J. Microbiol. Biotechnol. (2015), 25(6), 903–909
http://dx.doi.org/10.4014/jmb.1408.08047

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Expression of Auxin Response Genes *SlIAA1* and *SlIAA9* in *Solanum lycopersicum* During Interaction with *Acinetobacter guillouiae* SW5

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Received: August 22, 2014
Revised: January 2, 2015
Accepted: January 12, 2015

First published online
January 15, 2015

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pISSN 1017-7825, eISSN 1738-8872

Indole-3-acetic acid (IAA) production is a typical mechanism of plant growth promotion by some rhizobacteria. However, a functional genomic study is necessary to unravel the function and mechanism of IAA signaling during rhizobacteria-plant interactions. In this study, the expression of *SlIAA1* and *SlIAA9* among the auxin response genes in tomato was examined during the interaction between IAA-producing *Acinetobacter guillouiae* SW5 and tomato plants. When 3-day grown tomato seedlings were treated for 30 min with 10~100 μM of IAA produced by bacteria from tryptophan, the relative mRNA levels of *SlIAA1* and *SlIAA9* increased significantly compared with those of the control, demonstrating that IAA produced by this bacterium can induce the expressions of both genes. Inoculation of live *A. guillouiae* SW5 to tomato seedlings also increased the expressions of *SlIAA1* and *SlIAA9*, with more mRNA produced at higher bacterial density. In contrast, treatment of tomato seedlings with dead *A. guillouiae* SW5 did not significantly affect the expression of *SlIAA1*and *SlIAA9*. When 3-day bacterial culture in tomato root exudates was administered to tomato seedlings, the relative mRNA level of *SlIAA1* increased. This result indicated that the plant may take up IAA produced by bacteria in plant root exudates, which may increase the expression of the auxin response genes, with resulting promotion of plant growth.

Keywords: *Acinetobacter guillouiae*, auxin response genes, indole-3-acetic acid, tomato root exudate, tryptophan

Introduction

Phytohormone production may be one of the most important mechanisms of growth promotion and development of plants by plant growth-promoting rhizobacteria (PGPR). Plants can synthesize phytohormones and also take up and utilize exogenous sources, such as microbially produced phytohormones [22]. There have been many reports on phytohormone production by various PGPR in culture media. However, little is known concerning bacterial production of phytohormones in the rhizosphere or plants either *in vitro* or *ex vitro* [7]. Although the effects of PGPR on hormone concentrations in plant root and subsequently on plant growth and function are well known, uptake of bacteria-produced phytohormones by plants and signaling pathways activated by phytohormones have not been reported [7, 25].

Auxin is a typical phytohormone, whose effects, action mechanisms, and biosynthetic pathways in plants and some bacteria are established. Indole-3-acetic acid (IAA) is the most important natural auxin [23]. Although auxin also acts as a signal molecule in bacteria-plant interactions, studies on auxin have mainly focused on the inhibition of the expression of *vir*, the virulence gene in *Agrobacterium tumefaciens* that is active in crown gall formation and formation of N-fixing nodules in legume by rhizobia [23]. The role of auxin as a key factor in bacterial phytogenesis has been reported [22]. The auxin receptor, auxin signaling and auxin-regulated genes in plant cells have been extensively studied [3, 6, 26]. Plant auxin-inducible genes, whose expressions increase within 30 min after exposure to synthetic auxin, are composed of several families, including *GH3*, *SAUR*, and *Aux/IAA* [3]. Among them, the presence of roles of the *Aux/IAA* gene family in tomato

plants has been reported [2, 26]. However, the role of bacteria-produced auxin in the signaling involved in plant growth promotion has not been investigated. Thilmony *et al*. [24] reported the changes of expression of auxin-related genes in *Arabidopsis thaliana* infected by a phytopathogenic strain of *Pseudomonas syringae*. The study just showed the repression or activation of auxin-related genes in the plant after infection by bacterial pathogen.

Interactions between soil bacteria and plants are very important because they may deleteriously or positively affect plant production in pathogenic interactions or symbiotic interactions, respectively. A better understanding of these interactions requires knowledge of bacteria-plant signaling processes. To date, studies have been mainly limited to the interaction between plants and phytopathogens [4, 5, 24]. Studies on the signaling processes between plants and PGPR demonstrated an involvement of some phytohormones and biochemicals, such as polysaccharides and quorum sensing molecules, at the initial stage of interaction [8]. The plant genes involved in the initial signaling process triggered by PGPR have not been studied [25].

