Cooperative Research Centre
for National Plant Biosecurity

Final Report

CRC20080

Diagnostic technologies for phosphine resistance management

Author/s

David Schlipalius

30 June 2010

Project Leader contact details:

Name: David Schlipalius
Address: Ecosciences Precinct, GPO Box 267, BRISBANE QLD 4001
Phone: 07 3255 4443
Email: david.schlipalius@deedi.qld.gov.au

CRCNPB contact details:

Cooperative Research Centre for National Plant Biosecurity
LPO Box 5012
Bruce ACT 5012

Phone: +61 (0)2 6201 2882
Fax: +61 (0)2 6201 5067
Email: info@crcplantbiosecurity.com.au
Web: www.crcplantbiosecurity.com.au

Table of contents

1. Executive Summary

Phosphine is an inexpensive, versatile fumigant, accepted on world markets as a residue-free treatment. It is relied on heavily throughout the Australian grain industry to maintain freedom from insect infestation - an important aspect of grain quality for export markets.

Research spanning many years has not identified a suitable alternative for phosphine. This has placed pressure on efforts to maintain phosphine by minimising the development and spread of phosphine resistance among grain insects.

In order to contribute to the management of phosphine resistance, this project set out to determine the feasibility of developing a molecular diagnostic test for phosphine resistance.

The development of a molecular test is seen as an important step in providing industry and researchers with a new test to complement the existing method to determine the resistance status of insects which relies on bioassays.

Currently, bioassays to determine phosphine status in insects have a number of limitations. These include:

1. An inability to identify resistance genes
2. An inability to detect relevant genotypes as the current method can only detect homozygous resistance and therefore cannot detect incipient resistance
3. A relatively high cost

The limitations in current bioassay methods are particularly an issue in efforts to study the development and evolution of resistance to phosphine in grain storage insects.

To evaluate the feasibility of a molecular test the project initially focused on the development of gene-specific diagnostic markers for phosphine resistance in two key pest species; *Rhyzopertha dominica* and *Tribolium castaneum*. This work identified that strong resistance is mediated by two major genes in both species.

These genes have been named *rph1* and *rph2* (i.e. ***r****esistance to* ***ph****osphine 1* and *2*). These genes are incompletely recessive and individually confer weak resistance, i.e. ~30X or ~12X, for *rph1* and *rph2* respectively in *R. dominica*, and ≤4X for both *rph1* and *rph2* in *T. castaneum*, when homozygous for the resistance mutation. The project also found that the two genes were synergistic in effect and confer strong resistance (>250X in *R. dominica* and >100X in *T. castaneum*) when both are present and homozygous for the resistance alleles.

Genetic crossing experiments determined that the genes are expressed in all insect life stages (egg, larva, adult) suggesting a constitutively expressed resistance factor that does not appear to be 'switched off' at any stage of development.

Further research using high-throughput sequencing and genetic linkage techniques discovered that resistance in the *rph2* gene in both target species is based on several mutations occurring independently in different strains.

To build on this, the project characterised gene function and expression and identified that the phosphine resistance gene *rph2* is highly conserved between the two target species and is a basic metabolic gene integral to the Krebs (or TCA) cycle.

Subsequent research led to development of individual diagnostic tests for each of the known mutations. In addition to their value as research tools, the individual diagnostic tests may also form the basis for development of regionally specific tests for phosphine resistance.

However as research also identified that these mutations vary across populations of the two target species, the opportunity to develop a single molecular diagnostic test for phosphine resistance that could be universally applied across all Australian grain growing areas is likely to be limited.

Research on the *rph1* gene narrowed down its location to a very small number of candidate genes (about six) for *T. castaneum*. However the location for *R. dominica* is less clear with a region of about 100 genes identified as the source area.

Finally gene expression profiling was achieved in both target species using two different technologies. However the results show that gene expression profiling is not suitable as the basis for development of a diagnostic test for resistance as the known resistance genes are not differentially expressed in resistant and sensitive strains and do not change expression in response to phosphine.

2. Aims

To evaluate the feasibility of a molecular diagnostic test for phosphine resistance in key grain storage insect pests, the project had the following three aims:

Aim 1: To develop gene-specific diagnostic markers for phosphine resistance in *R. dominica* and *T. castaneum*.

