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Identification of a Gene Involved in the Negative Regulation of Pyomelanin Production in *Ralstonia solanacearum* S

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Ralstonia solanacearum causes bacterial wilt in a wide variety of host plant species and produces a melanin-like blackish-brown pigment in stationary phase when grown in minimal medium supplemented with tyrosine. To study melanin production regulation in *R. solanacearum*, five mutants exhibiting overproduction of melanin-like pigments were selected from a transposon (Tn) insertion mutant library of *R. solanacearum* SL341. Most of the mutants, except one (SL341T), were not complemented by the original gene or overproduced melanins. SL341T showed Tn insertion in a gene containing a conserved domain of eukaryotic transcription factor. The gene was annotated as a hypothetical protein, given its weak similarity to any known proteins. Upon complementation with its original gene, the mutant strains reverted to their wild-type phenotype. SL341T produced 3-folds more melanin at 72 h post-incubation compared with wild-type SL341 when grown in minimal medium supplemented with tyrosine. The chemical analysis of SL341T cultural filtrate revealed the accumulation of a higher amount of homogentisate, a major precursor of pyomelanin, and a lower amount of dihydroxyphenylalanine, an intermediate of eumelanin, compared with SL341. The expression study showed a relatively higher expression of *hppD* (encoding hydroxyphenylpyruvate dioxygenase) and lower expression of *hmgA* (encoding homogentisate dioxygenase) and *nagL* (encoding maleylacetoacetate isomerase) in SL341T than in SL341. SL341 showed a significantly higher expression of tyrosinase gene compared with SL341T at 48 h post-incubation. These results indicated that *R. solanacearum* produced both pyomelanin and eumelanin, and the novel hypothetical protein is involved in the negative regulation of melanin production.

Keywords: Melanin overproduction, negative regulation, pyomelanin, *Ralstonia solanacearum*

Introduction

The word "melanin" is derived from *melanos*, the Greek word for "dark" [1]. Melanin is defined as a group of negatively charged hydrophobic macromolecules, formed by oxidative polymerization of phenolic or indolic substances [2, 3]. Melanin is an amorphous compound that lacks an exact structure but exists in a range of colors like black, brown, and red. Chemically, it is insoluble in organic solvent and resistant to acid and bleaching by strong oxidizing agents [4]. Melanin is produced by most of the living organisms, from eukaryotes to prokaryotes [5]. Although melanin is not essential for the growth of the producing organism, it provides some advantages for its producer against different environmental challenges, such as protection from ultraviolet radiation, binding with toxic metals, oxidative stress response, and protection from heat and cold [6–9]. Therefore, melanin has received much attention for numerous biotechnological applications in the cosmetic, pharmaceutical, electronic, and food processing industries [10, 11].

Melanin can be classified into four categories: eumelanin,

pheomelanin, allomelanin, and pyomelanin [10, 11]. Eumelanin is produced during tyrosine catabolism via oxidative reaction from tyrosine to *o*-dihydroxyphenylalanine (DOPA). Pheomelanin is also produced from DOPA, but DOPA first undergoes cysteinylation, followed by polymerization. Pyomelanin is produced during the catabolism of tyrosine via the oxidation of homogentisate (HGA). Allomelanin is formed via the oxidation and polymerization of dihydroxynaphthalene through the pentaketide pathway. Bacteria usually produce either DOPA- or HGA-based melanins such as eumelanin or pyomelanin. Bacteria that produce DOPA-based melanin include *Bacillus, Rhizobium, Streptomyces*, and *Marinomonas* [12–14], whereas those that produce HGA-based pyomelanin include *Pseudomonas aeurginosa, Vibrio, Shewanella, Xanthomonas*, and *Burkholderia* [15–19].

Ralstonia solanacearum is a soilborne plant pathogen that causes destructive wilt disease in more than 200 different plant species worldwide, including many commercially important plants [20]. It has already been established that the genome of *R. solanacearum* GMI1000 possesses genes encoding tyrosinase (*tyr*) for eumelanin production and a pathway for HGA-based pyomelanin synthesis [21]. Previously, two tyrosinase genes were identified and it was suggested that *R. solanacearum* produces eumelanin [22]. In our previous study, we identified three different regulatory genes along with a gene encoding 4-hydroxyphenyl pyruvate dioxygenase (*hppD*) involved in pyomelanin synthesis. We reported that *R. solanacearum* produced pyomelanin, and identified a mutation in *hppD* responsible for attenuated pigment production in *R. solanacearum* [23].

