Host-specific signal perception by PsaR2 LuxR solo induces *Pseudomonas syringae* pv. *actinidiae* virulence traits

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ABSTRACT

Plant-associated bacteria, including pathogens, recognise host-derived signals to activate specific responses. The genome of *Pseudomonas syringae* pv. *actinidiae* (Psa), the aetiological agent of bacterial canker of kiwifruit, encodes for three putative LuxR-like receptors. Proteins of this family are usually involved in the quorum sensing system, through the perception of autoinducers (AHLs) produced by a cognate LuxI. However, Psa does not produce AHLs according to the lack of LuxI-encoding gene. It has been proposed that the so-called LuxR solos may be involved in the perception of environmental stimuli. We thus hypothesised that Psa LuxR-like receptors could be involved in host-derived signal sensing.

Psa virulence traits, i.e., biofilm formation, motility and endophytic colonisation, were stimulated by growing the pathogen in host plant extracts, but not in non-host plant extracts or rich medium. Moreover, the phenotypic analyses of Psa mutant strains lacking the LuxR solo-encoding genes, demonstrated that PsaR2 plays a major role in host recognition and induction of virulence responses. The heterologous expression of PsaR2, followed by affinity chromatography and fraction activity assessment, confirmed the specific recognition of plant-derived components by this sensor. Overall, these data provide a deeper understanding of the regulation of Psa virulence through interkingdom communication, which represents a interesting target for the development of tolerant/resistant genotypes or innovative control strategies.

1. Introduction

The *Pseudomonas syringae* species complex is a highly diversified group of bacteria, including well-known plant pathogens. To date, over 50 pathological variants (pathovars) (Nowell et al., 2016), carrying a diverse array of effectors and virulence factors, have been described (McCann et al., 2008). Bacterial pathogens are more virulent when freshly isolated from their hosts, or when grown in apoplast-mimicking conditions, rather than after being cultured in a rich medium, suggesting that the perception of the apoplast environment and/or plant-derived factor(s) are required for the full expression of their pathogenic arsenal. Bacteria communicate both intra- and inter-specifically, which allows them to regulate their metabolism according to the environmental conditions, nutrient availability and crowding conditions

(*quorum sensing*, QS) (Checcucci and Marchetti, 2020). The canonical system based on N-acyl homoserine lactones (AHLs), found in many Gram-negative bacterial species, is one of the best-studied and understood QS systems. It consists of a LuxI-LuxR protein pair, with the LuxI being responsible for AHL biosynthesis and the LuxR being the sensor/transcriptional regulator, which perceives AHLs and regulates downstream target genes (Venturi and Keel, 2016). Besides the regulation of bacterial behaviour, this system is also involved in 'interkingdom signalling', since bacterial AHLs can also influence plant immunity, growth and development (Bauer and Mathesius, 2004; González and Venturi, 2013). From their side, plants can produce AHL-mimicking compounds, acting as agonists or antagonists of bacterial QS systems (Palmer and Blackwell, 2008; LaSarre and Federle, 2013).

Pseudomonas syringae pv. *actinidiae* (Psa), the causal agent of

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bacterial canker of kiwifruit, has been one of the main phytosanitary concerns in kiwifruit growing areas since the 2008 pandemic outbreak (Donati et al., 2014; Vanneste, 2017). The molecular characterisation of the Psa genome revealed that this pathovar is closely related to the *P. syringae* pathovars *avellanae* and *theae* (pathogens of hazelnut and tea plant, respectively) and it is further classified into five lineages, named biovar 1, 2, 3, 5 and 6 (Vanneste et al., 2013; Fujikawa and Sawada, 2016; Sawada et al., 2016), which differ in their genomic and biochemical properties, toxin production and virulence induction (Vandelle et al., 2021). While all of them can cause the bacterial canker disease, biovar 3 is recognised as the most virulent lineage, responsible for the recent worldwide disease outbreak (Ferrante and Scortichini, 2010; Vanneste et al., 2010).

Psa can infect both *Actinidia chinensis* var. *chinensis* and *Actinidia chinensis* var. *deliciosa* (henceforth *A. chinensis* and *A. deliciosa*) (Donati et al., 2014), penetrating through wounds, flowers or natural openings, such as stomata or lenticels (Spinelli et al., 2011; Donati et al., 2018). Once inside the apoplast, it is able to move into the plant vascular system, spreading systemically, and it can rapidly cause plant death (Spinelli et al., 2011; Donati et al., 2020). Typical symptoms include leaf spots, cankers, twig dieback and flower blight (Scortichini, 1994; Chapman et al., 2012).

Effective control options for Psa are limited and mostly rely on the use of copper-based pesticides and resistance inducers (Vanneste et al., 2011), which however present several drawbacks. For instance, the development or selection of copper resistance traits in Psa has been observed (Nakajima et al., 2002; Marcelletti et al., 2011; Colombi et al., 2017). Secondly, the use of plant resistance inducers, such as acibenzolar-S-methyl, may limit the development and severity of bacterial canker by stimulating hormonal signalling and activating defence responses (Cellini et al., 2014; Michelotti et al., 2018), but it is not likely to achieve a complete protection against the pathogen, particularly when the pathogen has already become established in the orchard. Finally, a complete genetic resistance to Psa has not been found so far in any *Actinidia chinensis* genotype, while other *Actinidia* species (such as *A. arguta*) are considered tolerant, i.e., they can host a resident Psa population without developing the disease (Tahir et al., 2019).

The implementation of more effective control measures against Psa may, therefore, take advantage from a deeper understanding of the molecular mechanisms, signal molecules and target genes involved in host-plant cross-talk between this pathogen and kiwifruit plants. Psa does not produce AHLs and does not contain a complete canonical AHL-based QS system, but its genome contains the genes for three LuxR-like receptors (LuxR solos), designated PsaR1, PsaR2, PsaR3 (Patel et al., 2014). Two of them (namely PsaR1 and PsaR3) seem to respond to exogenous AHLs and may thus be involved in inter-species microbial communication (Cellini et al., 2020). By contrast, PsaR2 belongs to a sub-family of LuxR solos observed only in plant-associated bacteria, likely involved in the perception of plant low-molecular weight signals instead of AHLs, thus potentially playing a major role in various plant-bacteria interactions such as virulence, biocontrol, and symbiosis (Patel et al., 2013, 2014).

To clarify the molecular mechanisms and the signals involved in virulence induction in Psa, this study focused on the expression of phenotypes related to virulence, i.e. biofilm formation, motility and host colonisation (Cellini et al., 2020) in response to plant extracts, and the regulation of the genes responsible for these phenotypes. The level of host specificity and the conservation of recognition mechanisms across plant species was examined using the susceptible host plants *A. chinensis* and *A. deliciosa*, the tolerant one *A. arguta*, the host plants of closely related pathovars *Camellia sinensis* (tea plant), and *Corylus avellana* (hazelnut), and the non-host plant *Nicotiana tabacum*. Moreover, the involvement of Psa LuxR solos in the regulation of the phenotypic traits related to virulence was assessed using knock-out mutants. Finally, the putative plant signal receptor PsaR2 was produced as a recombinant protein to demonstrate its ability to bind factor(s) from the host plant

extracts eliciting host-induced responses in Psa.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Pseudomonas syringae pv. *actinidiae* (Psa) strains CFBP7286 and CRAFRU10.22, and the mutant strains carrying a deletion in the *psaR1, 2, 3* genes (Psa-mR1, Psa-mR2, Psa-mR3, respectively; Patel et al., 2014) were routinely grown in Lysogeny Broth (LB) medium at 27 °C under moderate shaking (120 rpm). Cell cultures used for phenotypic tests were grown in LB or a medium made of plant extracts/xylem saps (see below). The bacterial concentration was estimated by monitoring the optical density of the cultures. Optical density at 600 nm (OD_{600}) was spectrophotometrically measured and correlated to bacterial population, enumerated by plating tenfold serial dilutions of the same suspension. Colonies were counted after two days of incubation at 27 °C.

