

## Global Effect of Indole-3-Acetic Acid Biosynthesis on Multiple Virulence Factors of *Erwinia chrysanthemi* 3937<sup>∇</sup>

Shihui Yang,<sup>1</sup> Qiu Zhang,<sup>1</sup> Jianhua Guo,<sup>2</sup> Amy O. Charkowski,<sup>3</sup> Bernard R. Glick,<sup>4</sup> A. Mark Ibekwe,<sup>5</sup> Donald A. Cooksey,<sup>6</sup> and Ching-Hong Yang<sup>1\*</sup>

Department of Biological Sciences, University of Wisconsin—Milwaukee, Milwaukee, Wisconsin 53211<sup>1</sup>; Department of Plant Pathology, Nanjing Agricultural University, Nanjing, China<sup>2</sup>; Department of Plant Pathology, University of Wisconsin—Madison, Madison, Wisconsin 53706<sup>3</sup>; Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1<sup>4</sup>; USDA-ARS-George E. Brown Jr. Salinity Laboratory, Riverside, California 92507<sup>5</sup>; and Department of Plant Pathology, University of California—Riverside, Riverside, California 92521<sup>6</sup>

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**Production of the plant hormone indole-3-acetic acid (IAA) is widespread among plant-associated microorganisms. The non-gall-forming phytopathogen *Erwinia chrysanthemi* 3937 (strain Ech3937) possesses *iaaM* (ASAP16562) and *iaaH* (ASAP16563) gene homologues. In this work, the null knockout *iaaM* mutant strain Ech138 was constructed. The IAA production by Ech138 was reduced in M9 minimal medium supplemented with L-tryptophan. Compared with wild-type Ech3937, Ech138 exhibited reduced ability to produce local maceration, but its multiplication in *Saintpaulia ionantha* was unaffected. The pectate lyase production of Ech138 was diminished. Compared with wild-type Ech3937, the expression levels of an oligogalacturonate lyase gene, *ogl*, and three endopectate lyase genes, *pelD*, *pelI*, and *pelL*, were reduced in Ech138 as determined by a green fluorescent protein-based fluorescence-activated cell sorting promoter activity assay. In addition, the transcription of type III secretion system (T3SS) genes, *dspE* (a putative T3SS effector) and *hrpN* (T3SS harpin), was found to be diminished in the *iaaM* mutant Ech138. Compared with Ech3937, reduced expression of *hrpL* (a T3SS alternative sigma factor) and *gacA* but increased expression of *rsmA* in Ech138 was also observed, suggesting that the regulation of T3SS and pectate lyase genes by IAA biosynthesis might be partially due to the posttranscriptional regulation of the Gac-Rsm regulatory pathway.**

*Erwinia chrysanthemi* is an opportunistic phytopathogen that causes soft rot, wilt, and blight diseases on a wide range of plant host species (18, 39). Several virulence determinants have been discovered in *E. chrysanthemi* including the type III secretion system (T3SS) and well-studied extracellular enzymes such as pectate lyase and pectinase (35, 97).

In *E. chrysanthemi*, the pectinases (Pel), which are secreted through the type II secretion system, attack the plant cell wall pectin and lead to the loss of plant cell wall structural integrity (1, 44, 91, 93, 98, 99). The production of pectinases is induced by pectin and its derivatives including polygalacturonate (PGA) and is tightly regulated by environmental conditions (5, 39, 40). Several transcriptional regulators, including Fur, HNS, KdgR, PecS, PecT, cyclic AMP receptor protein, ExpR, and Pir, have been shown to modulate the expression of the genes encoding Pel enzymes (12, 13, 19, 20, 29, 39, 67, 68, 70, 74, 83, 84, 85, 86, 95). In addition, several interconnections between these regulatory systems have been observed (39, 67, 69, 71, 72, 73, 74). Consequently, this complex regulatory network allows *E. chrysanthemi* to use pectin as a sole carbon and energy source for growth as well as to finely adjust the synthesis of its virulence factors during pathogenesis.

The T3SS is considered one of the major pathogenic factors

in many bacterial pathogens (14, 21, 31, 32, 35, 103, 104). Recently, a T3SS regulatory pathway of strain Ech3937 was elucidated (105). Similarly to *Pseudomonas syringae* and *Erwinia* and *Pantoea* spp., the T3SS of Ech3937 is regulated by HrpX/HrpY, a two-component signal transduction system (TCSTS). The HrpX/HrpY system activates the enhancer HrpS. The HrpS protein activates the expression of an alternative sigma factor, HrpL (101, 105), and HrpL further activates the expression of genes encoding T3SS effectors such as DspE, structural components such as HrpA, and harpins such as HrpN.

Ech3937 also possesses an Rsm system, which plays a critical role in gene expression and has a profound effect on bacterial metabolism and behavior in many prokaryotic species. RsmA, *rsmB*, and RsmC are the major components of this global regulatory system. RsmA is a small RNA-binding protein that acts by repressing translation and by shortening the half-life of the mRNA species (24). *rsmB* is an untranslated regulatory RNA that binds RsmA and neutralizes its negative regulatory effect by forming an inactive ribonucleoprotein complex (24, 57). RsmC controls the production of RsmA and *rsmB* RNA by positively regulating *rsmA* and negatively controlling *rsmB* (26). The Rsm system has been reported to control the production of different extracellular enzymes like pectinases, proteases, and cellulases and secondary metabolites such as phytohormones, antibiotics, pigments, and polysaccharides (2, 9, 10, 15, 16, 25, 26, 36, 37, 46, 49, 53, 55, 57, 58, 65, 66, 80, 90, 100).

TCSTSs are used by organisms to respond to environmental

\* Corresponding author. Mailing address: Department of Biological Sciences, University of Wisconsin—Milwaukee, Milwaukee, WI 53211. Phone: (414) 229-6331. Fax: (414) 229-3926. E-mail: chyang@uwm.edu.

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stimuli and adapt to different environmental conditions. The TCSTS GacS/GacA has been reported to play important roles in various biological functions (17, 23, 38, 82). GacS is the putative sensor kinase, and GacA is the response regulator. GacS is suggested to activate GacA, and the activated GacA works as a transcriptional activator and further activates targeted genes. Many virulence factors including pectate lyase, exoprotease, and tabtoxin and syringomycin production are found to be regulated by GacS/GacA homologues in phytopathogens (17, 23). In *Pseudomonas syringae* pv. tomato, GacA acted as a master regulator of controlling regulatory RNA *rsmB*, several transcriptional activators, and alternative sigma factors (17). In *Erwinia carotovora* subsp. *carotovora*, GacA has been reported to stimulate the transcription of the pectate lyase, polygalacturonase, cellulase, and T3SS genes through the positive regulation of GacA on *rsmB* (14, 23, 42).