In this study, we hypothesized that *SlIAA1* and *SlIAA9*, members of subfamilies I and V in the *Aux/IAA* family, respectively [17], are involved in initial signal pathways in plants responding to PGPR-secreted auxin because they are induced immediately after IAA exposure. We investigated the interaction between tomato plants and IAA secreted by *Acinetobacter guillouiae* SW5 [12] by examining the expressions of the two auxin response genes.

Materials and Methods

Treatment of Tomato Seedlings with Synthetic and Bacterial IAA

Seeds of tomato (*Solanum lycopersicum* cv. Yegwang) were disinfected with 70% ethanol for 5 min and 0.2% hydrogen peroxide solution for 2 min, washed five times with sterile distilled water (DW), and placed on Whatman No. 2 filter paper soaked with 5 ml of DW in a sterile Petri dish. These seeds were germinated in a plant growth chamber for 7~14 days (30°C, light intensity of 97.5 μmol photons m^{-2} s^{-1} for 12 h, and 24°C, dark conditions for 12 h). Germinated tomato seedlings (20 g fresh wt) were treated with 0.1~300 μM of synthetic IAA (Sigma Chemical, USA) or 100 μM of L-tryptophan dissolved in 30 ml of DW. They were also treated with 30 ml of 30 μM bacteria-produced IAA solutions obtained from a culture of IAA-producing *Acinetobacter guillouiae* SW5 [12] grown in tryptophan-supplemented DW. The bacterial culture was centrifuged and the concentration of IAA in the supernatant was measured by high-performance liquid chromatography (HPLC) as described previously [11] and adjusted with DW. Tomato seedlings (20 g) were also inoculated with 30 ml of the bacterial suspension in DW, in which the density of washed bacterial cells grown in nutrient broth was adjusted to 10^3, 10^6, and 10^9 CFU/ml, with 10^6/ml of autoclaved cells also administered to tomato seedlings. All these treatments were done at 30°C in the dark for 30 min or 6 h. The expression of auxin response genes of tomato plants *SlIAA1* and *SlIAA9* in each treatment was examined.

Treatment of Tomato Seedlings with Bacterial Culture in Tomato Root Exudates

Eight tomato seedlings germinated for 3 days were cultivated on soaked filter paper in the plant growth chamber for 3 days. Tomato seedlings were removed after incubation and root exudates were extracted from the filter papers with 40 ml of sterile DW. The tomato root exudates solution was inoculated with 10^6 washed cells of *A. guillouiae* SW5 and incubated for 24 h at 30°C and 150 rpm in the dark. The culture was filtered through a membrane with a pore size of 0.2 μm and the concentration of IAA in the root exudates produced by bacteria was analyzed by HPLC. Germinated tomato seedlings (0.1 g) were treated with a 1-day bacterial culture in tomato root exudate for 30 min at 30°C in the dark, and the expression of *SlIAA1* in tomato plants was examined.

Isolation of Tomato Total RNA and cDNA Synthesis

Isolation of the total RNA from the tomato seedlings was performed with a slightly modified TRIzol method [20]. Tomato seedlings (20 g) reacted in each treatment were ground in a mortar with liquid nitrogen. One milliliter of TRIzol reagent (Invitrogen, USA) was added to 0.1 g of the ground seedlings and the mixture was vigorously mixed for 15 sec in a vortex mixer and retained at room temperature for 5 min. Two hundred microliters of chloroform was added, vigorously mixed for 15 sec, and centrifuged (10,000 ×*g*, 4°C, 15 min). Cold isopropyl alcohol (500 μl) was added to the supernatant retrieved, and the mixture was inverted and reacted for 15 min. After reaction, the solution was centrifuged for 10 min and the supernatant was discarded. For the washing of the remaining RNA pellet, 1 ml of 70% ethanol:30% diethyl pyrocarbonate (DEPC) solution was added and centrifuged for 5 min. The supernatant was discarded and the RNA pellet was dried at room temperature and redissolved with 44 μl of DEPC solution (1 ml DEPC in 1 L DW). Extracted RNA was identified with electrophoresis and cDNA was synthesized with oligo dT from 2 μg of total RNA using a TOPscript cDNA synthesis kit (Enzynomics, South Korea) according to the manufacturer's instructions. Synthesized cDNA was also identified by DNA electrophoresis.

