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Global Genomic Analysis of *Pseudomonas savastanoi* pv. savastanoi Plasmids▽†

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Pseudomonas savastanoi **pv. savastanoi strains harbor native plasmids belonging to the pPT23A plasmid family (PFPs) which are detected in all pathovars of the related species *Pseudomonas syringae* examined and contribute to the ecological and pathogenic fitness of their host. However, there is a general lack of information about the gene content of *P. savastanoi* pv. savastanoi plasmids and their role in the interaction of this pathogen with olive plants. We designed a DNA macroarray containing 135 plasmid-borne *P. syringae* genes to conduct a global genetic analysis of 32 plasmids obtained from 10 *P. savastanoi* pv. savastanoi strains. Hybridization results revealed that the number of PFPs per strain varied from one to four. Additionally, most strains contained at least one plasmid (designated non-PFP) that did not hybridize to the *repA* gene of pPT23A. Only three PFPs contained genes involved in the biosynthesis of the virulence factor indole-3-acetic acid (*iaaM*, *iaaH*, and *iaaL*). In contrast, *ptz*, a gene involved in the biosynthesis of cytokinins, was found in five PFPs and one non-PFP. Genes encoding a type IV secretion system (T4SS), type IVA, were found in both PFPs and non-PFPs; however, type IVB genes were found only on PFPs. Nine plasmids encoded both T4SSs, whereas seven other plasmids carried none of these genes. Most PFPs and non-PFPs hybridized to at least one putative type III secretion system effector gene and to a variety of additional genes encoding known *P. syringae* virulence factors and one or more insertion sequence transposase genes. These results indicate that non-PFPs may contribute to the virulence and fitness of the *P. savastanoi* pv. savastanoi host. The overall gene content of *P. savastanoi* pv. savastanoi plasmids, with their repeated information, mosaic arrangement, and insertion sequences, suggests a possible role in adaptation to a changing environment.**

Pseudomonas syringae strains and related pathogens, such as *Pseudomonas savastanoi* strains, infect a wide range of herbaceous and woody plants causing diverse symptoms, such as leaf spots and blights, soft rots of fruits, wilts, overgrowths, scabs, and cankers (18, 23). During the last decade, research on diseases caused by pseudomonads in herbaceous plants has progressed rapidly, and the application of molecular genetics and genome and plasmid sequencing (4, 13, 22, 31, 36, 39) has provided new insights into determinants of pathogenicity and virulence. However, there is a general lack of knowledge on virulence and pathogenicity determinants specific for infection of woody plants. Clear examples of this are the pathogens *P. savastanoi* pathovars nerii, fraxini, and savastanoi, which cause knots and galls on members of the various genera of the family *Oleaceae* and oleander (14). Symptoms on infected trees include hyperplasia formation on stems and branches and occasionally on leaves and fruit (23). At present, the only *P. savastanoi* determinants known to be involved in knot development are production of the phytohormones indole-3-acetic acid (IAA) and cytokinins (CKs) (1, 16, 30, 42) and biosynthesis of a functional type III secretion system (TTSS), encoded by the *hrp* and/or *hrc* gene clusters, which facilitate secretion of various protein effectors into the plant cell (34, 35).

Phytopathogenic species of the genus *Pseudomonas* usually carry native plasmids that encode, among others, sequences related to virulence and ecological fitness. In fact, in most oleander isolates of *P. savastanoi*, the genes responsible for the biosynthesis of phytohormones are usually located in plasmids, which have been called pIAA and pCK for the biosynthesis of IAA and CKs, respectively (5, 8, 25). In contrast, most olive isolates have been reported to carry these genes in the chromosome (5, 19). Native plasmids of *P. savastanoi* pv. savastanoi can differ in number (one to six plasmids) and size (few kilobases to approximately 100 kb) within the same strain (10, 29). Most native plasmids of *P. syringae* and related pathogens have been shown to belong to the pPT23A family, a phylogenetically related plasmid group characterized by the ubiquitous presence of a replication region containing the *repA* gene (15, 27, 37, 44). pPT23A family plasmids (PFPs) usually encode determinants related to virulence and pathogenicity, such as genes involved in the biosynthesis of the phytotoxin coronatine (2), effector proteins (31, 36, 43), and fitness determinants conferring resistance to copper and antibiotics as well as tolerance to UV radiation (3, 38, 40). The role of PFPs in pathogen virulence and growth in their specific hosts has been demonstrated using curing or disruption of some of these determinants in several *P. syringae* pathosystems (20, 26, 44).

Due to the importance of PFPs to the biology of *P. syringae*, several *P. syringae* plasmids have been sequenced recently in order to understand their role in pathogenesis as well as their

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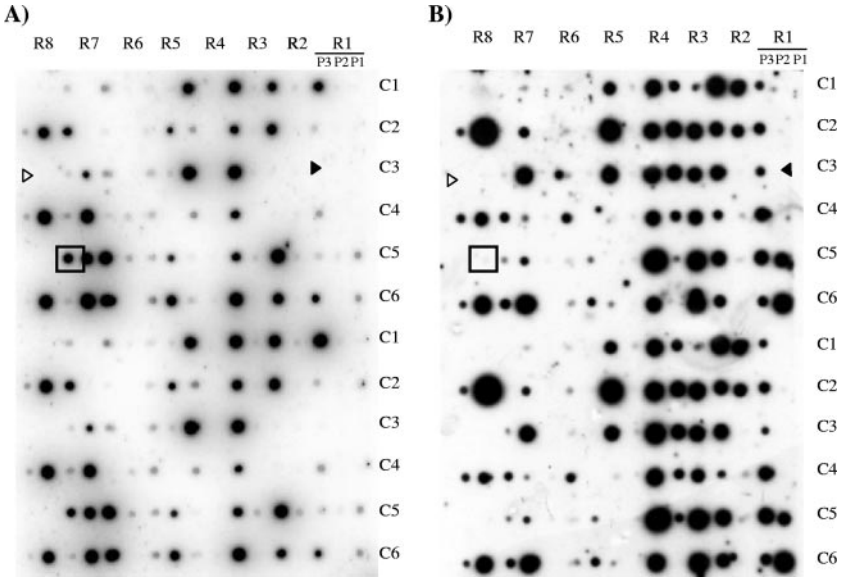

FIG. 1. Representative plasmid macroarray hybridization images for a *P. savastanoi* pv. savastanoi PFP and n-PFP. A total of 135 genes were printed in duplicate on a 7.5- by 11.5-cm membrane. The position of each gene on the array was labeled as a combination of P (plate), C (column), and R (row). (A) Array hybridized with PFP pPsv31C from strain Psv31 containing the type IVA secretion system genes; (B) array hybridized with n-PFP pPsv62C from strain Psv62 containing type IVA and type IVB secretion system genes. Closed and open arrowheads point to spots containing water and chromosomal genes, respectively, used as negative controls. *repA* gene hybridization spots are boxed. Positions C2 R7 P3 and C5 R3 P3 correspond to IS*Psy21* and IS*801*, respectively.

