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Original Article

Early changes in apoplast composition associated with defence and disease in interactions between *Phaseolus vulgaris* and the halo blight pathogen *Pseudomonas syringae* pv. *phaseolicola*

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ABSTRACT

The apoplast is the arena in which endophytic pathogens such as *Pseudomonas syringae* grow and interact with plant cells. Using metabolomic and ion analysis techniques, this study shows how the composition of *Phaseolus vulgaris* leaf apoplastic fluid changes during the first six hours of compatible and incompatible interactions with two strains of *P. syringae* pv. *phaseolicola* (*Pph*) that differ in the presence of the genetic island PHIGH-I. Leaf inoculation with the avirulent island-carrying strain *Pph* 1302A elicited effector-triggered immunity (ETI) and resulted in specific changes in apoplast composition, including increases in conductivity, pH, citrate, γ-aminobutyrate (GABA) and K⁺, that are linked to the onset of plant defence responses. Other apoplastic changes, including increases in Ca²⁺, Fe²⁺/³⁺, Mg²⁺, sucrose, β-cyanoalanine and several amino acids, occurred to a relatively similar extent in interactions with both *Pph* 1302A and the virulent, island-less strain *Pph* RJ3. Metabolic footprinting experiments established that *Pph* preferentially metabolizes malate, glucose and glutamate, but excludes certain other abundant apoplastic metabolites, including citrate and GABA, until preferred metabolites are depleted. These results demonstrate that *Pph* is well-adapted to the leaf apoplast metabolic environment and that loss of PHIGH-I enables *Pph* to avoid changes in apoplast composition linked to plant defences.

Key-words: apoplastic washing fluid; citrate; GABA; leaf apoplast; metabolic footprinting; metabolomics; plant defence response; plant–microbe interactions.

INTRODUCTION

The partially interconnected space between plant cell membranes, known as the apoplast, is a dynamic compartment housing many biological processes including nutrient and water transport, cell wall biosynthesis and defence responses to microbial pathogens (Sattelmacher 2001). The major structural component of the apoplast is the cell wall, which is surrounded by apoplastic fluid and a gaseous phase. Leaf apoplastic fluid contains a variety of proteins, metabolites and inorganic ions whose concentrations are controlled largely by the balance of transport processes with the xylem, phloem and symplasm of surrounding cells, although metabolic processes within the apoplast also contribute to control of metabolite concentrations (Grignon & Sentenac 1991). The composition of leaf apoplastic fluid remains poorly characterized overall but is known to respond to changing physiological conditions including nutrition, abiotic and biotic stress and diurnal cycles (López-Millán et al. 2000a; Sattelmacher 2001). Being one of the first compartments that encounter environmental stress, the apoplast is thought to play an important role in the perception of changing environmental conditions (Hoson 1998).

The apoplast also represents an environmental niche where many plant microbial pathogens establish a parasitic lifestyle. Among the best-studied occupants of the leaf apoplast are bacteria from the *Pseudomonas syringae* species complex, which gain access to the apoplast through stomata, hydathodes or wounds, and infect a wide variety of plants, causing necrotic symptoms in leaves, stems and fruit. The concentrations of nutrients in tomato, Arabidopsis and tobacco leaf apoplast extracts are sufficient to support rapid growth of *P. syringae* in vitro and it has been suggested that *P. syringae* strains are metabolically specialized to make efficient use of C and N sources that are abundant in their host’s apoplast (Melotto et al., 2008, Rico and Preston, 2008; Rico et al., 2009; Mithani et al. 2011). It is not known, however, whether any nutrients can become limiting to bacteria in the apoplast or whether the plant is able to sequester essential nutrients away from the apoplast as a defence mechanism.

Extensive study of *P. syringae*–host plant model systems has focused on dissecting the molecular mechanisms and co-evolution of pathogenesis and plant disease resistance. In particular, *P. syringae* use a type-III secretion system (T3SS) to suppress defence responses by secreting effector proteins directly into host plant cells. Successful suppression of plant defences is essential for the establishment of compatible *P. syringae*–host plant interactions which lead to a disease state.
However, resistant host plant varieties frequently express resistance proteins (R proteins) that recognize an effector protein or its biological activity and initiate further defence responses to block pathogen multiplication; this defines an incompatible *P. syringae*–host plant interaction. In this way, plant defence responses are generally regarded as two tiered (Jones & Dangl 2006). Initial recognition of pathogen associated molecular patterns (PAMPs), e.g. flagellin, elicits PAMP triggered immunity (PTI). Detection of bacterial effector proteins elicits effector triggered immunity (ETI). Effector triggered immunity often culminates in a form of localized programmed cell death known as the hypersensitive response (HR) (Tao et al. 2003; Jones & Dangl 2006; Thilmony et al. 2006; Truman et al. 2006).

