Phenylalanine 4-Hydroxylase Contributes to Endophytic Bacterium 
*Pseudomonas fluorescens*’ Melatonin Biosynthesis

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Melatonin acts both as an antioxidant and as a growth regulatory substance in plants. *Pseudomonas fluorescens* endophytic bacterium has been shown to produce melatonin and increase plant resistance to abiotic stressors through increasing endogenous melatonin. However, in bacteria, genes are still not known to be melatonin-related. Here, we reported that the bacterial phenylalanine 4-hydroxylase (PAH) may be involved in the 5-hydroxytryptophan (5-HTP) biosynthesis and further influenced the subsequent production of melatonin in *P. fluorescens*. The purified PAH protein of *P. fluorescens* not only hydroxylated phenylalanine but also exhibited L-tryptophan (L-Trp) hydroxylase activity by converting L-Trp to 5-HTP in vitro. However, bacterial PAH displayed lower activity and affinity for L-Trp than L-phenylalanine. Notably, the PAH deletion of *P. fluorescens* blocked melatonin production by causing a significant decline in 5-HTP levels and thus decreased the resistance to abiotic stress. Overall, this study revealed a possible role for bacterial PAH in controlling 5-HTP and melatonin biosynthesis in bacteria, and expanded the current knowledge of melatonin production in microorganisms.

Keywords: melatonin, endophytic bacteria, phenylalanine 4-hydroxylase, aromatic amino acid hydroxylases, 5-hydroxytryptophan

INTRODUCTION

Melatonin is a tryptophan-derived natural product and now recognized as a ubiquitous biomolecule synthesized in almost all species of animals, plants, and unicellular algae (Balzer and Hardeland, 1996; Hardeland and Poeggeler, 2003; Rodriguez-Naranjo et al., 2012; Arnao and Hernández-Ruiz, 2015). Melatonin is presumed to be an ancient molecule that appeared in primitive photosynthetic bacteria, and it originally functions as a free radical scavenger and antioxidant (Hardeland et al., 1995; Tan et al., 2015). The antioxidative function of melatonin is thought to be intrinsic and evolutionarily conserved in almost all organisms, including bacteria. During evolution, melatonin has been adopted by multicellular organisms to perform many other biological functions, such as acting as a signaling molecule to modulate mood, sleep, seasonal reproduction, and circadian rhythms in vertebrates (Hardeland, 2008; Pandi-Perumal et al., 2008; Reiter et al., 2009). In plants, melatonin has been reported to be involved in seed germination (Zhang et al., 2013; Zhang et al.,...
2014), flowering (Kolá et al., 2003), fruit maturation (Sun et al., 2016), root development (Pelagio-Flores et al., 2012), delaying chlorophyll degradation, leaf senescence (Zhang et al., 2016a), and tolerance against biotic and abiotic stress (Arnao and Hernández-Ruiz, 2010; Nawaz et al., 2016).

As shown in Supplementary Figure S1, the classic melatonin biosynthesis pathway in animals starts with tryptophan and involves four sequential reactions: tryptophan \( \rightarrow \) 5-HTP \( \rightarrow \) serotonin \( \rightarrow \) N-acetylserotonin \( \rightarrow \) melatonin. However, melatonin synthesis involves multiple pathways and can be regulated by up to six enzymes in plants (Back et al., 2016). Among the two enzymatic steps involved in the conversion of tryptophan to serotonin, 5-HTP is the first intermediate in animals catalyzed by tryptophan 5-hydroxylase (TPH) (Fitzpatrick, 2003); however, plants initially decarboxylate tryptophan via tryptophan decarboxylase to form tryptamine (Byeon et al., 2014). Currently, plants are also believed to catalyze tryptophan to 5-HTP, whereas the putative gene encoding TPH enzyme has yet to be identified (Back et al., 2016). The melatonin synthesis machinery in eukaryotes is presumed to have been inherited from bacteria via endosymbiosis (Tan et al., 2015). This is because primitive bacteria may be the precursors of mitochondria and chloroplasts, and the two organelles were proved to be the dominant sites for melatonin production in mitochondria and chloroplasts, and the two organelles were because primitive bacteria may be the precursors of mitochondria and chloroplasts, and the two organelles were because primitive bacteria may be the precursors of mitochondria and chloroplasts, and the two organelles were because primitive bacteria may be the precursors of mitochondria and chloroplasts, and the two organelles were.

