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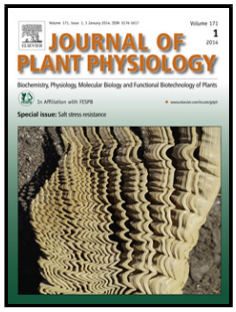

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PII: S0176-1617(18)30649-7
DOI: https://doi.org/10.1016/j.jplph.2018.09.014
Reference: JPLPH 52850

To appear in:

Received date: 4-1-2018
Revised date: 21-9-2018
Accepted date: 22-9-2018

Please cite this article as: Vilas JM, Romero FM, Rossi FR, Marina M, Maiale SJ, Calzadilla PI, Pieckenstain FL, Ruiz OA, Gárriz A, Modulation of plant and bacterial polyamine metabolism during the compatible interaction between tomato and *Pseudomonas syringae*, *Journal of Plant Physiology* (2018), https://doi.org/10.1016/j.jplph.2018.09.014

Modulation of plant and bacterial polyamine metabolism during the compatible interaction between tomato and *Pseudomonas syringae*

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Abstract

The polyamines putrescine, spermidine and spermine participate in a variety of cellular processes in all organisms. Many studies have shown that these polycations are important for plant immunity, as well as for the virulence of diverse fungal phytopathogens. However, the polyamines' roles in the pathogenesis of phytopathogenic bacteria have not been thoroughly elucidated to date. To obtain more information on this topic, we assessed the changes in polyamine homeostasis during the infection of tomato plants by *Pseudomonas syringae*. Our results showed that polyamine biosynthesis and catabolism are activated in both tomato and bacteria during the pathogenic interaction. This activation results in the accumulation of

putrescine in whole leaf tissues, as well as in the apoplastic fluids, which is explained by the induction of its synthesis in plant cells and also on the basis of its excretion by bacteria. We showed that the excretion of this polyamine by *P. syringae* is stimulated under virulence-inducing conditions, suggesting that it plays a role in plant colonization. However, no activation of bacterial virulence traits or induction of plant invasion was observed after the exogenous addition of putrescine. In addition, no connection was found between this polyamine and plant defence responses. Although further research is warranted to unravel the biological functions of these molecules during plant-bacterial interactions, this study contributes to a better understanding of the changes associated with the homeostasis of polyamines during plant pathogenesis.

Abbreviations: PAs, polyamines; Put, putrescine; Spd, spermidine; Spm, spermine; T3SS, type III secretion system; GABA, γ-amino-butyric acid; ODC, ornithine decarboxylase; ADC, arginine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase; dcSAM, decarboxylated S-adenosyl-methionine; SAMDC, S-adenosyl-methionine decarboxylase; DAO, diamine oxidase; PAO, polyamine oxidase; PuO, putrescine oxidase; PAT, putrescine transaminase; GPS, γ-glutamyl-Put synthases; PR1, pathogenesis-related protein 1; HrpL, RNA polymerase sigma factor HrpL; AvrPto, bacterial effector AvrPto; Pto, *P. syringae* pv. tomato DC3000; hpi, hours post-infiltration, AFs, apoplastic washing fluids.

Keywords: polyamines, tomato, *Pseudomonas syringae* pv. tomato DC3000, apoplast.

Introduction

Pseudomonas syringae is a phytopathogenic bacterium that causes disease on a broad range of economically important plant species (Mansfield et al., 2012). This bacterium survives saprophytically on the plant surface, and after the entering plant tissues through wounds or natural openings (such as stomata), it reproduces endophytically in the apoplastic space. As occurs with many other endophytic phytopathogens, a complex molecular dialogue is established at the site between the plant and bacteria, which turns the apoplastic space into the primary battleground for the pathogenic interaction (Pfeilmeier et al., 2016).

It has been shown that the infection of tomato plants by *P. syringae* induces remarkable changes in the concentration of different apoplastic components, such as aromatic amino acids, phenylpropanoids and the non-proteinogenic amino acid γ-amino-butyric acid (GABA) (Rico and Preston, 2008). Interestingly, phytopathogenic strains of *P. syringae* are well adapted to use these compounds as nutrients, a trait that is not present in non-pathogenic Pseudomonads (Rico et al., 2011). These results reinforce the paradigm that endophytic microorganisms assure their survival in plant tissues by adapting their metabolism to primarily utilize apoplastic nutrients. More recently, footprinting experiments have also shown that minor components of the apoplast are preferentially consumed before others. Thus, *P. syringae* pv. *phaseolicola* metabolizes malate, glucose and glutamate before switching its metabolism to the use of more abundant nutrients (O'Leary et al., 2016).

The apoplastic concentration of the polyamines (PAs), such as diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm) is also modulated during the interactions between plants and microorganisms. These ubiquitous compounds are involved in a wide range of cellular functions, probably due to their interaction with polyanions, such as proteins, phospholipids and nucleic acids (Michael, 2016). In plants and bacteria, the synthesis of PAs starts with the decarboxylation of the amino acids ornithine and arginine by the enzymes ornithine (ODC) and arginine (ADC) decarboxylase, respectively (1 and 2 in Figure 1). While ornithine decarboxylation directly results in Put, the decarboxylation of arginine produces agmatine, which is later metabolized to Put. The anabolic route continues with two consecutive aminopropylation reactions catalysed by the enzymes Spd and Spm synthase (SPDS and SPMS, respectively) that convert Put into Spd and Spm (3 and 4 in Figure 1). Two molecules of decarboxylated S-adenosyl-methionine (dcSAM) are used as aminopropyl donors in these reactions, which are obtained from the decarboxylation of SAM by the enzyme SAM decarboxylase (SAMDC).

In addition to their synthetic pathways, the catabolism of these compounds is essential for PA homeostasis. In plants, the oxidation of Put is conducted by copper-containing diamine oxidases (DAOs), while Spd and Spm are oxidized by PA oxidases (PAOs) that require FAD as a cofactor (Tavladoraki et al., 2016). In bacteria, our knowledge of the catabolism of Spd and Spm is limited, even though the routes involved in the degradation of Put have been studied in some depth. Interestingly, in addition to the existence of the Put oxidases (PuO, step 6 in Figure 1), such as those that occur in plants, two other alternative pathways were described for

the degradation of Put in these microorganisms. One of these pathways involves the action of Put transaminases (PAT, step 5 in Figure 1), which transfer the amino moiety from Put to either α-ketoglutarate or pyruvate, producing glutamate or alanine, respectively. Alternatively, Put could be oxidized after its conjugation with glutamic acid (step 7). The glutamylation of Put is conducted by γ-glutamyl-Put synthases (GPSs) at the expense of one molecule of ATP (step 8). All of these pathways converge in the production of GABA (Figure 1) that enters the tricarboxylic acid cycle after its conversion to succinate.

A substantial amount of research has been directed to the role played by PAs in the activation of plant defence responses (Jimenez-Bremont et al., 2014). Many studies have demonstrated that the increase of these amines in the apoplast plays an important role in this process. For instance, it was shown that the accumulation and oxidation of Spm in the apoplast of tobacco is conducive to the activation of protein kinases and induces the expression of pathogenesis-related genes. These responses are required to assure plant protection against several pathogens, such as the tobacco mosaic virus (TMV), *P. syringae* pv. *tabaci* and *Phytophthora parasitica* var. *nicotianae* (Moschou et al., 2008; Moschou et al., 2009; Yamakawa et al., 1998). Similarly, Marina et al. (2008) showed that apoplastic PAO activity is induced during biotic stress in tobacco leaf disks and that the inhibition of this activity prevented infections caused by the phytopathogenic bacterium *Pseudomonas viridiflava*.

In turn, PAs were demonstrated to be important for fungal phytopathogens. Thus, perturbation of PA biosynthesis affects the growth and differentiation of these microorganisms, which could lead to a reduction in their virulence (Bailey et al., 2000; Gárriz et al., 2004; Garriz et al., 2008; Guevara-Olvera et al., 1997; Mueller et al., 2001; Pieckenstain et al., 2001). A recent study by Lowe-Power *et al.* (2018) constitutes the first report using a phytopathogenic bacterium as a study model. Interestingly, these authors demonstrated that ODC activity is essential for the virulence of *Ralstonia solanacearum* in tomato, and most importantly, that the bacteria excrete Put as a virulence factor when multiplying in the xylem vessels. The treatment of plant roots and leaves with Put increased the spread of the pathogen and accelerated the appearance of disease symptoms. This previously unexplored aspect of the PAs produced by pathogens provides a new perspective on their functions during plant-microbe interactions. We envision that similar virulence strategies could be deployed by endophytic phytopathogens, such as *P. syringae*.