2.2. Collection of xylem sap and plant extracts for in vitro bioassays

Plant extracts were obtained from in vitro microcuttings of *A. deliciosa* (cv. Hayward) and *A. chinensis* (cv. Hort16A), and from fully expanded leaves of 1-year-old plants of *A. arguta* cv. Jumbo, *C. sinensis*, *C. avellana* and *N. tabacum*. Briefly, 2–3 g of green plant tissue were grinded at 4 °C with mortar and pestle in 100 mL phosphate buffered saline (PBS, 10 mM, pH 7.2) containing 1 mM ascorbic acid and 1% (w/v) polyvinylpolypyrrolidone (Sigma-Aldrich, St. Louis, MO, USA). The homogenate was filtered through a clean gauze and centrifuged (12,000 rpm, 20 min, 4 °C). The supernatant was filter-sterilised through a 0.22 μm pore membrane (Millipore, Billerica, MA, USA), maintained at 4 °C, and used within 24 h. Xylem sap was harvested as previously described (Nardozza et al., 2015) from *A. deliciosa* (cv. Hayward) and *A. chinensis* (cv. Hort16A) plants in orchards at the bleeding sap stage.

2.3. Phenotypic characteristics linked with virulence

Virulence traits (motility, biofilm production and endophytic colonisation) were analysed as previously described (Cellini et al., 2020). Briefly, motility was assessed with Psa suspensions at a density of approx. 10^7 CFU mL^{-1}; media not allowing a sufficient growth of Psa were directly inoculated with a bacterial suspension at 10^7 CFU mL^{-1}. Each treatment was replicated on 18–40 plates. Biofilm formation was evaluated after three days of growth in the indicated plant extract or xylem sap. Experiments were performed in biological triplicates, each including 6 technical repeats. For endophytic colonisation, Psa was grown for two days in plant extracts obtained from *A. deliciosa*, *A. chinensis*, *A. arguta*, *C. sinensis*, *C. avellana* and *N. tabacum*. Subsequently, the bacteria were precipitated by centrifugation and resuspended in 10 mM $MgSO_4$ to a density of approx. 10^7 CFU mL^{-1}. Five samples per treatment, consisting of micropropagated *A. deliciosa* cv. Hayward (unless stated otherwise) plants, were dip-inoculated with the bacterial suspension, and endophytic Psa population was measured 5 days later. After growing Psa on LB or *A. deliciosa*/*A. chinensis* extracts, mortality rates were determined on *A. deliciosa* and *A. chinensis* microcuttings (n = 20) 10 days post-inoculation. Plant death was defined as extensive browning, affecting plant stems and apical meristems.

2.4. Gene expression analysis

The expression of genes involved in biofilm formation, bacterial motility, virulence signalling and quorum sensing was analysed in bacteria grown in triplicates in LB or plant extracts. The genes (Table 1) were selected based on their function, previously determined in Psa or related bacteria. Effects of bacterial density were considered by performing the experiments with *A. deliciosa* and *A. chinensis* plant extract or xylem saps with bacterial cultures in the exponential growth phase

Table 1

Genes selected from several bacterial species and their relative function. Gene identity refers to the *Pseudomonas syringae* pv. *actinidiae* sequence homology to the corresponding gene of the reference organism.

	Gene	Identity	Organism	Function (s)	Reference
Biofilm	*algD*	99.3	*Pseudomonas syringae* pv. *tomato*	Synthesis of alginate	Penaloza-Vazquez et al. (2010)
	wspR	85.2	*Pseudomonas fluorescens*	Diguanylate cyclase, involved in the regulation of colony morphology and cellular aggregation	D'Argenio et al. (2002);Ude et al. (2006); Wolska et al. (2016)
	wssB	68.3	*Pseudomonas fluorescens*	Cellulose synthase, catalytic subunit	Spiers et al. (2013)
	mdoH	99.1	*Pseudomonas syringae* pv. *tomato*	Production of exopolysaccharide (membrane-derived oligosaccharides)	Penaloza-Vazquez et al. (2010)
Motility	*fliP*	81.9	*Pseudomonas putida*	Flagellar assembly	Segura et al. (2001);Ward et al. (2018)
	pilA	92.4	*Pseudomonas syringae* pv. *tabaci*	Pilum structural protein (pilin precursor)	De Souza et al. (2004);Hospenthal et al. (2017)
	pilC	90.4	*Pseudomonas syringae* pv. *tomato*	Pilum assembly/motor complex	De Souza et al. (2004);Hospenthal et al. (2017)
	pilO	74.9	*Pseudomonas aeruginosa*	Pilum assembly/structural protein (basal body)	Martin et al. (1995);Hospenthal et al. (2017)
Virulence effectors	*avrPto1*	–	*Pseudomonas syringae* pv. *actindiae*	Inhibition of plant defence signalling (interference with receptor kinase)	Lin et al. (2006);Marcelletti et al. (2011)
	hopD1	–	*Pseudomonas syringae* pv. *actindiae*	Inhibition of plant defence signalling (interaction with transcritprion factors)	Marcelletti et al. (2011);McCann et al. (2013); Block et al. (2014)
	hopQ1	–	*Pseudomonas syringae* pv. *actindiae*	Interaction with 14–3–3 proteins	Marcelletti et al. (2011);McCann et al. (2013); Li et al. (2013)
	hopR1	–	*Pseudomonas syringae* pv. *actindiae*	Not known	Marcelletti et al. (2011);McCann et al. (2013)
	hopS2	–	*Pseudomonas syringae* pv. *actindiae*	Not known (suppression of hypersensitive response)	Marcelletti et al. (2011);McCann et al. (2013)
	hopZ5	–	*Pseudomonas syringae* pv. *actindiae*	Putative acetyltransferase; typical of pandemic Psa strains	McCann et al. (2013);Jayaraman et al. (2017)
	virB4	–	*Pseudomonas syringae* pv. *actindiae*	ATPase, required for type 4 secretion system	McCann et al. (2013);Sgro et al. (2019)
QS, signalling and regulation	*gacA*	99.1	*Pseudomonas syringae* pv. *syringae*	Under GacS activation, regulates QS, motility, virulence, stress resistance, biofilm formation	Heeb and Haas (2001)
	gacS	100	*Pseudomonas syringae* pv. *syringae*	Membrane-bound sensor kinase, activates GacA	Heeb and Haas (2001);Pernestig et al. (2001)
	clpP	87.6	*Pseudomonas fluorescens*	Protease involved in biofilm regulation, stress resistance, virulence and regulation	O'Toole and Kolter (2008)
	psaR1	–	*Pseudomonas syringae* pv. *actindiae*	LuxR-like receptor, involved in inter-species signalling	Patel et al. (2014);Cellini et al. (2020)
	psaR2	–	*Pseudomonas syringae* pv. *actindiae*	LuxR-like receptor, predicted to bind plant-derived compounds	Patel et al. (2014)
	psaR3	–	*Pseudomonas syringae* pv. *actindiae*	LuxR-like receptor, involved in inter-species signalling	Patel et al. (2014);Cellini et al. (2020)
	rpoN	60.0	*Vibrio alginolyticus*	Alternative sigma factor, involved in signalling, biofilm and adhesion	Sheng et al. (2012)
	rpoS	69.4	*Vibrio alginolyticus*	Alternative sigma factor, involved in QS and virulence regulation	Tian et al. (2008)
	lysR	93.5	*Pseudomonas syringae* pv. *tabaci*	Transcriptional regulator	Maddocks and Oyston (2008)

(10^5 CFU mL^{-1}) or in the early stationary phase (10^7 CFU mL^{-1}). Primer design and qPCR analyses were performed as described previously (Cellini et al., 2020).

2.5. Hierarchical clustering of gene expression data

Normalised expression values from qPCR experiments were imported as data matrices into MeV (Howe et al., 2011). The data were adjusted as median centre genes/rows and clustered using the hierarchical clustering module. Gene and/or sample trees were clustered with optimised gene and sample leaf orders using Pearson correlation and average linkage clustering. The trees were subsequently cut into clusters using a distance threshold (0.5–1) empirically adjusted to highlight the most relevant features of the trees.

2.6. Cloning of psaR2, protein purification and plant extract fractioning

The gene *psaR2* was amplified with high fidelity Pfu DNA Polymerase (Promega, Madison, WI, USA), using primers (Table 2) designed for specific digestion sites at 5' and 3' in order to insert the amplicon in the expression plasmid pH6HTN His6HaloTagT7 vector (Promega). The amplicons and purified plasmid were digested with *Sac*I and *Xba*I (New England Biolabs, Ipswich, USA) following manufacturer's protocol, and ligated with Quick Ligation Kit (New England Biolabs) in a insert:vector ratio of 1:6 in a total volume of 15 µL. Two µL of the ligation reaction were used to transform electrocompetent *E. coli* strain DH5α (Green and Sambrook, 2012) using a 2-mm cuvette (Gene Pulser Cuvette, Bio-Rad, Hercules, CA, USA) with the following settings: 25 µF, 2.5 KV, 200 Ω. Transformant cells were plated and selected on LB supplemented with ampicillin (100 µg mL^{-1}). The growing colonies were checked for presence of *psaR2* using the same primers and PCR settings used for the cloning. The plasmid was subsequently purified using QIAprep miniprep kit (Qiagen, Hilden, Germany) and used to transform electrocompetent *E. coli* strain BL21, as previously described.