As shown in Supplementary Table S1, the melatonin-synthesizing endophytic bacterium P. fluorescens RG11, previously isolated from the roots of V. vinifera cv. Red Globe (Ma et al., 2017), was aerobically grown in beef extract-peptone (BP) liquid medium (3 g L\(^{-1}\) beef extract, 10 g L\(^{-1}\) tryptone, 5 g L\(^{-1}\) NaCl; pH 7.4) at 28°C. E. coli DH5α and BL21 (DE3) were used as the host for PAH gene cloning and expression, respectively. The plasmid pMD18-T (TaKaRa, Dalian, China) was used as a vector for gene cloning, and pET-30a (+) (Novagen, Darmstadt, Germany) was used for gene expression. E. coli strains were grown in Luria-Bertani (LB) medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl at 37°C. Purified agar (1.0%; Pronadisa, Madrid, Spain) was added for solid LB medium. When required, the medium was supplemented with kanamycin or ampicillin at final concentrations of 50 and 100 mg/L, respectively.

**Phenylalanine 4-Hydroxylase Gene Cloning, Phylogenetic Tree Construction, and Sequence Alignment**

Genomic DNA of P. fluorescens RG11 was extracted with the EZNA Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA, United States) as recommended by the manufacturer. The full-length gene encoding PAH was amplified with the primers pah-f (5' - ATGAAGCGAGCGAAATCGTG-3') and pah-r (5' -TCACGC GGTTCGGTTTT-3') based on sequence information (GenBank accession no. NC_007492). The PCR products were cloned into the pMD18-T vector for sequencing. Plasmids were routinely isolated from E. coli DH5α using the TIANprep Rapid Mini Plasmid Kit (Tiangen, Beijing, China).

Protein sequences were obtained by querying the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/) for “phenylalanine 4-hydroxylase/monoxygenase,” “tyrosine 3-hydroxylase,” and “tryptophan 5-hydroxylase” as the keywords. In total, 38 AAAH amino acid sequences derived from animals (Homo sapiens, Mus musculus, Gallus gallus, and Bos taurus) and prokaryotic bacteria were randomly selected, and play a pivotal role in melatonin biosynthesis in P. fluorescens RG11. However, this putative melatonin-biosynthesizing mechanism needs to be tested experimentally.

To prove the hypothesis, the PAH-encoding gene from P. fluorescens RG11 was cloned and expressed the PAH protein in Escherichia coli for functional identification. A mutant strain of P. fluorescens lacking functional PAH was also generated to investigate the effect of PAH on bacterial melatonin biosynthesis. Taken together, this work demonstrated a correlation between bacterial PAH and melatonin biosynthesis, and it expands the available information on the role of melatonin in microorganism.
a phylogenetic tree was constructed with the neighbor-joining method using MEGA X software (Kumar et al., 2018). Some TPHs and PAHs were aligned using ClustalX version 2.1, and the figure was created using ESPript3.0 (http://escript.ibcp.fr/ESPript/ESPript) (Robert and Gouet, 2014).

Detection of PiPAH Gene Expression
Total RNA was isolated from P. fluorescens RG11 cultured in BP liquid medium using an E.Z.N.A. Bacterial RNA Kit (OMEGA Bio-Tek, United States) according to the manufacturer’s instructions. Following quantification with the NanoDrop ND-1000 (Thermo Scientific, United States), RNA was reverse transcribed into cDNA with the HiScript III 1st Strand cDNA Synthesis Kit with gDNA wiper (Vazyme, Nanjing, China) and used as a template for RT-PCR. Primers pah-f/pah-r were used to assess transcription of the PAH-encoding gene by amplifying the full-length sequence. To eliminate contributions from contaminating DNA in the DNase-treated samples, control experiments were performed in the absence of reverse transcriptase.

