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TECHNICAL NOTE

Use of intercellular washing fluid to investigate the secreted proteome of the rice–*Magnaporthe* interaction

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Abstract Early interactions between invading penetration hyphae of the pathogenic fungus *Magnaporthe oryzae* and rice cells occur at the apoplast, the free diffusional space outside the plasma membrane of leaves. After initial colonization, intercellular hyphae are again in intimate contact with the rice apoplast. While several studies have looked at proteomics in rice–*Magnaporthe* interactions, none have focused on apoplast localized proteins. We adjusted a protocol for intercellular washing fluids (IWF) to rice leaves infected with *Magnaporthe oryzae* for proteomic analysis. In our IWF extract, we identified several proteins associated with compatible or incompatible pathogen interactions. Three DUF26 domain proteins were identified as changing in abundance 12 h after inoculation, confirming DUF26 domain-containing proteins are among early, pathogen stress-responsive proteins induced by infection with *Magnaporthe oryzae*. A *Magnaporthe* cyclophilin, previously identified as a virulence factor was also identified in the intercellular washing fluid.

Keywords 2D-PAGE · *Magnaporthe* · Protein extraction · Rice · Secreted proteins

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Introduction

Magnaporthe oryzae is a fungal pathogen that causes blast disease in rice. The infection of rice tissues begins as a biotrophic interaction. Elaboration of a germ tube from a conidium on the plant surface is followed by appressorium formation. High internal turgor pressure drives penetration into plant tissue (Talbot 2003). At the same time, changes in rice cellular biochemistry driven by the fungus remodel membranes and other structures, allowing accommodation of fungal hyphae within the apoplast, the free diffusional space outside the plasma membrane. Subsequently, plant membrane surrounds the intracellular hyphae, containing them entirely within the cytoplasm, before the hyphae penetrate the walls of the invaded cell to colonize adjoining cells, possibly by exploiting plasmodesmatal connections (Kankanala et al. 2007). After initial colonization, further infection hyphae process intercellularly to rapidly colonize new areas of plant tissue. These intercellular hyphae are again in direct contact with the rice apoplast.

A number of proteomic studies have identified *Magnaporthe* or rice proteins induced during infection (Kim et al. 2003, 2004, 2009), although in rice there is a general lack of information on extracellular proteins. The examination of sub-cellular proteomes can give more specific and relevant information about biological processes than whole extract studies e.g. (Bayer et al. 2006).

The proteome of secreted proteins (secretome) can be easily analysed from cell-cultured *M. oryzae* as demonstrated in a recent study on the response to nitrogen starvation (Wang et al. 2011). In that case 89 differentially expressed protein spots could be detected using two-dimensional gel electrophoresis coupled with mass spectrometry analysis (Wang et al. 2011). However, for the analysis of the secretome in *M. oryzae*–rice interaction a system is required that reflects the situation during the infection process in rice leaves. In biotrophic interactions such as the *Magnaporthe*–rice example it is useful to think of a specialized tissue derived from the infection, with its own proteome. In a previous work (Kim et al. 2009) the interaction secretome was examined by using proteins secreted into the culture medium of rice calli treated with *Magnaporthe* or elicitor. This approach is limited to those proteins which are freely secreted into the extracellular environment and stable in the culture medium. We have developed a method to make an intercellular washing fluid extract from infected rice leaves which is enriched in apoplast localized proteins. Examination of the protein complement of these extracts has the potential to identify important factors in rice–*Magnaporthe* interactions.

Materials and methods

Inoculation of rice leaves with *Magnaporthe oryzae*

4 leaf stage plants of *Oryza sativa* ssp. Japonica cv Kakehashi were fixed to flexible plastic sheets with rubber bands so that the leaf surfaces were available for inoculation. Suspensions of conidiospores of *Magnaporthe oryzae* isolate Ken54-20 (Race 003) Incompatible (IC) with *Oryza sativa* ssp. Japonica cv Kakehashi, or Ina186-137 (Race 007) Compatible (C) with *Oryza sativa* ssp. Japonica cv Kakehashi were sprayed onto the rice leaves at a concentration of 5×10^5 spores/ml containing 0.05% Tween-20 and the plants were sealed in a box in the dark at high humidity for the duration of the treatment. Mock inoculations were performed using distilled water containing 0.05% Tween-20.