In the present study, we selected a melanin-overproducing mutant and discovered a new gene that is involved in the negative regulation of pyomelanin production in SL341. The chemical analysis of mutant SL341T and wild-type SL341 revealed a higher pyomelanin production and a lower eumelanin production in SL341T compared with SL341. To correlate the chemical data with genetic data, we investigated the expression of *hppD*, a gene encoding homogentisate dioxygenase (*hmgA*), and a gene encoding maleylacetoacetate isomerase (*nagL*) involved in pyomelanin pathway, and *tyr* involved in the eumelanin pathway in SL341 and SL341T.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely cultured in Luria-Bertani (LB) medium at 37°C with shaking at 200 rpm. When necessary, antibiotics were added to the medium at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 10 μg/ml; ampicillin, 100 μg/ml. *R. solanacearum* strain SL341 of race 1 (phylotype I) was used [24], routinely cultured in casamino acid-peptone-glucose (CPG) [25] or CPG broth supplemented with 0.005% (w/v) 2, 3, 5-triphenyltetrazolium chloride (TTC) and 1.5% agar [26]. Selection of melanin-overproducing mutants was

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Characteristics	Reference
R. solanacearum		
SL341	Wild type, isolated from tomato plants, Race 1, Biovar 4	[24]
SL341T	*tfam* (RSc2080):: Tn5; Kan^r	This study
SL341TC	Transconjugant of SL341T carrying pRKT, complementation of SL341T; Kan^r, Tc^r	This study
E. coli		
DH5α	F^-ϕ80 *lac*ZDM15 D(*lacZYA-argF*) *U* 169 deoR *recA*1 *endA*1 *hsdR*17 (rk^-,mk^+) *phoA supE44λ- thi*-1 *gyrA*96 *relA*1	[29]
HB101	F-*thi-1hsdS20* (rB^-, mB^-) *supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 rpsL20* (str^r) *xyl-5 mtl-1*	[31]
Plasmid		
pUC119	Ap^r; cloning vector	[28]
pGEM-T Easy	Ap^r; T/A cloning vector	Promega
pGEMT	Ap^r; pGEM-T Easy carrying 904 bp fragment of *tfam* gene of *R. solanacearum* SL341	This study
pRK415	Tc^r; PK2-derived broad-host-range cloning vector	[30]
pRKT	Tc^r; pRK415 carry 904 bp EcoR1 fragment of pGEMT containing *tfam* gene of *R. solanacearum* SL341	This study
pRK2013	Km^r; mobilization helper plasmid for triparental mating	[32]

Kan^r, chromosomal kanamycin resistance; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance.

performed in MG minimal medium containing 1% mannitol, 0.2% L-glutamic acid, 0.05% KH_2PO_4, 0.02% NaCl, and 0.02% $MgSO_4$ supplemented with 500 μg/ml tyrosine solution. The following antibiotics were used in the CPG, TTC, and MG media: kanamycin, 25 μg/ml; tetracycline, 10 μg/ml. Yeast extract-dextrose-$CaCO_3$ medium, containing 1% yeast extract, 2% glucose, 2% $CaCO_3$, and 1.5% agar, was used for triparental mating between *R. solanacearum* mutant strain and *E. coli* donor and helper strains.

Screening of Melanin-Overproducing Mutants and Identification of Transposon Insertion Site

A previously constructed transposon insertion mutant library of SL341 [27] was screened to select melanin-overproducing mutants to compare with wild-type strain SL341. Mutants grown on MG agar plates were randomly selected and inoculated on 96-well culture plates containing 200 μl of minimal medium supplemented with 500 μg/ml of tyrosine solution. The 96-well plates were incubated at 30°C in a shaking incubator at 200 rpm for 48 h. The mutant strain showing a higher pigmented phenotype compared with wild-type SL341 was selected and further confirmed by plating on MG medium containing tyrosine. The transposon insertion site in the selected mutant was identified according to a method described previously [27]. Briefly, genomic DNA was extracted from the transposon mutant and randomly digested with SacI. Digested DNA was ligated into pUC119 [28], which was already digested with the same restriction enzyme. After completion of the ligation reaction, the ligation mixture was transformed into *E. coli* DH5α [29] and transformants were selected on LB agar plates containing kanamycin and ampicillin. Positive clones were selected from the plates and the recombinant plasmid was sequenced using the Tn5 transposon-specific primer in order to identify the transposon-flanking DNA sequences.

