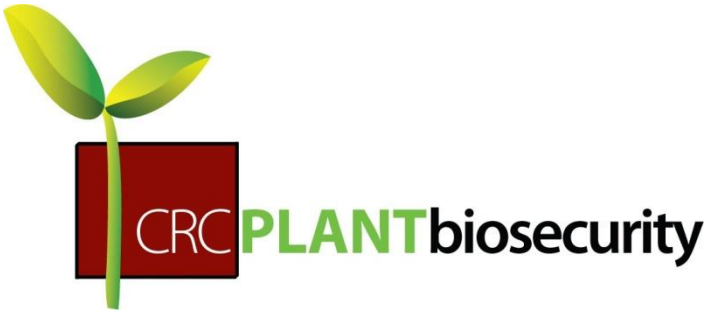

Cooperative Research Centre
for National Plant Biosecurity

Final Report

CRC20057

Phosphine Resistance-Proteomics

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1. Executive Summary

Phosphine is the main fumigant used in Australia to control insect pests in grain storages; both bulk grain handlers and farmers rely on phosphine for the control of insects and more than 80% of grain is fumigated with phosphine during storage. However, insect resistance to phosphine is increasing in most grain growing areas. To manage this resistance, a rapid and sensitive method for identifying phosphine resistance is required. The current detection method for phosphine resistance relies on time-consuming laboratory bioassay procedures (more than 7 days required).

A previous study (Park et al., 2008) had suggested that certain proteins, displayed by two-dimensional electrophoresis (2D-PAGE), from whole *Rhyzopertha dominica,* differed between resistant and susceptible insects and might be developed as a rapid diagnostic tool.

This project initially aimed to identify the genes encoding those differing proteins as a first step towards developing a diagnostic tool. However, when we used a larger number of strains of *R. dominica* and a more robust method the results of the previous study were not supported. We then proposed that this approach to biomarker discovery might yet be successful if we were to concentrate on a more appropriate subset of proteins for proteomic analysis.

Various lines of evidence suggested that mitochondria are a site of important differences associated with phosphine resistance. A proteomic comparison of mitochondrial proteins from susceptible and resistant *Tribolium castaneum* (chosen for its completed genome sequence) was therefore conducted. The study did not reveal any significant differences in the expression of the more abundant mitochondrial proteins between resistant and susceptible insects. The aim of identifying differentially expressed proteins that are diagnostic for phosphine resistance has been shown to be beyond the scope of this project.

2. Aims and objectives

This study aimed to identify biomarkers for phosphine resistance leading to the development of a rapid diagnostic test for phosphine resistance. This would have assisted the grain industry to make best use of phosphine and alternative fumigations though the use of the most appropriate dosage and exposure times.

3. Key findings

Proteomic assessment of resistance to the fumigant phosphine in the lesser grain borer, *Rhyzopertha dominica*.

This work proceeded from a report by Park et al. (2008) that certain prominent proteins in *R. dominca* varied between resistant and susceptible insects. In particular they nominated the amount of the enzyme arginine kinase as a key difference that might be developed in a diagnostic test. This was particularly attractive as this enzyme is believed to play a role in enhancing the efficiency of respiration, phosphine toxicity depends on respiration, and various resistant animals show reduced respiration.

The project started with the aim of isolating the genes responsible for the reported protein difference. However, that previous result was not supported when we used a larger number of strains and a more robust method. Indeed only two proteins out of hundreds

displayed by 2D-PAGE showed a small difference between resistant and susceptible insects, not enough for a diagnostic (Campbell, 2008).

Biological replicates of adult beetles from two strains with 'strong' resistance, one with 'weak' resistance and three phosphine-susceptible strains were homogenised and their proteins extracted for display by two-dimensional polyacrylamide electrophoresis (2D-PAGE). The Differential In-Gel Electrophoresis (DIGE) technique was used to visualise the protein spots, align the images of gels, and perform statistical analyses. Figure 1 shows one of the gels and proteins identified from that gel by mass spectral analysis of peptides from digestion of the protein spots with trypsin. As indicated in the figure legend, some of the proteins could be identified by their similarity to proteins/genes known from other species even though there were few protein sequences from this species in public databases.