In this study, we conducted an integrative examination of the changes associated with the metabolism of PAs during the interaction between tomato and *P. syringae* pv. tomato DC3000 (Pto). We found that Put accumulates at the plant apoplast during bacterial colonization, which is associated with the activation of the metabolism of PAs in both interacting organisms. Interestingly, we showed that Pto, as well as *R. solanacearum*, secretes Put to the extracellular environment, even though this process does not seems to be related to the development of the pathogenic process. The implications of such findings are discussed.

Materials and Methods

Biological material and growth conditions

The seeds of the tomato cultivar Río Grande and the lines of *A. thaliana* used in this study were germinated in a vermiculite-soil-sand- mix (1:1:1) and cultivated in a growth chamber with a 16 h day/8 h night photoperiod (photon flux density of 200 μmol m s^{-1} provided by cool-white fluorescent lamps), 24/21 ± 2°C and 55/65 ± 5% day/night temperature and relative humidity, respectively. The plants were regularly watered with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Four week-old plants were used in all the experiments. *Arabidopsis thaliana* ecotype Columbia-0 and a T-DNA insertion mutant in the *AtAO1* gene (SALK_077391) were provided by Dr. A. A. Rodriguez (UB1-IIB-INTECh, UNSAM-CONICET, Argentina).

Pseudomonas syringae pv. *tomato* strain DC3000 (Pto) was routinely grown in LB media at 28°C. Inocula were prepared by growing the bacteria in LB agar for 48 h and scraping the cells into sterile water. When indicated, the bacterial cells were inactivated by heat treatment. This was achieved by incubating the cells at 70°C for 15 min. The hrp-inducing minimal medium (HIM) was prepared as described by Kim et al. (2009). In all cases, the media were supplemented with rifampicin at a final concentration of 50 μg mL^{-1}. For *in vitro* cultures, 3 mL of LB, HIM or apoplastic washing fluids (AFs) were inoculated with Pto at a final OD_{600} of 0.01 and incubated at 28°C with shaking for 6 h.

Plant inoculation and recovery of the AFs

Tomato or *A. thaliana* leaves were detached from the plants and immediately infiltrated with bacterial suspensions under vacuum pressure as described by Katagiri et al (2002) for *Arabidopsis* plants. Mock-inoculated controls were infiltrated with water. Infiltrated leaves

were maintained in water agar (0.8%) in a growth chamber, and the samples were removed after 6 and 24 h. *In planta* bacterial growth was evaluated after 48 h as described by Marina *et al*. (2008). Briefly, three to five discs (0.5 cm in diameter) were cut from each leaf and ground in 250 μL distilled water. These extracts were serially diluted and plated onto LB agar media. The number of CFU was determined after 24 h incubation at 28°C. Leaf discs infiltrated with distilled water were used as the controls.

The AFs were obtained as described by O'Leary (2014) with some modifications. Infiltrated leaves were rolled-up and inserted into 20 mL-syringes, which were then introduced into 50-mL polyethylene tubes. Syringes in tubes were centrifuged at 2,000 x g for 20 min at 4°C. The AFs recovered were subjected to additional centrifugation at 12,000 x g for 10 min to remove the cells or particulate matter and stored at -20°C until use. Cytosolic contamination was measured by determining the concentration of the chlorophyll. Based on the values of the chlorophyll concentrations, we determined that under our experimental conditions, the cytosolic contamination of the AFs were approximately 0.6 and 1% in mock-inoculated and Pto-inoculated samples, respectively.

Free PA quantification

Free PAs were extracted from whole leaves by grinding the samples in 5% perchloric acid (200 mg FW in 600 μL). Bacterial pellets were resuspended in 100 μL of 5% perchloric acid and incubated in an ice bath for 24 h. All the samples were centrifuged at 10,000 x *g* for 15 min, and aliquots of 60 μL of the supernatants or the same volume of the AFs were removed for the derivatization procedure. Six microlitres of 100 μm 1,7-diamino heptane were added as the internal standard. Sixty microlitres of saturated Na_2CO_3 and 75 μL of dansylchloride (10 mg mL^{-1} acetone) were added, and the mixture was incubated in the dark at 55ºC for 2 h. The reaction was terminated by adding 25 μL proline (100 mg mL^{-1}). After 30 min, dansylated PAs were extracted in 200 μL toluene. The organic phase was separated and vacuum-evaporated. PA standards were treated in the same way as the samples. Dansylated PAs were dissolved in 40 μL acetonitrile and analysed using HPLC (Marcé et al., 1995). The protein concentration in the plant and bacterial samples was determined by conducting a Bradford assay using BSA as the standard (Bradford, 1976).

Gene expression profiling

Total RNA was extracted from the tomato leaves or bacterial cells using the TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) using the manufacturer's instructions. Total RNA was then reverse–transcribed using the following mixture: 2 μg of total RNA, 0.5 mM dNTPs, 1 μL of Moloney murine leukaemia virus RT (200 units μL^{-1}; Promega Corporation, Madison, WI, USA), 5 μL reaction buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, and 50 mM DTT, pH 8.3), Ten picomoles of oligo (dT) primer (Biodynamics SRL, CABA, Argentina) and distilled water were brought to a total volume of 25 μL. The reaction mixture was incubated at 37 °C for 1 h. One microlitre of a 1:5 dilution of cDNA was diluted to 7.5 μL with primer mix (300 nM final concentration), and the same volume of FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) was added to complete a final volume of 15 μL. The primers used in these reactions are listed in Table S1. Real–time PCR was performed on a Mx3005P qPCR System using the MxPro qPCR Software 4.0 (Stratagene, La Jolla, CA, U.S.A). The following programme was used: 94°C for 10 min, and 40 cycles of 94°C for 30 s followed by 60°C for 1 min. Relative quantification was performed using the comparative cycle threshold method with EF-1α and GyrB genes as endogenous controls in the plant and bacterial samples, respectively.

Determination of fluorescence transient of chlorophyll a and gas exchange

The leaves were infiltrated with water (controls), a 1 mM solution of Put, and suspensions of Pto (OD_{600}=0.001) in water (Pto) or Put 1 mM (Pto/Put). Infiltrated leaves were placed in Petri dishes containing water agar (0.8% w/v) and incubated in a growth chamber for 24 h. The chlorophyll a fluorescence transient (OJIP tests) was measured using a portable Plant Efficiency Analyzer (PEA, Hansatech Instrument Ltd., UK) as described by Cheng *et al* (2016). All the leaves were dark-adapted before measurements for 20 min. and then exposed for 3 s to light at an intensity of 3500 mol m^2s^{-1}. Stomatal conductance was measured with the use of a TPS-2 (Portable Photosynthesis, MA, USA) using an LED light with an intensity of 1780 mol $m^{-2}s^{-1}$ as described by Babuin *et al*. (2014). Measurements were performed in six leaves per treatment.

Statistics

Each experiment was conducted at least twice with similar results, and representative experiments are shown. The results are presented as the means ± standard deviation or standard

error, as indicated in the corresponding figures. The data were analysed using Student's t test. The QRT-PCR results were analysed using REST© software V 2.0.7 (Pfaffl et al., 2002).

Results

Tomato and bacterial PA metabolism genes are induced during infection

We first analysed the variation in the steady-state mRNA levels during bacterial infection of some of the most important tomato genes participating in the synthesis and catabolism of PAs. We analysed the *ADC, ADC1* and *ODC* genes previously studied by Song *et al.* (2015), as well as other transcripts encoding SAMDC, SPMS, DAO and PAO that we found in the tomato genome sequence using known plant sequences as queries. As shown in Figure 2, bacterial infiltration of the leaves induced the expression of all the genes examined. These results suggest that a high rate of PA turnover is induced during pathogenic conditions. In particular, we found a notable up-regulation of the biosynthetic genes *ADC1, ODC* and *SAMDC* (40-, 60- and 53-fold increase, respectively). In addition, we verified the activation of the plant defence responses by assessing the expression of the transcript encoding pathogenesis-related protein 1 (PR1), a well-known molecular marker associated with the salicylic acid response. Our results showed a 5-fold activation of PR1 in the tomato leaves upon pathogen attack (Figure 2).

