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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 witnessed a large number 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 prevented the pathogen from benefitting from the scientific progress in molecular biology that has elevated the foliar pathogens of the *P. syringae* complex to supermodels. A number of studies in recent 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 nonpathogenic 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, which 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 obtain 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*, *photiniae*, *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 × 1.0–3.0 μm, aerobic. Motile by one to four polar flagella, rather slow growing, optimal temperatures for growth of 25–30 °C; oxidase negative, arginine dihydrolase negative; elicits the hypersensitive response on tobacco; most isolates are fluorescent and levan negative, although some isolates are nonfluorescent and levan positive.

Host range: *P. savastanoi* pv. *savastanoi* causes tumours in cultivated and wild olive and ash (*Fraxinus excelsior*). Although strains from olive have been 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* are differentiated from pathovar *savastanoi* mostly in their host range, and were not formally recognized until 1996. Literature before about 1996 generally names strains of the three pathovars as *P. syringae* ssp. *savastanoi* or *P. savastanoi* ssp. *savastanoi*, contributing to confusion on the 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 with nonpathogenic bacteria, both epiphytically and endophytically, and have been demonstrated to form mutualistic consortia with *Erwinia toletana* and *Pantoea agglomerans*, which could result in increased bacterial populations and disease symptoms.

Disease control: Based on preventive measures, mostly sanitary and cultural practices. Integrated control programmes benefit

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from regular applications of copper formulations, which should be maintained for 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; Parkinson *et al*., 2011; Young, 2010).

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). Remarkable exceptions include a few pathovars producing aerial tumours in woody plants, such as *P. savastanoi* pv. *savastanoi*. *Pseudomonas 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 is considered to be one of the most important diseases of olive (CMI, 1987; Quesada *et al*., 2010a; Young, 2004). Diverse research groups worldwide have made substantial contributions towards the understanding of 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 promoted 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 and Collmer, 2010). Extensive recent research efforts have focused on *Pseudomonas* diseases of herbaceous plants, with knowledge on the virulence and pathogenicity determinants specific for the 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 up the way for the application of high-throughput molecular tools to the analysis of the molecular basis of bacterial adaptation to woody hosts.

TAXONOMY AND POPULATION BIOLOGY

Despite significant advances in molecular phylogeny and taxonomy, the nomenclature and classification of *P. savastanoi* pv. *savastanoi* are 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). *Pseudomonas savastanoi* pv. *savastanoi* was included in genomospecies 2, together with 16 other *P. savastanoi–P. syringae* pathovars (see 'Summary') and the species *P. amygdali*, *P. ficuserectae* and *P. meliae*; when genomospecies 2 is formally named, however, the species should be designated *P. amygdali* and not *P. savastanoi* (Gardan *et al*., 1999). Multilocus sequence analyses have shown 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, see Supporting Information) (Parkinson *et al*., 2011; Sarkar and Guttman, 2004). These studies support the genomospecies 2 grouping and indicate that it might encompass at least nine further pathovars (*broussonetiae*, *castaneae*, *cerasicola*, *cunninghamiae*, *daphniphylli*, *fraxini*, *nerii*, *rhaphiolepidis* and *retacarpa*) plus *P. tremae* (Parkinson *et al*., 2011; Sarkar and Guttman, 2004). Therefore, what name should be used for this bacterium? Although *P. savastanoi* is being widely used in the literature, the *P. syringae* designation helps to avoid the false idea that this pathogen is a different species from, 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. Quesada *et al*., 2008; Sisto *et al*., 2007). 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 nonfluorescent and produce levan, in contrast with the majority of other isolates (Marchi *et al*., 2005). Amplified fragment length polymorphism