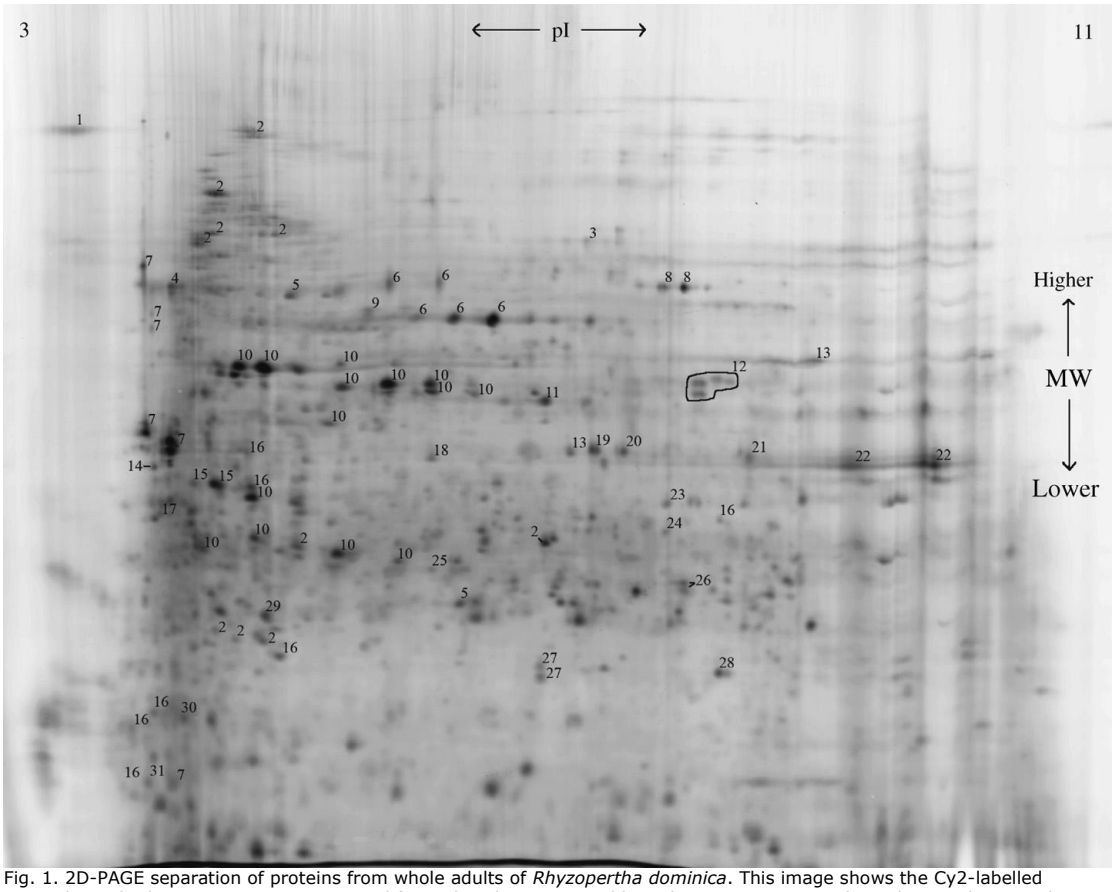

Fig. 1. 2D-PAGE separation of proteins from whole adults of *Rhyzopertha dominica*. This image shows the Cy2-labelled internal standard comprising proteins mixed from phosphine-susceptible and resistant insects. This gel was subsequently stained with Coomassie dye and a selection of the spots was identified by LC/MS/MS of tryptic peptides, some of which are indicated here. Except where indicated otherwise the label is above and to the right of the indicated spot. Spot no. 1, glycosyl hydrolase; 2, myosin components; 3, succinate dehydrogenase; 4, α-amylase; 5, HSP60; 6, enolase; 7, tropomyosin; 8, aldehyde dehydrogenase; 9, Zn-dependent peptidase; 10, actin; 11, arginine kinase; 12, fructose-1,6-bisphosphate aldolase; 13, citrate synthase; 14, HSP90; 15, a-tubulin; 16, various ATP synthase subunits; 17, 14-3-3 epsilon; 18, pyrophosphatase; 19, cytosolic malate dehydrogenase; 20, pyruvate dehydrogenase; 21, receptor for activated protein kinase C (RACK1); 22, glyceraldehyde-3-phosphate dehydrogenase; 23, ETF-α; 24, phosphoglycerate mutase; 25, CG7445-like; 26, arginine kinase; 27, actin-binding muscle protein 20-like; 28, thioredoxin-dependent peroxidase; 29, ferritin; 30, β-tubulin; 31, unidentified.

Of the hundreds of protein spots shown in Fig.1 only two differed significantly between susceptible and resistant insects (Fig. 2), one of which was identified. However, the magnitude of the differences were small (less than 3-fold) and unlikely to form the basis of a diagnostic test.

Several of the proteins that Park et al. (2008) reported as differing between a susceptible strain (CRD2) and a resistant strain (QRD569/CRD343), arginine kinase, HSP60, enolase and tropomyosin, were identified here among the proteins that did not show significant differences between an expanded set of resistant and susceptible strains. In particular, Park et al. (2008) suggest arginine kinase might be used in a kit for the detection of phosphine resistance. In common with Park et al. (2008) we found arginine kinase at two distinct locations on our 2D gel (Fig. 1). At the higher molecular weight/lower pI location (spot no. 11) the intensities of the spots were quite variable with up to 10-fold differences among the biological replicates of individual strains. However, the mean for the susceptible strains was the same as for the resistant strains. At the other location (spot no. 26) intensities differed less than two-fold among all samples.

These results have been published (Campbell, 2008).

