

Dual metabolomics: A novel approach to understanding plant–pathogen interactions

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ABSTRACT

One of the most well-characterised plant pathogenic interactions involves *Arabidopsis thaliana* and the bacteria *Pseudomonas syringae* pathovar *tomato* (*Pst*). The standard *Pst* inoculation procedure involves infiltration of large populations of bacteria into plant leaves which means that metabolite changes cannot be readily assigned to the host or pathogen. A plant cell–pathogen co-culture based approach has been developed where the plant and pathogen cells are separated after 12 h of co-culture via differential filtering and centrifugation. Fourier transform infrared (FT-IR) spectroscopy was employed to assess the intracellular metabolomes (metabolic fingerprints) of both host and pathogen and their extruded (extracellular) metabolites (metabolic footprints) under conditions relevant to disease and resistance. We propose that this system will enable the metabolomic profiling of the separated host and pathogen (i.e. ‘dual metabolomics’) and will facilitate the modelling of reciprocal responses.

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1. Introduction

Plant resistance to pathogens is often dependent on a localised cell death – the hypersensitive response (HR) – which forms at the site of attempted penetration of the host (Mur et al., 2008). The HR is elicited following the interaction of the products of a host-encoded resistance (*R*) gene and pathogen-encoded avirulence (*Avr*) gene. Disease symptoms develop in the absence of an *Avr*–*R* interaction (Bent and Mackey, 2007). Many plant–pathogen interaction studies have been based on a model pathosystem involving *Arabidopsis* and bacterial pathogens, particularly, *Pseudomonas syringae* pathovar *tomato* (*Pst*) (Preston, 2000). With such bacterial pathogens, *Avr* gene products have been found to be delivered into the plant cell via a type III secretion system (TTSS), encoded by *hypersensitive response and pathogenicity* (*hrp*) genes. In the absence of *R* gene interaction, *Avr* gene products have been revealed to be members of a battery of virulence proteins which are delivered into the host to initiate disease. *Pst* strains mutated in the *hrpA* genes will therefore abolish both the HR and disease formation on different hosts (Collmer et al., 2000). This host-dependent action for *Avr*/Vir proteins has led to their redefinition as TTSS effectors (Lindeberg et al., 2005). A key feature of bacterial plant pathogens

is that they are not internalised into the cells as is often the case with bacterial infections of animal cells (Dramsi and Cossart, 1998).

With the development of post-genomic technologies, the local and systemic plant responses against bacterial infection have been characterised at the levels of the transcriptome and proteome (Desikan et al., 2001a; Truman et al., 2006, 2007). These generate sequence based datasets allowing individual elements to be assigned to either host or pathogen. Metabolomics has been suggested to be the ultimate level of post-genomic analyses as it reflects both transcriptional and post-transcriptional regulation (Fiehn, 2001, 2002; Hall et al., 2002; Allwood et al., 2008). Plant defensive metabolism can occur through the constitutive accumulation of anti-microbial metabolites – phytoanticipins – which include cyanogenic glycosides and glucosinolates (Morrissey and Osbourn, 1999). On pathogen attack, changes in primary metabolism involve the mobilisation of photoassimilates so that tissues which have previously been sugar-importers (“sinks”) become exporters (“sources”) (Berger et al., 2004). Although this is clearly advantageous to any attacking pathogen, it is also necessary for the host to rapidly mobilise sugars to provide the energy required for a resistant response (Heil and Bostock, 2002). Rapid and major changes also occur in secondary metabolism, with the generation of defence hormones for example, salicylic acid and jasmonates, changes in antioxidant status, and the production of phenylpropanoids, hydroxycinnamic acids, monolignols and flavanoids (Dixon

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and Paiva, 1995). As with primary metabolism similar changes occur during resistance and disease development but are more rapid in the former case (Tao et al., 2003).

Taking pathogen responses to the plant into consideration, factors involved in the induction of virulence effectors in *P. syringae* have been extensively studied. These include carbon source (Huynh et al., 1989), levels of nitrogen, osmolarity, pH (Rahme et al., 1992) and iron concentration (Kim et al., 2009). These have been mostly established using chemically defined media which makes any association with the *in situ* environment very difficult to draw. However, some studies have utilised plant–bacterial co-culture approaches in targeted analyses of bacterial gene expression (for example, Brito et al., 1999; Kim et al., 2009).

This latter approach recognises that pathogenic attack involves an interaction between two partners that can only be partially assessed if focusing only on one partner. In metabolomic terms this reciprocal interaction will encompass: (i) constitutive metabolism that occurs within the host and pathogen cells; (ii) metabolite changes occurring within either host or pathogen in response to the interaction, including metabolites produced within one partner being excreted and then internalised within the other partner (metabolite cross-talk or the interactome); and (iii) the metabolites that are excreted by each of the interacting partners and which remain within the extracellular environment. The established method of bacterial inoculation into *Arabidopsis*, involves infiltration of leaf air spaces with suspensions of $\sim 10^6$ bacteria mL^{-1} , to give rise to large areas of near-synchronously responding tissue. As a result, the sample material contains very heterogeneous mixes of pathogen cells as well as infected and non-infected plant cells. As such a metabolomic analysis cannot discriminate either the exact origins of a given metabolite or the relative contribution from either interacting partner. To a certain extent, this may be circumvented by isotopic labelling of, most easily, the bacterial pathogen prior to inoculation (Godin et al., 2007), although dilution of the isotope through metabolism can mean that only immediate metabolite transformations are detected. Alternatively, the use of pathogens can be avoided altogether and bacterially-derived defence elicitors could be utilised (Zhao et al., 2005). However, this is at best a partial approach since this removes the entire metabolome of one of the interacting partners.