For complementation, a full-length copy of the mutated gene (RSc2080) was amplified from SL341 genomic DNA using specific primers (Table S1). Polymerase chain reaction (PCR) amplification was performed according to the following conditions: first denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at the specified temperature (Table S1) for 30 sec, and extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. The amplified PCR product was first cloned in the pGEM-T Easy vector (Promega, USA) (Table 1) and verified by DNA sequencing. Each gene from the pGEM-T Easy vector was digested with specific restriction enzymes (Table 1), and subsequently subcloned into pRK415 under the control of the *lac* promoter [30]. The recombinant plasmid carrying the gene of interest (Table 1) was introduced into the mutant strain through triparental mating using *E. coli* HB101 [31] harboring pRK2013 as a helper plasmid [32]. The triparental mating was performed as previously described [27].

Homology Modeling and Multiple Alignments

To analyze the homology of *R. solanacearum* hypothetical protein RSc2080 with a known homologous structure, SWISS-MODEL was used. SWISS-MODEL is a fully automated protein structure homology-modeling server accessible via the ExPASy Web server (http://swissmodel.expasy.org/). Multiple alignment analysis was performed using BioEdit software in combination with GeneDoc. The search for conserved domain and key protein residues was also performed based on the known protein conserved residues [33].

Spectrophotometric Analysis of Melanin Production

To investigate melanin production in SL341 and SL341T, both strains were grown in minimal medium supplemented with 500 μg/ml of tyrosine solution at 30°C with shaking at 200 rpm. Bacterial culture sample was collected from the main culture of these strains after 24 h and centrifuged at 13,000 ×*g* for 5 min. Cell-free supernatants were collected and their absorbance was examined at 400 nm using a spectrophotometer (Beckman Coulter, USA). The experiment was performed in triplicate. The number of viable bacterial cells was examined by dilution plate counting on TTC medium.

High-Performance Liquid Chromatography (HPLC)

HPLC analysis of the culture filtrates of both SL341 and SL341T was performed for detection of DOPA and HGA. Bacterial strains were grown in minimal medium supplemented with tyrosine at 30°C in a shaking incubator at 200 rpm. The bacterial culture sample was initially collected after 24 h from the wild-type SL341 and mutant SL341T. Samples were then collected from each strain at every 12 h until 72 h. Culture samples were centrifuged at 13,000 ×*g* for 5 min and supernatants were collected and filtered through a sterilized membrane filter (0.2 μm pore-sized; Corning Costar, USA). Twenty microliters of the sample was used in the Agilent 1100 series HPLC system fitted with a Zorbax Eclipse Plus C18 column (5 μm particle size; 250 mm 4.6 mm, Agilent Technologies, USA) and photodiode array detector. HPLC for detection of DOPA and HGA was performed as previously described [34]. Briefly, for elution, the mobile phase was 10 mM acetic acid-methanol (90:10 (v/v)). The elute flow rate was 1 ml/min. The total time for separation was 15 min. Tyrosine and DOPA were detected at 260 nm, whereas HGA was detected at 290 nm. Commercially available tyrosine, DOPA, and HGA (Sigma-Aldrich, USA) were used as standards.

Reverse Transcription-Quantitative PCR (RT-qPCR)

The expression levels of *hppD*, *hmgA*, *nagL*, and *tyr* (RSc1501) were examined in SL341 and SL341T by RT-qPCR. Total RNA was extracted from the 1 ml culture of SL341 and SL341T collected at 48 h, using a RNA Hybrid-R extraction kit (GeneAll Bio Inc., Korea). RNA was eluted in 50 μl of RNase-free water and directly used as a template for cDNA synthesis using a Tetro cDNA synthesis kit (Bioline, UK), following the manufacturer's instructions. Before cDNA synthesis, each RNA sample was treated with DNaseI to remove any trace of DNA contamination. The concentration and purity of cDNA of each sample were measured by using a