Phenylalanine 4-Hydroxylase Overexpression and Protein Purification
The prokaryotic expression vector pET-30a (+) was used for C-terminal His-tagged expression. The full-length phhA gene, encoding the P. fluorescens’ PAH, was PCR-amplified with the following primers: 5’-CATACATATGAAACAGGCAAA TAGTG-3’ (forward primer; the NdeI site is underlined) and 5’-GTGCTCGAGCCGGTCTTCTGTTTTT-3’ (reverse primer; the XhoI site is underlined). The amplified products were then purified, digested with NdeI and XhoI, gel-purified, and ligated into pET-30a. The resulting recombinant plasmid, named pET-30a-phhA, was transformed into E. coli BL21 (DE3) competent cells.

The E. coli BL21-pET-30a-phhA strain was cultured in LB medium supplemented with 50 μg/ml kanamycin at 37°C. When the OD600 reached approximately 0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO, to induce protein expression. After a 4-h incubation at 24°C when the OD600 reached approximately 0.6, isopropyl-

Measurement of Hydroxylation Activity Towards L-tryptophan and L-phenylalanine
The enzymatic activity of purified PiPAH-(His)6 was measured at a standard concentration of reactants (0.5 ml total) in 50 mM NaOH-HEPES buffer (pH 7.5) containing 500 U of catalase, 5 mM dithiothreitol, 0.5 mM tetrahydrobiopterin (BH4), and 100 μM FeSO4, as described previously (Kino et al., 2009) with some modifications. Briefly, the reactions were initiated by adding 0.25 μg of protein and variable concentrations of substrates, including L-Trp (0.4–2 mM) or L-Phe (0.1–2 mM). After incubation for 10 min at 35°C, the reaction was stopped by the addition of 0.5 ml of methanol. The amount of synthesized 5-HTP or L-tyrosine was measured using UPLC-MS/MS as described in our previous study (Jiao et al., 2016) with the quantification transitions of m/z 182→136 and m/z 221→204. The kinetic parameters for both substrates were calculated using Michaelis–Menten analysis. The effect of temperature on L-Trp hydroxylase activity for PiPAH was determined by pre-incubating the reaction mixture at temperatures from 10 to 55°C for 5 min. The reactions were initiated by adding 20 mM L-Trp into the standard mixture above and incubating for 10 min at the same temperature. All experiments were performed in triplicate.

Generation of Phenylalanine 4-Hydroxylase Deletion Mutants
As the first step toward obtaining PAH deletion mutants (ΔphhA), fragments upstream and downstream of phhA were amplified using primer pairs phhA-F1/phhA-R1 and phhA-F2/phhA-R2, respectively (sequences shown in Supplementary Table S2). The two PCR products were gel-purified and ligated using splicing by overlap extension PCR with the primer pairs phhA-F1/phhA-R2. The resulting fragment was cloned into the pMD18-T vector and then sub-cloned into the suicide vector pK18mobSacB (Schäfer et al., 1994) to produce pK18-ΔphhA. Gene replacement was performed through double homologous recombination. In brief, pK18-ΔphhA was introduced into the P. fluorescens RG11 strain by triparental mating using a helper strain E. coli DH5α containing the plasmid pRK600 (Finan et al., 1986). BP selection medium containing 1.5% agar and 50 μg/ml kanamycin was used to screen the first recombination events. The second crossover event was then selected by incubating kanamycin-resistant clones into a liquid medium supplemented with kanamycin for 24 h, and then plating onto BP plates containing 10% (w/v) sucrose. Double crossover recombinants were confirmed by PCR and sequenced with phhA-F1/phhA-R2 primers.