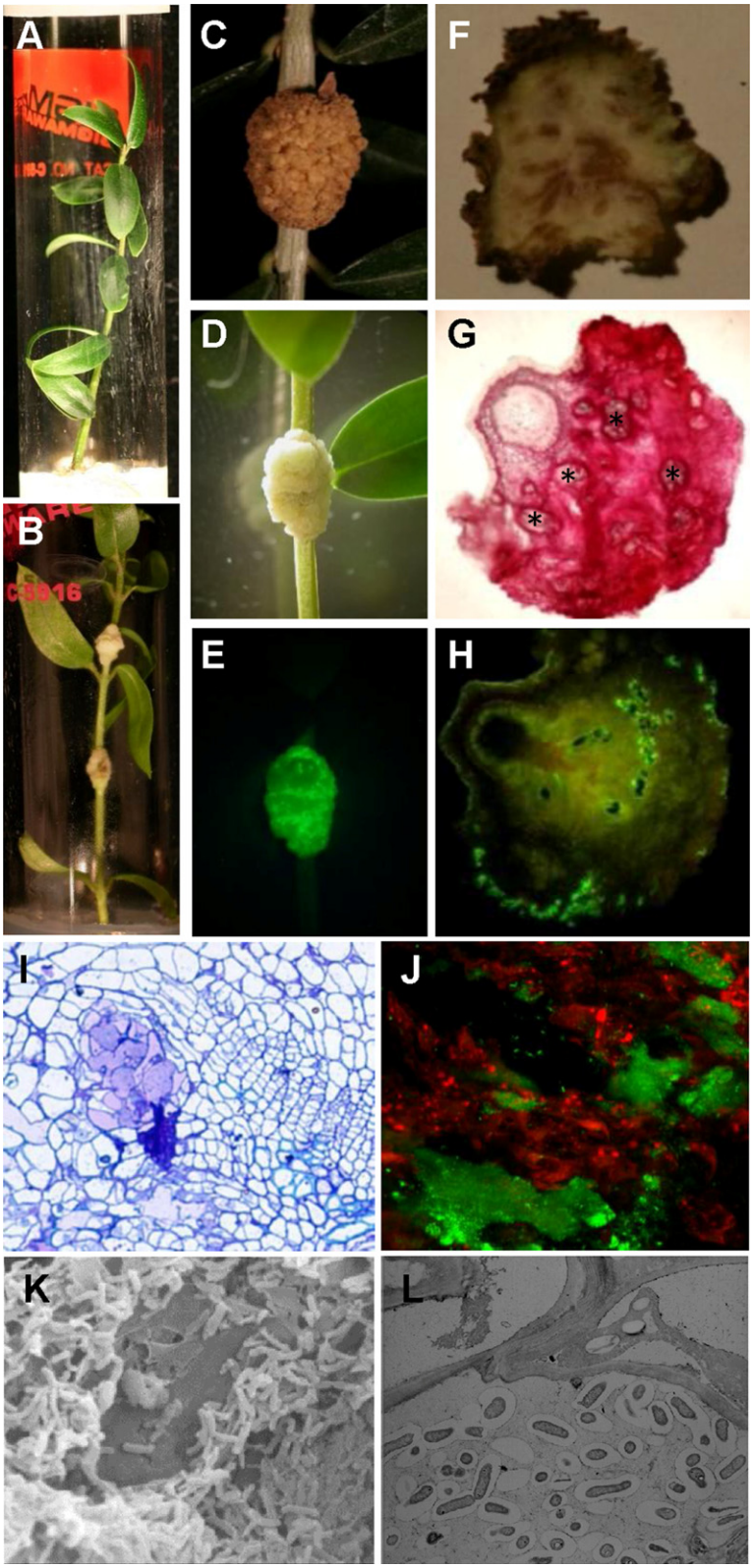

Fig. 1 Symptoms produced by *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 in olive plants and pathogen visualization within knots. *In vitro* micropropagated olive plants not inoculated (A) and inoculated (B). (C) Knot induced on a 2-year-old olive plant 90 days post-inoculation (dpi). Real-time monitoring of green fluorescent protein (GFP)-tagged *P. savastanoi* infection of a young micropropagated olive plant at 30 dpi (D) and complementary epifluorescence microscopy image (E). (F) Cross-section of the knot exposed in (C) showing necrosis associated with infection of the stem. (G) Cross section of a 30-dpi knot, stained with methylene blue–picrofuchsin; asterisks indicate newly formed bundles of xylem vessels. (H) 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. (I) Semithin cross-section of a knot stained with toluidine blue. Stained primary and secondary walls show dark and light blue in colour, respectively. (J) Scanning confocal electron microscopy (SCLM) image of a knot induced by GFP-tagged NCPPB 3335. (K) Scanning electron micrograph showing a group of rod-shaped *P. savastanoi* cells. (L) Transmission electron micrograph of ultrathin section of a knot showing pathogen cells colonizing the intercellular spaces of the host tissue.

Table 1 Phenotypic and genetic differences among selected pathovars of *Pseudomonas savastanoi**.

	Plant host†				Genomic location of hormone biosynthesis genes‡		
P. savastanoi pv.	Ash	Oleander	Olive	Spanish broom	*iaaMH*	*iaaL*	*ptz*
fraxini	c	–	c	–	nd	uk	uk
nerii	K	K	K	–	P	P	P
savastanoi	K	–	K	–	Ch	Ch	Ch
retacarpa	–	–	–	K	uk	uk	uk

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

†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.

‡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 i*aaMH* 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.

(AFLP) data cluster these levan-positive isolates separately from most of the common levan-negative isolates. Arbitrarily primed polymerase chain reaction (PCR) (Krid *et al*., 2009; Scortichini *et al*., 2004), AFLP (Sisto *et al*., 2007) and typing with IS*53* (Quesada *et al*., 2008) have revealed high levels of polymorphism; in addition, AFLP clearly differentiates pathovar *savastanoi* from pathovars *fraxini* and *nerii*. In general, genetic variability associates with geographical origin, with strains from the same area having a closer genetic relationship than those from different areas (Krid *et al*., 2009; Matas *et al*., 2009; Quesada *et al*., 2008; Sisto *et al*., 2007), suggesting a preference for clonal colonization of olive orchards; indeed, the spread of bacteria from inoculated to noninoculated trees in an olive orchard, where they produce tumours, has been documented in less than 1 year (Quesada *et al*., 2010a). Typing with IS*53* 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).