The gene-for-gene relationships determining infection outcomes between strains of *P. syringae* pathovar phaseolicola (*Pph*) and different cultivars of their host plant *Phaseolus vulgaris* (common bean) have been well studied (Taylor et al. 1996). The two strains used in this study, *Pph* 1302A and *Pph* RJ3, are directly related, differing only in the presence of a 106 kb genomic island, PPHGI-1, that can be excised or integrated into the genome in planta, thus interconverting the two strains (Jackson et al. 2000; Pitman et al. 2005; Godfrey et al. 2011; Lovell et al. 2011). Encoded on this genomic island is an effector gene, *avrPphB* (also called *hopARI*), whose product induces ETI and the HR in *P. vulgaris* cultivar Tendergreen, which encodes the R3 resistance protein (Jackson et al. 2000; Shao et al. 2003). As a result, these two genetically very similar *Pph* strains differ in their interaction with cultivar Tendergreen plants: *Pph* 1302A contains PPHGI-1 and elicits the HR; *Pph* RJ3 lacks PPHGI-1, and establishes a compatible interaction by inhibiting plant defence responses (Jackson et al. 2000).

Within the first hours after inoculation of plant leaves with pathogenic bacteria rapid transcriptional, physiological and metabolic changes occur that determine the outcome of this interaction (Jones et al. 2006; Thilmony et al. 2006; Truman et al. 2006; Ward et al. 2010; Etalo et al. 2013, Yu et al. 2013, Mitchell et al. 2014). Much of this action is likely to cause rapid changes to conditions within the apoplast, e.g. the downregulation of photosynthesis, production of reactive oxygen species (ROS) and changes in membrane potential and ion fluxes. However, in contrast to whole leaf tissue studies, few studies have examined changes to leaf apoplast composition during infection, and these have mostly focused on proteomic changes (De launois et al. 2014; Petriccione et al. 2014). In this study we employed a robust infiltration–centrifugation apoplastic fluid extraction technique to generate apoplastic washing fluid (AWF) samples at early timepoints during compatible and incompatible interactions of *Pph* with *P. vulgaris* cultivar Tendergreen leaves. Quantitative analyses of AWF metal ions and polar metabolites, combined with metabolite footprinting experiments that use AWF as a *Pph* growth medium, reveal rapid changes to apoplastic composition in both compatible and incompatible interactions that suit the distinct nutritional requirements and metabolic organization of the invading *Pph* strains. Further previously undescribed changes in apoplastic metabolite composition were observed to occur following inoculation with the ETI-eliciting strain 1302A, and prior to any detectable restriction of pathogen growth. We discuss the potential role of these changes in apoplastic processes in the induction and early onset of plant defence responses.

## RESULTS

### The conductance and pH of the *P. vulgaris* leaf apoplast increases following elicitation of ETI

It is known that the onset of ETI is associated with changes in membrane transport processes and membrane integrity that may substantially affect apoplastic composition. To determine when these changes occurred in our experimental system we observed the time-dependent progression of *Pph* interactions with *P. vulgaris* cv. Tendergreen following routine leaf infiltrations with 2 × 10⁸ colony forming units (cfu)/m of *Pph* RJ3 or *Pph* 1302A. Leaf inoculation with *Pph* 1302A led to an ETI response, and a clear HR at the site of infection was visible by 24 h post inoculation (hpi; Fig. S1). In contrast, inoculation with *Pph* RJ3 led to a disease state characterized by water soaked lesions visible by 24 hpi. Inoculation of entire leaves with *Pph* 1302A, but not *Pph* RJ3, led to tissue wilting beginning at 8 hpi. Bacterial population size in planta was estimated by performing colony counts on homogenized leaf discs (Fig. 1A). Both *Pph* RJ3 and 1302A populations increased similarly for 8 hpi, after which time the *Pph* 1302A population grew more slowly and then declined, presumably because of the onset of ETI (n = 3).

Major changes in the composition of infected leaf apoplast are linked to changes in solute movement across adjacent plasma membranes. To estimate when major changes in leaf apoplast composition occurred after inoculations with *Pph*, the conductance of AWF extracted from leaf discs was monitored over time (Fig. 1B). During infection with *Pph* 1302A, the conductance of the AWF increased rapidly between 4 and 8 hpi; this increase preceded the onset of tissue wilting, which subsequently precluded further measurements, as AWF could not be extracted from wilted leaves. With *Pph* RJ3 or water infiltrated leaves, AWF conductance did not change significantly for 8 hpi.

Leaf apoplastic fluid is also known to undergo an increase in pH during defence responses to pathogens (Felle et al. 2004, 2005). Here, a non-destructive microscopy-based imaging approach using the pH sensitive *F*₅₄₀/F₄₄₀ fluorescence ratio of Oregon Green 488-dextran allowed in planta recordings of apoplastic pH for 15 h following infection of *P. vulgaris* leaves with *Pph* RJ3 and *Pph* 1302A. Representative timecourse pH measurements are shown in Fig. 1C. Leaf tissue infected with *Pph* 1302A displayed a transient increase in apoplastic pH from 4.8 ± 0.05 to 5.1 ± 0.04 which began approximately 5 hours (301 ± 50 minutes) after inoculation and became most alkaline 8–9 h (530 ± 50 minutes) after inoculation before partially recovering (n = 6). An increase in pH during *Pph* RJ3 inoculations occurred later than with *Pph* RJ3 and was not consistently captured by the 15 h experiment (n = 3). In control leaves infiltrated with water, apoplastic pH stabilized at 4.6 ± 0.1 during the timecourse (n = 3). The observed timing of conductance and pH changes indicated that...
for subsequent experiments AWF extractions performed at 4 and 6 hpi would be appropriate to capture major changes in apoplast composition following infection with Pph 1302A and induction of ETI.