Primer Design and Sequence Analysis for *SlIAA1* and *SlIAA9*

Primers for amplification of the *SlIAA1* and *SlIAA9* auxin response genes in tomato were designed using Primer3 (Input ver. 0.4.0) software (http://frodo.wi.mit.edu/) based on the sequence information for *SlIAA1* (SGN No. U323670; GenBank Accession No. JN379431) and *SlIAA9* (SGN No. U313802; GenBank Accession

Table 1. The primer sequences of the tomato genes *SlIAA1* and *SlIAA9* for PCR and real-time PCR.

Aux/IAAs	SGN number	Primer	Sequence
SlIAA1	SGN-U323670	IAAstd1F	5′-GGAGGAGAGAAAGAAAATG-3′
		IAAstd2F	5′-GGATTTCATTTGGTGTTTTGG-3′
		IAAstd1R	5′-GGCTTGGGATGTGGAGTT-3′
		IAAstd2R	5′-ATCCCAAGCCTCTTGCTTCT-3′
		IAA1F	5′-ATGATGTTTTTCCTGTTAGATCTCACT-3′
		IAA1R	5′-TGAATCTAAGTCAAGTTCTGATCATGTC-3′
SlIAA9	SGN-U313802	IAA9-f	5′-TGGCCACCCATTCGATCTTTAG-3′
		IAA9-rf	5′-AGACAAACTCCAATATCAAACGG-3′
		IAA9-r2	5′-CAGGTTCTGAGGTCCACTTTC-3′

F, f: forward primer; R, r: reverse primer.

No. JN379437) provided by Sol Genomics Network (SGN; http://solgenomics.net/) and the National Center for Biotechnology Information (NCBI GenBank; http://www.ncbi.nlm.nih.gov/). PCR primers were designed to make a product exceeding 500 bp for the sequence analysis. Primers for real-time PCR were designed to make a product less than 200 bp within the >500 bp product range, to accommodate a short denaturation time (Table 1). PCR was performed with PTC DNA Engine System (BioRad, USA) using previously synthesized cDNA as a template and forward primers IAA1std1F and IAA1std2F, and reverse primers IAA1std1R and IAA1std2R for amplification of the *SlIAA1* and *SlIAA9* genes, respectively. The reaction mixture contained 10× Taq polymerase buffer 2.5 µl, 2.5 mM dNTP 2 µl, 10 pmol primer 1 µl each, Ex-taq polymerase (Takara, Japan) 0.125 µl, and DW to make a final volume of 25 µl. PCR conditions were initial temperature 94°C for 5 min, followed by 25 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 90 sec, and a final extension at 72°C for 5 min. The sizes of the PCR products were identified by electrophoresis, and DNA was extracted according to the manufacturer's instructions using a MinElute gel extraction kit (Qiagen, Germany). Purified DNA product was ligated into the *p*GEM-T Easy vector (Promega Corp., USA), and the ligated product was mixed with competent *Escherichia coli* (DH5α), and reacted for the transformation. The transformed product (42 µl) was inoculated in LB medium containing 50 µl/ml of ampicillin and incubated at 37°C and 200 rpm for 90 min. After centrifugation (10,000 ×*g*, 25°C, 10 sec), 100 µl of supernatant was discarded and the remaining 142 µl was inoculated in ampicillin LB medium containing 100 µl of isopropyl-1-thio-D-galactopyranoside and 10 µl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and incubated at 37°C and 200 rpm for 24 h. Plasmid DNA was extracted as described using the HiYield plasmid mini kit (RBC Bioscience, Taiwan). To confirm the proper insertion of the product into the plasmid DNA, *Eco*RI and *Sal*I digestion was done and the DNA bands were observed after electrophoresis. The sequences of the identified plasmid DNA were analyzed (Macrogen, South Korea) and compared with those of *SlIAA1* and *SlIAA9* provided by NCBI and SGN.