Here we describe an alternative approach based on the inoculation of plant cell cultures with isogenic strains of *Pst*, where at 12 h post inoculation (hpi) the cell types were separated by differential filtering, washing and centrifugation. The independent analysis of plant and bacterial cells allowed the metabolite changes within each interacting partner to be assessed, an outcome which we term 'dual metabolomics'. Analysis of the culture media (i.e. metabolic footprinting) allowed metabolites extruded by both cell types during their interaction to be described. This novel approach to metabolomic analyses of host–pathogen interactions will facilitate a greater understanding of both their independent metabolism and the metabolic cross-talk which represents the interactome.

2. Results and discussion

2.1. Variable responses of *Arabidopsis* suspension cultures to *Pst* strains

Plant cell cultures have been extensively used to characterise plant host responses to pathogens including defence gene expression, the generation of signal chemicals, and intracellular signalling events (Hahlbrock et al., 1995; Clarke et al., 2000; Desikan et al., 2001a,b; Ndimba et al., 2003). The reproducibility of plant cultures is such that they have served as the basis of several functional genomic studies (Desikan et al., 2001a; Pauwels et al., 2008; Lippert et al., 2009). *Arabidopsis* cultures have been proven to

exhibit variable responses to avirulent and virulent bacteria which are consistent with the elicitation of HR-type cell death or the initiation of a disease state respectively (Clarke et al., 2000).

In developing a plant host–pathogen co-cultivation system for metabolomic analyses, the responses of the *Arabidopsis* cell cultures to disease forming *Pst*, a transconjugant strain into which an avirulence gene had been introduced – *Pst avrRpm1*, and a *Pst* strain mutated in the *hrpA* gene which encodes a protein involved in forming the TTSS pilus (Fig. 1), were first assessed. These characterisations were undertaken using an inoculation procedure which would be subsequently employed for metabolomic sampling (*vide infra*). This preliminary work was also essential since intimate contact between the pathogen and host cell is vital for effective delivery of effector proteins across the cell wall (Collmer et al., 2000; Brown et al., 2001) and indeed is a requirement to activate *hrp* gene expression (Aldon et al., 2000). Thus, differential responses of the plant cell to each *Pst* genotype would indicate appropriate bacterial interactions with the plant cells leading to TTSS effector protein delivery.

One of the most readily assessable indicators of differential host responses, including within cell cultures (Levine et al., 1994), is the elicitation of cell death. Estimations of the levels of cell death at 6, 12 and 24 h post challenge were based on the retention of Evan's Blue stain by plant cell clusters. When inoculated with either of the *Pst* strains and at any time point, a given cluster could either exhibit no (Fig. 1A), partial (Fig. 1B) or total (Fig. 1C) staining with Evan's Blue. Quantification of Evan's Blue staining based on scoring clusters if they exhibited any sign of colouration indicated that by 6 hpi plant cells challenged with *Pst avrRpm1* already exhibited significantly ($p = 0.001$) greater cell death than cultures inoculated with the non-HR eliciting, non-pathogenic strain *Pst hrpA*. The extent of Evan's Blue staining with *Pst* never significantly differed from that with *Pst hrpA* (Fig. 1D). These outcomes were explicable within the context of the responses elicited by these *Pst* strains in *Arabidopsis* plants and indicated the successful delivery of TTSS effector proteins to the plant cells when in culture. Thus, *Pst avrRpm1* elicited a rapid HR, whilst *Pst* elicited the slower appearance of disease symptoms and *Pst hrpA* elicited, at most, the slow appearance of chlorosis over a period of days (typically > 3d; data not shown). Consistent with the elicitation of a rapid HR-type defence response, increased expression of the defence genes *pathogenesis related protein 1* (*PR1*) and defensin (*PDF1.2*) was observed in plant cultures only when inoculated with *Pst avrRpm1* (Fig. 1E). As *PR1* and *PDF1.2* are marker genes for signalling by the major defence hormones salicylic and jasmonic acid respectively (Mur et al., 2006), these data suggest differential metabolomic responses were being exhibited in the cultures inoculated with the various *Pst* strains.

2.2. Metabolic fingerprinting with Fourier transform infrared (FT-IR) spectroscopy for the assessment of whole cell metabolic signatures

The *Arabidopsis* cell culture and *Pst* sample collection procedure was modified to allow the metabolomes of host and pathogen to be individually sampled. To assess the success of these modifications fully, we chose to employ a metabolic fingerprinting approach. Although metabolic profiling by employing either mass spectrometry (MS) or nuclear magnetic resonance (NMR) offer the possibility of metabolite identification, it is currently impossible to detect all of the metabolites within a system due to variation in chemical complexity (molecular weight, polarity, and solubility), their physical properties (volatility) and major differences in metabolite concentrations within tissue extracts (Kopka et al., 2004). In contrast, with metabolic fingerprinting no attempt is made to distinguish metabolites but the resulting "signature" is indicative of the complete metabolome of a sample. Furthermore, FT-IR spectroscopy