Preparation of intercellular washing fluid

Infected rice leaves were harvested, washed briefly in distilled water and blotted dry, before being submerged in IWF buffer [50 mM Na–P buffer pH 7.5, 600 mM NaCl, 0.01% Tween-20, 0.1% β-mercapto ethanol] at 4°C in square petri dishes. A vacuum of −70 kPa was applied using a water aspirator and maintained for 5 min in a vacuum dessicator. The vacuum was slowly released. Leaves were re-submerged and the vacuum reapplied a further four times for 5 min each time. Leaves were then gently blotted dry with paper towels, and placed in the barrel of a 20 ml disposable syringe with the barrel removed. The syringe barrel was placed in a 50 ml Falcon tube and spun at 1,000*g* for 5 min in a benchtop centrifuge. IWF was collected in the bottom of the falcon tube. Proteins were precipitated using 10 volumes of 10% TCA in acetone overnight at −20°C, pelleted at 10,000*g* in a microfuge and washed twice with 100% ice-cold acetone. The precipitated proteins were resuspended in 7 M urea, 2 M thiourea and dialysed overnight using the Amersham minidialysis kit to remove excess salt from the preparation.

2D electrophoresis

Iso-electric focusing (IEF) was performed as first dimension using Immobiline Drystrip pH 3-11 NL, 11 cm (GE Healthcare). 150 μg of IWF was loaded into the strip by rehydration overnight in 200 μl of destreak rehydration solution (GE Healthcare). Focusing was performed at 3,500 V for 3 h on a Multiphor II Electrophoresis System (GE-Healthcare) according to the manufacturer's descriptions.

After IEF, strips were briefly rinsed in distilled water, then equilibrated in equilibration buffer [6 M urea, 75 mM Tris–HCl pH 8.8, 29.2% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue] containing 20 mM DTT for 15 min in glass tubes on a rocking platform. The buffer was changed to the same equilibration buffer containing 250 mM iodoacetamide and equilibration continued for a further 15 min.

For the second dimension, the 11 cm IEF strips were briefly rinsed in running buffer and placed at the top of 13 cm × 13 cm 15% polyacrylamide gels and sealed with 1% agarose containing 0.2% bromophenol blue. Gels were run at 10 mA overnight. Gels were fixed for 2 h to overnight in 10% methanol, 10% acetic acid solution, then rinsed twice in distilled water. Gels were sensitized for 2 min in 0.02% sodium thiosulphite solution then rinsed twice for 5 min each in distilled water before staining in 0.2% silver nitrate, 0.076% formaldehyde solution for 30 min. After two 1 min distilled water washes, gels were developed in 2% sodium carbonate, 0.05% formaldehyde solution for 4–8 min until spots were sufficiently stained. Development was stopped in 1% acetic acid, and gels stored at 4°C in fresh 1% acetic acid.

Mass spectrometry

Proteins spots were cut from the gel using a scalpel blade, and washed in water for three times 15 min. 200 μl of 200 mM ammonium bicarbonate, pH 8 was added and the tubes shaken for 15 min. The supernatant was discarded and 200 μl of 50:50 (v/v) 200 mM ammonium bicarbonate:

acetonitrile was added and the tubes were shaken gently for 15 min. The supernatant was discarded and 200 µl HPLC grade acetonitrile was added and the tubes shaken for 5 min. The supernatant was discarded and each gel piece was rehydrated at 4°C in 10–20 µl 0.02 µg/µl of Promega Sequencing Grade Modified Trypsin prepared in 50 mM ammonium bicarbonate as required. If additional solvent was required to cover the gel piece, extra 50 mM ammonium bicarbonate was used. The microcentrifuge tubes were closed tightly and incubated overnight at 37°C. To recover the peptides from the gel particles three extractions were performed. For the first extraction, 2 volumes of water were added and the tubes were vortexed for 10 min. For subsequent extractions, 5% formic acid/50% acetonitrile was used. Extracts were combined in siliconized tubes and dried using a rotary evaporator. Peptides were desalted using Millipore m-C18 Zip-tips. Samples were dissolved in 10 ml 5% formic acid. Zip-tips were activated by rinsing 3 times with 10 µl acetonitrile, then washed in 0.1% trifluoroacetic acid. Peptides were bound by pipetting up and down 10 times. Zip-tips were washed 3 times in 0.1% Trifluoroacetic acid and the sample was eluted in 3 ml 5% formic acid/50% acetonitrile and 12 µl 5% formic acid was added. The sample was then used in LC–MS/MS analysis.