Auxin regulates almost every aspect of plant growth and development in various biological processes including cell division, elongation, and differentiation; root initiation and elongation; vascular system patterning; somatic embryogenesis; apical dominance; flower development; fruit ripening; and tropisms (28, 46, 48, 106). In the plant, indole-3-acetic acid (IAA) can be derived from either of two tryptophan-independent pathways, which may utilize indole-3-glycerol phosphate or indole as a precursor, or four tryptophan-dependent pathways including the indole-3-pyruvic acid (IPyA) pathway, the indole-3-acetonitrile pathway, the indole-3-acetaldoxime pathway, and the tryptamine pathway (41, 106). IAA can be conjugated to amino acids, sugars, and carbohydrates. IAA conjugates have been implicated in several important plant processes including IAA storage, transport, protection from enzymatic destruction, and targeting of the IAA for catabolism. The bioactive form of IAA is believed to be free IAA. Plants maintain the IAA concentration by a complex network of pathways through the interplay of IAA biosynthesis, conjugate formation, hydrolysis, and irreversible oxidation (11, 75).

Similar to plant IAA production, microorganisms also possess several different IAA biosynthetic pathways. The metabolic routes are classified in terms of their intermediates as the indole-3-acetamide (IAM), IPyA, indole-3-acetonitrile, and tryptamine pathways (22). One major route, the IAM pathway, is employed mostly by pathogenic bacteria including the gall-forming bacterium *Pseudomonas syringae* pv. *savastanoi*. First, oxidative decarboxylation of tryptophan leading to indole-3-acetamide is catalyzed by IaaM (tryptophan 2-monooxygenase). The conversion of indole-3-acetamide to IAA is catalyzed by IaaH (indole-3-acetamide hydrolase) (L-tryptophan→IAM→IAA) (Fig. 1) (77). Another common pathway, the IPyA pathway (Fig. 1), is the major IAA biosynthetic pathway used by plant growth-promoting bacteria including *Pseudomonas putida* GR12-2 (78). In many cases, a single bacterial strain may possess more than one pathway (59). The IAM pathway is involved primarily in gall formation, and the IPyA pathway enhances bacterial epiphytic fitness (4, 7, 8, 30, 60, 94).

Although the role of IAA biosynthesis by microorganisms is not fully understood, IAA provides bacteria with a mechanism to influence plant growth by supplementing the host plant's endogenous pool of auxin (3, 6, 78, 96, 102). In several cases, production of IAA by pathogenic bacteria is a major pathogenicity determinant in gall- and knot-forming bacterial species.

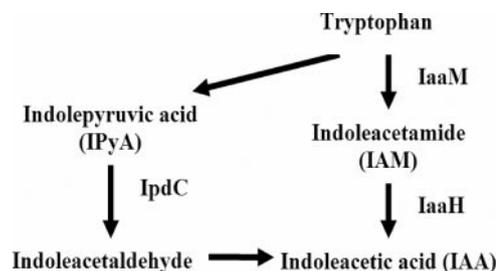


FIG. 1. IAM and IPyA metabolic routes of IAA biosynthetic pathways. IaaM, tryptophan-2-monooxygenase; IaaH, indole-3-acetamide hydrolase; IpdC, indole-3-pyruvate decarboxylase.

Recently, IAA has been shown to inhibit the growth of plant-associated pathogens (56). IAA was identified as a signature molecule for *Agrobacterium* transformation by down-regulating the expression of *virA* gene through the competition with the TCSTS VirA/VirG inducing signal acetosyringone after the transformation of T-DNA into the host by the bacterium (56).

The recently sequenced *E. chrysanthemi* 3937 genome revealed that the bacterium contains a complete set of IAA biosynthesis genes in the IAM pathway (unpublished results). Although the *iaaM* homologue of Ech3937 was discovered to be up-regulated in plant hosts (104), the ability of the bacterium to produce IAA and the role of IAA biosynthesis in Ech3937 pathogenesis are still unknown. In this study, we demonstrate that Ech3937 possesses the IAA biosynthesis gene homologues *iaaM* (ASAP16562) and *iaaH* (ASAP16563) and has the capacity to produce IAA through the IAM pathway. The expression of T3SS and pectinase genes under the wild-type Ech3937 and *iaaM* mutant background was examined using quantitative reverse transcription-PCR (qRT-PCR) and green fluorescent protein (GFP)-based fluorescence-activated cell sorting (FACS) promoter activity assays. Finally, the positive role of IAA biosynthesis for T3SS and exoenzymes through the Gac-Rsm posttranscriptional regulatory pathway was suggested in this work.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and chemicals.** The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type Ech3937 and mutant strains were stored at  $-80^{\circ}\text{C}$  in 15% glycerol and grown on LB agar and M9 minimal medium (MM) (92). Antibiotics ( $\mu\text{g/ml}$ ) used were as follows: ampicillin, 100; chloramphenicol, 25; kanamycin, 50; rifampin, 100; spectinomycin, 50; and tetracycline, 25. Primers used for PCR in this report are also listed in Table 1. IAA, IAM, L-tryptophan (Trp), and PGA sodium salt were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

**Mutant strain construction.** The *iaaM* (ASAP16562) deletion mutant Ech138 was constructed by a two-step PCR mutagenesis approach as described previously (103). Two sets of primers, *iaaM\_A\_Km* and *iaaM\_B\_Km* and primers *iaaM\_C\_Km* and *iaaM\_D\_Km*, which amplify the upstream and downstream flanking regions, respectively, of the *iaaM* gene were designed. The left PCR fragment (produced by primers *iaaM\_A\_Km* and *iaaM\_B\_Km*), right PCR fragment (produced by primers *iaaM\_C\_Km* and *iaaM\_D\_Km*), and kanamycin cassette were connected at their complementary, overlapping regions and cloned into the *SacI* and *KpnI* sites of the pRK415 vector (47), resulting in plasmid p $\Delta$ *iaaM*. The p $\Delta$ *iaaM* construct was further confirmed by DNA sequencing.

The plasmid construct p $\Delta$ *iaaM* was electroporated into Ech3937 competent cells. The Ech3937 cells containing p $\Delta$ *iaaM* were grown in LB broth, and the  $\Delta$ *iaaM* mutant was selected by resistance to kanamycin and screened by sensitivity to tetracycline. To confirm the mutation, chromosomal DNA was isolated from the putative mutant strains; PCR by primers flanking the target *iaaM* gene followed by DNA sequencing has been performed to locate the disruption site.