A freshly grown, single colony of *E. coli* strain BL21 harbouring the expression vector was cultured in 10 mL LB medium (37 °C, 150 rpm) to OD$_{600}$ = 0.5–0.6. Then, the culture was centrifuged for 10 min at 10,000 × *g* at room temperature and the pellet was resuspended in the same volume of LB containing 1% IPTG. After 5 h at 37 °C and shaking at 150 rpm, the cells were precipitated (10,000 × *g*, 15 min at 4 °C),

Table 2
Primers used in this work for qPCR analysis and *psaR2* cloning. Restriction sites are underlined.

	Gene	Forward primer	Reverse primer
Biofilm			
	algD	GACCTGGAACTGGACTACATC	TGCTGCGAACCACGATAG
	wspR	ACGACTATCTGGTCAAACTG	ATAGGCTTCATCACGCTG
	wssB	CGCTGGTGATGATGATGGT	CTGACGCTCAACGCTGTG
	mdoH	ACGGTAACCTTGAACTTGC	CACCATCGTTCTGCTGTT
Motility			
	fliP	TCAAGACGGCGTTTCAGA	CGGCGAGAGCATCATCAT
	pilA	GCCATTCCTTCCTATCAA	GTAAGACCATTGCTCCAG
	pilC	CGCTGGACATCGCATTCT	GCACCTTCGGCAATGATG
	pilO	CCTACAGAAAGCAGATGGA	GTGATGTCTTCAAGCAGTC
Virulence			
	avrPto1	GGAGCGAATCTTGCCATT	GGAGCGATATGCGTGAAG
	hopD1	CAGTAGACAGCAGTAGCC	CGGGTTATCGGAAACAAG
	hopQ1	GGCATTCCACTTCGTATAG	CAACGCACTCTTCTGAAC
	hopR1	GACATAACTGCCGATGCT	TCCAGATAGGCTCGATCA
	hopS2	CCTTAAACGGCTGGCAGAG	CGAAGTGATGCTTGAGGTGAA
	hopZ5	TCAGGCTACAATACTTACGCATCA	CAGGAATAGAACGGAACTCAGGAT
	virB4	TTTGAAGACACCACTGTTTC	CTGCGTCACCTACTACTC
Signalling			
	gacA	GATGACCATGACCTTGTTC	TCTTCAGCGATTCCTCAC
	gacS	AGAACCTGGAAACCATCG	ATCTCGTGGCTCATGTTG
	clpP	CTTATATTCAGCAGAACTCT	GCGAATAGATGTCATAGG
	rpoN	GCACCGACTCCTGATTGA	GAATCCACAGAAGCCGAATAC
	rpoS	CGTCGCTCAAACAACACAAAT	GAGACAGCAGAGGGGAAAAC
	psaR1	ATACCTGGTCAGTAGTCTCA	GCAGCACTTCAAGTTCAC
	psaR2	ACTGTTTGACCAGAAGATG	CTGAACGGTTGAGTTGAT
	psaR3	GGTTCGCTCATTATCTGAT	GCAATGCTTGAGGATAGG
	lysR	TGCGGAAGTTGAAGCGGATTACG	ACCGAAATGTTGCTGCCTCCC
Reference genes			
	rpoD	CCGAGATCAAGGACATCAAC	GAGATCACCAGACGCAAGTT
	recA	CGCACTTGATCCTGAATACG	CATGTCGGTGATTTCCAGTG
psaR2 *cloning*			
		AATTCCGAGCTCATGCATATCAGGTTGTCGG	CTAGTCTAGATCAGTGGTCG AGTAAACGGT

resuspended in 1 mL homogenisation buffer (10 mM Tris-HCl, pH 7.5, 1 mM PMSF) and transferred into a 50 mL tube. For cell lysing, fresh lysozyme (1 mg mL^{-1} final concentration; Sigma-Aldrich) was added to the cell suspension, incubated at 37 °C for 30 min under gentle shaking (70 rpm). To complete cell lysis, the sample was frozen in liquid N$_2$ and subsequently thawed at 37 °C for 20 min. DNase (0.1 mg mL^{-1}; Sigma-Aldrich) was added to remove contaminant DNA. After 30 min shaking at room temperature and 150 rpm, the suspension was centrifuged at 10,000 × g at room temperature. Protein purification was performed by His-tag-based affinity chromatography applying cell lysate on a Ni-NTA resin (Qiagen) column pre-equilibrated with homogenisation buffer.

The purification of the recombinant protein was confirmed by ELISA (Supplementary Fig. S1), using mouse monoclonal anti-His$_6$ primary antibodies (1:1000 in PBS plus 20 g L^{-1} skim milk) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibodies (1:800 in PBS plus 20 g L^{-1} skim milk) in presence of BM Chemiluminescence ELISA Substrate for peroxidase (Roche Diagnostics GmbH, Mannheim, D).

Purified PsaR2 protein was linked to HaloTag resin (Promega) following manufacturer's protocol, and plant crude extract was fractionated by affinity chromatography. Column flow-through (i.e., compounds not bound to PsaR2) and wash (i.e., loosely bound compounds) were collected separately. The PsaR2-bound fraction was eluted with PBS containing 1 M NaCl.

One mL of raw extract, or the corresponding volume of each PsaR2 affinity fraction, were independently added to Psa cultures in 15 mL LB for gene expression analysis.

2.7. hrpA1 promoter activity measurement

The activation of *hrpA1* promoter was monitored in wild-type CRA-FRU10.22 and Psa-mR2 mutant strains transformed with the reporter plasmid *hrpA1*::*gfp*, according to previous works (Rico and Preston, 2008; Vandelle et al., 2017). Bacterial cells, resuspended in *hrp*-inducing medium (HIM) at OD$_{600}$ of 0.1, were treated with raw kiwifruit leaf extract or the fractions from PsaR2-affinity experiment (1% final concentration). Fluorescence intensity was measured for 4 h using the fluorometer plate reader Tecan Infinite M Plex (Tecan Trading AG, Switzerland).

2.8. Statistical analysis

The significance of differences in gene expression and biofilm data was calculated by ANOVA followed by Tukey's test. Bacterial populations were log-transformed before statistical analysis. Marascuilo's procedure was applied to motility percentage data. Statistically significant differences were assumed for $p \leq 0.05$. The software STATISTICA 7.0 (StatSoft Inc., Tulsa, OK, USA) was used for statistical elaboration.

3. Results

3.1. Psa growth kinetics in plant extracts

The growth of Psa in *A. deliciosa* extract and xylem sap was relatively slow in the first 72 h, reaching a population density 1-log lower (approx. 10^7 CFU mL^{-1}) than when grown in LB medium (Fig. 1A). Similar values were observed for Psa grown in *A. chinensis* extract and xylem sap (data not shown). On the other hand, Psa population density did not significantly increase when grown in extracts of non-host plants (*C. avellana*, *C. sinensis*, *N. tabacum*) or tolerant host plant (*A. arguta*), reaching a maximum of 10^4 CFU mL^{-1} after 96 h (Fig. 1B).
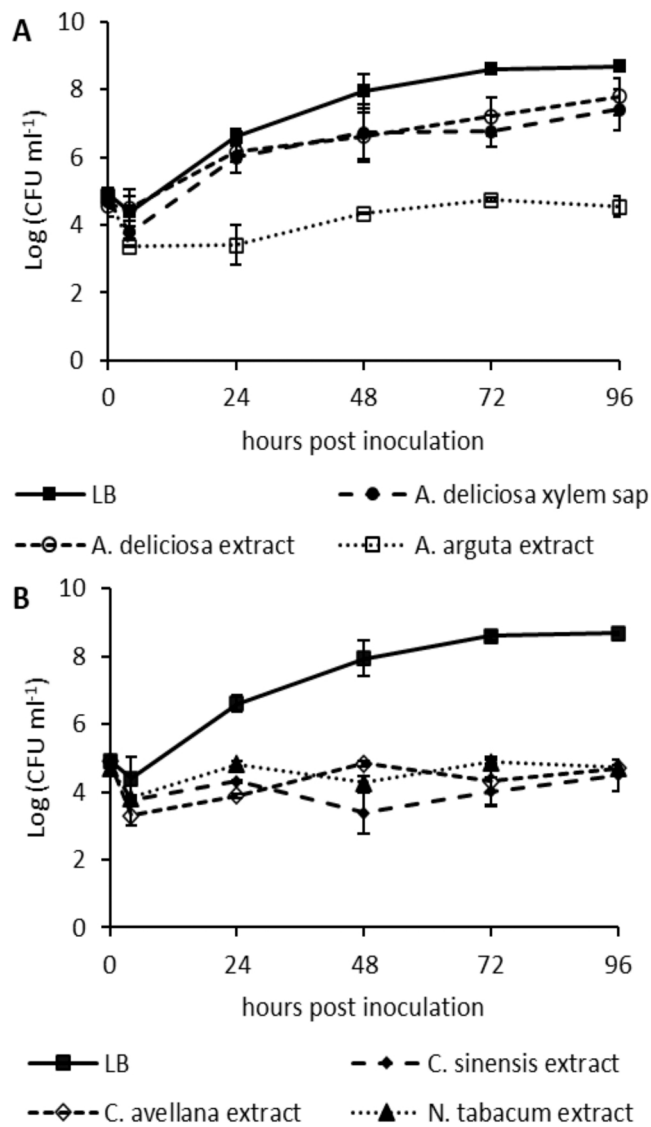
Fig. 1. Growth kinetics of *Pseudomonas syringae* pv. *actinidiae* in media obtained from (A) the susceptible host, *Actinidia deliciosa* (plant extract and xylem sap) or the tolerant host, *A. arguta*, and (B) from non-host plants *Camellia sinensis*, *Corylus avellana* and *Nicotiana tabacum*. The error bar represents the SEM calculated from three independent experiments.