EPIDEMIOLOGY AND CONTROL

Pseudomonas 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 can be disseminated by rain, wind-blown 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 1 year (Quesada *et al*., 2010a). The size of *P. savastanoi* pv. *savastanoi* populations is highly variable, even by 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 nonpathogenic bacterial species is found colonizing olive leaves or closely associated with knots produced by *P. savastanoi* pv. *savastanoi*, and the sizes of their populations are often positively correlated (Ercolani, 1978; Marchi *et al*., 2006; Moretti *et al*., 2011; Ouzari *et al*., 2008; Quesada *et al*., 2007; Rojas *et al*., 2004). Several of these species can synthesize large amounts of indoleacetic acid (IAA), which may favour the 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 to be associated with *P. savastanoi* pv. *savastanoi* populations, its growth being 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 can apparently lead to either an increase in virulence or a decrease in pathogen populations (Hosni *et al*., 2011; Marchi *et al*., 2006). As described below (see 'Other virulence factors'), a recent study has demonstrated that both *Erwinia toletana* and *P. agglomerans* can form stable communities *in planta* (Hosni *et al*., 2011).

The literature is elusive with regard to the crop losses caused by olive knot, which greatly depend on the 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 can the size and quality of the fruits (Quesada *et al*., 2010a; Schroth *et al*., 1973). 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 (Quesada *et al*., 2010a, b; Young, 2004). Methods should aim to avoid the

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 the wounding of trees and by reducing epiphytic populations of the pathogen. Detection and diagnosis of the pathogen can be performed using diverse rapid and highly sensitive PCR methodologies (see Table S1, see Supporting Information), some of which allow the differentiation of pathovars *fraxini*, *nerii* and *savastanoi*. Pivotal to efficient disease management is a carefully planned and executed pruning, which should always start with healthy trees and be avoided in wet weather. Chemical control with copper compounds has been traditionally used in both nurseries and the field (Teviotdale and Krueger, 2004; Young, 2004). An extensive and systematic study (Quesada *et al*., 2010b) reported a significant reduction in 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 programme that includes the regular application of two copper treatments per year. This schedule produced the greatest difference 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 in disease symptoms.