Subsequently, the genome of Pto was analysed to identify the genes encoding enzymes for the PA metabolism. We identified homologous genes for the biosynthetic enzymes *ADC* and *ODC*, as well as the catabolic enzymes PAT and PuO. In addition, we identified three genes with homology to GPSs, the enzymes involved in the conjugation of Put to glutamic acid. Unfortunately, a large proportion of the primers designed to analyse these genes resulted in the co-amplification of plant sequences under our experimental conditions, which negated the study of bacterial gene expression in the plant tissues. Despite this, we were able to assess the *in planta* expression of the catabolic genes *GPS1, GPS2, GPS3* and *PuO* (Figure S1). This analysis indicated that even though *GPS1* expression is reduced at 24 and 48 hpi, the expression of all the other catabolic genes is up-regulated, suggesting that Put degradation by bacteria is activated during plant colonization.

To study the regulation of the bacterial PA metabolism during pathogenesis in more detail, we compared gene expression levels in bacterial cells growing in LB media with those in cells cultivated under pathogenicity-inducing conditions. This was accomplished by growing

the cells in culture media, such as HIM (*hrp*-inducing medium) or AFs, which are known to stimulate common traits associated with pathogenicity (Rico and Preston, 2008). We corroborated this by assessing the induction of the T3SS-associated genes *hrpL* and *avrPto* (encoding an alternative sigma factor and a bacterial effector, respectively, see Figure 3). Interestingly, most of the genes encoding catabolic enzymes, such as *GPS2*, *GPS3*, *PuO* and *PAT*, as well as the biosynthetic gene *ODC*, were induced in the AFs, while comparable effects were observed in the HIM-growing cells. These results suggest that the PA metabolic genes are regulated by stimuli that participate in the elicitation of virulence.

Pto infection provokes the accumulation of Put in the plant apoplast

We evaluated the modifications of the PA contents in plant tissues during bacterial infections. For this purpose, tomato leaves were infiltrated with water (controls) or a suspension of Pto (OD_{600}=0.001), and samples were removed after 6 and 24 h to determine the free PAs. Under these conditions, the leaves remained symptomless throughout the whole experiment.

Spd was the most abundant PA in the total leaf extracts of the control, followed by Put and Spm (Figure 4A, 0 hpi). Infiltration with water or Pto resulted in a 40% reduction in Spd at 6 hpi. However, the initial values of this PA were recovered at 24 hpi. In addition, a small but significant reduction in the levels of Spm was detected at 24 hpi in Pto-treated leaves. Put concentrations were not altered by any treatment at 6 hpi, although a significant increase was observed at 24 hpi (4-fold increase compared to levels at 0 hpi). Therefore, Put and Spd represented the most abundant PAs in the control AFs (Figure 4B, 0 hpi). Interestingly, the concentration of Put increased 2- and 4-fold after infiltration with Pto at 6 and 24 hpi, respectively, while the concentration of the other PAs was not altered at any time.

To attempt to assess the contribution of the plant PA metabolism to the increase of the Put levels, we determined the whole leaf PA contents after the infiltration with heat-inactivated Pto cells (hiPto). Two different cell densities were used: 1 x 10^6 cells mL^{-1} ($hiPto_{0.001}$) and 2 x 10^7 cell mL^{-1} ($hiPto_{0.02}$). We used these cell suspensions, because they represent approximately the same bacterial levels that we usually found in tomato leaves at the beginning and end following 24 h of infection, respectively. It is worth noting that the amounts of the PAs in the suspensions of the hiPto cells were minor compared to the levels of the PAs in the AFs (data not shown). As shown in Figure S2, leaves infiltrated with hiPto show similar PA levels to those in the control leaves. These results suggest that Put only accumulates in apoplasts in the

presence of actively multiplying bacteria and that this phenomenon does not depend on plant responses triggered by pathogen-associated molecules.

Since the apoplast is the primary localization of Pto in plants, we hypothesized that the bacterial metabolism could contribute to increased Put at this site. To investigate this hypothesis, we grew bacterial cells *in vitro* in LB, HIM and AFs and determined the concentration of the PAs in the cells, as well as in the extracellular medium. As shown in Figure 5A, no differences were observed in the intracellular PA concentrations between the cells growing in the LB and HIM media. Consequently, the intracellular PAs were drastically reduced in the cells growing in the AFs. Considering that the tomato AFs provide a complete medium for Pto (Rico and Preston, 2008) and that the multiplication rate in this medium is relatively lower but close to that shown by cells growing in LB (Figure S3), we concluded that the reduction in the intracellular levels of PAs is due to a physiological response elicited by the culture medium rather than nutrient depletion. A very interesting scenario was observed when the extracellular PAs were assessed (Figure 5B). In this case, our experiments showed that cells are able to secrete Put when growing in LB or the AFs. Thus, this PA reached a concentration of approximately 24 μmoles mL^{-1} and 82 μmoles mL^{-1} in the LB and AFs media, respectively, while the concentrations of Put in the non-inoculated media were 1.3 μmoles mL^{-1} for LB and 14.3 μmoles mL^{-1} for the AFs. In addition, Spd and Spm did not show any changes between the inoculated and non-inoculated media, suggesting that these PAs are not consumed or secreted during bacterial growth. The extracellular PAs were absent in the HIM after bacterial cultivation, which indicates that the excretion of these compounds is not activated by the same mechanisms that regulate T3SS expression.

In summary, these results suggest that Put excretion by bacteria could substantially contribute to the increased apoplastic Put contents during the tomato-Pto interactions.

Put does not modulate tomato immunity or bacterial pathogenesis

To test the effects of Put on the hormonal signalling networks implicated in plant defence, we infiltrated leaves with this PA and assessed the expression of *PR1*, *PinII*, *Le4* and *GluB*. These genes are known to respond to defence hormones, such as salicylic acid, jasmonic acid, abscisic acid and ethylene, respectively (Martinez-Medina et al., 2013). As shown in Figure 6A, the expression of these genes was not regulated after Put infiltration, suggesting

that this molecule does not have a significant role in the elicitation of plant defence responses in tomato.

We also verified the effects of Put in the different processes associated with bacterial virulence. Thus, we first determined the effect of Put on the expression of *avrPto* in cells growing in LB and HIM. As shown in Figure 6B, the transcription of this gene was not altered by the amendment of the PA, indicating that it does not regulate the expression of the T3SS. In addition, this PA had no effect on the emission of fluorescence by a bacterial strain expressing the *GUS* reporter gene under the control of the *hrpA* promoter, a known regulator of the expression of *hrp* genes (data not shown). Additionally, the amendment of Put to the culture media did not affect any other important processes associated with bacterial virulence, such as biofilm formation or swimming (data not shown). These results indicate that bacterial virulence is not regulated by the external concentrations of Put.

Photosynthesis inhibition is not ameliorated by Put

The assessment of transient chlorophyll *a* fluorescence has been used as an *in vivo* non-intrusive method to evaluate the functionality of phostosystem II (PSII) under different stress conditions (Baron et al., 2016). It has been demonstrated that phytopathogens perturb the activity of this system, which is related to a reduction in the maximum quantum yield of primary PSII photochemistry (Fv/Fm). In turn, PAs were demonstrated to be effective in maintaining thylakoid membrane integrity and retarding the loss of the light-harvesting chlorophyll protein complex from PSII (Walters et al., 2008). Thus, we performed an OJIP analysis to clarify the effect of Put on PSII activity during the interaction between the tomato and Pto. As shown in Figure 7A, leaf infiltration with Put did not alter any of the photosynthetic parameters examined, demonstrating that it does not effect the photosynthetic system. In contrast, infiltration with Pto or the co-infiltration of Pto plus Put (Pto/Put) resulted in remarkable and similar negative effects on PSII activity, as demonstrated by increases in the values of the dissipated energy flux (DI_0/RC), as well as the reduction in the performance index (PIabs), Fv/Fm, and electron transport (ET_0/Rc) (Figures 7B and C).

Alternatively, stomatal conductance (gs) was also quantified to determine the effect of Put on gas exchange efficiency (Figure 7D). Our results demonstrate that treatment with Put has no effect on gs and that this parameter increases to similar degrees in both Pto- and Put/Pto-infiltrated leaves. These results are consistent with previous studies demonstrating that Pto

actively opens plant stomata by producing the phytotoxin coronatine, a virulence factor that facilitates entry into the host tissues (Melotto et al., 2008).

