

Pathogen profile

***Pseudomonas savastanoi* pv. *savastanoi*: Some like it knot**

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SUMMARY

Pseudomonas savastanoi pv. *savastanoi* is the causal agent of olive (*Olea europaea*) knot disease and an unorthodox member of the *P. syringae* complex, causing aerial tumours instead of the foliar necroses and cankers characteristic of most members of this complex. Olive knot is present wherever olive is grown; although losses are difficult to assess, it is assumed that olive knot is one of the most important diseases of the olive crop. The last century has witnessed a good deal of scientific articles describing the biology, epidemiology and control of this pathogen. However, most *P. savastanoi* pv. *savastanoi* strains are highly recalcitrant to genetic manipulation, which has effectively left the pathogen out of the scientific progress in

molecular biology that has elevated the foliar pathogens of the *P. syringae* complex to supermodels. A series of studies in the last years have made significant advances in the biology, ecology and genetics of *P. savastanoi* pv. *savastanoi*, paving the way for the molecular dissection of its interaction with other non-pathogenic bacteria and their woody hosts. The selection of a genetically pliable model strain was soon followed by the development of rapid methods for virulence assessment with micropropagated olive plants and the analysis of cellular interactions with the plant host. The generation of a draft genome of strain NCPPB 3335 and the closed sequence of its three native plasmids has allowed for functional and comparative genomic analyses for the identification of its pathogenicity gene

complement. This includes 34 putative type III effector genes and genomic regions, shared with other pathogens of woody hosts, that encode metabolic pathways associated with the degradation of lignin-derived compounds. Now, the time is right to explore the molecular basis of the *P. savastanoi* pv. *savastanoi*-olive interaction and to get insights into why some pathovars like it necrotic and why some like it knot.

Synonyms: *Pseudomonas syringae* pv. *savastanoi*

Taxonomy: Kingdom Bacteria; Phylum Proteobacteria; Class *Gammaproteobacteria*; Family *Pseudomonadaceae*; Genus *Pseudomonas*; included in genomospecies 2 together with at least *P. amygdali*, *P. ficuserectae*, *P. meliae* and 16 other pathovars from the *P. syringae* complex (aesculi, ciccaronei, dendropanacis, eriobotryae, glycinea, hibisci, mellea, mori, myricae, phaseolicola, photinae, sesami, tabaci, ulmi, and certain strains of lachrymans and morsprunorum); when a formal proposal is made for the unification of these bacteria, the species name *P. amygdali* would take priority over *P. savastanoi*.

Microbiological properties: Gram-negative rods, 0.4-0.8 by 1.0-3.0 μm , aerobic. Motile by one to four polar flagella, rather slow growing, optimal temperatures for growth of 25–30 $^{\circ}\text{C}$, oxidase negative, arginine dihydrolase negative, elicits the hypersensitive response on tobacco, most isolates are fluorescent and levan negative although some isolates are non-fluorescent and levan positive.

Host range: *P. savastanoi* pv. *savastanoi* causes tumours in cultivated and wild olive and ash (*Fraxinus excelsior*). Although strains from olive were reported to infect oleander (*Nerium oleander*), this is generally not the case; however, strains of *P. savastanoi* pv. *nerii* can infect olive. Pathovars *fraxini* and *nerii* differentiate from pv. *savastanoi* mostly in their host range, and were not formally recognized until 1996. Literature previous to about 1996 generally name strains of the three pathovars as *P. syringae* subsp. *savastanoi* or *P. savastanoi* subsp. *savastanoi*, contributing to confusion about host range and biological properties.

Disease symptoms: Symptoms of infected trees include hyperplastic growths (tumorous galls or

knots) on the stems and branches of the host plant and, occasionally, on leaves and fruits.

Epidemiology: The pathogen can survive and multiply on aerial plant surfaces, as well as in knots, from where it can be dispersed by rain, wind, insects and human activities, entering the plant through wounds. Populations are very unevenly distributed in the plant, and suffer drastic fluctuations throughout the year, with maximum numbers of bacteria occurring during rainy and warm months. Populations of *P. savastanoi* pv. *savastanoi* are normally associated to non-pathogenic bacteria, both epiphytically and endophytically, and were demonstrated to form mutualistic consortia with *Erwinia toletana* and *Pantoea agglomerans* that could result in increased bacterial populations and disease symptoms.

Disease control: Based on preventive measures, mostly sanitary and cultural practices. Integrated control programs benefit from regular applications of copper formulations, which should be maintained at least a few years for maximum benefit. Olive cultivars vary in their susceptibility to olive knot, but there are no known cultivars with full resistance to the pathogen.

Useful websites: <http://www.pseudomonas-syringae.org/>; <http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl>; ASAP access to the *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome sequence

<https://asap.ahabs.wisc.edu/asap/logon.php>.

INTRODUCTION

Pseudomonas syringae is an economically important pathogen and one of the most relevant models for the study of plant-microbe interactions (e.g., Mansfield, 2009, Mansfield *et al.*, 2012). The species is currently a taxonomic conundrum and has been pulled together with *P. amygdali*, *P. avellanae*, *P. cannabina*, *P. caricapapayae*, *P. ficuserectae*, *P. meliae*, *P. savastanoi*, *P. tremae* and *P. viridiflava* into a group designated as the *P. syringae* complex, which could correspond to at least nine different species (Gardan *et al.*, 1999, Young, 2010, Parkinson *et al.*, 2011).

Pathovars of the *P. syringae* complex generally exploit the plant apoplast as a parasitic

niche and cause foliar necrosis in diverse plant hosts, with a minority of strains causing other types of symptoms, such as vascular diseases on woody plants (Agrios, 2005). A remarkable exception are a few pathovars producing aerial tumours in woody plants, including *P. savastanoi* pv. *savastanoi*. *P. savastanoi* pv. *savastanoi* is the causal agent of olive (*Olea europaea*) knot disease, whose symptoms include hyperplastic growths (tumorous galls or knots) on the stems and branches of the host plant and, occasionally, on leaves and fruits (Fig. 1). Olive knot is present worldwide, wherever olive is grown, and it is considered one of the most important diseases of olive (CMI, 1987, Young, 2004, Quesada *et al.*, 2010a). Diverse research groups worldwide have made substantial contributions towards understanding the biology, epidemiology and control of this pathogen; however, most strains of *P. savastanoi* pv. *savastanoi* are highly recalcitrant to genetic manipulation (Pérez-Martínez *et al.*, 2007), which has significantly slowed down their molecular analysis.

The growing availability of microbial genomes has spurred a new research era in the field of plant-microbe interactions, leading to the identification of potentially comprehensive repertoires of putative virulence genes and the emergence of unified models of interaction between prototypical pathogens and plant hosts (Lindeberg *et al.*, 2008, Mansfield, 2009, Schneider & Collmer, 2010). Extensive recent research efforts have focused on *Pseudomonas* diseases of herbaceous plants, with knowledge on the virulence and pathogenicity determinants specific for infection of woody plants, including those of tumour-inducing strains, lagging far behind. The selection of strain *P. savastanoi* pv. *savastanoi* NCPPB 3335 as a research model (Pérez-Martínez *et al.*, 2007) has opened the door for the application of high-throughput molecular tools to the analysis of the molecular basis of bacterial adaptation to woody hosts.