LC–MS/MS analysis was performed with an HCTultra ESI-ion-trap mass spectrometer (Bruker Daltonics, Leipzig, Germany) equipped with an Agilent 1100 CapLC system (Agilent, Wilmington, DE, USA). The digested peptides were loaded into a reversed-phase column (Zorbax 300S-C18, 3.5 µm, 150 × 0.3 mm, Agilent) in a CapLC, eluted in a linear gradient from 10 to 65% (v/v) of acetonitrile in 0.1% (w/w) formic acid over a period of 30 min at a flow rate of 4 µl min^{-1} after a hold at 10% (v/v) of acetonitrile for 5 min, and introduced into a mass spectrometer. A mass spectrum with a range of 200 and 1,800 m/z was acquired using the Esquire Control program (version 6.1). MS/MS spectra were searched against the protein database of the National Center for Bio-technology Information using the MASCOT MS/MS ion search server (Matrix Science, http://www.matrixscience.com). The searches were performed with the following parameters: allowed one missed cleavage, fixed modifications for carbamidomethyl (C), variable modifications for phospho (ST/Y) and oxidation (M), peptide tolerance of ±0.5 Da, MS/MS tolerance of ±0.3 Da, and peptide charge of 1+, 2+ and 3+; and instrument, ESI-TRAP.

Results

First we set out to establish a protocol to isolate an intercellular washing fluid enriched in apoplastic proteins with a relatively low level of contamination of cytoplasmic proteins. IWF was recovered as detailed in materials and methods. A key to successful extraction was a reduction in detergent concentration in the IWF buffer to compensate for Tween-20 included in the *Magnaporthe* inoculation solution.

IWF extracts compared with whole cell extracts were compared by SDS polyacrylamide gel electrophoresis (Fig. 1). We could observe that the IWF extract had a substantially different protein complement to the whole cell extract (although there were some bands in common). In addition we could observe several changes in protein abundance between compatible, incompatible and control extracts in the IWF extracts which were not observable in the whole cell extracts.

As the purpose of these extracts was to observe changes during infection when compared to non-infected controls, we were not concerned to eradicate all cytoplasmic or organellar contamination. Rather for this type of experiment it is better to be inclusive, than to exhaustively avoid contamination but exclude some important proteins. We therefore reasoned that the 1D SDS-PAGE analysis was satisfactory and could move to detailed examination of protein profiles by 2D electrophoresis.

We examined extracts taken at two time points after inoculation of rice leaves with *Magnaporthe oryzae*. At 12 h the infections are at the stage of appressorium formation and early penetration, comprising early recognition, signaling and defense events in either incompatible or compatible interactions. At 72 h, we expect to see intercellular procession of infecting hyphae in a mature infection in the compatible case. In the incompatible case we

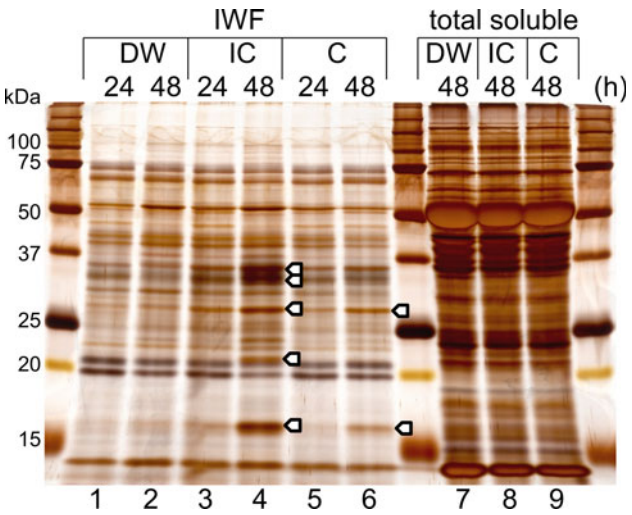

Fig. 1 SDS polyacrylamide gel analysis of intercellular washing fluids vs total soluble protein extracts. *Arrows* indicate protein changes after infection with *Magnaporthe oryzae* detectable in intercellular washing fluids. DW, distilled water control; IC, incompatible *Magnaporthe*; C, compatible *Magnaporthe*

