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Pseudomonas savastanoi pv. savastanoi Contains Two *iaaL* Paralogs, One of Which Exhibits a Variable Number of a Trinucleotide (TAC) Tandem Repeat▽†

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In this study, *Pseudomonas savastanoi* pv. savastanoi isolates were demonstrated to contain two *iaaL* paralogs, which are both chromosomally located in most strains. Comparative analysis of *iaaL* nucleotide sequences amplified from these two paralogs revealed that one paralog, $iaaL_{Psn}$, is 100% identical to *iaaL* from *P. savastanoi* pv. nerii, while the other paralog, $iaaL_{Psv}$, exhibited 93% identity to *iaaL* from *Pseudomonas syringae* pv. tomato ($iaaL_{Pto}$). A 3-nucleotide motif (TAC) comprised of 3 to 15 repeats, which remained stable after propagation of the strains in olive plants, was found in $iaaL_{Psv}$. Based on the observed nucleotide sequence variations, a restriction fragment length polymorphism assay was developed that allowed differentiation among $iaaL_{Psn}$, $iaaL_{Psv}$, and $iaaL_{Pto}$. In addition, reverse transcriptase PCR on total RNA from *P. savastanoi* pv. savastanoi strains demonstrated that both $iaaL_{Psv}$ and $iaaL_{Psn}$ containing 14 or fewer TAC repeats are transcribed. Capillary electrophoresis analysis of PCR-amplified DNA fragments containing the TAC repeats from $iaaL_{Psv}$ allowed the differentiation of *P. savastanoi* pv. savastanoi isolates.

Infections with *Pseudomonas savastanoi* pv. savastanoi (46), pv. fraxini, and pv. nerii (13) result in knots and galls on members of the Oleaceae family, and *P. savastanoi* pv. nerii also causes galls on oleander. Symptoms of infected trees include hypertrophy formation on the stems and branches and occasionally on the leaves and fruits. In the olive, the disease is considered to reduce both olive yield and productivity (41, 42); however, quantitative data on the impact of the disease on crop yield or crop quality are not available (19). Nevertheless, losses can be caused directly by localized infections that inhibit flowering and affect fruit development, as well as taste, and can be caused indirectly by weakening immature main leader branches that result in later damage to the tree frame (48).

Currently, the only molecular *P. savastanoi* determinants known to be involved in knot development are the phytohormones indoleacetic acid (IAA) and cytokinins (1, 15, 33, 38, 45), as well as biosynthesis of a functional type III secretion system, encoded by the *hrp-hrc* gene clusters (43, 44). Recently, a global genomic analysis of *P. savastanoi* pv. savastanoi plasmids allowed the identification of several putative virulence factors in the olive pathogen, including several type III secretion system protein effectors and a variety of genes encoding known *Pseudomonas syringae* virulence factors (32).

The genetic determinants of *P. savastanoi* that are involved in the conversion of tryptophan (Trp) to IAA are Trp monooxygenase (encoded by the *iaaM* gene), which converts Trp to indoleacetamide (IAM), and IAM hydrolase (encoded by the *iaaH* gene), which catalyzes transformation of IAM to IAA (28). IAA can be further metabolized in *P. savastanoi* to an amino acid conjugate, 3-indole-acetyl-ε-L-lysine (IAA-lysine), through the action of the *iaaL* gene (14). In *P. savastanoi* pv. savastanoi and pv. nerii, *iaaM* and *iaaH* are organized in an operon with the *iaaM* locus first in the gene cluster (12). The IAA operon and the *iaaL* gene reside on a plasmid (pIAA) in *P. savastanoi* strains isolated from oleander galls (5, 7, 8, 14); however, the direction of *iaaL* transcription has been determined to be opposite to IAA operon transcription (14). In contrast, most olive isolates have been reported to carry all three IAA genes on the chromosome (5, 8, 32). In a recent study, *P. savastanoi* pv. savastanoi strains carrying a pIAA plasmid were also suggested to contain chromosomally encoded copies of all three *iaa* genes (32); however, the exact copy numbers for the three genes in *P. savastanoi* isolates have not yet been estimated.