Characterization of the quality of whole leaf AWF extractions

Quality control assays were established to verify minimal cytoplasmic contamination of AWF extracts from whole P. vulgaris leaves. All AWF extracts contained detectable protein concentrations and malate dehydrogenase (MDH) activities, which did not differ significantly between inoculation treatments (p < 0.05). However, leaves that visibly sustained even slight physical damage during extraction produced AWF with marked increases in both protein concentration and MDH activity that were attributed to cytoplasmic contamination. To discriminate between cytoplasmic contaminated and uncontaminated AWF, a subjective cut-off was imposed such that only samples with less than 0.25 mg/mL protein and less than 0.4 U/mL MDH activity were considered acceptable for further analyses. The average protein concentration of analysed AWF extracts was 0.18 ± 0.01 mg/mL and the average MDH activity was 0.25 ± 0.02 U/mL (n = 64). Glucose-6-phosphate was also used as an indicator of cytoplasmic contamination for samples subjected to GC-MS metabolite analysis. No uncontaminated AWF samples (as determined by the above criteria) contained a detectable signal for glucose-6-phosphate except for samples from 6 h infections with Pph 1302A wherein five of seven samples contained trace amounts (<1 μM), indicating that plant cell membrane leakiness increased with time during this interaction. The AWF extracts from the 6 h Pph 1302A infections were coloured a pale yellow, whereas all other AWF extracts were colourless. Using indigo carmine dye the extent of apoplast dilution caused by infiltration was determined spectrophotometrically to be 2.9 ± 0.2 fold (n = 6), and this value was used to correct all concentrations measured from AWF samples.

AWF composition undergoes specific metabolite and cation changes during early compatible and incompatible interactions

i) GC-MS metabolite analysis

GC-MS analysis was used to identify and quantify freely soluble, polar metabolites across five AWF sample types: 4 h control inoculations with water, and 4 and 6 h infections with either Pph 1302A or Pph RJ3 (n = 6). Manual inspection of the GC-MS data identified 60 compound peaks. A further nine major peaks that could not be definitively identified are listed as unknowns with retention times in minutes. Standard curves were generated for 35 identified compounds allowing estimations of the in planta apoplastic concentration of these metabolites (Fig. 2, Table S1). Compounds with no standard curve, or where one or more values were below the linear range of quantification, are listed in Table S2 as percentages of the control inoculation signal. The most abundant metabolites

Figure 1. Timing of changes in conductance and pH in P. vulgaris leaf apoplast during an incompatible interaction. A) Growth of Pph 1302A (closed circles) and Pph RJ3 (open circles) populations following leaf inoculation. Error bars represent standard error. B) Conductance measurements performed on diluted AWF collected from leaf discs following leaf inoculations with water (closed circles), Pph 1302A (closed triangles) and Pph RJ3, (open circles). Error bars represent standard error. Asterisks denote a significant difference from control inoculations (ANOVA, p < 0.05, n = 4). C) Representative in vivo ratiometric pH measurements performed on leaf tissue following inoculation with Oregon green dye and water (closed circles), Pph 1302A (closed triangles) and Pph RJ3, (open circles). Error bars represent technical standard error calculated by dividing the infected areas into 12 subsections.
detected in the AWF samples were di- and tri-carboxylates: malate, malonate, maleate, citrate and fumarate. Sucrose was the most abundant sugar and GABA was the most abundant amino acid. In total sixteen amino acids were detectable in control AWF; five of these amino acids were non-proteinogenic, including an unexpected abundance of β-cyanoalanine (37 μM; an intermediate of the plant cyanide detoxification pathway).

The differences in metabolite concentrations in AWF samples following Pph infection were remarkable. Principal component analysis revealed that samples from both 6 h infections clearly clustered away from control samples (Fig. 3A). This separation, captured mostly by the first principal component (PC), reflects an increase in most compounds that was generally more severe in Pph 1302A infected leaves (Fig. 3B). Nine quantified compounds significantly increased (p < 0.01) in the Pph 1302A 6 hpi samples compared to control and Pph RJ3 6 hpi samples (Fig. 2, Table S1). In particular, citrate and GABA displayed large absolute increases (from 298 to 2871 μM and 85 to 740 μM, respectively) during the Pph 1302A interaction that were not paralleled in the Pph RJ3 interaction. Conversely, no compounds significantly increased (p < 0.01) in the Pph RJ3 infected leaves compared to Pph 1302A infected leaves at either timepoint. Notably, malate was never observed to differ significantly between any treatments. A single unknown compound (unknown 29.466 min; spectrum consistent with a 3-(hydroxyphenyl)propanoate) significantly decreased upon infection with both Pph strains, whereas malonate decreased significantly only during RJ3 infection (Table S2).

