Chapter 3

Separating the Inseparable: The Metabolomic Analysis of Plant–Pathogen Interactions

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Abstract

Plant–microbe interactions—whether pathogenic or symbiotic—exert major influences on plant physiology and productivity. Analysis of such interactions represents a particular challenge to metabolomic approaches due to the intimate association between the interacting partners coupled with a general commonality of metabolites. We here describe an approach based on co-cultivation of *Arabidopsis* cell cultures and bacterial plant pathogens to assess the metabolomes of both interacting partners, which we refer to as dual metabolomics.

Key words: Plant–microbe interactions, Pathogen, *Arabidopsis*, Plant suspension cultures, Co-cultivation, Dual metabolomics

1. Introduction

Plant interactions with microbes play a major role in defining physiology and development. Plants are continually under attack from pathogens of various species and the deployment of diverse defences represents a substantial cost to the host (1). Equally, pathogens can act as selective agents driving the selection of resistant germplasm (2). From an anthropogenic point of view, pathogen attack represents a considerable source of crop loss (3). However, plant–microbe interactions are not solely pathogenic; many symbiotic relationships exist which improve nutrient assimilation by the plant and hence improve productivity. The interaction between nitrogen-fixing bacteria and legume plants is especially well characterised (4, 5), but due to their wider host-species range, interactions with mycorrhizal fungi are possibly more important. Mycorrhizal fungi establish hyphal connections with cells in the root and extend into the surrounding soil, thereby

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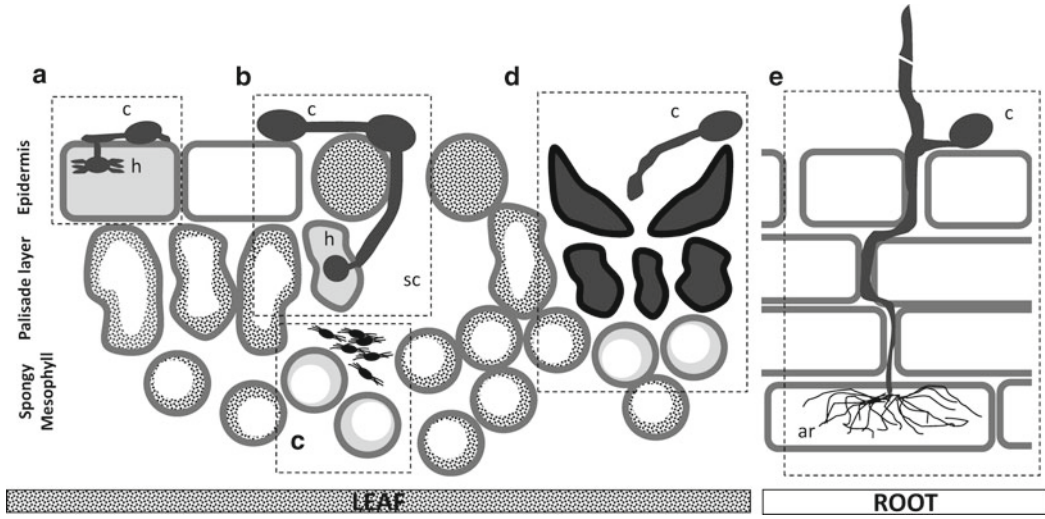

Fig. 1. Tissue heterogeneity as a result of various plant pathogen interactions. A schematic transverse section through a plant leaf and root illustrating interactions with a range of microbes. Green and healthy plant cells are filled with *dots*, and those which ware exhibiting disease symptoms are shown in *light grey*, whilst those which are dead are in *dark grey*. (**a**) The germinated condium (c) ultimately forms a digitate feeding structure—the haustorium (h)—which does not penetrate beyond the epidermal layer but supplies nutrients from the host to ectopic fungal development. (**b**) The infection structure of Rust fungal pathogens which target open stomata, penetrating into the substomatal cavity (sc). Within this area, the fungus forms haustoria-like feeding structures and elaborates *in planta* hyphal development until sporulation, where the rust-clusters of conidiophores burst through the epidermal surface (not shown). (**c**) Biotrophic bacterial pathogens (i.e. those which live off living plant tissue for extended periods) tend to infect via stomata or opportunistically at wound sites. They multiply within the apoplastic space surrounding the cells. The amphitrichous flagellate *Pseudomonas syringae* is shown. (**d**) A pathogenic interaction involving a necrotrophic fungus is shown. Host death arises through toxin production and/or enzymatic attack originating from the pathogen. Note that no obvious infection structure is observed with necrotrophic fungal pathogens. (**e**) A symbiotic interaction with an arbuscular mycorrhiza (plural mycorrhizae or mycorrhizas) is shown where the fungus (Phylum Glomeromycota) penetrates to the cortical cells of the roots of a vascular plant. This interaction is characterised by the formation of arbuscules (ar) and significant fungal growth from the root into the surrounding soil (indicated by a *broken hypha* in the diagram).

improving nitrogen and phosphate uptake (4) and encourage association with plant-growth-promoting rhizobacteria (6, 7).

A key feature of all such plant–microbe interactions is an intimate association between both partners (Fig. 1). It is important to note that reciprocal responses in both host and microbe results in altered molecular and physiological status so that it is distinct to their situations when considered in isolation. Further, when investigating these interactions it is often difficult as they frequently involve only a few participating plant cells, which themselves can show considerable spatial heterogeneity in their responses. Sampling therefore often includes large numbers of non-, or differently, responding cells so that any localised responses may be difficult to discern. Equally, it is often difficult to gather sufficient material from the microbial partner to make analysis possible. Therefore, there are considerable technical problems in investigating plant–microbe interactions.

If the aim is only to consider the host response and the focus is on gene transcript or proteomic changes, it is valid to simply ignore