Aim 2: To characterise phosphine resistance gene function and expression for both *T. castaneum* and *R. dominica* to validate gene-specific markers

Aim 3: To explore gene expression profiling as the basis for development of a diagnostic test for resistance

3. Key findings

3.1 Summary of key findings:

Key findings from this project are that:

1. Phosphine resistance is mediated by two major genes in both *T. castaneum* and *R. dominica.* These two genes have been named r*ph1* and *rph2* (i.e. ***r****esistance to* ***ph****osphine 1* and *2*).

2. The two genes are incompletely recessive and individually confer weak resistance when homozygous for the resistance mutation.

3. The two genes are synergistic in effect and confer strong resistance in *R. dominica* and *T. castaneum* when both are present and homozygous for the resistance alleles.

4. The two genes are expressed in all insect life stages suggesting a constitutively expressed resistance factor that does not appear to be 'switched off' at any stage of development.

5. The gene *rph2* is highly conserved between *R. dominica* and *T. castaneum* and is a basic metabolic gene integral to the Krebs (or TCA) cycle.

6. The resistance mutations in the *rph2* gene are based on several independently occurring mutations in different strains.

7. The mutations in *rph2* vary between populations of *R. dominica* and *T. castaneum.* This indicates that the opportunity to develop a single universal molecular diagnostic test for phosphine resistance is limited. However, tests that combine multiple regionally-specific markers could be developed.

8. The location of the *rph1* gene has been narrowed down to a very small number of candidate genes (about six) for *T. castaneum*. However the location for *R. dominica* is less clear with a region of about 100 genes identified as the source area.

9. While gene expression profiling was achieved for *R. dominica* and *T. castaneum* the results indicate the technique is not suitable as the basis for development of a diagnostic test for resistance as the known resistance genes are not differentially expressed in resistant and sensitive strains and do not change expression in response to phosphine.

3.2 Summary of research results:

The following is intended as a summary of the research data; a more detailed explanation of methods and results can be sourced from the publications arising from this project.

3.2.1 Identifying resistance genes in *T. castaneum*

3.2.1.2 Classical genetics

We initiated a series of single pair intercross and backcross experiments between sensitive (QTC4), weak resistant (QTC1012) and strong resistant (QTC931) *T. castaneum* strains. Through classical genetic analysis of these crosses we determined that two genes responsible for strong resistance in *T. castaneum*, similar to the situation seen in *R. dominica* (Figure 1). These genes are both individually very weak (~4X), but are strongly synergistic when homozygous for both genes (>100X).

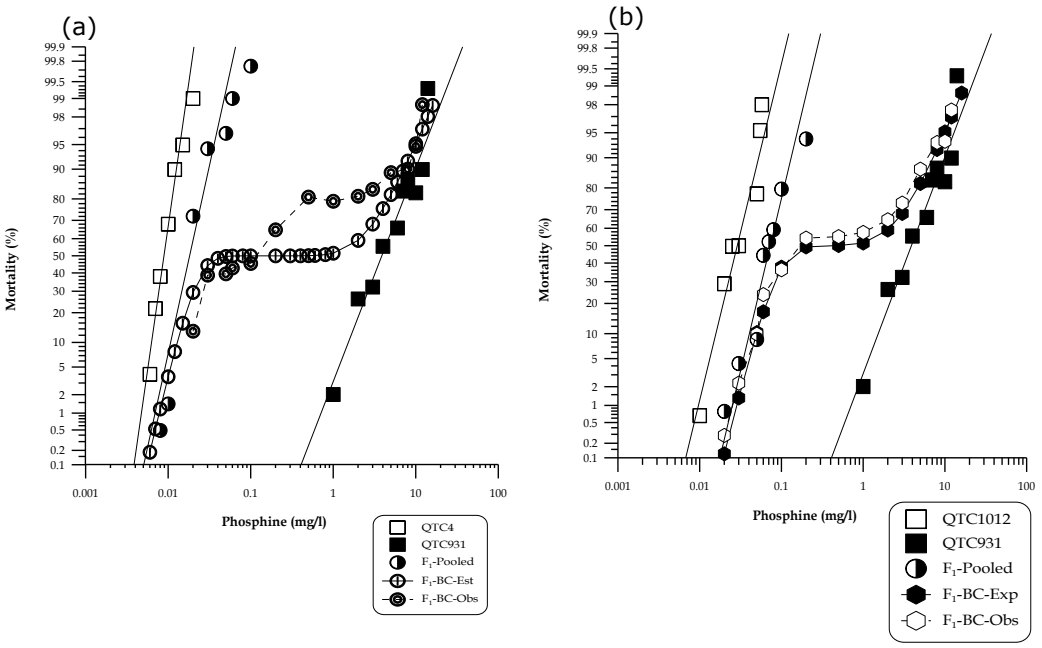

Figure 1. Mortality-response graphs showing (a) a backcross between susceptible and strong resistant strains and (b) a backcross between weak resistant and strong resistant strains of *T. castaneum*. The graph in (a) is typical of a two gene response , and the graph in (b) is typical of a single gene response, showing that one weak resistance gene is shared between the weak and strong resistant strains