Reduction of host susceptibility, including the use of resistant cultivars, is the 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 the 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

Pseudomonas savastanoi pathogenicity and virulence are generally tested on 1–3-year-old olive plants (Glass and Kosuge, 1988; Hosni *et al*., 2011; Iacobellis *et al*., 1994; Penyalver *et al*., 2006; Pérez-Martínez *et al*., 2007; Sisto *et al*., 2004). Apart from the space required, this often results in a large variability in the size and number of knots that develop. *In vitro* techniques have been widely used to study the pathogenicity and virulence of animal bacterial pathogens and can also be conveniently applied in plant pathology. Several techniques have been described for the micropropagation of a vast number of fruit trees, including several olive varieties, facilitating the 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 the pathogenicity and virulence of *P. savastanoi* strains isolated from olive and oleander knots (Rodríguez-Moreno *et al*., 2008). As observed previously 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 (Marchi *et al*., 2009; Rodríguez-Moreno *et al*., 2008, 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 the 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. In addition, scanning and transmission electron microscopy can be used for 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 the *in vivo* analysis of *P. savastanoi* pv. *savastanoi* NCPPB 3335 mutants affected in virulence (Bardaji *et al*., 2011; Pérez-Martínez *et al*., 2010).

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, has allowed the identification of the virulence gene complement of this tumour-inducing pathogen of woody hosts (Bardaji *et al*., 2011; Rodríguez-Palenzuela *et al*., 2010).

Phytohormones

In *P. savastanoi*, IAA is synthesized from tryptophan in two steps catalysed by the products of the genes *iaaM* (tryptophan monooxygenase) and *iaaH* (indoleacetamide hydrolase) (Comai and Kosuge, 1982; Palm *et al*., 1989). In addition, *P. savastanoi* pv. *nerii* (oleander isolates) also converts IAA to IAA-lysine through