Molecular detection of *P. savastanoi* pv. savastanoi in olive plants is usually performed by an enrichment-PCR assay based on amplification of an internal 454-bp fragment of the *iaaL* gene followed by HaeIII digestion (29) or by nested PCR followed by dot blot hybridization (2). Although *iaaL* is widespread among plant-associated bacteria (10, 16), this assay has

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TABLE 1. Bacterial strains used in this study

Name and strain[a]	Country (province)[b]	*iaaL* hybridization		$iaaL_{Psv}$ size (bp)[e]	$(TAC)_n$[f]	Reference or source
		No.[c]	Classification[d]			
P. syringae pv. tomato						
DC3000	United Kingdom	1C			3	9
P. savastanoi pv. nerii						
ITM519	Italy	2P			3	45
LPVM Psn2	Italy	1P			3	S. Tegli
P. savastanoi pv. savastanoi						
NCPPB 1506	Italy	2C	II	122	3	29
ITM 317[h]	Italy	2C		122	3	45
PVFi 1	Italy	2C		122	3	18
IVIA 2733-1a	Italy	2C		122	3	35
CFBP 1020	France	2C	II	122	3	29
CFBP 2074[h]	Algeria	2C		122	3	29
NCPPB 1342	United States	2C	II	128	5	29
NCPPB 1344[h]	United States	2C	II	128	5	29
NCPPB 3335[h]	France	2C		128	5[g]	29
IVIA 1628-3[h]	Spain (Valencia)	2C		128	5[g]	29
IVIA 2743-3	Spain (Badajoz)	2C	II	128	5	35
GUMA Psv160	Spain (Jaén)	2P (25–95)	III	131	6	This study
GUMA Psv185	Spain (Málaga)	2C	I	131	6	This study
LSV C3.01	Spain (Sevilla)	2C	I	131	6[g]	32
IVIA 2445-4	Spain (Jaén)	1P (70)/1C	II	131	6	35
IVIA 1624-b1[h]	Spain (Valencia)	1P (75)/1C	II	131	6	29
NCPPB 2327	Italy	1P (60)/1C	II	131	6	29
NCPPB 1479	Serbia	1P (65)/1C	II	131	6	29
NCPPB 639[Th]	Serbia	1P (90)/1C	II	131	6	16
LSV C1.01	Spain (Sevilla)	2C	II	137	8	32
LSV B15.00	Spain (Sevilla)	2C	I	137	8	32
IVIA 1649-1[h]	Spain (Castellón)	2C	I	137	8[g]	29
LSV C2.01	Spain (Sevilla)	2C	I	140	9	31
IVIA 1657-a2	Spain (Alicante)	2C	I	140	9	29
IVIA 1637-B3[h]	Spain (Alicante)	2C	III	143	10[g]	29
IVIA 1629-1a	Spain (Valencia)	2C	I	149	12	29
IVIA 1637-C1	Spain (Alicante)	2C	I	151	13[g]	29
IVIA 1651-c15	Spain (Alicante)	2C	I	155	14[g]	29
NCPPB 64[h]	Portugal	2C	I	155	14	29
IVIA 1657-b8[h]	Spain (Valencia)	2C	I	158	15[g]	29

[a] ATCC, American Type Culture Collection, Manassas, VA; CFBP, Collection Francaise des Bacteries Phytopathogenes, Angers, France; IVIA, Instituto Valenciano de Investigaciones Agrarias Collection, Valencia, Spain; LPVM, Laboratorio di Patologia Vegetale Molecolare Collection, Firenze, Italy; LSV, Laboratorio de Sanidad Vegetal Collection, Sevilla, Spain; NCPPB, National Collection of Plant Pathogenic Bacteria, York, United Kingdom; GUMA, Área de Génetica, Universidad de Málaga Collection, Málaga, Spain.

[b] The province of isolation is indicated only for Spanish strains.

[c] Number of chromosomal (C) and plasmid (P)-borne copies of *iaaL*. The size (in kilobases) of *iaaL*-hybridizing plasmids is indicated in parentheses.

[d] Classification groups for *iaaL* hybridization patterns of *P. savastanoi* pv. savastanoi strains exhibiting a common BglII hybridization band of approximately 8 kb. I, banding pattern exhibits a second hybridization band at 20 kb (Fig. 1, lanes 8 and 10 to 14); II, banding pattern exhibits an additional hybridization band larger than 9.4 kb and smaller than 20 kb (Fig. 1, lanes 6, 7, 16, and 17); and III, banding pattern exhibits an additional hybridization band larger than 23 kb (Fig. 1, lane 9).

[e] Size of PCR-amplified $iaaL_{Psv}$ fragments calculated by capillary electrophoresis.