Microbial Cultivation With 15N Double-Labeled L-Tryptophan and UPLC-MS/MS Analysis
Wild-type and ΔphhA mutant RG11 strains were individually transferred to 20 ml of BP liquid medium and cultivated for 8–12 h at 28°C to a final OD600 of approximately 1.0. After
harvesting at 6,000 × g for 10 min, the cells were re-suspended to 1 × 10^8 cells ml^−1 in 0.9% sterilized saline solution using a Petroff-Hauser counting chamber (Hauser Scientific Company, Horsham, PA, United States). The resulting bacterial inocula (1 ml) were added to individual 100-ml brown bottles containing 50 ml of BP liquid medium with 500 mg L^−1 15N double-labeled l-Trp (Cambridge Isotope Laboratories, Andover, MA, United States). The cultures were incubated in a rotary shaker at 32°C with 160 rpm agitation in the dark under aerobic conditions. Three replicates were performed for each treatment.

Samples were obtained every 6 h for viable cell counts using the plate counting method after sufficient dilutions. The bacterial culture (1 ml) was diluted 1:1 with methanol and ultrasonicated (300 W, ice water) for 10 min to assist the extraction of melatonin. The resulting mixture was filtered using a 0.22-μm filter and then used to quantify 15N-tryptamine, 15N-5-HTP, 15N-serotonin, 15N-5-methoxytryptamine, 15N-N-acetylserotonin, and 15N-melatonin with UPLC-MS/MS as described in our previous study (Ma et al., 2017). All non-labeled standards were purchased from Sigma-Aldrich (St. Louis, MO, United States). The experiment was repeated in triplicate. The Student’s t-test was used to identify significant differences in melatonin levels and its precursors between wild-type and ΔphhA strains.

The Abiotic Stress Tolerance of ΔphhA Mutant and Wild-Type of P. fluorescens

The strain of ΔphhA mutant and wild-type of P. fluorescens were routinely transferred to LB medium and cultivated for 12–16 h at 37°C with 150 rpm of agitation to the final desired bacterial concentration of 1.5 × 10^8 CFU ml^−1 (OD600 of about 1.0). The cultures were harvested at this stage and used as the inoculum in the following experiments.

To investigate the abiotic stress tolerance of wild-type P. fluorescens toward NaCl, drought, and heat, 1 ml of inoculum was inoculated into 150 ml of LB and then treated with different conditions: (i) control (37°C); (ii) 400, 600, 1,000, 1,500, and 2,000 mM NaCl at 37°C; (iii) drought [20%, 30%, 40%, 50%, and 60% polyethylene glycol 6000 (PEG6000) at 37°C]; and (iv) high temperature (44°C, 48°C, 52°C, and 56°C). Then, the flasks were incubated in a thermostat shaker set to 150 rpm. Cultures were collected after 12 h, and viable cells (log10 CFU/ml) were counted by serial dilution plating on LB plates in triplicate. The experiment was repeated three times.

To test the effect of PAH on bacterial abiotic stress resistance, the cultures of ΔphhA mutant and wild-type of P. fluorescens were treated with NaCl (1.5 M), drought (50% PEG6000), or high temperature (52°C). An aliquot of 0.5 ml was taken at 1-h intervals, diluted in PBS buffer, and plated on LB plates in triplicate. The number of colonies appearing on the plates after 24 h of incubation at 37°C were counted.

RESULTS

Identification of the Phenylalanine 4-Hydroxylase Gene in P. fluorescens RG11 and Sequence Analysis

The phhA gene, encoding a PAH ortholog, was found in genomes of most P. fluorescens strains by querying "phenylalanine 4-monoxygenase" in the NCBI nucleotide database. In the previously sequenced chromosomal DNA of P. fluorescens SBW25 (NC_012660; Figure 1A), the phhA gene had been designated as the ORF PFLU_RS21870, and mapped between the ORFs of phhR and phhB. RT-PCR analysis confirmed that phhA was expressed in the RG11 strain after culturing in BP liquid medium (Figure 1B).