NanoDrop2000 spectrophotometer (Thermo Scientific, USA). Samples were normalized and amplification reactions were performed using the CFX384 Real-Time PCR Detection System (Bio-Rad, USA). Primers used for qPCR amplification reaction are listed in Table S1. Each reaction mixture contained SYBR Green supermix (Bio-Rad), 1 μl of diluted cDNA template, 10 μM of both forward and reverse primers, and RNase-free water. Thermal cycling included two reaction steps: an initial preheat for 3 min at 95°C, followed by 39 cycles at 95°C for 5 sec, 55°C for 10 sec, and 72°C for 35 sec. The qPCR data were analyzed using the CFX Manager software ver. 3.1 (Bio-Rad). Each reaction was performed in triplicate. The V3 region of the 16S rRNA gene of *R. solanacearum* was used as a reference gene to normalize the expression level of each gene. RT-qPCR results were evaluated using the iCycleriQ Real-Time PCR Detection System (Bio-Rad). The cycle threshold (C(t)) values of each qPCR product were used to determine the target cDNA concentration based on the comparison with V3 gene expression. The Student *t*-test was used to compare the expression of *hppD*, *hmgA*, *nagL*, and *tyr* in wild-type SL341 and mutant SL341T.

Results

Selection of Melanin-Overproducing Mutants of SL341

R. solanacearum SL341 (phylotype 1) produces melanin-like blackish brown pigment in tyrosine-supplemented minimal medium [23]. Using SL341 as a reference, we screened a total of 4,000 mutants to identify melanin-overproducing mutants. Based on the initial screening, five mutants with high-pigmented phenotype were selected and subsequently analyzed.

Identification of a Gene Encoding a Negative Regulator Involved in Melanin Production

To identify the disrupted gene in the selected mutant strains, subclones of each mutant were sequenced with Tn-specific primers. The sequence comparison revealed that one mutant (SL341T) exhibited mutation in a gene coding for hypothetical protein (RSc2080), whereas the other four mutants showed mutations in genes encoding gyrase inhibitor, glutamate racemase, a membrane transporter protein (TolQ), and peptidoglycan-associated lipoprotein. Among these mutants, we chose the SL341T mutant strain showing mutation in hypothetical protein RSc2080 because of its consistent and high-pigmented phenotype. In fact, none of the mutants complemented with its original gene, except SL341T. Even though RSc2080 did not share significant amino acid sequence identity with any other functional protein, its homology modeling revealed close structural similarity with a transcriptional factor A of human (*Homo sapiens*) and mouse (*Mus musculus*) (Fig. S1). Multiple alignment analysis of the protein encoding transcription factor A displayed 22% identity with the amino acid sequences of RSc2080 (Fig. 1) [33]. Moreover, RSc2080 shared