Real-Time PCR Analysis of Expression of Auxin Response Genes

The relative expression of *SlIAA1* and *SlIAA9* in each treatment of tomato seedlings was examined by real-time PCR. Copy numbers of *SlIAA1* and *SlIAA9* plasmid DNA were calculated from the size and concentration of the plasmid DNAs using Peak Scanner ver. 1.0 software (Applied Biosystems, USA), and adjusted to 2×10^3/100 µl. *SlIAA1* and *SlIAA9* plasmid DNAs were amplified with 5 µl of the plasmid DNA solution as a template using real-time PCR, and the standard curves were drawn with the cycle threshold (Ct) obtained. The concentration of cDNA synthesized with the total RNA extracted from the tomato seedlings was adjusted to 20 ng/µl with the addition of RNase-free water using a model Infinite M200 microplate reader (Tekan, Germany), and was used as a template. Quantitative PCR (qPCR) was performed with the StepONE Real-time PCR system (AB Applied Biosystems, USA) using primers IAA1F and IAA1R for the *SlIAA1* gene and IAA9-f and IAA9-r2 for the *SlIAA9* gene. Reactions contained 5 µl of template, 1 µl each of 10 *p*mol primers, 12.5 µl of Maxima SYBR Green qPCR Master Mix 2× (Fermentas, USA), and DW to make a final volume of 25 µl. qPCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min and 1 cycle of 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Each reaction was run in triplicate and Ct was calculated using the standard curve and expressed as the relative mRNA level of each sample.

Analysis of variance (ANOVA) was performed on all experimental data, and means were compared using the SYSTAT software (ver. 10; SPSS, USA) followed by the Bonferroni post-hoc test. The significance level was $p < 0.05$.

Results

Isolation of RNA and Design of Primers

Electrophoretic analysis of tomato RNA showed that the total RNA was efficiently extracted from both uninoculated control and inoculated tomato seedlings. These synthesized were also identified by the band sizes. When PCR was

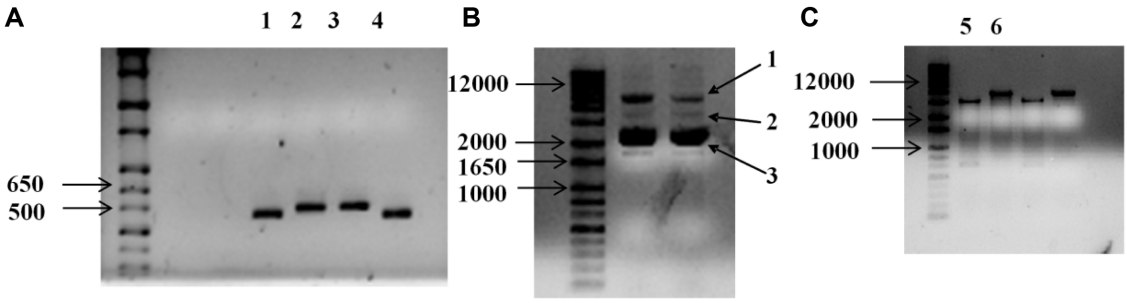

Fig. 1. Tomato cDNA PCR band of *SlIAA1* gene using four primers (**A**), plasmid DNA (**B**; 1: nicked circular DNA; 2: circular DNA; and 3: supercoiled DNA) and enzyme cutting band (**C**).

The primers used and sequence length in each lane are as follow; lane 1: IAA1std1F, IAA1std1R (595 bp); lane 2: IAA1std2F, IAA1std2R (638 bp); lane 3: IAA1std2F, IAA1std1R (646 bp); lane 4: IAA1std1F, IAA1std2R (587 bp). Restriction enzymes of lanes 5 and 6 were *Eco*RI and *Sal*I, respectively. The left lane in each figure shows the DNA size marker (bp).

performed with the cDNA as a template and the four primer combinations for tomato gene *SlIAA1*, the sizes of the bands in the electrophoresis coincided with the size information expected to be amplified by each primer (Fig. 1A). Insertion of the DNA extracted from the bands in Fig. 1A into the *p*GEM T Easy vector was identified after restriction enzyme treatment of plasmid DNA, followed by electrophoresis (Fig. 1C). The DNA sequences analyzed showed 100% similarity compared with those of *SlIAA1* and *SlIAA9* deposited in NCBI and SGN databases (data not shown).