Fig. 2. Expression of virulence-related phenotypes of *Pseudomonas syringae* pv. *actinidiae* grown in media derived from susceptible hosts (*Actinida deliciosa*, *A. chinensis*), tolerant host (*A. arguta*) and non-host plants (*Camellia sinensis*, *Corylus avellana* and *Nicotiana tabacum*). (A) Percentage of colonies showing swarming motility (positive observations over total replicates are indicated over each bar). (B) Estimation of biofilm production. (C) Endophytic population in *A. deliciosa* microcuttings 5 days post-inoculation. Different letters indicate significant differences among treatments according to Marascuilo's procedure (A) or ANOVA followed by Tukey's test (B and C).

3.2. Influence of plant extracts on Psa motility, biofilm formation and in planta growth

The extracts from *A. deliciosa* and *A. chinensis*, but not those obtained from non-host or tolerant host plants, stimulated Psa swarming and biofilm formation in vitro (Fig. 2A-B). Moreover, the colonisation of *A. deliciosa* plants was enhanced when Psa had been incubated in *A. deliciosa* or *A. chinensis* extract prior to inoculation (Fig. 2 C).

The host specificity of Psa virulence induction was further evaluated on micropropagated plants. The relative virulence-priming effect of *A. deliciosa* and *A. chinensis* extracts was determined by inoculating *A. deliciosa* or *A. chinensis* plants with Psa previously incubated in either plant extract or LB as a negative control. Both plant extracts were more effective than LB in stimulating endophytic plant colonisation by Psa (Fig. 3), although Psa grown in *A. deliciosa* extract and inoculated in the same plant variety grew to a significantly higher level compared to the other inoculum/host combinations (Fig. 3A). Similarly, plant mortality 10 days after inoculation was highest when Psa had been incubated in *A. deliciosa* extracts prior to plant inoculation (Fig. 3B).
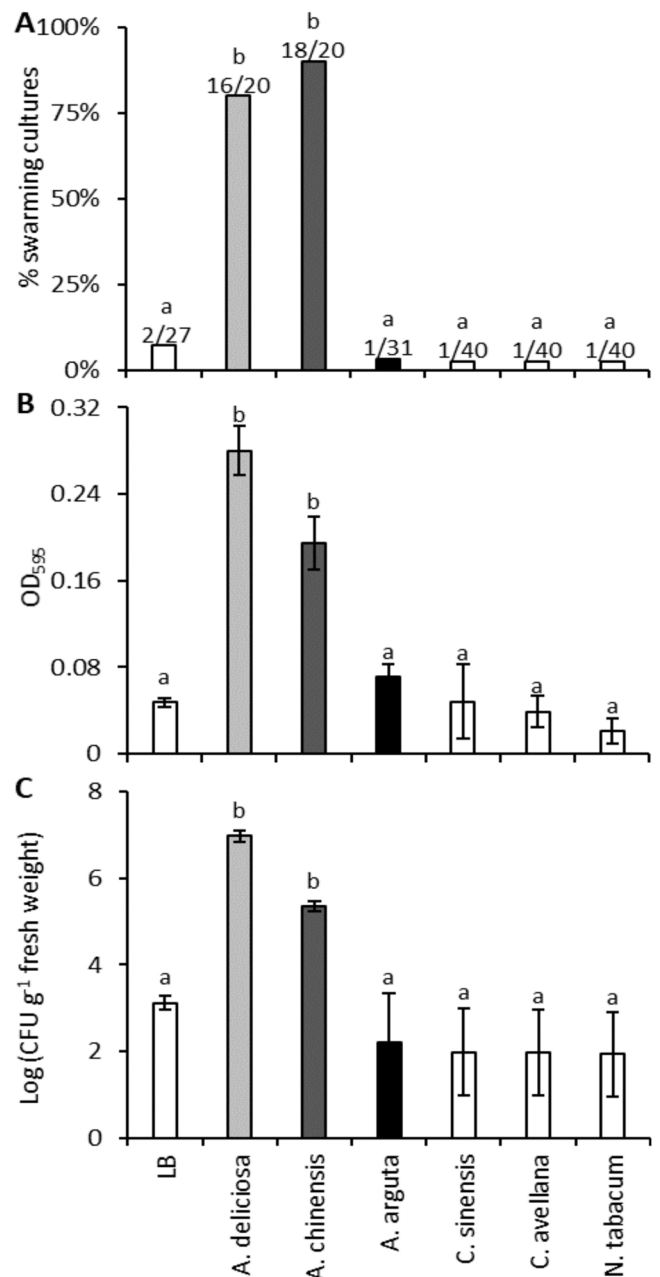
3.3. Psa gene expression profiles in response to plant extracts

To discriminate between the effects of the growing media and bacterial density, bacteria were grown in LB or host plant extracts to a density of 10^5 or 10^7 CFU mL^{-1}, prior to analyse the expression of genes involved in different virulence-related phenotypes (Table 1). Most of the genes related to biofilm formation, virulence effectors and signalling/ regulatory pathways were up-regulated in Psa grown in plant extracts, with a major effect observed at high bacterial density (Fig. 4 and Supplementary Fig. S2). By contrast, motility-related gene expression was
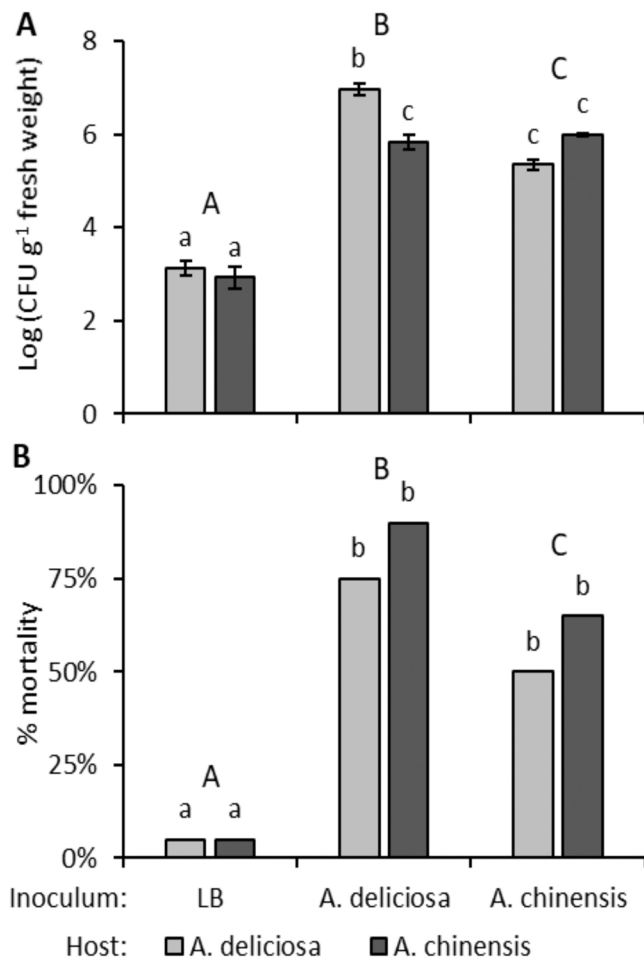
Fig. 3. Endophytic population of *Pseudomonas syringae* pv. *actinidiae* 5 days post inoculation (A) and mortality 10 days post inoculation (B) of host micro-cuttings (*Actinidia deliciosa*, *A. chinensis*) inoculated with bacteria grown in LB, *A. deliciosa*- or *A. chinensis*-derived extract. Significant differences, calculated by two-way ANOVA followed by Tukey's test (A) or by Marascuilo's procedure (B), are indicated by lower-case (for host × inoculum interaction) or upper-case (for inoculum alone) letters.

higher at low bacterial concentration. Only one gene (*clpP*) was regulated only by cell density but not by growing medium. Moreover, among LuxR solos-encoding genes, only the expression of *psaR2* was significantly promoted by plant extracts.