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Bacterial strain, plasmid, or primer	Characteristic(s) <sup>a</sup> or sequence <sup>b</sup> (5' to 3')	Reference or source
<b>Bacterial strains</b>		
Ech3937	<i>E. chrysanthemi</i> 3937 wild type	N. Hugouvieux-Cotte-Pattat
Ech138	<i>iaaM</i> homologue (ASAP16562) null knockout mutant; Km <sup>r</sup>	This work
Ech139	<i>iaaM</i> and <i>iaaH</i> (ASAP16562 and ASAP16563) double knockout mutant; Km <sup>r</sup>	This work
Ech138(pLiaaM)	Ech138 containing pLiaaM; Km <sup>r</sup> Sp <sup>r</sup>	This work
Ech138(pLiaaMH)	Ech138 containing pLiaaMH; Km <sup>r</sup> Sp <sup>r</sup>	This work
Ech139(pLiaaMH)	Ech139 containing pLiaaMH; Km <sup>r</sup> Sp <sup>r</sup>	This work
<b>Plasmids</b>		
pCL1920	Low-copy-number vector; Sp <sup>r</sup>	50
pRK415	Low-copy-number vector; Tc <sup>r</sup>	47
pGEMT-Easy	PCR cloning vector; Ap <sup>r</sup>	Promega
pGΔiaaM	pGEM T-Easy derivative with a 3-kb <i>iaaM</i> deletion construct PCR fragment; Ap <sup>r</sup>	This work
pΔiaaM	pRK415 derivative with a 3-kb EcoRI fragment from pGΔiaaM; Tc <sup>r</sup>	This work
pGΔiaaMH	pGEM T-Easy derivative with a 3-kb <i>iaaMH</i> deletion construct PCR fragment; Ap <sup>r</sup>	This work
pΔiaaMH	pRK415 derivative with a 3-kb EcoRI fragment from pGΔiaaMH; Tc <sup>r</sup>	This work
pTiaaM	pGEM T-Easy derivative with a 2.4-kb PCR fragment; <i>iaaM</i> <sup>+</sup> Ap <sup>r</sup>	This work
pTiaaMH	pGEM T-Easy derivative with a 3.7-kb PCR fragment; <i>iaaM</i> <sup>+</sup> <i>iaaH</i> <sup>+</sup> Ap <sup>r</sup>	This work
pIaaM	pCL1920 derivative with a 2.4-kb SacI-KpnI fragment from pTiaaM; <i>iaaM</i> <sup>+</sup> Sp <sup>r</sup>	This work
pIaaMH	pCL1920 derivative with a 3.7-kb SacI-KpnI fragment from pTiaaMH; <i>iaaM</i> <sup>+</sup> <i>iaaH</i> <sup>+</sup> Sp <sup>r</sup>	This work
<i>PdspE</i>	pProbe-AT derivative with PCR fragment containing <i>dspE</i> promoter region; Ap <sup>r</sup>	79
<i>PhrpL</i>	pProbe-AT derivative with PCR fragment containing <i>hrpL</i> promoter region; Ap <sup>r</sup>	This work
<i>PhrpN</i>	pProbe-AT derivative with PCR fragment containing 396-bp <i>hrpN</i> promoter region; Ap <sup>r</sup>	This work
<i>PgacA</i>	pProbe-AT derivative with PCR fragment containing 788-bp <i>gacA</i> promoter region; Ap <sup>r</sup>	This work
<i>PrsmA</i>	pProbe-AT derivative with PCR fragment containing 424-bp <i>rsmA</i> promoter region; Ap <sup>r</sup>	This work
<i>PrsmC</i>	pProbe-AT derivative with PCR fragment containing 870-bp <i>rsmC</i> promoter region; Ap <sup>r</sup>	This work
<i>Pogl</i>	pProbe-AT derivative with PCR fragment containing 534-bp <i>ogl</i> promoter region; Ap <sup>r</sup>	This work
<i>PpelD</i>	pProbe-AT derivative with PCR fragment containing <i>pelD</i> promoter region; Ap <sup>r</sup>	79
<i>Ppell</i>	pProbe-AT derivative with PCR fragment containing 1,145-bp <i>pell</i> promoter region; Ap <sup>r</sup>	This work
<i>PpeLL</i>	pProbe-AT derivative with PCR fragment containing 609-bp <i>pell</i> promoter region; Ap <sup>r</sup>	This work
<b>Primers</b>		
<i>iaaM_A_Km</i>	<b>AGAGCTCTCTAGAGGATCCAAAAACAGCGGGTCTGTATTGCC</b>	This work
<i>iaaM_B_Km</i>	<b>GGGACTCTGGGGTTCGAAATCTAGACAACACAAGGCACCTGAATTGGCTA</b>	This work
<i>iaaM_C_Km</i>	<b>CCAGTAGCTGACATTCATCCCTCGAGGGTTGTGTCCAGACTATTAGGTTTC</b>	This work
<i>iaaM_D_Km</i>	<b>TGGTACCCTCGAGAAGCTTGTTTAAAGTGACGGCACAGCAG</b>	This work
<i>iaaMH_C_Km</i>	<b>CCAGTAGCTGACATTCATCCCTCGAGGCCACCCGTATAGAAACCATCA</b>	This work
<i>iaaMH_D_Km</i>	<b>TGGTACCCTCGAGAAGCTTGATGCAATAGCAATCAGAGAGG</b>	This work
<i>iaaM_F</i>	<b>AGAGCTCTCTAGAGGATCCTCACCCGCCGCTGGATGACTA</b>	This work
<i>iaaM_R</i>	<b>TGGTACCCTCGAGAAGCTTCGATTAACCATGCCACTCTTGC</b>	This work
<i>phrpN_F</i>	<b>CGATACCTACCCGCAAGTGA</b>	This work
<i>phrpN_R</i>	<b>TGGAACCCAGGGATGACGT</b>	This work
<i>phrpL_F</i>	<b>CTGTTTCTGGTTCGGGTCGGT</b>	This work
<i>phrpL_R</i>	<b>GCCACTTCCAACGCATCGTC</b>	This work
<i>Pogl_F</i>	<b>ACATAAAGCATCAACTGGAGC</b>	This work
<i>Pogl_R</i>	<b>CAGATAACATCGGGAGGAGT</b>	This work
<i>PpelI_F</i>	<b>GCGTGAAAAAGATGCTGGGATA</b>	This work
<i>PpelI_R</i>	<b>TTGGGCGGCGAATGAAGG</b>	This work
<i>PpelL_F</i>	<b>ATGCGGTAATGCGGGGAT</b>	This work
<i>PpelL_R</i>	<b>GGCCAGAAGTATGTACTGT</b>	This work
<i>PgacA_F</i>	<b>TGTTGTTTCATAGCGTCTCCTG</b>	This work
<i>PgacA_R</i>	<b>CACCATCTGACCGCATCTT</b>	This work
<i>PrsmA_F</i>	<b>AGCAGGCGGCAGTGATGT</b>	This work
<i>PrsmA_R</i>	<b>GCCAACTCGACGAGTCAAAT</b>	This work
<i>PrsmC_F</i>	<b>AGAGCTCTGTGAACAGGGGCGCTTAAC</b>	This work
<i>PrsmC_R</i>	<b>AGAGCTCGCAATGGTGGCGGGTAT</b>	This work

<sup>a</sup> Ap<sup>r</sup>, ampicillin resistance; Km<sup>r</sup>, kanamycin resistance; Sp<sup>r</sup>, spectinomycin resistance; Tc<sup>r</sup>, tetracycline resistance.

<sup>b</sup> Restriction sites in primers are in boldface, and barcodes are underlined. Barcodes are random computer-designed DNA sequences which provide a complementary region for overlapping PCR products.

An *iaaM* and *iaaH* (ASAP16562 and ASAP16563) double deletion mutant, Ech139, was constructed in a similar manner using the primer sets *iaaM\_A\_Km* and *iaaM\_B\_Km* and *iaaMH\_C\_Km* and *iaaMH\_D\_Km* to generate the up-stream and downstream PCR fragments, respectively.