Subsequently, the phhA homologue was cloned from P. fluorescens RG11, translated it into a 263-aa protein (PfPAH), and constructed a phylogenetic tree to reflect the evolutionary separation between prokaryotic PAHs and animal aromatic amino acid hydroxylase (AAAHS). As shown in Figure 2, the tree formed two separate, well-defined clades of animal AAAHSs and prokaryotic PAHs. As expected, PfPAH localized to the clade of prokaryotic PAHs. Animal AAAHSs were divided into three subgroups (PAHs, TPHs, and THs), among which PAHs displayed a closer phylogenetic relationship with TPHs.

The PfPAH polypeptide was 32.81%–33.08% identical to human and rat PAH. It showed high identity to some bacterial PAHs that have been structurally characterized, including Pseudomonas aeruginosa (85.50%), C. violaceum (44.44%), and Legionella pneumophila (48.99%). From secondary structure prediction, the Biopterin-H domain, a signature of bioperin-dependent aromatic amino acid hydroxylases, was observed in all AAAHS sequences examined. As underlined by the black line in Supplementary Figure S2, animal TPHs and PAHs harbor an N-terminal ACT (aspartate TyrA) domain that was not found in PfPAH and other bacterial PAHs. Furthermore, two His residues and a Glu residue that cooperatively bind the catalytic iron atom (closed triangles in Supplementary Figure S2), as well as the cofactor binding sites (open triangles in Supplementary Figure S2), were relatively conserved within the biopterin-H domain.

In Vitro Expression and Enzymatic Analysis of PfPAH

To describe the enzymatic properties of the PfPAH, a prokaryotic expression vector pET-30a-phhA was constructed to express recombinant PfPAH-(His)_6 in vitro. After Ni²⁺ affinity chromatography and SDS-PAGE, the purified PfPAH protein had a molecular weight of ~30 kDa (Figure 3A), and was close to its theoretical size [31.14 kDa for the PfPAH-(His)_6].

To investigate the hydroxylation activities of PfPAH towards l-Phe and l-Trp, the reactions were performed at the optimum temperature (35°C; Figure 3B). In the hydroxylation of l-Phe, kinetic analyses showed that the K_m and V_max values were 0.73 ± 0.07 mM and 3.37 ± 0.13 μmol tyrosine/min/mg protein (Table 1;
mutant strains during bacterial culture with UPLC-MS/MS, tryptophan could be monitored in both wild-type and

compared to hydroxylate L-Phe. The lower activity and weaker substrate preference for L-Trp as PfPAH had tryptophan hydroxylase activity, but displayed lower activity and weaker substrate preference for L-Trp (Table 1; Figure 3D). In addition, the catalytic efficiency ($K_{cat}/K_m$) of PfPAH for L-Phe hydroxylation (2.40 s$^{-1}$ mM$^{-1}$) was 8.0-fold higher than that for L-Trp hydroxylation (0.30 s$^{-1}$ mM$^{-1}$). The results indicate that PfPAH had tryptophan hydroxylase activity, but displayed lower activity and weaker substrate preference for L-Trp as compared to hydroxylate L-Phe.

PfPAH Is Necessary for the Biosynthesis of 5-HTP and Melatonin in *P. fluorescens* RG11

PfPAH hydroxylates L-Trp into 5-HTP *in vitro*, suggesting that phhA may play a potential role in the biosynthesis of 5-HTP and melatonin *in vivo*. To test this functionality, a ΔphhA mutant RG11 strain that specifically lacks the intact phhA gene was generated. Notably, some melatonin precursors such as tryptamine, 5-HTP, and N-acetylserotonin were present in the fresh BP liquid medium at concentrations of 3.46–167.42 μg/L (Supplementary Figure S3), suggesting that these components could be present in the medium composition (beef extract or tryptone). Therefore, the *P. fluorescens* may assimilate and use these compounds in the early culture stages as melatonin intermediates. To prevent interference, 15N double-labeled L-Trp (15N-Trp) was added into the medium as the precursor. Furthermore, the flow of 15N-labeled tryptophan could be monitored in both wild-type and mutant strains during bacterial culture with UPLC-MS/MS analysis.