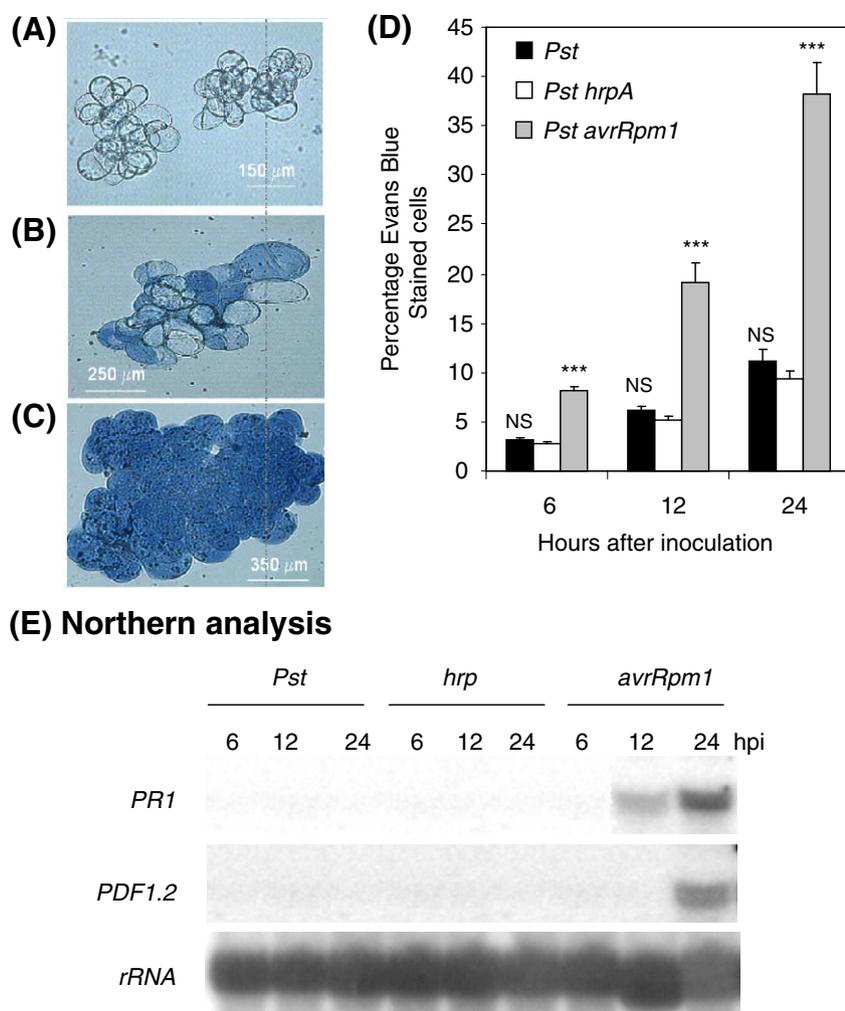


Fig. 1. The responses of cell cultures of *Arabidopsis thaliana* to *Pseudomonas syringae* pathovar tomato (*Pst*). *Arabidopsis thaliana* cell suspension cultures at 24 hpi with virulent *Pst*, avirulent *Pst avrRpm1*, or non-virulent *Pst hrpA*, were stained with 0.25% (w/v) Evans Blue to reveal the level of cell death. Plant cell clusters either: (A) failed to retain any stain, (B) were partially stained, or (C) were fully stained. Images were obtained using light microscopy at 400 \times magnification. (D) Clusters within 100 μ L samples ($n = 5$ per inoculation) were assessed for the percentage of cells retaining Evans Blue stain. Indicated are significance levels of differences to *Pst hrpA* data. NS = non-significant, *** = $P = 0.001$. (E) RNA was extracted from *Arabidopsis* challenged by either *Pst*, *Pst hrpA* or *Pst avrRpm1*, northern blotted and sequentially screened with probes to the defence genes *PR1* and *PDF1.2*. The membrane was finally screened with a probe for ribosomal RNA (*rRNA*) to demonstrate equal loading. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

requires minimal sample processing, only resuspension in water and spotting onto aluminium plates. Hence, extractable metabolites, polymers (for example cell wall associated polymers) and proteins are assessed within each sample using FT-IR (Ellis et al., 2007). We have frequently demonstrated the utility of FT-IR metabolic fingerprinting to generate biologically relevant data (Johnson et al., 2003, 2007; Kaderbhai et al., 2003; Allwood et al., 2006). FT-IR therefore, offered the ability to test the responses of *Arabidopsis* and *Pst* strains and the extent of variation under differing susceptible and resistant scenarios.

2.3. Establishment of reproducible pre-infection metabolomes of the *Arabidopsis* cell cultures and *Pst* isogenic strains

A major requirement of our dual metabolomic strategy was that each of the three *Pst* strains used should not differ significantly prior to inoculation into *Arabidopsis* culture. The ideal scenario would have been to establish three continuous cultures so that the growth and thus metabolic status of each could have been synchronised (Winder et al., 2008). However, this was not performed as this was not a capability that is available in many laboratories. Instead a semi-batch approach was employed where the three

strains were each cultured to mid-log phase before re-inoculation into fresh media. This was repeated three times following which the bacteria were sampled and separated from the culture medium by centrifugation. Following three washing steps using an iso-osmotic 0.85% NaCl (w/v) solution (physiological saline) the three bacterial strains were analysed using FT-IR spectroscopy. The results of two separate experiments were analysed simultaneously (Fig. 2A). The results were indistinguishable when examined by principal component analysis (PCA), however, we have frequently found that application of supervised discriminant function analysis to the previously obtained PC values (PC-DFA) derived from the metabolomic data is required to discriminate between separate experimental classes (e.g., Allwood et al., 2006). In this case, it was not possible to distinguish between bacterial strains or between experiments (Fig. 2A). This indicated that the metabolomes of the three bacterial strains were substantially equivalent prior to inoculation into *Arabidopsis* cultures and could be accurately reproduced between experiments. Variation between the plant cell cultures was another consideration. To reduce this, 15 separate 200 mL cultures of plant cells were pooled to establish a 3 L culture which would serve as the common source of 20 mL sub-cultures which could be inoculated with different *Pst* strains. This large

