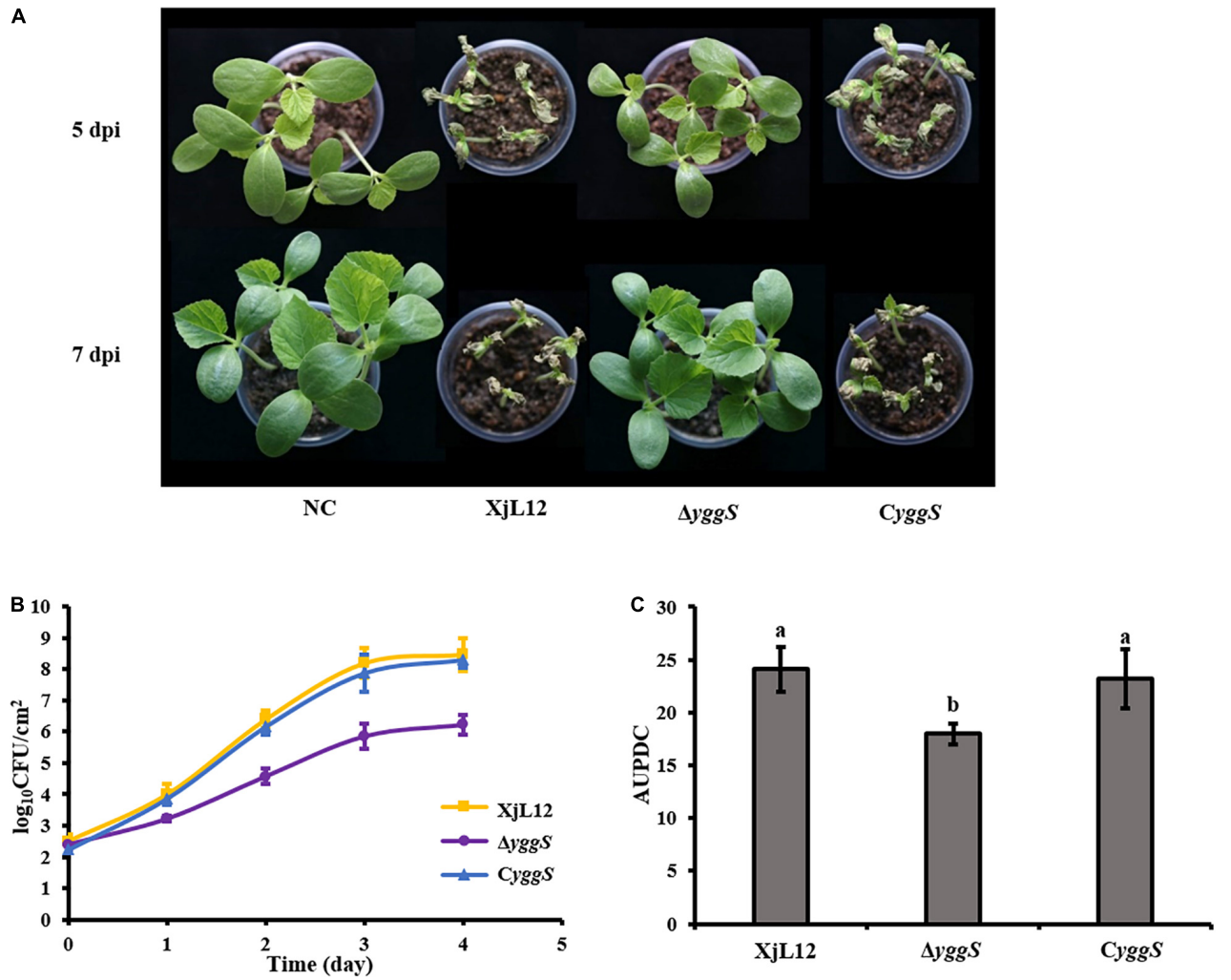

FIGURE 3 | Effect of *yggS* on the ability of *A. citrulli* to colonize in melon cotyledons. **(A)** *A. citrulli* cell suspensions (~1×10^4 CFU/mL) were injected into the cotyledons of melon seedlings. The images represent seedling symptoms at 5 and 7 days post-inoculation (dpi). **(B)** The melon cotyledons were injected with bacterial suspensions (~1×10^5 CFU/mL) and the bacterial populations were quantified for five days. **(C)** Histogram of area under population dynamics curve (AUPDC) calculated for *A. citrulli* colonization of cotyledons. In the graph, the data points represent means and the tines represent the standard errors of the mean. In the histogram, the bars represent means and lines indicate the standard errors of the mean. XjL12: wild-type strain, Δ*yggS*: *yggS* deletion mutant; C*yggS*: complemented strain of Δ*yggS*. Letters above the bar represent significant differences as determined by the LSD test ($P < 0.05$).

was significantly less than that of XjL12 and C*yggS* in cotyledons ($p < 0.05$) (**Figure 3C**).

yggS Is Required for Seed-to-Seedling Transmission of BFB

In the seed-to-seedling transmission assay, melon seeds infiltrated with *A. cirtulli* strains were planted at 20 seeds per pot and the percentage of dead seedlings was calculated one week after planting. Most seedlings from seeds inoculated with wild-type strain XjL12 and complemented strain C*yggS* were died, while all the seedlings inoculated with Δ*yggS* survived without visible BFB symptoms (**Figure 4A**). The reduced seed-to-seedling transmission indicated that it is likely that the loss of *yggS* impaired the colonization ability in melon seeds. So, we assayed the population dynamics of *A. citrulli* in germinating seeds and the bacterial population in inoculated seeds were quantified per 24 h after inoculation (**Figure 4B**). The average populations of XjL12, Δ*yggS* and C*yggS* were approximately 1.07×10^8, 5.12×10^5, 5.37×10^7 CFU/g of seed by one week after inoculation, respectively. AUPDC data showed that the populations of Δ*yggS* was significantly less than XjL12 and C*yggS* in seeds ($p < 0.05$) (**Figure 4C**). These results suggest that *yggS* plays an important role in seed-to-seedling transmission of BFB and seed colonization by *A. citrulli*.

