Variant Linkage Analysis Using *de Novo* Transcriptome Sequencing Identifies a Conserved Phosphine Resistance Gene in Insects

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ABSTRACT Next-generation sequencing methods enable identification of the genetic basis of traits in species that have no prior genomic information available. The combination of next-generation sequencing, variant analysis, and linkage is a powerful way of identifying candidate genes for a trait of interest. Here, we used a comparative transcriptomics [RNA sequencing (RNAseq)] and genetic linkage analysis approach to identify the *rph1* gene. *rph1* variants are responsible for resistance to the fumigant phosphine (PH_3) that is used to control insect pests of stored grain. In each of the four major species of pest insect of grain we have investigated, there are two major resistance genes, *rph1* and *rph2*, which interact synergistically to produce strongly phosphine-resistant insects. Using RNAseq and genetic linkage analyses, we identified candidate resistance (*rph1*) genes in phosphine-resistant strains of three species: *Rhyzopertha dominica* (129 candidates), *Sitophilus oryzae* (206 candidates), and *Cryptolestes ferrugineus* (645 candidates). We then compared these candidate genes to 17 candidate resistance genes previously mapped in *Tribolium castaneum* and found only one orthologous gene, a *cytochrome b5 fatty acid desaturase* (C*yt-b5-r*), to be associated with the *rph1* locus in all four species. This gene had either missense amino acid substitutions and/or insertion/deletions/frameshift variants in each of 18 phosphine-resistant strains that were not observed in the susceptible strains of the four species. We propose a model of phosphine action and resistance in which phosphine induces lipid peroxidation through reactive oxygen species generated by dihydrolipoamide dehydrogenase, whereas disruption of *Cyt-b5-r* in resistant insects decreases the polyunsaturated fatty acid content of membranes, thereby limiting the potential for lipid peroxidation.

KEYWORDS pesticide; insecticide resistance; transcriptome; linkage

NEXT-GENERATION sequencing methods offer unprecedented opportunities to identify the genetic basis of traits in species that have no prior genomic information available. Whole-genome sequencing and *de novo* assembly of uncharacterized species comes with some major challenges. Genomes for uncharacterized species assembled from short reads can be highly heterozygous and fragmentary, which impacts on determining sequence quality and the annotation of genes. The power of genome-wide sequencing or genetic variant analysis of populations becomes most apparent when combined with determination of genetic linkage as this does not depend of the quality of the genome assembly.

In fact, it is possible to dispense with detailed genome assembly and annotation and focus on the transcriptome while including a genetic approach. By focusing on the transcriptome, the complexity of the data being studied is reduced, resulting in much higher sequence coverage and more accurate sequence assembly. Working with expressed sequences assembled from uncharacterized species also facilitates the

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identification and annotation of protein-coding sequences, which in turn assists in preliminary analysis of the functional significance of single-nucleotide polymorphisms (SNPs). We have used RNA sequencing (RNAseq) methods to scan the genomes of specific genetic crosses of several pest insect species that diverged from each other > 120 MYA (Zhang *et al.* 2018) and are resistant to an important industrial chemical, phosphine fumigant (PH_3).

Phosphine is currently the most important fumigant used for disinfestation of stored products worldwide, but resistance to phosphine in insect pests threatens its efficacy (Jagadeesan *et al.* 2016). The strong resistance phenotype results from two loci that work in synergy, *rph1* and *rph2* (*r*esistance to *ph*osphine 1 and 2), and are responsible for resistance in every species that we have examined (Schlipalius *et al.* 2002, 2008; Jagadeesan *et al.* 2013a; Nguyen *et al.* 2015, 2016). These two loci are recessive and when individually homozygous only confer a weak resistance phenotype (~5–30×) (Schlipalius *et al.* 2002; Jagadeesan *et al.* 2012; Nguyen *et al.* 2015, 2016). However, in combination the two homozygous loci result in a strongly resistant phenotype (100 to ≥ 1000×) (Schlipalius *et al.* 2002; Jagadeesan *et al.* 2012, 2013a; Mau *et al.* 2012a; Nguyen *et al.* 2015, 2016).