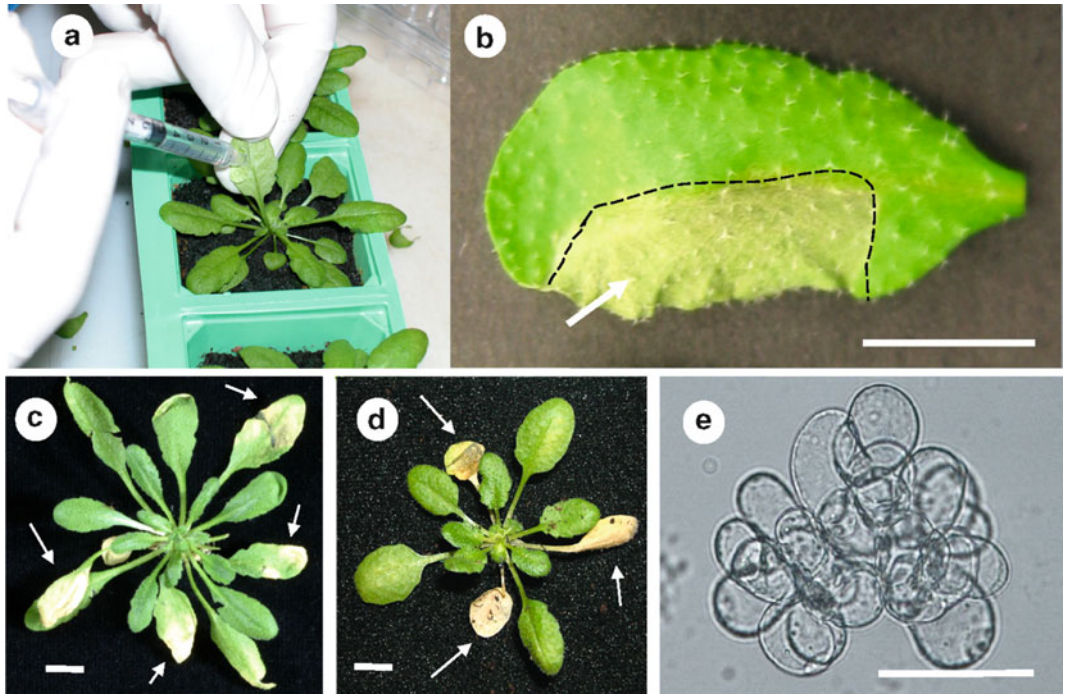

Fig. 2. Approaches to assess changes in plant microbe interactions. (**a**) A widely used approach to inoculate *Arabidopsis thaliana* with bacterial pathogens involves the infiltration of the intracellular spaces of leaves with bacterial suspensions in 10 mM $MgCl_2$ (~10^6 cell/mL). Typically, the bacterial suspensions are infiltrated using a syringe via the stomata of the lower epidermal surface. Alternative approaches can involve dipping or spraying *Arabidopsis* with high titres of bacterial suspensions. Infiltration of leaf spaces has the advantage of producing a large area of synchronously responding plant tissue which reflects the nature of the interaction. (**b**) Inoculation with *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst*) *avrRpm1* rapidly elicits cell death (a Hypersensitive Response (HR)) within the inoculated area (encompassed by the dashed lines and arrowed) (bar = 1 cm). Disease and elicitation of the HR is dependent on the delivery of bacterial protein effectors into the host, the nature of the response being dependent on the plant genotype. The bacterial effectors may be cloned and fused to an inducible promoter and introduced into *Arabidopsis* plants to generate transgenic lines. Two examples are given. (**c**) The *HopAB2* bacterial effector gene is fused to the glucocorticoid responsive promoter. This, along with the mammalian glucocorticoid receptor/transcriptional activator protein gene, was introduced into *Arabidopsis.* Application of glucocorticoid to *HopAB2* transgenic plants resulted in the elaboration of symptoms (*arrowed*) analogous to disease symptoms. Details of the inducible system can be found in ref. (65). (**d**) The *avrPpiA1* avirulence gene which elicits a HR in *RPM1* encoding *Arabidopsis* Col-0. The *avrPpiA1* gene was fused to the *Aspergillus nidulans niger* alcohol dehydrogenase (*alcA)* promoter. This, along with the alcohol responsive transcriptional activator (*AlcR*) protein gene, was introduced into *Arabidopsis* (66). Application of alcohol to *avrPpiA1* transgenic plants resulted in the rapid elicitation of cell death (*arrowed*)—which was reminiscent of the HR. (**e**) Plant–pathogen interactions can also be investigated in plant suspension cell cultures inoculated with bacterial pathogens. Illustrated is an *Arabidopsis* cell cluster from a suspension cell culture. (Bar = 200 μm).

the interacting microbe, since RNA transcript or protein sequence allows changes to be targeted to a specific partner. However, metabolites tend not to exhibit such specificity and therefore this poses a significant challenge to metabolomic analyses. This problem is further compounded if interactions of bacterial pathogens with, for example, *Arabidopsis thaliana* are being examined. In such cases, a commonly used approach is to infiltrate the intracellular species of leaves with high titres of bacterial suspension (Fig. 2a). This offers an excellent source of large areas of synchronously

responding tissue, thereby mitigating the problem of tissue limitation referred to above. Depending on host and pathogen genotypes, inoculated areas can exhibit disease symptoms or a form of plant cell death—the Hypersensitive Response (HR; Fig. 2b)—which is localised to the point of attempted infection and is often linked to resistance (8). However, the high bacterial population makes a considerable contribution to the metabolome of a sample from plant–pathogen interactions so that the origin of a given metabolite may defy attribution.

A number of approaches exist that may possibly circumvent this problem. It may be assumed that the biomass of the interacting microbe is so low that its contribution to the metabolome will be insignificant and will therefore represent only that of the plant host. If such a strategy is followed, it would be necessary to confirm that no significant metabolite contribution to the samples has occurred. This necessitates screening for microbe specific metabolite biomarkers. In the case of fungal microbes, this may involve assaying for the steroid ergosterol (9) or the phospholipid acyl chain, arachidonic acid (C20:4; (10)), both of which are absent from plants. Alternatively, the metabolome of one or indeed both interacting partners can be isotopically labelled (11–13). Thus, comparison of labelled and non-labelled interactions will allow the relative contribution of the interacting partners to the metabolome to be defined.

Another approach is to avoid any plant-interacting metabolome at all, for example, by focusing on plant viruses. For example, metabolomic changes associated with tobacco infected with tobacco mosaic virus have been described (14). There is, however, no need to limit oneself to viral pathogens. The use of pathogen-derived elicitors to investigate plant defence responses is a very well-established approach (15). There are a range of elicitors available from pathogens from various kingdoms which can be used to examine resistance mechanisms associated with or without limited host cell death (Table 1). It is also possible to focus on mutants in model plant species which show the constitutive activation of plant defences. For example, *lesions simulating disease 1* (*lsd1*), exhibits the spontaneous exhibition of necrotic lesions, which is frequently equated with a HR (16, 17). There are many examples of such cell death mutants (18). Other mutants show constitutive exhibition of defence-associated signalling events linked to, for example, the hormones salicylic acid (SA), jasmonates (JA), or ethylene (19).

Yet another approach is suggested from the pathogenesis mechanisms of bacterial pathogens, particularly those of *Pseudomonas syringae* and *Xanthomonas* spp. In these species, bacteria deliver large numbers of effector proteins into plant cells to cause disease, but in certain plant genotypes an effector is recognised by a resistance gene to elicit the HR (20). In the latter situation, the recognised effectors are referred to as avirulence gene products. Bacterial effectors can be fused to an inducible promoter

Table 1
Some microbial elicitors of plants defence or symbiotic responses

Elicitor	Origin	Action	References
Chitin oligosaccharide	Fungal cell walls	General defence initiation. No cell death	(55)
13-Pep	*Phytophthora sojae*	General defence initiation. No cell death	(56)
Flagellin/flg22	Bacterial pathogens	Initiator of basal defences	(57)
Lipopolysaccharide (LPS)	Bacterial	Initiator of basal defences	(58)
Harpin	Bacterial pathogens	Initiator of cell death	(59)
Cryptogein	*Phytophthora cryptogea*	Initiator of defences including cell death	(60)
Victorin	*Cochliobolus*	Initiator of cell in *Vb* genotypes of oat	(61)
NIP1	*Rhynchosporium secalis*	Initiator of cell in *Rrs1 genotypes of barley*	(62)
Nod factors (*lipochito-Oligosaccharides*)	*Rhizobium* spp.	Root deformation *in legumes*	(63)

Plant responses to pathogens may be crudely designated as cell death associated (often equated with the Hypersensitive Response, HR) or defences without initiation of macroscopic cell death. The table lists some of the elicitors of each form of defence that are available. Elicitors of plant defence leading to the induction of defence gene expression, but not cell death, include chitin and 13-Pep. Other elicitors initiate a subset of plant defences, which are linked to basal/innate resistance, which are displayed against any microbes and not only plant pathogens. Well-characterised examples of basal resistance are elicited by flagellin—flg22—or the bacterial LPS. Defences associated with cell death may be investigated by the addition of NIP1 (Necrosis inducing protein 1) which is secreted by the fungus *Rhynchosporium secalis* (the causal agent of scald disease) on *Rrs1* genotypes of barley or the general cell death elicitor harpin, which is an ionophore isolated from Pseudomonad bacterial pathogens. Other cell death elicitors are produced by pathogens which actively kill as part of their pathogenesis mechanism, but these are taken as parallels of the HR. These include cryptogein, a 98-amino acid proteinaceous elicitor from *Phytophthora cryptogea*—which elicits cell death on many plant species and victorin, a host-selective toxin produced by the fungus *Cochliobolus victoriae*, which acts only on *Vb* oat (*Avena sativa*) genotypes. Considering symbiotic interaction, although nitrogen fixation in legume-*Rhizobium* bacteria interactions takes place within root, some early aspects can be induced by pathogen-secreted Nod factors. Nod factors are lipochitooligosaccharides consisting of an acylated chitin oligomeric backbone with differing moieties conferring different host specificities (63, 64)

and introduced into plants to generate transgenic lines (Fig. 2c, d). This offers a substantial source of responsive tissue that can be linked to the action of particular bacterial cell effectors and be used to examine metabolomic changes.