The Absence of *yggS* Causes Delay in *Acidovorax citrulli* Growth, Which Can Be Restored by Exogenous PL

For most organism but *Pseudomonas aeruginosa*, YggS is dispensable for growth (Rusmini et al., 2014; Vu et al., 2020). To investigate the role of YggS in *A. citrulli* growth, the ability of the *A. citrulli* strains Δ*yggS*, wild-type XjL12 and C*yggS* complement to grow in LB medium was compared by measuring the optical density of cell suspensions. Δ*yggS* reached the exponential growth phase after 14 h and reached the stationary phase at 26 h. On the other hand, the wild-type and complemented strains reached the exponential and stationary phases approximately at 12 h and 24 h, respectively (**Figure 5A**). The date indicated that *yggS* also plays a dispensable role for *A. citrulli* despite of the slight reduction in growth of Δ*yggS*. In addition, Δ*yggS* cultured in the LB broth amended with 1 μM PL returned to wild-type

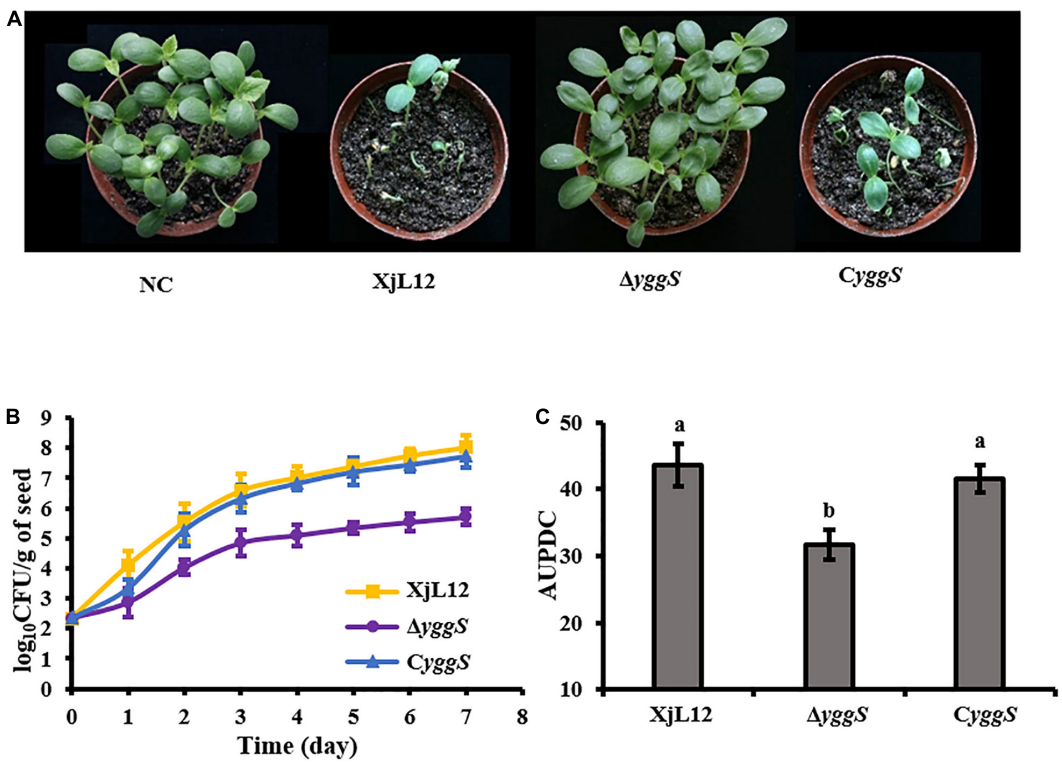

FIGURE 4 | Role of *yggS* in seed-to-seedling transmission BFB in melon and in colonization in melon seeds by *A. citrulli*. **(A)** Effect of *yggS* on seed-to-seeding transmission of *A. citrulli* on melon. Melon seeds (n = 20) were innoculated by soaking in bacterial cell suspensions (~1×10^8 CFU/mL) and then planted. One week after planting, BFB symptoms were observed and photographed. **(B)** Germinating melon seeds were inoculated with approximately 5 uL of bacterial suspension (~1×10^5 CFU/mL) and the bacterial populations in seeds were quantified each day for seven days. Each strain was used to inoculate five seeds and each experiment was repeated three times. **(C)** Histogram of AUPDC calculated bacterial colonization of melon seed. In the graph, the data points represent means and the tines represent the standard errors of the mean. In the histogram, the bars represent means and lines indicate the standard errors of the mean. XjL12: wild-type strain, Δ*yggS*: *yggS* deletion mutant; C*yggS*: complemented strain of Δ*yggS*. Letters above the bars represent significant differences as determined by the LSD test ($P < 0.05$).

