New insights into the role of indole-3-acetic acid in the virulence of Pseudomonas savastanoi pv. savastanoi

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Abstract
Indole-3-acetic acid (IAA) is a widespread phytohormone among plant-associated bacteria, including the tumour-inducing pathogen of woody hosts, Pseudomonas savastanoi pv. savastanoi. A phylogenetic analysis of the iaaM/iaaH operon, which is involved in the biosynthesis of IAA, showed that one of the two operons encoded by Pseudomonas savastanoi pv. savastanoi NCPPB 3335, iaaM-1/iaaH-1, is horizontally transferred among bacteria belonging to the Pseudomonas syringae complex. We also show that biosynthesis of the phytohormone, virulence and full fitness of this olive pathogen depend only on the functionality of the iaaM-1/iaaH-1 operon. In contrast, the iaaM-2/iaaH-2 operon, which carries a 22-nt insertion in the iaaM-2 gene, does not contribute to the production of IAA by this bacterium. A residual amount of IAA was detected in the culture supernatants of a double mutant affected in both iaaM/iaaH operons, suggesting that a different pathway might also contribute to the total pool of the phytohormone produced by this pathogen. Additionally, we show that exogenously added IAA negatively and positively regulates the expression of genes related to the type III and type VI secretion systems, respectively. Together, these results suggest a role of IAA as a signalling molecule in this pathogen.

Introduction
Production of the phytohormone indol-3-acetic acid (IAA) is common among plant-associated bacteria, and this phytohormone can interfere with plant development by disturbing the auxin balance in plants. Moreover, IAA has been described as a signalling molecule that can influence microbial gene expression, including that of bacterial phytopathogens (Spaepen & Vanderleyden, 2010; Patten et al., 2012). The best characterized IAA biosynthetic pathway in phytopathogenic bacteria is the indole-3-acetamide pathway. In this pathway, the genetic determinants involved in the conversion of tryptophan (Trp) to IAA are Trp monoxygenase (encoded by the iaaM gene), which converts Trp to indoleacetamide (IAM), and IAM hydrolase (encoded by the iaaH gene), which catalyses the conversion of IAM to IAA. Furthermore, the oleander pathogen Pseudomonas savastanoi pv. merii (Psn) is also able to convert IAA to a less biologically active compound, the amino acid conjugate IAA–lysine (IAA–Lys), through expression of the iaal gene (Glass & Kosuge, 1986).

The iaaM/iaaH genes, which are best characterized in phytopathogenic bacteria such as Agrobacterium spp. and Pseudomonas savastanoi, have been proposed to have originated from common ancestor genes in these two species based on their amino acid sequences (Gielen et al., 1984; Yamada et al., 1985, 1986; Inze et al., 1987). In Psn, these two genes are encoded on plasmids. They are co-transcribed in the same transcriptional unit, and they are essential for gall formation in oleander and olive (Comai & Kosuge, 1980; Yamada et al., 1985; Palm et al., 1989; Gaffney et al., 1990). In contrast, P. savastanoi pv. savastanoi (Psv, olive isolates) usually carries two copies of both IAA genes on its chromosome (Pérez-Martínez et al., 2008). Psv NCPPB 3335 causes olive knot disease and is a model bacterium in which to study the molecular basis of pathogenesis and tumour formation in woody hosts (Matas et al., 2012). This strain contains two chromosomally encoded copies of the iaaM/iaaH operon.
genes (Rodríguez-Palenzuela et al., 2010), which are organized into two operons called iaaM-1/iaaH-1 and iaaM-2/iaaH-2. Additionally, the iaaM-2 gene has been suggested to be a pseudogene that contains a premature termination codon yielding a product of 142 amino acids (Ramos et al., 2012). However, the specific role of each of these two IAA operons in the pathogenicity and virulence of Psv strains has not been reported to date.