ii) AAS of AWF cation concentrations

The abundance of metal cations in the apoplast can impact upon plant-microbe interactions, and K+ efflux from the cytosol is a hallmark of plant defence responses (Atkinson et al. 1985; Atkinson & Baker 1987; Demidchik et al. 2014). To quantify changes in apoplastic metal cation concentrations upon infection, atomic absorption spectroscopy (AAS) for Ca2+, K+, Mg2+, Na+, Ni2+, Fe2/3+, and Zn2+ was performed on aliquots from the same AWF extracts used for GC-MS metabolite analysis (Fig. 4). At 4 hpi, significant (p < 0.05) increases compared to control were only observed for K+ and Ni2+ within Pph 1302A samples (though Ni2+ was not significantly increased at 6 hpi). By 6 hpi all other metals except for Na+ had significantly increased by 6 hpi. The largest change in absolute concentration was that of K+ in the Pph 1302A samples, which increased from 18 ± 3 mM in control samples to 55 ± 7 mM by 6 hpi. The detected concentration of K+ from healthy leaf apoplast is similar to previous measurements from Vicia faba (Mühling & Sattelmacher 1997). Lastly, Ca2+ and Mg2+ levels were highly correlated across all samples (R2 = 0.78).
incubations were performed where bacteria were initially cultured in AWF rather than M9 minimal media to observe whether the initial culture conditions affected the metabolic activity of the bacteria in AWF. The patterns of metabolite accumulation and depletion were indistinguishable when Pph 1302A was initially cultured in M9 minimal media or AWF (n = 5).

Certain metabolite levels, e.g. sucrose and amino acids, changed in the control incubations in the absence of Pph and these changes were attributed to the activity of endogenous apoplastic enzymes (Fig. 5, Table S3). Sucrose was depleted in a linear fashion in control and Pph incubations. This was likely because of endogenous invertase activity in the AWF and related to concomitant increases in glucose and fructose. Most proteinogenic amino acids increased linearly during the control incubation to varying degrees; the exceptions were glutamate, which decreased in concentration and aspartate and asparagine which did not change. Conversely, among non-proteinogenic amino acids, only GABA increased in concentration during the control incubation. These patterns indicate protein hydrolysis by proteases and possible GABA formation from glutamate as endogenous enzyme activities in the AWF. Organic acid levels were relatively stable in control incubations with the exception of 2-oxoglutarate which decreased by 80% over 10 h.

Amid these background changes, Pph inoculated samples clearly differed from control samples, and these differences are attributable to bacterial export, uptake and metabolism. Among the various organic acids distinct patterns of accumulation and depletion are apparent (Figs 5 and 6, Table S3). Some dicarboxylates (including dicarboxylic amino acids) were preferred substrates for the bacteria, and were consumed sequentially, with malate and maleate depletion being most rapid, followed by glutamate, aspartate, fumarate and succinate, then citrate and malonate. Interestingly, both 2-oxoglutarate and pyruvate increased sharply in abundance before beginning to be depleted (Figs 5 and 6). The levels of all amino acids, except homo-cysteine, decreased during Pph incubations compared with control incubations. The rate of depletion of amino acids varied and the bacteria displayed a preference for the uptake and catabolism of glutamine, glutamate, aspartate and β-cyanoalanine (Fig. 5). The hexose sugars glucose and galactose were the predominant carbohydrates metabolized by Pph. Sugar alcohols, with the exception of glycerol, were not appreciably metabolized during the incubation (Fig. 5, Table S3). Gluconate, presumably derived from glucose oxidation by bacterial gluconate dehydrogenase, displayed by far the largest fold change in abundance of any compound, accumulating greatly in the media for 6 or 8 h before being rapidly consumed. Quantification of the gluconate increase revealed a higher maximum of 1077 ± 16 μM at 8 h for Pph RJ3 incubations compared to 216 ± 13 μM at 6 h within Pph 1302A incubations (Fig. 5, Table S3).

Carbon substrate utilization profiles were also evaluated for Pph 1302A and RJ3 on 95 predetermined metabolites using Biolog GN2 plates (Table S4). These assays determined the capability of Pph to oxidize carbon compounds towards the production of reductant, and can therefore infer the presence or absence of metabolic and transport pathways. The preferred compounds for consumption during the metabolite footprinting
timecourse, e.g. glucose, dicarboxylates and asparagine, were also all strongly metabolized during the Biolog plate assays (some compounds, e.g. malate and glutamine, are not present in Biolog GN2 plates). Conversely, 2-oxoglutarate, which was excreted during the metabolite footprinting timecourse, was only weakly utilized in the Biolog assays. Some other compounds, in particular sucrose and inositol, were used when presented as sole C substrates during the Biolog assays but not appreciably metabolized by Pph from the AWF media, further indicating the preferential use of certain carbon compounds in the apoplast. Biolog assay results were generally similar for Pph 1302A and RJ3, although Pph RJ3 tended to display a higher metabolism rate for organic acids, while Pph 1302A displayed a higher metabolism rate for several sugars.

**DISCUSSION**

**Changes in apoplast composition differ between compatible and incompatible interactions**

This study sought to increase knowledge regarding the chemical composition of the leaf apoplast during the early stages of compatible or incompatible bacterial infections. It has previously been established that induction of the HR by a race 1
strain of Pph in P. vulgaris cv. Red Mexican requires approximately 4 h, and that marked alteration of cell ultrastructure, which progresses towards cell collapse, is visible beginning at 8 hpi (Roebuck et al. 1978). This matches the results reported here, where increases in apoplast conductivity, pH and K+ efflux (Figs 1 and 4) commenced near 4–5 hpi in the incompatible interaction, consistent with previous measurements (Atkinson et al. 1985; Atkinson & Baker, 1987; Felle et al. 2004, 2005), but a negative effect of ETI on bacterial population growth in the leaf was not observed until after 8 hpi. The compatible interaction with Pph RJ3 did not cause a similar increase in pH, conductance or K+ over the first 6–8 hpi. The delayed and dampened defence response in compatible versus incompatible interactions is well established in the literature (Bonfig et al. 2006; Jones & Dangl 2006) and is further confirmed in our metabolomic analyses. The major differences in leaf apoplastic composition observed between 4 and 6 hpi with Pph 1302A versus RJ3 are therefore most readily attributed to changes in metabolism and membrane transport processes associated with the induction of ETI, rather than direct inhibition of pathogen growth, and include large increases in citrate and GABA. Conversely, changes in apoplastic composition that occurred in both compatible and incompatible Pph interactions compared to control inoculations are likely to be attributable to bacterial activity and PTI, and include increases in Mg2+, Ca2+, Fe2+/3+, β-cyanoalanine and sucrose as discussed below.