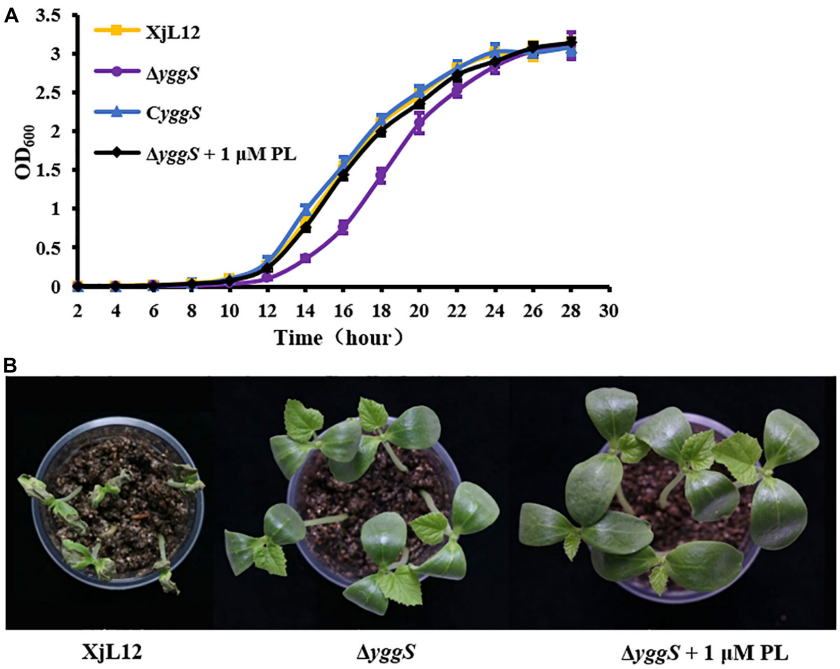

FIGURE 5 | Effect of pyridoxal (PL) on Δ*yggS* growth in LB and virulence on melon seedlings. **(A)** Assay of *A. citrulli* growth rate. All strains were cultured overnight, diluted to OD_{600} = 0.1 with sterilized water, and then transferred to fresh LB at a ratio of 1:100. Optical density of cell suspensions was measured at 2 h intervals. The data points represent means and the tines represent the standard errors of the mean for three experiments. Each experiment was repeated three times. **(B)** Effect of *yggS* on virulence of *A.citrulli* on melon cotyledons through exogenous addition of PL. Δ*yggS* suspension (~1 × 10^5 CFU/mL) mixed 1 μM PL was injected into cotyledons of seedlings, and XjL12 and Δ*yggS* were used as controls. The images represent the seedlings at 5 days post-inoculation. XjL12: wild-type strain, Δ*yggS*: *yggS* deletion mutant; C*yggS*: complemented strain of Δ*yggS*.

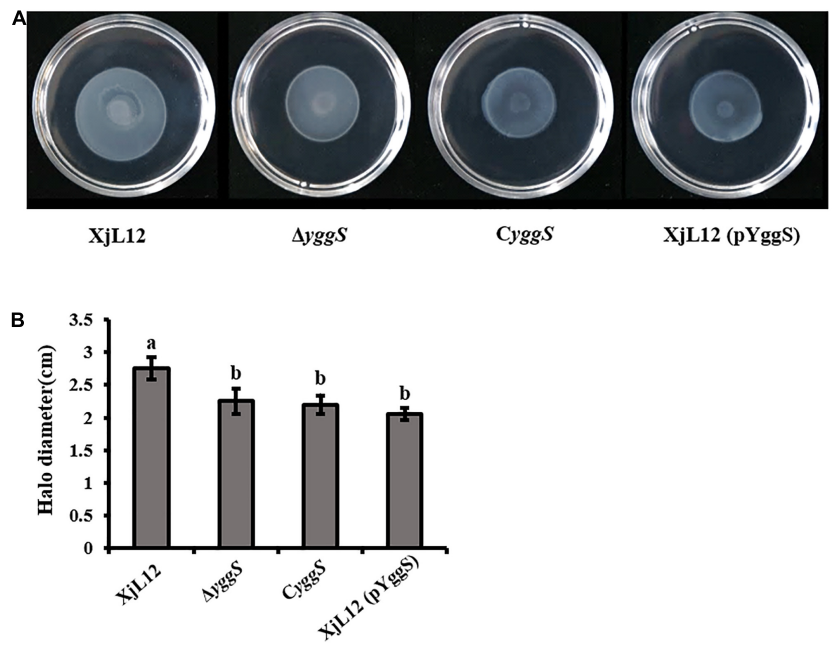

FIGURE 6 | Effect of *yggS* on *Acidovorax citrulli* motility. **(A)** Swimming motility of *A. citrulli* strains. Bacterial suspensions diluted to 3 × 10^8 CFU/mL with sterilized water were deposited 5 μL of suspensions in the center of the 0.3% agar plate. The swimming colonies were photographed after 48 h. **(B)** The swimming colonies diameter of *A. citrulli* strains were measured after 48 h. The vertical bars in the histogram represent standard errors of the mean for three experiments. Each experiment was repeated three times. XjL12: wild-type strain, Δ*yggS*: *yggS* deletion mutant; C*yggS*: complemented strain of Δ*yggS*. XjL12 (pYggS): *yggS*-overexpressed strain. Letters above the bars represent significant differences as determined by the LSD test ($P < 0.05$).

growth (**Figure 5A**), while PL was unable to enhance the growth of wild-type or complemented strain (**Supplementary Figure 2**).

To determine if PL could restore compromised virulence, Δ*yggS* was syringe-inoculated into melon cotyledons along with 1 μM PL. Although PL restored growth of Δ*yggS*, it did not restore pathogenicity on melon cotyledons (**Figure 5B**). This result showed that the effect of *yggS* on bacterial growth may not be related to virulence attenuation.