However, such approaches are limited as the metabolome of the interacting microbe is absent. One way of assessing the complicated metabolomic changes associated with plant microbial interactions is to exploit the possibilities offered by in situ imaging of metabolites and thereby assigning key changes to one interacting partner or the other. For example, there have been recent advances in imaging metabolites based on matrix-assisted laser desorption ionisation

(MALDI) imaging techniques. In this approach, the MALDI matrix is applied to thin sections of sample and the laser desorbed proteins, peptides, or small molecules are assessed by Mass Spectrometry (MS). The spatial patterns of metabolites can be mapped using a range of suitable imaging processing software and related to an optical image of the sample. A particularly attractive aspect of this technique is the ability to describe the distribution of tentatively identified metabolites. MALDI-MS imaging has been used to map the distribution of a modified form of the CLAVATA3 (CLV3) secreted peptide hormone in *Arabidopsis* callus slices (21), the movement of pesticides on the leaves and within the stems of soya plants (22), and the distribution of carbohydrate in wheat stems (23). Most pertinent to this chapter, MALDI was used in conjunction with Fourier Transform-Mass Spectrometry (FT-MS) to compare the spectra of leaves from a healthy *Prunus persica* (peach) tree to those infected with the fungus *Taphrina deformans*. This approach identified differences in the abundance of various phospholipids (24).

In an allied approach, microspectroscopic techniques are now being applied to visualise biochemical changes in plant systems, although identification of actual metabolites is difficult. Infrared (IR) mapping with a synchrotron source using a focal plane array (FPA) allowed the analysis of Eucalyptus (25), aleurone cell walls in wheat grain (26), and maize seeds (27). Raman spectroscopy relies on inelastic scattering in monochromatic light (28) and has proven to be particularly useful for imaging lignin structure (29).

Although we have exploited FT-IR microspectroscopic approaches (30), here we describe an alternative approach based on the co-culture of *Arabidopsis* suspension cell clusters (Fig. 2f) and bacteria pathogens. This approach offers a ready source of plant material that is often used as a model for plant responses (12, 31–33). Further, as phytopathogenic bacteria are not phagocytosed, as occasionally occurs with disease in animals (34), it is possible to separately elucidate the microbial metabolome. This determination of interacting host and microbial metabolomes we refer to as dual metabolomics.

2. Materials

1. *Arabidopsis* cell cultures (see Note 1).
2. AT3 medium: Murashige–Skoog salts with vitamins 4.41 g/L, sucrose 30 g/L, NAA 0.5 mg/L, kinetin 0.05 mg/L. Sterilised by autoclaving.

3. Bacterial strains: *Pseudomonas syringae* pathovar *tomato* DC3000 (*Pst*), *Pst avrRpm1*, and *Pst hrpA* (see Note 1).
4. Nutrient Agar (NA) 5 g/L peptone, 3 g/L beef extract, 15 g/L agar; adjusted pH to 7.0. Sterilised by autoclaving.
5. Nutrient Broth (NB 5 g/L peptone, 3 g/L beef extract, adjusted pH to 7.0). Sterilised by autoclaving.
6. Whatman No 1 filter paper (pore size ~11 πm) or equivalent.
7. Vacuum Pump.
8. Laminar Flow Cabinet.
9. Antibiotics. The antibiotics used will reflect the bacterial strains and plasmids under selection. In the experiments described here, the following antibiotics and concentrations were used: rifampicin (100 πg/mL) and kanamycin (10 πg/mL).
10. $MgCl_2$ used at 10 mM concentration in de-ionised (for example, by distillation) water. Sterilised by autoclaving.
11. Evans Blue strain (Sigma Pharmaceuticals Ltd) is used at 0. 25% (w/v) in water.
12. NaCl was used at 0.85% (w/v) concentration in de-ionised (for example, by distillation) water. Sterilised by autoclaving.
13. A mix of chloroform (Fisher Scientific, UK), methanol (Fisher Scientific, UK), and de-ionised (for example, by distillation) and sterile (sterilised by autoclaving) water in a proportion of 1:2.5:1 (v/v).
14. Commercially prepared ultra-pure dH_2O (Fisher Scientific, UK): Sterilised by autoclaving.
15. Acetonitrile (Fisher Scientific, UK) and 0.2% formic acid (Fisher Scientific, UK) in de-ionised (for example, by distillation) and sterile (sterilised by autoclaving) water (v/v) were mixed in a proportion of 1:1 (v/v).
16. Methanol (Fisher Scientific, UK): used at 80% and 60% (v/v) dilution in de-ionised (for example, by distillation) and sterile (sterilised by autoclaving) water.
17. Propan-2-ol (Fisher Scientific, UK): used at 70 dilution (v/v) in de-ionised (for example, by distillation) and sterile (sterilised by autoclaving) water.
18. Acetonitrile (Fisher Scientific, UK): used at 10 dilution (v/v) in de-ionised (for example, by distillation) and sterile (sterilised by autoclaving) water.

3. Methods

3.1. Establishing the Host and Pathogen Metabolomes

3.1.1. Culture of Arabidopsis Suspension Cells

1. The *Arabidopsis* Landsberg *erecta* (L *er*) suspension was first derived from callused stem cells developed by May and Leaver (35).
2. The plant culture regime should be standardised and well established in the investigators group prior to commencing with dual metabolomic studies. Maintain *Arabidopsis* suspension as 200 mL AT3 medium at 24°C on a long day 16 h light cycle at 25 μmol/m^2/s. Cultures should be aerated by shaking on an orbital shaker at 140 rpm. Subculturing should occur after no more than 7 days by transferring ~3 mL of 7 day culture into 200 mL of fresh AT3 in a laminar flow cabinet. The suspension cells should be free of contamination and exposed to minimal stress (see Notes 2 and 3).
3. Maintain large numbers of 200 mL cultures. For each experiment 15 × 200 mL cultures are pooled (see Subheading 3.2); hence, multiple cultures will allow ready inoculation of large numbers of AT3 cultures (see Note 4).

3.1.2. Culture of Bacterial Strains

Whilst it is perfectly valid to examine metabolite changes within a single bacterial strain interacting with a host, the value of the dual metabolomic approach is increased if the responses of different bacterial strains are compared. However, this requires that either the starting metabolomes of each strain be well defined or ideally, be substantially equivalent. The latter can be achieved by growing each strain in chemostats. However, in many laboratories this may not be possible; hence, the following protocol details a semi-batch approach where *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*—which is virulent on *Arabidopsis*), *Pst avrRpm1* (which is "avirulent"—in that it can elicit a HR from *Arabidopsis*), and *Pst hrpA* (which is non-virulent and is unable to elicit a HR) were grown.