During Pph infection of P. vulgaris, citrate displayed the largest increase in absolute abundance of any metabolite.
detected and became the most abundant metabolite in AWF. The increase in citrate may reflect an electric charge balancing anion flux from the cytosol to the apoplast accompanying K\(^+\) efflux, and this is consistent with the increase of several other organic acids in the AWF (Fig. 2). However, in light of recent studies discussed below a further signalling role for apoplastic citrate during bacterial infection is possible and deserves further study. Firstly, Arabidopsis leaf discs immersed in 1 mM citrate displayed marked changes in transcript abundance that were not mirrored by immersion in malate, and which most resembled changes associated with biotic stress induction by \textit{P. syringae} (Finkemeier et al. 2013). Secondly, cytosolic NADP isocitrate dehydrogenase knockouts in \textit{Arabidopsis} that accumulate citrate in their leaves display increased expression of defensive PR genes and decreased \textit{P. syringae} growth during infection (Mhamdi et al. 2010). Thirdly, Anderson et al. (2014) observed a dose-dependent signalling effect of citrate on \textit{P. syringae} pv. tomato (Pto) DC3000 T3SS gene expression: citrate strongly promoted the expression of the T3SS-secreted effector protein \(\alpha\)-AvrPto between 50 and 200 \(\mu\)M but completely inhibited expression at 1 mM. These observations may be physiologically important given that apoplastic citrate levels were found in this study to increase from 300 \(\mu\)M in healthy apoplast to greater than 1 mM at 4 hpi with \textit{Pph} (Fig. 2, Table S2).

This study revealed a large increase in the extracellular accumulation of GABA during the incompatible interactions between \textit{P. vulgaris} and \textit{Pph}. GABA levels have previously been reported to increase in response to tomato leaf infection by \textit{Cladosporium fulvum} (Solomon & Oliver 2001), and in response to infection of \textit{Arabidopsis thaliana} with \textit{Pto} DC3000 (Ward et al. 2010). Although GABA is a potential source of both carbon and nitrogen for \textit{Pph} and other pseudomonads (Rico and Preston 2008), here we show that GABA is not a preferred nutrient (Fig. 5). In plants GABA has long been known to be a stress-induced metabolite whose accumulation is associated with a range of physiological functions (Shelp et al. 2006); and GABA, like citrate, has been shown to suppress \textit{P. syringae} T3SS expression when present at high concentrations (Park et al. 2010). Nevertheless, the function of extracellular GABA accumulation during the defence response requires elucidation.

**Metal ion accumulation in the apoplast is associated with \textit{Pph} infection**

There were large correlated increases in Mg\(^{2+}\) and Ca\(^{2+}\) at 6 hpi in both compatible and incompatible interactions. The dynamic influx of Ca\(^{2+}\) from the apoplast to the cytosol via Ca\(^{2+}\) channels occurs at the onset of and continues throughout plant defence responses (Grant et al. 2000; Felle et al. 2004; Shelp et al. 2006; Ma et al. 2012). These Ca\(^{2+}\) influxes cause apoplastic levels of free (unchelated) Ca\(^{2+}\) to decrease (Felle et al. 2004), while here it was observed that total Ca\(^{2+}\) levels increased in AWF extractions following infection. The increase in total Ca\(^{2+}\) could be because of release from the vacuole (the main storage compartment for Ca\(^{2+}\) in eudicots (Conn et al. 2011)) or alternatively could be because of chelation and redistribution of Ca\(^{2+}\) by extracellular chelators such as citrate and alginates, a polysaccharide secreted by \textit{P. syringae} (Aslam et al. 2008). The production of an insoluble material, possibly containing extracellular polysaccharides, was clearly visible by 10 hpi when \textit{Pph} was grown in full-strength AWF in vitro. The chelation of divalent cations would also explain the increase in Mg\(^{2+}\) and could support \textit{P. syringae} cell wall and biofilm integrity, which require Mg\(^{2+}\) and Ca\(^{2+}\) (Vaara 1992; Banin et al. 2006).

Iron is known to be a major limiting nutrient for \textit{Pseudomonas} in minimal media cultures (Kim et al. 2009), and has the potential to be limiting in the apoplast as well. Total iron in the AWF extracts was measured at 7 \(\mu\)M in healthy leaves, which is consistent with previous measurements in sugar beet leaves (López-Millán et al. 2000b). Iron levels increased substantially to 16–20 \(\mu\)M in infected leaves, which are not likely to be limiting for \textit{Pph} growth in the apoplast (Bronstein et al. 2008; Kim et al. 2009; Jones & Wildermuth 2011).