The Absence of *yggS* Reduces Swimming Motility in *Acidovorax citrulli*

Previous studies have confirmed that bacterial motility plays a key role in pathogenicity of *A. citrulli* (Bahar et al., 2009b; Bahar et al., 2011). *A. citrulli* strains produced near circular colonies *via* swimming motility using the soft agar plate assay (**Figure 6A**). The diameters of XjL12, Δ*yggS*, C*yggS*, and XjL12 (pYggS) were 2.75 ± 0.17, 2.25 ± 0.19, 2.20 ± 0.14, and 2.05 ± 0.09 cm, respectively (**Figure 6B**). The diameters of the swimming motility colony produced by Δ*yggS* and overexpressed strain XjL12 (pYggS) were significantly smaller than the colony produced by wild-type strain ($p < 0.05$) (**Figure 6B**). However, the complemented strain with pBBRMCS-5, containing the *yggS* gene, failed to restore the swimming motility phenotype.

Δ*yggS* Is More Susceptible to Pyridoxine (PN), H_2O_2 and BCDA

In *E. coli*, excess PN led to a zone of inhibition with Δ*yggS*, but not with the wild-type strain. We observed a similar phenomenon with *A. citrulli* (**Figure 7A**). The mean diameter of the inhibition zones produced by Δ*yggS* (2.42 ± 0.12 cm) was greater ($p < 0.05$) than the diameters of the wild type XjL12 (1.50 ± 0.08 cm) and the complementation strain C*yggS* (1.41 ± 0.06 cm) (**Figure 7B**). This result suggested that the deletion of *yggS* increases the sensitivity to PN. However, the sensitivity to PN could not be rescued by exogenous addition of PL, and even with a range of PL concentrations; this was different to the observations reported for *E. coli* (**Figure 7A**; Prunetti et al., 2016). To determine if YggS is involved in oxidative stress tolerance in *A. citrulli*, we measured the sensitivity to H_2O_2 (**Figure 7C**). Compared to the wild-type and complemented strains, the diameters of inhibition zone for the mutant were greater ($p < 0.05$) at 5 and 10% H_2O_2; conversely, the diameter of C*yggS* was not significantly different to that of XjL12 (**Figure 7D**). The results suggest that deletion of *yggS* increases sensitivity to H_2O_2 and that *yggS* contributes to oxidative stress tolerance of *A.citrulli*. In order to determine whether *yggS* inactivation affects the sensitivity of *A. citrulli* to antibiotics, we tested 7 common antibiotics, including D-cycloserine (DCS) and β-chloro-D-alanine (BCDA) targeting alanine racemase. As shown in **Figures 7E,F**, the *yggS* mutant was more sensitive than XjL12 and C*yggS* to BCDA, but not to the other antibiotics (**Supplementary Figure 3**). Further, the sensitivity of mutant to BCDA was reduced by exogenous addition of L-alanine or D-alanine (**Figure 7E**). These results suggest that *yggS* contributes to the resistance of *A. citrulli* to BCDA.

yggS Is Involved in HR Induction

To determine whether *yggS* contributes to the *A. citrulli* type III secretion system (T3SS), we examined HR induction by wild-type XjL12 and *yggS* mutant Δ*yggS* strains on *N. tabacum* (**Figure 8**). At 12 hpi, XjL12 and C*yggS* induced HR, while Δ*yggS* showed no cell death (similar to the negative control). However, HR induction was observed for Δ*yggS* at 24 hpi. These observations suggest that the deletion of *yggS* delays HR induction on *N. tabacum* and the deletion of *yggS* potentially impairs the T3SS function. In addition, the effect of reduced growth in Δ*yggS* on HR induction is inevitable.

RNA-Seq Analysis Revealed That *yggS* Is Involved in the Regulation of T3SS *in vitro*

To investigate the cause of attenuated virulence observed for Δ*yggS*, the differences in the transcriptomes between Δ*yggS* and XjL12 were analyzed by RNA-Seq. A list containing the expression of all genes including the differentially expressed genes (DEGs) is provided in **Supplementary File 2**. The RNA-Seq results were validated by qPCR assay with ten chosen DEGs (**Supplementary Figure 4**). Compared with wild-type strain, there were 971 DEGs in the mutant strain: 506 genes were upregulated and 465 were downregulated (**Supplementary Figure 5**). In agreement with the previous abovementioned phenotypes, the DEGs were involved with *A. citrulli* motility, T3SS and anti-oxidative stress (**Table 1**).

Among these DEGs listed in **Table 1**, *in vitro* condition, a variety of T3SS genes including that encode indispensable apparatus subunit HrcC, HrcJ, et al., transcriptional regulator HrpX directly mediating the expression of most T3SS and T3Es genes significantly lowly-expressed. Correspondingly, the expressing level of many T3Es genes was decreased (**Table 2**). In addition to the T3Es revealed by annotation of genome, we also summarized the genes with high similarity to known T3Es from other plant pathogens based on the results reported by Jiménez-Guerrero et al. (2020). The homologues of *Aave_2177*, *Aave_1555*, *Aave_1244,* and *Aave_3960* have been validated as T3Es in *A. citrulli* strain M6 by translocation assay (Jiménez-Guerrero et al., 2020). Gene ontology (GO) analysis showed that down-regulated DEGs were enriched in terms related to secretion system (e.g., protein secretion, peptide secretion, secretion by cell) and regulations of these pathways were downregulated significantly in Δ*yggS* (**Supplementary Figure 6A**). Based on these factors, it is possible that YggS regulates a variety of pathogenic factors, and that regulation of secretion systems, especially T3SS, contributes to attenuated virulence on melon.