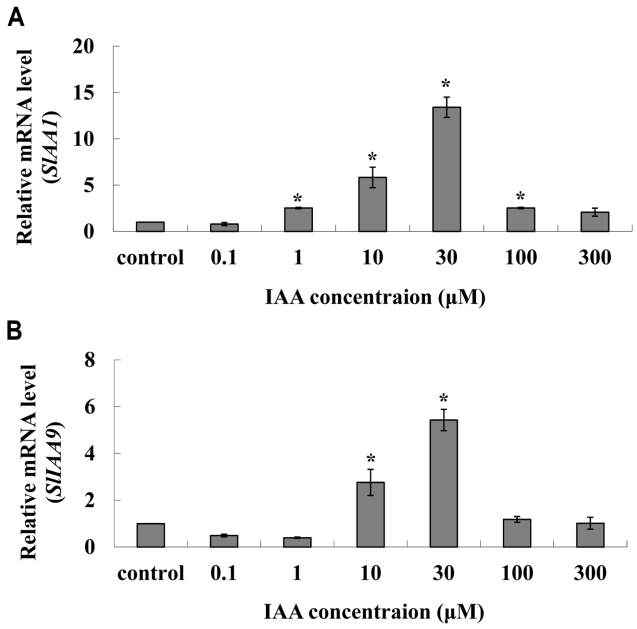

Fig. 2. Real-time PCR analysis of the relative mRNA levels of tomato genes *SlIAA1* (**A**) and *SlIAA9* (**B**) after 30 min treatment of tomato seedlings with synthetic IAA.

Detection of *SlIAA* Expression in Tomato Seedlings After IAA Treatment

The expression levels of *SlIAA1* and *SlIAA9* were examined by real-time PCR analysis to study the interaction between tomato plant and PGPR *A. guillouiae* SW5. When synthetic IAA was administered to tomato seedlings at various concentrations for 30 min, expression of both genes increased at 10~100 μM and showed the highest relative mRNA levels at 30 μM of IAA (Fig. 2). Treatment of 30 μmol of IAA produced by *A. guillouiae* SW5 from tryptophan for 30 min also significantly increased the expression of *SlIAA1* 10.9-fold compared with the control, which was almost the same, displaying an 11.5-fold increase following treatment with synthetic IAA (Fig. 3). Treatment with 100 μM IAA produced a relatively weaker effect compared with 30 μM of IAA, and addition of 100 μM of tryptophan, a precursor of IAA, did not have any significant effect on the expression of *SlIAA1*. Relative mRNA levels of *SlIAA1* and *SlIAA9* in tomato seedlings treated for 6 h with 30 μM of

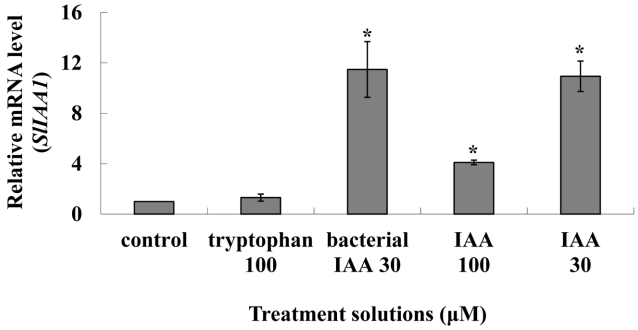

Fig. 3. Real-time PCR analysis of the relative mRNA levels of tomato gene *SlIAA1* after 30 min treatment of tomato seedlings with tryptophan, synthetic IAA, and bacteria-produced IAA.