To construct an *iaaM* complementary plasmid, a fragment with the *iaaM* gene and its promoter was PCR amplified using the primer pair of *iaaM\_F* and

*iaaM\_R*. The PCR product was ligated into a low-copy-number plasmid vector, pCL1920 (50). The resultant plasmid, pIaaM, was further confirmed by PCR and DNA sequencing with the primer pair of *iaaM\_F* and *iaaM\_R*. The plasmid was then electroporated into the *iaaM* mutant strain Ech138 for complementation. A similar approach was used to construct a complementary plasmid, pIaaMH, using the primers *iaaM\_F* and *iaaMH\_D\_Km*, which contained the *iaaM* and

*iaaH* genes and the promoter regions. To confirm that pIaaM and pIaaMH were reintroduced into the *iaaM* mutant Ech138, in addition to the plasmid-derived spectinomycin resistance obtained by the *iaaM* mutant Ech138, the plasmids from the bacterial cells were purified and digested with restriction enzymes. Restriction patterns and sizes of the DNA fragments of the digested plasmids were analyzed.

**IAA quantification, exoenzyme, and pectate lyase production assays.** IAA quantification was performed with the Fe-H<sub>2</sub>SO<sub>4</sub> reagent (34, 78). Briefly, bacterial strains were propagated overnight in 5 ml of LB broth and then 50- $\mu$ l aliquots were transferred into 5 ml of M9 minimal medium supplemented with L-tryptophan. The density of each culture was measured spectrophotometrically at 600 nm, and then the bacterial cells were removed from the culture medium by centrifugation. A 1-ml aliquot of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (150 ml of concentrated H<sub>2</sub>SO<sub>4</sub>, 250 ml of distilled H<sub>2</sub>O, 7.5 ml of 0.5 M FeCl<sub>3</sub> · 6H<sub>2</sub>O), and the absorbance at 535 nm was measured with a Cary 100 UV-Vis spectrophotometer (Varian Inc., CA). The concentration of IAA in each culture medium was determined by comparison with a standard curve (78).

Plating and the spectrophotometric assays for activity of Pel were carried out as reported by Matsumoto et al. (61). Briefly, a 30- $\mu$ l sample of bacterial culture or sonicated culture supernatant (optical density at 600 nm [OD<sub>600</sub>] of 0.5 [ca. 5 × 10<sup>8</sup> CFU/ml]) was used in the plate assay, and a 10- $\mu$ l sample of 0.5-OD<sub>600</sub> bacterial culture or sonicated culture supernatant was added into 990  $\mu$ l pectate lyase reaction buffer (0.1 M Tris-HCl, pH 8.5, 0.05% PGA, 0.1 mM CaCl<sub>2</sub>) for the spectrophotometric assay.

**FACS assays.** The *gacA*, *rsmA*, and *rsmC* genes of Ech3937 were identified based on homologues in *E. carotovora* (unpublished data). The promoter regions of T3SS genes *dspE*, and *hrpN*; pectin catabolic genes *ogl*, *pelD*, *pell*, and *pellL*; and regulator genes *hrpL*, *gacA*, *rsmA*, and *rsmC* of Ech3937 were cloned into a GFP reporter vector, pPROBE-AT, to produce *PdspE*, *PhrpN*, *Pogl*, *PpelD*, *Ppell*, *PpellL*, *PhrpL*, *PgacA*, *PrsmA*, and *PrsmC*, respectively (Table 1). The promoter activity of Ech3937 and Ech138 carrying the GFP reporter plasmid was examined in MM for T3SS genes and T3SS regulators or in MM supplemented with 1% PGA for pectin catabolic genes and regulators. GFP intensity was determined by FACS (Becton Dickinson, San Jose, CA) as described previously (79). Three types of gene expression parameters were measured, including average GFP fluorescence intensity of total bacterial cells ("Total"), average GFP fluorescence intensity of GFP-expressing bacterial cells ("GFP<sup>+</sup> mean"), and GFP-expressing bacterial cells as a percentage of the total bacterial cells ("GFP<sup>+</sup> %") (31).

For in planta promoter activity assay, the bacterial strains were grown in LB broth at 28°C. A volume of 1 ml of bacterial suspension from each strain was inoculated into the leaves of the African violet cv. Gauguin and incubated at 28°C. The leaves were sliced into small pieces, placed into centrifuge tubes, and centrifuged at 5,000 rpm for 10 min. The intercellular fluids from leaf tissues that contained bacterial cells were separated by centrifugation. The cell pellets were washed with 1× phosphate-buffered saline as described previously (79). To precisely locate the *E. chrysanthemi* bacterial cells, the test samples were stained with BacLight Red bacterial stain (Molecular Probes, OR) prior to being run on the flow cytometer. Three biological replicates were performed for each treatment.

**RT-PCR and qRT-PCR analysis.** A TRI reagent method (Sigma, St. Louis, MO) was used to isolate total RNA from the bacteria. The isolated RNA was treated with Turbo DNA-free DNase kits (Ambion, Austin, TX), and 0.5  $\mu$ g of the treated RNA was used as template to synthesize cDNA by using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). For qRT-PCRs to quantify the cDNA level of target genes in different samples, the Opticon 2 system (Bio-Rad) was used to collect data. *rplU* was used as the internal control to normalize the cDNA input of each sample. The primer pairs used in this study are RplUsF (5' GCG GCA AAA TCA AGG CTG AAG TCG 3') and RplUsR (5' CGG TGG CCA GCC TGC TTA CGG TAG 3') for *rplU*, HrpLsF (5' GAT GAT GCT GCT GGA TGC CGA TGT 3') and HrpLsR (5' TGC ATC AAC AGC CTG GCG GAG ATA 3') for *hrpL*, *dspE* (5' GAT GGC GGA GCT GAA ATC GTT C 3') and *dspEr* (5' CCT TGC CGG ACC GCT TAT CAT T 3') for *dspE*, *hrpAf* (5' CAG CAA TGG CAG GCA TGC AG 3') and *hrpAr* (5' CTG GCC GTC GGT GAT TGA GC 3') for *hrpA*, and *iaaMf* (5' GGC GCG TAA GGC ATG GCA 3') and *iaaMr* (5' GCC ACG GGA CGC CTC C 3') for *iaaM*.

**Local maceration assay, growth kinetics, and in planta pectate lyase production.** The local leaf maceration assay was carried out as described previously (104). Briefly, wild-type bacterial cells and Ech138 cells were syringe infiltrated in the middle of each symmetric side of the same leaf with approximately 50  $\mu$ l of a bacterial suspension at the concentration of 10<sup>6</sup> CFU/ml. Phosphate buffer (50 mM, pH 7.4) was used to suspend the bacterial cells. Three replicate plants

with a total of 12 leaves were inoculated. Inoculated plants were kept in growth chambers at 28°C with 95% relative humidity and a photoperiod of 16 h, with regular water misting to provide humid conditions. The area of maceration on plant leaves caused by the bacterial strains was precisely measured with the ASSESS image analysis software (APS Press, The American Phytopathological Society, St. Paul, MN).