[f] Estimated number of TAC repeats in the *iaaL* gene. For *P. savastanoi* pv. savastanoi strains, the number of TAC repeats refers to the $iaaL_{Psv}$ paralog.

[g] Strain with both *iaaL* paralogs sequenced. Accession numbers of $iaaL_{Psv}$ fragments are as follows: NCPPB 3335 (EU616802), IVIA 1628-3 (EU616795), LSV C301 (EU616796), IVIA1649-1 (EU616800), IVIA 1637-B3 (EU616799), IVIA 1637-C1 (EU616798), IVIA 1651-c15 (EU616801), and IVIA 1657-b8 (EU616797).

[h] Strain with partially sequenced *gyrB* and *rpoD* genes. Nucleotide sequences exhibited 100% identity with those of *P. savastanoi* pv. savastanoi strain NCPPB 639 (accession numbers AB039514 and AB039469 for *rpoD* and *gyrB*, respectively).

been designed using the published sequence of *iaaL* from an oleander isolate (37) and appears to be specific for *P. savastanoi* (29).

The aim of this study was to develop an improved method for the detection and differentiation of *P. savastanoi* pv. savastanoi strains based on the existence of nucleotide sequence variations in the *iaaL* gene. First, we determined the copy numbers of plasmid- and chromosome-borne *iaaL* sequences for a collection of 30 *P. savastanoi* pv. savastanoi strains that were isolated in different countries. Then, nucleotide sequence differences, including short tandem repeat sequences (STRs), between *iaaL* paralogs were analyzed. Based on the observed variations, a PCR-restriction fragment length polymorphism (RFLP) assay was developed that allowed differentiation between *iaaL* paralogs and transcriptional analysis of paralogs by reverse transcriptase (RT) PCR, followed by RFLP. Capillary electrophoresis analysis of PCR-amplified STRs within the *iaaL* gene allowed the differentiation of *P. savastanoi* pv. savastanoi isolates.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and media. The *Pseudomonas* strains used in this study (Table 1) were grown at 28°C in Luria-Bertani medium (26) or King's medium B (21), as specified below. *P. savastanoi* pv. savastanoi identifi-

cation was performed by LOPAT biochemical tests (22) and olive plant inoculations (30) for all strains included in this study. In addition, amplification and analysis of *gyrB* and *rpoD* nucleotide sequences (36, 47) were performed for 10 of the strains (Table 1).

General molecular techniques. Agarose gel electrophoresis and other standard recombinant DNA techniques were performed by standard procedures as described previously (40). Genomic DNA was extracted using the Jet Flex Extraction Kit (Genomed, Löhne, Germany), digested with the appropriate restriction enzymes, and separated by electrophoresis, as described by Pérez-Martínez and coworkers (32). Plasmid DNA minipreps from *P. savastanoi* were performed and separated through 0.7% agarose gels in 1× Tris-acetate-EDTA, as previously reported (31, 32). A DNA fragment of the *iaaL* gene, amplified from *P. savastanoi* pv. savastanoi strain NCPPB 3335 using primers IAAL-F and IAAL-R (29), was labeled with a nonradioactive digoxigenin kit (Roche Diagnostics) and used as a probe, as previously described (32).

PCR-RFLP analysis of *iaaL* paralogs. PCR amplification of an internal fragment of the *iaaL* gene was performed using *GoTaq* DNA polymerase (Promega Corporation, Madison, WI) and primers IAAL-F and IAAL-R, as previously described (29), with slight modifications. Briefly, the amplification conditions consisted of an initial denaturation step at 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min and a final extension step at 72°C for 5 min in a Perkin Elmer GeneAmp PCR System 2400 thermocycler. In order to reduce nonspecific DNA products, 5% formamide was added to the reaction mixture. The amplified products were separated by electrophoresis through a 3% Tris-borate-EDTA 1× agarose gel for 4 hours at 80 V. Under these conditions, the target *iaaL* fragment was amplified not only from *P. savastanoi* pv. savastanoi and pv. nerii, but also from *P. syringae* pv. tomato, pv. maculicola, pv. tabaci, and pv. pisi (data not shown). The PCR products were digested with HaeIII, which recognizes GGCC sites, and the resulting fragments were separated by electrophoresis through a 3% agarose gel for 6 hours at 80 V. After electrophoresis, the gels were stained with ethidium bromide.