TAXONOMY AND POPULATIONS BIOLOGY

Despite significant advances in molecular phylogeny and taxonomy, the nomenclature and classification of *P. savastanoi* pv. *savastanoi* is still a source of confusion. This bacterium is part of

the *P. syringae* complex, encompassing at least 60 pathovars and several other *Pseudomonas* species (Bull *et al.*, 2010, Young, 2010). A study limited to a few taxa, formally classified pathovars *glycinea*, *phaseolicola* and *savastanoi* into the new species *P. savastanoi* (Gardan *et al.*, 1992), to which pathovars *fraxini*, *nerii* and *retacarpa* were later added (Bull *et al.*, 2010). DNA-DNA hybridization distributed *P. syringae* into at least nine separate genomospecies (Gardan *et al.*, 1999, Young, 2010). *P. savastanoi* pv. *savastanoi* was included in genomospecies 2, together with 16 other *P. savastanoi*-*P. syringae* pathovars (see Taxonomy, above) and the species *P. amygdali*, *P. ficuserectae* and *P. meliae*; when genomospecies 2 is formally named, however, the species should be designated *Pseudomonas amygdali* and not *P. savastanoi* (Gardan *et al.*, 1999). Multilocus sequence analyses show that *P. savastanoi* pv. *savastanoi* NCPPB 3335 is evolutionarily closer to *P. syringae* pathovars *aesculi* 2250 and NCPPB 3681, *tabaci* ATCC 11528 and *phaseolicola* 1448A (genomospecies 2) than to *P. syringae* pv. *tomato* DC3000 (genomospecies 3) or *P. syringae* pv. *syringae* B728a (genomospecies 1) (Fig. S1) (Sarkar & Guttman, 2004, Parkinson *et al.*, 2011). These studies support the genomospecies 2 grouping and indicate that it might encompass at least 9 further pathovars (*broussonetiae*, *castaneae*, *cerasicola*, *cunninghamiae*, *daphniphylli*, *fraxini*, *nerii*, *rhapiolepidis* and *retacarpa*) plus *P. tremae* (Sarkar & Guttman, 2004, Parkinson *et al.*, 2011). Then, what name should be used for this bacterium? Whereas *P. savastanoi* is being widely used in the literature, the *P. syringae* designation helps avoid the false idea that this pathogen is a different species than, for example, *P. syringae* pv. *tabaci*.

Natural isolates of *P. savastanoi* pv. *savastanoi* are heterogeneous, both phenotypically and genotypically (Table 1), although they tend to generate clonal populations in colonized areas (e.g., Sisto *et al.*, 2007, Quesada *et al.*, 2008). There is an important variation in virulence, with strains showing either low, intermediate or, most commonly, high virulence to diverse olive cultivars (Penyalver *et al.*, 2006), and also variation in the size and morphology of tumours in artificial inoculations (Pérez-Martínez *et al.*, 2007). Certain isolates in Central Italy are non-fluorescent and produce

levan, in contrast with the majority of other isolates (Marchi *et al.*, 2005). AFLP data clustered these levan-positive isolates separately from most of the common levan-negative isolates. Arbitrarily-primed PCR (Scortichini *et al.*, 2004, Krid *et al.*, 2009), AFLP (Sisto *et al.*, 2007), and typing with IS53 (Quesada *et al.*, 2008) revealed high levels of polymorphism; also, AFLP clearly differentiated

pv. *savastanoi* from pvs. *fraxini* and *nerii*. In general, genetic variability associates with geographic origin, with strains from the same area having a closer genetic relationship than those from different areas (Sisto *et al.*, 2007, Quesada *et al.*, 2008, Krid *et al.*, 2009, Matas *et al.*, 2009), suggesting a preference for clonal colonization of olive orchards; indeed, the spread of bacteria