Assays for growth kinetics in planta were carried out in African violet cv. Gauguin as described previously (104). Briefly, leaves were syringe infiltrated with approximately 50  $\mu$ l bacterial suspension at 10<sup>6</sup> CFU/ml with a 1-ml syringe. Leaf discs (4 mm in diameter) around the maceration area were harvested at different intervals following infiltration and ground in 50 mM phosphate buffer (pH 7.4). The bacterial populations (CFU/cm<sup>2</sup>) were determined by plating serial dilutions of leaf extracts on LB agar plates. A spectrophotometric assay was used to monitor the pectate lyase production of Ech3937 and Ech138 during the in planta growth. A 10- $\mu$ l supernatant of the plant juice from African violet leaves inoculated with the bacteria was added into 990  $\mu$ l Pel reaction buffer, and the Pel production was quantified using the spectrophotometric assay (61). Pectate lyase production was the ratio of the OD<sub>230</sub> unit to the log unit of the bacterial population [U/log(CFU/cm<sup>2</sup>)]. Six leaves from six replicate plants were used in each sampling time for the in planta pectate lyase production and bacterial growth kinetics assays.

## RESULTS

**Genes ASAP16562 and ASAP16563 of Ech3937 involved in IAA biosynthesis.** The *iaaM* (ASAP16562) homologous mutant, Ech138, was constructed using the direct two-step PCR approach (103). An RT-PCR was used to confirm the *iaaM* mutation. As expected, *iaaM* mRNA can be detected in wild-type Ech3937 but was not detectable in *iaaM* mutant Ech138 within 30 PCR cycles (data not shown). There was only basal-level IAA production of cells grown in MM only; the IAA production of Ech3937 required the addition of tryptophan (data not shown). Compared with wild-type Ech3937 in MM supplemented with L-tryptophan at a concentration of 500  $\mu$ g/ml, the IAA biosynthetic ability of Ech138 was reduced at 48 h of growth (Fig. 2). Similarly, the *iaaM* and *iaaH* double mutant, Ech139, also showed reduced IAA biosynthetic ability (Fig. 2). The IAA biosynthesis ability of Ech138 was only partially restored by introducing the *iaaM*-expressing plasmid, pIaaM, into the mutant (Fig. 2), suggesting that the *iaaM* mutation may have a polar effect on downstream *iaaH*. When the *iaaM*- and *iaaH*-expressing plasmid, pIaaMH, was introduced into Ech138, the IAA production ability of the mutant was restored (Fig. 2). The IAA production ability of Ech139 was also restored when pIaaMH was introduced into the mutant (Fig. 2). The higher production of IAA in the pIaaMH-complemented strains of Ech138 and Ech139 than in wild-type Ech3937 may be due to the copy number effect of the plasmid.

**IAA biosynthesis of Ech3937 influenced pectate lyase production.** In the MM broth, wild-type Ech3937 and Ech138 had similar growth kinetics (data not shown). Since pectate lyase is one of the key virulence determinants in Ech3937 (43, 88), a spectrophotometric assay was applied to quantify the pectate lyase production (U/OD<sub>600</sub>) in the Ech138 mutant (61). With a pectate lyase-inducing condition (MM supplemented with 1% PGA), higher extracellular and total pectate lyase activities of Ech3937 was observed in comparison to Ech138 after 12 h of culture. The extracellular and total pectate lyase activities of Ech3937 were 58.5 ± 1.0 and 63.0 ± 0.9, respectively, which were about 163% and 97%, respectively, more than those of Ech138 (22.2 ± 2.4 and 31.9 ± 1.6, respectively). In addition, compared to the pectate lyase production of *iaaM* mutant Ech138 (7.8 ± 0.7) grown in the IAA biosynthesis-inducing

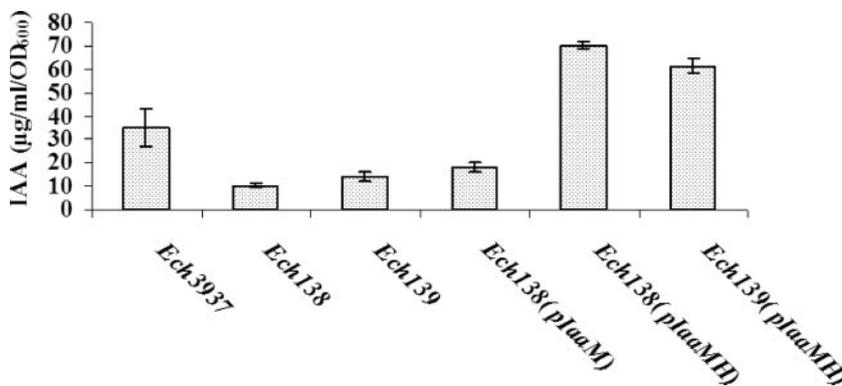


FIG. 2. IAA production abilities of Ech3937, Ech138, Ech139, Ech138(pLiaaM), Ech138(pLiaaMH), and Ech139(pLiaaMH) in MM broth supplemented with 500 µg/ml L-tryptophan. IAA was quantified with the Fe-H<sub>2</sub>SO<sub>4</sub> reagent as described previously (34, 78). Bacterial strains were incubated at 28°C with shaking at 200 rpm for 48 h.

medium of MM supplemented with tryptophan, the pectate lyase production of the complemented strain Ech138(pLiaaMH) was 12.8 ± 1.0, which is almost restored to the wild-type Ech3937 level of 15.2 ± 3.3.

To further confirm the effect of IAA biosynthesis on pectate lyase production by the bacterium, the pectate lyase production by Ech138 in MM, MM supplemented with IAM, or MM supplemented with IAA was quantified using the spectrophotometric assay. Compared with Ech138 grown in MM alone, the production of pectate lyase by Ech138 was increased at least twofold in MM supplemented with IAM or IAA (Table 2), which is comparable to the pectate lyase production by wild-type Ech3937 in MM only (Table 2). In addition, compared with Ech3937 grown in MM alone, a higher pectate lyase production in Ech3937 was observed when the bacterial strain was supplemented with IAM or IAA (Table 2).

Since Ech138 exhibited reduced pectate lyase and the supplementation of IAA or IAM increased the pectate lyase production (Table 2), the effect of IAA biosynthesis on the expression of genes involved in pectin degradation of Ech3937 was investigated. The GFP intensities of bacterial cells of Ech3937 and Ech138 carrying the GFP promoter probe reporter plasmids *Pogl*, *PpelD*, *Ppell*, and *PpellL* were examined using FACS after 12 h of growth in MM supplemented with PGA. The *ogl* gene encodes an oligogalacturonate lyase, and the *pelD*, *pell*, and *pellL* genes encode endopectate lyases.

TABLE 2. Spectrophotometric quantification of pectate lyase activity of wild-type *E. chrysanthemi* 3937 (Ech3937) and *iaaM* deletion mutant (Ech138) in MM, MM supplemented with 10 µg/ml IAM, and MM supplemented with 100 µg/ml IAA

Treatment	Bacterial strain	Pectate lyase activity <sup>a</sup> (U/OD <sub>600</sub> )
MM	Ech3937	6.8 ± 0.7
	Ech138	3.9 ± 1.2
MM-IAM	Ech3937	21.2 ± 0.3
	Ech138	10.7 ± 2.5
MM-IAA	Ech3937	12.7 ± 1.3
	Ech138	7.7 ± 2.6

<sup>a</sup> Pectate lyase activity of bacterial strains grown for 48 h in the medium was determined as described previously (61). One unit of Pel activity was equivalent to an OD<sub>230</sub> increase of 0.001 in 1 min. The data represent the averages of two experiments with the standard deviations shown.