Sequencing of *iaaL* fragments. Primers IAAL-PSN-F (5′-CATATGACTGCCTACGATATGG-3′) and IAAL-PTO-F (5′-CATATGACTGCCTACGATGTA-3′) were designed to specifically amplify a fragment from the *iaaL* paralog $iaaL_{Psn}$ or $iaaL_{Psv}$ (see below), respectively, in combination with IAAL-R (29). Amplicons obtained using both primer pairs from eight different *P. savastanoi* pv. savastanoi isolates (Table 1) were sequenced using primers IAAL-F and IAAL-R (29). The amplified DNA fragments were sequenced on both strands by the dideoxy sequencing termination method using an ABI Prism 310 (Applied Biosystem) sequencer. Contiguous DNA sequences were assembled using Seqman (DNAStar) and compared with sequences in public databases using NCBI BLAST.

Capillary electrophoresis to assess the copy numbers and stabilities of tandem repeats. Determination of the number of tandem TAC repeats in the $iaaL_{Psv}$ allele and stability analysis of repeats in *P. savastanoi* pv. savastanoi strains was performed by capillary electrophoresis as follows. Total-DNA samples isolated from *P. savastanoi* pv. savastanoi strains or enriched bacterial cultures recovered from olive knots were used as a template in a PCR with primers IAAL-F (29) and 6-carboxyfluorescein (6-FAM)-labeled primer IAAL-6-FAM (5′-6-FAM-TTGGGCAGCGATCAC-3′). The amplification products, which varied in size from 122 bp to 158 bp depending on the *iaaL* allele and strain, were analyzed using an ABI3130 genetic analyzer, a LIZ500 size standard, and GeneMapper v. 3.7 software (Applied Biosystems). To determine the stability of the number of tandem TAC repeats in plants, *P. savastanoi* pv. savastanoi strains were inoculated into olive plants as previously described (31). After a period of 7 (IVIA 1628-3, IVIA 2445-4, IVIA 2733-1a, IVIA 2743-3, and NCPPB 639) or 12 (CFBP 2074, ITM 317, NCPPB 64, and NCPPB 1344) months, *P. savastanoi* pv. savastanoi strains were recovered from the developed knots, as described by Quesada et al. (34, 35), and grown overnight in LB medium for bacterial enrichment.

Preparation of RNA and RT-PCR–RFLP. Pure bacterial cultures were grown overnight at 28°C in either LB or minimal SSM medium (25), diluted in the same medium to an optical density at 600 nm of 0.1, and incubated at the same temperature until the cultures reached a turbidity of 0.4 (exponential phase) or approximately 1.0 (stationary phase). Isolation of RNA from bacterial cultures was performed as described by Castillo and coworkers (6). The RNA concentration was determined using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and RNA integrity was assessed by agarose gel electrophoresis. RT-PCR was performed with 100 ng RNA in a final volume of 50 μl using the Tital OneTube RT-PCR system, according to the manufacturer's instructions (Roche Diagnostics), with primers IAAL-F and IAAL-R. A 40-cycle amplification program (94°C for 30 s, 57°C for 30 s, and 68°C for 1 min) was performed, followed by a final extension cycle at 68°C for 7 min. Positive control reactions, containing total DNA isolated from the corresponding bacterial strain, were included in all assays.

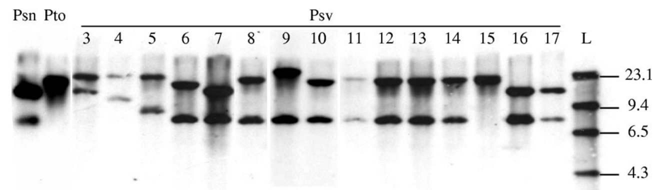

FIG. 1. Detection of *iaaL* gene sequences in *P. savastanoi* strains. Shown is Southern blot analysis of BglII-digested total DNA using an *iaaL* probe. Lane 1 (Psn) is *P. savastanoi* pv. nerii ITM 519, and lane 2 (Pto) is *P. syringae* pv. tomato DC3000. Lanes 3 to 17 (Psv) are *P. savastanoi* pv. savastanoi strains. Lanes: 3, CFBP 2074; 4, PVF 1; 5, IVIA 1628-3; 6, NCPPB 1344; 7, IVIA 1624-b1; 8, LSV C2.01; 9, IVIA 1637-B3; 10, IVIA 1637-C1; 11, IVIA 1629-1a; 12, IVIA 1651-c15; 13, NCPPB 64; 14, IVIA 1657-b8; 15, IVIA 2733-1a; 16, IVIA 2743-3; and 17, IVIA 2445-4. L, HindIII-digested λ phage DNA. The positions (in kilobases) of the molecular size markers are indicated on the right of the gel.