3.2.1.3 Re-sequencing the T. castaneum genome

In order to identify regions that are linked to phosphine resistance in *T. castaneum*, we created two separate single-pair genetic crosses between a sensitive strain (QTC4) and a strongly resistant strain (QTC931). The resistance genotypes in these strains were then segregating for the resistance alleles, i.e. they were a mixture of genotypes containing both sensitive and resistant alleles. One strain was selected for high-level resistance at the F_4 generation, and the other was selected at the F_{19} generation. DNA was extracted from the resistant survivors of these selections as well as unselected progeny from the same generation.

The extracted DNA from both the F_4 and the F_{20} was then sequenced using a high-throughput sequencing technology, Illumina GAII sequencing. The resulting sequences were mapped to the existing reference *T. castaneum* genome (version 3.0 from NCBI). Single nucleotide polymorphisms (SNPs) were then detected and tabulated from each of the chromosomes for each set, selected/resistant and unselected/segregating of the generation (F_4 and F_{20})

Through this analysis, we identified two gene regions that became homozygous in the selected resistant progeny, but remained heterozygous in the unselected progeny. The two loci were located on chromosome 8 (Chr8) and chromosome 9 (Chr9). We also saw homozygosity on several unplaced scaffolds from the genome project, which were later mapped to a 'gap' on Chr9.

From the comparison of F_{20} sequence data from insects either selected for resistance toward phosphine or unexposed to phosphine, we were able to map regions of DNA that were likely to harbour a resistance gene. By this approach, we were able to narrow our search to two genomic regions, one of which spanned approximately 200Kb on Chr8 (Figure 2), while the other was restricted to just 22Kb on a short, unaligned sequence fragment referred to as Unknown Group 7 (Unk7) (Figure 3). Unk7 was later determined to

reside on Chr9.Figures 2 & 3 show the mapping of homozygosity along the chromosomes that was used to define the genomic regions containing the phosphine resistance genes.

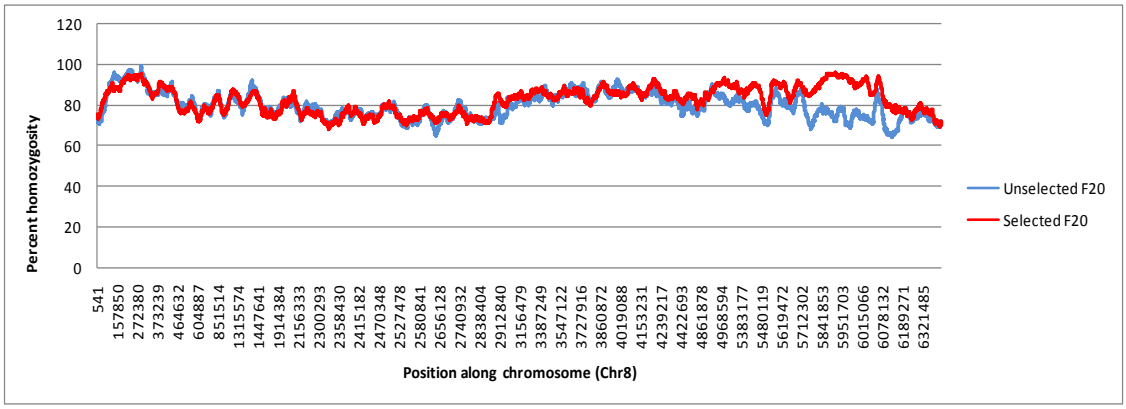

Figure 2. A graph showing the measured homozygosity of the variant regions seen on Chromosome 8, averaged across groups of 300 SNPs. The peak seen on the right defines the only region on this chromosome linked to resistance and spans a region approximately 200Kb.