The chemical structures and chromatograms of standards (40–60 ng ml$^{-1}$) and their corresponding 15N-metabolites are presented in Supplementary Figures S4A,B, respectively. As for 15N-5-HTP, the 15N-isotope increases two and one molecular mass units for the parent and daughter ions, respectively, but the retention time was identical to that of the unlabeled 5-HTP standard. So, the production of 15N-5-HTP was judged to have occurred when a peak was obtained at the transitions of m/z$^{-}$ 223→205. Similarly, 15N-serotonin, 15N-5-methoxytryptamine, 15N-N-acetylserotonin, and 15N-melatonin were all detected and confirmed in wild-type RG11 strain cultures using the process described above. However, no peak was detected at the correct retention time for 15N-tryptamine at the transitions of m/z$^{-}$ 163→145 throughout the experiments. These findings suggest that 5-HTP may be a key intermediate of melatonin biosynthesis in the RG11 strain.

For the wild-type strain, 15N-5-HTP and 15N-N-acetylserotonin were detectable at 6 h and showed an increase in concentration over the course of incubation (Figure 4). Similar trends were observed for 15N-serotonin, 15N-5-methoxytryptamine, and 15N-melatonin, but their levels declined slightly at 30 h post-incubation. Moreover, we observed that PAH mutants displayed a significant decrease in 15N-5-HTP production of 1.54–7.28 μg/L (~8- to 19-fold lower than the wild type). In addition, 15N-serotonin, 15N-5-methoxytryptamine, and 15N-N-acetylserotonin were found in small quantities at 12–30 h, but the 15N-melatonin was absent in ΔphhA mutant cultures, suggesting that PAH loss of function caused a significant decline in the levels of 5-HTP and the subsequent melatonin precursors. The findings indicate that PAH was functional in 5-HTP and melatonin biosynthesis in the RG11 strain.
Phenylalanine 4-Hydroxylase Loss-of-Function Decreases the Tolerance to Abiotic Stress in P. fluorescens

The tolerance to different range of stressors for P. fluorescens RG11 and ΔphhA mutant was also evaluated. As shown in Figure 5A, wild-type RG11 strain reached the bacterial count of 10.82 log10 CFU/ml under the control condition (37°C) after incubation for 16 h. On the other hand, the overheating condition (>44°C) and the addition of salt (>400 mM) and PEG6000 (20–50%, w/v) inhibited bacterial growth. The cells of ΔphhA mutant strain exposed to 52°C, 1.5 M salt or 50% PEG6000 showed a significant reduction in viable cells than those of wild type strain after 20 min treatment (Figures 5B–D). Notably, an increase in survival of cells was observed when the ΔphhA strain was cultured under the extreme condition supplemented with 500 µg/L 5-HTP. The results suggest that the loss of function of PAH decreased the tolerance to abiotic stress in P. fluorescens.

DISCUSSION

The ability of melatonin biosynthesis may be a new biological function for endophytic bacteria in plant–microbe interactions. Several species of endophytic bacteria that synthesize melatonin were isolated previously (Jiao et al., 2016), and P. fluorescens RG11 is one of these endophytic bacteria and was further observed to enhance the level of endogenous melatonin in grape roots (Ma et al., 2017). However, almost all of the genes involved in the bacterial melatonin biosynthesis were not identified. The present study confirmed that PAH from P.
fluorescens hydroxylated l-Trp to form 5-HTP and thus affected melatonin production downstream, which filled a knowledge gap in current melatonin research in microorganisms.

