A Na\(^+\)/Ca\(^{2+}\) exchanger of the olive pathogen *Pseudomonas savastanoi* pv. *savastanoi* is critical for its virulence

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**SUMMARY**

In a number of compatible plant-bacterium interactions, a rise in apoplastic Ca\(^{2+}\) levels is observed, suggesting that Ca\(^{2+}\) represents an important environmental clue, as reported for bacteria infecting mammalians. We demonstrate that Ca\(^{2+}\) entry in *Pseudomonas savastanoi* pv. *savastanoi* (*Psav*) strain DAPP-PG 722 is mediated by a Na\(^{+}\)/Ca\(^{2+}\) exchanger critical for virulence. Using the fluorescent Ca\(^{2+}\) probe Fura 2-AM, we demonstrate that Ca\(^{2+}\) enters *Psav* cells foremost when they experience low levels of energy, a situation mimicking the apoplastic fluid. In fact, Ca\(^{2+}\) entry was suppressed in the presence of high concentrations of glucose, fructose, sucrose or adenosine triphosphate (ATP). Since Ca\(^{2+}\) entry was inhibited by nifedipine and LiCl, we conclude that the channel for Ca\(^{2+}\) entry is a Na\(^{+}\)/Ca\(^{2+}\) exchanger. *In silico* analysis of the *Psav* DAPP-PG 722 genome revealed the presence of a single gene coding for a Na\(^{+}\)/Ca\(^{2+}\) exchanger (*cneA*), which is a widely conserved and ancestral gene within the *P. syringae* complex based on gene phylogeny. Mutation of *cneA* compromised not only Ca\(^{2+}\) entry, but also compromised the Hypersensitive response (HR) in tobacco leaves and blocked the ability to induce knots in olive stems. The expression of both pathogenicity (*iaaM*, *hrpA*, and *iaaM*) and virulence (*ptz*) genes was reduced in this *Psav-cneA* mutant. Complementation of the *Psav-cneA* mutation restored both Ca\(^{2+}\) entry and pathogenicity in olive plants, but failed to restore the HR in tobacco leaves. In conclusion, Ca\(^{2+}\) entry acts as a ‘host signal’ that allows and promotes *Psav* pathogenicity on olive plants.

**Keywords:** calcium, β-galactosidase assay, host detection, Na\(^{+}\)/Ca\(^{2+}\) exchanger, olive knot disease, pathogenicity factor, *Pseudomonas savastanoi* pv. *savastanoi*.

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**INTRODUCTION**

Cytosolic calcium (Ca\(^{2+}\)) has essential functions in eukaryotic signalling as secondary messenger. The cytosolic Ca\(^{2+}\) levels are influenced by the difference in its intracellular-to-extracellular concentration (Berridge et al., 2000; Bhosale et al., 2015; Islam, 2012). In particular in mammals, Ca\(^{2+}\) signalling is well understood with a central role in nearly all the known cellular processes ranging from egg-cell fertilization to programmed cell death (Brini et al., 2013; Rajagopal and Ponnamys, 2017), impacting gene expression levels, heart and muscle contraction, neurotransmission and synaptic plasticity, secretion of hormones and their action, blood coagulation and other motility processes, to diverse metabolic pathways involved in the generation of cell fuels (Sharma et al., 2017). Furthermore, Ca\(^{2+}\) acts both as a messenger and cofactor to coordinate many intracellular signalling pathways (Rajagopal and Ponnamys, 2017). Noteworthy, it can already activate different cellular responses only by differences in the amplitude, frequency and duration of the intracellular Ca\(^{2+}\) concentration (Rajagopal and Ponnamys, 2017). Located predominantly in the extracellular environment, Ca\(^{2+}\) entry relies in animals on membrane depolarization resulting from action potentials, where it then can perform its regulatory functions. In these eukaryotes, most ion channels as well as transporters, pumps, binding proteins and L-type voltage-dependent calcium channels have the capacity to transport Ca\(^{2+}\) across the depolarized membrane (Cai and Lytton, 2004; Carafoli, 1987; Norris et al., 1996; Tsien and Tsien, 1990). In plants, Ca\(^{2+}\) is present in high concentrations in the apoplast (i.e. intercellular spaces and xylem) (Fishman et al., 2018) and Ca\(^{2+}\) influx can for example, activate plant defences (Aslam et al., 2008). Furthermore, Ca\(^{2+}\) signalling plays an essential role in pollen tube elongation, seed germination, hypersomatic and oxidative stresses (Sanders et al., 1999; White and Broadley, 2003).