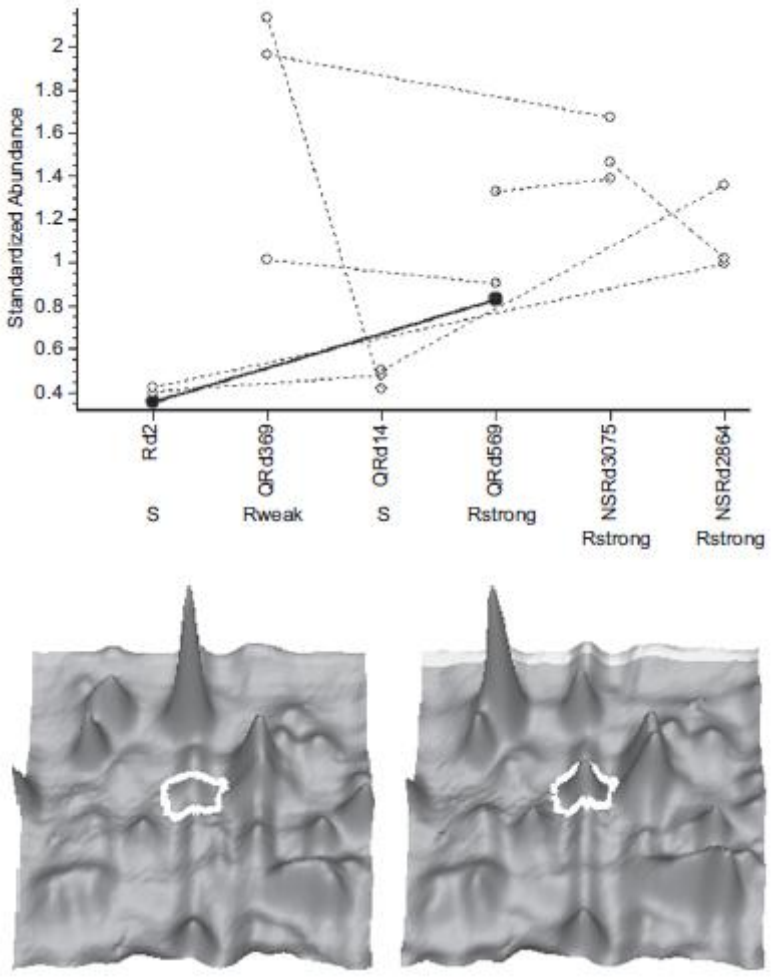

Fig. 2. Detail from the analysis of spot no. 24. This figure shows the relative intensities of spot no. 24 (Fig. 1) in each of the three samples from six strains after normalisation with the internal standard. Resistance status is indicated under the strain names. Lines join the data points from samples that were separated on the same gel. The filled circles are the data corresponding to the detailed 3D images of the spot and the surrounding region.

Comparison of the mitochondrial proteomes of phosphine-susceptible and -resistant *Tribolium castaneum*.

A frequent technical hurdle for proteomic approaches to biomarker discovery is seeing only the 'iceberg' tip of uninformative 'housekeeping' proteins and not the less abundant but informative proteins. A common strategy is to concentrate an appropriate subset of less abundant proteins for proteomic analysis. Various lines of evidence suggested that mitochondria are a site of important differences associated with resistance. For example, the toxicity of phosphine requires, and then inhibits, aerobic respiration which occurs in the mitochondria and several pest beetles were reported to have reduced respiration in resistant strains (Pimentel et al., 2007). We also changed to using *Tribolium castaneum* because its phosphine resistance resembles that of other pest species but the identification of proteins would be made easier by its completely sequenced genome.

Protein extracts of mitochondria were analysed as above by 2D-PAGE with DIGE analysis. A selection of the displayed proteins were identified confirming that the samples contained predominantly mitochondrial proteins (Fig. 3). Fluorescence images of the protein spots from replicates of susceptible and resistant strains were compared. Few proteins varied among any of the strains and none differed consistently between resistant and susceptible strains.

Phosphine resistance could be induced in a nematode, *Caenorhabditis elegans*, by down-regulation of many of the genes encoding proteins of the respiratory chain (Zuryn et al., 2008) and some of the corresponding proteins were detected here although they did not differ with resistance status. Differences might be invisible to the methods employed here if the function of a protein has been changed by a mutation that does not alter its molecular weight, isoelectric point or abundance. Alternatively, differences may involve yet lower abundance proteins.

A recent analysis suggests that mitochondrial proteomes may be much larger than previous estimates with about 1,500 proteins suggested for *Drosophila melanogaster* (Smith and Robinson, 2009). In this study we have displayed only about 10% of that number. The hypothesis tested here could be pursued further, firstly through biochemical studies to define more precisely the differences in respiration between susceptible and resistant beetles, and secondly through more exhaustive or complementary proteomic methods.

