Phytochemistry 71 (2010) 590–597

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

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

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ARTICLE INFO

Article history:
Received 6 October 2009
Received in revised form 29 December 2009

Keywords:
Dual metabolomics
Arabidopsis thaliana
Pseudomonas syringae pv. *tomato*
Fourier transform infrared (FT-IR) spectroscopy

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.

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 lead 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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doi:10.1016/j.phytochem.2010.01.006

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 avrRpm*1, 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 intra*). 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 avrRpm*1 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 avrRpm*1 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 (*PR*1) and defensin (*PDF*1.2) was observed in plant cultures only when inoculated with *Pst avrRpm*1 (Fig. 1E). As *PR*1 and *PDF*1.2 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