Although the molecular mechanisms that cause the cytosolic fluctuations of Ca\(^{2+}\) levels are well understood for eukaryotic cells,
much remains to be discovered for prokaryotes. Nevertheless, there is a growing amount of evidence that Ca\(^2+\) also plays an important regulatory role in the physiology of prokaryotes (Fishman et al., 2018). However, due to their small cell size, the selective permeability of their cell walls and cell membrane and the toxicity of many chelators used in these Ca\(^2+\) studies, it remains complex to monitor Ca\(^2+\) concentrations inside bacterial cells, which is nevertheless indispensable to increase our understanding of the connection between Ca\(^2+\) influx and other cellular processes. The use of the Ca\(^2+\) reporters aequorin (Watkins et al., 1995) and Fura 2 (1-[2-(5-carboxyoxazol-2-yl)-6-amino-benzofuran-5-oxyl]-2-(2'-amino-5'-methylphenoxy) ethane-N,N,N',N'-tetraacetic acid) (Gangola and Rosen, 1987; Tisa and Adler, 1995) revealed that variations in cytosolic Ca\(^2+\) levels also regulate many important bacterial cellular processes. For example, Ca\(^2+\) acts in bacteria, including plant pathogenic bacteria, as a versatile intracellular messenger involved in the maintenance of cell structure (Domínguez et al., 2015), motility (Cruz et al., 2012; Fishman et al., 2018; Gode-Potratz et al., 2010; Guragain et al., 2013; Parker et al., 2015; Tisa and Adler, 1995), cell division (Domínguez et al., 2015), gene expression (Domínguez et al., 2015), type III secretion (Dasgupta et al., 2006; DeBord et al., 2003; Fishman et al., 2018; Gode-Potratz et al., 2010), exopolysaccharide production (Kierrek and Watnick, 2003; Kim et al., 1999; Patrauchan et al., 2007), iron scavenging (Domínguez et al., 2015; Patrauchan et al., 2007), quorum sensing (Werthén and Lundgren, 2001), biofilm formation (Cruz et al., 2012; Das et al., 2014; Parker Jennifer et al., 2016; Patrauchan et al., 2005; Rinaudi et al., 2006; Sarkisova et al., 2005; Zhou et al., 2013) or biofilm suppression (Bilecen and Yildiz, 2009; Shukla and Rao, 2013). Furthermore, Ca\(^2+\) appears to determine the virulence of the facultative human pathogen *Pseudomonas aeruginosa* (Guragain et al., 2016; Patrauchan et al., 2007; Sarkisova et al., 2014) and of all species of *Yersinia* (Mekalanos, 1992). Hardly any information is available on the role of Ca\(^2+\) for virulence of phytopathogenic bacteria. It was recently demonstrated that a two-component system induced by Ca\(^2+\) controls virulence of the model plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Fishman et al., 2018). Here, we report on the role of Ca\(^2+\) for virulence of *Pseudomonas savastanoi* pv. *savastanoi* (referred to as *Psav*), the causal agent of olive knot disease.

Olive knot disease is characterized by knots or gall outgrowths on mainly twigs and young plant branches, while leaf and fruit infections are rare and only develop during wet summers. *Psav* survives as an epiphyte in the phyllosphere penetrating its host through wounds (Lavermicocca and Surico, 1987). Once inside host plants, the bacterium colonizes the apoplast and due to its ability to secrete the plant hormones indole-3-acetic acid (IAA) and cytokinins, it stimulates olive cells to produce new tissue giving rise to knot development and tissue overgrowth (Glass and Kosuge, 1988; Powell and Morris, 1986; Quesada et al., 2012; Ramos et al., 2012; Rodríguez-Moreno et al., 2008; Surico et al., 1985; Temsah et al., 2008). The switch from an epiphytic to endophytic (apoplastic) life style is an abrupt transition for the bacterium that requires: (i) a remarkable adaptation to an environment that is extremely different in pH, osmotic pressure, carbon sources and oxygen availability, (ii) the ability to suppress basal and induced plant defences (Rico et al., 2009). Although the bacterial signals (e.g. flagellin, elongation factors) that the plant perceives through specific receptors and via which it activates plant immunity have been extensively studied in the last decades (Buonaurio, 2008; Chisholm et al., 2006; Dangl et al., 2013; Jones and Dangl, 2006; Silva et al., 2018), little is known on the molecular signals that the phytopathogenic bacteria perceive during this transition to the apoplast. We here reveal that Ca\(^2+\) influx in *Psav* is stimulated by low energy situations and that it requires a Na\(^+\)/Ca\(^2+\) exchanger that is essential for *Psav* virulence on olive plants.

### RESULTS

**Ca\(^2+\) entry in *Psav* cells is promoted under starvation conditions and is not influenced by exogenous indole-3-acetic acid**

Our understanding of molecular signalling in the early phases of plant bacterial infection is limited, while this early signalling largely defines the onset of bacterial disease. Since Ca\(^2+\) is a well-known signalling molecule in plants and animals, we here investigated the role of Ca\(^2+\) signalling for a bacterial pathogen. We chose the olive—*Psav* pathosystem and used a biochemical approach to study if Ca\(^2+\) signalling is important for pathogenicity and virulence. First, we assessed if the cytosolic Ca\(^2+\) concentration of *Psav* is influenced by external Ca\(^2+\). To this end, we measured in *Psav* DAPP-PG 722 cells under basal conditions (i.e. Hanks’ Balanced Salt Solution, HBSS buffer) whether an increase in external Ca\(^2+\) resulted in an increase in the cytosolic Ca\(^2+\) concentrations in *Psav*. We find that the cytosolic Ca\(^2+\) concentrations rapidly increase in response to external Ca\(^2+\) concentration in the medium (Fig. 1). This trend was suppressed when different carbon sources (glucose, fructose or sucrose) or ATP were added in a combination with Ca\(^2+\) (Fig. 1). Since IAA is produced by *Psav* to stimulate plant cell proliferation and knot formation, we also investigated whether IAA or its precursor (L-tryptophan) influences Ca\(^2+\) entry. However, addition of IAA or L-tryptophan to the incubation buffer did not significantly alter Ca\(^2+\) entry in *Psav* DAPP-PG 722 cells (Fig. 1). Combined, these data suggest that *Psav* actively controls Ca\(^2+\) entry rather than that this Ca\(^2+\) influx represents a passive process.