Put treatment and inhibition of its synthesis does not affect bacterial growth in plant tissues

Since Put abundance is increased in the apoplast during Pto infection, we hypothesized that it could play an important role in the interaction between this bacterial strain and tomato plants. To evaluate this assumption, we compared the CFU levels recovered from the leaves infiltrated with inocula prepared with and without 1 mM Put. In addition, to test whether the oxidation of this PA is important, we evaluated the effect of the same concentration of the amino oxidase inhibitor 2-bromo-ethylamine. As shown in Figure 8, neither of these treatments affects the rate of multiplication of the bacteria. To address this issue in more detail, we tested the susceptibility of an *Arabidopsis* mutant line to Pto. The mutant contained a T-DNA insertion in the *atao1* gene, which encodes an apoplastic DAO isoform (Planas-Portell et al., 2013). Consistent with the results described above, our experiments showed that the phenotype of this line does not differ from that of the wild type Col-0 line (Figure S4), suggesting that plant Put oxidase activity is dispensable for disease development.

Discussion

A growing body of research demonstrates that PAs play important roles in the regulation of plant defence responses against microbial pathogens (Jimenez-Bremont et al., 2014). Most of the effects of these compounds are explained on the basis of the production of H_2O_2 through PA oxidation (Angelini et al., 2010), even though oxidation-independent mechanisms were also described (Gonzalez et al., 2011; Yamakawa et al., 1998). Strikingly, the majority of the studies on this field disregarded the participation of the microbial metabolism in the modifications of the levels of PAs or enzyme activities detected in plant-pathogen systems. In some cases, it is valid to assume that the contribution of microbes is negligible, but researchers should be cognizant that microorganisms could produce significant levels of endogenous PAs (and enzymes with high catalytic activities) that, if not considered, may lead to erroneous conclusions. This issue can be more important when compartments of plant tissues are under assessment, i.e., niches colonized by pathogenic bacteria, such as the apoplast and xylem vessels. We addressed this matter by integrating the analysis of the

metabolism of PAs during the interaction between the tomato plants and Pto, together with the assessment of the plant responses to non-viable cells and bacteria growing in virulence-inducing media. These strategies could help to discern the individual mechanisms influencing the levels of PAs in pathosystems.

PA metabolism in the tomato – *P. syringae* pathosystem

Our results demonstrate that PA biosynthetic and catabolic genes are induced in tomato plants during the interaction with *P. syringae*. These findings are consistent with previous studies showing that plants induce the synthesis and degradation of PAs in response to pathogens, which appears to be instrumental for protection (Jimenez-Bremont et al., 2014). For example, ADC, ODC, SAMDC and DAO activities were reported to be up-regulated in a TMV resistant tobacco line during viral infection, while no change was observed in a susceptible line (Marini et al., 2001). In addition, the over-expression of the genes from the PA metabolism has been used as a strategy to promote plant tolerance to pathogens. Therefore, the over-expression of the oat *ADC* gene in eggplants leads to an increase in the concentration of PAs and the induction of DAO activity, which was associated with resistance to fungal wilt caused by *Fusarium oxysporum* (Prabhavathi and Rajam, 2007). In addition, the constitutive expression of *SAMDC* and *PAO* from cotton in *Arabidopsis* increased its tolerance to *Verticillium dahliae* (Mo et al., 2015; Mo et al., 2016), while an *Arabidopsis* line over-expressing the endogenous *SPMS* gene exhibited higher tolerance to *Pseudomonas viridiflava* (Gonzalez et al., 2011). In contrast, silencing or deleting these genes increased the susceptibility of the plants (Mo et al., 2015; Mo et al., 2016). In addition, we demonstrate that the tomato PA genes are not induced by infiltration with heat-inactivated Pto bacteria in this study. Thus, it appears that the recognition of common pathogen-derived molecules is not sufficient to engage a full response at the level of the plant PA metabolism. However, since the defence gene PR1 was also not regulated under these experimental conditions, it is possible that earlier changes in gene expression could have been missed. A more thorough analysis of gene expression should help to discern whether the plant PA metabolism participates in defence responses elicited by common microbial effectors.

Interestingly, our analysis also showed that bacterial genes from the PA biosynthetic and catabolic pathways are up-regulated under virulence-inducing conditions, such as cell culture in the AFs. This finding suggests that PA turnover is required for pathogenesis in *P.*

syringae. The connection between the metabolism of PAs and pathogenesis has been described in others bacteria. Thus, the induction of *ADC* was associated with the adaptation to acid stress in *Salmonella typhimurium* and *Helicobacter pylori* (Alvarez-Ordonez et al., 2010; Valenzuela et al., 2014); ADC activity was demonstrated to be essential for biofilm formation in *Bacillus subtilis* and swarming in *Proteus mirabilis* (Burrell et al., 2010; Sturgill and Rather, 2004), while both *ADC* and *ODC* expression were involved in biofilm formation in *Yersinia pestis* (Wortham et al., 2010). In addition, an *ODC*-deficient strain of *Bordetella* spp. was demonstrated to be perturbed during the production of the siderophore alcaligin, which is critical for successful host colonization (Brickman and Armstrong, 1996, 2007). Importantly, an *odc* mutant strain in *R. solanacearum* also showed reduced virulence in tomato plants (Lowe-Power et al., 2018). The characterization of *P. syringae* mutant strains in the PA metabolic genes would be an interesting approach to unravel the importance of these compounds in bacterial virulence.

Overall, the quantification of the PA levels in our experimental model demonstrated that Put accumulated in the leaf tissues and AFs during plant infection. This could be a consequence of a higher synthesis rate and/or a reduction in its catabolism by both plant and bacterial enzymes. The expression of the plant PA metabolic genes is consistent with the first explanation, since a considerable up-regulation in the *ADC*, *ADC1* and *ODC* genes was detected upon infection. Even though the catabolic gene *DAO* was also up-regulated, the level of induction was much lower than that in the biosynthetic genes. Similarly, the genes from the bacterial PA metabolic pathway were up-regulated, but this response was relatively less significant than in the plant genes. Therefore, our results suggest that both organisms activate Put biosynthesis to increase its concentration in the plant tissues, even though the plant metabolism probably contributes to a much higher extent than the bacterial metabolism.

Put is not involved in the defence of tomato plants to *P. syringae*

Studies conducted in different plant pathosystems showed that the rise in free PA levels that occurs after pathogen recognition is accompanied by the concomitant increase in conjugated PAs (Rodríguez-Kessler et al., 2008; Walters, 2003). Importantly, PAs conjugated to organic acids were shown to play important roles in plant resistance due to their antimicrobial activity and ability to reinforce plant cell walls (Martin-Tanguy, 2001; Muroi et al., 2009; Walters et al., 2001). Whether a rise in the concentration of conjugated PAs occurs

during tomato-*P. syringae* interactions merits additional research. Apoplastic PAs may also have a remarkable significance for plant defence. In particular, the roles played by apoplastic Spm have been deeply investigated, demonstrating that it plays a pivotal role in the defence of tobacco against TMV. For instance, Yamakawa et al. (1998) showed that viral infection induces the accumulation of apoplastic Spm in tobacco, which was shown to be required for the up-regulation of acidic and basic pathogenesis-related proteins. In addition, treatment with exogenous Spm in different plant species leads to the up-regulation of a wide array of genes involved in pathogen perception and defence (Gonzalez et al., 2011). A few studies have also assigned a key role for Put in the elicitation of plant defence responses. Therefore, Kim *et al* (2013) demonstrated that the susceptibility of an *adc2* deletion mutant in *Arabidopsis* to *P. syringae* is attenuated by Put, suggesting that this PA can stimulate plant protection. However, our analysis indicates that Put may have little incidence in the tolerance of tomato plants to *P. syringae* invasion. This is based on the observation that the Put treatment was unable to activate the expression of the key genes from the defence hormone pathways. However, since this experiment was conducted in plants unchallenged with the pathogen, the possibility that Put could participate in the elicitation of plant defence responses under pathogenic conditions cannot be ruled out. In fact, Pto-infiltration activated the induction of the plant *SPDS* and *PAO* genes, implying that only under these conditions could Put be transformed into Spd, which could be later oxidized to release H_2O_2 (an essential trait in the elicitation of defence). Another possibility is that Put could be oxidized by the DAO enzymes localized in the apoplast, enabling the production of H_2O_2 similarly to studies in *Arabidopsis*. However, this possibility seems unlikely, since we were unable to detect DAO activity in the AFs from *P. syringae*-infected tomato leaves (data not shown). In addition, the treatment of tomato leaves with the DAO inhibitor 2-bromomethylamine has no effect on bacterial colonization. Consistent with this, we also showed that the absence of the *atao1* gene (encoding an apoplastic DAO isoenzyme) in *Arabidopsis* did not alter the susceptibility to the plant to *P. syringae*.