We have previously characterized the genetics of resistance in four insect species that are major pests of stored grain—*Rhyzopertha dominica* (Schlipalius *et al.* 2002, 2008), *Tribolium castaneum* (Jagadeesan *et al.* 2012, 2013a), *Sitophilus oryzae* (Nguyen *et al*. 2015, 2016), and *Cryptolestes ferrugineus* (Jagadeesan *et al.* 2016)—and noticed that strong resistance to phosphine appears to follow a similar etiology with two genetic loci (*rph1* and *rph2*) primarily responsible for the strong resistance phenotype. The similar features of the resistance trait between species led to the discovery of the *rph2* locus as dihydrolipoamide dehydrogenase (DLD) in *R. dominica*, *T. castaneum*, and *S. oryzae* (Schlipalius *et al.* 2012). Phosphine resistance at the *rph2* locus is associated with specific mutations near the active site of the encoded protein that must retain at least partial function, as DLD is an essential enzyme in energy metabolism. The *rph1* locus was not previously identified.

Resistance alleles at the *rph1* locus were already widespread in field populations of grain pest insects at the time of the discovery of strongly resistant insects, which, in addition to being homozygous resistant at *rph1*, also carried a homozygous resistance allele at *rph2* (Schlipalius *et al.* 2008). The *rph1* locus seemingly works by a different mechanism to *rph2*/DLD, as the effects of the two loci are synergistic rather than additive, as would likely be the case if they were working via the same mechanism (Raymond *et al.* 1989; Schlipalius *et al.* 2008). In *T. castaneum*, the locus of *rph1* was mapped to a candidate region containing 17 genes; these genes were predicted to be involved in lipid metabolism and chitin synthesis, as well as membrane permeability and maintaining ion gradients. The available information did not allow us to identify the resistance gene or determine which of these biochemical processes, when disrupted, was responsible for resistance (Jagadeesan *et al.* 2013a).

Given the apparent conservation of genetics of strong resistance to phosphine among insect species, we hypothesized that the genetic identity of the *rph1* locus would likely be conserved across species as well. Thus, we took a comparative genomic approach using transcriptome sequencing to identify *rph1* in four key species of grain pest that plague food grains across the globe: *R. dominica*, *S. oryzae*, *C. ferrugineus*, and *T. castaneum*.

Materials and Methods

Strains used

The strains used in this study and their phenotypes are outlined in Supplemental Material, Table S4 in File S1. *R. dominica* and *S. oryzae* strains were maintained on whole wheat at 30° 70% relative humidity (RH) and 25° 60% RH, respectively. *C. ferrugineus* strains were maintained on media containing rolled oats, sorghum, and flour (Jagadeesan *et al.* 2013b) at 30° 70% RH.

Fumigation

Phosphine gas generation and most fumigation bioassays were performed for 48 hr at 25° in sealed airtight desiccators as described previously (Schlipalius *et al.* 2008; Jagadeesan *et al.* 2012; Daglish *et al.* 2014), except where stated otherwise in the text.

Genetic crosses

***R. dominica* crosses:** An *R. dominica* introgressed strain, QRD_Introgressed, was developed by repeated backcrossing of a strain to the original susceptible parental line as per the scheme outlined in Figure S1. The backcrossing was performed using 50 virgin females of the susceptible strain mated to 50 selected male progeny of the first cross. The progeny were then allowed to mate for two generations before selection and this process was repeated for three more rounds of backcrossing. To further reduce the genetic complexity of the introgressed strain, the final backcross was performed using a single-pair mating. As each backcross should remove ~50% of the genetic background of the resistant strain, we expected the final selected introgressed strain to have > 97% genetic background similar to the susceptible parental strain. As a result of the selection for the resistance genes, we also expected the regions around the resistance loci to be homozygous for variants that arose exclusively from the resistant parents.

***S. oryzae* crosses:** A single-pair intercross between a susceptible (LS2) virgin female and a weakly resistant (QSO335) virgin male was developed as per the crossing scheme outlined in Figure S2. A single gene is responsible for phosphine resistance in the weakly resistant strain QSO335 (Daglish *et al.* 2014; Nguyen *et al.* 2015). F_6 progeny were selected at 0.1 mg/liter phosphine for 20 hr with a 7-day recovery period. Total RNA was extracted from survivors of phosphine fumigation as well as from unexposed siblings.