**Ca\(^2+\) entry in *Psav* cells is mediated by the Na\(^+\)/Ca\(^2+\) exchanger CneA**

To determine if Ca\(^2+\) entry depends on an ion channel, *Psav* DAPP-PG 722 cells were pre-treated with nifedipine, an inhibitor
of the L-voltage channels responsible for the entry of the extracellular Ca\textsuperscript{2+} in mammals (Sorkin et al., 1985) or LiCl that, in substitution of Na\textsuperscript{+} in the buffer, inhibits Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (Yanagita et al., 2007). Since Ca\textsuperscript{2+} entry was inhibited by both nifedipine and LiCl (Fig. 2), we conclude that a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is potentially involved in the entry of extracellular Ca\textsuperscript{2+} in Psav. In silico analysis of the genome of Psav DAPP-PG 722 (Moretti et al., 2014) revealed the presence of a single gene coding for a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (here designated as cneA; MK408668), which belongs to the ChaA antiporter superfamily (Shijuku et al., 2002). This cneA gene encodes for a protein (CneA) that encompasses the PRK10599, caca2 and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger protein domains (Marchler-Bauer et al., 2017). In the genome of Psav DAPP-PG 722, two genes are located directly upstream of cneA gene, which encode for a guanine deaminase and hydroxydechloroatrazine ethylaminohydrolase, while

Fig. 1 Increase of cytosolic Ca\textsuperscript{2+} levels in Pseudomonas savastanoi pv. savastanoi DAPP-PG 722 cells incubated in HBSS medium alone (basal conditions; open circles) or in the presence of glucose, fructose, sucrose, ATP, indole 3 acetic acid (IAA) or tryptophan (closed squares) over a concentration range extracellular calcium chloride. Each point is the mean of 10 independent experiments ± SE.
Calcium and *P. savastanoi* pv. *savastanoi* virulence

downstream we find a gene encoding an iron(III) dicitrate transport system. A comparative phylogenetic analysis of the nucleotide sequences of the *cneA* gene was performed using the Geneious resource (Kearse et al., 2012). Sequences of this gene were retrieved from a series of strains that belong to the seven primary (monophyletic) phylogroups (PGs) described for the *P. syringae* complex. Homologs of the *cneA* gene were found to be widely distributed across the *P. syringae* complex. However, the branching of the *cneA* gene tree was not fully consistent with the previously reported phylogeny of the *P. syringae* species complex that is based on a multilocus sequence analysis (MLSA) of housekeeping genes (Baltrus et al., 2017). This suggests that the *cneA* gene has undergone horizontal gene transfer between species in this bacterial complex. For example, although PG2, PG3 and PG6 are equally distributed in a common branch in both the *cneA* and the MLSA phylogeny, some PG3 pathovars (e.g. *P. syringae* pathovars *cunninghamiae*, *castaneae*, *photinae* and *myricae*, amongst others) have a different position in the *cneA* gene tree than in the MLSA tree (Fig. S1).

A *Psav-cneA* mutant is inhibited in Ca\(^{2+}\) entry and is unable to induce both the hypersensitive response (HR) in *Nicotiana tabacum* and formation of knots on olive plants

In order to investigate the role of the *Psav-cneA* gene in Ca\(^{2+}\) entry, a *Psav* DAPP-PG 722 *cneA* mutant was constructed and its ability to transport Ca\(^{2+}\) into the cytosol was tested in comparison to the wild-type strain. For this purpose, *Psav* cells were incubated in basal conditions or in the presence of glucose. The uptake of Ca\(^{2+}\) was strongly impaired in the mutant cells incubated under basal condition (Fig. 3). It is worth mentioning that the in vitro growth rate of *Psav-cneA* mutant cells was identical to that of the *Psav* wild-type strain in KB medium (Likelihood ratio test, P-value = 0.85; Fig. S2).

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**Fig. 2** Increase of cytosolic Ca\(^{2+}\) levels in *Pseudomonas savastanoi* pv. *savastanoi* DAPP-PG 722 cells pre-treated with nifedipine, Lithium chloride (squares) and the negative control (circles) after which the cells were incubated in HBSS medium at different concentrations of extracellular calcium chloride. Each point is the mean of 10 independent experiments ± SE.

**Fig. 3** Increase of cytosolic Ca\(^{2+}\) levels in *Pseudomonas savastanoi* pv. *savastanoi* wild type (closed squares) and *Psav-cneA* mutant (open circles) cells incubated in HBSS medium at different concentrations of extracellular calcium chloride. Each point is the mean of 10 independent experiments ± SE.
To examine whether Ca\(^{2+}\) entry was involved in \(P. sav\) pathogenicity and virulence, both the \(P. sav\) DAPP-PG 722 wild type and \(P. sav\)-cneA mutant were: (i) infiltrated in the non-host tobacco, (ii) inoculated on 1-year-old wounded olive plants. When infiltrated in tobacco leaves, the \(P. sav\)-cneA mutant was unable to induce an HR (Fig. 4A). Likewise, \(P. sav\)-cneA mutant was significantly affected in the ability to induce knots on olive (Fig. 4B). In fact, olive plants inoculated with the \(P. sav\)-cneA mutant showed a drastic reduction in knot overgrowth (Fig. 4C). It must be pointed out that the residual stem overgrowth seen on the \(P. sav\)-cneA mutant inoculated plants was due to the formation of cicatrisation callus as a consequence of the wounding (incisions). Moreover, we found that the \(P. sav\)-cneA mutant strain was unable to proliferate in olive plants in comparison with the \(P. sav\) wild type (Fig. 4D).