A subset of the selected genes was tested with non-host (*C. sinensis*, *C. avellana*, *N. tabacum*) or tolerant host (*A. arguta*) plant extracts. Even considering the low Psa density reached in such extracts (Fig. 1B), virulence-related gene expression was overall only slightly affected compared to cultures in susceptible host extracts at a similarly low density. Only *fliP*, *hopZ5*, *psaR2* genes (and marginally *algD* and *wspR*) were up-regulated in the *A. arguta* extract compared to the low-density LB cultures (Fig. 5 and Supplementary Fig. S2).

3.4. Psa responsiveness to xylem saps

To confirm the reliability of the data obtained with Psa grown in plant extracts, the same set of experiments including Psa swarming motility, biofilm formation, *in planta* growth and transcriptional regulation was repeated with Psa cultured in raw *Actinidia* xylem saps obtained from orchard conditions (Fig. 6). Similarly to plant extracts from the same plant varieties, *A. deliciosa* and *A. chinensis* xylem saps stimulated swarming motility, biofilm formation and *in planta* growth (Fig. 6A-C). However, virulence-related genes were up-regulated to a
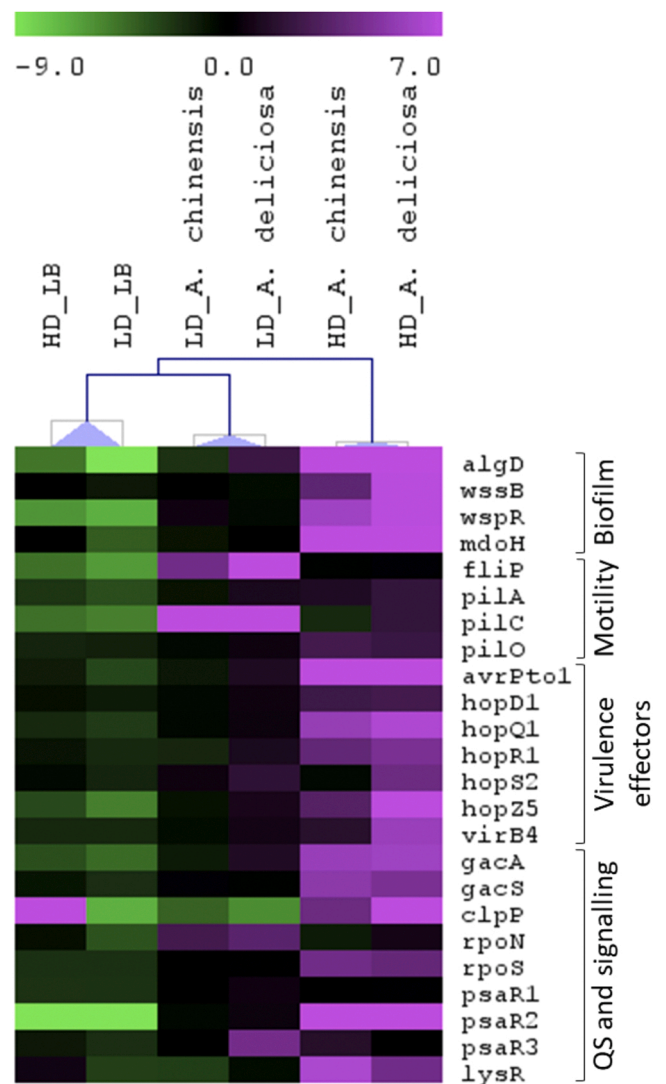

Fig. 4. Effects of bacterial density and host-derived extracts on the expression of genes related to biofilm formation, bacterial motility, virulence factors and signalling in *Pseudomonas syringae* pv. *actinidiae*. The experiment was performed at bacterial densities of 10^5 (low density, LD) or 10^7 (high density, HD) CFU mL^{-1}. The hierarchical clustering of the relative expression was performed using the average values of three biological replicates.

lower extent (Fig. 6D and Supplementary Fig. S2) compared to the induction observed in the corresponding fresh plant extracts. Moreover, *psaR2* gene expression, although significantly increased at a bacterial density of 10^7 CFU mL^{-1}, was barely detectable at 10^5 CFU mL^{-1}.

3.5. Phenotypic characterisation of psaR1, psaR2 and psaR3 knock-out mutants

In order to establish the role of Psa LuxR-like sensors in plant-microbe cross-talk, biofilm formation, swarming motility and *in planta* growth were evaluated in Psa strains carrying a knock-out mutation in *psaR1*, *psaR2* or *psaR3* genes (Psa-mR1, Psa-mR2 and Psa-mR3, respectively) cultured in *A. deliciosa* plant extract.

Psa-mR2 and Psa-mR3 mutants showed a strong reduction of biofilm formation and swarming motility compared to the wild-type strain (Fig. 7 A, B). In contrast, Psa-mR1 mutant was able to form biofilm and swarm to the same extent as the wild-type. The three mutants were all impaired in the colonisation of plant microcuttings, with endophytic populations, measured 5 days after inoculation, reduced from 2 (Psa-
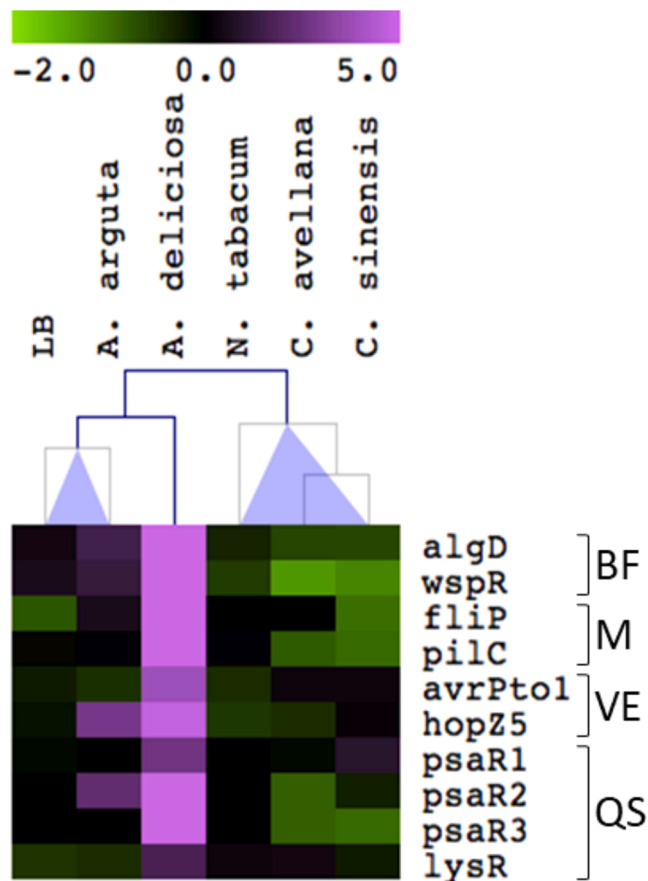
and Supplementary Fig. S2). In addition, the fraction bound to PsaR2 strongly induced the activity of the *hrpA1* promoter in apoplast-like conditions. The *hprA1* promoter was more than twice induced in presence of the PsaR2-bound fraction than when in prensece of the raw extract, suggesting that the signal molecule(s) may have been concentrated during extract fractionation (Fig. 8 C).