In addition to the contribution of IAA in bacterial phytopathogens in circumventing plant defence responses, IAA can also directly affect bacterial physiology and survival during plant infection (Spaepen & Vanderleyden, 2010). Examples of the signalling role of IAA in plant-associated bacteria include the upregulation of the type III secretion system (T6SS) in the bacterial phytopathogen Dickeya dadantii (Yang et al., 2007) and the rhizosphere bacterium Azospirillum brasilense (Van Puyvelde et al., 2011), respectively. However, the role of IAA as a signalling molecule in the Pseudomonas syringae complex, which also includes P. savastanoi, has only been reported for the synthesis of the virulence determinant syringomycin (Mazzola & White, 1994).

In the present study, we investigated the functionality of the two iaaM/iaaH operons present in the genome of Psv NCPPB 3335 and their individual roles in virulence using single and double ΔiaaMH mutants constructed by gene replacement. Moreover, we report the effect of exogenously added IAA on the transcription of virulence-related genes, including the T3SS (hrpA and hrpL) and the T6SS (vgrG).

**Materials and methods**

**Bacterial strains and culture conditions**

Strains and plasmids used in this study are listed in Table 1. The strains of Psv NCPPB 3335 and *Escherichia coli* were grown at 28 and 37 °C, respectively, in Luria–Bertani (LB) medium (Miller, 1972) or super optimal broth (SOB; Hanahan, 1983). When required, antibiotics were added at

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<th>Strain/plasmid</th>
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<tr>
<td>Strain</td>
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<td><em>P. savastanoi</em> pv. savastanoi</td>
<td>Wild-type strain</td>
<td>Pérez-Martínez et al. (2007)</td>
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<td>NCPPB 3335</td>
<td>iaaM/iaaH-1 mutant (Km&lt;sup&gt;R&lt;/sup&gt;)</td>
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<td>ΔiaaMH-1</td>
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<td>DH5α</td>
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<td>GM2929</td>
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<td>Plasmid</td>
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<td>pGEM-T easy</td>
<td>Cloning vector containing ori f1 and lacZ (Ap&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Promega, Madison, WI</td>
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<td>pGEM-T-KmFRT- Xhol</td>
<td>Contains Km&lt;sup&gt;R&lt;/sup&gt; from pKD4 and Xhol sites (Ap&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Zumaquero et al. (2010)</td>
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<td>pGEM-T-KmFRT- HindIII</td>
<td>Contains Km&lt;sup&gt;R&lt;/sup&gt; from pKD4 and HindIII sites (Ap&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;)</td>
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<td>pFLP2</td>
<td>Contains a flipase gene (Ap&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Hoang et al. (1998)</td>
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<td>pMP220</td>
<td>Broad-host-range, low-copy-number promoter probe vector, IncP replicon, lacZ (Tc&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Spaink et al. (1987)</td>
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<tr>
<td>pGEM-T-ΔiaaMH1-Km</td>
<td>pGEM-T derivative containing c. 1.2 kb on each side of the iaaM/iaaH-1 operon from NCPPB 3335 interrupted by the kanamycin resistance gene nptI (Ap&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;).</td>
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<td>pGEM-T-ΔiaaMH2-Km</td>
<td>pGEM-T derivative containing c. 1.2 kb on each side of the iaaM/iaaH-2 operon from NCPPB 3335 interrupted by the kanamycin resistance gene nptI (Ap&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;).</td>
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<td>pMP220-P&lt;sub&gt;hrpA&lt;/sub&gt;</td>
<td>Contains a fragment of 270 bp corresponding with the <em>hrpA</em> promoter from NCPPB 3335 directionally cloned in pMP220 using EcoRI</td>
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<td>pMP220-P&lt;sub&gt;hrpL&lt;/sub&gt;</td>
<td>Contains a fragment of 245 bp corresponding with the <em>hrpL</em> promoter from NCPPB 3335 directionally cloned in pMP220 using EcoRI</td>
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the following concentrations. For *E. coli*: ampicillin 100 μg mL⁻¹, kanamycin 50 μg mL⁻¹ and tetracycline 10 μg mL⁻¹. For *P. savastanoi*: ampicillin 300 μg mL⁻¹, kanamycin 10 μg mL⁻¹, and tetracycline 10 μg mL⁻¹. When relevant, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to a final concentration of 0.02%.