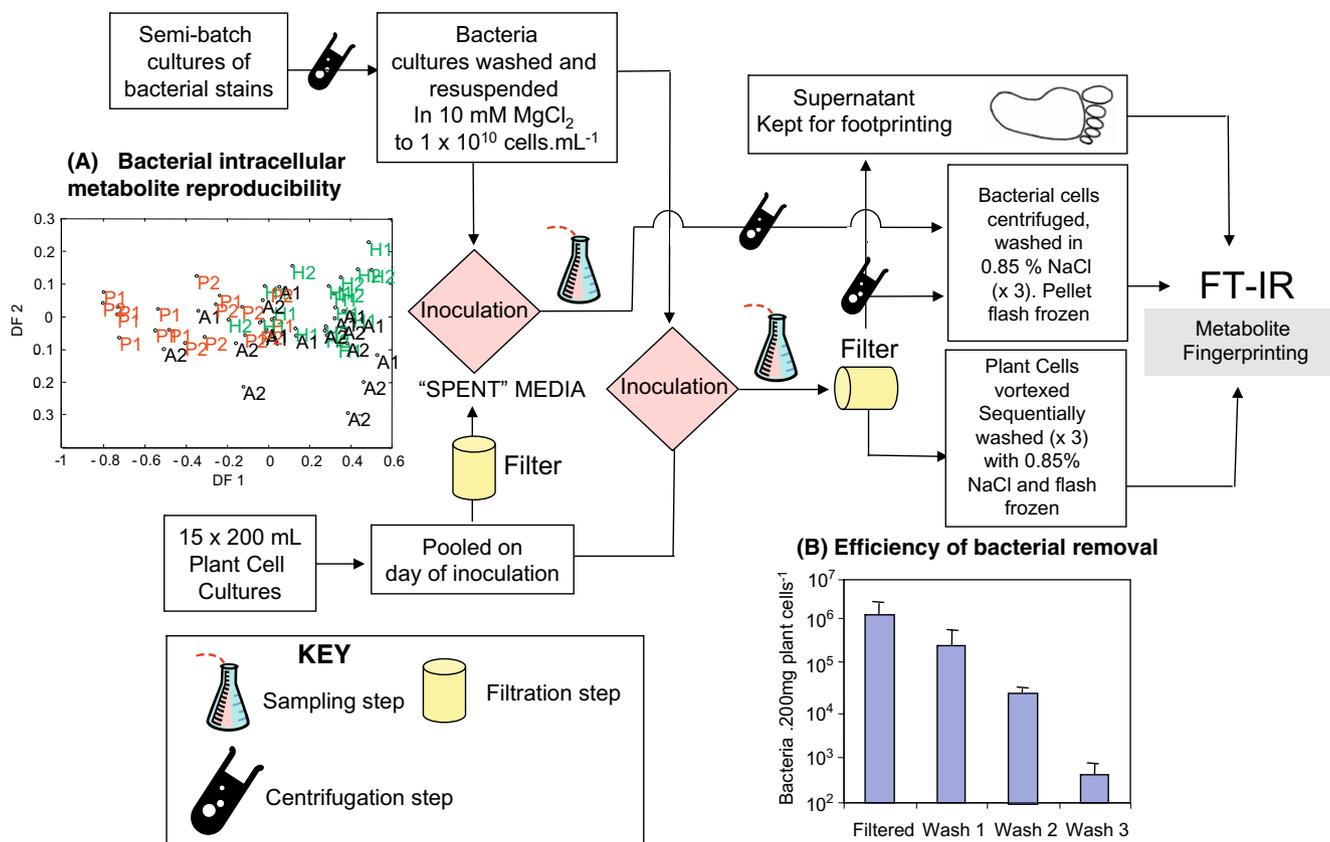


Fig. 2. Work flow for dual metabolomic analyses of the *Arabidopsis thaliana*–*Pseudomonas syringae* pathovar *tomato* interaction. A work flow for, the generation of material, inoculation of *Arabidopsis* and control cultures with *Pst*, *Pst avrRpm1*, and *Pst hrpA*, and preparation of samples for a dual metabolomics experiment is presented. The bacterial strains were cultured and 1×10^{10} cells mL⁻¹ inocula were prepared as detailed in Section 4.5. (A) Samples of bacterial cultures were first assessed by Fourier transform infrared (FT-IR) spectroscopy to indicate that their pre-infection metabolic fingerprints were indistinguishable. Samples of *Pst* were designated as a red “P”, *Pst avrRpm1* black “A” and *Pst hrpA* as a green “H”. The numbers 1 and 2 refer to samples from the two replicate experiments. FT-IR spectra were interrogated by discriminant function analysis (DFA) based upon the first 12 principal components (PCs) accounting for 99.96% of the total explained variance (i.e. PC-DFA). The *a priori* group structure consisted of nine replicates (three biological replicates \times three analytical replicates) per each of the six experimental classes. *Arabidopsis* cells were continuously maintained as described in Section 4.2. After 7 days culture, 15 \times 200 mL cultures were pooled into a 3 L culture. To provide a source of spent AT3 media, 1.5 L of the suspension culture was filtered and the cells discarded. Bacterial inoculates were added to 20 mL aliquots of spent media, fresh media, and *Arabidopsis* cultures, to give a density of 1×10^8 cell mL⁻¹ (refer to Section 4.6). Sampling of spent and fresh AT3 control cultures or bacteria–*Arabidopsis* cell cultures (sampling stages shown by conical flasks on the figure) occurred at 12 hpi as described in Section 4.7. Briefly, the culture was filtered and the plant cells harvested and washed with 0.85% (w/v) NaCl. The bacterial pellets were harvested from the filtrate by centrifugation followed by washing in 0.85% NaCl. After the final washing step, plant and bacterial samples were flash frozen in liquid N₂ and stored at -80 °C until analysis by FT-IR. (B) To assess the efficiency of the washing steps to remove bacteria from plant cells, the number of bacterial cells retained in a plant sample (~ 100 mg dry weight) after each wash with 0.85% (w/v) NaCl was assessed by plating out onto rifampicin-supplemented nutrient agar. Three washes with 0.85% NaCl proved sufficient to reduce bacterial contamination to a few hundred bacterial cells per plant sample.

scale pooling of 15 separate plant cell cultures proved to yield plant cells whose metabolomes did not differ between experiments when assessed using FT-IR spectroscopy coupled with PC-DFA (data not shown).