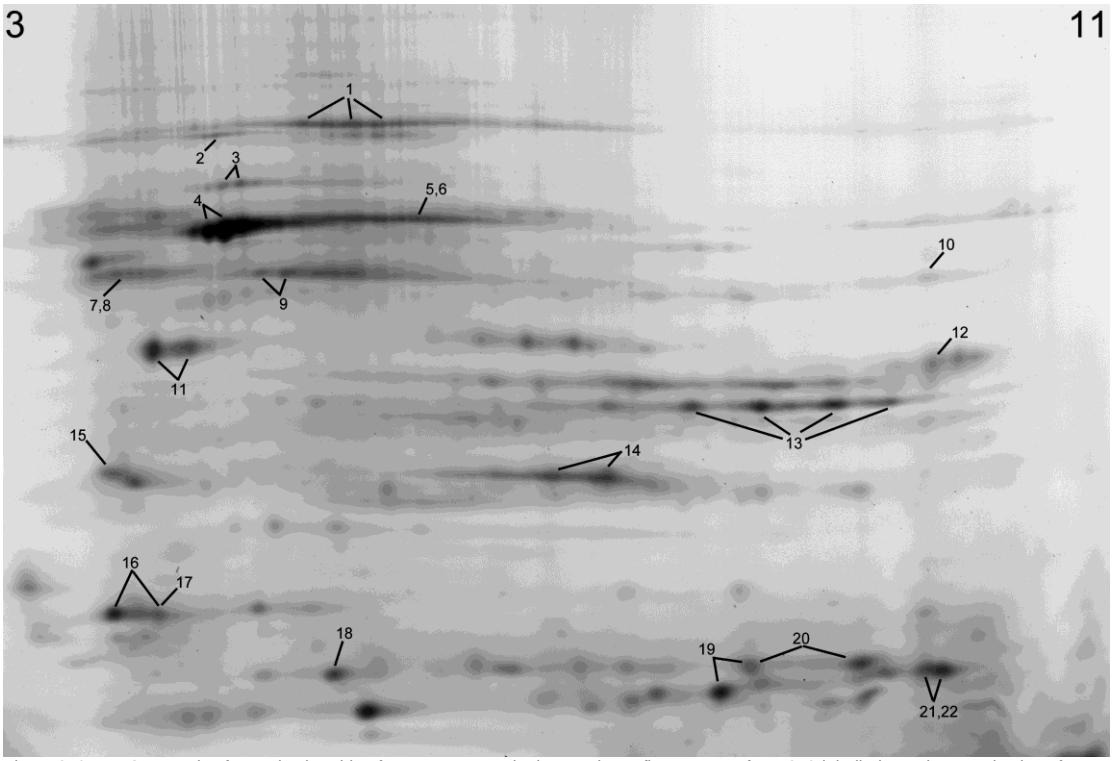

Figure 3. 2D-PAGE proteins from mitochondria of *T. castaneum*. The image shows fluorescence from Cy2-labelled proteins. A selection of protein spots were excised and identified as follows. Except where noted the annotation was 'similar to (the name given)' and the species was T. castaneum. 1) N-terminal region (peptide coverage up to residue 709) of Retinoid and Fatty Acid Binding Protein, 2) Heat Shock Protein 70, 3) Heat Shock Protein 60, mitochondrial precursor, 4) H+-transporting ATP Synthase, beta subunit, 5) AGAP005134-PA, (F0F1 ATP Synthase, alpha subunit), 6) Dihydrolipoamide Succinyltransferase Component of 2-Oxoglutarate Dehydrogenase (minor component of the spot), 7) Isocitrate Dehydrogenase, 8) Citrate Synthase, 9) Actin (strong identification with various Drosophila sequences but not T. castaneum), 10) AGAP006099-PA isoform 2 (Zn-dependant peptidase domain, Ubiquinol-Cytochrome C Reductase, core protein II), 11) Tropomyosin, 12) Malate Dehydrogenase, 13) Voltage-Dependent Anion-Selective Channel Isoform 1, 14) Mitochondrial F0 ATP Synthase D Chain, 15) Myosin light chain, 16) ATP Synthase Delta Chain, Mitochondrial, 17) Cytochrome C Oxidase, Subunit Va, 18) Ubiquinol-Cytochrome C Reductase Complex, 14kD subunit, 19) ATPase Inhibitor-Like Protein, 20) AGAP008724PA (Cytochrome C oxidase, subunit Vb), 21) CG14235 (Cytochrome C oxidase, subunit VIb), 22) AGAP001502-PA (Chaperonin 10 Kd subunit).

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Campbell, P.M. 2008. Proteomic assessment of resistance to the fumigant phosphine in the lesser grain borer, *Rhyzopertha dominica* (F.). Journal of Stored Products Research 44, 389–393.