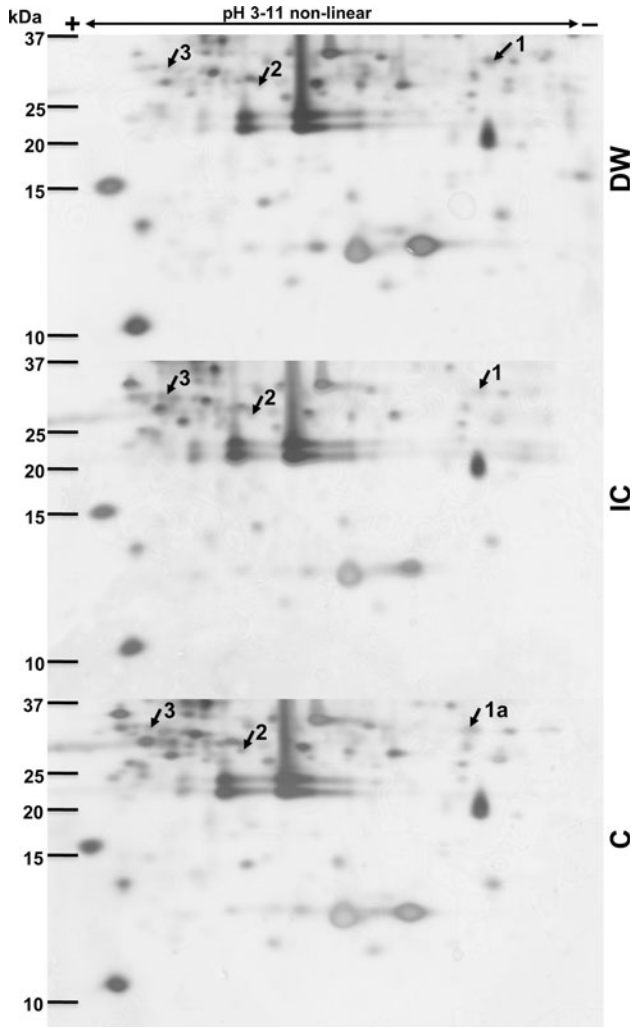

Fig. 2 2D electrophoresis of IWF extracts from rice leaves 12 h after inoculation with distilled water control (DW), incompatible *Magnaporthe* (IC) or compatible Magnaporthe (C)