origin, evolution, and coexistence—they use the same replicon—within the same strain (4, 22, 31, 36, 39). These genomic resources and previous experimental data have enabled comparative analyses of the gene content of PFPs from different pathovars of *P. syringae*. Nevertheless, complete understanding of the biology of this important plasmid family from *P. syringae* pathovars and their related pathogens affecting woody hosts requires more sequencing and comparative genomic analysis (46). The aim of this study was to determine and compare the general genetic content of the native plasmids of *P. savastanoi* strains isolated from olive knots. Ten different *P. savastanoi* pv. savastanoi strains were selected according to their pathogenicity, geographical origin, and accessibility to genetic manipulation (29). We analyzed the gene content of both PFPs and plasmids not hybridizing with a *repA* probe from pPT23A, named here non-PFPs (n-PFPs). To our knowledge, this is the first comparative genetic analysis of the overall plasmid content conducted in *P. savastanoi* pv. savastanoi. The results presented suggest that both plasmid types may contribute to the ecology and pathogenesis of *P. savastanoi* pv. savastanoi.

MATERIALS AND METHODS

Bacterial strains, plasmids, probes, and growth media. *P. savastanoi* pv. savastanoi strains and plasmids used in this study are listed below in Table 1. *Pseudomonas* strains were grown overnight at 28°C in Luria-Bertani (LB) medium. *P. syringae* strains used for amplification of DNA probes have been described previously (46).

DNA extraction and purification. Agarose gel electrophoresis and other recombinant DNA techniques were carried out by standard procedures (32). Plasmid DNA minipreparations from *P. savastanoi* pv. savastanoi strains were performed using 1.5-ml overnight LB cultures by a modified alkaline lysis procedure (29, 47). Several steps were taken to minimize the isolation of chromosomal DNA: TENS (10 mM Tris [pH 8], 1 mM EDTA, 100 mM NaOH, 0.5% sodium dodecyl sulfate [SDS]) and NaOH were freshly prepared before isolation, mixing of cells with solutions was done gently, lysis was allowed to proceed for 5 min at 0°C in an ice bath, and tubes were maintained for at least an hour on ice after adding ice-cold 3 M sodium acetate. After two steps of centrifugation for precipitating proteins and DNA, plasmids were resuspended in Tris-EDTA (TE) buffer. Undigested plasmid DNA was separated in 0.7% agarose gels for 8 hours at room temperature and 70 V; after electrophoresis, DNA was stained with ethidium bromide. Care was taken not to boil the agarose excessively, which was usually the cause of poor resolution.

For large-scale plasmid preparations, plasmid DNAs were extracted from 500-ml 0.5× LB cultures as previously described (46). For purification, plasmids were separated in 0.7% agarose gels and then isolated from the agarose gel using the QIAEX II agarose gel extraction kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Genomic DNA was extracted using the Jet Flex extraction kit (Genomed; Löhne, Germany).

Design of PFP gene macroarray and hybridization with *P. savastanoi* pv. savastanoi plasmids. We constructed a modified version of the macroarray reported previously (46) with 112 of the original 161 genes included. Hypothetical and unknown proteins and the majority of chromosomally encoded genes present on the original macroarray were eliminated. Additionally, a fragment from *ptz* (*P. savastanoi* pv. nerii) and 22 fragments amplified from plasmid-borne genes of *P. syringae* pv. phaseolicola strain 1448A were also included (GenBank accession numbers NC_007274 and NC_007275). A total of 135 fragments of genes known to be carried on plasmids in *P. syringae* were amplified from plasmids pDC3000A and pDC3000B of *P. syringae* pv. tomato DC3000 (accession numbers NC_004633 and NC_004632, respectively), pPSR1 of *P. syringae* pv. syringae A2 (accession number NC_005205), pFKN of *P. syringae* pv. maculicola M6 (accession number NC_002759), and from native plasmids of *P. savastanoi* pv. savastanoi and other pathovars of *P. syringae* as described below. Genes were divided into seven functional groups, including type IV secretion system (T4SS) genes, type IVA (12 genes), and type IVB (25 genes); plasmid-specific functions (18 genes); transcriptional factors (10 genes); genes encoding hypothetical proteins known to be encoded on PFPs (13 genes); avirulence and virulence genes and fitness-related genes, including insertion sequences (IS) (44 genes) and other genes (13 genes). All genes printed on the macroarray and their brief descriptions are listed in Table S1 in the supplemental material.

Macroarray printing. Oligonucleotide primer sequences for the amplification of DNA fragments from six PFPs were selected from the published sequences of plasmid pFKN of *P. syringae* pv. maculicola M6, pPSR1 of *P. syringae* pv. syringae A2, pDC3000A and pDC3000B of *P. syringae* pv. tomato DC3000, and p1448A-A and p1448A-B of *P. syringae* pv. phaseolicola 1448A (4, 22, 31, 39). Primers used to amplify other genes on the chromosome or plasmids of *P. syringae* were

generated using sequences in the National Center for Biotechnology Information databases. All of the oligonucleotide primer sequences used in this study and the expected sizes of the PCR products are listed in Table S1 in the supplemental material. The expected size and purity of each sequence utilized were confirmed by gel electrophoresis, and PCR products were gel purified prior to use. PCR products of selected genes were diluted 1:1 in a denaturing solution (1 M NaOH, 5 M NaCl) and placed in a 96-well microtiter plate immediately prior to printing. The denatured PCR products were then deposited in duplicate on a positively charged 7.5- by 11.5-cm nylon membrane with the Beckman 96-pin high-density replicating tool (Biomek 2000 laboratory automation workstation; Beckman Coulter, Inc., Fullerton, CA). After spotting was completed, the membranes were denatured again in 1.5 M NaCl-0.5 M NaOH and neutralized in 1.5 M NaCl–0.5 M Tris-HCl (pH 7.5)–1 mM EDTA. DNA fragments were then cross-linked to the membrane with a UV transilluminator (120 mJ) (Stratalinker; Stratagene, La Jolla, CA). Macroarray printing was performed at the Genomics Technology Support Facility, Michigan State University.