4. Discussion

4.1. Specificity of host-induced phenotypes

The endophytic, disease-inducing lifestyle of bacterial pathogens is a specialised survival strategy, involving the activation of their virulence arsenal upon host recognition, and thus it is not limited to the mere ability to grow in host tissues. Specific host plant molecules (Yan et al., 2019) as well as host-derived media (Zaini et al., 2009; Shi et al., 2013; Duge de Bernonville et al., 2014) can induce virulence-related phenotypes, including swarming motility and biofilm formation.

Psa was able to grow in extracts or xylem saps derived from susceptible hosts, and higher endophytic bacterial populations were observed in *A. deliciosa* plants infected with Psa previously grown in host extracts, although endophytic population did not appear strictly correlated with plant death rate. Indeed, the highest Psa density was observed in infected *A. deliciosa* plants, while the death rate was higher in *A. chinensis* plants. It may be assumed that *A. deliciosa* plants are able to induce a stronger defence response, leading to weaker cell damages and death, and, possibly, that Psa displays a stronger virulence induction by extracts obtained from *A. deliciosa*.

High population densities induce Psa virulence-related phenotypes and improve host colonisation by Psa (Cellini et al., 2020). Thus, since Psa populations reached only approximately 10^4 CFU mL, when grown in extracts obtained from the tolerant host (*A. arguta*) or non-host plants, the inability of Psa to stimulate motility and biofilm production in these conditions may be due to the lower Psa growth (Fig. 1). However, this hypothesis can be ruled out, since susceptible host extracts induce the up-regulation of virulence-associated genes in Psa grown only up to low density (10^5 CFU mL^{-1}), whereas LB does not, even when Psa was grown to high density (Figs. 4, 5). Therefore, though bacteria density influences Psa responsiveness to plant molecule(s), host recognition and specificity are more important than population density per se in triggering the expression of virulence-related traits.

Psa virulence induction by host plant signal(s) was confirmed both in xylem saps and whole plant extracts, supporting their relevance to Psa pathogenesis in nature and suggesting that the recognised plant-derived signal(s) are freely diffusible in the xylem. While xylem fluids have been generally regarded as a poor nutrient medium for bacteria, their composition is influenced by seasonal, environmental and metabolic changes, and certain plant-associated microbes (including pathogens) specialise in xylem colonisation (Lowe-Power et al., 2018). Psa, for instance, can colonise xylem vessels, leading to twig dieback in spring (Spinelli et al., 2011). In this work, *A. deliciosa* and *A. chinensis* xylem saps were sampled in early spring, preceding leaf expansion (bleeding sap stage), i.e. when the highest nutrient contents are observed due to the remobilisation of winter carbon reserves to sustain vegetative growth for the new season (Ferguson et al., 1983; Nardozza et al., 2015). These conditions may contribute to the higher Psa growth rate in xylem saps obtained at this development stage (Nardozza et al., 2015) and, somehow, could account for the fact that early spring corresponds to the most critical period for Psa infection, together with environmentally conducive conditions of temperature and humidity (Serizawa and Ichikawa, 1993; Donati et al., 2014).

Plant sugars have been shown in some cases to regulate the virulence of phytopathogenic bacteria, such as *Agrobacterium tumefaciens* (Subramoni et al., 2014). Among sugars reported in *Actinidia* species, the rare sugar planteose plays a role as a major short-term storage carbohydrate and is present in phloem exudate (Klages et al., 2004), and may also act
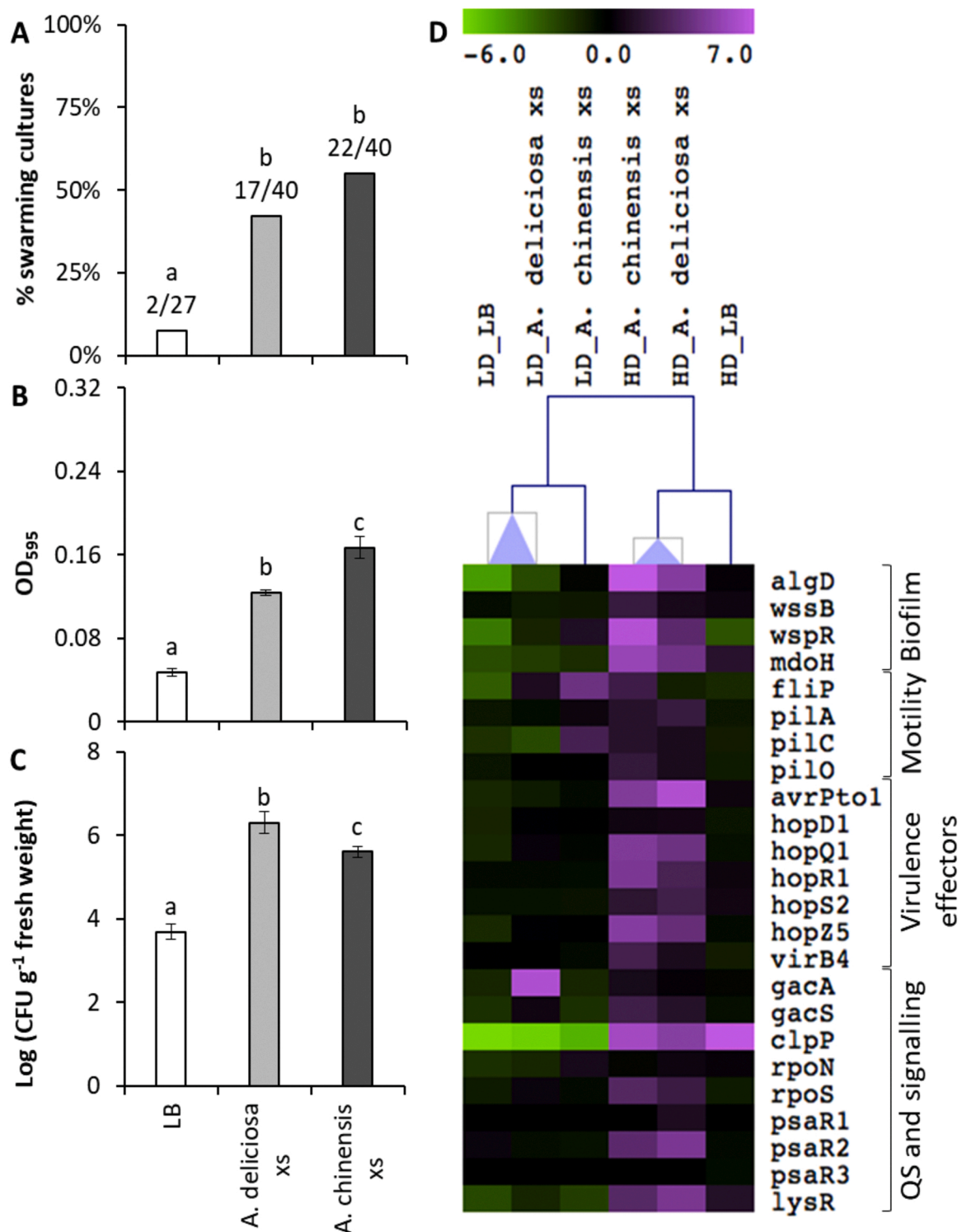

Fig. 5. Effects of tolerant host (*Actinidia arguta*) or non-host-derived growing medium on the expression of *Pseudomonas syringae* pv. *actinidiae* genes related to biofilm formation (BF), bacterial motility (M), virulence effectors (VE) and quorum sensing or signalling (QS). The hierarchical clustering of the relative expression was performed using the average values of three biological replicates.

mR1 and Psa-mR3) to 3 (Psa-mR2) logs compared to the wild-type (Fig. 7 C). Finally, the expression of selected genes was significantly affected by the deletion of *psaR2* or *psaR3* genes. In particular, Psa-mR2 showed down-regulation of several genes involved in all the analysed categories. On the other hand, Psa-mR3 showed a more complex expression pattern, with down-regulation of genes related to biofilm and motility and up-regulation of genes involved mainly in signaling, including *psaR2* (Fig. 7D and Supplementary Fig. S2).