C. ferrugineus crosses: A single-pair intercross between a susceptible (CF31) virgin female and a weakly resistant (CF37) virgin male was developed as per the crossing scheme outlined in Figure S2. F_4 progeny were selected at 0.02 mg/liter phosphine for 20 hr. After a 7-day recovery period, total RNA was extracted from survivors of phosphine fumigation as well as from unexposed siblings from the same generation.

RNA extraction and RNAseq: For each strain sequenced, total RNA was extracted from 60 pooled insects per strain and sent to the Australian Genome Research Facility for RNAseq. A summary of the strains sequenced and the sequencing protocols used is outlined in Table S5 in File S1.

Bioinformatic analysis: For *R. dominica*, a reference transcriptome was created *de novo* using the 75-bp reads from the susceptible strain (QRD14). The Velvet algorithm (Zerbino and Birney 2008) was employed with k-mer length set to 21, maximum coverage set to 500×, and minimum coverage set to 10×. Redundant contigs were removed. Sequencing reads from the QRD_Introgressed, QRD14, QRD369, and QRD569 strains were each mapped to the *de novo R. dominica* transcriptome using the read mapping algorithm of the CLC Genomics Workbench v7.0 software. Read mapping was performed with the following default parameters: length fraction = 0.5, similarity fraction = 0.8, mismatch cost = 2, deletion cost = 3, and insertion cost = 3.

For *S. oryzae*, a reference transcriptome was assembled from reads of the LS2 susceptible strain. The CLC Genomics Workbench v 7.0 *de novo* assembly algorithm was used with bubble size set to 50, word size set to 23, and minimum contig length set to 200. Sequence reads of the strains LS2, QSO335, Santai, and Sangrur, as well as from the phosphine-selected and -unselected F_6 progeny of the LS2×QSO335 cross, were mapped separately to the *S. oryzae de novo* transcriptome using the same parameters as were used for *R. dominica* above.

For *C. ferrugineus*, a reference transcriptome was assembled using the CLC Genomics Workbench v 7.0 *de novo* assembly algorithm with bubble size set to 200, word size set to 15, and minimum contig length set to 200. Sequence reads of the strains CF31, CF37, and CF73, as well as from the phosphine-selected and -unselected F_4 progeny of the CF31×CF37 cross were mapped separately to the *C. ferrugineus de novo* transcriptome using the same parameters as for *R. dominica* above. A summary of all the strains and sequencing methods used for each of the species is outlined in Table S5 in File S1.

SNP detection

Quality-based SNP detection in CLC Genomics Workbench is based on a neighborhood quality standard (NQS) algorithm. For a SNP to be called, it was required to meet the default quality specifications: neighborhood radius (5), maximum number of gaps and mismatches (2), minimum average quality of surrounding bases (15), and minimum quality of the central base (20). We used a minimum coverage filter of 6 and minimum variant frequency of 20% to exclude sequencing artifacts. For each assembled transcriptome, the frequency of the variants (*i.e.*, count/coverage expressed as a percentage) was averaged across each contig using the R statistical package.

Comparative SNP analysis

R. dominica: Candidate genes were selected by filtering for contigs from the resistant introgressed strain that contained one or more variant nucleotides relative to the *de novo* reference transcriptome assembled from the sequence of a fully susceptible strain. We required that the variants be present in essentially 100% of the relevant sequence reads that made up the contig to indicate that it had been selected to homozygosity in the population that survived phosphine exposure. The cutoff was actually set to accept contigs that had a variant frequency of $>$ 95% of the sequence reads to allow for a low frequency of sequence artifacts. This resulted in the identification of contigs from the introgressed strain (that had been selected for phosphine resistance) that carried variants that had originated from the parental resistant QRD569 strain but that were absent from the susceptible reference strain.

S. oryzae: Candidate genes were selected by filtering for contigs that had average variant frequencies across the length of the contig of $>$ 95% (*i.e.*, homozygous and strongly selected) in the contig sequences from the individuals of the F_6 generation that had been selected as phosphine-resistant and $<$ 90% (*i.e.*, heterozygous) in the unselected F_6 population.

C. ferrugineus: Sequence generation and analysis for *C. ferrugineus* was carried out as described for *S. oryzae*. Progeny of an F_4 intercross between a susceptible and a resistant strain of *S. oryzae* were either selected for phosphine resistance or unselected. The resulting transcriptome sequence was analyzed as described for *S. oryzae*.