DNA labeling and hybridization experiments. For macroarray hybridizations, probes were generated from whole plasmids as the DNA template. Purified plasmids were first denatured by boiling at 95°C for 10 min. The denatured plasmids were labeled with [^{32}P]dCTP using the Random Primers DNA labeling system (Megaprime DNA labeling system; Amersham Biosciences) according to the manufacturer's instructions. Macroarray hybridizations were carried out by standard DNA hybridization techniques. Hybridization was performed at 65°C, followed by high-stringency washes (30 min with 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% SDS, 30 min with 1× SSC and 0.1% SDS, and 30 min with 0.5× SSC and 0.1% SDS, with all of the washes done at 65°C). Hybridization of macroarrays, with ^{32}P-labeled whole plasmids as probes, yielded consistent and repeatable results; examples of hybridization results obtained for a PFP and a n-PFP are shown in Fig. 1. Hybridization of macroarrays was scored on the basis of the degree of the hybridization signal compared to the background signals obtained from blank spots and chromosomal genes used as negative controls.

DNA fragments from *iaaL*, *iaaM*, *iaaH*, *ptz*, and *repA* genes were labeled individually with a nonradioactive digoxigenin kit (Roche Molecular Biochemicals) according to the supplier's instructions for use as probes and were hybridized at 65°C with plasmid preparations blotted on separate nylon membranes. DNA fragments used as probes and their sizes are shown below in Table 2.

RESULTS

***P. savastanoi* pv. savastanoi strains carry one to several PFP plasmids.** The molecular sizes of native plasmids contained in the different *P. savastanoi* pv. savastanoi strains were estimated by comparing their migration with those isolated from strain Psv62 (10, 42, 48, 63, 73 and 88 kb), previously predicted by restriction analysis of individual plasmids (29, 42). The 10 strains examined contained a total of 32 plasmids that varied in size from about 10 to 95 kb, and all of the strains studied contained between one and four PFPs (Table 1 and Fig. 2 and 3). There were no plasmids smaller than 35 kb hybridizing to the *repA* probe (Table 1 and Fig. 2). n-PFP plasmids were present in six of the strains tested varying in number between one (Psv29, Psv32, Psv31, Psv35, and Psv37) and four (Psv62) (Fig. 2). The size of n-PFPs ranged from about 10 kb (Psv62) to 88 kb (Psv35). Of the total 32 plasmids examined, 23 were PFPs and 9 were n-PFPs (Table 1).

Most *P. savastanoi* pv. savastanoi plasmids encoding IAA or CK biosynthetic genes are PFPs. Probes for *iaaM*, *iaaH*, and *iaaL* (genes involved in the biosynthetic pathway of indole-3-acetic acid) and *ptz* (involved in the biosynthesis of cytokinins in *P. savastanoi*) were obtained by PCR (Table 2) using digoxigenin labeling and hybridized to small-scale plasmid preparations to determine the localization of these genes within the replicons of the *P. savastanoi* pv. savastanoi strains tested.

Only 3 (Psv31, Psv36, and Psv416) out of the 10 *P. savastanoi* pv. savastanoi isolates harbored *iaaL*, *iaaH*, and *iaaM* sequences in one of their high-molecular-weight PFP plasmids (Table 1 and Fig. 3A and C). In these three strains, the three *iaa* gene probes (Table 2) hybridized with a single plasmid. However, the size of the pIAA plasmid was different for each of the strains: Psv31 (pPsv31B [65 kb]), Psv36 (pPsv36B [75 kb]), and Psv416 (pPsv416A [95 kb]). None of the tested n-PFP plasmids hybridized with any of these three probes (Table 1 and Fig. 3A and C). Hybridization of *iaa* probes with total DNA isolated from the other seven strains revealed that all of them harbored at least one copy of those three genes located in the chromosome (data not shown).

Hybridization analysis of plasmids with a *ptz* probe (Table 2) revealed that 6 of the 10 *P. savastanoi* pv. savastanoi strains analyzed contained a native plasmid, five PFPs (pPsv31A [93 kb], pPsv32A [73 kb], pPsv36A [93 kb], pPsv47A [85 kb], and pPsv48A [73 kb]) and one n-PFP (pPsv29B [54 kb]), that hybridized with this probe (Table 1 and Fig. 3B and D). Hybridization of plasmid preparations and total DNA isolated from 25 different *P. savastanoi* pv. savastanoi isolates increased to approximately 72% the number of strains containing one plasmid hybridizing with the *ptz* probe (data not shown). The other 28% of the strains either presented sequences hybridizing with this gene in their chromosomal DNA (i.e., Psv35 and Psv62) or did not hybridize with this probe (i.e., Psv37). To our knowledge, this is the first time that the absence of sequences hybridizing with a *ptz* probe is reported for *P. savastanoi* strains.

Related to the biosynthesis of ethylene, the *efe* gene from pETH2 encoding an ethylene-forming enzyme was included on the macroarray (see Table S1 in the supplemental material). However, no homology to this gene was observed on the 32 plasmids studied.

Most *P. savastanoi* pv. savastanoi PFPs encode at least one putative TTSS effector and other virulence and fitness-related factors. All of the strains examined except Psv29 contained at least one homolog of various type III secretion effector genes in one of their PFPs. The total number of effector sequences detected on PFPs varied from one (pPsv32A, pPsv36C, pPsv37A, pPsv47B, pPsv48A, and pPsv48B) to six (pPsv36A). Positive hybridizations were distributed within the same strain either among different plasmids (i.e., strain Psv35) or most of them located on one plasmid (i.e., strain Psv36). Some of the strains also harbored multiple copies of specific effector genes on different plasmids, i.e., Psv35, which carries *hopQ1* on two different plasmids, pPsv35A and pPsv35C (Table 3). Only 8 of the 17 effectors included in the macroarray were detected in *P. savastanoi* pv. savastanoi strains, and the most widely distributed effectors were *hopAB1*, *hopQ1*, and *hopAW1* (Table 3).

Other genes related to virulence or fitness detected in *P. savastanoi* pv. savastanoi PFPs were A0129 (*Yersinia*/*Haemophilus* virulence surface antigen) and A0080 (methyl-accepting chemotaxis protein), both present in p1448A-A, and *bip* (putative bacteriocin immunity protein, similar to the pyocin S3 immunity protein of *Pseudomonas aeruginosa*) present on pPSR1. Those three genes, A0129, A0080, and *bip*, were found in four, five, and four different strains, respectively. Only strain Psv47 did not contain any of these three putative virulence genes on any of its PFP plasmids. In contrast, strain Psv416 harbored these three genes on PFPs. Homologs of *rulA*, which in combination with *rulB* confers resistance to UV light, were found in six PFPs (pPsv31A, pPsv35B, pPsv36B, pPsv47B,

TABLE 1. *P. savastanoi* pv. savastanoi strains isolated from the olive plant and plasmids used in this study