In the pectate lyase-inducing condition, the average GFP fluorescence intensity of bacterial cells of Ech3937(*Pogl*), Ech3937(*PpelD*), Ech3937(*Ppell*), and Ech3937(*PpellL*) was higher than that of Ech138(*Pogl*), Ech138(*PpelD*), Ech138(*Ppell*), and Ech138(*PpellL*), respectively, at 12 h postinoculation (Table 3).

**IAA biosynthesis influences the expression of T3SS genes.**

To detect the effect of IAA biosynthesis on the expression of T3SS genes of Ech3937, the bacterial cells were grown in MM and the GFP intensities of *PdspE* and *PhrpN* in Ech3937 and Ech138 cells were compared. The *dspE* gene is a putative T3SS effector, and *hrpN* is a T3SS harpin, a T3SS substrate. A lower GFP intensity of total bacterial cells (total) was observed in Ech138(*PdspE*) and Ech138(*PhrpN*) than in Ech3937(*PdspE*) and Ech3937(*PhrpN*) (Table 4). In addition, compared with Ech3937(*PdspE*) and Ech3937(*PhrpN*), lower average GFP fluorescence intensity of GFP-expressing bacterial cells (GFP<sup>+</sup> mean) and a lower percentage of GFP-expressing bacterial cells relative to the total bacterial cells (GFP<sup>+</sup>%) were observed in Ech138(*PdspE*) and Ech138(*PhrpN*), respectively (Table 4).

TABLE 3. *ogl*, *pelD*, *pell*, *pellL*, *gacA*, *rsmA*, and *rsmB* promoter activities of wild-type *E. chrysanthemi* 3937 (Ech3937) and *iaaM* deletion mutant (Ech138) grown in MM supplemented with 1% polygalacturonate

Gene promoter	Avg fluorescence intensity for bacterial cells <sup>a</sup> :			
	Ech3937		Ech138	
	Total cells	GFP-expressing cells	Total cells	GFP-expressing cells
<i>Pogl</i>	132 ± 8	137 ± 8	103 ± 5	106 ± 5
<i>PpelD</i>	1,135 ± 52	1,183 ± 40	693 ± 81	709 ± 89
<i>Ppell</i>	57 ± 2	58 ± 3	48 ± 7	49 ± 7
<i>PpellL</i>	60 ± 2	62 ± 2	45 ± 2	46 ± 2
<i>PgacA</i>	892 ± 16	897 ± 17	598 ± 33	619 ± 40
<i>PrsmA</i>	273 ± 5	279 ± 5	368 ± 28	373 ± 30
<i>PrsmC</i>	99 ± 0	105 ± 1	168 ± 18	171 ± 19

<sup>a</sup> The promoter activities were compared after 12 h of culture in medium. GFP intensity was determined on gated populations of bacterial cells by flow cytometry. The percentage of cells expressing GFP (GFP<sup>+</sup>%) is around 95 to 100%. Values (mean fluorescence intensity) are representative of two experiments. Three biological replicates were used in this experiment.

TABLE 4. *dspE* and *hrpN* promoter activities of wild-type *E. chrysanthemi* 3937 (Ech3937) and *iaaM* deletion mutant (Ech138) grown in MM

Time of culture (h)	Activity for gene and promoter <sup>a</sup> :																	
	Ech3937( <i>PdspE</i> )			Ech138( <i>PdspE</i> )			Ech3937( <i>PhrpL</i> )			Ech138( <i>PhrpL</i> )								
	Total	GFP <sup>+</sup> mean	GFP <sup>+</sup> %	Total	GFP <sup>+</sup> mean	GFP <sup>+</sup> %	Total	GFP <sup>+</sup> mean	GFP <sup>+</sup> %	Total	GFP <sup>+</sup> mean	GFP <sup>+</sup> %						
6	70 ± 7	131 ± 1	52 ± 5	4 ± 0	43 ± 7	1 ± 0	16 ± 4	143 ± 2	9 ± 3	3 ± 0	69 ± 23	1 ± 0	11 ± 0	20 ± 1	37 ± 1	7 ± 0	16 ± 0	16 ± 0
12	107 ± 2	148 ± 2	72 ± 1	7 ± 0	48 ± 1	8 ± 1	46 ± 6	126 ± 4	35 ± 6	12 ± 0	106 ± 5	9 ± 1	14 ± 1	23 ± 1	46 ± 2	9 ± 0	18 ± 0	27 ± 1

<sup>a</sup>The promoter activities were compared after 6 and 12 h of culture in MM. GFP intensity was determined on gated populations of bacterial cells by flow cytometry. Values (mean fluorescence intensity) are representative of two experiments. Three biological replicates were used in this experiment. "Total" represents the average GFP fluorescence intensity of total bacterial cells, "GFP<sup>+</sup> mean" represents the average GFP fluorescence intensity of GFP-expressing bacterial cells, and "GFP<sup>+</sup>%" represents GFP-expressing bacterial cells as a percentage of the total bacterial cells.

To confirm our FACS results showing the positive effect of IAA biosynthesis on T3SS gene expression, the relative mRNA levels of *dspE* and *hrpN* of Ech3937 and Ech138 were examined by qRT-PCR. Compared with Ech3937, a lower amount of *dspE* and *hrpN* mRNA was observed in Ech138 (Fig. 3). The levels of *dspE* and *hrpA* mRNAs produced by Ech138 were ca. 0.6- and 0.2-fold those of Ech3937, with a *P* value for the Student *t* test of less than 0.05 (Fig. 3).

**Regulatory network of IAA biosynthesis controlling pectate lyase and T3SS gene expression.** Since GacA, RsmA, and RsmC have been reported to regulate the pectate lyase gene expression of *Erwinia* spp. (16, 23, 24, 26, 42, 52, 57), we examined the regulatory pathway of IAA biosynthesis for pectate lyase gene expression. For this purpose, the GFP intensities of *PgacA*, *PrsmA*, and *PrsmC* in Ech3937 and Ech138 grown in MM supplemented with 1% PGA were compared at 12 h. A higher average GFP fluorescence intensity of total bacterial cells (Total) was observed in Ech138(*PrsmA*) and Ech138(*PrsmC*) than in Ech3937(*PrsmA*) and Ech3937(*PrsmC*), indicating that IAA biosynthesis down-regulated *rsmA* and *rsmC* (Table 3). A lower average GFP fluorescence intensity of total bacterial cells (Total) was observed in Ech138(*PgacA*) than in Ech3937(*PgacA*), suggesting that IAA biosynthesis up-regulated *gacA* in the pectinase-inducing condition.

Similarly, since HrpL has been reported to regulate the T3SS gene expression of *Erwinia* spp. (14, 23, 26, 64), to further elucidate the regulatory network of IAA biosynthesis on T3SS genes, the GFP intensities of *PhrpL* in Ech3937 and Ech138 grown in the *hrp*-inducing MM were compared at 12 h. A higher average GFP fluorescence intensity of total bacterial cells (Total) and a higher percentage of GFP-expressing bacterial cells (GFP<sup>+</sup>%) were observed in Ech3937(*PhrpL*) than in Ech138(*PhrpL*), indicating that IAA biosynthesis up-regulated *hrpL* (Table 4). Consistent with the FACS result, the level of *hrpL* mRNA produced by Ech138 was ca. 0.7-fold that of Ech3937, with a *P* value for the Student *t* test of less than 0.05 (Fig. 3).