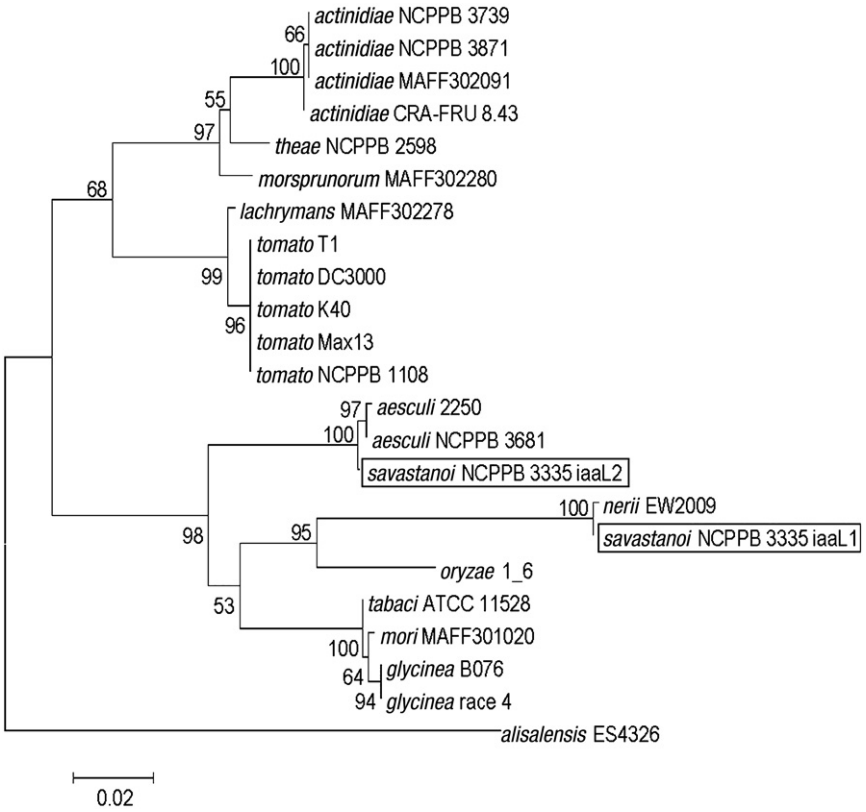

Fig. 2 Unrooted neighbour-joining (NJ) tree of *iaaL* nucleotide sequences from strains of the *Pseudomonas syringae* complex. (See Fig. S1 for methodology and Table S3 for accession numbers.) Only the pathovar name and strain designation are shown; all strains belong to *P. syringae*, except *P. cannabina*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

the action of the *iaaL* gene (Glass and 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-lysine has not been detected in culture filtrates of *P. savastanoi* strains isolated from olive (Evidente *et al*., 1986; Glass and 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 coding sequence (CDS).

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) provides evidence of 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, see Supporting Information) (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% amino acid identity), but high identity with putative monooxygenase and amidase genes common in the *P. syringae* complex (e.g. 99%/89% amino acid identity with PSA3335_4651/PSA3335_4172 from NCPPB 3335), and whose role in IAA biosynthesis has not been demonstrated. The limited data available also suggest the horizontal transfer of *iaaMH* within the *P. syringae* complex, which is 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; Pérez-Martínez *et al*., 2008; Silverstone *et al*., 1993). 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 (Bardaji *et al*., 2011; Iacobellis *et al*., 1994; Rodríguez-Moreno *et al*., 2008) 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, as 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).

Type III secretion system (T3SS) and effectors

Cluster analysis of HrpS protein sequences (Inoue and Takikawa, 2006) has shown 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 an *hrpA2* gene, which is highly similar to those of *P. syringae* pathovars *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) has allowed a prediction of *hop* genes, including 19 putative T3SS effectors with amino acid identities of 65%–80% to previously described effectors. In addition, 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.

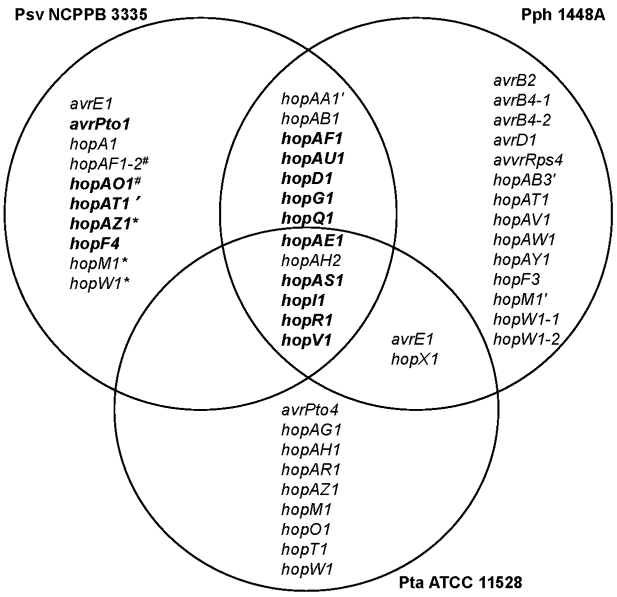

Fig. 3 Updated and corrected comparison of the type III effector gene complements of *Pseudomonas savastanoi* pv. *savastanoi* (Psv) NCPPB 3335 and other sequenced plant-pathogenic pseudomonads. Pph, *P. syringae* pv. *phaseolicola*; Pta, *P. syringae* pv. *tabaci*. Gene *hopAF1-2*, plasmid encoded in 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 bold type. #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).