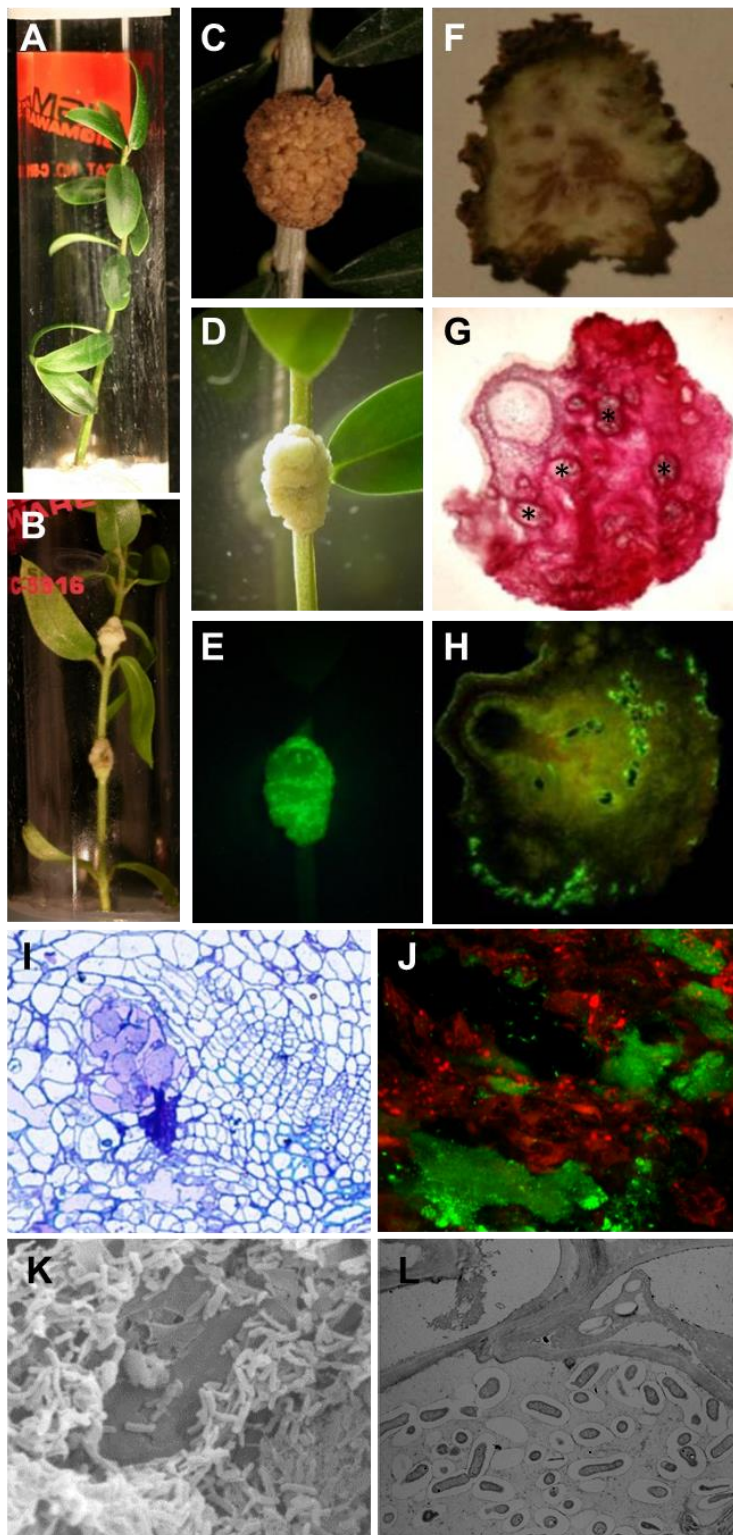


Fig. 1 Symptoms produced by *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 in olive plants and pathogen visualization within knots. *In vitro* micropropagated olive plants not inoculated (A) and inoculated (B). Knot induced on a two-years-old olive plant 90 days post inoculation (dpi) (C). Real-time monitoring of GFP-tagged *P. savastanoi* infection of a young micropropagated olive plant at 30 dpi (D) and complementary epifluorescence microscopy image (E). Cross section of the knot exposed in (C) showing necrosis associated with infection of the stem (F). Cross section of a 30 dpi knot, stained with methylene blue-picric acid; asterisks indicate newly formed bundles of xylem vessels (G). Transverse section of a knot, induced by GFP-tagged NCPPB 3335, showing GFP emission within the lumen of xylem vessels, in the internal cavities and at the periphery of the tumour tissue (H). Semithin cross-section of a knot stained with toluidine blue. Stained primary and secondary walls show dark and light blue colour, respectively (I). SCLM image of a knot induced by GFP-tagged NCPPB 335 (J). SEM micrograph showing a group of rod-shaped *P. savastanoi* cells (K). TEM micrograph of ultrathin section of a knot showing pathogen cells colonizing the intercellular spaces of the host tissue (L).

from inoculated to non-inoculated trees in an olive orchard, where they produced tumours, was documented in less than one year (Quesada *et al.*, 2010a). Typing with IS53 revealed higher

diversity than any of the other techniques, and could be used to track strains in the environment because many strains display unique patterns (Quesada *et al.*, 2008).

Table 1 Phenotypic and genetic differences among selected pathovars of *P. savastanoi*^a

<i>P. savastanoi</i> pv.	Plant host ^b				Genomic location of hormone biosynthesis genes ^c		
	Ash	Oleander	Olive	Spanish broom	<i>iaaMH</i>	<i>iaaL</i>	<i>ptz</i>
fraxini	c	-	c	-	nd	uk	uk
nerii	K	K	K	-	P	P	P
savastanoi	K	-	K	-	Ch	Ch	Ch
retacarpa	-	-	-	K	uk	uk	uk

^a Modified from (Janse, 1982, Iacobellis *et al.*, 1998, Pérez-Martínez *et al.*, 2008).

^b ash, *Fraxinus excelsior*; oleander, *Nerium oleander*; olive, *Olea europaea*; Spanish broom, *Retama sphaerocarpa*; c, cankers accompanied by wart-like excrescences; K, knots; -, no visible symptoms.

^c Symbols indicate that the gene is located in the chromosome (Ch) or in plasmids (P) in at least 70% of the strains examined; nd; not detected; uk, unknown. Genes *iaaMH* and *iaaL* are involved in the biosynthesis of indoleacetic acid and indoleacetic acid lysine, respectively, whereas *ptz* codes for an isopentenyl transferase, involved in the biosynthesis of cytokinins.

EPIDEMIOLOGY AND CONTROL

P. savastanoi pv. *savastanoi* does not survive for long in soil, and is normally found as an epiphyte and also endophytically, being able to migrate to produce secondary knots in new wounds (Ercolani, 1978, Penyalver *et al.*, 2006, Quesada *et al.*, 2007). Epiphytic life allows the build-up of populations for plant colonization and also fosters the interaction with other microbial communities in the phyllosphere. The pathogen is usually introduced to new areas through infected plant material. The bacterium can survive and multiply as a saprophyte on plant surfaces (Ercolani, 1978, Quesada *et al.*, 2007), as well as inside knots, from where it could be disseminated by rain, windblown aerosols, insects and cultural practices, such as pruning. It enters the plant through any type of wound, such as leaf scars or those caused by pruning, harvesting, frost and hail. The presence of knots in even a single tree normally leads to the rapid infection of the whole orchard because the pathogen is very rapidly and efficiently disseminated, with significant colonization of healthy trees in as little as a year (Quesada *et al.*, 2010a). The size of *P. savastanoi* pv. *savastanoi* populations is highly variable, even of several orders of magnitude between different leaves of the same shoot (Quesada *et al.*, 2007), with the highest

populations occurring in rainy months with moderate temperatures (10-20 °C).

A plethora of non-pathogenic bacterial species are found colonizing olive leaves or closely associated to knots produced by *P. savastanoi* pv. *savastanoi*, and the sizes of their populations are often positively correlated (Ercolani, 1978, Rojas *et al.*, 2004, Marchi *et al.*, 2006, Quesada *et al.*, 2007, Ouzari *et al.*, 2008, Moretti *et al.*, 2011). Several of these species could synthesize large amounts of IAA, which could favour proliferation of the pathogen and colonization of the plant (Cimmino *et al.*, 2006, Marchi *et al.*, 2006, Ouzari *et al.*, 2008). *Pantoea agglomerans* is the species most frequently found associated to *P. savastanoi* pv. *savastanoi* populations, its growth stimulated in the presence of active populations of the pathogen (Marchi *et al.*, 2006, Quesada *et al.*, 2007). Their interaction is not completely understood, and it can apparently lead to either an increase in virulence or a decrease of the pathogen populations (Marchi *et al.*, 2006, Hosni *et al.*, 2011). As described below (see "Other virulence factors"), a recent study demonstrated that both *Erwinia toletana* and *P. agglomerans* can form stable communities in planta (Hosni *et al.*, 2011).