Strain name[a]	Original name[b]	Origin	Source	Plasmid[c]	Plasmid size (kb)[d]	Hybridization with probe[e]	
						iaa	*ptz*
Psv29	IVIA 1628-3	Spain	IVIA	pPsv29A	75	−	−
				pPsv29B*	54	−	+
Psv31	NCPPB 2327	Italy	G. L. Ercolani	pPsv31A	93	−	+
				pPsv31B	65	+	−
				pPsv31C	42	−	−
				pPsv31D	35	−	−
				pPsv31E*	15	−	−
Psv32	IVIA 1657-b8	Spain	IVIA	pPsv32A	73	−	+
				pPsv32B	48	−	−
				pPsv32C*	35	−	−
Psv35	CFBP 2074	Algeria	M. M. Yahiaoui	pPsv35A	95	−	−
				pPsv35B*	88	−	−
				pPsv35C	40	−	−
Psv36	IVIA 1624-b1	Spain	IVIA	pPsv36A	93	−	+
				pPsv36B	75	+	−
				pPsv36C	35	−	−
Psv37	NCPPB 64	Portugal	M. d'Oliveira	pPsv37A	80	−	−
				pPsv37B	44	−	−
				pPsv37C*	40	−	−
Psv47	NCPPB 1344	United States	E. Wilson	pPsv47A	85	−	+
				pPsv47B	68	−	−
Psv48	NCPPB 3335	France	D. E. Stead	pPsv48A	73	−	+
				pPsv48B	42	−	−
Psv62	ITM317	Italy	N. Iacobellis	pPsv62A	88	−	−
				pPsv62B	73	−	−
				pPsv62C*	63	−	−
				pPsv62D*	44	−	−
				pPsv62E*	42	−	−
				pPsv62F*	10	−	−
Psv416	CFBP 1670	Serbia	D. A. Cooksey	pPsv416A	95	+	−
				pPsv416B	75	−	−
				pPsv416C	44	−	−

[a] Name at the Section of Genetics Collection, University of Malaga, Malaga, Spain.
[b] IVIA, Instituto Valenciano de Investigaciones Agrarias Collection, Valencia, Spain; NCPPB, National Collection of Plant Pathogenic Bacteria, York, United Kingdom; CFBP, Collection Française des Bacteries Phytopathogenes, Angers, France.
[c] n-PFP plasmids are indicated with an asterisk.
[d] Estimated by comparing their migration with those isolated from strain Psv62 (ITM317) (29, 42).
[e] Hybridization with i*aaM*, *iaaH*, and *iaaL* (genes involved in the biosynthetic pathway of IAA) probes or hybridization with *ptz* probe (involved in the biosynthesis of CKs). Symbols: +, hybridization; −, no hybridization.

pPsv416A, and pPsv416B) belonging to five different *P. savastanoi* pv. savastanoi strains (data not shown). Additional hybridization data showing the detection of genes encoding transcriptional regulators, conserved hypothetical proteins, and plasmid-specific genes on *P. savastanoi* pv. savastanoi plasmids is presented in Table S1 in the supplemental material.

Most *P. savastanoi* pv. savastanoi PFPs encode a complete or partial set of type IVA secretion system genes. Hybridization results showed that variability with regard to the gene content encoding T4SSs was high, and examples of different combinations of genes were found (Table 4). As described before for PFPs of several *P. syringae* pathovars (46), *P. savastanoi* pv. savastanoi native plasmids can be classified into four different groups based on the presence of type IVA genes (*vir* genes), type IVB genes (*tra* and/or *trb* genes), genes for both type IV systems (*vir* and *tra* and/or *trb* genes), or a complete absence of any of these genes (Table 4). We found that *vir* genes were more commonly present on PFPs, and 7 of 16 plasmids hybridized with all 12 *vir* genes (Table 4). Only 1 of the 10 strains analyzed (Psv29) did not carry any *vir* genes on their PFPs.

Ten of 23 PFPs analyzed presented type IVB secretion system genes; however, only 4 of them (pPsv29A, pPsv32A, pPsv35C, and pPsv48A) contained the type IVB secretion system as the only representative group (Table 4). Only 1 of these 10 plasmids (pPsv35A) hybridized with all *tra* and/or *trb* genes composing the putative type IVB secretion system. This plas-

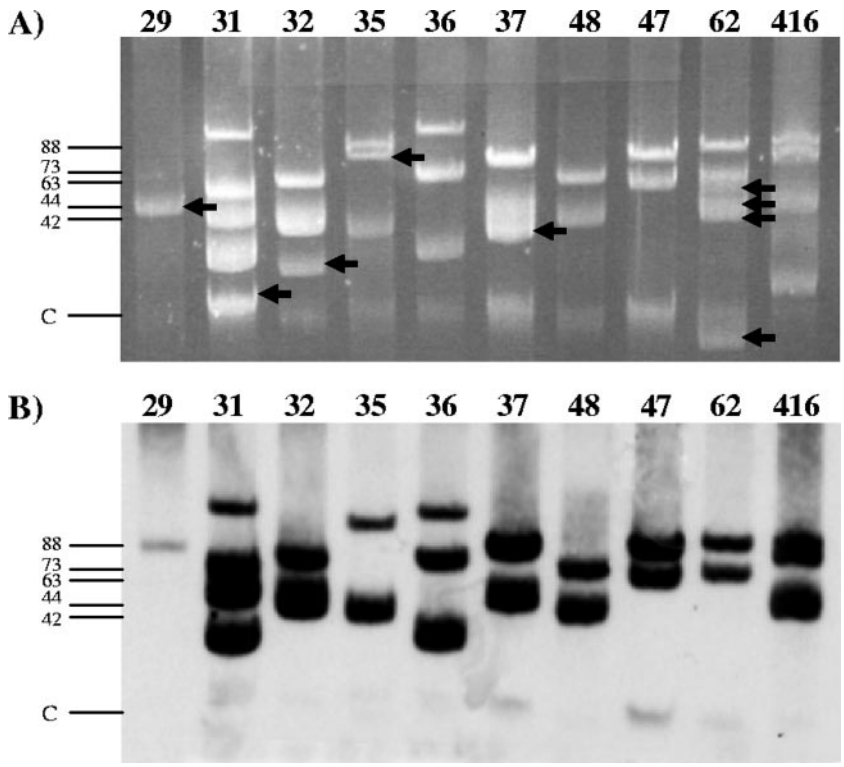

FIG. 2. Detection of PFP plasmids in *P. savastanoi* pv. savastanoi strains. (A) Gel electrophoresis of native plasmids isolated from the indicated *P. savastanoi* pv. savastanoi strains (Psv29 [29] to Psv416 [416]). (B) Southern blot analysis of plasmid profiles shown in panel A using a digoxigenin-labeled *repA* probe from plasmid pPT23A (Table 2). Plasmids giving a negative hybridization signal (n-PFPs) are indicated by black arrows. Hybridization results were confirmed using the PFP DNA macroarray. The positions of molecular size markers (in kilobases) are indicated to the left of the gels. C, chromosomal DNA.

mid, along with five others, hybridized to genes from both T4SSs. The presence of complete sets of genes encoding both T4SSs has been previously described for other *P. syringae* PFPs (46). To our knowledge, this is the first time that the presence of *tra* and/or *trb* genes in *P. savastanoi* is reported. Only three of 23 PFPs analyzed did not hybridize to any of the T4SS genes, with the exception of *trbB*, which was detected in 31 of the 32 plasmids analyzed.