During infection, pathogens encounter the large generation of reactive oxygen species as part of the oxidative burst associated with plant defense response, making response to and protection against oxidative stress an important aspect for infection (Apel and Hirt, 2004; Burbank and Roper, 2014). The DEGs related to oxidative stress response partly listed in **Table 1** indicated the perturbation in antioxidative response as a result of *yggS* mutation. FlgM and PilA have been conformed as a key factor responsible for flagellum and TFP assembly, respectively, in

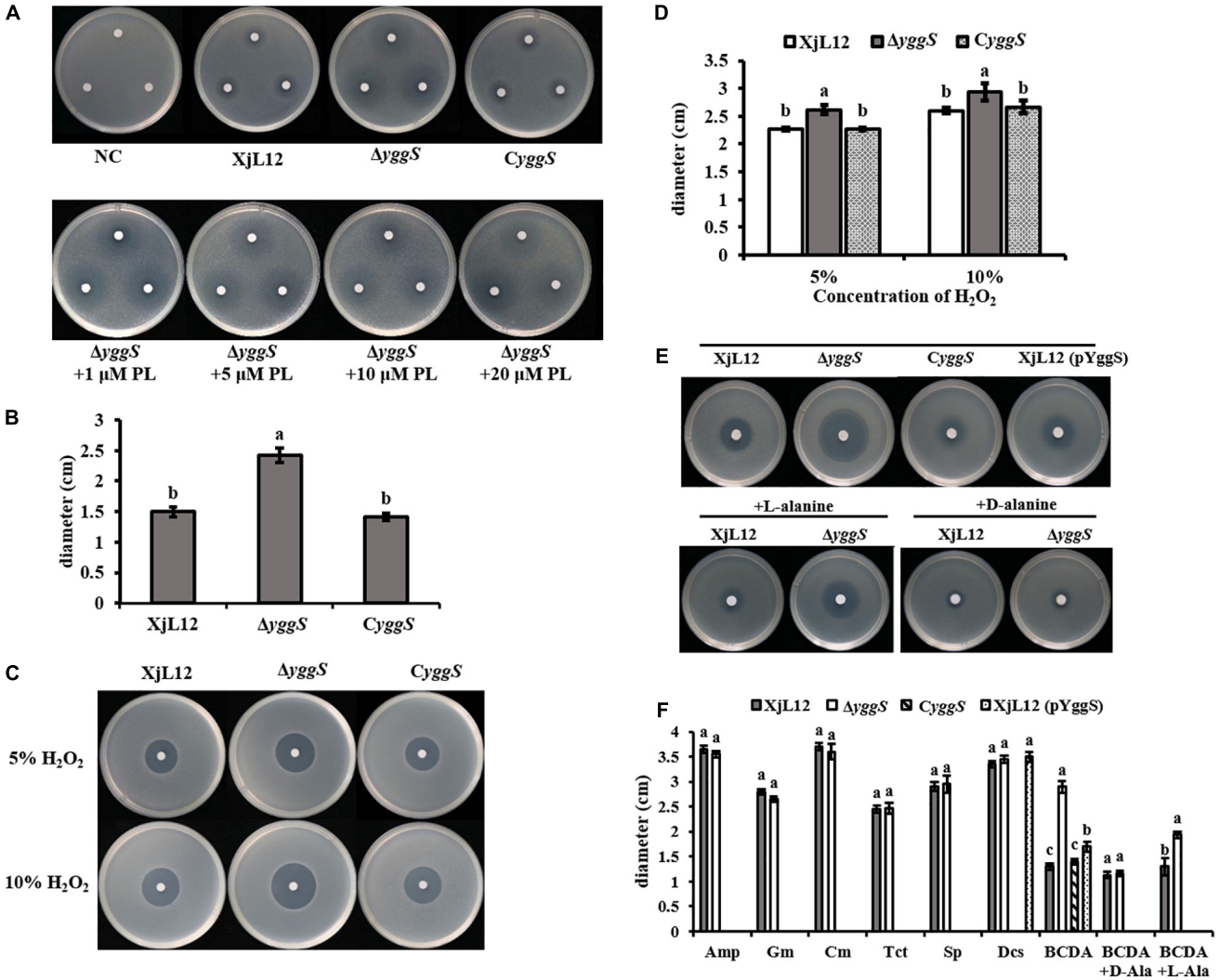

FIGURE 7 | The role of *yggS* in the sensitivity of *Acidovorax citrulli* to selected stressors. **(A)** The toxicity of pyridoxine (PN) (in the absence or presence of pyridoxal (PL)) to *A. citrulli* strains. **(B)** The diameter of inhibition zones caused by PN. All phenotypes were observed after incubation for 48 h. **(C)** Sensitivity of *A. citrulli* strains to H_2O_2. Two different concentrations of H_2O_2 were dropped in the center of the plate containing *A. citrulli* strains. **(D)** H_2O_2 sensitivity was measured by the diameter of the zone of bacterial growth inhibition. **(E)** Representative images illustrating the sensitivity of *A. citrulli* strains to BCDA (in the absence or presence of L-alanine or D-alanine). **(F)** Diameters of *A. citrulli* inhibition zones for each antibiotic were represented by histogram. The bars in the histogram represent mean inhibition zone and lines represent standard errors of the mean of three experiments. XjL12: wild-type strain, Δ*yggS*: *yggS* deletion mutant; C*yggS*: complemented strain of Δ*yggS*. XjL12 (pYggS): *yggS*-overexpressed strain. Letters above the bars represent significant differences as determined by the LSD test ($P < 0.05$). Amp = ampicillin, BCDA = β-chloro-D-alanine, Cm = chloramphenicol, DCS = D-cycloserine, Gm = gentamicin, Sp = spectinomycin, Tct = tetracycline.