The literature is elusive regarding crop losses caused by olive knot, which greatly

depend on geographical location and olive cultivar, although it is generally accepted that it is one of the most important diseases affecting the olive crop (Young, 2004). Tree vigour, growth and yield can be moderately or severely reduced, as well as the size and quality of fruits (Schroth *et al.*, 1973, Quesada *et al.*, 2010a). Olive knot cannot be eradicated once it is established in a tree or orchard, and therefore its control is based on preventive measures, mostly sanitary and cultural practices (Young, 2004, Quesada *et al.*, 2010b, Quesada *et al.*, 2010a). Methods should aim to avoid introduction and dissemination of the pathogen, for instance by using certified pathogen-tested trees and rootstocks to start new olive groves (EPPO, 2006), by minimizing wounding of trees and by reducing epiphytic populations of the pathogen. Detection and diagnosis of the pathogen can be done using diverse rapid and highly sensitive PCR methodologies (see Table S1), some of which allow differentiation of pathovars *fraxini*, *nerii* and *savastanoi*. Pivotal for an efficient disease management is a carefully planned and executed pruning, which should always start with the healthy trees and be avoided in wet weather. Chemical control with copper compounds has been traditionally used both in nurseries and in the field (Teviotdale & Krueger, 2004, Young, 2004). An extensive and systematic study (Quesada *et al.*, 2010b), reported a significant reduction of pathogen populations from the very first application of copper compounds, either copper oxychloride or cuprocalcic sulphate plus mancozeb. Nevertheless, treatments should be part of an appropriate integrated control program that includes the regular application of two copper treatments per year. This schedule produced the greatest differences with respect to the untreated control in the third year, after five copper treatments, resulting in a significant reduction in the average number of knots per plant (Quesada *et al.*, 2010b). Conversely, acibenzolar-S-methyl treatments did not result in a significant reduction of the disease symptoms.

Reduction of host susceptibility, including the use of resistant cultivars, is a most effective method for the integrated control of plant diseases; unfortunately, there are no known olive cultivars that are completely resistant to the pathogen. Early comparative studies showed a

considerable degree of phenotypic variation among olive cultivars, ranging from high susceptibility to a certain resistance (reviewed in Young, 2004). A larger assay evaluated the effect on symptom development of diverse variables—cultivar, plant age, development of secondary knots, inoculum dose and strain virulence—, and proposed a standardized method to assess cultivar susceptibility (Penyalver *et al.*, 2006). These authors demonstrated large differences in disease response with small variations in the inoculum dose, which might explain discrepancies in cultivar assessment among different studies, and classified 29 cultivars in three categories of high, medium and low susceptibility to the pathogen.

PSEUDOMONAS SAVASTANOI PV. SAVASTANOI: LIFE INSIDE THE KNOT

P. savastanoi pathogenicity and virulence is generally tested on 1- to 3-year-old olive plants (Glass & Kosuge, 1988, Iacobellis *et al.*, 1994, Sisto *et al.*, 2004, Penyalver *et al.*, 2006, Pérez-Martínez *et al.*, 2007, Hosni *et al.*, 2011). Aside from the space required, this often results in large variability in the size and number of knots that develop. *In vitro* techniques are widely used to study pathogenicity and virulence of animal bacterial pathogens and can also be conveniently applied in plant pathology. Several techniques have been described for micropropagation of a vast number of fruit trees, including several olive varieties, facilitating mass production of clonal and disease-free plants that can easily be maintained under controlled conditions in growth chambers. The use of *in vitro* micropropagated olive plants has been established as a fast and inexpensive method to study pathogenicity and virulence of *P. savastanoi* strains isolated from olive and oleander knots (Rodríguez-Moreno *et al.*, 2008). As previously observed with older olive plants, symptom development in micropropagated olive plants is highly dependent on both the olive variety and the strain. Nevertheless, histological modifications observed in *in vitro* olive plants after infection by *P. savastanoi* pv. *savastanoi* strains (Rodríguez-Moreno *et al.*, 2008, Marchi *et al.*, 2009, Rodríguez-Moreno *et al.*, 2009) are very similar to those in older olive plants (Smith, 1920, Surico,

1977, Temsah *et al.*, 2008), further confirming the suitability of this model system.

Tagging of *P. savastanoi* pv. *savastanoi* NCPPB 3335 with the green fluorescent protein (GFP), in combination with the use of *in vitro* olive plants and epifluorescence microscopy, allows real-time monitoring of disease development at the whole-tumour level, as well as the monitoring of bacterial localization inside knots at the single-cell level by scanning confocal electron microscopy (SCLM). Additionally, scanning and transmission electron microscopy (SEM and TEM, respectively) can be used for a detailed ultrastructural analysis of tumour histology, as well as for the visualization of the *P. savastanoi* pv. *savastanoi* lifestyle within knot tissues (Fig. 1) (Rodríguez-Moreno *et al.*, 2009). A combination of these microscopy techniques was used for *in vivo* analysis of *P. savastanoi* pv. *savastanoi* NCPPB 3335 mutants affected in virulence (Pérez-Martínez *et al.*, 2010, Bardaji *et al.*, 2011).

GENOMIC INSIGHTS INTO *P. SAVASTANOI* PV. *SAVASTANOI* PATHOGENICITY AND VIRULENCE

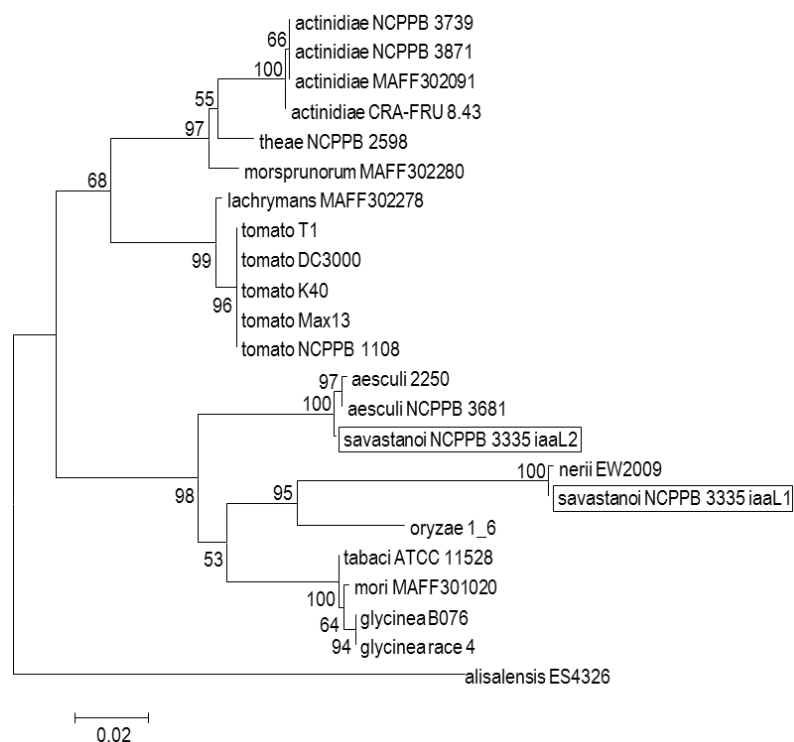
In this section we review how the recent sequencing of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 draft genome and the complete sequence of its three plasmid complement, allowed the identification of the virulence gene

complement of this tumour-inducing pathogen of woody hosts (Rodríguez-Palenzuela *et al.*, 2010, Bardaji *et al.*, 2011).