Insertion sequences IS*801* and IS*Psy21* are encoded in most *P. savastanoi* pv. savastanoi PFPs. All *P. savastanoi* pv. savastanoi PFPs analyzed hybridized to one to several transposases of IS elements. IS sequences included in the macroarray were IS*Ps1-a*/*b* from pPSR1 (*P. syringae* pv. syringae A2), IS*Psy4* from pDC3000A (*P. syringae* pv. tomato DC3000), IS*801* from pPG4180A (*P. syringae* pv. glycinea), and IS*Psy16*, IS*Psy17*, IS*Psy19*, IS*Psy21*, and IS*Psy24* from p1448A-A (*P. syringae* pv. phaseolicola 1448A). The 23 PFPs hybridized with IS*801*, and 22 of them hybridized with ISPsy*21* (Table 5); these two IS elements were clearly the most common, and they both can be found in the same plasmid giving a very strong hybridization signal (Fig. 1).

***P. savastanoi* pv. savastanoi non-PFP plasmids encode virulence and fitness-related genes as well as IS elements.** Because of the common presence of n-PFP plasmids in *P. savastanoi* pv. savastanoi (>28% of the total plasmids analyzed), we decided to analyze their genetic content using the same macroarray used for the analysis of PFPs. Five of nine plasmids, four of which presented T4SS genes, hybridized with one to several type III effector genes as well as other virulence-related genes. For example, both pPsv29B and pPsv35B hybridized with up to five virulence genes, such as *avrD1*, *avrB2*, *hopAB1*, and *ptz*. Additionally, these two plasmids hybridized with the shikimate kinase gene sequence, which corresponds to a putative 6,456-bp open reading frame (ORF) encoded on plasmid p1448A-A. Virulence-related genes, such as A0129, A0080, and *bip*, found in PFPs, were also detected in n-PFPs on two plasmids (pPsv29B and pPsv35B) (Table 6).

Five of nine plasmids hybridized to genes coding type IV secretion systems and contained either a complete (pPsv37C) or partial (pPsv31E, pPsv35B, pPsv62C, and pPsv62D) suite of type IVA genes. Moreover, only hybridization with *virD4* was lacking for plasmids pPsv31E and pPsv35B. Three of these five plasmids (pPsv35B, pPsv62C, and pPsv62D) contained genes from both T4SSs; however, none of them showed type IVB genes as the only representative of the T4SS. Note that two of these three plasmids belonging to the same strain (pPsv62C and pPsv62D) contained a complete set of *tra* and/or *trb* genes (type IVB) (Table 6).

As detected on all PFPs analyzed, insertion sequences IS*801* and IS*Psy21* were also present in all n-PFP plasmids. In fact, those two sequences plus *trbC* and A0034 genes were the only ones detected for plasmids pPsv32C (42 kb) and pPsv62F (10 kb), respectively, among the 135 gene fragments included in the macroarray (Table 6). These results suggest that n-PFP *P. savastanoi* pv. savastanoi plasmids could harbor a set of novel genes not

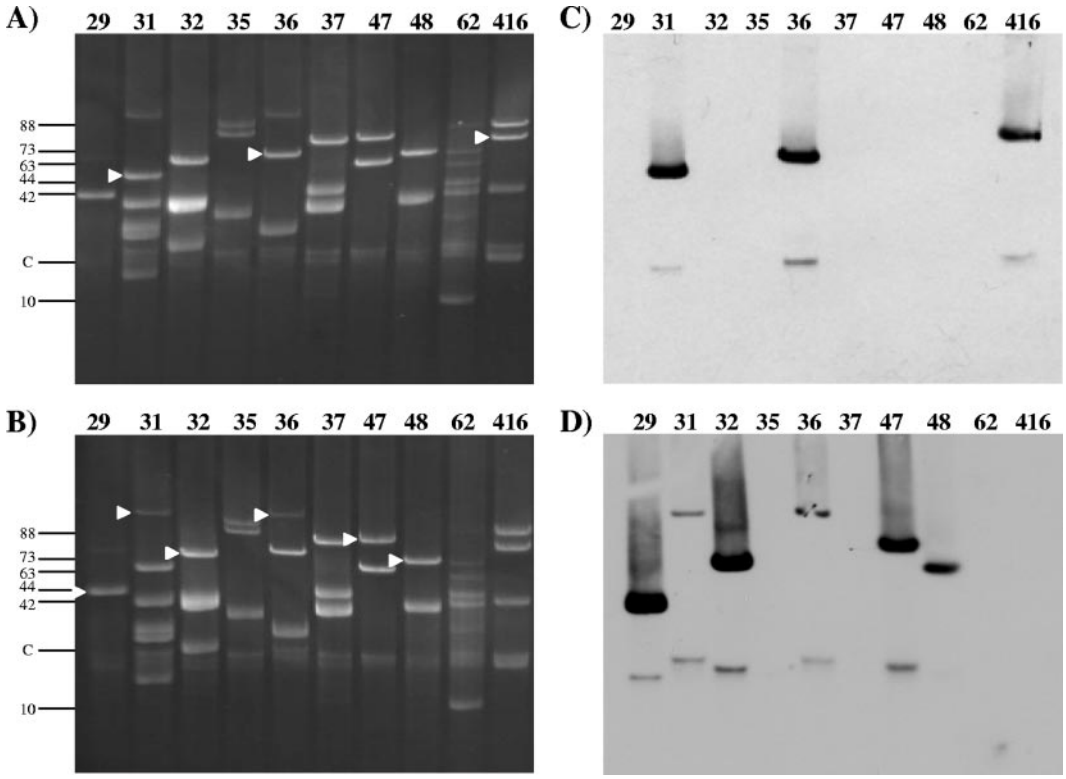

FIG. 3. Detection of phytohormone biosynthetic genes (*iaa* and *ptz*) on *P. savastanoi* pv. savastanoi plasmids. (A and B) Gel electrophoresis of native plasmids isolated from the indicated *P. savastanoi* pv. savastanoi strains. (C and D) Southern blot analysis of plasmid profiles shown in panels A and B using *iaaM* and *ptz* probes, respectively. Southern blot analysis of plasmid profiles shown in panel A using *iaaH* and *iaaL* probes (Table 2) resulted in hybridization patterns identical to those in panel C. Plasmids giving positive hybridizations are indicated by white arrows. The positions of molecular size markers (in kilobases) are indicated to the left of the gels. C, chromosomal DNA.

encoded on any of the sequenced *P. syringae* PFPs. With the exception of plasmid pPsv35B, hybridizing with a putative bacteriocin immunity protein gene (*bip*), no hybridization with fitness-related genes, such as *rulA*, was found for n-PFP plasmids.