In the \(P. sav\)-cneA mutant expression of genes involved in type III secretion and phytohormone production are suppressed

In order to investigate the expression of genes involved in pathogenicity and virulence of \(P. sav\), the promoter activity of the hrpL, hrpA, iaaM and ptz genes was determined via transcriptional fusions of their gene promoters with the promoterless lacZ gene. Although \(\beta\)-galactosidase levels associated to the hrpL and hrpA promoters were very low under the conditions tested (Fig. 5A and B), transcription from the hrpL promoter was significantly reduced in the cneA mutant grown either in KB or HBSS media (Fig. 5A). When the \(P. sav\)-cneA mutant strain was grown in Hrp medium (Huynh et al., 1989), the activity of the hrpA promoter was reduced in comparison to that obtained for wild-type \(P. sav\) (Fig. 5B). In addition, hrpA promoter activity...
Calcium and *P. savastanoi* pv. savastanoi virulence was significantly lower in *Psav DAPP-PG 722* than in *Psav NCPPB 3335* (Fig. 5B). In *Psav-cneA* mutant cells, the activity of the *iaaM* promoter was also low in all media tested (Fig. 5C). Nevertheless, the activity of this promoter was significantly lower in the *cneA* mutant than in the wild-type strains in Hrp medium, HBSS and HBSS+CaCl₂ media. As a negative control, *Psav* wt and *Psav-cneA* mutant strains transformed with a promoterless β-galactosidase were used. For comparison, *hrpA* promoter activity in *Psav NCPPB 3335* strain (yellow column) was included. Each column is the mean of one experiment with three replicates ± SE. For each medium, values recorded in the *Psav-cneA* mutant are statistically different (*P* < 0.05) respect to that of *Psav* wt, according to the Student’s t-test. Columns capped with different letters, in Figure 5B, are significantly different (*P* < 0.05) according to the Duncan’s multiple range test.

**Psav-cneA** mutant was restored by gene complementation

Complementation of the *Psav-cneA* mutant was performed using both a plasmid encoding the *cneA* gene expressed from the *E. coli* lac promoter (*Psav-cneA* mutant [pBBR::cneA]) or a mini-Tn7 transposon encoding *cneA* from its own promoter and inserted in the chromosone of the mutant strain (*Psav-cneA* mutant [miniTn7::cneA]). Ca²⁺ entry into the complemented strains was restored to more than 60%, in the absence (Fig. 6) of glucose. Next, we assessed the virulence of the complemented strains on olive plants. The knot overgrowth generated by *Psav-cneA* mutant (pBBR::cneA) was not significantly different to that of *Psav* wild type (Fig. 7A), but it was significantly higher compared to that of *Psav-cneA* mutant (Fig. 7A). Also bacterial proliferation of the *Psav-cneA* mutant (pBBR::cneA) in olive plants was comparable to the *Psav* wild type (Fig. 7B). Similar results were obtained for *Psav-cneA* mutant (miniTn7::cneA) (Fig. 7C and D). This means that gene complementation restored bacterial pathogenicity and virulence on olive plants to wild-type levels.

Other phenotypic characteristics of the *Psav-cnaA* mutant

In order to determine if the *Psav-cneA* mutant is impaired in other phenotypic traits important for its epiphytic and endophytic lifestyles (Ramos et al., 2012; Rodriguez-Moreno et al., 2009), several phenotypic characters were tested (Table 1). The mutant was impaired in the production of exopolysaccharides (EPSs), both in KB and LBS media, and N-acyl homoserine lactones (Fig. 8A and B).
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lactones (AHLs), and it showed a higher swimming motility than the wild-type strain. No difference in proteolytic activity, siderophore production and swarming motility was observed between wild type and Psav-cneA mutant. At 24 h neither wild type nor the mutant formed biofilms under shaking or static conditions. The same results were obtained after 48 h incubation in shaking conditions. However, biofilm formation (similar to those of Pseudomonas putida KT2440; positive control) was detected in the Psav-cneA mutant strain 48 h after incubation at static conditions, while no formation was detected in the wild-type strain under these conditions (Fig. 8). Amongst the phenotypic characteristics examined, the complemented strains (Psav-cneA mutant [pBBR::cneA], Psav-cneA mutant [miniTn7::cneA]) were not able to swim as the Psav wild type under the conditions tested, and they only recovered partially the capacity to produce EPSs (Table 1). Other phenotypes that were not restored in the complemented strains include the induction of the HR on tobacco plants, the production of AHLs (Table 1) and the inability to form biofilms under the conditions tested (Fig. 8).