**Construction of Psv mutants**

To construct the single mutants ΔiaaMH-1 and ΔiaaMH-2, a fragment of c. 2 kb was removed from the *iaaM/iiaH* operon. To construct the plasmids pGEM-T-ΔiaaMH1 and pGEM-T-ΔiaaMH2, the upstream and downstream fragments of the region to be deleted were cloned into the pGEM®-T Easy Vector (Table 1) as described by Matas et al. (2014). Later, the *nptII* kanamycin resistance gene obtained from pGEM-T-KmFRT-XhoI and pGEM-T-KmFRT-HindIII (Table 1) was introduced into these plasmids, yielding pGEM-T-ΔiaaMH1-Km and pGEM-T-ΔiaaMH2-Km, respectively (Table 1). Finally, for marker exchange mutagenesis (Supporting Information, Fig. S1), each plasmid was transformed by electroporation into NCPPB 3335 as described previously (Pérez-Martinez et al., 2007). Screening and verification of the mutants was carried out as previously described (Matas et al., 2014).

The double mutant ΔiaaMH-1.2 was generated using the ΔiaaMH-1 single mutant, in which the kanamycin gene was removed using the pFLP2 plasmid (Zumaquero et al., 2010). Then, a ΔiaaMH1-Km^r^ clone was transformed with the pGEM-T-ΔiaaMH2-Km plasmid for marker exchange mutagenesis to obtain the double mutant.

**Transcription initiation mapping by 5′cRACE**

The transcription start point of the *iaaM-1/iiaH-1* operon was determined using a system for rapid amplification of cDNA ends (5′cRACE; Filiatrault et al., 2011). Total RNA from wild-type Psv NCPPB 3335 was obtained as previously described (Matas et al., 2014). One microgram of this RNA was used as a template to synthesize the first-strand cDNA, using the synthesis kit SMART® RACE cDNA Amplification (Clontech, Mountain View, CA) and *iaaM-1*-specific primers designed to anneal within the coding region of this allele and not *iaaM-2*. All reactions were performed according to the manufacturer’s instructions. The amplification products were cloned into the pGEM®-T Easy Vector and sequenced.

**Quantification of IAA production**

For the IAA extraction for HPLC, 2 mL of the supernatant from liquid cultures grown in LB medium without added tryptophan was collected and acidified to pH 2.5–3.0 with 1 M HCl. Then, the supernatant was mixed with the same volume of ethyl acetate and incubated under shaking conditions for 30 min at room temperature. Finally, the extracted ethyl acetate fraction was evaporated at room temperature, and the pellet was resuspended in 1 mL MeOH/H₂O (10 : 90; v/v), filtered (0.2 μm) and injected into the HPLC machine. The analyses were carried out using a SunFire HPLC system with C18 analytical column, 5 μm particle size, 2.1 × 100 mm (Waters). The chromatographic system was interfaced to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). MASSLYNX NT version 3.4 (Micromass) was used to process the quantitative data from the calibration standards and bacterial samples. IAA quantities were normalized using the dry weight (DW) of a 2-mL bacterial pellet, which was obtained by complete drying for 1 h at 80 °C.

**Plant infection and isolation of bacteria from olive knots**

Olive plants were micropropagated, rooted and maintained as previously described (Rodriguez-Moreno et al., 2008). Micropropagated plants were infected in the stem wound with a bacterial suspension (c. 10⁷ CFU) and incubated for 30 days in a growth chamber as described by Rodriguez-Moreno et al. (2008). The morphology of the olive plants infected with bacteria was visualized using a stereoscopic microscope (Leica MZ FLIII; Leica Microsystems, Wetzlar, Germany). Knot volume was quantified using a three-dimensional scanner, and MINIMAGICS 2.0 software. The competitive index (CI) between the wild-type and the *iaaMH* mutants was determined as described previously (Rodriguez-Moreno et al., 2008; Matas et al., 2012). To analyse the pathogenicity of *P. savastanoi* isolates in 1-year-old olive explants (lignified plants), the plants were maintained and inoculated according to previously described methods (Penyalver et al., 2006; Pérez-Martinez et al., 2007; Matas et al., 2012). Morphological changes, scored at 90 days post-infection (d.p.i.), were captured with a high-resolution digital camera (Canon D600; Canon Inc., Tokyo, Japan). Knot volume was calculated as previously reported (Moretti et al., 2008; Hosni et al., 2011). Statistical data analysis was performed by *ANOVA* followed by Student’s *t*-test (*P* ≤ 0.05).