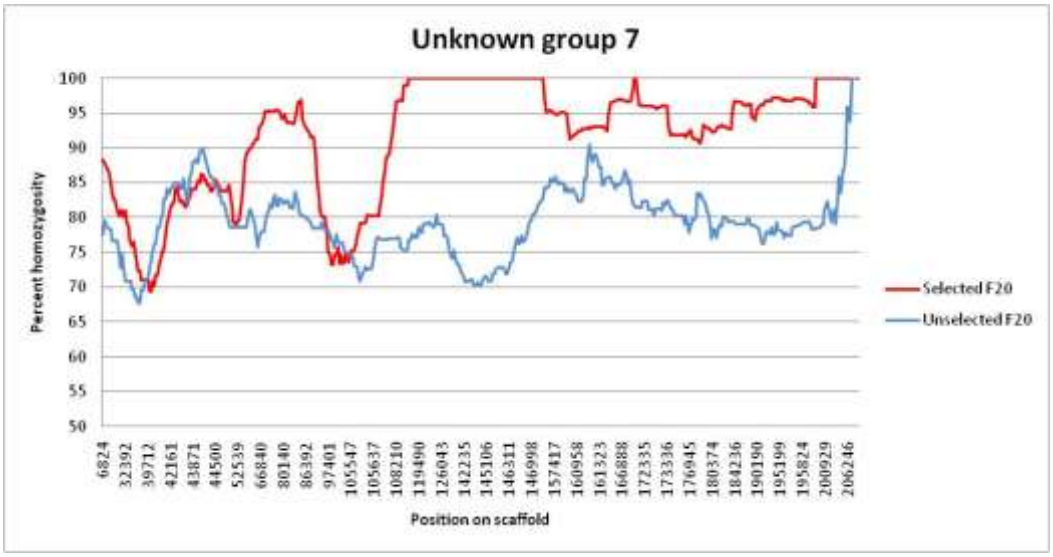

Figure 3. A graph showing the measured homozygosity of the variant regions seen on Unknown group 7 using mapped 75bp sequencing data in the F_{19}, averaged across groups of 40 SNPs. One region has a clear linkage to resistance

Our list of candidate resistance genes within the interval defined by the DNA sequencing on Chr8 comprises 18 genes. Subsequent fine scale mapping with specific DNA markers has reduced the list to six genes.

The region defined for the locus on Unk7 is somewhat narrower than that for Chr8, being approximately 22Kb and containing one gene. There is a highly conserved metabolic gene located within this region that appears in the candidate gene list of *R. dominica* and is very closely linked to the *rph2* locus (see section 3.4.5). Therefore we have labelled the gene on Unk7 (Chr9), *rph2*, and the locus on Chr8, *rph1*. We do not yet know, however, if the gene referred to as *rph1* in *T. castaneum* is the same as the gene referred to as *rph1* in *R. dominica*.

3.2.2 Fitness cost analysis for strong resistance in *T. castaneum*

3.2.2.1 Fitness analysis by dose-response

We determined the LD_{50} and LD_{90} for 20 hour exposure to phosphine on crosses between sensitive and strongly resistant strains (QTC4xQTC931), as well as sensitive and weak resistant strains (QTC4xQTC1012) on several generations of progeny, i.e. F_5, F_{10}, F_{15} and F_{19} generations (Figure 4). Resistance levels remained very stable in each of the three populations, over 15 generations, providing no indication of a strong fitness cost to resistance.