Phytohormones

In *P. savastanoi*, indoleacetic acid (IAA) is synthesized from tryptophan in two steps catalysed by the products of genes *iaaM* (tryptophan monooxygenase) and *iaaH* (indoleacetamide hydrolase) (Comai & Kosuge, 1982, Palm *et al.*, 1989). Additionally, *P. savastanoi* pv. *nerii* (oleander isolates) also converts IAA to IAA-lysine through the action of the *iaaL* gene (Glass & Kosuge, 1988), which is also present in most *P. syringae* complex pathovars (Glickmann *et al.*, 1998). Although *P. savastanoi* pv. *savastanoi* strains contain two *iaaL* alleles (Matas *et al.*, 2009), IAA-Lys has not been detected in culture filtrates of *P. savastanoi* strains isolated from olive (Evidente *et al.*, 1986, Glass & Kosuge, 1988). Two chromosomally encoded *iaaM*, *iaaH* and *iaaL* alleles were also found in the genome of *P. savastanoi* pv. *savastanoi* NCPPB 3335; however, the *iaaM-2* and *iaaH-1* alleles appeared to be pseudogenes (Rodríguez-Palenzuela *et al.*, 2010). Resequencing of these two loci has recently confirmed that, in fact, *iaaM-2* is a pseudogene whereas *iaaH-1* encodes a complete CDS.

Fig. 2 Unrooted NJ trees of *iaaL* nucleotide sequences from strains of the *P. syringae* complex. See Fig. S1 for methodology and Table S3 for accession numbers. Only pathovar name and strain designation are shown; all strains belong to *P. syringae*, except *P. cannabina* pv. *alisalensis* ES4326, which was previously designated as *P. syringae* pv. *maculicola*.



Gene *iaaL* is widely distributed within the *P. syringae* complex (Glickmann et al., 1998), and its phylogeny (Fig. 2) is largely congruent with the phylogeny deduced from housekeeping genes (Fig. S1), suggesting that *iaaL* is ancestral to the complex. However, clustering of *iaaL* from *P. syringae* pv. *oryzae* 1_6 (genomospecies 4) with genomospecies 2 (Fig. 2) evidences horizontal transfer. This is not surprising because *iaaL* is often found in several copies and located in plasmids (Glickmann et al., 1998, Matas et al., 2009), although the transfer appears to preferentially occur within the *P. syringae* complex (not shown). Conversely, highly conserved *iaaMH* alleles are present in only a handful of *P. syringae* complex strains (Table S2) (Glickmann et al., 1998); nevertheless, diverse pathovars contain CDSs (Baltrus et al., 2011) whose deduced products show very low identity to those of *iaaMH* (e.g. PSPTO_0518/PSPTO_4204; 29.3/29.7% aa identity), but high identity with putative monooxygenase and amidase genes common in the *P. syringae* complex (e.g. 99/89% aa identity with PSA3335_4651/PSA3335_4172 from NCPPB 3335), and whose role in IAA biosynthesis has not been demonstrated. The limited data available also suggests horizontal transfer of *iaaMH* within the *P. syringae* complex, which are also less related to the corresponding genes of other organisms (Table S2).

Genes for phytohormone biosynthesis have a disparate genomic localization in different tumour-inducing strains of *P. savastanoi* (Table 1), with those for the biosynthesis of cytokinins (CKs) preferentially located in plasmids of the pPT23A-family in *P. savastanoi* pv. *savastanoi* (Macdonald et al., 1986, Silverstone et al., 1993, Pérez-Martínez et al., 2008). The *ptz* gene, encoding an isopentenyl transferase and characterized by a low G+C content (43.4% G+C), was found in a potential genomic island located in plasmid pPsv48A of *P. savastanoi* pv. *savastanoi* NCPPB 3335 (Bardaji et al., 2011). Knots induced in olive plants by *P. savastanoi* strains cured of plasmids containing *ptz* are smaller (Iacobellis et al., 1994, Rodríguez-Moreno et al., 2008, Bardaji et al., 2011) and show a lower presence of spiral vessels (Bardaji et al., 2011) than those induced by wild-type strains. Another gene putatively involved in the

biosynthesis of CKs, gene *ipt*, encoding a putative isopentenyl-diphosphate delta-isomerase, was found in plasmid pPsv48C of *P. savastanoi* pv. *savastanoi* NCPPB 3335; however, its role in virulence has not been tested, since derivatives lacking pPsv48C are not yet available (Bardaji et al., 2011).

Apparently, *P. savastanoi* pv. *savastanoi* does not belong to the group of 2-oxoglutarate-dependent ethylene producers, a pathway dependent on gene *efe* in several *P. syringae* pathovars (Weingart et al., 1999). First, no homology to an *efe* probe was found by hybridization analysis of 32 different *P. savastanoi* pv. *savastanoi* plasmids (Pérez-Martínez et al., 2008). Second, proteins homologous to ethylene forming enzymes from *P. syringae* pv. *phaseolicola*, pv. *glycinea* and pv. *pisi* have not been found in the *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome (Rodríguez-Palenzuela et al., 2010).

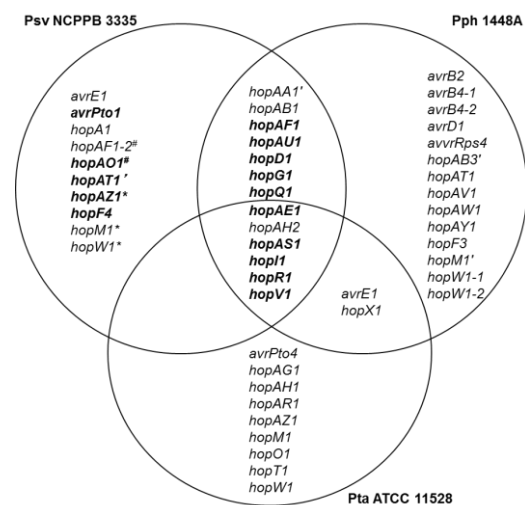


Fig. 3 Updated and corrected comparison of the type III effector gene complements of *P. savastanoi* pv. *savastanoi* (Psv) NCPPB 3335 and other sequenced plant-pathogenic pseudomonads. Pph, *P. syringae* pv. *phaseolicola*; Pta, *P. syringae* pv. *phaseolicola*. Gene *hopAF1-2*, plasmid encoded in NCPPB 3335, shows 73%-74% amino acid identity with *hopAF1* from Psy B728a, Pph 1448A and Pto DC3000. Psv NCPPB 3335 effectors included in the Hop database (http://www.pseudomonas-syringae.org/pst_func_gen2.htm) are indicated in boldface. #, Plasmid-encoded gene; asterisks indicate putative pseudogenes; *hop* genes truncated by a frameshift or a premature stop codon are indicated by a single quotation mark (Lindeberg et al., 2005).