We also explored the impact of Put on the activity of PSII, since positive effects of PAs on the photosynthetic rate have been reported in different plant species (Demetriou et al., 2007; Shu et al., 2012). Thus, it could be argued that Put accumulation could enhance plant performance during pathogenesis by ameliorating the negative effects of infection on PSII activity. However, our results show that the PA does not affect the photosynthetic rate under control conditions, and it is unable to avoid the inhibitory effects provoked by the bacterial

invasion. Therefore, we conclude that the increase in the concentration of Put in the plant tissues could still be important for plant fitness or immunity, but without direct relation to the photosynthetic process.

Put does not promote *P. syringae* invasion

The observation that Pto contributes to elevate the extracellular concentration of Put in the apoplast also suggests that it could be important for bacterial fitness. Consequently, it is tempting to speculate that the PA would act as a signal molecule modulating important processes associated with bacterial virulence. However, this hypothesis does not seem to be plausible, since the addition of Put to the culture media could not stimulate *hrp* gene expression in the bacterial cells, swimming or biofilm formation.

Alternatively, the stimulation in Put export could favour the adaptation of the pathogen to the apoplastic space. Consistent with this, a recent study by Lowe-Power *et al.* (2018) has shown that Put excretion at the xylem vessels is important for the virulence of *R. solanacearum*. These authors hypothesized that the secretion of this PA possibly acts by attenuating the negative effects generated by the reactive oxygen species produced by plants as a defence response. Consequently, it has been shown that extracellular PAs exert positive effects on bacterial viability. For instance, exogenously added PAs induced the expression of the genes required for the tolerance to bactericidal antibiotics and oxidative stress in *E.coli* (Tkachenko et al., 2012). Similarly, El-Halfawy and Valvano (2014) demonstrated that the synthesis and excretion of Put alleviated the effects of the oxidative stress induced by antibiotics in the human pathogen *Burkholderia cenocepacia*. If this is also true in our experimental model, Put would protect *P. syringae* cells during the colonization of the apoplast and favour disease development. However, our results showed that the growth of *P. syringae* was not stimulated further in the leaves infiltrated with Put. It should be considered that the amounts of Put accumulated in the plant tissues might be sufficient to fulfill the roles played by this PA during the pathogenic interaction. Thus, increasing the concentration of Put beyond those levels would not necessarily affect bacterial multiplication. Therefore, further studies should focus on the phenotype of the Pto mutant strains unable to synthesize and secrete Put to the extracellular space.

Concluding remarks

Since plants and microbes can produce PAs and need to preserve PA homeostasis for survival and stress responses, there is a remarkable complexity underlying the study of this metabolism in plant-microbe interactions. We found that plant and bacterial PA metabolism are activated during the interaction between tomato and *P. syringae*, which leads to the accumulation of Put in plant tissues. However, although different approaches were combined in our study, the identification of the roles played by this PA in the pathosystem still remains elusive. In addition, we do not know the degree of contribution of the plant and bacterial metabolism to these changes. Therefore, future studies should address this issue using a combination of biochemical and genetic strategies that enable the independent study of PA pathways in plants and microbes during the establishment of biological interactions. For example, potential experiments include the inoculation of plants with bacterial elicitors, the evaluation of transgenic plants expressing proteins from microbial origins, and the use of bacterial and plant mutants unable to express key genes from the PA metabolism.

Acknowledgements

F. R. Rossi, M. Marina, S. Maiale, F. L. Pieckensatin, O. A. Ruiz and A. Gárriz are members of the Research Staff of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). J. M. Vilas and F. M. Romero are fellows from the Agencia Nacional de Promoción Científica y Técnica (ANPCyT, Argentina). This study was partly funded by PIP 114-201101-00367 (CONICET), PIP 112 201001 00395 (CONICET), PICT2011-0943 (ANPCyT), PICT 2012-1716 (ANPCyT) and PICT2013-0477 (ANPCyT). We would also like to thank Dr. Andrés Rodríguez for the A. thaliana Δ*ata1* mutants.

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Figure legend

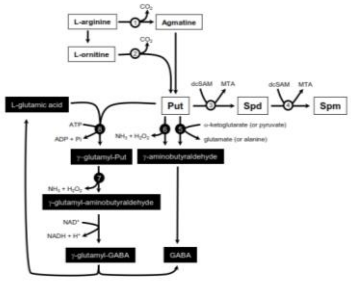

Figure 1. Schematic representation of the PA metabolic pathways. White rectangles correspond to the biosynthetic pathway, while black rectangles depict Put catabolism. 1, arginine decarboxylase (ADC); 2, ornithine decarboxylase (ODC); 3, spermidine synthase (SPDS); 4, spermine synthase (SPMS); 5, putrescine aminotransferase (AT); 6, putrescine oxidase (PuO); 7, γ-glutamyl-putrescine oxidase (GPuO), and 8, γ-glutamyl-putrescine synthase (GPS).

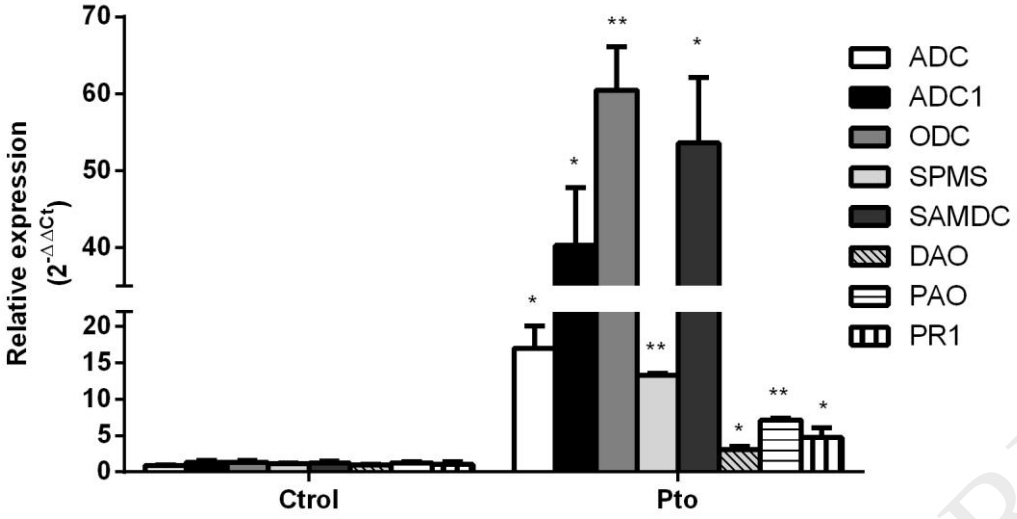

Figure 2. Gene expression in tomato leaves infiltrated with water (control) or Pto. The quantitative real-time polymerase chain reaction was used to analyse the abundance of transcripts from the PA metabolism in detached tomato leaves after 24 hpi. The results are the means of five replicates + standard error. To compare the samples, relative gene expression in control plants was defined as 1. Statistically significant differences in gene expression between the control and bacterial-infiltrated leaves are shown as $*P \leq 0.05$ and $**P \leq 0.01$. ADC, arginine decarboxylase; ODC, ornithine decarboxylase; SPMS, spermine synthase; SAMDC, S-adenosyl-methionine decarboxylase; DAO, diamine oxidase; PAO, polyamine oxidase; PR1, pathogenesis-related protein 1.