3.6. Gene expression-inducing activity of plant extract fractions

Since PsaR2 shares similarities with other plant-associated bacteria LuxR solos, and is required for Psa virulence induction in presence of host plant extract, it represents a promising candidate as a putative plant signal sensor (González and Venturi, 2013; Patel et al., 2014). To demonstrate the possible interaction of PsaR2 with plant-derived compound(s), *A. deliciosa* plant extract was fractionated based on PsaR2 affinity. For this purpose, the recombinant PsaR2 protein was expressed in *E. coli*, fixed on affinity chromatographic resin, and used as a bait for the separation of the molecular components of plant extract (Fig. 8 A). The fractions, corresponding to different levels of affinity to PsaR2, were tested for their ability to induce virulence-related genes expression. The specific PsaR2-binding fraction, but not the loosely bound (wash) or unbound (flow-through) fractions or the elution buffer alone, stimulated the expression of the selected genes in Psa grown in LB medium, similar to the raw plant extract, or in some cases even to a higher extent (Fig. 8B

Fig. 6. Effects of growing *Pseudomonas syringae* pv. *actinidiae* in *Actinidia deliciosa* or *A. chinensis* xylem saps (XS) on virulence-related phenotypes and gene expression. (A) Percentage of colonies showing swarming motility (positive observations over total replicates are indicated over each bar). (B) Estimation of biofilm production. (C) Endophytic population in *A. deliciosa* microcuttings 5 days post-inoculation. (D) Expression of genes related to biofilm formation, bacterial motility, virulence factors and signalling at 10^5 (LD) or 10^7 (HD) CFU mL^{-1} population density. Different letters indicate significant differences among treatments according to Marascuilo's procedure (A) or ANOVA followed by Tukey's test (B-C).

as a host-specific signal. In agreement with the abundance of planteose not only in susceptible hosts, but also in *A. arguta* (Boldingh et al., 2015), several Psa genes (including *psaR2*) were observed to be up-regulated in *A. arguta*-derived extract. This suggests that Psa could recognise *A. arguta* as a host, but other biochemical features of this species, such as

high contents of organic acids and phenolics (Sciubba et al., 2020) may restrict bacterial growth and virulence induction.
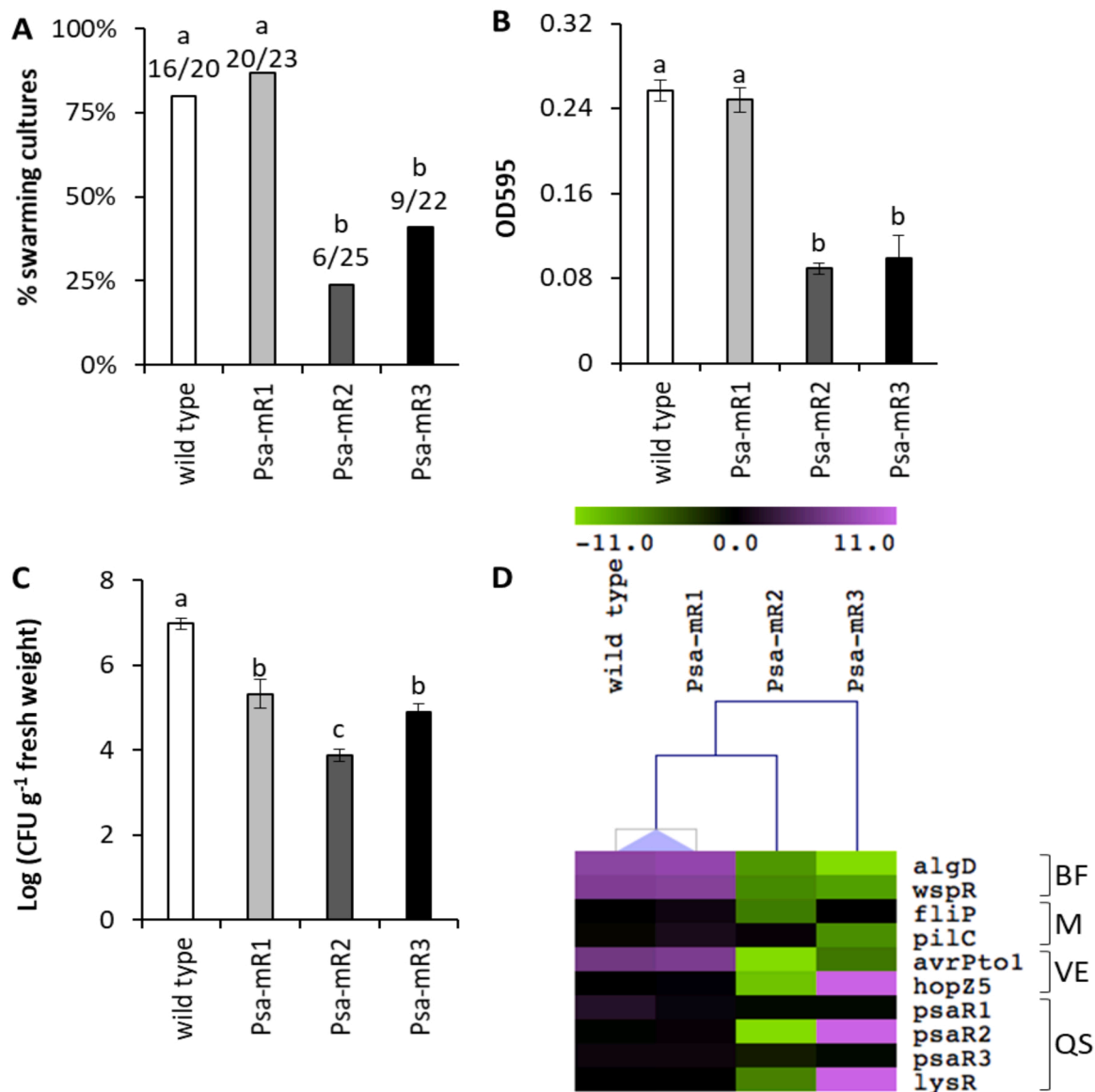
Fig. 7. Effects of cultivation in *Actinidia deliciosa* plant extract on virulence-related phenotypes and gene expression of *Pseudomonas syringae* pv. *actinidiae* PsaR1,2,3 defective mutants. (A) Percentage of colonies showing swarming motility (positive observations over total replicates are indicated over each bar). (B) Estimation of biofilm production. (C) Endophytic population in *A. deliciosa* microcuttings 5 days post inoculation. (D) Expression of genes related to biofilm formation (BF), bacterial motility (M), virulence effectors (VE) and quorum sensing or signalling (QS) at 10^7 CFU mL^{-1} population density. Different letters indicate significant differences among treatments according to Marascuilo's procedure (A) or ANOVA followed by Tukey's test (B-C).

4.2. Host-induced effects on Psa virulence

Biofilm formation, motility and virulence effectors contribute to virulence in phytopathogenic bacteria, including strains belonging to the *P. syringae* complex (Yu et al., 2013). In agreement, an enhancement of such virulence traits, along with increased endophytic population after host infection, was observed after growing Psa in host-derived extracts. This result was further confirmed by gene expression analysis, with the up-regulation of genes involved in exopolysaccharide biosynthesis, such as *algD* (alginate), *wssB* (cellulose) and *mdoH* (UDP-glucose polymerisation) (Penaloza-Vazquez et al., 2010; Spiers et al., 2013). Moreover, the diguanylate cyclase/response regulator, WspR, known to be regulated by cell density (D'Argenio et al., 2002; Cellini et al., 2020), was also induced in these conditions, further supporting the crucial role of c-di-GMP in governing cell aggregation, biofilm production, motility and virulence (Kazmierczak et al., 2015; Jenal et al., 2017; Pérez-Mendoza et al., 2019; Vandelle et al., 2021), in