Type III secretion system and effectors

Cluster analysis of HrpS protein sequences (Inoue & Takikawa, 2006) showed that *P. savastanoi* pv. *savastanoi* NCPPB 3335 belongs to group I, which comprises exclusively proteins from *P. syringae* pathovars from genomospecies 2 (Gardan *et al.*, 1999). In relation to HrpA, *P. savastanoi* pv. *savastanoi* NCPPB 3335 contains a *hrpA2* gene, which is highly similar to those of *P. syringae* pvs. *phaseolicola*, *glycinea* and *tabaci* (Pérez-Martínez *et al.*, 2010).

In agreement with Sisto *et al.* (2004), a T3SS mutant of strain NCPPB 3335 was also unable to multiply in olive tissues and induce the formation of knots in woody olive plants. Interestingly, tumours induced by the T3SS mutant on young micropropagated olive plants did not show the necrosis and internal open cavities observed in knots induced by the wild-type strain (Pérez-Martínez *et al.*, 2010).

Bioinformatic analysis of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome sequence (Rodríguez-Palenzuela *et al.*, 2010) allowed a prediction of *hop* genes, including 19 putative T3SS effectors with amino acid identities of 65-80% to previously described effectors. Additionally, a further 11 candidate genes do not share sequence similarity with known effectors (Rodríguez-Palenzuela *et al.*, 2010). A later revision of this genome sequence identified four new candidate effectors, AvrPto1, HopAT1', HopAZ1 and HopF4 (Hops Database, <http://www.pseudomonas-syringae.org/home.html>) (Fig. 3). Furthermore, sequencing of the three-plasmid complement of this strain revealed that two of the T3SS effector genes are plasmid encoded, *hopAF1* (plasmid pPsv48A) and *hopAO1* (plasmid pPsv48B) (Bardaji *et al.*, 2011). Figure 3 shows an updated and corrected comparison of the T3SS effector gene complements of *P. savastanoi* pv. *savastanoi* NCPPB 3335 and other sequenced plant-pathogenic pseudomonads. Translocation analysis of the T3SS effector repertoire of *P. savastanoi* pv. *savastanoi* NCPPB 3335 is currently in progress.

Other virulence factors

Pathogenicity of *P. savastanoi* pv. *savastanoi* in olive critically depends on quorum sensing (QS) regulation. The QS system of *P. savastanoi* pv.

savastanoi strain DAPP-PG 722 consist of a *luxI* homolog (*pssI*) and a *luxR* homolog (*pssR*) (Hosni *et al.*, 2011). However, the lack of signal production in a *pssI* mutant of this pathogen has been shown to be complemented *in planta* by the presence of wild-type *Erwinia toletana*, a non-pathogenic bacterium that is very often found associated with the olive knot pathogen (Hosni *et al.*, 2011). *E. toletana* produced the same N-acyl-homoserine lactone molecules than *P. savastanoi* pv. *savastanoi*; moreover, populations of *E. toletana* significantly declined over time after inoculation in olive tissues, but increased upon co-inoculation with a strain of *P. savastanoi* pv. *savastanoi*. This relationship is mutualistic, because the populations of *P. savastanoi* pv. *savastanoi* also increased significantly when the pathogen was co-inoculated with *E. toletana*; additionally, the size of knots also increased, reflecting an increase in virulence (Hosni *et al.*, 2011). The mechanism underlying this relationship is not fully clear, but it appears to result at least in part from sharing QS signalling mediated by N-acyl-homoserine lactones.

Other known virulence determinants in plant-pathogenic *Pseudomonas* include phytotoxins, cell wall-degrading hydrolytic enzymes, extracellular polysaccharides, iron-uptake systems, resistance to plant-derived antimicrobials, adhesion, as well as the general processes of motility and chemotaxis. Annotation of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 draft genome revealed the existence of 551 genes potentially involved in several processes that could contribute to virulence, most of which are conserved in *P. syringae* pv. *phaseolicola* 1448A. However, the subset of *P. savastanoi* pv. *savastanoi* NCPPB 3335-specific genes (not found in 1448A), includes a cellulase, a pectate lyase and a putative filamentous hemagglutinin (Rodríguez-Palenzuela *et al.*, 2010). Genes for levansucrase, the enzyme responsible for biosynthesis of the exopolysaccharide levan, are found in the genome of all sequenced *P. syringae* strains, although their numbers vary from three to one (O'Brien *et al.*, 2011). Only a single levansucrase-coding gene (PSA3335_2033) was identified in *P. savastanoi* pv. *savastanoi* NCPPB 3335 (Rodríguez-Palenzuela *et al.*, 2010), probably because *P. savastanoi* pv. *savastanoi* strains are in general levan-negative whereas the

P. syringae pathovars of LOPAT subgroup 1a are all levan-positive (Lelliott & Stead, 1987). The relevance of all these putative virulence factors in *P. savastanoi* has not been reported to date.

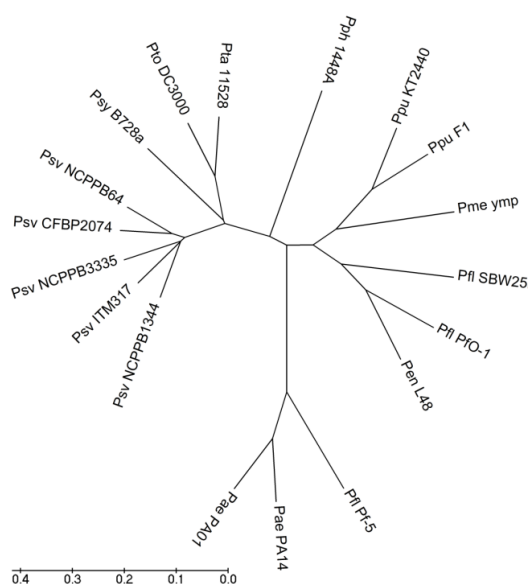


Fig. 4 Unrooted UPGMA tree based upon nutrient utilization data of *P. savastanoi* pv. *savastanoi* (Psv) and other pseudomonads. Metabolic activities of Psv strains, tested using Biolog GN2 plates, were compared with carbon utilization data reported for *P. syringae* strains and non-plant pathogenic species of *Pseudomonas* (Rico & Preston, 2008). The tree was constructed using MEGA5 (Tamura *et al.*, 2011) and is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the dendro-gram. Distances were computed using the Maximum Composite Likelihood method and are in the units of the number of substrate utilization per site. Abbreviations are *P. syringae* pathovars syringae, Psv; tomato, Pto; tabaci, Pta; phaseolicola, Pph; and *P. putida*, Ppu; *P. entomophila*, Pme; *P. fluorescens*, Pfi; and *P. aeruginosa*, Pae.