**DISCUSSION**

Based on our data, we propose that in the early phases of the Psav infection and in particular when the bacterium reaches the apoplast (intercellular spaces and xylem), the abundant presence of Ca\(^{2+}\) (Stael et al., 2011) and the low concentration of sugars (Rico et al., 2009) therein permit Ca\(^{2+}\) entry into the bacterial cells via the Na\(^+/Ca^{2+}\) exchanger cneA, which in turn induces the expression of Psav pathogenicity and virulence genes. Although the level of Ca\(^{2+}\) in olive apoplast has not been reported, its concentration is likely sufficient to guarantee Ca\(^{2+}\) influx in the Psav cells. In fact, the Ca\(^{2+}\) concentrations used in this study are consistent with those reported in plant apoplast (Hepler, 2005; Plieth and Vollbehr, 2012), which range from 10 µM to 10 mM. In addition, during the early phase of bean infection with avirulent and virulent Pseudomonas savastanoi pv. phaseolicola strains, an increase in apoplastic Ca\(^{2+}\) was documented (O’Leary et al., 2016). Our biochemical experiments demonstrated that Ca\(^{2+}\) entry in Psav cells is inhibited by glucose, fructose or sucrose. Although the concentration of these sugars in olive apoplast has not been documented, their concentrations in the apoplast of other plants is low (Preston, 2017) and decrease during the early phase of a bacterial infection (O’Leary et al., 2016). Even though the level of these sugars in the olive apoplast should attenuate Ca\(^{2+}\) entry, we have to consider that minimal changes in cytosolic Ca\(^{2+}\) concentration can modulate gene expression (Borowiec et al., 2014; Domínguez, 2004). We therefore, hypothesize that a sugar starvation status can facilitate the entry of Ca\(^{2+}\) inside Psav cells. A high degree of starvation already occurs during the epiphytic phase of Psav, which is able to live on olive leaf surfaces exploiting the poor nutrients there present (Ramos et al., 2012). We cannot exclude that in this ecological niche, Ca\(^{2+}\) present in water and stored in EPSs enters into the Psav cells to regulate important processes that control in the epiphytic life style. The starvation experience during the epiphytic phase of the life cycle, is mitigated as soon as the bacteria enter the apoplast; however, a limited amount of starvation is always present in the apoplast that is considered a nutrient-limited environment (Rico et al., 2009), supporting the existence of certain starvation conditions also in this niche.

The importance of Ca\(^{2+}\) for the virulence of a phytopathogenic bacterium was recently reported by Fishman et al. (2018), who characterized a two-component system of P. syringae pv. tomato DC3000 that is responsive to Ca\(^{2+}\) and necessary for virulence of this bacterium. Through the use of a Na\(^+/Ca^{2+}\) exchanger mutant, we now identify for a related bacterium, Psav, an exchanger that is essential for Ca\(^{2+}\) influx. In corroborate, we demonstrated at the biochemical and pharmacological level that Ca\(^{2+}\) enters Psav bacterial cells via this Na\(^+/Ca^{2+}\) exchanger that belongs to the ChaA antiporter superfamily.
Fig. 7 Effect of plasmidic and chromosomal complementation of the calcium exchanger mutant (Psav-cneA mutant) on knot formation (A and B) and in planta population dynamics (C). (A) Knot formation, expressed as stem overgrowth observed 60 dpi, in olive (cv. Frantoio) inoculated plants with Pseudomonas savastanoi pv. savastanoi (Psav, wild type [wt]), Psav-cneA mutant, and Psav-cneA mutant (pBBR::cneA) (plasmidic complemented mutant). Each column represent the mean of four replicates ± S.E. Columns capped with different letter are significantly different (P < 0.01) according to the Duncan’s multiple range test. (B) Knot formation, expressed as stem overgrowth observed 60 dpi, in olive (cv. Frantoio) inoculated plants with Psav wt, Psav-cneA mutant, and Psav-cneA mutant (miniTn7::cneA) (chromosomal complemented mutant). Each column represent the mean of four replicates ± S.E. Columns capped with different letter are significantly different (P < 0.01) according to the Duncan’s multiple range test. (C) Population dynamics of Psav wt (closed squares), Psav-cneA mutant (open circles), Psav-cneA mutant (pBBR::cneA) (closed triangle), and Psav-cneA mutant (miniTn7::cneA) (grey triangle) in inoculated olive (cv. Frantoio) plants. Each point is the mean of four replicates ± SE.

Table 1 Phenotypic characterization of Pseudomonas savastanoi pv. savastanoi (wild type), Psav-cneA mutant, and two complementation lines Psav-cneA (pBBR::cneA) and Psav-cneA mutant (miniTn7::cneA).

|                          | Wild type | Psav-cneA | Psav-cneA (pBBR::cneA) | Psav-cneA (miniTn7::cneA) |
|--------------------------|-----------|-----------|------------------------|--------------------------|
| Hypersensitive reaction  | +         | −         | −                      | −                        |
| Proteolytic activity     | −         | −         | −                      | −                        |
| Siderophore production   | +         | +         | +                      | +                        |
| EPS production           | +         | −         | +/−                    | +/−                      |
| Swimming                 | −         | +         | −                      | −                        |
| Swarming                 | −         | −         | −                      | −                        |
| AHL production           | +         | −         | −                      | −                        |

+, positive; −, negative; +/−, weak positive.
IAA does not alter Ca²⁺ entry into the Psav cells, suggesting our results demonstrate that the presence of L-tryptophan or during infection. Our data imply that Ca²⁺ entry regulates other that there is no feedback regulation by the auxin pathway

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 2. Bacterial strains were grown at 27 °C in Luria-Bertani (LB) medium (Miller, 1972), King’s B (KB) medium (King et al., 1954) or Nutrient Agar (NA). Escherichia coli was grown at 37 °C. Antibiotics were added, when required, at the following final concentrations: ampicillin 100 μg/mL, nitrofurantoin (Nitrof) 100 μg/mL, kanamycin (Km) 100 μg/mL and gentamicin (Gm) 10 μg/mL.