DISCUSSION

Our DNA macroarray analysis highlighted the gene diversity contained within indigenous *P. savastanoi* pv. savastanoi plasmids (23 PFPs and 9 n-PFPs). In general, the sets of plasmids harbored by specific *P. savastanoi* pv. savastanoi isolates are so unique that their plasmid profiles usually allow strain differentiation (11). In fact, each of the 10 strains analyzed here contains a completely different suite of plasmids clearly differentiable from one another (Table 1 and Fig. 2).

TABLE 2. Probes used in this study for individual hybridizations to plasmids

Gene	Probe size (bp)	Original gene (%)[a]
repA	1,399	100
ptz	690	98
iaaL	454	38
iaaM	1,674	100
iaaH	1,368	100

[a] Percentage of the ORF gene sequence included in the amplified fragment to be used as a probe. The *iaaL* probe was designed according to reference 28.

Although it is well-known that most plasmids of *P. syringae* belong to the PFP family, there are few reports relating *P. savastanoi* plasmids with this family (40). Previous analyses of the genetic content of *P. savastanoi* pv. savastanoi plasmids have demonstrated that they can carry virulence genes related to the biosynthesis of the phytohormones indole-3-acetic acid (*iaaM*, *iaaH*, and *iaaL* genes) (17, 33, 41) and cytokinins (25, 30). Southern hybridization analysis of *P. savastanoi* pv. savastanoi plasmids using *repA* and *iaa* gene probes clearly demonstrated that all three pIAA plasmids analyzed in this study (pPsv31B, pPsv36B, and pPsv416A) belong to the PFP family (Table 1 and Fig. 2 and 3A and C). In agreement with data previously reported (5), the other seven *P. savastanoi* pv. savastanoi strains analyzed contain at least one copy of all three *iaa* genes located on the chromosome (data not shown).

Hybridization results with a *ptz* probe revealed a higher presence of plasmid encoded CK-related genes in *P. savastanoi* pv. savastanoi than previously reported (5); 18 of 25 (72%) olive strains isolated in different countries presented this gene on one of their plasmids. Figure 3B and D show the results obtained for the 10 strains included in this study, 6 of these strains carried *ptz* on one of their plasmids. These results are in agreement with those reported by Caponero et al. (5) for Italian *P. savastanoi* pv. savastanoi strains, where *ptz* was detected in all 13 strains analyzed and 6 of them carried this gene on a plasmid. In addition, our results show that although most

TABLE 3. Detection of homologs of *hrp*-dependent outer proteins (*hop*) genes and avirulence (*avr*) genes on *P. savastanoi* pv. savastanoi PFPs

Strain	Plasmid[a]	Hybridization[b] with the following gene(s):								
		hopAB1	*avrD1*	*avrB2*	*hopQ1*	*hopAU1*	*hopAV1*	*hopD1*[c]	*hopAW1*	9 *avr* genes[d]
Psv31	A	−	−	−	+	+	+	−	−	−
	D	+	−	−	−	−	−	+	−	−
Psv32	A	−	−	−	−	−	−	−	+	−
Psv35	A	−	+	−	+	+	−	−	−	−
	C	+	−	+	+	+	−	−	+	−
Psv36	A	+	+	+	+	+	−	−	+	−
	C	−	−	−	−	−	−	+	−	−
Psv37	A	+	−	−	−	−	−	−	−	−
Psv47	B	−	−	−	−	−	−	−	+	−
Psv48	A	−	−	−	−	−	−	−	+	−
	B	−	−	−	−	−	−	+	−	−
Psv62	A	−	−	+	−	−	+	−	−	−
Psv416	A	+	−	−	−	−	−	−	−	−
	B	+	+	−	+	−	+	−	−	−
	C	+	+	−	−	−	−	−	−	−
None	8 plasmids[e]	−	−	−	−	−	−	−	−	−

[a] Plasmid names incorporate the name of the host strain. pPsv31A and pPsv31D are shown as A and D, respectively.
[b] +, hybridization; −, no hybridization.
[c] *hopD1* from *P. syringae* pv. phaseolicola 1448A.
[d] *hopAM1-2*, *hopX1*, *avrA*, *avrRpm1*, *avrE1*, *avrRps4*, and *hopD1* from *P. syringae* pv. glycinea, *avrRps4* from *P. syringae* pv. pisi, and *hopW1-1* from *P. syringae* pv. maculicola.
[e] pPsv29A, pPsv31B, pPsv31C, pPsv32B, pPsv36B, pPsv37B, pPsv47A, and pPsv62B.

P. savastanoi pv. savastanoi pCKs are PFPs (five plasmids), *ptz* can also be found in n-PFPs (one plasmid, pPsv29B) (Table 1 and Fig. 2, 3B, and D). The high frequency of chromosomally encoded *iaa* and *ptz* genes found in *P. savastanoi* olive isolates could be a response to the need to stabilize important ecological determinants through mechanisms of integration in the host chromosome. In fact, maintenance and evolution of PFPs are probably based on horizontal transfer of these plasmids

TABLE 4. Distribution of homologs of *virB-virD4* and *tra* genes on *P. savastanoi* pv. savastanoi PFPs

T4SS system and plasmid	Type IVA secretion system gene(s) (*virB1-virB11* and *virD4*)	Type IVB secretion system gene(s) (*traH-traY* and *trbA-trbC* and type IV pilus gene)
Type IVA		
pPsv31A	*virB1-virB11*, *virD4* (complete)	*trbB*
pPsv31B	*virB8-virB11*	*trbB*
pPsv31C	*virB1-virB11*, *virD4* (complete)	*trbB*
pPsv36A	*virB7-virB11*, *virD4*	*trbB*
pPsv36B	*virB8-virB11*	*trbB*
pPsv37B	*virB1-virB11*, *virD4* (complete)	*trbB*
pPsv47B	*virB1*, *virB2*, *virB4*	*trbB*
pPsv48B	*virB1-virB11*	*trbB*
pPsv416A	*virB1-virB11*, *virD4* (complete)	*trbB*
pPsv416B	*virB1-virB4*	*trbB*
Type IVB		
pPsv29A	None	*traT-traY*, *trbA-trbC*
pPsv32A	None	*traT-traY*, *trbA-trbC*, pilus gene
pPsv35C	None	*trbB*, *trbC*
pPsv48A	None	*traT-traY*, *trbA-trbC*
Both T4SSs		
pPsv32B	*virB1-virB11*, *virD4* (complete)	*traH*, *traI*, *traO-traQ*, *traT-traY*, *trbA-trbC*, pilus gene
pPsv35A	*virB1-virB11*, *virD4* (complete)	*traH-traY*, *trbA-trbC*, pilus gene (complete)
pPsv37A	*virB1*, *virB6*, *virB8*, *virB9*, *virB11*	*traT-traY*, *trbA-trbC*, pilus gene
pPsv47A	*virB1*, *virB2*, *virB4*	*traT-traY*, *trbA-trbC*
pPsv62A	*virB1-virB11*, *virD4* (complete)	*traH*, *traI*, *traM*, *traO*, *traP*, *traQ*, *traT*, *traU*, *traX*, *traY*, *trbA-trbC*, pilus gene
pPsv62B	*virB1*, *virB3-virB11*, *virD4*	*traH* to *traC*, *traM* to *traQ*, *traU* to *traY*, *trbA-trbC*, pilus gene
No T4SS		
3 PFPs[a]		*trbB*

[a] pPsv31D, pPsv36C, and pPsv416C.