**Bacterial nutrition is well supported by the composition of the apoplast throughout the early stages of infection**

The two metabolomic analyses performed in this study (the quantification of AWF metabolites throughout early infection and the metabolic footprinting experiment using AWF as a...
and/or toxins still elicit the accumulation of apoplastic nutrients. Whether amino acids and organic acids, supporting bacterial nutrition. 

increased extracellular pH and K+ concentration (Atkinson 2010, 2012). Lastly, limitation of sucrose and other nutrient activation of extracellular invertases, causes apoplastic accumulate in the apoplast. Atkinson may be altered in favour of nutrient accumulation in the apoplast. Atkinson first showed that sucrose efflux to the leaf apoplast increased during infection in a manner related to increased extracellular pH and K+ concentration (Atkinson 1987). Fourthly, Pto DC3000 infection of Arabidopsis has been reported to cause the upregulation of plasma membrane sugar transport proteins (SWEETs), so it is possible that P. syringae effectors manipulate sugar efflux to the apoplast (Chen et al. 2010, 2012). Lastly, limitation of sucrose and other nutrient export from infected leaves because of callose deposition and activation of extracellular invertases, causes apoplastic accumulation of sugars and other exported metabolites, (Scharte et al. 2005). These mechanisms may or may not operate in concert, but one result of P. syringae infection, as observed here, is a marked increase in apoplastic metabolites such as sucrose, amino acids and organic acids, supporting bacterial nutrition. In future work it would be of interest to systematically determine whether P. syringae strains deficient in specific effector proteins and/or toxins still elicit the accumulation of apoplastic nutrients. (Roitsch & González 2004; Bolton 2009). Within infected photosynthesizing leaves a metabolic transition from photosynthesis to respiratory metabolism, stimulated by the inhibition of sucrose export and the activation of hexose producing cell wall invertases, is likely necessary to initiate a full defence response (Scharte et al. 2005; Swarbrick et al. 2006; Kocals et al. 2008; Bonfig et al. 2010). It is clear from the disappearance of sucrose in the metabolic footprinting experiments reported here that invertase activity was present in apoplastic extracts. However, although sucrose levels increased during both compatible and incompatible interactions, the levels of hexoses (fructose, glucose, galactose) did not show a pronounced increase. Scharte et al. (2005) also reported a delay in the accumulation of apoplastic hexoses beyond the accumulation of sucrose and extractable invertase activity. This could be because of rapid and increasing uptake of hexoses by infected leaf cells to fuel respiration and defence compound biosynthesis (Fotopoulos et al. 2003), or alternatively because of post-translational control of apoplast invertases (Bonfig et al. 2010).

We observe from the metabolic footprinting experiment that the Pph strains studied do not appreciably consume sucrose or alter the rate of sucrose hydrolysis. The bacteria do rapidly produce gluconate, which is most obviously produced from glucose oxidation by the periplasmic bound enzyme glucose dehydrogenase (Fig. 7). Gluconate excretion by P. aeruginosa into liquid medium has been previously shown and would directly contribute electrons to the bacterial electron transport chain, as would further oxidation of gluconate to 2-ketogluconate (Behrends et al. 2013). Glucose oxidation to gluconate therefore provides a rapid source of energy to P. syringae in planta. The metabolic footprinting experiment revealed clear evidence of catabolite suppression in line with what has previously been observed for Pseudomonads: that the dicarboxylic acids repress the uptake and metabolism of sugars by the Entner–Doudoroff pathway (Rojo 2010; Behrends et al. 2013). Alleviation of catabolite repression is likely responsible for the sudden reversal of gluconate accumulation in the AWF media that occurred around the same time that preferred dicarboxylic acids were exhausted (Figs 4 and 5; Behrends et al. 2013). Furthermore, pyruvate and 2-oxoglutarate displayed patterns of rapid accumulation and depletion synchronous with those of gluconate. The excretion of pyruvate and 2-oxoglutarate by pseudomonads has been commonly observed and associated with inhibition of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (Behrends et al. 2013; Lundgren et al. 2014). Therefore, we hypothesize that upon exhaustion of preferred organic acid substrates Pph undergoes a general re-organization of metabolic flux that involves increased flux through the Entner–Doudoroff pathway and initiation of an altered flux through the citric acid cycle (Fig. 7).

As previously shown for Pph 1448A (Rico & Preston 2008), Pph 1302A and RJ3 contain an authentic frameshift mutation in the gene encoding the C5-dicarboxylic acid transporter (kgtP), responsible for 2-oxoglutarate import. This mutation is consistent with the poor oxidation of 2-oxoglutarate by Pph in Biolog assays and indicates that import rather than metabolism is limiting. Interestingly, extracellular accumulation of pyruvate and 2-oxoglutarate by Pseudomonads has been
implicated in both cyanide (CN) and ROS detoxification as these α-keto acids react non-enzymatically with CN to form nitriles and with H₂O₂ to form decarboxylation products (Andrae et al. 1985; Kunz et al. 1998; Mailloux et al. 2009). As ROS production is a hallmark of the plant defence response, the benefit to *P. syringae* of excreting keto-acids as additional antioxidants is obvious but remains to be directly tested in planta.