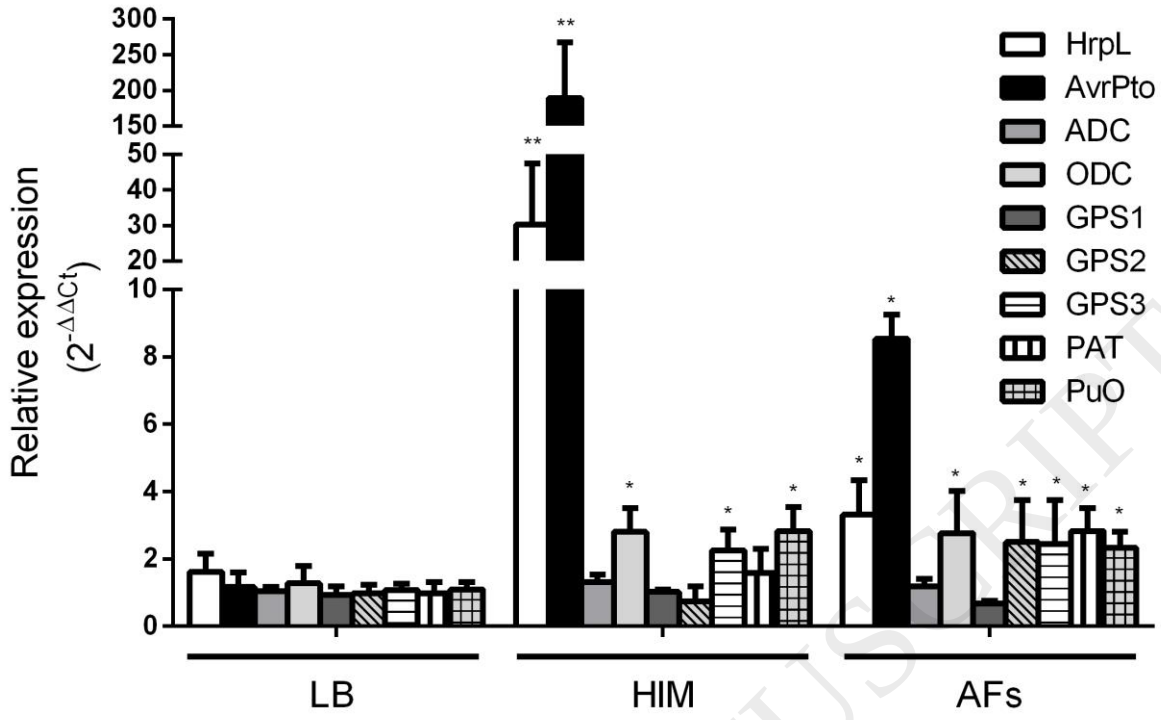

Figure 3. Expression of the bacterial PA metabolic genes in bacterial cells. The cells were cultured in LB, HIM and AFs for 6 h and harvested by centrifugation. Transcript levels were normalized to the average signal intensities in LB, which were assigned a value of 1. Results are the means of five replicates + standard error, and statistically significant differences in gene expression between LB and HIM or AFs are shown as $*P \leq 0.05$ and $**P \leq 0.01$. HrpL, RNA polymerase sigma factor HrpL; AvrPto, bacterial effector AvrPto; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; GPS, γ-glutamyl-Put synthases; PAT, putrescine transaminase; PuO, putrescine oxidase.

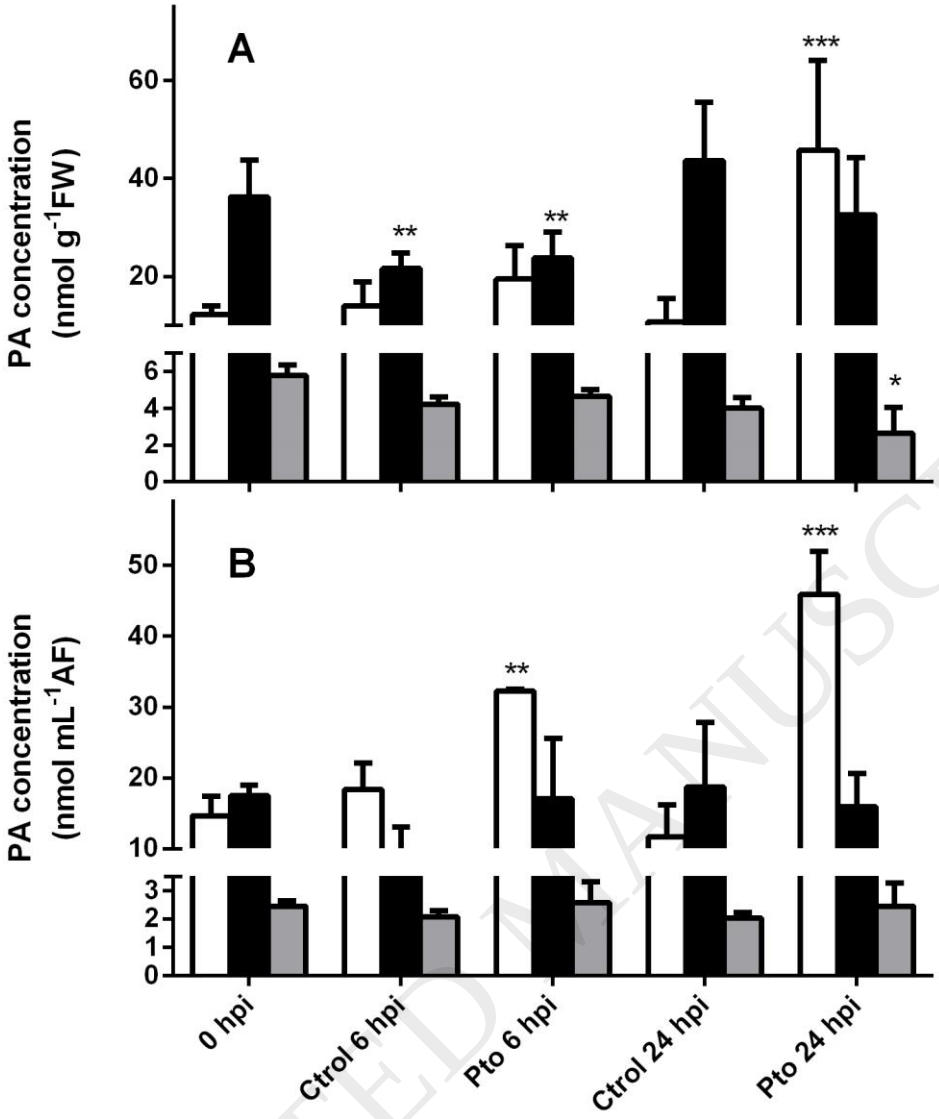

Figure 4. Free PA concentrations in tomato total leaf extracts (**A**) and AFs (**B**) during Pto infections. Leaves were detached from the plants, vacuum-infiltrated with water (control) or Pto at OD_{600} 0.001 and incubated in a growth chamber. Samples of leaf extracts and AFs were immediately removed (0 hpi), 6 and 24 h after infiltration. Free PA levels were determined using HPLC after their derivatization with dansyl chloride. The values are reported in nanomoles relative to leaf fresh weight (FW) in **A** and to the volume of AFs in **B**. Data represent the means + SD. Statistical differences in the concentration of each PA with the values at 0 hpi are shown using the Student's t test as: *, P≤0.05; **, P≤0.01; ***, P≤0.001. ☐ Put, ■ Spd, and ▭ Spm.