**Recombinant DNA techniques**

DNA digestions with restriction enzymes (Xhol, Spel and EcoRI), agarose gel electrophoresis, DNA fragment purification, ligation with T4 ligase, end filling using the Klenow enzyme and E. coli transformation were performed as described by Sambrock et al. (1989). Plasmids were purified using the GenElute™ Plasmid...
Miniprep Kit (Sigma-Aldrich, MO, Saint Louis, USA). The genomic DNA was extracted with the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, MO, Saint Louis, USA). Triparental mating between E. coli and Psav DAPP-PG 722 was performed using a helper E. coli strain carrying plasmid pRK2013 (Figurski and Helinski, 1979).

### Determination of the cytosolic Ca²⁺ levels

Cytosolic Ca²⁺ levels were determined using a fluorimetric method, which employed the fluorescent probe Fura 2-AM (Fura 2-acetoxy methyl ester; Sigma-Aldrich, MO, Saint Louis, USA). The genomic DNA was extracted with the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, MO, Saint Louis, USA). Triparental mating between E. coli and Psav DAPP-PG 722 was performed using a helper E. coli strain carrying plasmid pRK2013 (Figurski and Helinski, 1979).

### Table 2  Bacterial strains, plasmids and primers used in this study.

| Strains | Relevant characteristics* | References |
|---------|---------------------------|------------|
| *Pseudomonas savastanoi pv. savastanoi (Psav)* | | |
| DAPP-PG 722 (wild type) | Olive knot (Italy) | Moretti et al. (2014) |
| Psav-cneA mutant | Interruption cneA mutant (NitrofR – KmR) of Psav DAPP-PG 722 | This study |
| Plasmid complemented strain | Psav-cneA mutant (pBBR::cneA) | * |
| Chromosomal complemented strain | Psav-cneA mutant (miniTn7::cneA) | * |
| *Escherichia coli* | | |
| DH5α | F-, φ80d lacZ M15, (lacZYA-argF) U169, deoR, recA1, endA, hsdR17 (k- m- r-, phoA, supE44, thi-1, gyrA96, relA1) | Hanahan (1983) |
| **Plasmids:** | | |
| pKNOCK-Km | Conjugative suicide vector; KmR | Alexeyev (1999) |
| pKNOCK-cneA | Internal PCR EcoRV cneA fragment of Psav cloned in pKNOCK-Km | This study |
| pBBR MCS-5 | Broad-host-range cloning vector; GmR | Kovach et al. (1995) |
| pBBR MCS-5-cneA | pBBRMCS5 with 1.1 kb XhoI - SpeI fragment containing the cneA gene of Psav | This study |
| pGEM®-T Easy vector | Cloning vector; AmpR | Promega, Fitchburg, WI, USA |
| pUC18R6KT-miniTn7BB-Gm | Cloning vector; GmR | Caballero and Govantes (2011) |
| pUC18R6KT-miniTn7BB-cneA-Gm | pUC18R6KT-miniTn7BB-Gm containing the cneA gene of Psav | This study |
| **Primers:** | | |
| cneA For | 5′-GGCGAGCAGTCTCTATAACGAT-3′ | This study |
| cneA Rev | 5′-ACACCGATGCAATGTGACA-3′ | * |
| cneA compl 1 | 5′-CTCAGAGAGAGATTGCGCTTGCTCAAGC-3′ | * |
| cneA compl 2 | 5′-CCTAGGCTTAAGCCCACAGACAGG-3′ | * |
| PromAP_Fw | 5′-CAGAAAGCTGAATCGTGAAAA-3′ | * |
| AP_Rev | 5′-TGGGAGCGATAGGCAATA-3′ | * |
| glmS_savastanoi | 5′-AACCTGGGCAAGTGGGAC-3′ | * |
| Tn7Rev | 5′-CAGCATAACTGGACTTACAG-3′ | * |
| **Primers for β-galactosidase activity:** | | |
| iaaM For | 5′-ACTCATGGGATGACATTGCGATGC-3′ | Aragón et al. (2014) |
| iaaM Rev | 5′-ACTCATGGGATGACATTGCGATGC-3′ | * |
| ptz For | 5′-ACTCATGGGATGACATTGCGATGC-3′ | * |
| ptz Rev | 5′-ACTCATGGGATGACATTGCGATGC-3′ | * |
| hpaA For | 5′-GACGAAATTGCGTTGCGATGC-3′ | * |
| hpaA Rev | 5′-TACGATCGCCGCTGTCATCAG-3′ | * |
| hpl For | 5′-CACCGATCCGGTCGATGACT-3′ | * |
| hpl Rev | 5′-CCCTAGGATCCGGTCGATGACT-3′ | * |

*Nitrof, nitrofurantoin; Km, kanamycin; Gm, gentamycin; Amp, ampicillin.
USA). Approximately $5 \times 10^6$ cells of *Psav DAPP-PG* 722 grown at 27 ± 1 °C for 16 h in LB broth to a stationary phase, were suspended in 0.12 M Tris (pH 7.8) and 2 mM EGTA. At 200 s after incubation at 25 °C, 2 mM CaCl$_2$ was added to stop the EGTA effect as reported by Grynkiewicz et al. (1985). Then the cells were incubated for 2 h in basal condition i.e. HBSS buffer (140 mM NaCl, 5.3 mM KCl, 25 mM HEPES, pH 7.4) supplemented with 2 mM Fura 2-AM (dissolved in DMSO) or in HBSS buffer supplemented with 2 mM Fura 2-AM and different carbon sources (glucose, fructose or sucrose, 5 mM) or ATP 50 µM. The fluorescence intensities of Fura 2-AM (Ex. = 335 nm, Em. = 505 nm) were monitored with a spectrophotometer (Perkin-Elmer, Waltham, Massachusetts, USA). The cytosolic Ca$^{2+}$ concentration was calculated following the formula reported by Grynkiewicz et al. (1985).