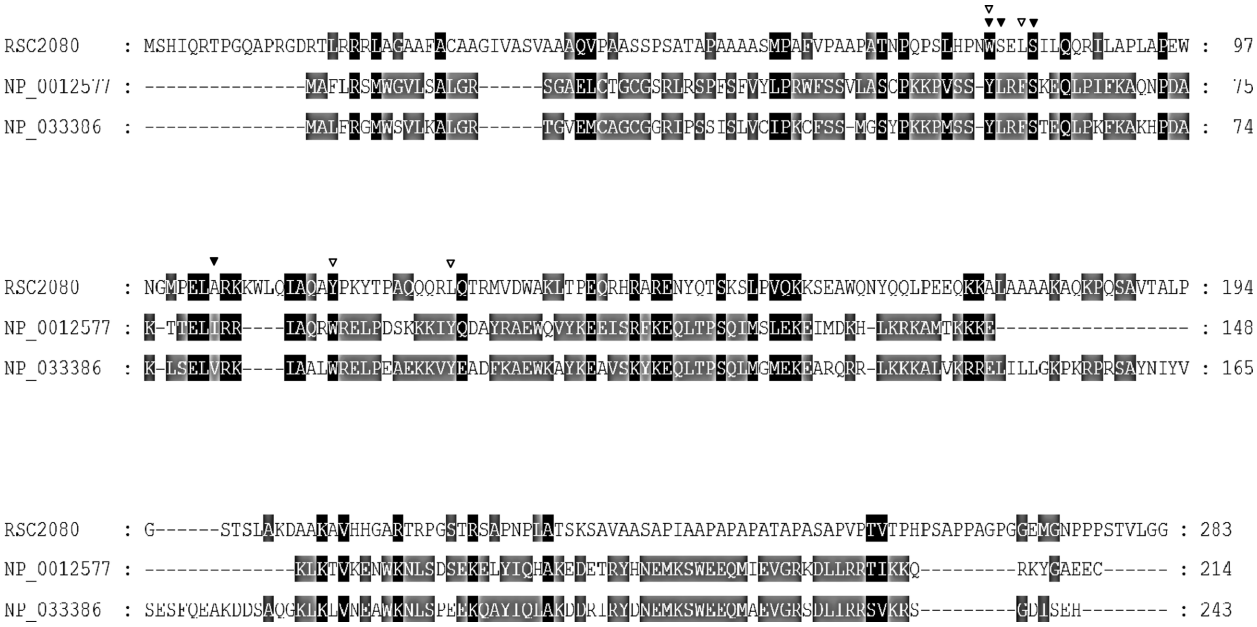

Fig. 1. Multiple alignment of hypothetical protein RSc2080 with NP_ 0012577 (*Homo sapiens*) and NP_033386 (*Mus musculus*). The amino acid residues interacting with DNA are indicated as inverse closed triangles, and the residues that are involved in core formation and stabilization are indicated by open inverse triangles. The core includes tyrosine (Y)57 (replaced by W), phenylalanine (F)60 (replaced by leucine (L)), tryptophan (W)88 (replaced by Y), and Y99 (replaced by L).

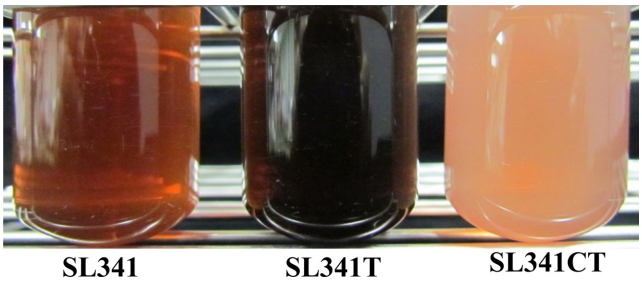

Fig. 2. Complementation of the melanin-overproducing mutant strain SL341T and complemented strain SL341CT with restored wild-type phenotype (photo taken at 72 h post incubation).
The bacterial strains were grown in minimal medium supplemented with 500 µg/ml of tyrosine solution at 30°C with shaking at 200 rpm.

conserved residues involved in key protein functions (Fig. 1), including amino acid residues interacting with DNA and those involved in core formation and stabilization. Even though most of the key protein residues were similar, substitutions in RSc2080 were detected in the core-forming region, such as phenylalanine (F)60 (replaced by leucine (L)), tryptophan (W)88 (replaced by tyrosine (Y)), and Y99 (replaced by L). To verify the role of high pigmentation related to the disrupted gene, complementation of SL341T (*tfam*$^{-}$) was conducted by transferring the original full-length mutated gene into SL341T, which resulted in reversion of the pigmentation pattern (Fig. 2).

Quantitation of Melanin Production and Accumulation of Melanin Intermediates

To quantify melanin production in SL341 and SL341T, we analyzed the culture filtrate of each strain at different time points until 72 h incubation. Bacterial growth of SL341T was slightly delayed in the early stage of bacterial growth compared with SL341. However, the number of viable cells of SL341T was equivalent to those of SL341 at 72 h post-incubation (Fig. 3A). In contrast to the equivalent bacterial growth between wild-type strain SL341 and mutant SL341T, SL341T produced a smaller amount of melanin pigment compared with SL341 until 24 h post-incubation; however at 48 h post-incubation, melanin production by SL341T increased dramatically (Fig. 3B). Melanin production by SL341T increased as much as 3-folds at 72 h post-incubation.