1. Maintain the bacterial strains on solid nutrient agar (NA) plates. Derive single colonies by streaking across the agar surface using a sterile wire loop. Supplement the medium with appropriate antibiotics to maintain any plasmids within the strains.
2. Add a single colony from the bacterial plate to 10 mL of NB and incubate at 28°C in an orbital shaker at ~200 rpm for ~12 h. Use an aliquot of 5 mL of this culture to inoculate 400 mL NB and incubate at 28°C in an orbital shaker at ~200 rpm (~10 × *g*) (see Note 4).
3. The bacteria used for inoculation of *Arabidopsis* cultures should be in a mid-exponential growth phase. Assess samples (1 mL) from the culture for culture turbidity using a spectrophotometer.

An absorbance of 0.01 at 600 nm with a 1 cm path length represents ~2×10^7 cell/mL and indicates that the bacteria are in mid-exponential phase.

3.2. Inoculation Procedure

When inoculating the *Arabidopsis* cultures, consideration must be given to the time required to subsequently collect and process the samples (see Subheading 3.4). In large experiments, the difference in time between processing the first and last samples can be considerable and can complicate interpretation of the results. In our hands, we staggered the bacterial inoculation of individual cultures by 5 min, but this can differ depending on the experience of the researcher(s) and should be practised and assessed in advance. The speed and repeatability of the sample processing steps can have a massive effect on the analytical reproducibility of replicate samples.

When following the subsequent protocol, readers are also referred to the work flow diagram shown in Fig. 3.

1. Once at mid-exponential phase, pellet the bacteria from the 400 mL cultures by a 3 min centrifugation at 17,000 × *g* at 20°C. Resuspend the pellet in 40 mL sterile 10 mM $MgCl_2$ and transfer to 50-mL sterile tubes. To remove any contaminating media, the suspension should be centrifuged at 6,000 × *g* for 3 min at 20°C to pellet the bacteria. Discard the supernatant and resuspend the pellet in 40 mL sterile 10 mM $MgCl_2$. Repeat this pelleting and resuspension step twice, with the final resuspension again being in 10 mM $MgCl_2$. At the final resuspension, the bacterial density should be 1×10^{10} cells/mL. It is imperative that at each step where the supernatant is decanted, the bacteria are not contaminated; hence, these steps should be undertaken in a laminar flow cabinet.
2. To reduce experiment-to-experiment variation in the plant metabolome, large numbers of 7-day-old 200 mL *Arabidopsis* suspension cultures should be pooled. Our approach is to pool 15 × 200 mL cultures into a sterile 5-L conical flask in a laminar flow cabinet to maintain sterility.
3. Compare the responses of the bacterial strains to AT3 medium that had been used to grow plant cells for 7 days (designated as "spent" medium). Thus, at this stage, split the pooled culture into 2 × 1.5 L cultures in sterile conical flasks. To isolate spent medium from one 1.5 L subculture, filter through Whatman No.1 filter paper using a Buchner funnel linked to an electric vacuum pump in a laminar flow cabinet.
4. Transfer 20 mL aliquots of both the 1.5 L of *Arabidopsis* suspension cell culture and 1.5 L of spent medium to 50-mL sterile tubes in a laminar flow cabinet. Inoculate these with 200 μL of a given *Pst* strain, to yield a final bacterial cell density of 1×10^8 cells/mL.

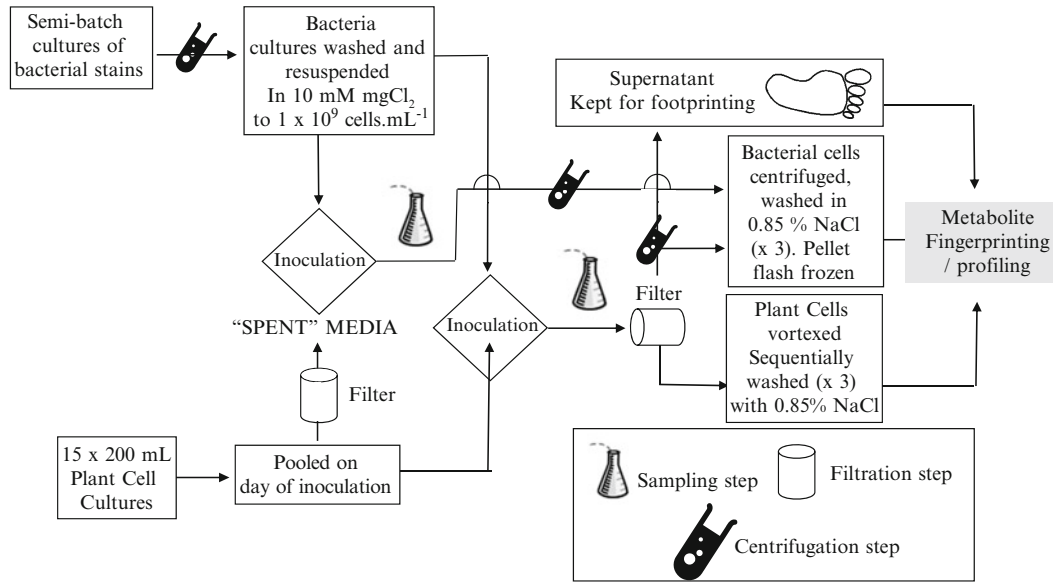

Fig. 3. Work flow for dual metabolomic analyses of the *Arabidopsis thaliana–Pseudomonas syringae* pv. *tomato* interaction. Each of the bacterial strains used in these analyses, the virulent *Pseudomonas syringae* pv. *tomato* (*Pst*), the hypersensitive response (HR) eliciting *Pst avrRpm1* and the non-HR and non-virulent strain *Pst hrpA* were used in an identical manner. The strains were initially grown on nutrient agar plates from which a single colony was used to inoculate 400 mL Nutrient Broth (NB). Once the cell density of the cultures had reached 1×10^9 cells/mL (typically ~ 24 h), 300 µL was used to inoculate 400 mL of fresh NB. This procedure was repeated a further two times once the subcultures had reached the indicated cell density. To prepare the bacteria for inoculation, the cultures were centrifuged, washed in 10 mM $MgCl_2$, re-centrifuged, the supernatant discarded, and finally resuspended in 10 mM $MgCl_2$ to a final concentration of 1×10^{10} cells/mL. *Arabidopsis* cells were continuously maintained as 200 mL cultures of AT3 media and grown at 24°C on a long day 16-h light cycle on an orbital shaker at 140 rpm (~$8 \times g$). To prepare *Arabidopsis* cells for bacterial inoculation, ~3 mL of 7 day culture was added to 200 mL. After 7 days, 15 cultures were pooled into one 3 L culture. To provide a source of spent AT3 medium, 1.5 L of the suspension culture was filtered through Whatman No. 1 filter paper using a Buchner funnel and 500 mL side arm flask connected to a vacuum pump. The filtered cells were discarded. Bacterial suspensions were added to 20 mL aliquots of this spent medium in 50-mL centrifuge tubes to give a density of 1×10^8 cell/mL. Sampling of bacteria-spent AT3 cultures or bacteria-*Arabidopsis* cell cultures (sampling stages shown by conical flasks on the Figure) occurred at 12 h post inoculation (hpi). The culture was filtered through Whatman No. 1 paper and the plant cells harvested and sequentially washed with 0.85% (w/v) NaCl. The bacterial pellet was harvested from the filtrate by centrifugation and washed three times in 0.85% NaCl. After the final washing step, plant and bacterial samples were flash-frozen in liquid N_2 and stored at −80°C until metabolomic analysis.