**Phylogenetic analysis of the Na$^+/Ca^{2+}$ exchanger gene**

A comparative phylogenetic analysis of the nucleotide sequences of the *cneA* gene coding for the Na$^+/Ca^{2+}$ exchanger was performed using the Geneious resource (Kearse et al., 2012). Blast searches were used to retrieve the close homologs of the *cneA* gene from different *Pseudomonas* species. Phylogenetic and molecular evolutionary analysis was conducted using MEGA 7 (Kumar et al., 2016) and the maximum likelihood method. Clade stability was assessed by 1000 bootstrap replications.

**Construction of a *P. savastanoi* pv. *savastanoi* knockout mutant of the Na$^+/Ca^{2+}$ exchanger gene *cneA***

A genomic null mutant of the Na$^+/Ca^{2+}$ exchanger gene (referred to as *cneA* gene) was created as follows. An internal 305 bp fragment of the *cneA* gene was amplified from *Psav* DAPP-PG 722 genomic DNA using the primers *cneA* For and *cneA* Rev (Table 2). The amplified PCR product was cloned in plasmid pKNOCK-Km (Alexeyev, 1999), generating pKNOCK-cneA (Table 2). A *Psav-cneA* knockout mutant (Table 2) was generated by homologous recombination (Alexeyev, 1999) after transformation of pKNOCK-cneA in *Psav DAPP-PG* 722 as a suicide delivery system. Transformants were selected on KB-Nitrof $+\text{Km}$ plates. Interruption of *cneA* was verified by PCR using primers specific to the pKNOCK-Km vector and to the genomic DNA sequences upstream and downstream of the targeted gene. The amplicons were sequenced at Macrogen Europe (Amsterdam, Netherlands; http://www.macrogen.com).

**Plasmid and chromosomal complementation of *Psav-cneA* mutant***

Complementation of *Psav-cneA* mutant with a plasmid encoding the *cneA* gene was performed as follows. The complete sequence of the *cneA* open reading frame (ORF) with its ribosome binding site was amplified from *Psav DAPP-PG* 722 genomic DNA using primers *cneA* compl 1 and *cneA* compl 2 (Table 2) and Q5® High-Fidelity DNA Polymerase (New England Biolabs, Hitchin, UK). The amplified fragment was purified from an agarose gel using the EuroGOLD Gel Extraction Kit (EuroClone, Milan, Italy) following the instructions of the manufacturer. After A-tailing (Promega, Fitchburg, WI, USA), the fragments were cloned in pGEM-T Easy vector (Promega, Fitchburg, WI, USA) and sequenced at Macrogen Europe. Having verified the correctness of the sequence, the *cneA* ORF was excised from pGEM-T Easy Vector using *XhoI* and *SpeI* and cloned in the corresponding sites of the plasmid pBBR MCS-5. The resulting plasmid (pBBR MCS-5-cneA; Table 2) was purified using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, MO, Saint Louis, USA) and transformed in *Psav-cneA* by electroporation, generating *Psav-cneA* mutant (pBBR-::cneA) (Table 2).

Chromosomal complementation of the *Psav-cneA* mutant was performed using the Tn7 transposon vector pUC18R6KT-miniTn7BB-Gm. The complete ORF of the *cneA* gene, including its own promoter, was amplified from *Psav DAPP-PG* 722 chromosomal DNA using the Expand High Fidelity PCR System (Roche, Mannheim, Germany) and the primers *PromAP_Fw* and *AP_Rev* (Table 2). The amplified DNA fragment was cloned in the pGEM-T Easy Vector (Promega, WI, Fitchburg, USA) and sequenced at GATC Biotech (Konstanz, Germany). Once verified the correctness of the sequence, the *cneA* gene was excised from pGEM using EcoRI and cloned in the corresponding site of the plasmid pUC18R6KT-miniTn7BB-Gm, yielding pUC18R6KT-miniTn7BB-cneA-Gm (Table 2) that was electroporated in *Psav-cneA*. Selection of the transconjugants in KB-Gm plates yielded the complemented strain *Psav-cneA* mutant (miniTn7::cneA) (Table 2). Insertion of the Tn7 transposon into the correct site was verified using the primers GlmS_savastanoi (hybridizing at the 3′ of the *glmS* gene) and the Tn7Rev primer (hybridizing at the Tn7R end of the integrated plasmid) (Table 2). Only in the case of integration, a 165 bp fragment was amplified.

**Phenotypic characterization of the *Psav-cneA* mutant and its complemented strains***

*In vitro* bacterial growth dynamics of wild-type *Psav* and the *Psav-cneA* mutant strains were carried out in KB liquid medium at 27 °C. Bacterial growth was spectrophotometrically followed every hour for 24 h at OD$_{660}$ and through colony counts at 4, 8, 20, 24 and 28 h post-incubation (hpi). For each bacterial strain, the relationship between the number of cells (log$_{10}$ transformed) and the hpi was investigated by means of a second-order polynomial model. Likelihood ratio test was used to assess the differences between wild type and *Psav-cneA* mutant strains under R statistical environment (R Core Team, 2018).

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**Note**

This text is a natural representation of the document. It does not include any additional notes or references beyond what is already included in the document.
The HR assay was carried out in Nicotiana tabacum (cv. Havana 425) plants. To prepare the inoculum, the strains were grown in NA at 27 °C for 48 h, resuspended in sterile deionized water and spectrophotometrically adjusted to 10^6 CFU/mL. About 10 µL of the bacterial suspensions or water (control) was infiltrated into the mesophyll of tobacco leaves using a needleless syringe. The appearance of the HR was scored at 24 hpi.