Candidate genes from each species were aligned by tBLASTx against the *Drosophila melanogaster* official gene set (release 6.02) and the *T. castaneum* official gene set (release 3.0) to identify homologs of previously mapped candidate resistance genes. The *D. melanogaster* gene set was used to enable more comprehensive functional insight of identified candidates.

Linkage mapping by marker analysis in R. dominica

A readily assayed genetic marker was identified that eliminated a restriction enzyme recognition site located within the 14-bp DNA fragment in the *rph1* gene that is deleted in both QRD369 and QRD569 (see Figure 2). This marker was used against a previously published mapping population of a QRD14×QRD569 cross (Schlipalius *et al.* 2002, 2008).

A fragment of the *R. dominica Cyt-b5-r* gene that encompasses a region that contains the 14-bp deletion was amplified using the primers FADS_1_fwd (5′-TTTCTTGTCCTGCAGTCATT-3′) and Rd-FADS_3_rev (5′-AACGACCCTTTCGACCTAAAC-3′).

The PCR reaction mixture contained TERRA (Clontech) PCR 2× buffer 12.5 μl, forward primer (10 μM) 1.25 μl, reverse primer (10 μM) 1.25 μl, water 7.5 μl, Taq polymerase 0.5 μl, and 2 μl of DNA template (~5 ng). Temperature cycling conditions were as follows: 98° for 2 min, followed by 40 cycles of 98° 20 sec, 55° 30 sec, and 68° for 60 sec, with a final extension at 68° for 60 sec, after which the temperature was held at 4° for 10 min.

The resulting fragments were digested with *Bst*NI (New England Biolabs, Beverly, MA) under the following conditions: 10× NEBuffer (3.1) 2.5 μl, water 1 μl, and *Bst*NI enzyme 0.5 μl at 60° overnight. The DNA was subsequently electrophoresed on a 2% agarose gel in 1× TAE. Digested DNA was indicative of a phosphine-susceptible genotype at the *rph1* locus.

Data availability

Insect strains are maintained at the Queensland Department of Agriculture and Fisheries and are available upon request. Sequence data of the *Cyt-b5-r* homologs for each species are available at GenBank (accession numbers MG254892-MG254895). The RNAseq data are deposited at the European Nucleotide Archive under the project accession number PRJEB24430. The determined phenotypes of each of the strains sequenced are in Table S4 in File S1.

Results

T. castaneum candidate genes

The *rph1* locus in *T. castaneum* was mapped previously to a region on chromosome 8 containing 17 genes (Jagadeesan *et al.* 2013a). We sequenced cDNA from 15 of these predicted candidate genes from five phosphine-resistant strains. The sequences were compared to the *T. castaneum* (v3.0 release) genome (Tribolium Genome Sequencing Consortium 2008) for SNP analysis and to predict amino acid changes within the coding regions. Of the 15 genes in the mapped region, only the TC006231 predicted protein showed amino acid changes in every resistant strain sequenced, but not in the susceptible strain (QTC4) relative to the reference GA2 genome (Tribolium Genome Sequencing Consortium 2008). Thus, the results of the SNP analysis strongly suggested that TC006231 was the most likely candidate to be the *rph1* gene in *T. castaneum*. The TC006231 predicted gene was identified as a cytochrome-b5 fatty acid desaturase by BLAST analysis against the National Center for Biotechnology Information nonredundant database (http://www.ncbi.nlm.nih.gov/) and the predicted protein shared a 44% sequence similarity (*E*-value = 1.34×10^{-115}) with the *D. melanogaster Cyt-b5-r* gene (CG13279, FBgn0000406).

We proceeded to test the hypothesis that the *Cyt-b5-r* homolog was a phosphine resistance gene by taking an unbiased comparative transcriptomic approach. Currently, there are no published genomic resources available for the other phosphine-resistant species we used in this study. Therefore, our comparative approach entailed sequencing and assembling the transcriptomes of each species. We first created genetic crosses between resistant and susceptible strains of each species and selected resistant progeny. We then sequenced the transcriptome and identified transcripts with nucleotide variants from the resistant parent, but not the susceptible parent, that were absolutely associated with the resistance phenotype. The identity of the proteins encoded by each of these candidate transcripts was then determined by sequence homology, which allowed us to determine whether the *Cyt-b5-r* ortholog was associated with resistance in each species (Figure 1).