Other virulence factors

The 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 consists of a *luxI* homologue (*pssI*) and a *luxR* homologue (*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 *E. toletana*, a nonpathogenic bacterium that is very often found to be associated with the olive knot pathogen (Hosni *et al*., 2011). *Erwinia toletana* produces the same *N*-acyl-homoserine lactone molecules as *P. savastanoi* pv. *savastanoi*; moreover, populations of *E. toletana* significantly decline over time after inoculation in olive tissues, but increase on co-inoculation with a strain of *P. savastanoi* pv. *savastanoi*. This relationship is mutualistic, because the populations of *P. savastanoi* pv. *savastanoi* also increase significantly when the pathogen is co-inoculated with *E. toletana*; in addition, the knot size also increases, 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 the sharing of 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, and 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 haemagglutinin (Rodríguez-Palenzuela *et al*., 2010). Genes for levansucrase, the enzyme responsible for the 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 and Stead, 1987). The relevance of all these putative virulence factors in *P. savastanoi* has not been reported to date.

METABOLIC VERSATILITY AND ADAPTATION TO WOODY HOSTS

Pseudomonas syringae pathovars are nutritionally specialized for growth in the plant environment relative to nonpathogenic pseudomonads (Rico *et al*., 2011). Biolog GN2 MicroPlate Technology (Bochner *et al*., 2001) has 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 nonpathogenic pseudomonads (Mithani *et al*., 2011; Rico and Preston, 2008) shows that the metabolic activities of *P. savastanoi* pv. *savastanoi* 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), despite the fact 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 as a result of host-specific features or pathogen evolutionary history.

The 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. Indeed, 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*

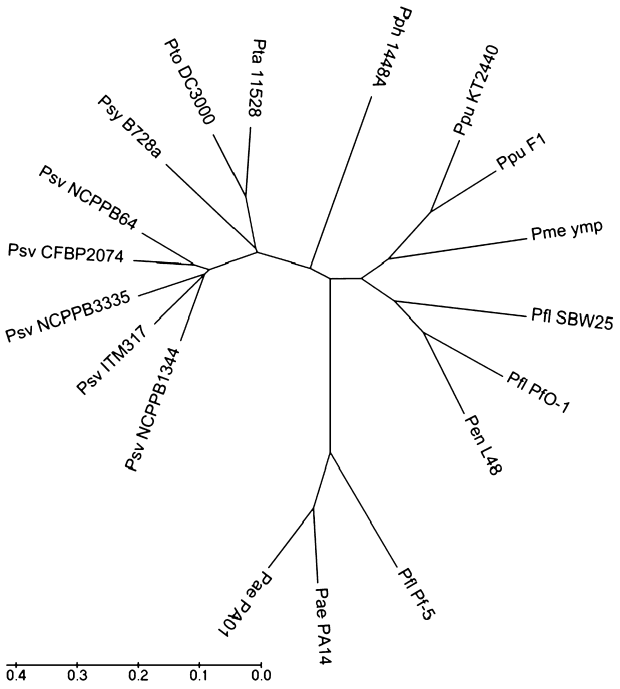

Fig. 4 Unrooted unweighted pair group method with arithmetic mean (UPGMA) tree based on nutrient utilization data of *Pseudomonas savastanoi* pv. *savastanoi* (Psv) and other pseudomonads. Metabolic activities of Psv strains, tested using Biolog GN2 plates, were compared with carbon utilization data reported for *P. syringae* strains and nonplant pathogenic species of *Pseudomonas* (Rico and 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 dendrogram. Distances were computed using the maximum composite likelihood method and are in units of the number of substrate utilizations per site. *P. syringae* pathovars: *syringae*, Psy; *tomato*, Pto; *tabaci*, Pta; *phaseolicola*, Pph. *Pseudomonas putida*, Ppu; *P. entomophila*, Pme; *P. fluorescens*, Pfl; *P. aeruginosa*, Pae.