particular once within host tissues. Coordinated motility phenotypes, mediated by flagella or type IV pili, have been implicated in exploration of and/or the adhesion to host surfaces, and are thus considered crucial for virulence (De Souza et al., 2004). In particular, pili may initiate the formation of biofilms, or facilitate apoplast migration and endophytic colonisation (Kazmierczak et al., 2015). The expression of both *fliP*, encoding an integral membrane component of the flagellar type III secretion apparatus (Ward et al., 2018), and *pilC*, encoding a type IV pilus biogenesis protein (Hospenthal et al., 2017), was also triggered by growing Psa in host plant extract, in agreement with the increased incidence of swarming phenotype on agar plates. The induction of genes involved in the first steps of flagellum and pilus biosynthesis, including structural components of the secretion apparatus, implies that host-derived signals likely control early movement regulators. Moreover, it is well known that flagellum-related type III secretion system (T3SS), including FliP, is related to the type III injectisome (Erhardt et al., 2010; Ward et al., 2018), used by bacteria to release virulence
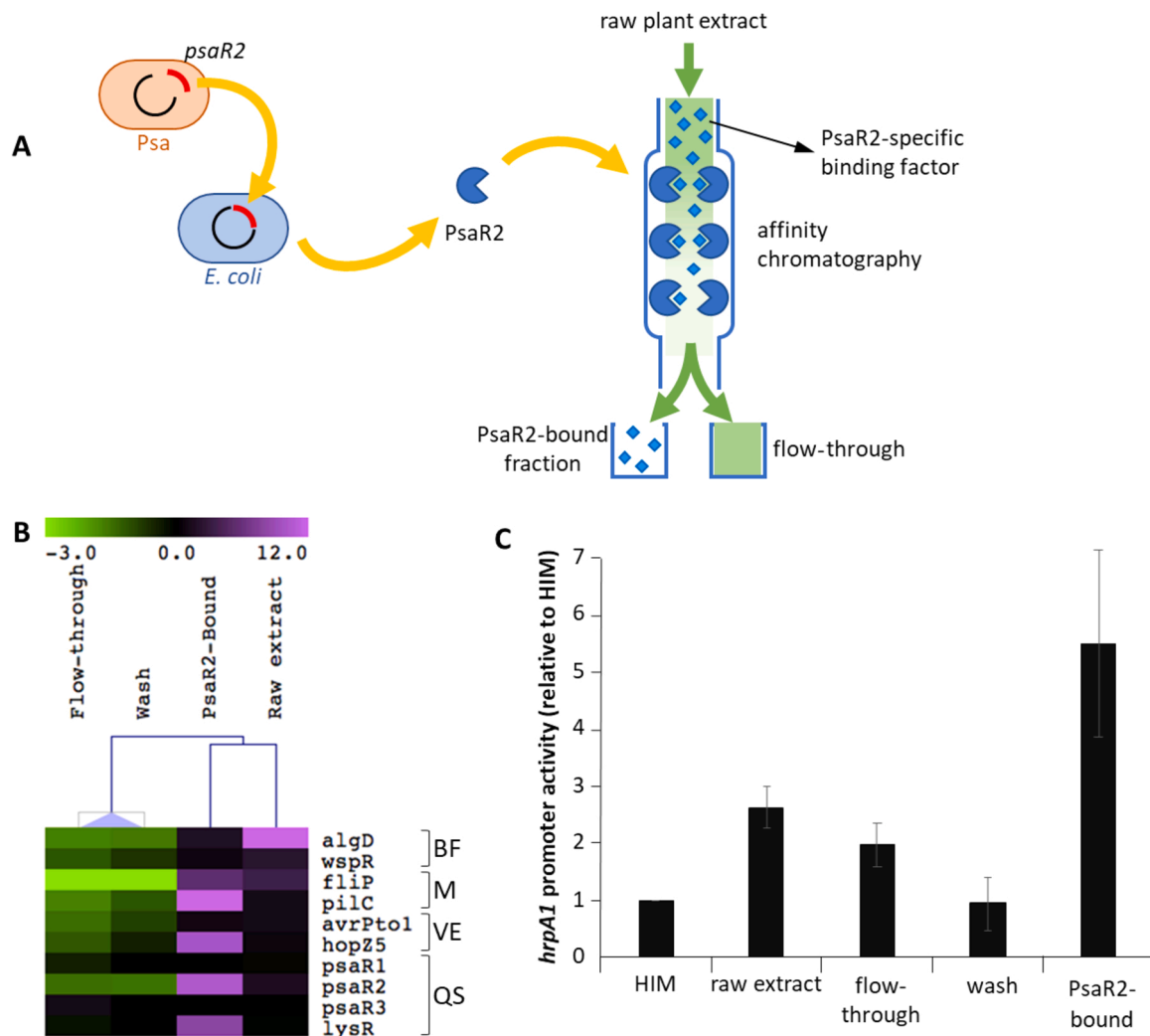
Fig. 8. (A) Experimental procedure for heterologous expression of PsaR2 and purification of the specific ligand. (B) Effects of addition of *Actinidia deliciosa* extract and the PsaR2-binding fraction on the expression of genes related to biofilm formation (BF), bacterial motility (M), virulence effectors (VE) and quorum sensing or signalling (QS) of *Pseudomonas syringae* pv. *actinidiae*. (C) Induction of *hrpA1* promoter activity by the plant extract and PsaR2-binding fraction.

effectors into host cells, and recognised to be regulated by plant signal(s), also in Psa (Vandelle et al., 2017, 2021). It could be thus assumed that plant signal(s) may regulate swarming motility mediated by flagella, at least partly, through the regulation of the flagellum-related T3SS, as a general regulation mechanism targeting type III secretion machineries. A positive link between motility and T3SS may be further provided by the up-regulation of *pilO* and *pilA* genes. Indeed, mutants of *P. syringae* pv. *tabaci* lacking one of these genes were shown to be impaired, not only in swarming motility (while twitching was retained), but also in the expression of *hrp* genes, encoding the T3SS, thus suggesting a co-regulation of type IV pili (T4P) and the T3SS apparatus (Taguchi and Ichinose, 2011), likely through the perception of host signal(s). Accordingly, a type IV pilus biogenesis protein (VirB4) as well as type III effectors (AvrPto1, HopD1, HopQ1, HopR1 and HopZ5) (Lin et al., 2006; Marcelletti et al., 2011; Li et al., 2013; McCann et al., 2013; Block et al., 2014; Jayaraman et al., 2017; Sgro et al., 2019) displayed a transcript level increase in presence of host plant extracts.

In addition to genes directly linked to the observed phenotypes, or involved in their regulation, several genes involved in bacterial signalling were also stimulated by the host, such as genes encoding receptors potentially involved in the perception of environmental and QS signals (GacA/GacS system), alternate sigma factors (RpoN, RpoS) responsible for transcriptional reprogramming, or transcriptional regulators (ClpP, LysR) previously linked to virulence in bacteria, including *Pseudomonas*

spp. (Heeb and Haas, 2001; Pernestig et al., 2001; Maddocks and Oyston, 2008; O'Toole and Kolter, 2008; Tian et al., 2008; Sheng et al., 2012).

4.3. Role of PsaR1, PsaR2, PsaR3 in host recognition

Recently, the Psa LuxR solos PsaR1 and PsaR3 were implicated in the perception of bacterial signals, such as AHLs, playing a role in interspecies signalling among bacteria. Instead, *psaR2* deletion did not affect bacterial communication, but dramatically reduced host colonisation (Cellini et al., 2020).

The up-regulation of *psaR2* in plant host extracts-derived media (Figs. 4–6), the impairment of virulence-related phenotypes in Psa-mR2 bacteria (Fig. 7), and the similar stimulation of gene expression by raw *A. deliciosa* extract and the PsaR2-bound fraction (Fig. 8) demonstrate that PsaR2 plays a role in interkingdom communication, allowing plant host recognition by Psa and subsequent induction of virulence genes. Interestingly, the Psa-mR3 mutant was also partially hindered in plant-induced (i.e., non-AHLs-mediated) swarming and biofilm formation, suggesting a function for PsaR3 in plant signal perception. Thus, PsaR receptors play specific roles in Psa virulence regulation. On the one hand, PsaR1 and PsaR2 present a high specificity for bacterial- and host-derived signals, respectively, presumably regulating Psa epiphytic (PsaR1) and endophytic (PsaR2) metabolism. On the other hand, PsaR3

shows a dual function, responding to plant extract similarly to PsaR2 (this work), while displaying opposite effects compared to PsaR1 in response to bacterial signals (Cellini et al., 2020). Thus, virulence in Psa is at least partially determined by the integration of different stimuli that converge to the same signalling pathway(s).

4.4. Conclusions and unresolved questions

This study highlights that Psa establishes a specific communication with its host plants, responding in a specific way only to their xylem saps and extracts for inducing virulence. Psa LuxR solos, namely PsaR2 and PsaR3, play a role in such interkingdom dialogue, as demonstrated in the corresponding mutants. While the precise function of PsaR3 in this mechanism remains to be elucidated, the use of a recombinant protein, for affinity chromatography analyses, demonstrated that PsaR2 directly binds to host molecule(s) that induce Psa virulence traits. Future research will thus aim at identifying and characterising the specific plant-derived ligands and their integrated signal cascades.

CRediT authorship contribution statement

Antonio Cellini: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Giampaolo Buriani:** Conceptualization, Investigation. **Cristiana Correia:** Investigation, Writing – review & editing. **Luca Fiorentini:** Investigation. **Elodie Vandelle:** Conceptualization, Investigation, Writing – review & editing. **Annalisa Polverari:** Resources. **Conceição Santos:** Supervision. **Joel L. Vanneste:** Conceptualization, Writing – review & editing. **Francesco Spinelli:** First conceptualization, Writing – review & editing, Project administration, Funding acquisition.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2022.127048.

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