**β-Galactosidase assays**

The transcriptional fusions of the T3SS-related genes, *hrpA* (structural gene encoding Hrp pilin) and *hrpL* (a regulatory gene encoding an alternative sigma factor)
promoters, to *lacZ* were constructed by PCR amplification using NCPPB 3335 genomic DNA and the forward and reverse primers shown in Supporting Information, Table S1. The resulting DNA fragments were EcoRI-digested and cloned into pMP220 to generate the plasmids pMP220- *P*/*hrpA* and pMP220- *P*/*hrPL* (Table 1). PCR using the forward primer specific for each promoter and pMP220-R was carried out to confirm that the promoters were directionally cloned in pMP220. Preinocula of bacterial strains harbouring the relevant plasmids were grown overnight in LB. The next day, the cells were diluted to an OD<sub>600</sub> of 0.05 and grown to an OD<sub>600</sub> of 0.5. At this point, total cells were pelleted, washed twice with 10 mM MgCl<sub>2</sub> and resuspended in 5 mL of Hrp-inducing medium (Huynh et al., 1989) in the presence or absence of 1 mM IAA. No changes in the pH of this medium (pH 5.7) were observed after addition of IAA. β-Galactosidase activity was measured after 2, 6 and 24 h of incubation in this medium, as previously described (Miller, 1972).

**Quantitative RT-PCR assays**

Quantitative real-time PCR (qRT-PCR) assays in Psv NCPPB 3335 were performed in the same conditions as those used for the β-galactosidase assays. RNA extraction was carried out after 6 h of incubation in the Hrp-inducing medium. The cells were pelleted and processed for RNA isolation using TRIzol Reagent (Roche Applied Science, Mannheim, Germany) as described previously (Matas et al., 2014). cDNA synthesis and qRT-PCR assays were as described by Matas et al. (2014). Transcriptional data were normalized to the housekeeping gene gyrA. qRT-PCR reactions were performed in triplicate.