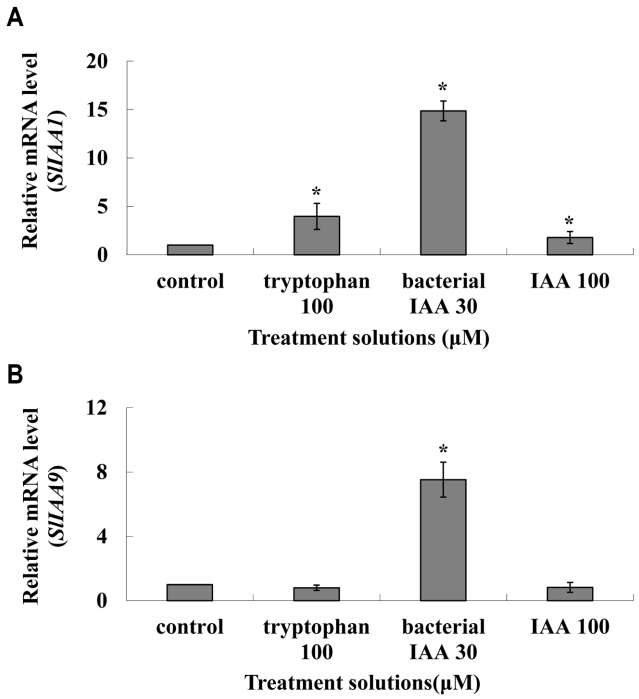

Fig. 4. Real-time PCR analysis of the relative mRNA levels of tomato genes *SlIAA1* (**A**) and *SlIAA9* (**B**) after 6 h treatment of tomato seedlings with tryptophan, synthetic IAA, and bacteria-produced IAA.

bacteria-produced IAA were increased 14.9-fold and 7.5-fold, respectively, compared with those of untreated control (Fig. 4). At the same time, addition of 100 μM of tryptophan produced a 3.9-fold increased production of only *SlIAA1* mRNA; the effects of 100 μM synthetic IAA were less than those of the 30 min treatment.

Detection of *SlIAA* Expression in Tomato Seedlings After PGPR Treatment

When the tomato seedlings were treated with washed cells of *A. guillouiae* SW5 for 30 min, the relative mRNA levels of *SlIAA1* were significantly increased at 10^6 and 10^9 CFU/ml compared with those of the uninoculated control (Table 2). The expression of *SlIAA9* increased significantly at all bacterial densities. Although the relative mRNA levels of *SlIAA9* compared with the control were higher than those of *SlIAA1*, the copy number of *SlIAA9* of the control was less than one-quarter that of *SlIAA1*. Inoculation of autoclaved cells (10^6 ml^{-1}) of *A. guillouiae* SW5 to tomato seedlings did not produce significant increases of the relative mRNA levels of both genes. When tomato seedlings were treated for 30 min with the 1-day bacterial culture in tomato root exudates, the relative mRNA level of *SlIAA1* increased by 3.4-fold compared with that of the uninoculated control. This bacterial culture contained 4.06 ± 0.13 μM of IAA produced from the root exudates of tomato seedlings by *A. guillouiae* SW5.

Discussion

The expression levels of auxin-inducible genes increase within 30 min after treatment of synthetic auxin. The genes contain several families, including *GH3*, *SAUR*, and *Aux/IAA* [3]. Among them, the presence, roles, and activities of the *Aux/IAA* gene family have been reported from *Arabidopsis thaliana* [1, 19] and recently from tomato [2, 17, 27]. However, the role and signaling of auxin produced by PGPR have not been studied at the molecular level. In this study, the expression of *Aux/IAA* genes in tomato, *SlIAA1* and *SlIAA9*, was investigated in tomato plants treated with IAA produced by *A. guillouiae* SW5 and the bacterial cells of *A. guillouiae* SW5, which promote the growth of tomato plants [14]. When 10~100 μM of synthetic IAA was administered to tomato seedlings, the expression of *SlIAA1* and *SlIAA9* increased significantly (Fig. 2). Increases of expression of several *Aux/IAA* genes in tomato or other plants have also been reported in this IAA concentration range [1, 9, 17]. Although the reason for this expression increase is still unknown, plant growth enhancement has also been reported at the same range of IAA concentration [21, 28]. The maximal expression of *SlIAA1* after auxin treatment occurs in 60 min or less, and is grouped as fast class *Aux/IAA* genes [1]. However, the precise response of *SlIAA9* has not been reported, and the relatively weaker response of *SlIAA9* compared with *SlIAA1* after a 30-min IAA treatment was also similar to prior results [17].

Table 2. The relative mRNA levels of tomato genes *SlIAA1* and *SlIAA9* after 30 min treatment of tomato seedlings with *A. guillouiae* SW5.

Gene	Uninoculated control	Autoclaved cells (10^6 ml^{-1})	Live cells (10^3 ml^{-1})	Live cells (10^6 ml^{-1})	Live cells (10^9 ml^{-1})
SlIAA1	1.00	1.31 ± 0.05	1.67 ± 0.80	5.93 ± 2.61*	135.34 ± 97.74*
SlIAA9	1.00	1.17 ± 0.15	5.01 ± 2.35*	7.36 ± 1.55*	265.21 ± 149.62*

Average ± SD, *: $p < 0.05$ in contrast to uninoculated control by Bonferroni post hoc test.