A. citrulli (Rosenberg et al., 2018; Yang et al., 2018), and both were upregulated in mutant (**Table 1**). Surprisingly, a gene *Aave_3811*, accounting for synthesizing acyl homoserine lactones-QS signal molecules (Fan et al., 2011), were negatively regulated. The expressing level of genes related to T2SS and T6SS was also changed in Δ*yggS*.

Ribosomes carrying out protein synthesis is required for cell growth (Lempiäinen and Shore, 2009). However, the biosynthesis of ribosomes is energy-consuming, thus the overproduction of ribosomal proteins is detrimental to cell proliferation (Jorgensen et al., 2004). GO analysis and KEGG pathway analysis revealed that the ribosome was most affected in *yggS* mutation (**Supplementary Figures 6, 7**). **Supplementary Table 3** displayed the expression profile of the DEGs encoding 30S or 50S ribosomal proteins and all the genes were upregulated, indicating that inactive *yggS* caused increasing ribosomal biosynthesis, which may result in the growth defect. Previous reports have demonstrated the *yggS*-deficient *E. coil* impacts amino acids homeostasis, such as valine and isoleucine (Ito et al., 2013, 2019). According to KEGG pathway analysis, the pathway in valine, leucine and isoleucine degradation, β-Alanine metabolism was significantly regulated by *yggS*. In addition to both pathways, **Supplementary Table 4** displayed the expression profile of the DEGs related to arginine and proline metabolism,

FIGURE 8 | Effect of *yggS* on the ability of *Acidovorax citrulli* to induce a hypersensitive response (HR) on *Nicotiana tabacum*. All strains were cultured in LB overnight and infiltrated into the *N. tabacum* leaves at a concentration of OD_{600} = 0.3. *N. tabacum* was grown under greenhouse conditions at 24°C and observed for HR at 12 h and 24 h post infiltration. 1 = *yggS* deletion mutant Δ*yggS*, 2 = wild-type strain XjL12, 3 = negative control (NC) = double-distilled H_2O (ddH_2O), 4 = complemented strain C*yggS*.

TABLE 1 | Differentially expressed genes related to *Acidovorax citrulli* pathogenicity.

Gene Id	log_2FC	Description	*p* value
T2SS			
Aave_2725	−0.68704	type II secretion pathway component PulD-like protein	1.21E-07
Aave_2721	−0.55174	type II secretion system F family protein	0.000568
Aave_4150	0.27564	type II secretion system secretin GspD	0.007403
T3SS			
Aave_0444	−0.57401	AraC family transcriptional regulator HrpX (Zhang et al., 2018)	4.94E-05
Aave_0468	−2.1809	HrpB1 family type III secretion system apparatus protein	9.42E-20
Aave_0479	−1.2729	HrpB1 family type III secretion system apparatus protein	1.09E-05
Aave_0464	−1.4722	HrpE/YscL family type III secretion apparatus	6.38E-10
Aave_0446	−0.89909	type III secretion regulatory protein HpaB	0.000252
Aave_0447	−1.514	type III secretion regulatory protein HpaA	1.91E-21
Aave_0463	−1.0533	type III secretion system ATPase HrcN	7.86E-12
Aave_3948	−0.62563	type III secretion system chaperone	0.000666
Aave_0450	−2.6199	type III secretion system cytoplasmic ring protein HrcQ	5.92E-20
Aave_0449	−1.8577	type III secretion system export apparatus subunit HrcR	5.78E-08
Aave_0452	−2.1077	type III secretion system export apparatus subunit HrcV	8.67E-14
Aave_0466	−1.6779	type III secretion inner membrane ring lipoprotein HrcJ	3.32E-09
Aave_0474	−3.1228	type III secretion system outer membrane ring subunit HrcC	1.08E-21
T6SS			
Aave_0497	−0.75883	type VI secretion system tip protein VgrG	1.73E-14
Aave_4009	−0.4222	type VI secretion system tip protein VgrG	0.000394
Oxidative stress			
Aave_0348	0.30046	Peroxiredoxin	0.002919
Aave_1172	0.47563	peroxide stress protein YaaA	4.60E-05
Aave_1235	0.31254	Peroxiredoxin	0.002603
Aave_1375	0.33467	peroxiredoxin	0.000697
Aave_1376	0.40729	alkyl hydroperoxide reductase subunit F	0.000155
Aave_2599	−1.6937	superoxide dismutase family protein	2.11E-27
Aave_3047	0.56971	oxidative damage protection protein	3.57E-05
Aave_3991	−1.2794	Catalase	3.37E-16
Aave_4272	−1.2075	organic hydroperoxide resistance protein	2.35E-07
Motility			
Aave_4418	0.29599	flagellar biosynthesis anti-sigma factor FlgM	0.005056
Aave_4679	0.50058	pilin, PilA	1.85E-07
QS			
Aave_3811	−0.55673	acyl homoserine lactone synthase LuxI	0.003906

Differentially expressed genes (DEGs) related to secretion system (T2SS, T3SS, and T6SS), oxidative stress, motility, quorum sensing (QS) in ΔyggS compared to wild-type strain XjL12 were listed in the table. Gene Id: the locus tags of DEGs that identified by hits in a Blastn search against the A. citrulli AAC00-1 genome (NC_008752). FC: fold change.

TABLE 2 | Differentially expressed genes related to type III-secreted effectors.