**Results and discussion**

**Horizontal transfer of the *iaaM/iaaH* operon in the *P. syringae* complex**

IAA is synthesized in *P. savastanoi* through the indole-3-acetamide pathway, which involves the genetic determinants *iaaM* and *iaaH* (Fig. 1a). Two different alleles of each of these genes were found in the chromosome of Psv NCPPB 3335, organized in two operons (*iaaM-1/iaaH-1* and *iaaM-2/iaaH-2*; Rodríguez-Palenzuela et al., 2010; Fig. 1c), which are located at a distance of about 670 kb (data not shown). A nucleotide sequence alignment of the complete *iaaM/iaaH* operons (from the start codon of the *iaaM* gene to the stop codon of the *iaaH* gene) encoded by NCPPB 3335 revealed 92% identity between them (the percentage identity between *iaaM-1* and *iaaM-2* and between *iaaH-1* and *iaaH-2* was 92% and 93%, respectively). In addition, a comparative phylogenetic study of the same nucleotide fragment was performed using all available bacterial genomes. Those sequences showing an identity lower than 40% with NCPPB 3335 were not included in this analysis. The *iaaM/iaaH* operon was found in five different groups of plant-pathogenic bacteria, i.e. the *Pseudomonas syringae* complex, *Pantoea* spp., *Burkholderia* spp., *Dickeya* spp. and *Agrobacterium* spp. In fact, all these bacteria have recently been included in Group I of *iaaM*-like sequences (Patten et al., 2012). According to these authors, Group I can be clearly distinguished from Group II, which includes many other bacterial species encoding a divergent Trp-monoxygenase. In general, the phylogeny of the *iaaM/iaaH* sequences is largely congruent with the phylogeny deduced from housekeeping genes (Ramos et al., 2012), suggesting that this operon is ancestral within these bacterial groups. However, the clustering of *iaaM-2/iaaH-2* from Psv NCPPB 3335 (genomospecies 2) with *P. syringae* pv. *aceris* M302273PT and *P. syringae* pv. *syringae* B728a (genomospecies 1) is consistent with the possibility of horizontal gene transfer. This result is not surprising given that the *iaaM/iaaH* operon is often found in several copies and located in plasmids (Caponero et al., 1995; Pérez-Martínez et al., 2008). In contrast, the clustering of the NCPPB 3335 *iaaM-1/iaaH-1* operon with *Psn* and *P. syringae* pv. *glycinea* (genomospecies 2) is consistent with the phylogeny of the *iaaL-1* gene reported by Ramos et al. (2012). This gene is located upstream of the *iaaM-1/iaaH-1* operon in NCPPB 3335 (Matas et al., 2009). Co-evolution and recent stabilization of the plasmid-encoded *Psn* fragment containing all three genes (*iaaM, iaaH* and *iaaL*) in the genome of Psv have been proposed (Matas et al., 2009). In agreement with this hypothesis, the transcription start site of the *iaaM-1/iaaH-1* operon in Psv NCPPB 3335, determined by 5′cRACE, was located within the ORF of an IS4 transposase gene 401 bp upstream of the *iaaM-1* start codon (Fig. 1c). This site is almost coincident (1 bp further upstream) with the transcription initiation site predicted for this operon in *Psn* (Gaffney et al., 1990). Moreover, the −10 and −35 promoter regions previously described in *Psn* (Gaffney et al., 1990) were found upstream of the +1 site identified for the *iaaM-1/iaaH-1* operon in Psv NCPPB 3335. Taking into account that the *iaaM-2* gene has been shown to be a pseudogene (Ramos et al., 2012), the transcription start site of the *iaaM-2/iaaH-2* operon was not identified in this study.

**Production of IAA in Psv NCPPB 3335 mainly depends on the *iaaM-1/iaaH-1* operon**

To determine the individual roles of the *iaaM/iaaH* operons in the biosynthesis of IAA by Psv NCPPB 3335,
single (\(\text{DiaaMH-1}\) and \(\text{DiaaMH-2}\)) and double (\(\text{DiaaMH-1.2}\)) knockout mutants were constructed by gene replacement (Fig. 1c). Psv NCPPB 3335 and all three mutants were grown on LB medium to the stationary phase, and IAA was extracted from the culture supernatants and quantified by HPLC. The level of IAA in the culture supernatants of the \(\text{DiaaMH-1}\) and \(\text{DiaaMH-1.2}\) mutants was c. 40 times lower than the level in the wild-type strain (5565 \(\mu\)g g\(^{-1}\) DW). However, a residual amount of IAA was found in the culture supernatants of these two strains. Moreover, no significant differences were found between the \(\text{DiaaMH-2}\) mutant and the wild-type strain (Fig. 2), suggesting that the \(\text{iaaM-2/iaaH-2}\) operon does not contribute to the pool of IAA produced by Psv NCPPB 3335. In fact, transcription analysis of the \(\text{iaaM-2}\) gene performed by qRT-PCR indicated that this gene is not expressed in Psv cells grown in LB medium (data not shown). Furthermore, the residual amounts of IAA (120 and 167 \(\mu\)g g\(^{-1}\) DW) detected in cultures of \(\text{DiaaMH-1}\) and \(\text{DiaaMH-1.2}\), respectively, might indicate the existence of an additional IAA biosynthetic pathway in Psv NCPPB 3335. Redundancy in IAA biosynthetic pathways has been previously predicted in other microorganisms after the inactivation of a single pathway (Patten et al., 2012).