3.3. Validation of the Outcome of Plant Interaction with the Pathogen

A crucial validation step in the dual metabolomic procedure must be establishing that the plant cells are responding in an appropriate manner. For plant–pathogen interactions, we suggest two methods, which in our hands have proven to be robust and easy to perform; the assessment of plant cell death using Evan's Blue Staining and the detection of defence gene expression. Although we highlight these here, the reader may wish to use other suitable indicators. These include the generation of reactive oxygen species (ROS) which may be detected using the indicator stain Amplex Red (36) or NO production detected, for example, using the oxy-haemoglobin method (37), an NO electrode (38), or staining using NO sensitive dyes (39).

3.3.1. Evans Blue Staining

1. Samples of 1 mL of bacterially inoculated plant cell cultures should be taken under sterile conditions.
2. To these samples add 0.5 mL of 0. 25% (w/v) Evans Blue (in water) and leave to absorb for 10 min.
3. Place a drop of each Evans Blue treated sample on a microscope slide with a coverslip. The sample may be viewed under white light using a microscope under 400× magnification. Counts of dead (blue stained cells) should be taken as a proportion of a total number of 100 cells. Cell viability counts should be averaged across three slides.
4. Typically, ~5% of *Arabidopsis* cell clusters should exhibit evidence of Evans Blue retention under unstressed conditions. Plant cultures where >20% of the cell clusters exhibit Evan's Blue staining should be considered to be responding to the bacterial inoculation.

3.3.2. Extracting RNA from Cultured Plant Cells and Assessment of Defence Gene Expression

The selection of suitable marker genes for defence responses should be influenced by the interaction under study. Generally, it can be assumed that responses to biotrophic pathogens can be indicated by increased expression of *pathogenesis related protein 1* (*PR1*, At2g14610), whilst responses to necrotrophic pathogens can be indicated by the defensin gene *PDF1.2* (Ar5g44420). Respectively, these are gene markers for the activation of salicylic acid and jasmonate/ethylene signalling pathways. Increased expression of defence genes will indicate that defence-associated metabolomic reprogramming is occurring. If required, cDNAs for these and other *Arabidopsis* genes may be obtained from http://www.arabidopsis.org.

The techniques of RNA extraction from plant cells are well established and commercial extraction kits are available, so it is not necessary to describe these here. Gene expression can be assessed by either northern blotting and DNA probe hybridisation or quantitative amplification by polymerase chain reaction (qPCR). Suitable protocols for these techniques are described in many places. Our approach follows those described by Sambrook and Russell (40).

In our experiments, *PR1* gene expression is detected 6 h after inoculation with *Pst avrRpm1* and at 12 h with *Pst*. With *PDF1.2* increased expression was detected at 12 h post inoculation with either *Pst avrRpm1* or *Pst*. No significant expression of either gene was detected when inoculated with *Pst hrpA*.

3.4. Sampling Procedure

The time at which the bacterially inoculated *Arabidopsis* cultures may be sampled is very much at the discretion of the investigator. We have concentrated on 12 h post inoculation, as this represents a time when cell death is not prominent in *Pst avrRpm1* challenged samples and yet increased defence gene expression is noted.

1. Filter the 20 mL cultures through Whatman No.1 filter paper to retain the plant cells using a Buchner funnel linked to an electric vacuum pump. Transfer the filtrate containing the bacterial cells to a 50-mL sterile tube and store in ice. This need not be undertaken in a laminar flow cabinet.
2. Resuspend the filtered *Arabidopsis* cells in 20 mL of 0.85% (w/v) NaCl and re-filter as in Subheading 3.4 step 1. Repeat this process twice. The *Arabidopsis* cells (~100 mg fresh weight) should be rapidly transferred to 2-mL microcentrifuge tubes each containing a single 5-mm stainless steel ball bearing (washed in acetone), flash-frozen in liquid N_2, and stored at −80°C.
3. Harvest the bacterial cells in the ice-stored filtrates by centrifugation at 3°C and 6,000 × *g* for 3 min. The supernatant can be transferred to 2-mL microcentrifuge tubes and stored at −80°C, as this represents the co-culture "footprint" (see Note 5). The remaining bacterial pellets should be resuspended in 5 mL 0.85% NaCl, re-centrifuged, and the supernatant discarded. This wash should be repeated twice further prior to flash-freezing the bacterial pellets in liquid N_2 and storage at −80°C. This process should also be followed to assess the effects of spent AT3 medium on bacterial cells.

3.5. Metabolite Profiling by Mass Spectroscopy

The extraction procedure used to extract metabolites from *Arabidopsis* and bacterial samples will depend on the platform used for metabolite profiling and also the chemical diversity across the metabolite groups of interest (41, 42). Our analysis was based on the Direct injection-Electrospray ionisation-Mass Spectroscopy (DI-ESI-MS). The extraction procedure for plant cells should essentially follow that of Fiehn et al. (43). Our approach is to separately extract polar and non-polar metabolite samples.

1. Grind the samples in liquid nitrogen, preferably using a ball mill for high-throughput preparation of large numbers of samples, but a mortar and pestle will suffice.
2. Add 1 mL of chloroform–methanol–sterile dH_2O (1:2.5:1) and vortex thoroughly in a cold room and place onto ice.
3. Add a volume of 0.5 mL of sterile ultra-pure dH_2O to each sample.
4. The polar and non-polar phases should be mixed with a vortex and then centrifuged at 3°C and 17,000 × *g* for 3 min.
5. The polar and non-polar phases are easily separated and the upper aqueous can be removed into a separate microcentrifuge tube using a pipette. Both polar and non-polar phases should be dried down in an environmental speed vacuum concentrator and stored at −80°C. However, it should also be noted that

the non-polar phase can be analysed directly and that lipids are proposed as being more stable over short storage periods when present in solution.

6. Bacterial samples are extracted into acetonitrile: 0.2% formic acid (1:1 (v/v)). The samples are vortexed for 30 s and centrifuged at 17,000 × *g* for 3 min to pellet any debris (44). This represents a rapid bacterial extraction method appropriate for DIMS (see Note 6).
7. Metabolite profiling of both the polar and non-polar plant extracts as well as bacterial extracts is carried out using DI-ESI-MS on a Micromass LCT mass spectrometer. Bacterial extracts may be introduced in their extraction buffer. Non-polar plant extracts should be reconstituted in 100 µL 80% (v/v) methanol and polar extracts in 100 µL 20% (v/v) methanol. Alternatively for non-polar extracts, 100 µL 70% (v/v) propan-2-ol or 10% (v/v) acetonitrile may be used, although in our hands these increased the signal-to-noise ratio. The actual MS conditions to use are described in the legend of Fig. 4.
8. Subsequent data analysis and comparisons can be performed as described in refs. (30, 45).

4. Notes

1. The described protocol allows for the analysis of bacterial pathogens simultaneously with *Arabidopsis* cell cultures; however, this approach can be readily adapted to study other plant–pathogen interactions. Clearly, suspension cultures of any plant species can be utilised. Well-established plant cell cultures which have been employed to examine plant defences include tobacco (*Nicotiana tabacum cv. BY-2*) BY2 cells (46), parsley (*Petroselinum crispum*) cells (47), and soybean (*Glycine* L. Max) cells (48). Considering potential pathogens, we suggest that our system is most appropriate for bacteria. Besides *Pseudomonas syringae*, *Xanthomonas* pathovars and *Ralstonia solanacearum* could be used, as well as necrotrophic pathogens such as *Erwinia carotovora*. Besides plant pathogens, interactions with endophytic bacteria could be assessed (49). However, we suggest that symbiotic interactions with nitrogen-fixing bacteria are not suited to analysis using this system, as this is governed by highly differentiated root tissue. For example, one of the earliest plant responses to *Rhizobium* is the corkscrewing of a root hair to encompass the interacting bacterium. Such complex responses cannot be adequately mimicked in liquid cultures.