2.4. Plant and bacterial cells can be efficiently and rapidly separated

Given that the metabolomes of dying rather than dead cells would be of most interest to plant pathologists, *Pst* bacteria were sampled at 12 hpi (Fig. 1D). It was necessary to establish that plant and bacterial cells could be efficiently and rapidly separated. In testing the efficiency of bacterial removal, inoculations with the *Pst hrpA* strain were used, as this was assumed to elicit the least potent defence from plants and therefore should exhibit the highest bacterial viability after co-cultivation with plant cells. At 12 hpi the plant cells were separated from the surrounding medium by filtration through filter paper and sequentially washed as described in the Section 4.7 (and see Fig. 2). After filtration and each wash, samples of the plant cells were ground down, serially diluted and plated onto rifampicin-supplemented NA to select for *Pst hrpA*. Based upon this the extent of bacterial contamination of the plant

cells was estimated (Fig. 2B). In freshly, filtered plant cells, even though these had been washed with 0.85% NaCl, bacterial contamination was considerable ($>10^6$ bacteria per plant sample). This undoubtedly reflected the close association between bacteria and host required to deliver the type III effectors (Collmer et al., 2000) and possibly encapsulation within the plant cell wall (Bestwick et al., 1995). Thus, the plant cells were transferred to sterile 50 mL tubes and washed further with 20 mL 0.85% NaCl and mixed using a vortex. This process was repeated three times; a process that took around 5 min to complete. After each wash, the number of contaminating bacteria was reduced and after the third wash was considered to be trivial, at a few hundred rifampicin-resistant, viable bacteria per 100 mg of plant tissue (Fig. 2B). At these low levels of contamination it may be possible, although highly unlikely, that the bacterial cells would make a contribution to the FT-IR spectra collected from the plant cell samples. With these data, the basic premise of the dual metabolomic approach (i.e. large scale separation of bacterial and plant cells followed by independent intracellular metabolomic analyses) was successfully established. Therefore the dual metabolomics methodology had been developed to a stage where it permitted the independent

analysis of intracellular metabolites from both host and pathogen cells.

2.5. Robust determination of the post-infection intracellular metabolomes of the *Arabidopsis* cell cultures and *Pst* strains

Following the development of the host–pathogen cell separation approach, the metabolomes of plant cells isolated after 12 h

co-cultivation with each of the *Pst* strains were assessed using FT-IR spectroscopy (Fig. 3A). PC-DFA indicated that the *Arabidopsis* cells exhibited distinctive responses to each bacterial strain. PC-DFA indicated that each *Pst* strain elicited metabolomic changes which were different to the controls where only 10 mM MgCl₂ was added to *Arabidopsis* cultures. Interestingly, although inoculation with *Pst* resulted in levels of cell death which did not differ significantly from those seen with *Pst hrpA* (Fig. 1B), the metabolome