**Contribution of the \(\text{iaaM-1/iaaH-1}\) operon to the virulence and fitness of Psv NCPPB 3335 in olive plants**

To determine the role of the Psv NCPPB 3335 operons in the virulence and fitness of this pathogen in olive plants, we analysed the ability of the single mutants (\(\text{DiaaMH-1}\) and \(\text{DiaaMH-2}\)) and the double mutant (\(\text{DiaaMH-1.2}\)) to cause olive knot symptoms and maintain fitness in the olive plant. Fitness attenuation and knot size reduction...
have been commonly used to describe hypovirulent mutants in Psv (Rodríguez-Moreno et al., 2008; Matas et al., 2012). Mixed infections using the wild-type strain and each of the mutants were prepared to calculate the CI on in vitro olive plants at 30 d.p.i. The results showed that the CI values obtained for the wild-type strain and each of the ΔiaaMH-1 and ΔiaaMH-1.2 mutants were significantly lower than 1, indicating that these mutants were outcompeted by the wild-type strain in planta. Conversely, this value was not significantly different from one in the competition assay between the ΔiaaMH-2 mutant and the wild-type strain, revealing that the ΔiaaM-2/iaaH-2 operon is not required for the full fitness of Psv NCPPB 3335 (Fig. 3).

All the strains were inoculated independently on in vitro-grown and 1-year-old olive plants, and disease severity was evaluated after 30 or 90 d.p.i., respectively. In both plant systems, the symptoms developed upon infection with the ΔiaaMH-1 and ΔiaaMH-1.2 mutants were visually less severe than those induced by the wild-type strain and the ΔiaaMH-2 mutant (Fig. 4a and b). Additionally, the knot volumes in olive plants infected with the ΔiaaMH-1 and ΔiaaMH-1.2 mutants were approximately eight times smaller (in vitro-grown olive plants, Fig. 4a, bottom) and 16 times smaller (lignified olive plants, Fig. 4b, bottom), respectively, of the knot volume of plants infected with the wild-type strain. Together, these results show that the ΔiaaM-2/iaaH-2 operon encoded by Psv NCPPB 3335 does not contribute to IAA biosynthesis or the induction of symptoms in olive plants. Moreover, these results suggest that the redundancy of IAA biosynthetic genes in some Psv strains (Pérez-Martínez et al., 2008) does not affect the total amount of IAA produced or the full virulence and fitness of the pathogen in olive plants. Nevertheless, a comparative virulence analysis with other Psv strains and a functional analysis of their ΔiaaM/iaaH operons is necessary to verify this hypothesis.

Fig. 2. IAA quantification in the ΔiaaMH mutants by HPLC. Quantification of total IAA produced by ΔiaaMH mutants and the wild-type strain after growing for 24 h in LB medium at 28 °C. Data are represented as μg IAA per g DW of bacterial cells. Each bar is the mean of three biological replicates, and the error bars represent standard deviation.

Fig. 3. Competition assays of ΔiaaMH mutants in olive plants. Competitive index values for mixed inoculations of Psv NCPPB 3335 and its derivative strains in micropropagated olive plants. Error bars indicate the standard error from the average of three different assays. Asterisks indicate values significantly different from 1. Statistical analyses were performed using Student’s t-test with a threshold of P = 0.05.