Gene Id	log_2FC	Description	*p* value
Aave_0454[a]	−1.7491	type III secretion protein	0.009104
Aave_0465[a]	−1.7458	type III secretion protein	5.26E-13
Aave_0467[a]	−1.611	type III secretion protein	2.01E-07
Aave_0473[a]	−1.8826	type III secretion protein	1.37E-28
Aave_2166[a]	−0.52149	type III secretion system YopJ family effector	0.000437
Aave_3621[a]	−0.29943	type III effector AopN (Zhang et al., 2020b)	0.006845
Aave_3452[a]	−2.1122	hypothetical protein	2.25E-15
Aave_4728[a]	−1.6464	hypothetical protein	1.94E-09
Aave_3462[a]	−0.84509	peptidase C55	6.10E-06
Aave_2177[b]	−1.6842	hypothetical protein	0.002479
Aave_1555[b]	−1.1729	hypothetical protein	5.49E-30
Aave_1244[b]	−0.97312	hypothetical protein	1.31E-12
Aave_3960[b]	−0.92878	hypothetical protein	6.16E-07
Aave_1090[c]	−1.7257	hypothetical protein	1.22E-27
Aave_4254[c]	−1.6907	hypothetical protein	8.08E-13
Aave_0458[c]	−1.6642	ribonuclease inhibitor	4.84E-09
Aave_4612[c]	−1.554	hypothetical protein	6.88E-13
Aave_4631[c]	−1.1544	hypothetical protein	2.41E-15
Aave_4632[c]	−1.1128	leucine-rich repeat domain-containing protein	6.28E-21
Aave_3847[c]	−0.63486	hypothetical protein	3.39E-07
Aave_2802[c]	−0.43537	hypothetical protein	0.000197
Aave_0310[c]	−0.35704	hypothetical protein	0.001025
Aave_3621[c]	−0.29943	hypothetical protein	0.006845

Differentially expressed genes (DEGs) related to putative type III-secreted effectors (T3Es) compared to wild-type strain XjL12 were listed in the table. Gene Id: the locus tags of DEGs that identified by hits in a Blastn search against the A. citrulli AAC00-1 genome (NC_008752). FC: fold change.

[a] *The DEGs whose products are annotated as T3Es in A. citrulli strain AAC00-1 (Eckshtain-Levi et al., 2014).*

[b] *The homologues of these hypothetical proteins have been validted as T3Es in A. citrulli strain M6 by experimental assay (Jiménez-Guerrero et al., 2020).*

[C] *The gene product is similar to known T3Es by BlastP analyse (Jiménez-Guerrero et al., 2020).*

alanine, aspartate and glutamate metabolism, glycine, serine and threonine metabolism, indicating that *yggS* also was involved in amino acids homeostasis in *A. citrulli*.

In addition, among the most GO enriched terms, only up-regulated DEGs were divided into terms belonging to cellular component domain, such as cellular anatomical entity, intracellular organelle, et al. (**Supplementary Figure 6B**). YggS appears to negatively affect pathway terms related to cellular component in *A. citrulli*.

DISCUSSION

YggS is a member of the highly conserved PLP-binding protein family classified as fold-type III family of PLP-dependent enzymes (Ito et al., 2019), which accounts for ∼4% of all activities classified by the Enzyme Commission (Percudani and Peracchi, 2003). PLP is involved in over 140 chemical reactions (Mooney et al., 2009) and is required for survival and virulence for some pathogens (Dick et al., 2010; Grubman et al., 2010; Xie et al., 2017). However, the role of YggS, which maintains PLP homeostasis, in pathogenicity remains poorly understood. To the best of our knowledge, this is first study of YggS in plant pathogenic bacteria and the first confirmation of its role in growth, secretion system, motility, oxidative stress response and virulence in *A. citrulli*.

A previous study indicated that high concentration of intracellular PNP is the root cause of partly pleiotropic phenotypes, e.g., toxicity of PN to *yggS* mutants and disordered amino acid metabolism (Ito et al., 2019). In the *yggS*-deficient strain, further accumulation of PNP induced by excess PN impacts the isoleucine/valine biosynthetic pathway, resulting in overproduction of valine, which is toxic to the cell. Because of a similar inhibitory effect of PN caused by *yggS*, it is possible that PNP was accumulated in the Δ*yggS*. Different from the reports in *E. coil* and *S. elongate* (Prunetti et al., 2016; Labella et al., 2017), in the case of *A. citrulli*, PL failed to suppress the toxicity of PN, but made up for the shortage in growth. The mechanism by which PL restore the Δ*yggS* growth needs to be studied in further detail.

The antibiotics DCS and BCDA are both peptidoglycan inhibitors that target alanine racemase, which is involved in the formation of D-alanine (Manning et al., 1974; David, 2001; Feng and Barletta, 2003). In contrast to the previous report on *S. elongate* (Labella et al., 2017), only sensitivity to BCDA was affected by deletion of *yggS* in *A. citrulli*. This difference may be because the targets of BCDA are not only alanine racemase, but also glutamate racemase (Prosser et al., 2016). In agreement with the observation described by Labella et al. (2017) in *S. elongate*, the sensitivity to BCDA caused by

inactive *yggS* could be suppressed by L-alanine and D-alanine in *A. citrulli*, indicating that YggS may play a key role in preventing BCDA from destroying the activity of targets by altering the metabolism of alanine in the cell. It is worth mentioning that the overproduction of alanine racemase confers the resistance to DCS and BCDA (Cáceres et al., 1997; Feng and Barletta, 2003). However, we did not observe resistance when YggS was overexpressed in *A. citrulli* (**Figure 7E** and **Supplementary Figure 3**). This result indirectly indicates that YggS may have no racemase activity in *A. citrulli* despite showing structural similarity to alanine racemase, which is in consistent with the report in *E. coil* (Ito et al., 2013).