RESULTS

***P. savastanoi* pv. savastanoi contains two paralogs of the *iaaL* gene.** Hybridization analysis of *P. savastanoi* plasmids with an *iaaL* probe revealed that 5 of the 30 *P. savastanoi* pv. savastanoi isolates tested, as well as the *P. savastanoi* pv. nerii strain LPVM Psn2, contained a native plasmid that hybridized with the probe. In addition, GUMA-Psv160 and *P. savastanoi* pv. nerii strain ITM 519 contained two different plasmids that hybridized to the probe (Table 1). While BglII-digested total DNA isolated from *P. syringae* pv. tomato DC3000 resulted in a single band of approximately 20 kb (Fig. 1), as expected from the published genome sequence of the strain (4), total DNA isolated from *P. savastanoi* yielded two clearly distinguishable hybridization bands for most of the strains tested (28 out of 30 *P. savastanoi* pv. savastanoi strains and *P. savastanoi* pv. nerii strain ITM 519) (Table 1 and Fig. 1). Interestingly, a common BglII hybridization band of approximately 8 kb was detected in 24 *P. savastanoi* pv. savastanoi strains (80%), indicating that most of these strains carry a copy of *iaaL* in similar or identical locations (Fig. 1). Three hybridization patterns were found within this group of strains: group I, composed of 11 strains isolated in the Iberian Peninsula (Fig. 1, lanes 8 and 10 to 14); group II, including all *P. savastanoi* pv. savastanoi strains carrying only one plasmid-borne *iaaL* sequence (Fig. 1, lanes 6, 7, 16, and 17); and group III, composed of two strains (Fig. 1, lane 9). The remaining six strains (20%) exhibited a strain-specific hybridization pattern that was different from those found in these three groups (Fig. 1, lanes 3, 4, 5, and 15, and Table 1).

Total isolated DNA from *P. savastanoi* pv. savastanoi strains yielding a single hybridization band (Fig. 1, lane 15) was digested with alternative restriction enzymes (BamHI and HindIII) and hybridized again with the *iaaL* probe. The hybridization results confirmed the existence of two different DNA fragments that hybridized to the probe for all 30 *P. savastanoi* pv. savastanoi strains tested (Table 1). All of these results indicate that most *P. savastanoi* pv. savastanoi isolates (24 out of 30

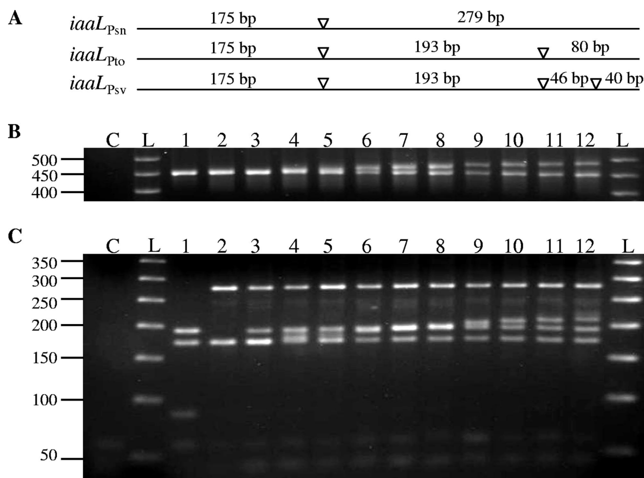

FIG. 2. PCR-RFLP differentiation of the *iaaL* gene sequences from *P. syringae* and *P. savastanoi* strains. (A) Schematic representation of *iaaL* DNA fragments amplified by PCR from the indicated strains using primers IAAL-F and IAAL-R. Target positions for HaeIII are indicated by arrowheads. $iaaL_{Psn}$, *P. savastanoi* pv. nerii EW-2009; $iaaL_{Pto}$, *P. syringae* pv. tomato DC3000; and $iaaL_{Psv}$, *P. savastanoi* pv. savastanoi ITM 317. (B and C) Gel electrophoresis (3% agarose) of *iaaL* amplicons and HaeIII-digested amplicons, respectively. Lane 1, *P. syringae* pv. tomato DC3000; lane 2, *P. savastanoi* pv. nerii strain LPVM Psn2. Lanes 3 to 12 are *P. savastanoi* pv. savastanoi strains. Lanes: 3, CFBP 2074; 4, IVIA 1628-3; 5, IVIA 1624-b1; 6, IVIA 1649-1; 7, LSV C2.01; 8, IVIA 1637-B3; 9, IVIA 1629-1a; 10, IVIA 1637-C1; 11, IVIA 1651-c15; and 12, IVIA 1657-b8. Lane C, negative control without template. A fragment of approximately 60 bp, most likely formed by nonspecific primer annealing, is visible in all samples, including C. Lane L, 50-bp DNA step ladder (Promega Co.); the positions (in base pairs) of the molecular size markers are indicated on the left of the gel.