Cyanide detoxification by invading bacteria is likely to be an important and understudied aspect of adaptation to the apoplast environment. Ethylene production increases during infection and ETI which produces an equimolar amount of CN, showing that bacterial electron transport chain. Glucosamine may be further oxidized to 2-ketogluconate. The uptake of glucose, glucosamine and 2-ketogluconate provides substrates for the Entner–Doudoroff pathway. In the second pathway, uptake of preferred dicarboxylic acids provides the major carbon and nitrogen sources for the rapidly growing *Pph* populations. The excretion of pyruvate and 2-oxoglutarate could represent metabolic endpoints which are because of the regulation of TCA cycle and associated enzymes; e.g., possible inhibition of the pyruvate dehydrogenase (Pdc) and 2-oxoglutarate dehydrogenase complexes. Transamination reactions from glutamate to create other amino acids would contribute to the production of 2-oxoglutarate (not shown). The extracellular accumulation of pyruvate, 2-oxoglutarate and glucosamine appeared to be under the control of catabolite repression by the preferred dicarboxylic acids as indicated by dashed lines.

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**Figure 7.** Simplified diagram depicting two major aspects of *Pph* metabolism predicted to be occurring within AWF media based on metabolic footprinting data. In the first pathway, extracellular hydrolysis of sucrose by apoplastic invertases (Inv) provides the substrate for the periplasmic conversion of glucose to glucosamine by membrane localized glucose dehydrogenase (Gcd) which in turn energizes the bacterial electron transport chain. Glucosamine may be further oxidized to 2-ketogluconate. The uptake of glucose, glucosamine and 2-ketogluconate provides substrates for the Entner–Doudoroff pathway. In the second pathway, uptake of preferred dicarboxylic acids provides the major carbon and nitrogen sources for the rapidly growing *Pph* populations. The excretion of pyruvate and 2-oxoglutarate could represent metabolic endpoints which are because of the regulation of TCA cycle and associated enzymes; e.g., possible inhibition of the pyruvate dehydrogenase (Pdc) and 2-oxoglutarate dehydrogenase complexes. Transamination reactions from glutamate to create other amino acids would contribute to the production of 2-oxoglutarate (not shown). The extracellular accumulation of pyruvate, 2-oxoglutarate and glucosamine appeared to be under the control of catabolite repression by the preferred dicarboxylic acids as indicated by dashed lines.

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**Summary**

This study provides a substantial dataset increasing our knowledge of the composition of the plant apoplast. Induction of ETI by *Pph 1302A* results in large changes in *P. vulgaris* apoplast composition in comparison to the changes observed in response to *Pph RJ3*, which has evolved to avoid elicitation of ETI through loss of the genomic island PPHGI-1. Providing mechanistic explanations for the distinctive early changes in the apoplastic metabolome of leaves undergoing defence responses, including large increases in GABA, citrate and β-cyanoalanine, will be of particular interest to determine the extent to which apoplast composition actively contributes to processes underpinning plant defences. In both interactions it is evident that the apoplast is replete with nutrients that can support pathogen growth and metabolism throughout the early stages of infection. However, *Pph* preferentially assimilates a subset of the metabolites present, despite possessing the metabolic capability to assimilate non-preferred metabolites, and this could be taken into account in considering resistance strategies targeting pathogen metabolism.

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**MATERIALS AND METHODS**

**Plant and bacterial growth conditions**

*P. vulgaris* cv. Tendergreen seeds were obtained from a commercial distributor (Chiltern Seeds). Seeds were sown on moist paper towels and, once germinated, transferred to soil pots (John Innes number 1 compost) and placed in a growth chamber with 18 h of light at 20 °C and 6 h of dark at 18 °C.

For inoculation into leaves, cultures of *Pph* were grown overnight in King’s Broth media (KB), pelleted and resuspended in sterile distilled water to an OD₆₀₀ of 0.15 (~2 × 10⁸ CFU/mL). Inoculations were performed by piercing the leaf surface with a fine point and using a 1 mL needleless syringe to infiltrate the entire apoplastic air-space with the bacterial suspension.

For *in planta* growth assays, a 1 cm diameter cork borer was used to harvest the inoculated leaf tissue. This tissue was homogenized in sterile distilled water, and following centrifugation for 10 s at 1300 rpm the supernatant was serially diluted and spread onto KB plates. For *in vitro* growth assays, diluted overnight culture was added to 200 μL of full strength AWF in a 96-well plate. Plates were incubated at 25 °C while shaking at 100 rpm. A 10 μL sample was taken at 0, 2, 4, 6, 8 and 24 h, serially diluted and spread onto KB plates. Total CFUs were calculated after incubation of the KB plates for 48 h at 25 °C.

**Leaf apoplast extractions**

AWF was extracted from the first true leaves of *P. vulgaris* cv. Tendergreen 3–4 weeks post germination and between 8 and 11 h into the light period using ddH₂O as the infiltration medium as previously described (O’Leary et al. 2014b). Biological replicates of AWF samples (indicated as n for each analysis) were collected over several days from one batch of plants grown under common conditions. To calculate the dilution of...
apoplast components during AWF extraction, parallel AWF extractions were performed with and without 50 μM indigo carmine in the infiltration fluid. Ratios of absorbance at A610 were used to calculate the apoplast dilution factor as described previously (O’Leary et al. 2014b). To approximate in vivo solute concentrations ‘full-strength’ AWF samples were generated by freeze-drying pooled AWF samples then redissolving the material in distilled water to a volume determined by the apoplast dilution factor. Full-strength AWF samples were stored at –80 °C.