Proteolytic activity, swarming and swimming were determined as reported by Huber et al. (2001). Qualitative analysis of EPSs was tested on KB and LB solid medium amended with 5% of sucrose (LBS). Single colonies, previously obtained from NA plates, were streaked on KB and LBS and then grown at 28 °C for 48 h. Colonies producing EPSs showed a fluidal, mucoid appearance. Production of AHLs was performed in T-streak analysis as described by Piper et al. (1993) using the C. violaceum CVO26 as AHL biosensor. To measure biofilm formation, overnight cultures of Psav DAPP-PG 722, Psav-cneA mutant and Psav-cneA mutant (pBBR::cneA) grown in KB broth, were diluted to OD_600nm = 0.1 and loaded in a 96-well plate (150 µL per well, eight wells per strain). Plates were incubated under static or shaking conditions and biofilm formation was quantified by measurement of the absorbance at 595 after 24 h and 48 h after crystal violet staining (O'Toole and Kolter, 1998).

Pathogenicity test on olive plants

Disease severity and bacterial growth were tested in 1-year-old olive (cv. Frantoio) plants inoculated with the strains Psav DAPP-PG 722, Psav-cneA mutant, Psav-cneA mutant (pBBR::cneA) and Psav-cneA mutant (miniTn7::cneA) (Table 2). To prepare the inoculum, bacteria were grown on NA at 27 °C for 48 h, resuspended in sterile deionized water and adjusted spectrophotometrically to approximately 1 x 10^6 CFU/mL. Also, 20 µL of bacterial suspension or water (control plant) was placed in wounds (five per plant) made in the bark of olive plants using a sterile scalpel as previously described (Moretti et al., 2008). Wounds in the inoculated and control plants were protected with parafilm (American National Can, IL, Chicago, USA) until the developing knots break it (14 to 21 days). Plants were maintained in transparent polycarbonate boxes to reach high RH values (90%–100%) and kept in a growth chamber at 22 °C to 24 °C with illumination at 70 µE/m²/s and 12 h light period. The Psav population density was calculated at 0, 7, 14, 21 and 60 dpi by serial dilution of the bacterial suspension obtained from inoculated sites excised and homogenized by mechanical disruption and plated in NA medium. Colony counts were calculated 24 h and 48 h after incubation at 27 °C. The disease severity was recorded at 60 dpi by determining the knot volume, by measuring the length, width and depth of every knot with a Vernier caliper (Moretti et al., 2008). Four plant replicates were included in each of the two in planta experiments performed.

Transcriptional analysis of Psav pathogenicity and virulence genes

To verify whether Ca^{2+} entry promotes the expression of pathogenicity (hrpL, hrpA and iaaM) and virulence (ptz) genes of Psav, transcriptional fusions of their promoters were constructed with LacZ reporter gene. For amplification of the iaaM and ptz promoters, the regions upstream of the iaaM and ptz ORFs (477 bp and 373 bp, respectively) were amplified by PCR using primers iaaM For, iaaM Rev, ptz For and ptz Rev (Table 2). Amplicons were cloned into pMP220 in order to obtain promoter fusions to LacZ. The resulting plasmids and those encoding the hrpL and hrpA promoters fused to lacZ (Aragón et al., 2014) were transferred by conjugation into both wild-type Psav DAPP-PG 722 and its cneA mutant. Cells carrying the plasmids grown overnight in KB media were diluted in the same media and incubated at 28 °C to OD_600 of 0.5 (time = 0). The cultures were harvested by centrifugation, washed twice with 10 mM MgCl2, and the cells were transferred to Hrp medium (Huynh et al., 1989), HBSS and HBSS amended with CaCl2. The cultures were adjusted to OD_600 of 0.5 and incubated for 6 h at 28 °C. β-galactosidase enzymatic activity was measured using the methods developed (Miller, 1972) and modified previously (Maloy, 1990). Psav DAPP-PG 722 and its cneA mutant transformed with pMP220 (encoding a promoterless lacZ) were used as negative controls. To determine the activity associated exclusively to the promoter fusions to lacZ, the background activity detected in the control strains was subtracted from those obtained for each of their corresponding transformants.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors have declared that no conflict of interest exists.

Research Involving Human Participants and/or Animals: This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

Informed consent: Informed consent was obtained from all individual participants included in the study.
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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Maximum likelihood tree based on the nucleotide sequence of the cneA gene showing the phylogenetic relation within the P. syringae complex. Phylogroup (PG) designations are indicated on the appropriate branches. Numbers at branching points are bootstrap percentages based on 1000 replications. Psy = Pseudomonas syringae; Psav = Pseudomonas savastanoi; Pca = Pseudomonas cannabina and P = Pseudomonas.

Fig. S2 In vitro growth on KB medium of Pseudomonas savastanoi pv. savastanoi (Psav) DAPP PG 722 (wild type [wt]) and the calcium exchanger Psav mutant (Psav cneA mutant). Number of cells (mean ± SE) and fitted polynomial models of wt (closed squares, solid line; fitted model: y = −0.004x² + 0.359x + 3.876) and Psav cneA mutant (open circles, dashed line; fitted model: y = −0.005x² + 0.410x + 3.664). Standard error bars are not visible in the plot as their values are smaller than the dimensions of the closed squares and open circles.