strains) contain at least two chromosomally located copies of *iaaL*.

Differentiation of *iaaL* paralogs in *P. savastanoi* pv. savastanoi by PCR-RFLP. The nucleotide sequences of *iaaL* genes that were currently annotated included only a plasmid-borne open reading frame from *P. savastanoi* pv. nerii strain EW2009 ($iaaL_{Psn}$; accession number M35373) and the chromosomally located ortholog from *P. syringae* pv. tomato strain DC3000 ($iaaL_{Pto}$; accession number NC_004578). The internal 454-bp fragment of this gene used for PCR identification of *P. savastanoi* pv. savastanoi (29) contains one and two HaeIII sites in $iaaL_{Psn}$ and $iaaL_{Pto}$, respectively (Fig. 2A). The observed differences between these two ortholog sequences were further confirmed by PCR-RFLP (Fig. 2).

PCR amplification of the 454-bp *iaaL* fragments from some of the tested *P. savastanoi* pv. savastanoi isolates yielded an additional fragment of variable size, which was always greater than 454 bp (Fig. 2B, lanes 6 to 12). Further HaeIII digestion of these amplicons revealed RFLP profiles of at least four fragments, with two of them identical to those generated by $iaaL_{Psn}$. Additionally, all *P. savastanoi* pv. savastanoi isolates exhibited a 193-bp fragment, also generated by $iaaL_{Pto}$, and a fragment of approximately 45 bp. Moreover, some of the tested strains exhibited an additional fragment of variable size (between 175 bp and 250 bp). Together, these results suggest that the *P. savastanoi* pv. savastanoi isolates contain two *iaaL* paralogs, one paralog with a PCR-RFLP profile that is identical to that of $iaaL_{Psn}$ and a second paralog with a variable profile among strains, which shows some similarities to that of $iaaL_{Pto}$ and is referred to hereafter as $iaaL_{Psv}$ (Fig. 2).

Identification of a trinucleotide repeat sequence, $(TAC)_n$, in the $iaaL_{Psv}$ paralog of *P. savastanoi* pv. savastanoi. DNA fragments from $iaaL_{Psn}$ and $iaaL_{Psv}$ were specifically amplified and sequenced for eight *P. savastanoi* pv. savastanoi strains (Table 1). While the nucleotide sequences of the $iaaL_{Psn}$ amplicons were 100% identical to that of $iaaL_{Psn}$ from *P. savastanoi* pv. nerii strain EW2009, $iaaL_{Psv}$ paralogs exhibited a variable number (n) of trinucleotide TAC tandem repeats, which were located in frame and immediately after a $(TAC)_3$ sequence encoding three consecutive tyrosine residues (Tyr78 to Tyr80) in both $iaaL_{Pto}$ and $iaaL_{Psn}$. Except for these additional TAC codons, the nucleotide sequences of all eight $iaaL_{Psv}$ paralogs were 100% identical and exhibited 93% identity to that of $iaaL_{Pto}$. Nucleotide variations found between $iaaL_{Pto}$ from *P. syringae* pv. tomato strain DC3000 and $iaaL_{Psv}$ from these eight *P. savastanoi* pv. savastanoi strains demonstrated the existence of an additional HaeIII site in $iaaL_{Psv}$, which is located 40 nucleotides upstream from the 3′ end of this amplicon (Fig. 2A). Accession numbers of $iaaL_{Psv}$ for these eight strains are shown in Table 1.