Malate dehydrogenase activity was measured by monitoring NADH oxidation at A340 in 200 μL reactions containing 100 mM HEPES pH 7, 10% glycerol, 0.15 mM NADH, 2 mM oxaloacetate and 20 μL of AWF. Protein concentrations were quantified using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories) according to the manufacturer’s instructions using bovine serum albumin as the protein standard.

Metabolic footprinting and carbon utilization assays

Metabolic footprinting timecourse incubations of Pph in full-strength AWF were performed using cultures grown overnight in M9 minimal media (Sambrook et al. 1989) or in AWF, both to mid log phase. Overnight cultures were pelleted and resuspended in distilled water to an OD600 of 0.15. Either 50 μL of diluted culture or water was added to 1 mL of full-strength AWF from healthy leaves and incubated in a 2 mL tube while shaking at 28 °C. At least five replicate AWF cultures were established for each bacterial strain and 150 μL aliquots were removed at 0, 2, 4, 6, 8 and 10 hpi and centrifuged at 10 000 g for 5 min to pellet the bacteria. The supernatant was removed, frozen in liquid N2 and stored at –80 °C before analysis by GC-MS. Protein concentrations were quantified using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories) according to the manufacturer’s instructions using bovine serum albumin as the protein standard.

Conductance assays

For measurements of conductance, AWF was extracted from P. vulgaris cv. Tendergreen leaf discs obtained using a 2 cm diameter cork borer. After rinsing and infiltration of the leaf discs with ddH2O, excess surface liquid was removed with a paper towel. Each leaf disc was then inserted into a 1 mL pipette tip which was inserted into a 1.5 mL tube and centrifuged at 600 g for 5 min. The eluted AWF was centrifuged again at 17 000 g for 2 min, then 25 μL of supernatant was then diluted to 3 mL with ddH2O and the conductance measured using a conductivity meter (Jenway).

GC-MS analysis

Samples were prepared for GC-MS analysis using a modified version of the method of Lisec et al. (2006). Frozen 150 μL samples of AWF or cell-free AWF media from bacterial culture were extracted in 700 μL of methanol containing 10 μg/mL of ribitol while shaking at 70 °C for 10 min, followed by centrifugation for 5 min at 11 000 g. Next, 700 μL of supernatant was removed and mixed sequentially by vortexing with 375 μL of cold chloroform, then 500 μL of ddH2O. Samples were centrifuged at 2200 g for 15 min, then 150 μL of supernatant was transferred to a fresh tube and dried in a vacuum concentrator without heat. The samples were derivitized in 29 μL of pyridine containing 20 mg/mL methoxyamine and 50 μL of N-Methyl-N-(trimethylsilyl)trifluoroacamide (MSTFA) as described previously (Lisec et al. 2006). GC-MS analysis was performed as described previously (O’Leary et al. 2014a). For absolute quantification of metabolites standard curves were generated from pure stock solutions that were extracted and derived as above. Multivariate analyses were performed with SIMCA software (Umetrics), with data mean-centred and scaled to unit variance.

Metal cation analysis

Metal cation concentrations within AWF were determined using a Beckman AAS equipped with graphite furnace in the case of Ni, Ca, Mg, Fe, Na, and Zn or an acetylene flame for K. Standard curves were created for each element and AWF samples were diluted in ddH2O to be within the linear range.

Ratiometric quantification of apoplastic pH

For in planta quantitation of leaf apoplastic pH, 25 μM of the ratiometric fluorescent pH indicator dye Oregon Green 488-dextran (Invitrogen GmbH, Darmstadt, Germany) was loaded with or without 2 × 106 CFU/mL of Pph into the leaf apoplast of intact plants using a needleless syringe. The 10 kDa dextran conjugation ensures that the dye does not access the cytoplasm from the apoplastic space (Geilfus & Mühling 2012). As established previously, all pH responses were monitored starting 2 hpi to ensure evaporation of excess infiltrated water and normal gas exchange within the apoplast (Geilfus & Mühling 2011). Fluorescence images at excitation wavelengths 440/20 and 490/10 nm were collected with a Leica inverted microscope via 50-fold magnification (0.15 numerical aperture, dry objective; voxel size = 0.002 mm; HCX PL FLUOTAR L, Leica Microsystems) with the optical and instrumental settings described elsewhere (Geilfus & Mühling 2011). Exposure time was 75 ms for both channels. The fluorescence ratio F490/F440 was obtained as a measurement of pH on a pixel-by-pixel basis using LAS AF software (version 2.3.5; Leica Microsystems). Background values were subtracted at each channel. For conversion of the fluorescence ratio data into apoplastic pH, an in vivo calibration was conducted (Fig. S2).

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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:

**Figure S1:** Infection of *P. vulgaris* leaves with *Pph* 1302A and *Pph* RJ3 leads to an incompatible and compatible interaction, respectively.

**Figure S2:** In vivo calibration of the ratiometric pH measurements.

**Figure S3:** Comparison of in vitro population growth of *Pph* 1302A versus RJ3 in full strength AWF.

**Table S1:** GC-MS determined absolute concentrations of compounds in leaf AWF extracts.

**Table S2:** GC-MS determined relative concentrations of compounds in leaf AWF extracts.

**Table S3:** Values for metabolic footprinting analysis of *Pph* incubated in AWF.

**Table S4:** Values for carbon utilization assays of *Pph* performed on GN2 Biolog plates.