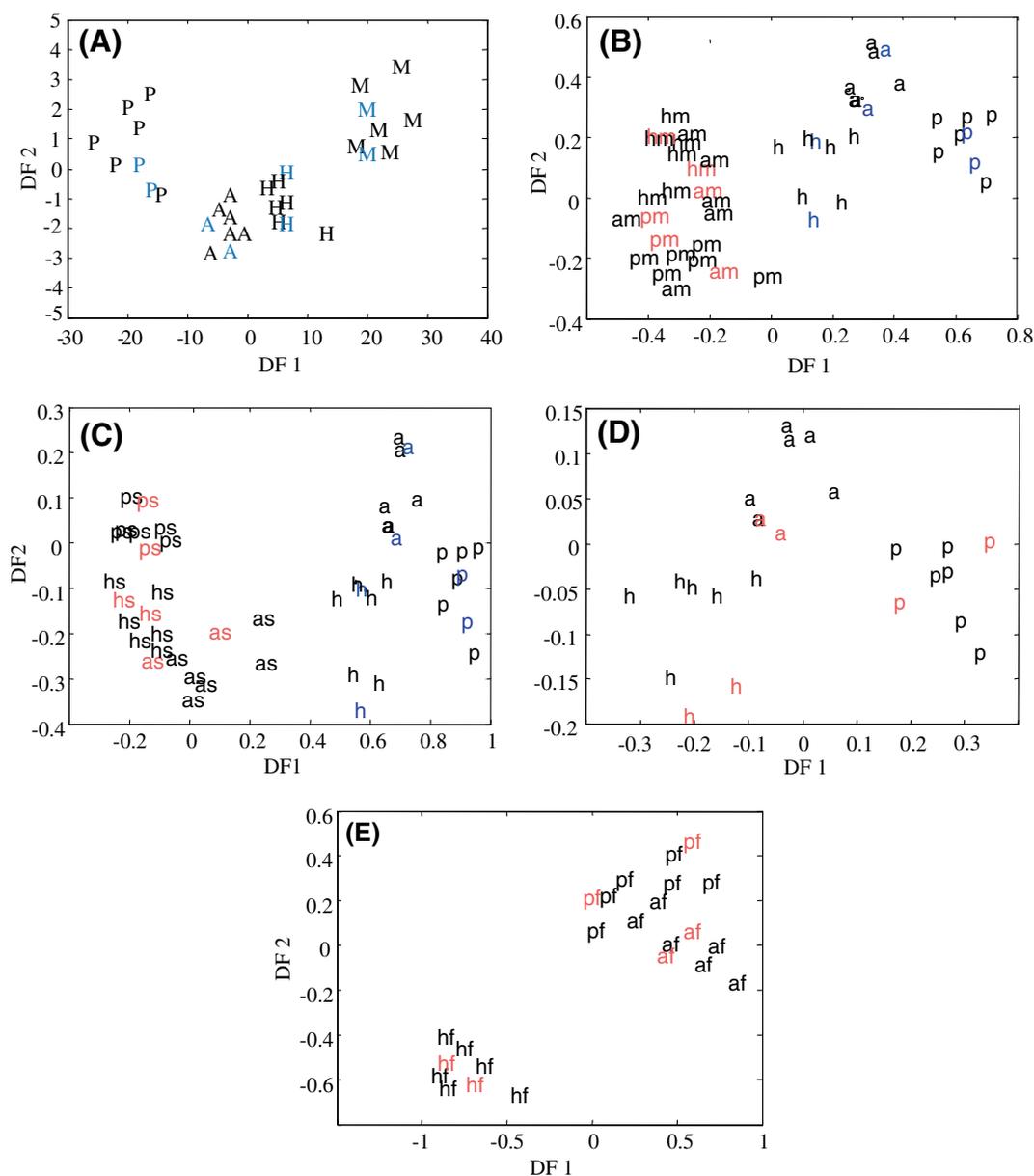


Fig. 3. Principal component-discriminant function analysis (PC-DFA) of Fourier transform infrared (FT-IR) data from the *Pseudomonas syringae* pv. *tomato*–*Arabidopsis thaliana* interaction. (A) PC-DFA of cultured *Arabidopsis* cells at 12 h post inoculation (hpi) with P = *Pst* (virulent strain), A = *Pst avrRpm1* (HR-eliciting strain), H = *Pst hrpA* (non-HR/non-virulent strain) or M = 10 mM MgCl₂. The PC-DFA model employed 10 PCs accounting for 99.32% of the total variance. Eight replicates of each biological category were used to generate the model. (B) PC-DFA comparing the FT-IR metabolite fingerprints of *Pst* (p), *Pst avrRpm1* (a) and *Pst hrpA* (h) bacteria incubated with fresh AT3 media (designed with “m” suffix) compared to bacteria co-cultured with *Arabidopsis* cells (no suffix). Eight replicates of each biological category were used to generate the model which accounted for 99.91% of the variance (10 PCs). (C) PC-DFA of FT-IR metabolite fingerprints of *Pst* (p), *Pst avrRpm1* (a) and *Pst hrpA* (h) bacteria incubated with spent AT3 media (designed with “s” suffix) compared to bacteria co-cultured with *Arabidopsis* cells (no suffix). Eight replicates of each biological category were used to generate the model which accounted for 96.53% of the variance (10 PCs). (D) A PC-DFA projection of FT-IR metabolite fingerprints of only *Pst* (p), *Pst avrRpm1* (a) and *Pst hrpA* (h) bacteria – cultured with *Arabidopsis* cells is presented to aid clarity. Eight replicates of each biological category were used to generate the model which accounted for 98.87% of the variance (10 PCs). (E) PC-DFA model of FT-IR spectra of the metabolite footprints – the plant and bacterial cell free media prepared after 12 h of *Arabidopsis*–*Pst* co-culture. Shown are the footprint data following inoculation with *Pst* (pf), *Pst avrRpm1* (af) and *Pst hrpA* (hf). Eight replicates of each biological category were used to generate the model which accounted for 97.34% of the variance (10 PCs). Please also note that all PC-DFA models were validated by the independent projection of two biological replicates from each experimental class (the test data set; blue or red) into the PC-DFA space of their remaining six replicates (the training data set; black).

of plants cells challenged with *Pst* proved to be more distinctive than the metabolomes of *Arabidopsis* challenged with either *Pst hrpA* or *Pst avrRpm1* (Fig. 3A), possibly due to commonalities between the *Arabidopsis* basal resistance responses to both *Pst hrpA* and *Pst avrRpm1*. Taking all other data together, and as would be predicted from the extensive use of this *Arabidopsis* cell culture line in defence related studies (Clarke et al., 2000; McCabe and Leaver, 2000; Desikan et al., 2001a,b), we have demonstrated that cultured plant cells were appropriate to determine phytopathogen relevant metabolomic changes.

In developing the co-cultivation method to reveal the pathogen metabolome, the possibility that the bacteria were responding primarily to AT3 media, or AT3 media that had supported plant cell growth for 7 d (“spent” media), had to be considered. The distinctiveness of the *Pst* strains co-cultivated with plant cells compared to fresh AT3 media was assessed. Each *Pst* strain was inoculated into 20 mL of fresh AT3 media and into 20 mL of pooled *Arabidopsis* cell cultures (Fig. 2). After 12 h, both the bacterial-fresh AT3 and bacterial-plant culture samples were filtered and the bacteria were isolated from the filtrates by centrifugation and sequentially washed with 0.85% NaCl (as detailed in the Section 4.7). Comparisons of the bacterial metabolomes by FT-IR spectroscopy coupled with PC-DFA revealed that the three bacteria responded differently to plant cell culture compared to fresh AT3 media (Fig. 3B). To investigate if the bacteria were responding primarily to the spent media an additional step was added at the stage when the *Arabidopsis* cell culture was pooled (Fig. 2). Immediately after pooling, the culture was split into 2 × 1.5 L samples, with one 1.5 L being aliquoted and taken forward for inoculation with bacterial strains. The other 1.5 L was filtered through Whatmann No. 1 filter paper with the filtrate providing the spent AT3 medium. Each bacterial strain was inoculated into 20 mL of this spent AT3 medium. It should be noted that the spent media analysis was directly comparable to the analysis of plant cultures subjected to bacterial challenge, where the initial plant cell culture and spent media had been attained from the same initial 3 L pool of plant cells. Following sampling at 12 hpi, FT-IR fingerprinting coupled with cluster analysis showed that each bacterial strain responded differently to plant cell cultures compared with spent AT3 (Fig. 3C). It was also relevant that the responses of each of the *Pst* strains to fresh or spent AT3 could not be differentiated, unlike when plant cells are also cultured with the bacterial cells (Fig. 3B and C). Hence, although the bacterial strains were responding to AT3 media, these responses could be distinguished from their responses to plant cells, and further, interaction specific effects could also be defined. A PC-DFA model for bacterial cells cultured only with plant cultures was re-plotted to highlight the distinctive metabolomic responses to plant cells which are elaborating either the beginning of a HR (in response to *Pst avrRpm1*), disease (in response to *Pst*) or a basal defence response which does not lead to either disease or a HR (in response to *Pst hrpA*; Fig 3D).