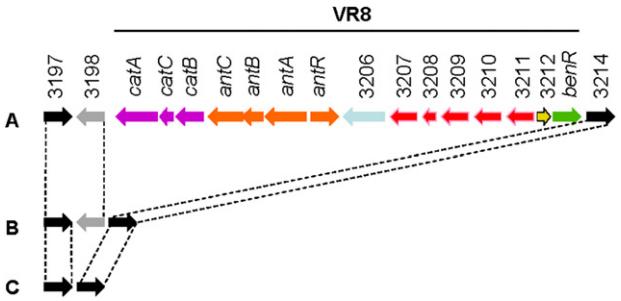

Fig. 5 Schematic map of variable region 8 (VR8) in the genomes of *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 and other sequenced *P. syringae* pathovars. (A) *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335, *P. syringae* pathovars *aesculi* strains 2250 and NCPPB 3681, *morsprunorum* MAFF302280 and *actinidiae* MAFF302091. (B) *Pseudomonas syringae* pathovars *tabaci* ATCC 11528, *mori* 301020, *phaseolicola* 1448A, *glycinea* race 4, *lachrymans* MAFF302278 and *japonica* MAFF301072. (C) *Pseudomonas 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*) and anthranilate (*antABC* and *antR*), respectively. PSA3335_3197, outer membrane protein; PSA3335_3198, ribosomal-protein-S5p-alanine acetyltransferase (*rimJ*); PSA3335_3206, aerotaxis receptor; PSA3335_3207, nitrilotriacetate 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.

pv. *savastanoi* NCPPB 3335 and all other strains encoding VR8, the genetic content and chromosomal location of this region are 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).

PLASMID GENETICS AND BIOLOGY

Plasmids are the main agents in the horizontal exchange of DNA amongst 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 and 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 method of strain tracking (Pérez-Martínez *et al*., 2007, 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 have been 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 *rulAB* genes for UV radiation tolerance. This could be significant because *rulAB* genes often appear to 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 and Kosuge, 1980). Unlike pathovar *nerii*, most strains of pathovar *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 CDSs; the majority (38 CDSs) have been annotated as hypothetical proteins, followed by 37 CDSs involved in DNA metabolism, including plasmid replication and maintenance. Each of the plasmids contain 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 an Hrp-box: pPsv48A contains a chimeric copy of gene *hopAF1*, included in the transposon effector IS*Psy30*, plus three copies of a large CDS found in many plant-associated proteobacteria, whereas pPsv48B contains gene *hopAO1* (*avrPphD2*). In addition, two genes putatively involved in CK biosynthesis, *ptz* (PSPSV_A0024) and *ipt* (PSPSV_C0024), are 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 (Jackson *et al*., 2011; Ma *et al*., 2007; Sundin, 2007). 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 participate 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; in addition, 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 because of the difficulties of genetic manipulation and 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 are related to the presence in these plasmids of genes for IAA and/or CK biosynthesis (Bardaji *et al*., 2011; Iacobellis *et al*., 1994; Rodríguez-Moreno *et al*., 2008; Silverstone *et al*., 1993). As 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 will 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 studies in recent years have made significant advances in the biology, ecology, genetics and genomics of *P. savastanoi* pv. *savastanoi*, which has 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 that will allow the 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

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

Fig. S1 Evolutionary relationships of *Pseudomonas savastanoi* pv. *savastanoi* and selected *P. syringae* pathovars. The tree was constructed by multilocus sequence analysis using a concatenated dataset (exactly 12 000 nucleotides) of *acnB*, *fruK*, *gapA*, *gltA*, *gyrB*, *pgi*, *recA* and *rpoD* genes. Phylogenetic groups 1, 2, 3 and 4 (Sarkar and 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 performed using MEGA5 (Tamura *et al.*, 2011); all positions containing gaps and missing data were eliminated using the option of complete deletion. Bootstrap values (1000 repetitions) are shown on the 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[a].

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

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