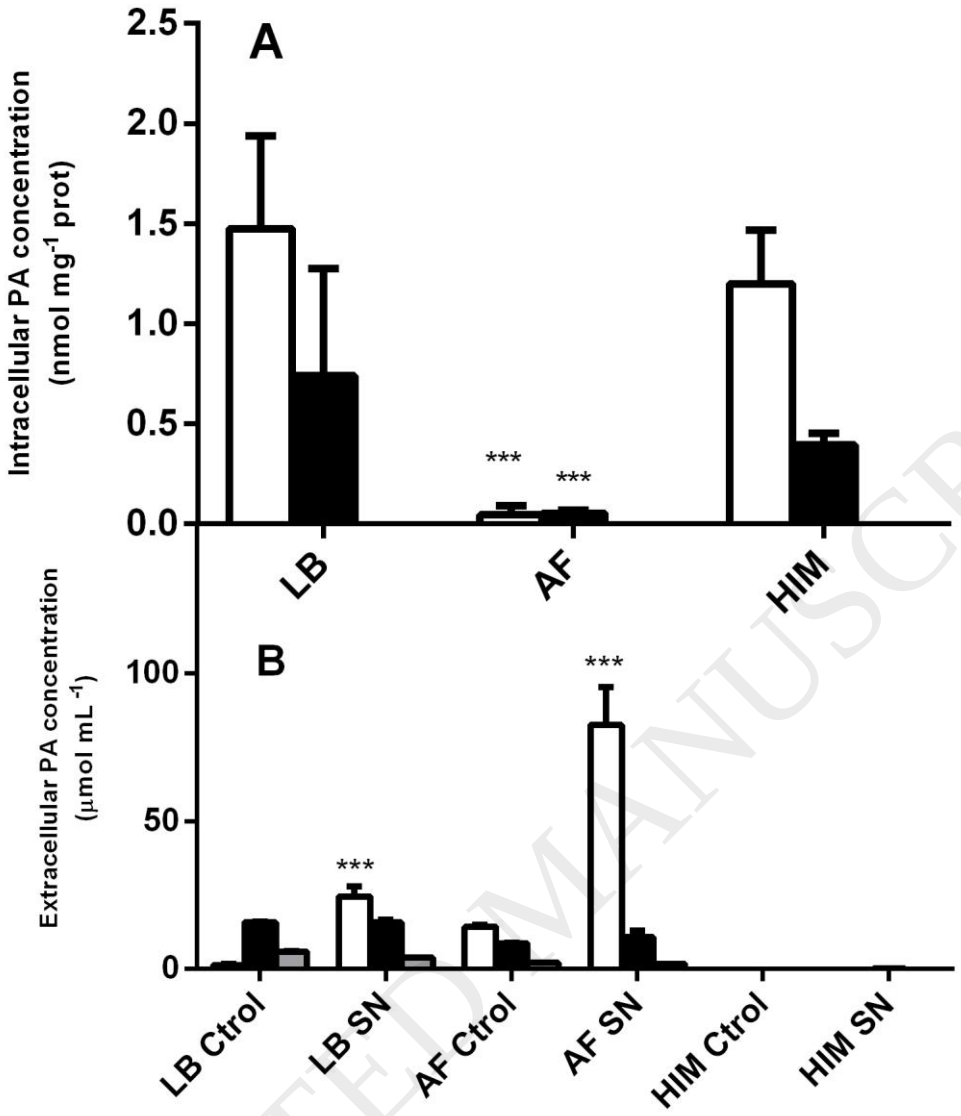

Figure 5. Intracellular (**A**) and extracellular (**B**) free PA concentrations in the bacterial cultures. The cells were cultured in LB, HIM and AFs for 6 h and harvested by centrifugation. Samples of supernatants and cell extracts were removed to determine the free PA levels using HPLC after their derivatization with dansyl chloride. The amount of PAs are reported relative to the protein contents in **A** and the volume of culture media in **B**. Data represent the means + SD. Statistical differences in the concentration of intracellular PAs between LB and HIM or AFs (**A**) or between control and Pto-inoculated media (**B**) are shown using the Student's t test as: ***, $P \leq 0.001$. ■ Put, □ Spd, and ▭ Spm.

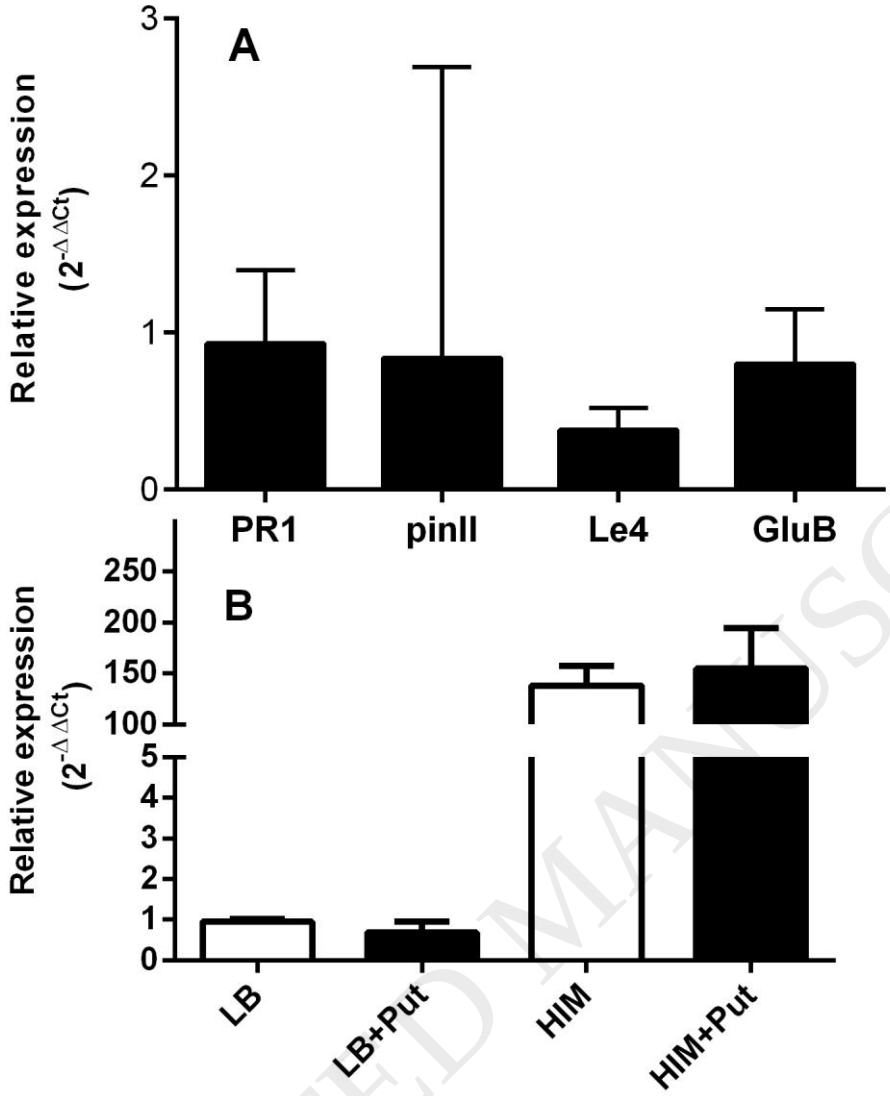

Figure 6. A. Defense gene expression in tomato leaves in response to Pto infections. The leaves were detached from the plants, vacuum-infiltrated with water or Pto at OD_{600} 0.001, and sampled after 24 h of incubation in a growth chamber. **B.** Effect of Put in the expression of *avrPto* by bacterial cells. The cells were cultured for 6 h in LB and HIM with and without added Put and harvested by centrifugation. The quantitative real-time polymerase chain reaction was used to analyse the abundance of bacterial and plant transcripts. In **A**, signal intensities were normalized to the average signal of water-infiltrated leaves. Subsequently, signal intensity in LB without the amendment of Put was used for normalization purposes in **B**. The results are the means of five replicates + standard error.

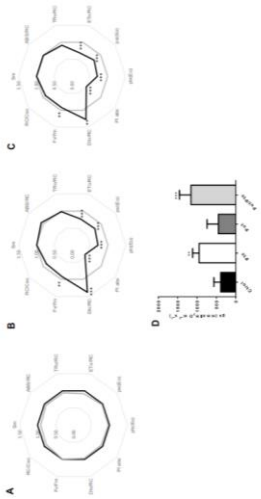

Figure 7. Response of selected functional OJIP test parameters to Pto infections. The leaves were detached from the plants and vacuum-infiltrated with water (controls), Put (**A**), a suspension of Pto (**B**), or a combination of these treatments (**C**). Fluorescence of chlorophyll was assessed using a portable Plant Efficiency Analyser (PEA, Hansatech Instrument Ltd., UK) after 24 h of incubation in a growth chamber. The parameters measured were plotted relative to controls (set as 1.0, grey lines). Fv/Fm, maximum quantum yield of primary PSII photochemistry; RC/Cso, number of reaction centres per cross section; Sm, energy required to close all reaction centres; ABS/Rc, apparent antenna size; TRo/RC, trapped energy flux per reaction centre; ETo/RC, electron transport flux per reaction centre; phi(Eo) and psi(Eo), maximum yield of primary photochemistry and efficiency, respectively; PIabs, performance index; DI_0/RC, dissipated energy flux. Figure **D** shows stomatal conductance (gs) measurements in the same leaves using a TPS-2 (Portable Photosynthesis, MA, USA) with a LED light with an intensity of 1780 mol $m^{-2}s^{-1}$. Data represent the means ± SD. Statistical differences with control leaves are shown using the Student's t test as: **, $P \leq 0.01$; ***, $P \leq 0.001$.