**IAA biosynthesis of Ech3937 influenced the local maceration symptoms but not the growth of the bacterium in plants.** The disruption of IAA biosynthesis reduced the expression of certain pectin catabolism-related genes like *ogl*, *pelD*, *pell*, and *pelL* (Table 3), T3SS genes like *dspE* and *hrpN*, in vitro (Table 4; Fig. 3). PelD was reported to play a major role in causing maceration in plant tissues (88). To test whether the influence of *iaaM* on *pel* gene expression holds true in planta, the transcription of *pelD* was investigated in planta using the GFP reporter. In addition, the expression of two T3SS genes, *dspE* and *hrpN*, was also examined in leaves of *Saintpaulia ionantha*. A lower GFP intensity of total bacterial cells (Total) and a lower percentage of GFP-expressing bacterial cells (GFP<sup>+</sup>%) of Ech138 carrying *PpelD*, *PdspE*, and *PhrpN* were observed in African violet than in Ech3937 carrying the same GFP reporter plasmid at 24 h (Table 5).

The T3SS genes and the exoenzymes, especially the pectate lyases, are the major virulence determinants in Ech3937. Since the disruption of IAA biosynthesis reduced the production of pectate lyases (Table 2) as well as the expression of certain pectin catabolism-related genes and T3SS genes in vitro (Tables 3 and 4; Fig. 3) and in planta (Table 5), the local maceration and population kinetics of wild-type Ech3937 and Ech138

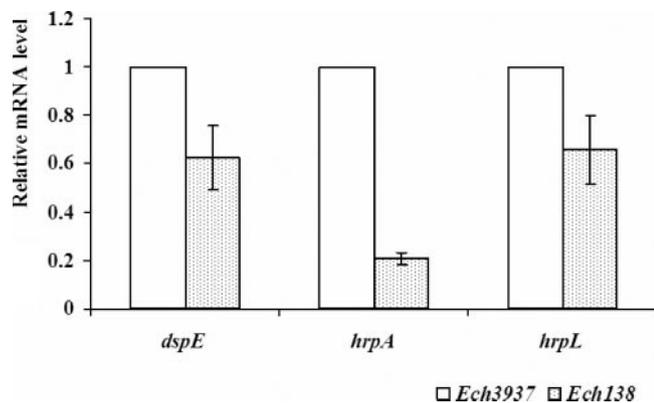


FIG. 3. Relative levels of *dspE*, *hrpA*, and *hrpL* mRNA in wild-type Ech3937 and *iaaM* mutant Ech138 grown for 12 h in a minimal medium. The amount of mRNA was examined by a qRT-PCR assay using the Real Master Mix (Eppendorf, Westbury, NY) in different samples. Reactions were run, and data were collected with the Opticon 2 system (Bio-Rad). The *rplU* gene was used as the internal control to normalize the cDNA input of each sample. Three replicates were used in this experiment, and the Student *t* test *P* value is less than 0.05.

strains in leaves of *S. ionantha* were further investigated in order to examine the influence of IAA biosynthesis on bacterial pathogenicity. Compared with wild-type Ech3937 (318 mm<sup>2</sup>), a significant reduction in the area of maceration of *S. ionantha* caused by Ech138 (176 mm<sup>2</sup>) ( $P = 0.002$  by paired-sample *t* test) was observed at 48 h postinoculation. Meanwhile, a lower pectate lyase activity (U/log [CFU/cm<sup>2</sup>]) was observed in plant leaves inoculated with Ech138 ( $1.0 \pm 0.3$ ) than in Ech3937 ( $2.2 \pm 0.1$ ) after 3 days postinoculation. Interestingly, the Ech138 strain had in planta multiplication abilities similar to those of wild-type Ech3937 in *S. ionantha* within 5 days postinoculation (Fig. 4).

## DISCUSSION

In microorganisms, the IAM pathway genes are generally plasmid borne, and the IPyA pathway genes are chromosomally encoded. For example, *iaaM* and *iaaH* genes are borne on a plasmid designated pIAA in *P. syringae* pv. savastanoi iso-

TABLE 5. *pelD*, *dspE*, and *hrpN* promoter activities of wild-type *E. chrysanthemi* 3937 (Ech3937) and *iaaM* deletion mutant (Ech138) grown in African violet cv. Gauguin (*Saintpaulia ionantha*)

Strain with gene promoter	Activity at 24 h <sup>a</sup>		
	Total	GFP <sup>+</sup> mean	GFP <sup>+</sup> %
Ech3937( <i>PpelD</i> )	68 ± 6	171 ± 14	35 ± 0
Ech138( <i>PpelD</i> )	23 ± 7	142 ± 17	12 ± 5
Ech3937( <i>PdspE</i> )	87 ± 42	184 ± 27	42 ± 17
Ech138( <i>PdspE</i> )	15 ± 4	111 ± 2	9 ± 2
Ech3937( <i>PhrpN</i> )	65 ± 16	239 ± 5	26 ± 6
Ech138( <i>PhrpN</i> )	14 ± 14	182 ± 14	6 ± 7

<sup>a</sup> The promoter activities were compared after 24 h of inoculation. GFP intensity was determined on gated populations of bacterial cells by flow cytometry. "Total" represents the average GFP fluorescence intensity of total bacterial cells, "GFP<sup>+</sup> mean" represents the average GFP fluorescence intensity of GFP-expressing bacterial cells, and "GFP<sup>+</sup>%" represents GFP-expressing bacterial cells as a percentage of the total bacterial cells. Values (mean fluorescence intensity) are representative of two experiments. Three biological replicates were used in this experiment.

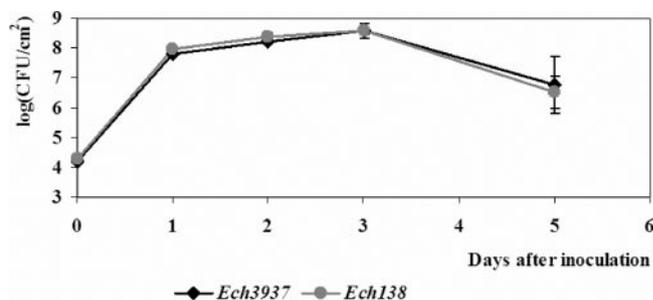


FIG. 4. Population kinetics of wild-type Ech3937 and *iaaM* mutant Ech138 in African violet cv. Gauguin (*Saintpaulia ionantha*). Leaves were inoculated with a 50- $\mu$ l bacterial suspension at 10<sup>6</sup> CFU/ml. Six leaves from three replicate plants were used at each sampling time for each bacterial strain for the population kinetics, with standard deviations shown.

lated from oleander galls (33). The IPyA pathway is also commonly distributed in higher plants, and the IAA supply is under stringent regulation (59). In contrast, the presence of the IAM pathway in higher plants is quite rare (45) and may be considered unique to bacteria (77). By utilizing the IAM pathway in plant hosts, the pathogen is able to build up large amounts of IAA for gall formation or to control the free IAA level.