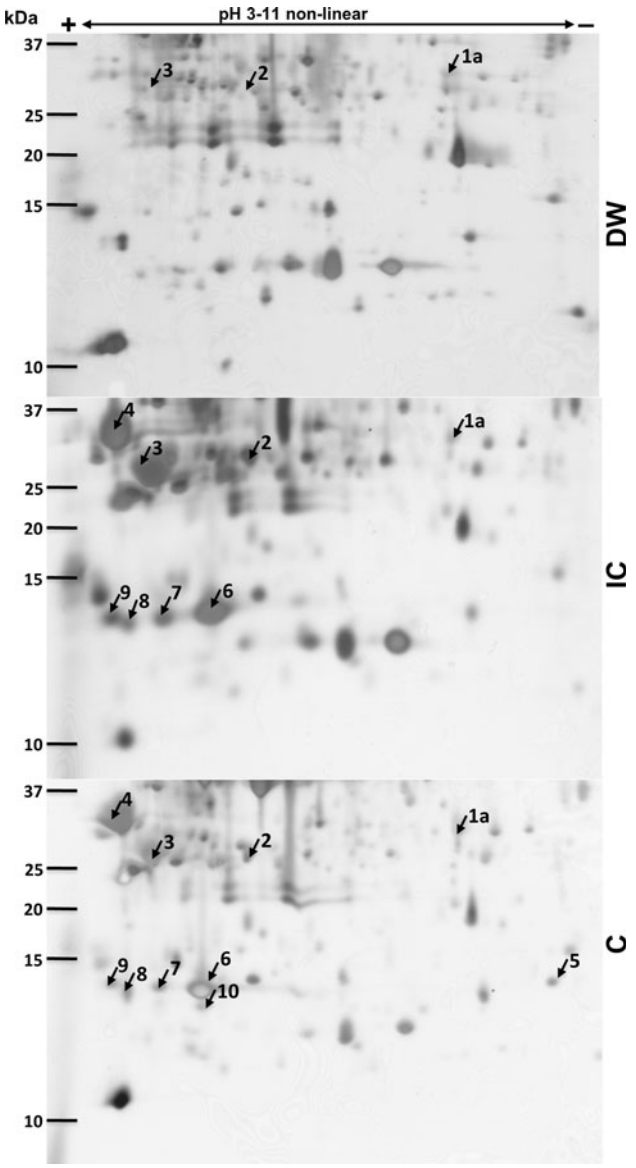

Fig. 3 2D electrophoresis of IWF extracts from rice leaves 72 h after inoculation with distilled water control (DW), incompatible *Magnaporthe* (IC) or compatible *Magnaporthe* (C)

would expect extensive defense reactions and protein changes associated with localized cell death and defense signaling. The time points were chosen to illustrate early, or late events in *Magnaporthe*–rice interactions. At both stages we would expect intimate contact between plant apoplast and *Magnaporthe* hyphae.

Figure 2 shows a comparison between control, incompatible or compatible interactions at 12 h post inoculation. Three proteins which changed in abundance, were identified by LC MS/MS as DUF26 domain containing cysteine rich repeat proteins. Initially we noted that the protein labeled 1a was present only in the compatible sample 12 h after inoculation. However further analysis of the protein labeled 1 in the control and incompatible reactions identified the same protein, corresponding to Q6PUW7 a DUF26 domain containing secretory protein corresponding to the gene Os04g0316200 in the database. The protein spot labeled 2 was identified as Q10N97 another DUF26 domain containing secretory protein corresponding to the gene Os03g0277700. The protein labeled 3 was identified as Q8S3P3 a DUF26 domain containing secretory protein corresponding to the gene Os04g0659300.

Figure 3 shows a comparison between IWF extracts from control, incompatible or compatible interactions after 72 h. In this case several proteins were dramatically changed in abundance, especially in the incompatible reaction, the majority of which have been previously identified as defense related proteins, including PR proteins, thaumatins and other similar proteins (spots 4, 6, 7, 8 and 9). The protein labeled 10 was induced only in the compatible reaction and was identified as a putative barwin

gene in rice. Also unique to the compatible interaction after 72 h we were able to identify one *Magnaporthe* protein labeled 5, the cyclophilin CYP1 which has previously been identified as a virulence factor in *Magnaporthe* infection (Kankanala et al. 2007).

Discussion

Several studies have examined proteins induced during *Magnaporthe*–rice interactions for example (Kim et al. 2009). The difficulty in obtaining a complete picture of proteins involved in the *Magnaporthe*–rice interaction results from the complexity of the interaction. The unique features of penetrating pathogenic fungi, as compared to pathogenic bacteria, or oomycetes mean that there are likely to be biochemical mechanisms unique to this class of pathogens which have not previously been discovered. For example genetic analyses are beginning to isolate effector proteins which bear little similarity with other known effectors (Yoshida et al. 2009). It is now clear that following initial penetration of the host cell, the invading *Magnaporthe* hyphae are completely surrounded by plant membrane, finally forming an amorphous haustorium type structure within the plant cell (Kankanala et al. 2007). Because of the nature of the interaction and the structures formed and maintained during invasion, the combined interaction proteome of *Magnaporthe* and rice is likely to be extremely complex. In order to understand complex interactions it can be useful to examine simplified sub-proteomes, for example using subcellular fractionation. Overall proteomic information can then be compiled from different sub-proteomes and used in conjunction with gene expression and genetic data in order to understand further the biological interaction. IWF has been prepared by De Wit and Spikman (1982) from *C. fulvum*-infected tomato leaves in which they discovered proteinaceous elicitors showing necrosis-inducing activity with specificity towards tomato genotypes with particular resistance genes. We adjusted the method of IWF preparation to rice leaves that have been sprayed with suspensions of conidiospores of *M. oryzae* to isolate proteins for 2D analysis.