METABOLIC VERSATILITY AND ADAPTATION TO WOODY HOSTS

P. syringae pathovars are nutritionally specialized for growth in the plant environment relative to non-pathogenic pseudomonads (Rico *et al.*, 2011). The Biolog GN2 MicroPlate technology (Bochner *et al.*, 2001) revealed that the carbon utilization profiles of five different *P. savastanoi* pv. *savastanoi* strains, including NCPPB 3335, are almost identical. However, comparative analysis with previously reported data for *P. syringae* pathovars and non-pathogenic

pseudomonads (Rico & Preston, 2008, Mithani *et al.*, 2011) shows that *P. savastanoi* pv. *savastanoi* metabolic activities are more similar to those shown by *P. syringae* pv. *tabaci* ATCC 11528, *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728a than to those observed for *P. syringae* pv. *phaseolicola* 1448A (Fig. 4), in spite that both strains ATCC 11528 and 1448A cluster together with *P. savastanoi* pv. *savastanoi* NCPPB 3335 by multilocus sequence analysis of housekeeping genes (Group 3, Fig. S1). Thus, nutritional divergence does not mirror phylogenetic divergence, possibly due to host-specific features, or pathogen evolutionary history.

Production of phenolic compounds, which provide a natural defence against pathogen attack, is greatly increased in olive knots induced by *P. savastanoi* pv. *savastanoi* (Cayuela *et al.*, 2006), suggesting that bacterial resistance to phenols could be of paramount importance in pathogenicity. The *P. savastanoi* pv. *savastanoi* NCPPB 3335 genome (Rodríguez-Palenzuela *et al.*, 2010) encodes a region of about 15 Kb, named VR8 (60.1% G+C), which is absent in all sequenced *P. syringae* strains infecting herbaceous plants but shared with *P. syringae* pathovars infecting woody hosts, such as *aesculi* (Green *et al.*, 2010), *morsprunorum* and *actinidiae* (Fig. 5), which are pathogenic to chestnut, cherry and kiwi, respectively. Among other genes encoded in this region, the *antABC* and *catBCA* operons are involved in the degradation of anthranilate and catechol, respectively, and could offer a selective advantage for growth in woody hosts. In fact, the *antABC* cluster is homologous to the anthranilate degradation genes found on plasmid pCAR1 of *Pseudomonas resinovorans* (Nojiri *et al.*, 2002, Urata *et al.*, 2004), a bacterium commonly found in the lubricating oils of wood mills. Other metabolic pathways involving the *cat* and/or *ant* genes included in the KEGG Pathway Database (<http://www.genome.jp/kegg/pathway.html>) are those related to the degradation of benzoate, fluorobenzoate, toluene, chlorocyclohexane and chlorobenzene. In *P. savastanoi* pv. *savastanoi* NCPPB 3335 and all other strains encoding VR8, the genetic content and chromosomal location of this region is identical (Fig. 5). However, genetic elements suggesting its possible acquisition by

horizontal transfer were not found bordering VR8 in *P. savastanoi* pv. *savastanoi* NCPPB 3335 (Rodríguez-Palenzuela *et al.*, 2010).

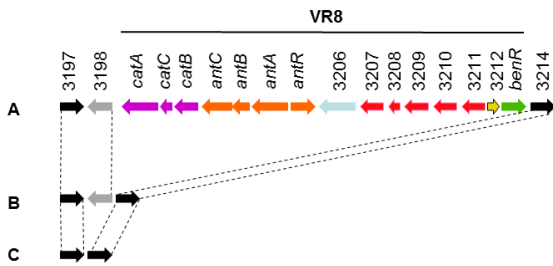


Fig. 5 Schematic map of variable region 8 (VR8) in the genomes of *P. savastanoi* pv. *savastanoi* NCPPB 3335 and other sequenced *P. syringae* pathovars. A) *P. savastanoi* pv. *savastanoi* NCPPB 3335, *P. syringae* pathovars aesculi strains 2250 and NCPPB 3681, morsprunorum MAFF302280, actini-diae MAFF302091; B) *P. syringae* pathovars tabaci ATCC 11528, mori 301020, phaseolicola 1448A, glycinea race 4, lachrymans MAFF302278, and japo-nica MAFF301072; C) *P. syringae* pathovars syringae B728a, tomato DC3000, and oryzae 1_6. Black and grey arrows indicate genes flanking VR8 in *P. savastanoi* pv. *savastanoi* NCPPB 3335 which are present (PSA3335_3197 and PSA3335_3214) or not (PSA3335_3198), respectively, in the genome of all the strains analysed. Pink and orange arrows indicate genes involved in the catabolism of catechol (*catBCA*) or anthranilate (*antABC* and *antR*), respectively. PSA3335_3197, outer membrane protein; PSA3335_3198, ribosomal protein S5p alanine acetyltransferase (*rimJ*); PSA3335_3206, aerotaxis receptor; PSA3335_3207, nitrotriacetate monooxygenase component B, flavin reductase; PSA3335_3208, protein involved in meta-pathway of phenol degradation; PSA3335_3209, putative oxygenase subunit; PSA3335_3210, short-chain alcohol dehydrogenase/reductase; PSA3335_3211, dienelactone hydrolase; PSA3335_3212, hypothetical protein; PSA3335_3214, voltage-dependent potassium channel protein.

PLASMID GENETICS AND BIOLOGY

Plasmids are the main agents in the horizontal exchange of DNA among bacteria, and the *P. syringae* complex contains a significant horizontal gene pool distributed in diverse native plasmids (Jackson *et al.*, 2011). Strains of *P. savastanoi* pv. *savastanoi* usually contain one to six plasmids (around 10 to >100 kb) (Murillo & Keen, 1994, Pérez-Martínez *et al.*, 2008). Most of these belong to the pPT23A-like family of plasmids (PFP), characterized for sharing a highly conserved replication module (Gibbon *et al.*,

1999), although strains might contain from zero to four non-PFP plasmids. As usual in the *P. syringae* complex, plasmid profiles are highly variable and often strain-specific, offering a simple way for strain tracking (Pérez-Martínez *et al.*, 2007, Pérez-Martínez *et al.*, 2008). Nevertheless, plasmid profiles of *P. syringae* complex strains are dynamic and often change in response to repeated subculture or interaction with the host (e.g. Lovell *et al.*, 2011).

PFP plasmids carry a panoply of genes involved in pathogenicity, virulence and adaptation to the environment, such as genes for T3SS effectors, type IV secretion systems, phytotoxins, phytohormones, and resistance to antibiotics and heavy metals, as well as an array of insertion sequences (Sundin, 2007). Similar types of genes were found in 32 native plasmids from 10 *P. savastanoi* pv. *savastanoi* strains using a macroarray containing 135 different genes, albeit with a limited presence of genes *ruIAB*, for UV radiation tolerance. This could be significant because *ruIAB* genes appear to often control the expression of integrases, and are predicted to facilitate the dispersal of associated T3SS effector genes (Jackson *et al.*, 2011). Native plasmids from *P. savastanoi* pv. *savastanoi* contain diverse virulence genes carried indistinctly by PFP and non-PFP plasmids (Pérez-Martínez *et al.*, 2008), although PFP plasmids have been traditionally recognized as the main, or the only, repository of valuable genes in the *P. syringae* complex. At least eight T3SS effector genes (Jackson *et al.*, 2002, Pérez-Martínez *et al.*, 2008) are frequently found on *P. savastanoi* pv. *savastanoi* plasmids. Other relevant virulence genes are those involved in the biosynthesis of phytohormones, which were the first plasmid-borne pathogenicity genes found in *Pseudomonas* spp. (Comai & Kosuge, 1980). Unlike pv. *nerii*, most strains of pv. *savastanoi* carry chromosomal copies of genes for the biosynthesis of IAA and CKs (Table 1).