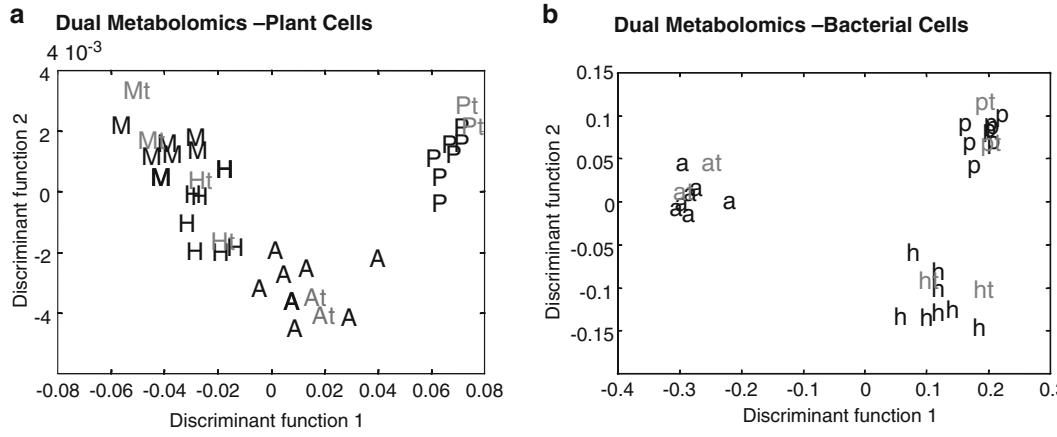

Fig. 4. Metabolomic analyses of *Arabidopsis* and *Pseudomonas syringae* cultures. Principal component-discriminant function analysis (PC-DFA) models of spectra derived from polar extracts of (**a**) *Arabidopsis* and (**b**) *Pseudomonas syringae* pv. *tomato* (*Pst*) strains following Direct injection-Electrospray ionisation-Mass Spectrometry(DI-ESI-MS) in positive ionisation mode. Cultured *Arabidopsis* cells were sampled at 12 h after inoculation (hai) with *Pst* (virulent strain;"**P**"), *Pst avrRpm1* (avirulent strain; "**A**"), *Pst hrpA* (non-avirulent and non-virulent; "**H**"). Control *Arabidopsis* cells were inoculated with 10 mM $MgCl_2$ ("M"). Ten 20 mL plant cultures were sampled per experiment. Sampling involved filtering the cultures through Whatman No.1 filter paper on a Buchner funnel linked to a vacuum pump. The filtered *Arabidopsis* cells were resuspended in 20 mL 0.85% (w/v) NaCl and re-filtered for a further two occasions. The *Arabidopsis* cells were transferred to 2-mL microcentrifuge tubes with stainless steel ball bearings (washed in acetone), flash-frozen, and stored at –80°C. The filtrate gathered, following filtration of the plant cells, contained either *Pst* ("**p**"), *Pst avrRpm1* (**"a**") and *Pst hrpA* ("**h**") These were pelleted by centrifugation at 3°C and 6,000 × *g* for 3 min. The pellets were resuspended in 1 mL 0.85% (w/v) NaCl re-centrifuged as before, the supernatant discarded, and the pellet stored at –80°C. The extraction of *Arabidopsis* and bacteria involved homogenisation in the ball mill in 1 mL of chloroform–methanol–sterile dH_2O (1:2.5:1) was added. The aqueous polar phases were extracted and dried down in a speed vacuum concentrator. Plant extracts were resuspended in 0.5 mL of sterile ultrapure dH_2O, whilst bacterial extracts were resuspended in 0.5 mL acetonitrile: 0.2% formic acid (1:1 (v/v)). The extracts were introduced by DI at a flow rate of 5 μL/min using a syringe pump in positive ionisation mode ESI-MS. The capillary voltage was always set at +3.0 kV. The desolvation and nebuliser gas flow rate was 400–480 L/h and 50–80 L/h, respectively. The source and desolvation temperatures were 120°C and 250°C, respectively. The cone voltage was 30 V (to minimise in-source fragmentation), the extraction voltage was 5 V, and the radio frequency voltage amplitude was 125 V. Data were acquired over the *m/z* range 65–1,000 Th (Thomson unit; for the physical quantity mass-to-charge ratio) for polar plant extracts and 65–1,500 Th for non-polar plant extracts and bacterial extracts. Data were exported in an ASCII format, binned and each sample aligned to form a data array to employ for PC-DFA and univariate analysis. The derived PC-DFA models were based on 10 PCs and accounted for either (**a**) 99.90% or (**b**) 99.71% of the total variance. Each PC-DFA model was validated by the independent projection of two biological replicates from each experimental class (the test data set (in *grey* and with a "t" suffix)) into the PC-DFA space of their remaining six replicates (the training data set in *black*). Note the discrete metabolomic responses of *Arabidopsis* and bacterial strains during each interaction type.

Conceivably our dual metabolomic approach could be adapted to investigate fungal or oomycete interactions with plants. Necrotrophic fungal pathogens, such as *Botrytis cinerea* (*B. cinerea*), are readily cultured and apparently need no specialised infection structure with which to interact with the host. However, it is more often the case that fungal/oomycete infections involve the formation of specialised infection and/or feeding structures. Hence, any attempt to develop a dual metabolomic model of fungal/oomycete interaction must be carefully and intensively validated.

2. It is essential that the plant suspension cell cultures are not senescent or contaminated in any way, and not stressed by any of the culture conditions, for example by the light levels. It should be noted that unlike many other plant suspension cultures (for example tobacco BY-2 cells), *Arabidopsis* cultures are photosynthetically active and therefore can readily experience light stress.

   Culture contamination will be readily indicated through a change in the colour or consistency of the culture. Cultures may become "milky" as a result of contaminant growth. If there is clumping of plant cells into large balls of tissue, this may also suggest contamination. Alternatively, a loss in the number of viable plant cells could be observed. The latter symptom can be revealed by staining with Evan's Blue prior to inoculation with the pathogen. Evidence of staining in >20% of plant cell clusters should be seen as evidence of either contamination or stress. However, plant stress need not be exhibited by increased cell death, but can still impact on the metabolome. Widely recognised markers for stress are increased generation of ROS and NO. These may be readily assessed using the indicator stain Amplex Red (for ROS) or an NO electrode or staining using NO sensitive dyes.

3. We recommend that, in case of suspension culture contamination, the *Arabidopsis* L *er* cells should be also regularly cultured on solid AT2 agar plates. In our hands, this involves fortnightly culture of *Arabidopsis* cell clusters isolated from liquid culture on Gamborg's B5 basal salts and vitamins 3.06 g/L, 2% (w/v) glucose, 20 g/L, MES 0.5 g/L, 2, 4-D 0.5 mg/L (2,4-Dichlorophenoxyacetic acid), Kinetin 0.05 mg/L (N6-furfuryladenine.), pH 5.5 with 1 M NaOH, 0.8% (w/v) plant tissue culture agar. After preparation of AT2 plates, ~1 cm^2 of a 7-day suspension culture is poured onto the surface under sterile conditions. The plant cells are allowed to settle and the excess liquid decanted away. The cell clusters readily form calli when grown under identical conditions as the liquid cultures. If required, inoculation of the friable calli into liquid culture allows the suspension culture to be established. The calli on solid media should remain green. If they become chlorotic or exhibit signs of microbial growth, they should be discarded.