Determination of the number of tandem TAC repeats and repeat stability in *P. savastanoi* pv. savastanoi $iaaL_{Psv}$. Determination of the number of tandem TAC repeats in the $iaaL_{Psv}$ allele for the remaining 22 *P. savastanoi* pv. savastanoi strains was performed using capillary electrophoresis. While the primers designed for this purpose, which are identical to the sequence flanking $(TAC)_n$ for both $iaaL_{Psn}$ and $iaaL_{Psv}$, amplified a fragment of 122 bp from $iaaL_{Psn}$, the sizes of the fragments amplified from $iaaL_{Psv}$ varied between 122 ($n = 3$) and 158 ($n = 15$) bp among *P. savastanoi* pv. savastanoi strains (Table 1). Figure S1A in the supplemental material shows the chromatograms obtained for 10 of the tested *P. savastanoi* pv. savastanoi strains. Interestingly, *P. savastanoi* pv. savastanoi strains containing eight or more TAC repeats (59%) were all isolated in the Iberian Peninsula (Spain, 10 strains; Portugal, 1 strain). In comparison, all six strains with a plasmid-borne *iaaL* gene contain six TAC repeats in $iaaL_{Psv}$ (Table 1). Purification of pIAA plasmids from the five strains containing only one plasmid-borne *iaaL* sequence, followed by PCR-RFLP analysis, demonstrated that $iaaL_{Psn}$ was found on a plasmid in all five strains (data not shown). Thus, $iaaL_{Psv}$ is located on the chromosome for these five strains.

The stability of the TAC repeats in olive knots was tested for nine *P. savastanoi* pv. savastanoi strains (see Materials and Methods). The sizes of the *iaaL* fragments amplified for all these strains were identical to those of the corresponding wild-type strains, indicating that the STR unit remains stable under the conditions tested. Figure S1B in the supplemental material shows the results obtained for four of these strains.

Transcriptional analysis of $iaaL_{Psn}$ and $iaaL_{Psv}$ in *P. savastanoi* pv. savastanoi. Transcriptional analyses of both $iaaL_{Psv}$ and $iaaL_{Psn}$ were performed for five different *P. savastanoi* pv. savastanoi strains. The expected product sizes for $iaaL_{Psn}$ (454 bp) and $iaaL_{Psv}$ (from 454 bp to 490 bp, depending on the number of TAC repeats) were obtained for all strains tested, and the transcript levels for each strain in LB medium (data not shown) and mini-

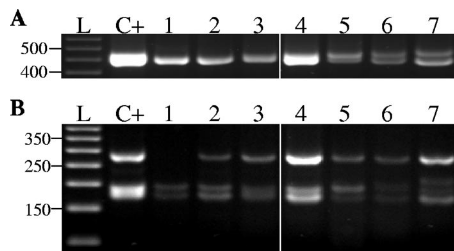

FIG. 3. Transcriptional analysis of $iaaL_{\mathrm{Psn}}$ *and* $iaaL_{Psv}$ in *P. savastanoi* pv. savastanoi. Gel electrophoresis (3% agarose) of RT-PCR amplicons of *iaaL* genes (A) and HaeIII-digested *iaaL* amplicons shown in panel A (B) are shown. Lane 1, *P. syringae* pv. tomato DC3000. Lanes 2 to 6 correspond to *P. savastanoi* pv. savastanoi strains. Lanes: 2, ITM 317; 3, NCPPB 3335; 4, NCPBB 639; 5, IVIA 1637-B3; 6, IVIA 1637-C1; and 7, NCPPB 64. Lane C+, positive control RT-PCR amplification reaction with total DNA of NCPPB 3335 as the template. Lane L, 50-bp DNA ladder (Promega Co.); the positions (in base pairs) of the molecular size markers are indicated on the left of the gel.

mal medium (Fig. 3) were similar. Digestion of RT-PCR products with HaeIII resulted in the expected RFLP profile for all strains tested, further demonstrating expression of both *iaaL* paralogs in these strains, regardless of the number of TAC repeats (from $n = 3$ to $n = 14$) contained in $iaaL_{\mathrm{Psv}}$. A higher intensity of the amplified RT-PCR product, reflected in the intensity of RFLP fragments corresponding to $iaaL_{\mathrm{Psn}}$ (279 bp and 175 bp), was observed for NCPPB 639. As $iaaL_{\mathrm{Psn}}$ is plasmid borne in this strain, these results suggest a higher level of expression of $iaaL_{\mathrm{Psn}}$ located on the plasmid than for the chromosomally located $iaaL_{\mathrm{Psv}}$. No differential expression between *iaaL* paralogs was found for strains containing both alleles located on the chromosome (Fig. 3).