2.6. Establishing the post-infection extracellular metabolome by metabolite footprinting

A further level of analysis is possible by comparing the media separated following filtration of plant cells and removal of bacterial cells by centrifugation. This is the metabolic footprint which has previously been used to monitor the metabolite secretion in bacteria, yeast and plant cultures (Kaderbhai et al., 2003; Carrau et al., 2008; Dowlatabadi et al., 2009). However, within this dual culture system it must be considered that the culture media will contain metabolites from both the plant host and the bacterial pathogen. Considering metabolite extrusion from the host, it will include species such as hexoses (Swarbrick et al., 2006) and anti-microbial terpenoids (Stukkens et al., 2005). Metabolites extruded from

Pseudomonas syringae will include toxins such as coronatine, and lipodepsipeptides (Bender et al., 1999).

PC-DFA analyses of the FT-IR spectra of the footprints of each bacterial interaction indicated that each was distinctive, but interestingly, the difference between responses to *Pst avrRpm1* and *Pst* (respectively HR and disease eliciting) were minor compared to that of *Pst hrpA* (Fig. 3E). This could have indicated that at 12 hpi, much of the metabolomic distinctiveness in TTSS-mediated elicitation of a HR or disease formation is intracellular (compare Fig. 3A, D and E).

3. Concluding remarks

Our results have shown that the co-cultivation of *Arabidopsis* cells with *Pst* strains produced discrete metabolomic changes in both host and bacteria. It is clear, that these can only partially mimic changes that occur *in planta* as plant cell cultures do not have the complicated syncytial links between cells and physiological interactions which are prevalent in a whole plant. Equally, *in planta* the pathogenic bacteria are not likely to be exposed to such nutrient rich conditions, and changes in ion balances and pH may also only poorly approximate to those occurring in an apoplast. However, against this, it should be noted that *Arabidopsis* cell cultures, and indeed those from other species, have proven to be vital in the definition of several defence responses, for example the oxidative burst and NO generation (Levine et al., 1994; Delledonne et al., 1998). In addition, plant cell cultures are employed for transcriptome and proteomic investigations of plant defence in several species (Desikan et al., 2001a,b; Contento et al., 2004; Pauwels et al., 2008). Further, we have demonstrated distinctive interaction specific responses in both host and pathogen, that will be further characterised in future research involving mass spectrometry based profiling for metabolite identification and quantification. In conclusion we believe that our dual metabolomic approach will reveal important features which can be subsequently tested *in planta*. It is also the case, that this carefully controlled approach here described would be readily amendable to systems level mathematical descriptions.

4. Experimental

4.1. General materials

Unless otherwise stated, all chemicals solvents and acids were obtained from Fisher Scientific Ltd. (Loughborough, UK) and plant culture media and vitamins from Duchefa (Haarlem, The Netherlands).

4.2. Propagation of plant cultures

The *Arabidopsis thaliana* Landsberg *erecta* (Ler) suspension cultures were first derived by May and Leaver (1993). Cultures were maintained in AT3 media (Touraev and Heberle-Bors, 1999) at 24 °C on a long day 16 h light cycle on an orbital shaker at 140 rpm in a controlled environment growth room. The suspension cultures were sub-cultured on a weekly basis by transferring ~3 mL of 7 day culture into 200 mL of fresh AT3.

4.3. Estimations of plant cell death

Plant cell death was estimated through the retention of Evan's Blue stain. Precisely 0.5 mL of 0.25% (w/v in water) Evans Blue was added to 1 mL of *Arabidopsis* culture and assessed under a microscope after 10 min.

4.4. Northern blotting

RNA extraction, blotting and probing with ^{32}P -labelled DNA probes was undertaken as described in Warner et al. (1994).

4.5. Propagation of bacterial cultures

Rifampicin-resistant strains *P. syringae* pv. *tomato* DC3000 (*Pst*), *Pst avrRpm1* and *Pst hrpA* were maintained on either solid nutrient agar (NA) or in liquid nutrient broth (NB) without antibiotic selection. NB was prepared from a preparatory mixture (Sigma–Aldrich Ltd., UK) following the manufacturer's instructions. NA was prepared by adding 28 g L^{-1} bacto-agar (Lab-M Ltd., UK) to the NB.

For dual metabolomics experiments the bacterial strains were prepared by sub-culturing. Starter cultures were generated from NB bacterial cultures at a mid-exponential growth phase (determined spectroscopically, $\text{Ab}_{600\text{nm}} = 0.1$, spectroscopic measurements were undertaken three times for accuracy). Next, 1 mL of this starter culture was inoculated into a fresh culture of 400 mL NB. Once the 400 mL NB cultures had reached a mid-exponential cell density (1×10^9 cells mL^{-1}), the bacteria were removed by centrifugation, cleaned three times and finally reconstituted in ~ 40 mL of 10 mM MgCl_2 (1×10^{10} cells mL^{-1}), thus providing inoculates for challenge of the *Arabidopsis* cultures.