Based on a series of virulence assays, we conclude that *yggS* is indispensable for *A. citrulli* virulence including seed-to-seedling transmission and melon tissue colonization. To elucidate the role of YggS in pathogenicity, several factors associated with YggS were determined and these phenotypes were supported by genome-wide expression analysis. Additionally, according to RNA-Seq DEGs, YggS also regulates *A. citrulli* QS, however, this result needs to be validated in more detail in subsequent studies.

Previous studies reported a crucial role of vitamin B6 in protecting cells from oxidative stress (Mooney et al., 2009; Vanderschuren et al., 2013). For *Cercospora nicotianae* and *Rhizoctonia solani*, blocking vitamin B_6 synthesis increased sensitivity to oxidative stress (Ehrenshaft et al., 1998; Samsatly et al., 2015). In addition, an *Actinobacillus pleuropneumoniae* mutant deficient in PLP synthase was sensitive to H_2O_2 and showed attenuated virulence (Xie et al., 2017). In the current study, increased sensitivity of Δ*yggS* to H_2O_2 demonstrated that YggS protects cell against oxidative stress. This can be explained by the result of the DEG analysis that showed genes related to oxidative stress response were differently expressed (**Table 1**). Based on the point that the level of PLP is controlled by YggS, it is possible that the normal oxidative stress response is impaired by disordered PLP or vitamin B_6 levels in Δ*yggS*.

In swimming assay, loss or overexpression of *yggS* reduced swimming motility (**Figure 6A**). According to transcriptome analysis, the expression of anti-sigma factor FlgM, a negative factor for flagellar assembly (Frisk et al., 2002), was increased and the overexpression of FlgM may lead to reduced swimming. In addition, consistent with reduced swimming, flagella-related genes (*fliA*, *fliC*, *fliS*) in XjL12 (pYggS) were downregulated (**Supplementary Figure 8**). Nonetheless, the effect of molecular manipulation in overexpression strain could not been excluded. These findings suggest that the YggS level maybe critical to *A. citrulli* swimming motility. Based on the reports that the regulation to flagellar motility is PLP-dependent in pathogens *Helicobacter pylori* and *Campylobacter jejuni* (Dick et al., 2010; Asakura et al., 2013), we postulate that the weakened swimming may be due to unbalanced PLP level caused by defective YggS. However, we failed to recover the swimming ability by complementation vector pBBR-*yggS*. Perhaps the level of YggS in C*yggS* was less optimal than in the wild type strain. On the other hand, the increased expression of *pilA* may imply the enhanced twitching caused by inactive YggS. Moreover, *yggS* is not involved in *A. citrulli* biofilm formation (**Supplementary Figure 9**), which is closely related to pathogenicity for pathogenic bacteria.

It is widely accepted that T3SS is a pivotal mechanism for many gram-negative bacteria infecting host plants and eliciting HR on non-host plants (Mudgett, 2005). *A. citrulli*, possessing a *hrp* gene cluster, relies on a functional T3SS for pathogenicity (Johnson et al., 2011; Eckshtain-Levi et al., 2014). Therefore, in order to enhance screening efficiency, a Tn5 mutant library was screened by inoculating tobacco leaves. In this study, delayed HR phenotype induced by inactive *yggS* suggests that the function of T3SS was impaired. In agreement with delayed HR, T3SS genes related to core subunits of T3SS apparatus, T3SS regulators, T3SS chaperone and effectors showed reduced expression *in vitro*. Among these DEGs, genes for assembling T3SS in *A. citrulli* including *hrcC*, *hrcJ*, *hrcR* and *hrcV*, an AraC-type transcriptional regulator HrpX and effector AopN were confirmed to be necessary for *A. citrulli* to exert pathogenicity or elicit HR (Bahar and Burdman, 2010; Burdman and Walcott, 2012; Zhang et al., 2018, 2020b). Gene products that comprise T3SS can be grouped into four classes: apparatus proteins, translocon proteins, effectors and type III chaperones (Bronstein et al., 2000). According to the established genome annotations in *A. citrulli*, DEGs covered three classes except for translocon proteins. Therefore, we postulate that the compromised virulence is due to impaired function of T3SS. However, mechanism by which YggS regulates T3SS is unclear and to date, and no studies report the relationship of PLP and T3SS. Additionally, the further study on the association between *yggS* and T3SS-related genes or effectors *in planta* needs to be carried out.

In summary, we identified a novel pathogenicity-associated factor YggS that is required for *A. citrulli* virulence and involved in motility, secretion, antibiotic resistance, oxidative stress response and growth, especially in T3SS when cultured in LB medium. Based on the existing literature on YggS or its homolog along with our *in vitro* study results, we are unable to elucidate the precise mechanism by which YggS regulates virulence. Perhaps, the imbalance of PLP homeostasis or amino acid metabolism caused by the deficiency of YggS results in reduced virulence. Finally, our finding uncovers a broader function of YggS and provides new insights into the pathogenesis of *A. citrulli*.

CONCLUSION

YggS, a pyridoxal 5′-phosphate binding protein that is conserved among the multiple organisms, is firstly reported as an indispensable factor for the virulence of *A. citrulli*, the causal agent of bacterial fruit blotch of cucurbits. The absence of YggS in *A. citrulli* reduces swimming motility, increases the sensitivity to H_2O_2, antibiotic BCDA and PN, and delays HR induction on *N. tabacum*. The RNA-Seq indicates that inactive YggS significantly impairs T3SS function and effectors translocation *in vitro*. The molecular details in the interaction between YggS and T3SS will be explored in the further study.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

YW, YT, and BH designed the experiments. YW, YZ, LC, BC, LX, WG, and YL performed the experiments and analyzed the data. YW wrote the manuscript. YZ, YT, and BH revised the manuscript. YT and BH provided guidance for the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.783862/full#supplementary-material

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