To elucidate the nature of the black pigment, culture filtrates of SL341 and SL341T were analyzed to detect either DOPA or HGA. The first culture filtrate was collected at 24 h post-incubation. Subsequent collections were carried out every 12 h until 72 h. Both SL341 and SL341T started to

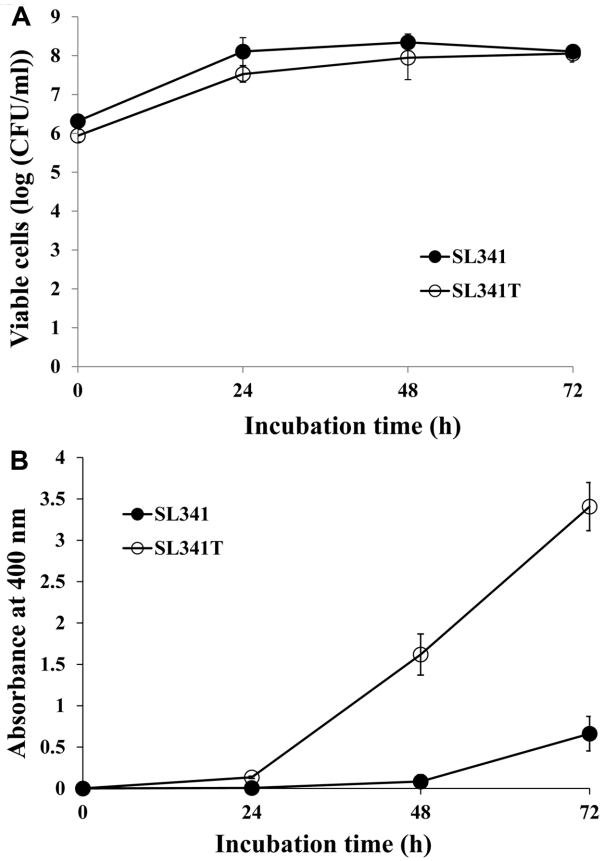

Fig. 3. Bacterial growth and comparison of melanin production between wild-type SL341 and mutant SL341T strains.
(**A**) Bacterial growth over time, measured by viable cell count, between SL341 and SL341T. (**B**) Melanin production estimated by measuring absorbance of cell-free supernatant of each strain at 400 nm over time. Vertical bar represents the standard deviation of three biological replicates.

produce DOPA at 36 h post-incubation, and melanin production ability was almost similar between the two strains until 60 h post-incubation (Fig. 4A). However, after 72 h, SL341 produced a relatively higher amount of DOPA compared with SL341T (Fig. 4A, Figs. S2C–S2E). After 24 h, SL341T produced high amounts of HGA, which increased steadily at 48 and 60 h post-incubation (Figs. 4B, S2C–S2F). A rather rapid decrease in HGA production at 72 h post-incubation was observed in the culture filtrate of SL341T, suggesting the rapid conversion of HGA to pyomelanin. In the culture filtrate of SL341, HGA production was detected at 48 h post-incubation, which slowly increased until 72 h post-incubation (Figs. 4B, S2C–S2F), indicating a slower and lower production of pyomelanin in SL341 during the stationary growth phase. The tyrosine catabolic activity of SL341 and SL341T was similar until 36 h. However,