The complete sequences of the three PFP plasmid complement of strain NCPPB 3335 (pPsv48A, 78 kb; pPsv48B, 45 kb; pPsv48C, 42 kb) (Bardaji *et al.*, 2011) contain 152 predicted coding sequences (CDSs); the majority (38 CDSs) were annotated as hypothetical proteins, followed by 37 CDSs involved in DNA metabolism, including plasmid replication and

maintenance. Each of the plasmids contained at least one putative toxin-antitoxin system, involved in plasmid maintenance, which is probably why we could not obtain derivatives cured of the three plasmids (Bardaji et al., 2011). The three plasmids contain seven putative virulence genes, five of which are putative type III effectors preceded by a Hrp-box: pPsv48A contains a chimeric copy of gene *hopAF1*, included in the transposon effector ISPsy30, plus three copies of a large CDS found in many plant-associated proteobacteria, whereas pPsv48B contains gene *hopAO1* (*avrPphD2*). Additionally, two genes putatively involved in CKs biosynthesis, *ptz* (PSPSV_A0024) and *ipt* (PSPSV_C0024), were also found in plasmids A and C, respectively.

Plasmids are very plastic and dynamic molecules, facilitating the exchange of sequences among them and with the chromosome (Ma et al., 2007, Sundin, 2007, Jackson et al., 2011). This is illustrated by plasmids pPsv48B and pPsv48C, which probably arose from a duplication event because their replication gene, *repA*, is 98.6% identical. However, they only share around 10 kb with at least 80% identity, implying they participated in an active exchange of DNA. Indeed, pPsv48B contains a complete type IVA secretion system and a well conserved origin of conjugational transfer, suggesting that it might be conjugative; additionally, pPsv48C also contains an origin of transfer and could be mobilizable by pPsv48B. Although, in principle, plasmids can be transferred to very distant organisms, they tend to propagate within a specific host clade (Jackson et al., 2011). A phylogenetic analysis of the *repA* gene from diverse PFP plasmids, and of other genes carried by them, indicate that they are actively exchanging DNA and moving amongst *P. syringae* complex pathovars (Ma et al., 2007).

The role of native plasmids in the life cycle of *P. savastanoi* pv. *savastanoi* has not been assessed in detail due to the difficulties for its genetic manipulation and for plasmid curing. Nevertheless, in diverse strains of *P. savastanoi* pv. *savastanoi* and pv. *nerii* certain native plasmids are essential for the expression of wild type symptoms, to reach high population densities *in planta* and for competitive fitness, all of which was related to the presence in those plasmids of genes for IAA and/or CKs biosynthesis (Silverstone et al., 1993, Iacobellis

et al., 1994, Rodríguez-Moreno et al., 2008, Bardaji et al., 2011). Since these effects are very drastic, they could conceivably have obscured more subtle roles in the pathogenic process of other plasmid-borne genes; however, the current availability of genetically tractable strains and plasmid sequences shall facilitate a more detailed analysis of their potential role.

FUTURE PROSPECTS

Diseases of woody plants caused by pathovars of the *P. syringae* complex are of major concern in fruit producing areas and nurseries worldwide and result in considerable economic losses (Kennelly et al., 2007). Undoubtedly, advances in the understanding of diseases caused by *P. syringae* pathovars on herbaceous plants, including the model plant *Arabidopsis*, are relevant to our understanding of fruit tree diseases, and vice versa. However, there is a pressing need for appropriate research model systems facilitating the identification and analysis of specific determinants involved in bacterial interactions with trees and shrubs. A series of works in the last years made significant advances in the biology, ecology, genetics, and genomics of *P. savastanoi* pv. *savastanoi*, which emerged as a powerful and uniquely valuable model for the study of the molecular basis of disease production and tumour formation in woody hosts. Analysis of the *P. savastanoi*-olive interaction, and comparison with the model systems of herbaceous plants, can provide insights into the interactions of other bacterial pathogens with woody hosts and address relevant unresolved questions, such as: What is the role of the T3SS system and its effectors during infection of woody tissues? Are there differences in the metabolic network required by bacterial pathogens for survival in woody hosts and herbaceous hosts? What virulence determinants are singularly required for infection of woody tissues? What factors are involved in tumour induction by *P. savastanoi* and what evolutionary advantage derives from producing them instead of necroses? To what degree do bacterial consortia influence disease incidence and severity, and can they be targeted for disease control? What traits govern host specificity in *P. savastanoi* pathovars? Comparative genomics among *P.*

syringae and *P. savastanoi* pathovars is generating workable hypotheses to critically investigate these questions. However, a great deal of research remains to establish genome-wide approaches allowing functional characterization of bacterial interactions with woody hosts and to develop effective control strategies for *Pseudomonas* diseases. Genetic dissection of the *P. savastanoi* pv. *savastanoi*-olive pathosystem is technically very challenging and requires the analysis of the always unfriendly woody plants but, as Osgood wisely summarized in the delightful Billy Wilder film, "Well, nobody's perfect".

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SUPPORTING INFORMATION

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Fig. S1 Evolutionary relationships of *P. savastanoi* pv. *savastanoi* and selected *P. syringae* pathovars. Tree was constructed by multilocus sequence analysis using a concatenated data set (exactly 12000 nt) of *acnB*, *fruK*, *gapA*, *gltA*, *gyrB*, *pgi*, *recA* and *rpoD* genes. Phylogenetic groups 1, 2, 3 and 4 (Sarkar & Guttman, 2004, Studholme, 2011) correspond to genomospecies (Gsp) 3, 1, 2 and 4 (Gardan et al., 1999), respectively. Sequence alignment using Muscle, determination of the optimal nucleotide substitution model and phylogenetic tree construction were done using MEGA5 (Tamura et al., 2011); all positions containing gaps and missing data were eliminated using the option of complete deletion. Bootstrap values (1,000 repetitions) are shown on branches. Similar or identical topologies were obtained by maximum likelihood. The scale bar represents nucleotide substitutions per site.

Table S1 Primers used for the detection of *Pseudomonas savastanoi* pv. *savastanoi*.

Table S2 Comparison of the deduced products of *iaaM*-1 (PSA3335_1475) and *iaaH*-1 (PSA3335_1476), from *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335, with their homologues in selected organisms.

Table S3 Accession numbers and coordinates of the nucleotide sequences used for the construction of the neighbour-joining tree shown in Fig. 2.