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Smith, A.C., Robinson, A.J., 2009 MitoMiner, an integrated database for the storage and analysis of mitochondrial proteomics data. Molecular and Cellular Proteomics 8, 1324–1337.

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4. Implications for stakeholders

No changes to the use of phosphine for fumigation nor the management of resistance can recommended as a result of this work.

5. Recommendations

The hypothesis that resistance to phosphine may be associated with a constitutive difference of expression of a mitochondrial protein associated with respiration remains plausible but it now seems unlikely that we would find a quantitative difference among the more abundant proteins that could lead easily to a diagnostic tool. A diagnostic tool might ultimately be developed, initially through biochemical studies to define more precisely the differences in respiration between susceptible and resistant beetles, and then through more targeted genetic and proteomic methods.

6. Abbreviations/glossary

ABBREVIATION	FULL TITLE
2D-PAGE	Two Dimensional Polyacrylamide Gel Electrophoresis. A technique for the separation of complex mixtures of proteins. Separation in one dimension is by isoelectric point, that is, electrophoresis in a pH gradient to the point where the protein has no net charge. Then the proteins are separated in a second dimension by SDS PAGE according to molecular size.
DIGE	Differential In-Gel Electrophoresis. A technique for the separation and display of hundreds of proteins simultaneously from two or three samples in one 2D-PAGE gel. Proteins from each sample are labelled with a different fluorescent dye before being mixed then separated by electrophoresis. In this case, each gel in a series contained a standard reference sample for internal calibration and alignment of images of different gels and two experimental samples.

7. Plain English website summary

CRC project no:	CRC20057
Project title:	Phosphine Resistance-Proteomics
Project leader:	Dr. Yong-Lin Ren
Project team:	Dr. Peter Campbell, Ms. Daphne Mahon.
Research outcomes:	Phosphine is the main fumigant used in Australia to control insect pests in grain storages; both bulk grain handlers and farmers rely on phosphine for the control of insects and more than 80% of grain is fumigated with phosphine during storage. However, insect resistance to phosphine is increasing in most grain growing areas. To manage this resistance, a rapid and sensitive method for identifying phosphine resistance is required. The current detection method for phosphine resistance relies on time-consuming laboratory bioassay procedures (more than 7 days required). A previous study (Park et al., 2008) had suggested that certain proteins, displayed by two-dimensional electrophoresis (2D-PAGE), from whole *Rhyzopertha dominica,* differed between resistant and susceptible insects and might be developed as a rapid diagnostic tool. This project initially aimed to identify the genes encoding those differing proteins as a first step towards developing a diagnostic tool. However, when we used a larger number of strains of *R. dominica* and a more robust method the results of the previous study were not supported. We then proposed that this approach to biomarker discovery might yet be successful if we were to concentrate a more appropriate subset of proteins for proteomic analysis. Various lines of evidence suggested that mitochondria are a site of important differences associated with phosphine resistance. A proteomic comparison of mitochondrial proteins from susceptible and resistant *Tribolium castaneum* (chosen for its completed genome sequence) was therefore conducted. The study did not reveal any significant differences in the expression of the more abundant mitochondrial proteins between resistant and susceptible insects. The aim of identifying differentially expressed proteins that are diagnostic for phosphine resistance has been shown to be beyond the scope of this project.
Research implications:	No changes to the use of phosphine for fumigation nor the management of resistance can be recommended as a result of this work.
Research publications:	Campbell, P.M. 2008. Proteomic assessment of resistance to the fumigant phosphine in the lesser grain borer, *Rhyzopertha dominica* (F.). Journal of Stored Products Research 44, 389–393.
Acknowledgements:	CSIRO Entomology would like to acknowledge the support of the Cooperative Research Centre for National Plant Biosecurity. D Schlipalius and P. Collins (Queensland Industries and Fisheries) supplied some of the insect strains.