DISCUSSION

In this study, we demonstrated that *P. savastanoi* pv. savastanoi isolates contain two *iaaL* paralogs that are both chromosomally located in most strains analyzed (80%) (Table 1). This high frequency of chromosome-borne *iaaL* genes in *P. savastanoi* olive isolates could be a response to the need for stabilization of the virulence factor (24, 49) through mechanisms of integration into the host chromosome. In fact, *P. savastanoi* pIAA plasmids that have been extensively characterized thus far belong to the pPT23A family of plasmids (32, 49), and the maintenance and evolution of these plasmids is probably based on horizontal transfer and recombination events, which result in either inclusion of plasmid-borne genes on the chromosome or gene duplications (24, 49). Interestingly, *iaaL*, *iaaM*, and *iaaH* seem to coevolve in concert, since all three genes have been found in the same replicon and copy number for some of the *P. savastanoi* pv. savastanoi strains included in this study (32). Most analyzed *P. savastanoi* pv. savastanoi strains (80%) contain an *iaaL* paralog at similar or identical chromosomal locations (Fig. 1 and Table 1). Moreover, purification of DNA fragments (6.5-kb to 9.0-kb) from BglII-digested total DNAs isolated from several of these *P. savastanoi* pv. savastanoi strains and further PCR-RFLP analysis demonstrated that this allele was $iaaL_{\mathrm{Psv}}$ (data not shown). This suggests that $iaaL_{\mathrm{Psn}}$, which was found on the pIAA plasmids of *P. savastanoi* pv. nerii and pv. savastanoi (Table 1), has stabilized more recently into the *P. savastanoi* pv. savastanoi genome.

Microsatellites, defined as tracts of DNA that are composed of multiple tandem repeats of a motif with a length between 1 and 6 nucleotides, are ubiquitous in prokaryotes and eukaryotes (11, 17, 23). Capillary electrophoresis analysis of STR numbers in the $iaaL_{\mathrm{Psv}}$ allele (Table 1; see Fig. S1 in the supplemental material) allowed the differentiation of *P. savastanoi* pv. savastanoi strains isolated from different geographical locations. For instance, strains containing eight or more TAC repeats were all isolated in the Iberian Peninsula (11 strains) (Table 1). Moreover, 7 of these 11 strains were isolated from the Valencian Community (Valencia, Alicante, and Castellón Provinces), and three were from the Sevilla Province ($n = 8$ or 9). However, no correlation between the number of TAC repeats and the cultivar or orchard of isolation (35) was found for strains isolated within the same Spanish province. Determination of the number of STRs in a higher number of *P. savastanoi* pv. savastanoi strains isolated from different geographical locations, as well as from *P. savastanoi* pv. savastanoi strains isolated from unknown olive knot samples, would be necessary in order to evaluate the potential of this marker for epidemiological studies of olive knot disease. The stability of the number of STRs at 7 to 12 months after inoculation in olive plants (see Fig. S1B in the supplemental material) further supports the suitability of this marker for strain differentiation. However, STR stability under various stress conditions, which is known to induce STR modification in other bacterial phytopathogens (20, 39), was not analyzed in this study.

The expansion and contraction of the number of STRs in protein-coding regions as a result of slipped-strand mispairing during DNA synthesis (3, 20) is related to bacterial adaptation to environmental changes. In fact, functional repeats have been found in or near antigenic-determinant genes or genes for other virulence factors in bacterial pathogens (27), including bacterial phytopathogens (20). Taking into account that the TAC motif identified in $iaaL_{\mathrm{Psv}}$ was found in frame for all strains analyzed and that both *iaaL* paralogs were transcribed (Fig. 3), translation of the corresponding mRNAs containing this motif would result in IAA-lysine synthetase enzymes that contain 3 to 15 consecutive tyrosine residues. Given that all data on this enzyme are restricted to proteins with only three TAC repeats and crude extracts of *P. savastanoi* oleander and olive strains (14, 15), which were not included in this study, the activities of IAA-lysine synthetase enzymes carrying a higher number of consecutive tyrosine repeats cannot be determined. Alteration and inactivation of IAA-lysine synthetase activity in *P. savastanoi* pv. savastanoi would probably have an effect on the amount of IAA and the virulence of this bacterial pathogen, as described for *P. savastanoi* pv. nerii strains (15). Comparative virulence analysis of *P. savastanoi* pv. savastanoi strains included in this study and determination of the levels of IAA produced by these strains would be necessary to verify this hypothesis.

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