4. We recommend that the reproducibility of bacterial and plant cultures prior to co-cultivation be exhaustively assessed to avoid misinterpretation of the results of a dual metabolomic experiment. We recommend a rapid metabolite fingerprinting approach, such as Fourier Transform InfraRed spectroscopy (FT-IR) or equivalent, be used. Space precludes a detailed description of the FT-IR approach, but this can be obtained from refs. (45, 50). FT-IR fingerprinting was typically used to

assess variation between cultures of bacterial strains, between plant cultures, and between experiments undertaken at different times.

5. The protocol includes a step where the metabolite footprints of the *Arabidopsis*–bacteria co-cultures are obtained. Metabolite footprinting with FT-IR spectroscopy has been used to monitor the metabolite secretion in bacteria, yeast, and plant cultures (51–53). In the case of this dual metabolomic approach, metabolites secreted from interacting plant and microbe cells will be revealed.
6. For GC- and LC-MS profiling, we recommend the extraction optimised by Winder et al. (54).

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References

1. M Heil, IT Baldwin: Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends in Plant Science* 2002, **7**: 61–67.
2. L Salvaudon, T Giraud, JA Shykoff: Genetic diversity in natural populations: a fundamental component of plant-microbe interactions. *Current Opinion in Plant Biology* 2008, **11**:135–143.
3. EC Oerke, H-W Dehne, F Schönbeck, A Weber: Crop production and crop protection—Estimated losses in major food and cash crops. Amsterdam: Elsevier; 1994.
4. M Parniske: Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews Microbiology* 2008, **6**:763–775.
5. KM Jones, H Kobayashi, BW Davies, ME Taga, GC Walker: How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature Reviews Microbiology* 2007, **5**:619–633.
6. V Bianciotto, P Bonfante: Arbuscular mycorrhizal fungi: a specialised niche for rhizospheric and endocellular bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 2002, **81**:365–371.
7. RP Ryan, K Germaine, A Franks, DJ Ryan, DN Dowling: Bacterial endophytes: recent developments and applications. *Fems Microbiology Letters* 2008, **278**:1–9.
8. LAJ Mur, P Kenton, AJ Lloyd, H Ougham, E Prats: The hypersensitive response; the centenary is upon us but how much do we know? *Journal of Experimental Botany* 2008, **59**:501–520.
9. C Mille-Lindblom, E von Wachenfeldt, LJ Tranvik: Ergosterol as a measure of living fungal biomass: persistence in environmental samples after fungal death. *Journal of Microbiological Methods* 2004, **59**:253–262.
10. D Choi, RM Bostock, S Avdiushko, DF Hildebrand: Lipid-derived signals that discriminate wound-responsive and pathogen-responsive isoprenoid pathways in plants – methyl jasmonate and the fungal elicitor arachidonic-acid induce different 3-hydroxy-3-methylglutaryl-coenzyme-a reductase genes and antimicrobial isoprenoids in *Solanum-tuberosum* L. *Proceedings of the National Academy of Sciences of the United States of America* 1994, **91**:2329–2333.
11. K Shimizu: Metabolic flux analysis based on C-13-labeling experiments and integration of the information with gene and protein expression patterns. In: *Recent Progress of Biochemical and Biomedical Engineering in Japan Ii*,

vol. 91. pp. 1–49. Berlin: SPRINGER-VERLAG BERLIN; 2004: 1–49.

12. TCR Williams, L Miguet, SK Masakapalli, NJ Kruger, LJ Sweetlove, RG Ratcliffe: Metabolic network fluxes in heterotrophic Arabidopsis cells: Stability of the flux distribution under different oxygenation conditions. *Plant Physiology* 2008, **148**:704–718.
13. MK Hellerstein: New stable isotope-mass spectrometric techniques for measuring fluxes through intact metabolic pathways in mammalian systems: introduction of moving pictures into functional genomics and biochemical phenotyping. *Metabolic Engineering* 2004, **6**:85–100.
14. YH Choi, HK Kim, HJM Linthorst, JG Hollander, AWM Lefeber, C Erkelens, JM Nuzillard, R Verpoorte: NMR metabolomics to revisit the tobacco mosaic virus infection in Nicotiana tabacum leaves. *Journal of Natural Products* 2006, **69**:742–748.
15. J Zhao, LC Davis, R Verpoorte: Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances* 2005, **23**:283–333.
16. RA Dietrich, MH Richberg, R Schmidt, C Dean, JL Dangl: A novel zinc finger protein is encoded by the arabidopsis *LSD1* gene and functions as a negative regulator of plant cell death. *Cell* 1997, **88**:685–694.
17. DH Aviv, C Rusterucci, BF Holt, RA Dietrich, JE Parker, JL Dangl: Runaway cell death, but not basal disease resistance, in *Isd1* is SA- and NIM1/NPR1-dependent. *Plant Journal* 2002, **29**:381–391.
18. S Lorrain, F Vailleau, C Balaque, D Roby: Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? *Trends in Plant Science* 2003, **8**:263–271.
19. JD Clarke, SM Volko, H Ledford, FM Ausubel, XN Dong: Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in Arabidopsis. *Plant Cell* 2000, **12**:2175–2190.
20. JR Alfano, AO Charkowski, WL Deng, JL Badel, T Petnicki-Ocwieja, K van Dijk, A Collmer: The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**:4856–4861.
21. T Kondo, S Sawa, A Kinoshita, S Mizuno, T Kakimoto, H Fukuda, Y Sakagami: A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis. *Science* 2006, **313**:845–848.
22. AK Mullen, MR Clench, S Crosland, KR Sharples: Determination of agrochemical compounds in soya plants by imaging matrix-assisted laser desorption/ionisation mass spectrometry. *Rapid Communications in Mass Spectrometry* 2005, **19**:2507–2516.
23. S Robinson, K Warburton, M Seymour, M Clench, J Thomas-Oates: Localization of water-soluble carbohydrates in wheat stems using imaging matrix-assisted laser desorption ionization mass spectrometry. *New Phytologist* 2007, **173**:438–444.
24. JJ Jones, S Mariccor, AB Batoy, CL Wilkins: A comprehensive and comparative analysis for MALDI FTMS lipid and phospholipid profiles from biological samples. *Computational Biology and Chemistry* 2005, **29**:294–302.
25. P Heraud, S Caine, G Sanson, R Gleadow, BR Wood, D McNaughton: Focal plane array infrared imaging: a new way to analyse leaf tissue. *New Phytologist* 2007, **173**:216–225.
26. F Jamme, P Robert, B Bouchet, L Saulnier, P Dumas, F Guillon: Aleurone cell walls of wheat grain: High spatial resolution investigation using synchrotron infrared microspectroscopy. *Applied Spectroscopy* 2008, **62**:895–900.
27. BO Budevska, ST Sum, TJ Jones: Application of Multivariate curve resolution for analysis of FT-IR microspectroscopic images of in situ plant tissue. *Applied Spectroscopy* 2003, **57**:124–131.
28. Z Movasaghi, S Rehman, IU Rehman: Raman Spectroscopy of Biological Tissues. *Applied Spectroscopy Reviews* 2007, **42**:493–541.
29. N Gierlinger, M Schwanninger: Chemical imaging of poplar wood cell walls by confocal Raman microscopy. *Plant Physiology* 2006, **140**:1246–1254.
30. AJ Lloyd, JW Allwood, CL Winder, WB Dunn, JK Heald, SM Cristescu, A Sivakumaran, FJM Harren, J Mulema, K Denby, R Goodacre, AR Smith, LAJ Mur: Metabolomic approaches reveal that cell wall modifications play a major role in ethylene-mediated resistance against *Botrytis cinerea*. *Plant Journal* 2011, **67**:852–868.
31. PF McCabe, CJ Leaver: Programmed cell death in cell cultures. *Plant Molecular Biology* 2000, **44**:359–368.
32. NM Cecchini, MI Monteoliva, F Blanco, L Holuigue, ME Alvarez: Features of basal and race-specific defences in photosynthetic Arabidopsis thaliana suspension cultured cells. *Molecular Plant Pathology* 2009, **10**:305–310.