Exogenous IAA affects the expression of virulence-related genes in Psv

IAA has been described as a signalling molecule that affects the expression of virulence factors, such as the T3SS and T6SS, and IAA biosynthesis in several plant-associated bacteria (Spaepen & Vanderleyden, 2010; Van Puyvelde et al., 2011; Patten et al., 2012). Previous studies on Psv NCPPB 3335 demonstrated that the T3SS-related genes, hrpA and hrpL, are required for infection establishment and knot formation on olive plants (Pérez-Martínez et al., 2010; Matas et al., 2014). To investigate a possible link between IAA and the T3SS in Psv, we monitored expression of the hrpA and hrpL genes using transcriptional fusions of their corresponding promoter regions to the lacZ gene. Psv NCPPB 3335 cells grown to the exponential phase expressing each of these fusions were transferred to Hrp-inducing medium lacking IAA or containing 1 mM IAA. The expression of T3SS-related genes in P. syringae and related pathogens is usually analysed in this medium, which is widely believed to mimic the apoplastic environment (Rico & Preston, 2008). However, the production of IAA by Psv NCPPB 3335 under these conditions was negligible (data not shown). Thus, a comparison of the expression of the T3SS promoters in the wild-type strain vs. the Δiaa mutants could not be performed. To circumvent this limitation, we analysed the expression of the promoters in the wild-type strain transferred to Hrp-inducing medium.
lacking IAA or supplemented with IAA. In agreement with expression analysis previously reported for other Psv genes (Matas et al., 2014), the highest differences were observed 6 h after transfer to Hrp-inducing medium. In the presence of IAA, the expression levels of hrpA and hrpl were c. 77 and seven times lower, respectively, than those in the absence of this compound, indicating that IAA had a negative effect on the expression of the T3SS genes (Fig. 5a). Similar differences in the β-galactosidase activities of these promoters were observed after 24 h (data not shown). Additionally, qRT-PCR experiments were carried out under the same culture conditions used for the β-galactosidase assays. In these assays, we also analysed the expression of hrpA and hrpl, as well as vgrG, a gene related to the T6SS, and iaaL, a gene involved in the biosynthesis of IAA-Lys in Psn (Glass & Kosuge, 1988), which contains an hrp box promoter sequence recognized by the alternative sigma factor HrpL (Fouts et al., 2002). In the presence of IAA, and in agreement with the β-galactosidase assays (Fig. 5a), the total amount of hrpA and hrpl transcripts was reduced 40- and 25-fold, respectively, compared with their levels in the absence of IAA (Fig. 5b). Interestingly, the level of expression of the iaaL gene was similar in the presence and absence of IAA (Fig. 5b). Taking into account the possible dependency of the expression of this gene on HrpL (Fouts et al., 2002),

Fig. 4. Virulence assay of iaaMH mutants on young micropropagated and lignified olive plants. Knots were induced in vitro (a) and on lignified (b) olive plants by the indicated strains 30 and 90 d.p.i., respectively. Below the pictures, the average volume of at least three knots is represented with their corresponding standard deviations. The total volume is indicated in mm$^3$.

Fig. 5. Differential expression of virulence-related genes in Psv NCPPB 3335 upon exogenous IAA treatment. (a) β-Galactosidase activity of P$_{hrpA}$ and P$_{hrpL}$ in Psv NCPPB 3335 6 h after transfer to Hrp-inducing medium without IAA [IAA (–)] and with 1 mM IAA [IAA (+)]. Psv NCPPB 3335 transformed with the empty vector was included as a negative control. The results were obtained from triplicate analysis. Error bars represent the standard deviation. (b) The expression of the indicated Psv NCPPB 3335 genes measured by qRT-PCR in the wild-type strain after transfer to Hrp-inducing medium with IAA vs. without IAA. The fold change was calculated after normalization using the gyrA gene as an internal control. The results represent the means from three independent experiments. Error bars represent standard deviations. Asterisks indicate significant differences ($P = 0.05$) between the values obtained for the different conditions.
the low level of transcripts of the hrpL gene observed in the presence of IAA does not seem to affect the levels of iaaL transcripts. Nevertheless, the expression and function of the iaaL gene remain to be investigated in Psv. Conversely, the transcript levels of vgrG doubled in the presence of IAA, indicating a positive effect of IAA on the expression of the T6SS genes has been reported in Azospirillum brasilense by the addition of exogenous IAA (Van Puyvelde et al., 2011).

Concluding remarks

The phytohormone IAA plays an essential role in the virulence of bacterial phytopathogens, including the olive pathogen Psv. Here we show that the biosynthesis of IAA in this pathogen mainly depends on one of its two chromosomally encoded iaaM/iaaH operons (iaaM-1/iaaH-1), a gene cluster horizontally transferred among bacteria belonging to the P. syringae complex. We also demonstrate that the full fitness and virulence of Psv in olive plants depends on the functionality of this operon. Moreover, we show that IAA acts as a signalling molecule in Psv, affecting the expression of other virulence-related genes such as the T3SS and the T6SS.

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Role of IAA in the virulence of P. savastanoi pv. savastanoi

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Strategy for the construction of the mutant ΔiaaM–1 in PsNCCPB 3335 by gene replacement.

Table S1. Oligonucleotides used in this study.

Table S2. Locus Tag/Accession numbers for the iaaM and iaaH genes used for the construction of the NJ tree shown in Fig. 1c.