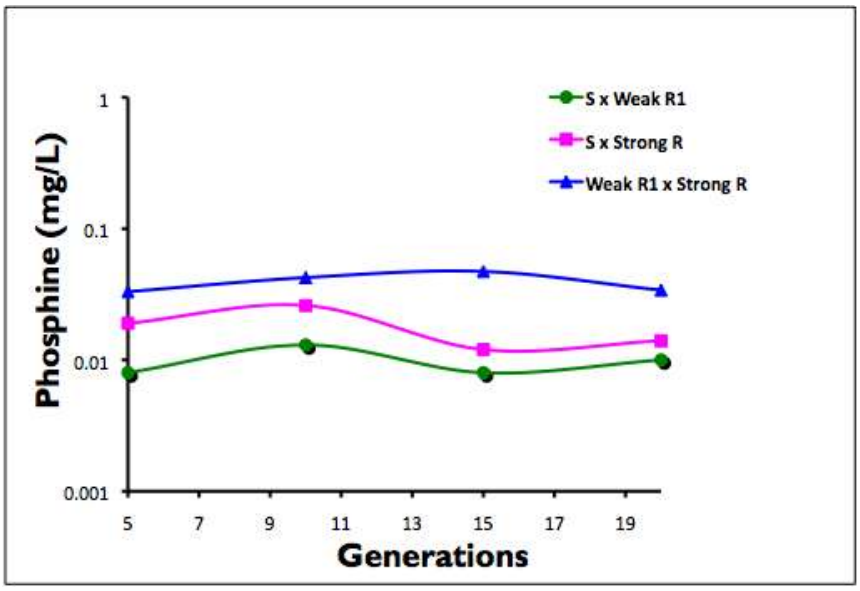

Figure 4. The change in calculated LD_{50} values for the crosses; Sensitive (S) X Weak-R, Sensitive (S) X Strong-R and Weak-R X Strong-R over multiple generations

3.2.2.2 Fitness analysis with molecular markers

Despite no apparent fitness cost associated with the resistance trait, a very different picture emerged when we analysed the underlying genes. Using molecular markers very closely linked to the two separate resistance loci, *rph1* and *rph2*, we found significant selection against the resistance genotype for *rph2* that was countered by selection for the resistance genotype at *rph1*. Table 1 shows the relative frequencies of the alleles over multiple generations.

This indicates that there may indeed be a fitness cost for the *rph2* allele in the field. This is the first time a fitness cost has been directly observed for a particular phosphine resistance genotype, as no resistance alleles appeared to carry a similar fitness cost in *R. dominica*.

Table 1. Estimating the change in allelic frequency of rph1 and *rph2* in strongly resistant *T. castaneum* using the markers SNP-LG8-597M and SNP-U7-138-279k respectively. A set of unselected individuals from the cross Sensitive X Strong-R (QTC4 X 931) was selected at discrete (F_2, F_5, F_{10}, F_{15} and F_{20}) generations and subjected marker analysis. The genotypes and the calculated allelic frequencies for both *rph1* and *rph2* were shown below.

Marker	*Generation*	*No. of insects tested*	*Genotypes*			*Allelic frequency*	
SNP-U7-138-279k (*rph2*)			*rr*	*rs*	*ss*	*p(resistant allele)*	*q(susceptible allele)*
	F_2	94	20	52	22	0.49	0.51
	F_5	96	5	54	37	0.33	0.67
	F_{10}	92	7	38	45	0.29	0.71
	F_{15}	96	0	28	64	0.15	0.85
	F_{20}	96	0	36	58	0.19	0.81
SNP-LG8-597M (*rph1*)							
	F_2	94	28	41	27	0.51	0.49
	F_5	94	34	43	17	0.59	0.41
	F_{10}	96	38	45	13	0.63	0.37
	F_{15}	95	35	46	14	0.61	0.39
	F_{20}	96	38	48	8	0.65	0.34

3.2.3 Gene expression studies of *T. castaneum*

We performed gene expression profiling by microarray analysis on several strains of *T. castaneum*, a sensitive (QTC4), strongly resistant (QTC931) and a reselected resistant strain derived from a cross between QTC4 and QTC931 (TC2ABPR). The reselected resistant strain was created to eliminate the effect of the genetic backgrounds of the sensitive and resistant strains on the analysis. The custom microarray chip design was obtained from a collaborator at Kansas State University, Dr. Yoonseong Park.

We used three different treatments for each of these strains, air (no dose) for 4 hrs, a 'low dose' (LC_{50} of QTC4) for 4 hrs, and 'high dose' (LC_{50} of QTC569) for 4 hrs. Because the high dose would effectively kill sensitive insects, even at 4 hours, we did not expose QTC4 to that treatment.

The results of the gene expression microarrays showed a dose-dependent response. The results were surprising, however, in that very little change in gene expression seemed to occur at low doses in any of the strains, including the sensitive strain. At high doses, some significant changes in gene expression were observed, much of it identifiable as a stress

response. Whether it is an oxidative stress response or a much more general stress response is still being evaluated, especially in light of the resistance gene identities. We expect to publish on this analysis later this year.

3.2.4 Identifying phosphine resistance genes in *Rhyzopertha dominica*

3.2.4.1 Classical genetics- life stage effects

Through a large detailed experiment involving crosses between sensitive (QRD14) and strongly resistant (QRD569) strains, we determined that genes for resistance have no particular dominance in most of the various *R. dominica* life stages, with one notable exception. We also determined that the resistance factors are expressed in all life stages (egg, larva, adult), and eggs and pupae have been confirmed to be the most tolerant life stages, even in resistant insects. This suggests a constitutively expressed resistance factor that does not appear to be 'switched off' at any stage of development.