Phylogenetic analysis showed that animal PAHs had a closer genetic relationship with TPHs than with THs (Figure 2), suggesting that both enzymes share a higher degree of sequence similarity. According to the phylogenetic evidence and sequence homology, Lin et al. (2014) and Gjetting et al. (2001) proposed that the prokaryotic PAH gene is a progenitor of a hydroxylase that subsequently diverged to form TPHs, PAHs, and THs in animals. Thus, the substrate specificities of PAHs and TPHs are not absolute. Indeed, human TPH and PAH readily hydroxylate both tryptophan and phenylalanine with comparable kinetics (Jeffrey et al., 2001). It is reasonable to infer that prokaryotic PAHs could hydroxylate l-Trp.

Traditionally, the role of PAH in Pseudomonas species, such as P. aeruginosa and P. putida, is to catabolize l-Phe as a source of carbon and nitrogen (Herrera and Ramos, 2007). In the present study, PAH from P. fluorescens not only hydroxylated l-Phe to produce tyrosine but also hydroxylated l-Trp to form 5-HTP. The function of l-Trp hydroxylation for PAH has been verified by previous reports, in which different prokaryotic PAH-encoding genes were utilized to produce 5-HTP via combinatorial protein and engineered E. coli (Sun et al., 2015) and Saccharomyces cerevisiae (Zhang et al., 2016b). However, prokaryotic recombinant PAH previously displayed lower activity and

![Figure 3](link_to_image)

**FIGURE 3** | PIPAH protein characterization. (A) PIPAH purification in E. coli. SDS-PAGE image shows molecular mass markers in Lane 1, total protein of bacterial cells without IPTG in Lane 2, total protein after IPTG treatment in Lane 3, and purified PIPAH in Lane 4. (B) Effect of temperature on PIPAH l-Trp hydroxylase activity measured with 20 mM l-Trp, 0.5 mM BH4, and 100 μM FeSO4. Each data point represents the mean ± standard deviation (n = 3). Different letters indicate significant difference by Tukey’s multiple comparison test (p < 0.05). (C) Effect of l-Phe concentration on PIPAH hydroxylase activity for l-Phe. (D) Effect of l-Trp concentration on PIPAH hydroxylase activity for l-Trp. The curves in Figures 3C,D were fit to the non-linear Michaelis–Menten equation.

**TABLE 1** | Steady-state kinetic parameters of PIPAH hydroxylase activity for amino acids.

| Substrates      | $V_{max}$ (μmol tyrosine/min/mg protein) | $K_{m}$ (mM) | $K_{cat}$ (s$^{-1}$) | $K_{cat}/K_{m}$ (s$^{-1}$ mM$^{-1}$) |
|-----------------|----------------------------------------|--------------|----------------------|--------------------------------------|
| l-Phenylalanine | 3.37 ± 0.13 b                          | 0.73 ± 0.07 b | 1.75 ± 0.07 a        | 2.40                                 |
| l-Tryptophan     | 0.96 ± 0.04 a                          | 1.67 ± 0.19 a | 0.50 ± 0.02 b        | 0.30                                 |

Values are the mean of three replicates ± standard deviation. In columns, different letters indicate significantly different at p < 0.05 according to Student’s t-test.
In vitro (Kino et al., 2009; Lin et al., 2014). The feature is consistent with our finding, in which the affinity \((K_m)\) of PfPAH for L-Trp was approximately 2.3-fold lower than that for L-Phe (Table 1). Additionally, PfPAH exhibited a much lower L-Trp hydroxylation activity than TPH in humans (Daubner et al., 2002). According to previous observation in C. violaceum (Kino et al., 2009), such L-Trp hydroxylase activity of PAH can be improved in vitro through mutagenesis of the amino acid sites that were expected to interact with cofactors and substrate. The L-Trp hydroxylation may be the initial step for melatonin biosynthesis in P. fluorescens. Therefore, it remains to be further verified whether site-specific mutagenesis of PfPAH can improve L-Trp preference and hydroxylase activity, thus enhancing 5-HTP and melatonin production.