In our experiments we have confirmed that DUF26 domain containing proteins are early stress responsive extracellular proteins induced by rice blast infection. Several reports have identified DUF26 domain containing secretory proteins (also called 33 kDa secretory proteins) in biotic or abiotic stress reaction (Kim et al. 2003, 2004, 2009; Zhang et al. 2009; Jiang et al. 2007). Kim et al (2009) identified five different DUF26 domain containing proteins induced by *Magnaporthe* or elicitor treatment of rice cell cultures. We identified a different subset of DUF26 proteins. In common were the proteins corresponding to Os04g0659300 and Os03g0277700. We additionally found the Os04g0316200 protein, but did not identify Os08g0136700 or 0s08g0136800, perhaps reflecting the different experimental systems used (leaf IWF as opposed to cell culture exudate) and the relative mobility of such proteins in the respective systems. The function of such DUF26 domain containing cysteine rich secretory proteins is unknown, but Os04g0659300 has been called *Root Meander Curling* due to the phenotype of its knockout, and implicated in jasmonate signaling and salt stress (Zhang et al. 2009; Jiang et al. 2007). Os04g0316200 has been reported as being under the transcriptional control of a NAC transcription factor (Nakashima et al. 2007) having effects on *Magnaporthe* resistance and salt tolerance.

We identified one *Magnaporthe* protein from our extracts, the CYP1 cyclophilin which has previously been identified as a virulence factor (Viaud et al. 2002). Although we found this protein in our apoplastic extract, it does not possess a predicted signal peptide and has predicted cytoplasmic location. It is interesting to ask the question whether this cyclophilin is a *Magnaporthe* secretory protein or whether its presence in the apoplastic fluid is as a result of the rupture of *Magnaporthe* hyphae during preparation of the IWF. The isoform identified by LC–MS/MS has been annotated as having cytoplasmic localization, so any secretion would have to be by a non standard secretory pathway. However, there are precedents for the extracellular presence of cyclophilins in other systems. For example Yokota et al. (2004) identified a cyclophilin secreted from Lily pollen tubes under low calcium conditions and there is some evidence of cyclophilin activity in intercellular communication in human cells (Bukrinsky 2002). A similar type of experiment with ours (Paper et al. 2007) found a *Fusarium graminarum* cyclophilin FG00777 when investigating secreted proteins produced during infection of wheat. Of the 23 predicted cyclophilin sequences in the *Magnaporthe oryzae* genome, the most closely related to FG00777 is the CYP1 found in our extracts. It is possible that in both systems cyclophilins are particularly abundant and stable in the extraction buffers used, when compared to other cytoplasmic proteins (This was the only *Magnaporthe* protein identified from our experiment). Alternatively, cyclophilin may have an as yet unknown extracellular role in plant pathogenic fungi. We confirmed by querying the publicly available microarray data that CYP1 is indeed expressed in infective hyphae (Barrett et al. 2011; Mosquera et al 2009). CYP1 was also expressed at a similar level in non-infective mycelia, however this is perhaps not surprising given that developmental roles for CYP1 have already been established (Viaud et al 2002). CYP1 shows significant identity with the N terminal part of a rice cyclophilin (LOC_Os07g08190) having 57% identity over 166 amino

acid residues. This rice cyclophilin is predicted to have a C terminal extension of unknown function which is absent in the *Magnaporthe* sequence. It is tempting to speculate whether the function of such a *Magnaporthe* protein could be to interfere with the function of such a rice protein, especially considering that according to rice microarray data, this cyclophilin is up-regulated 96 h after infection with virulent *Magnaporthe oryzae* (Ribot et al. 2008; Jung et al. 2008).

Characterization of the *Magnaporthe*–rice interaction remains an important aim in the understanding of fungal pathogen biology. Localization and translocation mechanisms of *Magnaporthe* effectors remain open questions which may be addressed, in part by further characterization of the infected plant apoplast.

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