We also found that there was a maternal effect on the egg stage, whereby the resistant female parent temporarily passed on a resistance factor to the egg (i.e. partially dominant), however that resistance factor was completely lost in the larvae and further stages. This is circumstantial evidence for the presence of a constitutively expressed factor from the mother, likely to be mitochondrial as they are inherited directly from the mother until the embryo starts expressing its own proteins.

3.2.4.2 Molecular genetics- sequencing R. dominica *genes*

In order to find informative sequence differences between resistant and sensitive strains of *R. dominica*, and since this insect does not yet have any reference genome, we used massively parallel sequencing methods to sequence all the expressed genes (the transcriptome) in *R. dominica* adults.

Using Roche GS-FLX, a high throughput DNA sequencing technology that produces sequence of the highest quality, we sequenced several strains of *R. dominica*, a sensitive strain (QRD14), a resistant strain (QRD569), an introgressed strain produced by backcrossing resistant genotypes into a sensitive background, and a strain of reselected F_{90} progeny of the original mapping cross started in 1999 (Schlipalius, Cheng et al. 2002). In total, more than 900,000 sequences of variable length were generated from this analysis.

We also sequenced the transcriptome of two strains, the sensitive strain and an introgressed resistant strain, using a complementary high throughput sequencing technology, the Illumina Genome Analyser (GAII). This technology produces much shorter sequences than the GS-FLX, but a much greater number of reads, thus giving greater sequence coverage. This was important in confidently identifying sequence differences, as well as assembling the whole transcriptome. In total more than 12-13 million sequences of 75bp/sequence were produced per sample, giving us more than 1.8Gb of sequence covering the *R. dominica* transcriptome, or an estimated 80X coverage.

3.2.4.3 Bioinformatics analysis

The assembly of the transcriptome was performed by the Centre for Comparative Genomics at Murdoch University, under the direction of Prof. Matthew Bellgard and Dr. Roberto Barrero.

It was observed that the majority of the genes in the *R. dominica* transcriptome were most closely related to *T. castaneum* sequences. This is unsurprising, as *T. castaneum* is the most complete beetle genome currently in the database, and it would be expected that the beetles are more closely related to each other than other orders of insects.

3.2.4.4 Identifying sequence differences- candidate gene list

Mapping (i.e. comparing) the sequencing reads from the sensitive and resistant strains against the reference gene set produced by the CCG, we were able to identify consistent sequence differences. These differences show us the genes which are linked to resistance across both loci (*rph1* and *rph2*). Our candidate gene list currently stands at about 100 genes, but we are as yet unable to assign linkage to a particular locus for most of these genes.

3.2.4.5 Identification of the rph2 candidate gene

We worked from the hypothesis that at least one of the genes between *T. castaneum* and *R. dominica* were likely to be conserved, and thus we checked to see if there was any overlap in homology between the candidate gene sets. That is, we checked if any of the candidate genes were the same in each species. One gene in the *R. dominica* candidate gene list had a highly conserved homology between the two species, moreover it appeared to be tightly linked to resistance in *T. castaneum* on the Unk7 scaffold. Through sequencing of cDNA, i.e. complementary DNA derived from RNA of the expressed genes, we determined the full sequence of the gene in resistant and sensitive insects from both species. Aligning the homologous sequences showed us a mutation that causes an amino acid change in similar positions (i.e. nearby, not exactly the same amino acid) in the structure of the protein.

Through linkage analysis and targeted genome sequencing we were able to confirm that this candidate gene is linked very closely with *rph2* in *R. dominica*, in fact it is physically next to the gene containing the STS5.11 marker that was previously identified as being the closest linked marker yet identified (Schlipalius, Chen et al. 2008).

There is reason in the literature to suspect that this candidate gene has a fundamental role in resistance and metabolism. The *rph2* candidate gene contributes to core energy metabolism within the mitochondria and is thought to be a major source of reactive oxygen species (ROS). This is consistent with the observed effects of phosphine, especially on mitochondria.

We already know that this gene is responsible for several outbreaks of phosphine resistance in *R. dominica* across eastern Australia (Mau 2008). This knowledge, together with our candidate gene sequence should make identifying genes responsible for phosphine resistance much easier in other species of grain pest exhibiting strong resistance to phosphine.