In assessing the bacterial contamination of the *Arabidopsis* cell clusters remaining after plant cell collection and processing, the plant cells were ground down in sterile 10 mM MgCl_2 and serially diluted prior to spreading on a NA selective media supplemented with rifampicin (50 mg mL^{-1}).

4.6. Dual metabolomics: inoculation procedure

Readers should also refer to Fig. 2. Bacterial inocula were prepared from 400 mL NB bacterial cultures following a 3 min centrifugation at 17,000g at 20 °C. The bacterial pellets resuspended in 40 mL 10 mM MgCl_2 , and transferred to 50 mL sterile tubes and washed three times. These wash steps involved centrifugation (3 °C, 6000g for 3 min) and resuspension of the pellet in 40 mL of 10 mM MgCl_2 . At the final resuspension the bacterial density was 1×10^{10} cells mL^{-1} .

To reduce experiment-to-experiment variation in the plant metabolome, 15 7-day-old 200 mL *Arabidopsis* suspension cultures were pooled into a sterile 3 L conical flask. This was split into 2×1.5 L cultures in sterile conical flasks. One 1.5 L culture was the source of 20 mL aliquots of *Arabidopsis* cells and these were transferred to 50 mL sterile tubes. These were inoculated with 200 μL of a given *Pst* strain to give a final bacterial cell density of 1×10^8 cells mL^{-1} . The other 1.5 L culture was filtered through Whatmann No. 1 filter paper (Whatman plc., UK) using a Buchner funnel linked to an electric vacuum pump. This provided a source of “spent” AT3 media from which 20 mL was similarly aliquoted into 50 mL sterile tubes and inoculated with 200 μL of a *Pst* strain to give final density of 1×10^8 cells mL^{-1} . Likewise, 20 mL aliquots of fresh AT3 in 50 mL sterile tubes were also inoculated with 200 μL of a *Pst* strain to give final density of 1×10^8 cells mL^{-1} . Given the time needed for subsequent sample processing each inoculation was staggered by 5 min.

4.7. Dual metabolomics: sample collection and preparation

The *Arabidopsis* inoculated cell suspension cultures were sampled after 12 h. The 20 mL cultures were first filtered through Whatmann No. 1 filter paper to retain the plant cells using a Buchner funnel linked to an electric vacuum pump. The filtrate containing the bacterial cells was transferred to a 50 mL sterile tube and stored on ice. The filtered *Arabidopsis* cells were resuspended in

20 mL 0.85% (w/v) NaCl, transferred to a 50 mL sterile tube, vortex mixed, and filtered as before. This process was repeated twice further. The *Arabidopsis* cells (~ 100 mg dry weight) were transferred into 2 mL microcentrifuge tubes containing stainless steel ball bearings (washed in acetone), flash frozen in liquid N_2 and stored at -80 °C.

The ice-stored filtrates were then centrifuged at 3 °C and 6000g for 3 min to pellet the bacteria. The supernatant was transferred to sterile 2 mL microcentrifuge tubes, flash frozen in liquid N_2 and stored at -80 °C. This represented the co-culture “footprint” (extracellular metabolome). The remaining bacterial pellets were resuspended in 1 mL 0.85% NaCl (w/v), vortex mixed, re-centrifuged as before, the supernatant discarded, and the wash procedure repeated twice further before the remaining bacterial pellet was flash frozen in liquid N_2 and stored at -80 °C.

For bacterial inoculations of fresh or spent AT3 media, bacteria were pelleted at 12 hpi by centrifugation at 3 °C and 6000g for 3 min. The pellets were next washed in 1 mL 0.85% NaCl (w/v) three times and the remaining pellet flash frozen in liquid N_2 and stored at -80 °C.

4.8. Fourier transform infrared (FT-IR) spectroscopy

Sample preparation was as described in Allwood et al. (2006). Plant material (~ 100 mg dry weight) was homogenised using a ball mill in 1 mL of sterile ultra pure dH_2O . Samples (15 μL) were loaded onto an acetone-washed 100 well aluminium sample plate (LNC Technology Ltd., Ystrad Mynoch, Hengoed, UK). Bacterial pellets were resuspended in 0.5 mL of sterile ultra pure dH_2O and 15 μL of the slurries were loaded onto a 100 well aluminium sample plate. Three machine replicates of each biological replicate were loaded onto a single FT-IR plate. Following oven drying (50 °C for 30 min) plates were scanned in reflectance mode using a Bruker IFS28 infrared spectrometer also as described in Allwood et al. (2006).

4.9. Chemometric data analysis

The FT-IR spectra that were obtained were exported in ASCII format into MATLAB version 6.5 (The MathWorks Inc., Natwick, MA, USA). The spectra were mined as described in Allwood et al. (2006). Pre-processing involved five-point smoothing using the Savitzky–Golay algorithm (Savitzky and Golay, 1964). Data were examined using principal components analysis (PCA) which derived principal components (PC) and thereby reduced the data dimensionality. Based on these PCs, discriminant function analysis (DFA) was used to attempt to discriminate between the experimental groups based on an *a priori* knowledge of experimental class structure (i.e. PC-DFA; Goodacre et al., 1998, 2004). Validation of PC-DFA involved projection of a randomly selected “test set” of a sub-population of replicates into the remaining replicates (‘the training set’). Close superimposition of test and training sets when plotted on the same PC-DFA ordination plot indicated highly reproducible data sets (Allwood et al., 2006).

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