At present, no substantial evidence exists that shows that prokaryotic PAHs is involved in bacterial melatonin biosynthesis. In the present study, \(^{15}\text{N}\)-tryptamine was not detected in the P. fluorescens RG11 cultures (Supplementary Figure S4), whereas the concentration of \(^{15}\text{N}\)-5-HTP increased throughout the incubation period (Figure 4). The production of \(^{15}\text{N}\)-5-HTP, as well as the subsequent production of \(^{15}\text{N}\)-serotonin, decreased to below detectability in the PAH loss-of-function strain. This suggests that serotonin was synthesized via L-Trp hydroxylation similar to that observed in animals or the unicellular organism Gonyaulax polyedra (Burkhardt and Hardeland, 1996) (Supplementary Figure S1). However, a low level of \(^{15}\text{N}\)-5-HTP was still detectable during the culture of \(\Delta\text{phhA}\) mutant strain, indicating that the carbon
skeleton of isotopic L-Trp flowed into 5-HTP by another enzyme with L-Trp hydroxylase activity, albeit operating at a lower activity. Tan et al. (2016) recently proposed an additional conversion pathway from serotonin to melatonin based on extensive evidence from plants, in which serotonin is first O-methylated to 5-methoxytryptamine and then N-acetylated to melatonin. This is because serotonin may be a more preferable substrate for plant N-acetylserotonin methyltransferase (ASMT), and is thus more easily O-methylated (Byeon et al., 2015). The preferred substrate for plant type serotonin N-acetyltransferases (SNATs) is 5-methoxytryptamine rather than serotonin (Lee et al., 2015). Consistently, 5-methoxytryptamine was also detected in the P. fluorescens RG11 strain; thus, P. fluorescens could produce melatonin by synthesizing 5-methoxytryptamine. The bacterial SNATs could be a member of the GCN5-related N-acetyltransferase (GNAT) family, which catalyzes the transfer of the acetyl group from acetyl coenzyme A to a number of compounds including histones, aminoglycosides, and arylalkylamines (Dyda et al., 2000). Given the success of identifying plant ASMT genes from the O-methyltransferase family of plants (Kang et al., 2011), it is fair to assume that bacterial ASMT may be discovered in the OMT family as well. Protein structure classification (Jia et al., 2021) could be used to find similar enzymes.

Overall, the study provided evidence that prokaryotic PAHs may contribute to the first step of melatonin biosynthesis in P. fluorescens. However, the finding does not suggest that all bacterial species rely on this protein to produce 5-HTP and melatonin. Further studies focusing on other genes, such as bacterial SNAT and ASMT, involved in the melatonin biosynthesis in P. fluorescens are still required. In addition, the conversion of L-Trp to tryptamine, which is one of the initial steps in melatonin biosynthesis in plants, is also an alternative pathway for indole-3-acetic acid biosynthesis in some endophytic bacteria (Hartmann et al., 1983; Carreño-Lopez et al., 2000). Therefore, it can be hypothesized that bacterial melatonin biosynthesis may be complex and species-dependent. Moreover, endophytes can interact with their plant hosts, perhaps both could synthesize melatonin by exchanging intermediates. Therefore, it is necessary to determine whether some endophytic bacteria might utilize the melatonin intermediates within plant host to produce melatonin in the symbiotic relationship.
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 below: NCBI (accession: KY652930).

AUTHOR CONTRIBUTIONS

JJ and YX designed and performed experiments and corote the manuscript. JJ conceived this project and supervised the experiments. YZ, XW, XZ, TB, SS, CS, and MW analyzed the data. CL and JP advised on experimental design, acquired the research funds, and provided some modifications. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.746392/full#supplementary-material

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