From the *E. chrysanthemi* genome-sequencing project, no obvious IPyA pathway gene homologue was found in Ech3937 (unpublished results). The *iaaM* (ASAP16562) and *iaaH* (ASAP16563) genes of wild-type Ech3937 are chromosomally encoded. The reduction of IAA production in Ech138 and Ech139 demonstrated that the IAM pathway serves as an IAA biosynthesis pathway in this bacterium. In this study, it appears that the IAA production is not totally abolished in Ech138 and Ech139. From the Ech3937 genome sequence, Ech3937 contains a gene (ASAP47082) exhibiting similarity to a nitrilase of *Arabidopsis thaliana*, which is involved in the IAA synthesis of *A. thaliana*. A reduction of IAA production was observed in the ASAP47082 mutant, suggesting that, along with the IAM pathway, gene ASAP47082 may also be involved in IAA biosynthesis (data not shown).

IAA biosynthesis by phytopathogenic bacteria may contribute to their ability to survive as epiphytes on plant surfaces (22, 30, 54). In addition, the IAA biosynthetic pathways of bacteria may also be involved in detoxification of tryptophan analogues, suppression of plant disease response gene expression, or inhibition of the plant hypersensitive response to facilitate bacterial invasion (22, 62, 89). Finally, various effects of IAA on plant tissues, including modification of electrochemical proton gradients across the host plasmalemma to promote solute uptake (81), alteration of respiration, protein synthesis in cells and enzyme secretion activities of various cell wall polymers, and the induction of host plant ethylene synthesis, have been reported elsewhere (30).

In this study, we demonstrate that the IAA biosynthesis pathway of Ech3937 modulates the expression of T3SS and pectinase genes of the bacterium. The disruption of the *iaaM* gene decreased the production of IAA and pectate lyase and the expression of T3SS genes (Fig. 2 and 3; Tables 3, 4, and 5). The addition of the IAA biosynthesis intermediate indoleacetyl-tryptophan (IAM, the product of the *IaaM* enzyme) as well as end

product IAA to the MM growth medium increases the pectate lyase production in the *iaaM* mutant Ech138 as well as the wild-type Ech3937 (Table 2), further demonstrating that IAA biosynthesis plays a positive regulatory role in pectate lyase production.

GFP reporters have been widely used to evaluate gene activity in several bacteria, and a numerical model was formulated to interpret the relationship between GFP intensity and gene promoter activity (51). In addition, the FACS-based approach has been used to investigate gene expression in bacteria at the single-cell level (27, 87). The promoter probe reporter vector, pPROBE-AT, used in this study produces an extremely stable GFP (63). Once the cells have produced GFP, they are fluorescent at later time points even though the cells have stopped generating GFP. In our work, the GFP intensity and percentage of cells expressing GFP at different time periods can be considered a sum of *E. chrysanthemi* cells that expressed GFP or are expressing GFP from the initial inoculation point until the cells are harvested (79). Rietsch and Mekalanos (87) recently demonstrated by using FACS analysis that the metabolic state of *P. aeruginosa* regulates the percentage of cells that are able to induce T3SS gene expression (87). In our study, under a homogenous growth environment (MM), distinct regulatory patterns of the IAA biosynthetic pathway of T3SS gene expression at the single-cell level were observed. Compared with wild-type Ech3937(*PdspE*) and Ech3937(*PhrpN*), both the GFP fluorescence intensity of bacterial cells and the percentage of GFP-expressing bacterial cells were reduced in Ech138(*PdspE*) and Ech138(*PhrpN*) at 12 h of growth in MM (Table 4). Bacterial intercellular communication, e.g., through biochemical signals, provides a mechanism for the regulation of gene expression resulting in coordinated population behavior. Several bacterial pathogens were discovered to use quorum sensing to regulate genes involved in virulence, such as pectinases, T3SS, and motility. At this stage, it is uncertain whether quorum sensing plays a role in this distinct gene regulation pattern of T3SS genes of Ech3937 among individual cells.

From our in planta assay, a decrease in local leaf maceration of Ech138 in *S. ionantha* was observed in comparison with wild-type Ech3937 2 days postinoculation. A reduced transcription of *pelD* and reduced pectate lyase production were also observed in Ech138 in African violet leaves in comparison with the wild-type Ech3937 (Table 5). The smaller local leaf maceration area observed in Ech138 might be due to the reduction of pectate lyase production of the mutant strain. Interestingly, the Ech138 strain did not reduce its growth in planta during the 5 days post-bacterial inoculation (Fig. 3), suggesting that the maceration ability of Ech3937 did not fully correspond to the ability of bacterial multiplication in plant tissues. Palva et al. demonstrated that although pectinases secreted by *E. chrysanthemi* can disassemble the plant cell wall and make the host tissue more accessible to bacteria, the pectate lyases themselves and the oligogalacturonides released through pectate lyase degradation may also trigger the plant defense responses (76). Consequently, a higher resistance response from the host plant might be encountered in the wild-type bacterium than in Ech138 due to a higher pectinase production during the infection process. A previous report demonstrated that an *hrpG* (encoding the T3SS gene product presumed to be involved in protein secretion) mutant of

Ech3937 had a reduced ability to multiply in African violet leaves (103). Mutation of *iaaM* of Ech3937 reduced but did not fully eliminate the expression of T3SS and pectinase genes (Tables 2, 3, 4, and 5; Fig. 3). The phenotype of the *iaaM* mutant Ech138 suggested that lower levels of pectinase and T3SS expression are sufficient for the multiplication of the bacterium in *S. ionantha* leaves during the first 5 days of bacterial invasion.

In this study, the expression of *dspE* and *hrpN* is decreased in Ech138. Since a lower promoter activity of *hrpL* was also observed in Ech138 (Table 4), the decrease of *dspE* and *hrpN* transcripts may be partially due to the reduction of HrpL in the mutant at the transcriptional level (Tables 4 and 5). At this time, it is uncertain whether the reduced expression of *hrpL* in Ech138 is through the HrpX-HrpY-HrpS regulatory pathway. In addition, a lower expression of *gacA* was observed in Ech138. The lower expression of *dspE* and *hrpN* in Ech138 might partially be due to a posttranscriptional regulation of the Gac-Rsm regulatory pathway. Indeed, compared with wild-type Ech3937, a smaller amount of mRNA of *rsmB* and *hrpL* in the *gacA* mutant in comparison with Ech3937 was discovered using qRT-PCR (C.-H. Yang et al., unpublished data). Finally, a greater amount of *rsmC* and *rsmA* transcript was observed in Ech138. Since RsmA is reported to negatively control the expression of extracellular enzymes (16), a smaller amount of extracellular enzyme production of Ech138 might be partially due to the higher expression of *rsmA* in the mutant. In conclusion, our data provide the evidence that the disruption of the *iaaM* gene has an effect on the local maceration pathogenicity on *S. ionantha* and interferes with the expression of *pel* genes and T3SS through the Gac-Rsm regulatory pathway.

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