REFERENCES

Mau, Y. (2008). Comparative genetic and toxicological analysis of phosphine resistance in the Lesser Grain Borer, Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae). School of Molecular and Microbial Sciences. St. Lucia, University of Queensland. **PhD**.

Schlipalius, D. I., W. Chen, et al. (2008). "Gene interactions constrain the course of evolution of phosphine resistance in the lesser grain borer, Rhyzopertha dominica." Heredity **100**(5): 506-516.

Schlipalius, D. I., Q. Cheng, et al. (2002). "Genetic linkage analysis of the lesser grain borer Rhyzopertha dominica identifies two loci that confer high-level resistance to the fumigant phosphine." Genetics **161**(2): 773-782.

4. Implications for stakeholders

Implication 1

The research has shown that there are two major genes that confer resistance in the two insect species studied and that both these genes are expressed in all insect life stages. This means that:

1. Selection for resistance can occur in all life stages of the two studied insects, and

2. There are no vulnerable life-stages that could have been targeted in attempts to manage resistance development

Implication 2

The finding that the two genes are synergistic in effect and confer strong resistance only when both genes are homozygous (for resistance) explains why strong resistance has taken a relatively long time to increase in frequency and appear in enough numbers to be detected. This is because you must have, in one individual, both resistance genes present and both homozygous. The chances of this occurring in random mating events in nature are not high.

Implication 3

It was found that the gene *rph2* is highly conserved between *R. dominica* and *T. castaneum.* The significance of this finding is that a similar mechanism for resistance could be common across all major grain storage pest species where phosphine is used for control.

Implication 4

The research has shown that mutations in *rph2* vary across populations of *R. dominica* and *T. castaneum.* This indicates that industry is unlikely to gain a universal molecular diagnostic test for phosphine resistance that could be applied across Australian grain growing areas.

5. Recommendations

Recommendation 1

In the previous section, an explanation was provided as to why strong resistance has been relatively slow to increase in frequency.

From this, it is recommended that future research compare populations of insects from different regions across Australia and where possible, from international sources.

This will provide critical information for industry as to whether increased rates of phosphine applications will be needed to control resistant *T. castaneum* and *R. dominica* insects.

Furthermore it has been determined that a similar mechanism for resistance could be common across all major grain storage pest species where phosphine is used for control.

Should this be verified as above, it would then be recommended that *T. castaneum* be considered as a model for resistance studies as there is currently a major research effort led by the USA to map the insect's genome, providing a highly valuable research resource.

Recommendation 2

The finding that *rph2* is highly conserved between *R. dominica* and *T. castaneum* suggested that a similar mechanism for resistance could be common across all major grain storage pest species where phosphine is used for control. Therefore it is recommended that this be tested by determining the presence / absence of the two genes in other grain storage insects. If they are indeed present, it would provide a single focus for industry to develop generic chemical approaches to manage phosphine resistance across all target pests.

Recommendation 3

The research has shown that mutations in *rph2* vary between populations of *R. dominica* and *T. castaneum.* This indicates that industry is unlikely to gain a universal molecular diagnostic test for phosphine resistance that could be applied across Australian grain growing areas.

Therefore it is recommended that efforts are focused on the development of regionally-specific diagnostic tests that could be combined in some form of platform to provide coverage across Australia.

To do this will require the developed platform or suite of individual tests to be placed in a format that is cost-effective for screening large numbers of individual insects.

This will be critical to overcome the current weaknesses inherent in the bioassay approach.

Recommendation 4

While gene expression profiling was achieved for *R. dominica* and *T. castaneum* the results indicate the technique is not suitable as the basis for development of a diagnostic test for resistance as the known resistance genes are not differentially expressed in resistant and sensitive strains and do not change expression in response to phosphine.

Therefore it is recommended that this technique not be explored any further, subject to the availability of substantial improvements in the technique, in efforts to develop a diagnostic test for phosphine resistance.

6. Abbreviations/glossary

ABBREVIATION	FULL TITLE
CRCNPB	Cooperative Research Centre for National Plant Biosecurity
DNA	Deoxyribonucleic acid
cDNA	complementary DNA
TCA	Tricarboxylic acid
rph1 & *rph2*	**R**esistance to **ph**osphine gene locus 1 and 2
CCG	Centre for Comparative Genomics (Murdoch University)
Kb	Kilobasepairs (1000 base pairs) of sequence
Gb	Gigabasepairs (1,000,000,000 base pairs) of sequence

7. Plain English website summary

Please complete table using plain English. This information will be published on CRCNPB's website for a public audience.