TABLE 5. Detection of insertion sequences on *P. savastanoi* pv. savastanoi PFPs

Strain	Plasmid[a]	Detection of insertion sequence[b]							
		IS*801*	IS*Psy21*	IS*Psy4*	IS*Psy17*	IS*Psy16*	IS*Psy24*	IS*Psy19*	IS*Psy1*-a/b
Psv29	A	+	+	−	+	+	−	−	−
Psv31	A	+	+	+	−	+	−	−	−
	B	+	+	−	−	+	−	−	−
	C	+	+	−	−	−	−	−	−
	D	+	+	−	−	−	−	−	−
Psv32	A	+	+	−	−	−	−	−	−
	B	+	+	−	−	+	−	−	−
Psv35	A	+	+	+	−	+	−	−	−
	C	+	+	−	−	−	−	−	−
Psv36	A	+	+	−	−	+	−	−	−
	B	+	+	+	−	−	−	−	−
	C	+	+	−	−	−	−	−	−
Psv37	A	+	+	−	−	+	−	−	−
	B	+	−	−	−	−	−	−	−
Psv47	A	+	+	−	+	−	−	−	−
	B	+	+	−	−	+	−	−	−
Psv48	A	+	+	−	−	−	−	−	−
	B	+	+	−	−	+	−	−	−
Psv62	A	+	+	−	−	+	−	−	−
	B	+	+	−	−	+	−	−	−
Psv416	A	+	+	+	−	+	−	−	−
	B	+	+	+	−	+	−	−	−
	C	+	+	−	−	−	−	−	−
Pma ES4326[c]	B	+	−	−	−	−	−	−	−
Pto DC3000[c]	A	−	−	+	−	−	−	−	−
	B	−	−	+	−	−	−	−	−
Pph 1448A[c]	A	+	+	−	+	+	−	+	−
	B	−	−	−	+	−	−	−	−
Psy A2[c]	pPSR1	−	−	−	−	−	−	−	+
Pma M6[c]	pFKN	+	−	−	−	−	−	−	−

[a] Plasmid names incorporate the name of the host strain. pPsv29A is shown as A, and plasmids pPsv31A, pPsv31B, pPsv31C, and pPsv31D are shown as A, B, C, and D, respectively.
[b] Symbols: +, hybridization; −, no hybridization.
[c] In silico analysis only. Pma, *P. syringae* pv. maculicola ES4326 and M6; Pto, *P. syringae* pv. tomato DC3000; Pph, *P. syringae* pv. phaseolicola 1448A; Psy, *P. syringae* pv. syringae A2.

and recombination events, resulting in either inclusion of plasmid-borne genes on the chromosome or gene duplications (24, 46).

Most *P. savastanoi* pv. savastanoi plasmids studied (25 of 32 plasmids) contained genes involved in the biosynthesis of the T4SS, one of the five major secretion systems present in gram-negative bacteria. T4SSs can be divided into two distinct subgroups, the type IVA system of *Agrobacterium tumefaciens* Ti plasmid, composed of *vir* genes, and the type IVB system of representative plasmid ColIb-P9 of *Shigella*, encoded by 21 *tra* and/or *trb* genes. Ancestrally related to conjugation systems of bacteria, T4SSs have been shown to be involved in pathogenesis and uptake and release of DNA, which involves horizontal transfer of genes and genome plasticity, as well as in delivery of effectors into host cells (6, 7). Recently, the involvement of T4SS in pathogenesis has been demonstrated in a plant-pathogenic bacterium (12). As previously described for *P. syringae* PFPs, *P. savastanoi* pv. savastanoi plasmids can be divided into four subgroups depending on the arrangements of the gene sets encoded, plasmids encoding complete or partial gene suites of type IVA or IVB secretion systems, plasmids containing genes encoding for both systems, and plasmids not containing any T4SS (46). Type IVA gene homologs are more frequently found among *P. savastanoi* pv. savastanoi plasmids, and only 1 of the 10 strains analyzed (Psv29) did not contain plasmids hybridizing to any of those genes (Table 4). Although several of the sequenced *P. syringae* PFPs encode a complete set of type IVA system genes, their role in processes of conjugation has not been studied. In addition, four *P. savastanoi* pv. savastanoi plasmids hybridized to type IVB secretion system genes, which are found in plasmids A and B of *P. syringae* pv. tomato DC3000 (Table 4). Although four PFP plasmids isolated from two different oleander isolates of *P. savastanoi* (named 0485-9 and 0693-10) were analyzed in a previous study using a PFP array (46), none of them hybridized to any *tra* and/or *trb* genes. Therefore, this is the first time that the presence of type IVB genes is reported for *P. savastanoi* plasmids.

Analysis of the macroarrays also revealed the presence of a variety of insertion sequences in *P. savastanoi* pv. savastanoi plasmids. Homologs for IS*801* and IS*Psy21* were detected in almost all plasmids analyzed (Table 5). These kinds of IS elements, usually present in several chromosomal and plasmid-encoded copies, have been postulated to have a role in driving the evolution of their hosts, allowing mechanisms of illegitimate recombination resulting in gene reorganizations, insertions, or deletions (37). Insertion sequences have also been implicated in loss of virulence, due to an insertion of IS*51* (also named IS*Psy21*) into the *iaaM* gene of a *P. savastanoi* oleander