Thirty-minute treatment of tomato plants with 30 μM of synthetic IAA and bacteria-produced IAA from IAA precursor tryptophan produced similar induction of the expression of *SlIAA* (Fig. 3), which suggests the bacterial IAA has the same effect on the plants as synthetic IAA. Addition of tryptophan did not significantly increase the expression of *SlIAA1*. Although the expression of the fast class *Aux/IAA* genes shows immediate induction, it may maintain expression levels even after 8 h of IAA treatment [17]. Expression levels of *SlIAA1* and *SlIAA9* were also pronounced after a 6-h treatment with IAA produced by *A. guillouiae* SW5 (Fig. 4). In contrast to the result in Fig. 3, the treatment of tryptophan for 6 h significantly increased the expression of *SlIAA1*, which might be induced by IAA formed by tomato plant in the 6-h reaction period. Induction of expression of *Aux/IAA* in plants by tryptophan has not been reported. However, plants can also synthesize IAA from tryptophan using several IAA biosynthetic pathways [16]. Expression of *Aux/IAA* may follow. The time period (30 min) of tryptophan treatment in Fig. 3 might have been too short for the conversion of tryptophan to IAA by tomato plants.

In addition to the bacteria-produced IAA from tryptophan, 30-min treatment with 10^3~10^9 CFU/ml of washed *A. guillouiae* SW5 also increased the expression of *SlIAA1* and *SlIAA9* in tomato seedlings (Table 2). PCR analysis (data not shown) confirmed the previously reported presence of *Aux/IAA* genes in plants and not in bacteria [19]. Gene expression increased according to the increase of bacterial cell density, which indicates that this expression may be related to the activity of *A. guillouiae* SW5. IAA can be synthesized by bacteria *via* several biosynthetic pathways, including tryptophan-dependent and -independent pathways [22]. Since the first observation of a tryptophan-independent pathway of IAA biosynthesis in *Arabidopsis* [15], the pathway has been reported in *Azospirillum brasilense* [18]. However, the precise precursors and enzymes involved in these pathways remain unclear. The expression of *SlIAA1* and *SlIAA9* in tomato plants might be due to the IAA produced by bacteria using one or more pathways of IAA synthesis, because *A. guillouiae* SW5 has several IAA synthetic pathways, including the tryptophan-independent pathway [12]. *A. guillouiae* SW5 may use tryptophan or other compounds in the exudates from tomato or bacterial cells and/or some dead cells of tomato and bacteria as precursors of IAA synthesis. Induction of expression of *SLIAA1* and *SLIAA9* mediated by live and autoclaved cells of *A. guillouiae* SW5 revealed an influence only by viable cells (Table 2), which suggests that the induction of *Aux/IAA* genes by *A. guillouiae* SW5 requires bacterial viability.

In addition to the bacteria-produced IAA solution and bacterial cells, cultures of *A. guillouiae* SW5 in tomato root exudates also enhanced *SlIAA* mRNA production. Owing to the low concentration of IAA (4.06 μM) produced in tomato root exudates by *A. guillouiae* SW5 [14], the increase of relative mRNA level was only 3.4-fold compared with that of the control. This level is likely to be achieved because it was within a range of relative mRNA levels of *SLIAA1* treated with 1 and 10 μM of synthetic IAA (Fig. 2). This result indicates that *A. guillouiae* SW5 can produce IAA using precursors contained in tomato root exudates, which is then taken up by tomato plants, with the subsequent induction of expressions of *Aux/IAA* genes. Various organic acids, carbohydrates, and amino acids, including tryptophan from tomato root exudates, are utilized for growth of some rhizobacteria and for IAA biosynthesis [10, 13]. Low levels of growth and IAA production of *A. guillouiae* SW5 in tomato root exudates have been described [14].

The present study is the first report on the signaling process between the bacteria-produced IAA from plant root exudates and plant cell at the molecular level. Because the auxin signaling network is quite complex and there are many other auxin-response genes, more studies are necessary for the complete investigation of the signaling of bacteria-produced IAA in plant cells.

Acknowledgments

This work was supported by CAER (Center for Aquatic Ecosystem Restoration) of Eco-STAR project from MOE (Ministry of Environment, Republic of Korea).

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