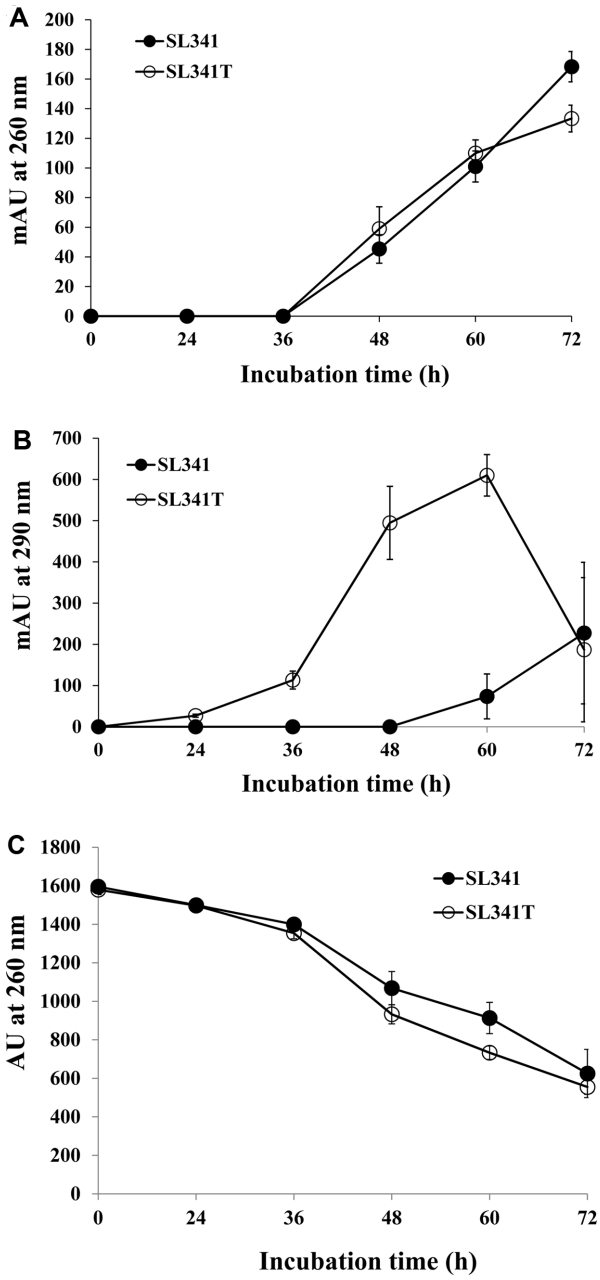

Fig. 4. Production of melanin intermediates and tyrosine catabolism over time in wild-type SL341 and mutant SL341T strains, assessed by high-performance liquid chromatography. (**A**) Accumulation of *o*-dihydroxyphenylalanine, an intermediate in eumelanin synthesis, over time between wild type and the mutant. (**B**) Production of homogentisate, an intermediate of pyomelanin synthesis, over time. (**C**) Tyrosine catabolism assessed in the culture filtrate of wild-type SL341 and mutant SL341T strains. The vertical bar represents the standard deviation of three biological replicates.

SL341T steadily consumed tyrosine at a higher rate than SL341 did until 72 h post-incubation (Fig. 4C).

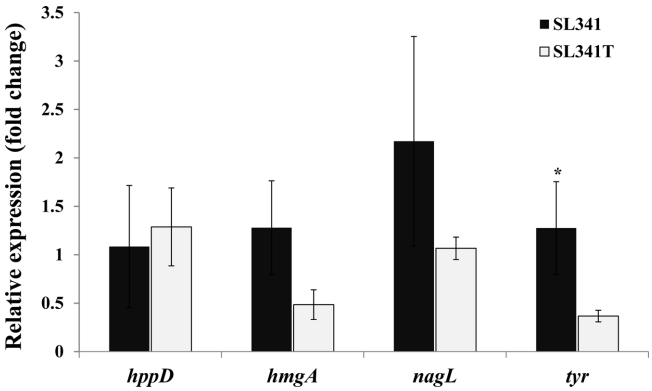

Fig. 5. Relative expression of *hppD*, *hmgA*, *nagL*, and *tyr* analyzed by reverse transcription-quantitative polymerase chain reaction in wild-type SL341 and mutant SL341T strains grown in tyrosine-supplemented minimal medium at 48 h post-incubation.

The expression level of *hppD*, *hmgA*, *nagL*, and *tyr* was normalized using expression of the V3 region of the 16S rRNA gene as a reference. Vertical bars represent standard deviations of three biological replicates. The asterisk represents significant difference among strains (*, $p < 0.05$ by *t*-test).

Gene Expression in the Regulatory Mutant

The expression level of *hppD*, *hmgA*, and *nagL* (involved in the pyomelanin pathway) as well as *tyr* (involved in the DOPA-melanin pathway) was investigated in SL341 and SL341T at 48 h post-incubation. The expression of *hppD* was relatively higher in SL341T than in SL341. The expression of *hmgA* and *nagL* was relatively lower in SL341T than in SL341 (Fig. 5). The expression of *tyr* was significantly higher in SL341 ($p < 0.05$) than in SL341T at 48 h post-incubation (Fig. 5).

Discussion

R. solanacearum produces a melanin-like blackish-brown pigment in the stationary phase in tyrosine-supplemented minimal medium. Although it is known that *R. solanacearum* produces two different types of melanin, eumelanin and pyomelanin [22, 23], the regulation process that leads to the production of these two different melanins in *R. solanacearum* has not been dissected.