TABLE 6. Genetic content of n-PFP plasmids in *P. savastanoi* pv. savastanoi

Plasmid	Size (kb)	T4SS[a]	TTSS and virulence gene(s)	ISs[b]	SK[c]	No. of genes hybridizing on the macroarray
pPsv29B	54		*hopQ1*, *hopAV1*, *hopAU1*, *ptz*, A0129	IS*801*, IS*Psy21*, IS*Psy16*	+	15
pPsv31E	15	A		IS*801*, IS*Psy21*	−	26
pPsv32C	42			IS*801*, IS*Psy21*	−	3
pPsv35B	88	A/B	*avrB2*, *hopAW1*, *avrD1*, *bip*, A0080	IS*801*, IS*Psy21*, IS*Psy16*, IS*Psy24*	+	33
pPsv37C	40	A complete		IS*801*, IS*Psy21*	−	18
pPsv62C	63	A/B IVB complete	*avrD1*	IS*801*, IS*Psy21*	−	49
pPsv62D	44	A/B IVB complete	*avrB2*	IS*801*, IS*Psy21*	−	55
pPsv62E	42		*hopAB1*	IS*801*, IS*Psy21*, IS*Psy16*	−	15
pPsv62F	10			IS*801*, IS*Psy21*	−	3

[a] A, type IVA secretion system, A/B, both type IVA and B secretion systems. Plasmids pPsv31E and pPsv35B hybridized with all *vir* genes except for *virD4*.
[b] IS, insertion sequences.
[c] SK, shikimate kinase.

strain (9). On the other hand, other IS elements, such as IS*52* and IS*801*, have been connected to the transfer of *iaa* genes between *A. tumefaciens* and *P. savastanoi* (45) or to movement of virulence effectors (*avrA* and *avrB2*) (21) in *P. savastanoi*.

While the distribution of certain *avr* and *hop* genes among PFPs is well-known for many *P. syringae* pathovars, the allocation of such TTSS effectors on n-PFPs is completely unknown. In addition, the distribution of effector genes on plasmids or in the chromosome in *P. savastanoi* pv. savastanoi is unknown except for *hopAB1* (formerly *virPphA*) and *avrB2* (formerly *avrPphC*) genes, previously identified on plasmids isolated from two different *P. savastanoi* pv. savastanoi strains (21). Widely distributed among *P. syringae* and *P. savastanoi* strains (23), *hopAB1* was the most common effector gene detected among the *P. savastanoi* pv. savastanoi plasmids analyzed (eight plasmids). This gene is also known to be present in plasmid p1448A-A of *P. syringae* pv. phaseolicola 1448A. In addition, five of the eight putative effector genes we have found in *P. savastanoi* pv. savastanoi plasmids are also encoded on plasmid p1448A-A. In contrast, three of these five genes (*hopD1*, *hopQ1*, and *avrD1*) are known to be on the chromosome of *P. syringae* pv. tomato DC3000. Although it has been reported that putative virulence genes located in *P. syringae* pv. phaseolicola plasmids are chromosomally encoded in *P. syringae* pv. tomato DC3000 (22), the significance of their specific genomic location has not been experimentally addressed. Hybridization to the *hopAB1* and *avrD1* genes has been previously described for plasmids isolated from oleander strains of *P. savastanoi*; however, *hopAM1-2*, also found on a *P. savastanoi* pv. nerii plasmid (46), was not detected in any of the *P. savastanoi* pv. savastanoi plasmids analyzed here.

As previously observed for other *P. syringae* pathovars (46), several effector genes (*hopAB1*, *avrD1*, *hopQ1*, and *hopAU1*) were located on more than one PFP plasmid within the same strain. Additionally, it was also common to find strains containing the same effector located on both a PFP and a n-PFP plasmid(s) (*avrB2*, *hopAW1*, *avrD1*, and *avrB2*) (Tables 3 and 6). Nevertheless, none of the eight effectors found on *P. savastanoi* pv. savastanoi plasmids were detected in all the strains analyzed; the possible localization of these genes on the chromosome of *P. savastanoi* pv. savastanoi strains was not studied here.

UV resistance genes *rulAB* are relevant for bacterial fitness in leaf surfaces. A complete study with 71 strains belonging to 15 *P. syringae* and *P. savastanoi* pathovars demonstrated that, in most cases, these genes were located in PFPs (40). In contrast, our results show that the presence of *rulA* in *P. savastanoi* pv. savastanoi plasmids is limited; only six plasmids out of the 32 analyzed hybridized with this gene. It could be possible that forces driving the movement of these genes into the genome have taken place in *P. savastanoi* pv. savastanoi strains. Alternatively, other genes also involved in protection against UV radiation could have replaced these genes in *P. savastanoi* pv. savastanoi.

Very little is known about the gene content of n-PFP plasmids in phytopathogenic *Pseudomonas* spp. and only 2 out of the 11 *P. syringae* plasmids already sequenced (pPMA4326D and pPMA4326E from *P. syringae* pv. maculicola strain ES4326) are n-PFPs (36). The role in virulence of these two plasmids, which contain only six ORFs, each of them encoding unknown hypothetical proteins, remains unknown thus far (36). Our macroarray detected the same kinds of genes in both n-PFPs and PFPs (Table 6). These results indicate that n-PFP plasmids may contribute to the virulence and fitness of the *P. savastanoi* pv. savastanoi host. However, this hypothesis needs to be proven experimentally, as other plasmids carrying putative virulence factors play no detectable role in fitness or virulence (4). Additionally, the presence of identical IS types in both kinds of plasmids implies a common evolution of these two plasmid types via recombination resulting in plasmid reorganizations. Examples of plasmids like pPsv62C and pPsv62D that hybridize to more than 50 genes found in PFPs (Table 6) suggest that they once belonged to the pPT23A family and lost the *repA* replication system perhaps due to incompatibility-mediated gene reassortments. In contrast, plasmids like pPsv32C, of approximately 42 kb, hybridized to practically none of the PFP genes included in the macroarray. This might be due to the presence on this plasmid of genes not yet described in *P. syringae* or new *P. savastanoi* genes involved in virulence or host specificity.

In summary, our study has allowed us to rapidly survey the distribution of 135 genes among 32 plasmids, 23 PFPs and 9 n-PFPs, isolated from 10 different *P. savastanoi* pv. savastanoi strains. According to the general genetic content of both

groups of plasmids, n-PFPs seem to be as important for the ecology and virulence of the host as PFPs are. Thus, it is tempting to speculate that the main difference between them could be the replication system. It will be very interesting to further investigate first, the mechanism of replication of this group of plasmids and second, whether they share a replication system and therefore could be grouped into a new plasmid family. On the other hand, the results obtained could also indicate that *P. savastanoi* pv. savastanoi strains are prone to stabilize in their chromosome important genes through mechanisms of reorganization. Some examples could be genes for the biosynthesis of IAA and *rulA*, which are found mostly in plasmids in several *P. syringae* plasmids and not in *P. savastanoi* pv. savastanoi plasmids. The large amount of transposase sequences detected seems to confirm the movement of genes, driving the evolution of the plasmid and chromosome and as a consequence, the speciation of *P. savastanoi*. Sequencing and functional experiments to evaluate the effect of these plasmid-borne genes in pathogenicity are needed for a better understanding of the role of *P. savastanoi* pv. savastanoi plasmids during the process of infection of olive trees.

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