CRC project no:	CRC20080
Project title:	Diagnostic technologies for phosphine resistance
Project leader:	David Schlipalius
Project team:	David Schlipalius (DEEDI) Andrew Tuck (DEEDI) Paul Ebert (UQ) Rajeswaran Jagadeesan (UQ) Ramandeep Kaur (UQ) Greg Daglish (DEEDI) Manoj Nayak (DEEDI) Richard Glatz (SARDI) Matthew Bellgard (CCG) Roberto Barrero (CCG) Paula Moolhuijzen (CCG)
Research outcomes:	Key outcomes of the research are that: 1. Phosphine resistance is mediated by two major genes in both *T. castaneum* and *R. dominica*. These two genes have been named r*ph1* and *rph2* (i.e. ***r****esistance to* ***ph****osphine 1* and *2*). 2. The two genes are incompletely recessive and individually confer weak resistance when homozygous for the resistance mutation. 3. The two genes are synergistic in effect and confer strong resistance in *R. dominica* and *T. castaneum* when both are present and homozygous for the resistance alleles. 4. The two genes are expressed in all insect life stages suggesting a constitutively expressed resistance factor that does not appear to be 'switched off' at any stage of development. 5. The gene *rph2* is highly conserved between *R. dominica* and *T. castaneum* and is a basic metabolic gene integral to the Krebs (or TCA) cycle. 6. The action of the *rph2* gene is based on several mutations. 7. The mutations in *rph2* vary across populations of *R. dominica* and *T. castaneum.* This indicates that the opportunity to develop a universal molecular diagnostic test for phosphine resistance that could be applied across Australian grain growing areas is

	limited. 8. The location of the *rph1* gene has been narrowed down to a very small number of candidate genes (about six) for *T. castaneum*. However the location for *R. dominica* is less clear with a region of about 100 genes identified as the source area. 9. While gene expression profiling was achieved for *R. dominica* and *T. castaneum* the results indicate that the technique is not suitable as the basis for development of a diagnostic test for resistance as the known resistance genes are not differentially expressed in resistant and sensitive strains and do not change expression in response to phosphine.
Research implications:	Implication 1 The research has shown that there are two major genes that confer resistance in the two insect species studied and that both these genes are expressed in all insect life stages. This means that: 1. Selection for resistance can occur in all life stages of the two studied insects, and 2. There are no vulnerable life-stages that could have been targeted in attempts to manage resistance development Implication 2 The finding that the two genes are synergistic in effect and confer strong resistance only when both genes are homozygous (for resistance) explains why strong resistance has taken a relatively long time to increase in frequency and appear in enough numbers to be detected. This is because you must have, in one individual, both resistance genes present and both homozygous and the chances of this occurring in random mating events in nature are not high. Implication 3 It was found that the gene *rph2* is highly conserved between *R. dominica* and *T. castaneum.* The significance of this finding is that a similar mechanism for resistance could be common across all major grain storage pest species where phosphine is used for control. Implication 4 The research has shown that mutations in *rph2* vary between

	populations of *R. dominica* and *T. castaneum.* This indicates that industry is unlikely to gain a universal molecular diagnostic test for phosphine resistance that could be applied across Australian grain growing areas.
Research publications:	DI Schlipalius, W Chen, PJ Collins, T Nguyen, PEB Reilly and PR Ebert (2008) Gene interactions constrain the course of evolution of phosphine resistance in the lesser grain borer, *Rhyzopertha dominica. Heredity*, 100 (5):505-516 Schlipalius, D.I., Jagadeesan, R., Mau, Y., Collins, P.J. and Ebert, P.R. (2008). DNA testing for phosphine resistance - the future of resistance monitoring and management. In: Daolin, G., Navarro, S., Jian, Y., Cheng, T., Zuxun, J., Yue, L., Yang, L. and Haipeng, W., Proceedings of the 8th International Conference on Controlled Atmosphere and Fumigation in Stored Products. *8th International Conference on Controlled Atmosphere and Fumigation in Stored Products*, Chengdu, China, (595-598). 21-26 September 2008. There are also several publications arising from this work currently in preparation for submission.
Acknowledgements:	The authors would like to acknowledge the support of the Australian Government's Cooperative Research Centres Program. We would also like to acknowledge the contributions of the University of Queensland which contributed to two PhD scholarships that worked on this project.