33. A Clarke, R Desikan, RD Hurst, JT Hancock, SJ Neill: NO way back: nitric oxide and programmed cell death in Arabidopsis thaliana suspension cultures. *Plant Journal* 2000, **24**:667–677.
34. P Cossart, PJ Sansonetti: Bacterial invasion: The paradigms of enteroinvasive pathogens. *Science* 2004, **304**:242–248.
35. MJ May, CJ Leaver: Oxidative stimulation of glutathione synthesis in *Arabidopsis-thaliana* Suspension-Cultures. *Plant Physiology* 1993, **103**:621–627.
36. MJ Zhou, ZJ Diwu, N PanchukVoloshina, RP Haugland: A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: Applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Analytical Biochemistry* 1997, **253**:162–168.
37. M Delledonne, YJ Xia, RA Dixon, C Lamb: Nitric oxide functions as a signal in plant disease resistance. *Nature* 1998, **394**:585–588.
38. IR Davies, XJ Zhang: Nitric oxide selective electrodes. *Globins and Other Nitric Oxide-Reactive Proteins, Pt A* 2008, **436**:63–95.
39. E Prats, LAJ Mur, R Sanderson, TLW Carver: Nitric oxide contributes both to papilla-based resistance and the hypersensitive response in barley attacked by Blumeria graminis f. sp hordei. *Molecular Plant Pathology* 2005, **6**:65–78.
40. Sambrook J., Russell D.: Molecular cloning : A laboratory manual: Cold Spring Harbor Laboratory Press; 2006.
41. JW Allwood, DI Ellis, R Goodacre: Biomarker metabolites capturing the metabolite variance present in a rice plant developmental period. *Physiologia Plantarum* 2008, **132**:117–135.
42. RD Hall: Plant metabolomics: from holistic hope, to hype, to hot topic. *New Phytologist* 2006, **169**:453–468.
43. O Fiehn, J Kopka, P Dormann, T Altmann, RN Trethewey, L Willmitzer: Metabolite profiling for plant functional genomics. *Nature Biotechnology* 2000, **18**:1157–1161.
44. S Vaidyanathan, DB Kell, R Goodacre: Flow-injection electrospray ionization mass spectrometry of crude cell extracts for high-throughput bacterial identification. *J Am Soc Mass Spectrom* 2002, **13**:118–128.
45. JW Allwood, DI Ellis, JK Heald, R Goodacre, LAJ Mur: Metabolomic approaches reveal that phosphatidic and phosphatidyl glycerol phospholipids are major discriminatory non-polar metabolites in responses by *Brachypodium distachyon* to challenge by *Magnaporthe grisea*. *Plant Journal* 2006, **46**:351–368.
46. V Houot, P Etienne, A-S Petitot, S Barbier, J-P Blein, L Suty: Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells, in a dose-dependent manner *J. Exp. Bot.* 2001, **52**:1721–1730.
47. E Kombrink, K Hahlbrock: Responses of cultured parsley cells to elicitors from phytopathogenic fungi : Timing and dose dependency of elicitor-induced reactions *Plant Physiol.* 1986, **81**:216–221.
48. A Levine, R Tenhaken, R Dixon, C Lamb: H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 1994, **79**:583–593.
49. C Lodewyckx, J Vangronsveld, F Porteous, ERB Moore, S Taghavi, M Mezgeay, D van der Lelie: Endophytic bacteria and their potential applications. *Critical Reviews in Plant Sciences* 2002, **21**:583–606.
50. R Goodacre, EM Timmins, R Burton, N Kaderbhai, AM Woodward, DB Kell, PJ Rooney: Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks. *Microbiology-Sgm* 1998, **144**:1157–1170.
51. NN Kaderbhai, DI Broadhurst, DI Ellis, R Goodacre, DB Kell: Functional genomics via metabolic footprinting: monitoring metabolite secretion by Escherichia coli tryptophan metabolism mutants using FT-IR and direct injection electrospray mass spectrometry. *Comparative and Functional Genomics* 2003, **4**:376–391.
52. FM Carrau, K Medina, L Farina, E Boido, PA Henschke, E Dellacassa: Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: effects of yeast assimilable nitrogen on two model strains. *Fems Yeast Research* 2008, **8**:1196–1207.
53. R Dowlatabadia, AM Weljie, TA Thorpe, EC Yeung, HJ Vogel: Metabolic footprinting study of white spruce somatic embryogenesis using NMR spectroscopy. *Plant Physiology and Biochemistry* 2009, **47**:343–350.
54. CL Winder, WB Dunn, S Schuler, D Broadhurst, R Jarvis, GM Stephens, R Goodacre: Global metabolic profiling of *Escherichia coli* cultures: An evaluation of methods for quenching and extraction of intracellular metabolites. *Analytical Chemistry* 2008, **80**:2939–2948.
55. J Fliegmann, A Mithofer, G Wanner, J Ebel: An ancient enzyme domain hidden in the putative beta-glucan elicitor receptor of soybean may play an active part in the perception of pathogen-associated molecular patterns during broad host resistance. *Journal of Biological Chemistry* 2004, **279**:1132–1140.

56. D Nennstiel, D Scheel, T Nurnberger: Characterization and partial purification of an oligopeptide elicitor receptor from parsley *(Petroselinum crispum*). *Febs Letters* 1998, **431**:405–410.
57. L Gomez-Gomez, T Boller: Flagellin perception: a paradigm for innate immunity. *Trends in Plant Science* 2002, **7**:PII S1360-1385(02)02261-6.
58. A Meyer, A Puhler, K Niehaus: The lipopolysaccharides of the phytopathogen *Xanthomonas campestris* pv. *campestris* induce an oxidative burst reaction in cell cultures of Nicotiana tabacum. *Planta* 2001, **213**:214–222.
59. J Lee, DF Klessig, T Nurnberger: A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene HIN1 independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. *Plant Cell* 2001, **13**:1079–1093.
60. S Bourque, MN Binet, M Ponchet, A Pugin, A Lebrun-Garcia: Characterization of the cryptogein binding sites on plant plasma membranes. *Journal of Biological Chemistry* 1999, **274**:34699–34705.
61. DA Navarre, TJ Wolpert: Victorin induction of an apoptotic/senescence-like response in oats. *Plant Cell* 1999, **11**:237–249.
62. M Rohe, A Gierlich, H Hermann, M Hahn, B Schmidt, S Rosahl, W Knogge: The race-specific elicitor, Nip1, from the barley pathogen, *Rhynchosporium-Secalis*, determines avirulence on host plants of the *Rrs1* resistance genotype. *Embo Journal* 1995, **14**:4168–4177.
63. W D'Haeze, M Holsters: Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology* 2002, **12**:79R–105R.
64. M Parniske, JA Downie: Plant biology – Locks, keys and symbioses. *Nature* 2003, **425**:569–570.
65. M de Torres, JW Mansfield, N Grabov, IR Brown, H Ammouneh, G Tsiamis, A Forsyth, S Robatzek, M Grant, J Boch: *Pseudomonas syringae* effector AvrPtoB suppresses basal defence in Arabidopsis. *Plant Journal* 2006, **47**:368–382.
66. RZ Li, XY Jia, X Mao: Ethanol-inducible gene expression system and its applications in plant functional genomics. *Plant Science* 2005, **169**:463–469.