Several genomes of *R. solanacearum* strains have been completely analyzed [21], showing the presence of metabolic pathways for pyomelanin and eumelanin biosynthesis. Previously, we have shown that three different regulatory genes along with *hppD* are responsible for pyomelanin production in SL341 [23]. For successful pyomelanin

production, the ABC transporter responsible for HGA export is essential in some bacteria such as human pathogenic *P. aeruginosa* [35], but it is not clear whether *R. solanacearum* also requires the transporter for pyomelanin production. In the present study, SL341T showing melanin overproduction was selected and investigated for its genetic and chemical characteristics of melanization.

The mutated gene by transposon insertion in SL341T exhibited 22% identity in the amino acid sequence with the eukaryotic transcription factor A, which is responsible for the expression, organization, and maintenance of mitochondrial DNA copy number in mammals [36]. Although the amino acid sequence identity with transcription factor A was very low, some key amino acids involved in protein stabilization and core formation were conserved in RSc2080. To verify if RSc2080 plays a role in regulation of gene expression in *R. solanacearum*, it will be necessary to purify RSc2080 and investigate if the protein binds DNA and regulates the expression of genes relevant for pyomelanin production.

The culture filtrates of *R. solanacearum* SL341 grown to stationary phase in liquid culture with tyrosine exhibited eumelanin and pyomelanin accumulation in almost equivalent amounts. Previously, production of two different kinds of melanin has been reported in *Aspergillus fumigatus* and *Aeromonas* [37-39]. Further study to understand the reason why *R. solanacearum* produced two different kinds of melanin is necessary. Our previous study on SL341D defective in *hppD* activity revealed the production of a trace amount of eumelanin, and SL341D grown in minimal medium with tyrosine did not exhibit any pigmentation due to pyomelanin production [23]. This result suggested that eumelanin production had a minor contribution in pigmentation in *R. solanacearum* compared with pyomelanin. It was suggested that pyomelanin production in *R. solanacearum* enhances the bacterial tolerance against oxidative stress [23].

DOPA production in both SL341T and SL341 was similar; however, SL341T produced a rather significantly high amount of HGA. Therefore, this result led us to the conclusion that RSc2080 may be involved in the regulation of gene expression in SL341. The high-pigmented phenotype of SL341T was mainly due to the accumulation of pyomelanin, since SL341T and SL341 produced almost the same amount of DOPA, whereas differential accumulation of HGA was the only difference between SL341 and SL341T. Therefore, we can speculate that this transcription factor-like protein might be involved in the negative regulation of pyomelanin production in *R. solanacearum*.

The expression study of selected genes of the pyomelanin pathway and eumelanin further elucidated the molecular basis of pyomelanin production in SL341T. A relatively high expression of *hppD* and low expression of *hmgA* in SL341T may result in accumulation of a sufficient amount of HGA in SL341T, which is subsequently oxidized, causing the production of higher amounts of pyomelanin. Lower expression of *nagL*, which is located downstream of *hmgA* in the pyomelanin pathway, confirmed our result, because a small amount of 4-maleylacetoacetate produced in tyrosine catabolism in SL341T may cause lower expression of *nagL* in SL341T compared with SL341. It is not clear whether RSc2080 directly regulates the transcription of *hppD* and *hmgA*. The tyrosinase activity of SL341T was also lower than that of SL341, suggesting the lower production of eumelanin in SL341T. In contrast, the HPLC analysis of bacterial culture filtrates showed the same level of DOPA accumulation in both SL341T and SL341 at 48 h post-incubation. Since the readily oxidizable nature of DOPA makes its quantitation difficult, eumelanin production in SL341T may not be directly quantified based on DOPA accumulation. However, it was clear that eumelanin production did not increase in SL341T compared with SL341, based on the lower expression of *tyr* and equivalent accumulation of DOPA in SL341T. This result clearly indicated that overproduction of melanin pigment in SL341T was due to pyomelanin production. Previously, we have shown that *R. solanacearum* produced pyomelanin under the positive transcriptional control of OxyR, RpoS, and HrpG, which suggested that this bacterium produced pyomelanin under different physiological conditions [23]. Since *R. solanacearum* normally does not produce melanin-like pigment during exponential growth, it is plausible to predict the presence of a negative regulator of pyomelanin production in *R. solanacearum*. Based on our results, we speculate that RSc2080, showing homology with eukaryotic transcription factor, may negatively regulate pyomelanin production in *R. solanacearum*.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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