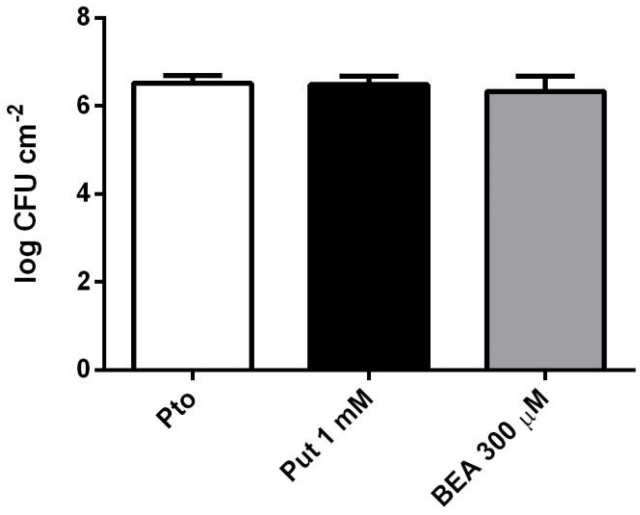

Figure 8. Measurement of *Pto* growth in tomato leaves after 48 h of infiltration. The leaves were detached from plants, vacuum-infiltrated with water (control) or Pto at OD_{600} 0.001 and incubated in a growth chamber. Five discs (0.5 cm diameter) were removed from infiltrated leaves after 48 h and processed for CFU analysis as described in the Materials and Methods. Data represent the means + SD. The Student's t test was used to compare the means and a difference of $p<0.05$ was considered to be significant.

Figure S1. Bacterial gene expression during leaf colonization. The leaves were detached from the plants, vacuum-infiltrated with water (control) or Pto at OD_{600} 0.001 and incubated in a growth chamber. The leaves were immediately sampled (0 hpi), 24 and 48 h after infiltration. The quantitative real-time polymerase chain reaction was used to analyse the abundance of the bacterial transcripts in tomato leaves. Transcript levels were normalized compared to mock-inoculated plants, which were assigned a value of 1. The results are the means of five replicates + standard error. Statistically significant differences in gene expression with 0 hpi are shown as $*P \leq 0.05$ and $**P \leq 0.01$.

Figure S2. Free PA concentrations in the AFs from Pto and hiPto-infiltrated leaves. The leaves were detached from the plants and infiltrated with distilled water (control), Pto OD_{600} 0.001, or heat-inactivated Pto cell suspensions of OD_{600} 0.001 and 0.02. After 24 h of incubation in a growth chamber, the AFs were obtained as described in the Materials and Methods. Free PA levels were analysed using HPLC after derivatization with dansyl chloride. The values are reported in nanomoles relative to the volume of AFs. Data represent the means + SD. Statistical differences in the concentrations of each PA between the treatments and controls are shown using the Student's t test as: ** $P \leq 0.01$. ▭ Put, ■ Spd, and ▭ Spm.

Figure S3. *In vitro* growth of Pto in LB, HIM and AFs. Liquid cultures were inoculated with bacteria at a final OD_{600} of 0.01 and incubated at 28°C with shaking for 6 h. Samples were removed after 3, 4.5 and 6 h, harvested by centrifugation, and the number of CFUs was determined by plating serially diluted samples onto LB media. Data represent the means + SD.

Figure S4. Growth of *Pto* within *A. thaliana* Col-0 and Δ*atao1* leaves. Leaves from *A. thaiana* Col-0 and *atao1* were detached from the plants, vacuum-infiltrated with water (control) or Pto at OD_{600} 0.001 and incubated in a growth chamber for 24 h. Three discs (0.5 cm diameter) were removed from the infiltrated leaves and processed for CFU analysis as described in the Materials and Methods. Data represent the means + SD. The Student's t test was used to compare the means, and a difference of $p<0.05$ was considered to be significant.

Table S1. List of primers used in this study

Primer	Gene (organism)	Sequence	Source
GyrA-F	*gyrA* (Pto)	GGCAAGGTCACCCGCTTCAAGGAAT	Vargas et al, 2011
GyrA-R	*gyrA* (Pto)	GACCGCCACGCTTGTACTCAGGGAAC	Vargas et al, 2011
ADCPto-F	*adc* (Pto)	TGTACAATTCGCTGAAAGCC	This study
ADCPto-R	*adc* (Pto)	GAACACCGAGAAGTTGCAGA	This study
ODCPto-F	*odc* (Pto)	TGAAGCTGATCAACATGGGT	This study
ODCPto-R	*odc* (Pto)	TCTTCCTTCAGAAAGCGGAT	This study
PATPto-F	*patA* (Pto)	TGATCTGTCGAACCTTCTGC	This study
PATPto-R	*patA* (Pto)	TCCACCAGTTCATCGATTTG	This study
GPS1Pto-F	*gps1* (Pto)	GATCGATGACCTCGACGAAT	This study
GPS1Pto-R	*gps1* (Pto)	CGTAGTCGCACGCTTTGATA	This study
GPS2Pto-F	*gps2* (Pto)	GCCGATCAGATTCTGGTGTT	This study
GPS2Pto-R	*gps2* (Pto)	TCTCGATGTCGATGATGCTC	This study
GPS3Pto-F	*gps3* (Pto)	TGTTCAAGCACCTGCTCAAG	This study
GPS3Pto-R	*gps3* (Pto)	CATCGCTGAACACGTTTTGT	This study
PAOPto-F	*pao* (Pto)	TTACTTCACCCAGGCTGACC	This study
PAOPto-R	*pao* (Pto)	AGGGCGAACAATGTGCTATT	This study
HrpL-F	*hrpL* (Pto)	ATGCGAAACCCGTCTCGAAT	Park et al, 2010
HrpL-R	*hrpL* (Pto)	TGTGCAGGAACTCTTCTTGCTG	Park et al, 2010
avrPto-F	*avrPto* (Pto)	GAACACTGGATATGGCGGACA	Park et al, 2010
avrPto-R	*avrPto* (Pto)	CGCTACGCATATCGTCCACAT	Park et al, 2010
DAOSl-F	*dao* (tomato)	GGAGAATCACCGCGAAGAAG	This study
DAOSl-R	*dao* (tomato)	ACCAACAGGGTTCCCAACTCT	This study
SAMdc1Sl-F	*samdc* (tomato)	CCTGCTTTTGCACTCATTGC	This study
SAMdc1Sl-R	*samdc* (tomato)	CCAGGCTCGACGAAAGAAAT	This study
SPDSSl-F	*spd synth* (tomato)	GGGAAGGTGCTTGTTTTGGA	This study
SPDSSl-R	*spd synth* (tomato)	ACGCAGGACACCACCATCTC	This study
SPMSSl-F	*spm synth* (tomato)	CATTATGCATGGGCCAGTGTT	This study
SPMSSl-R	*spm synth* (tomato)	CTTGAGTTCCCGCTGATGCT	This study
PAOSl-F	*pao synth* (tomato)	AAAATGCTTATCCGGGATCCA	This study
PAOSl-R	*pao synth* (tomato)	CGGTGGCATCAGGAATGTC	This study
ADCSl-F	*adc synth* (tomato)	TGCTTGAAGTGTCTCTTG	Song et al, 2014
ADCSl-R	*adc synth* (tomato)	GATTGCGGTCATAACATAAG	Song et al, 2014
ADC1Sl-F	*adc1* (tomato)	CACAAGGAAGAAGAAGTAGA	Song et al, 2014
ADC1Sl-R	*adc1* (tomato)	GCCAACACCAACAATATTC	Song et al, 2014
ODCSl-F	*odc* (tomato)	TAAGGGATTACCAGTTACC	Song et al, 2014
ODCSl-R	*odc* (tomato)	GGATAAGCATAAGCAAGG	Song et al, 2014
GluB-F	*β-1,3-glucanase* (tomato)	CCATCACAGGGTTCATTTAGG	Martínez Medina et al, 2013
GluB-R	*β-1,3-glucanase* (tomato)	CCATCCACTCTCTGACACAACT	Martínez Medina et al, 2013
Le4-F	*Desiccation protective protein* (tomato)	ACTCAAGGCATGGGTACTGG	Martínez Medina et al, 2013

Le4-R	*Desiccation protective protein* (tomato)	CCTTCTTTCTCCTCCCACCT	Martínez Medina et al, 2013
Pr1a-F	*Pathogenesis-related protein PR1a* (tomato)	GTGGGATCGGATTGATATCCT	Martínez Medina et al, 2013
Pr1-R	*Pathogenesis-related protein PR1a* (tomato)	CCTAAGCCACGATACCATGAA	Martínez Medina et al, 2013
PI II-F	*Proteinase inhibitor II* (tomato)	GAAAATCGTTAATTTATCCCAC	Martínez Medina et al, 2013
PI II-R	*Proteinase inhibitor II* (tomato)	ACATACAAACTTTCCATCTTTA	Martínez Medina et al, 2013