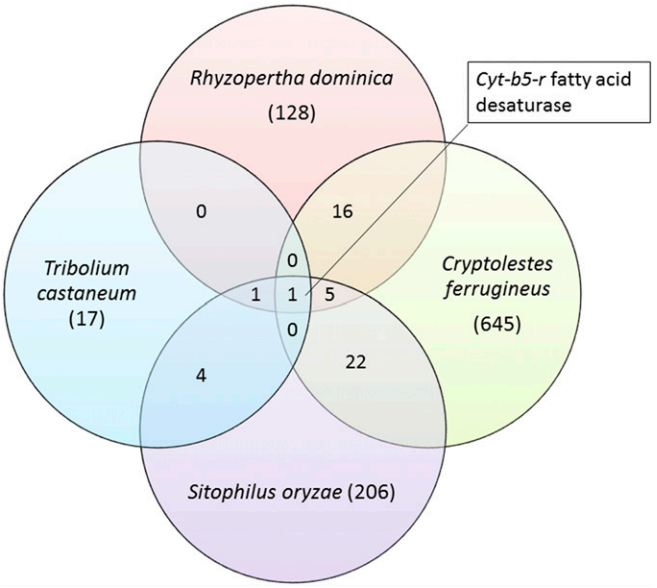

Figure 1 Venn diagram showing the number of shared homologs within the candidate gene sets for the four species. Homology was determined by best similarity by BLAST (Basic Local Alignment Search Tool) with *D. melanogaster* and *T. castaneum*.

R. dominica candidate gene identification

R. dominica currently does not have a published genome sequence. We therefore assembled *de novo* from next-generation sequence reads (RNAseq) using the Velvet (Zerbino and Birney 2008) algorithm, a reference transcriptome obtained from adults of a phosphine-susceptible strain (QRD14). There were 36,182 assembled sequences in the reference transcriptome, with an average length per sequence of 736 bp and a combined sequence length of 26.6 Mbp (Table 1). Compared to the *D. melanogaster* official gene set of 17,560 transcripts with an average length of 5754 bp per transcript, there is clearly fragmentation or incomplete assembly of the transcriptome. However, when we mapped the reads back against the transcriptome, ~92% of the reads from each sequenced strain mapped back to the reference genome, indicating 92% coverage of the expressed transcripts in adult *R. dominica*.

After the reference transcriptome assembly, we sequenced the transcriptome of a resistant strain having resistance alleles

Table 1 Summary bioinformatic statistics of reference transcriptome builds

	R. dominica length (bp)	*S. oryzae* length (bp)	*C. ferrugineus* length (bp)	*T. castaneum* official gene set (bp)	*D. melanogaster* official gene set (r-6.02)
Count	36,182	28,808	47,957	16,529	17,560
N75	538	894	424	—	—
N50	1,019	1,888	658	—	—
N25	1,726	3,346	1,301	—	—
Minimum	200	200	200	18	30
Maximum	25,026	19,047	10,371	330,447	1,668,527
Average	736	1,102	592	5,296	5,754
Total length	26,637,182	31,759,201	28,393,706	87,543,405	101,044,083

of *rph1* and *rph2* introgressed into the susceptible (QRD14) background (QRD_Introgressed) (see Figure S1). We then mapped the QRD_introgressed sequence reads against the assembled susceptible reference transcriptome, performed variant analysis, and took a bulk segregant approach to candidate transcript discovery. We reasoned that the introgressed strain would be homozygous for the recessive resistance allele at both the *rph1* and *rph2* resistance loci, and that the surrounding genes would likewise be homozygous based on their tight linkage to the selected loci. Therefore, to obtain a list of potential resistance genes, we identified all transcripts from the phosphine-resistant QRD_Introgressed strain that were homozygous for nucleotide variants that originated from the resistant parent across their entire length. Conceptually, this strategy is similar to the regional averaging of SNP homozygosity method used for to identify linked genomic regions in *T. castaneum* (Jagadeesan *et al*. 2013a) but uses transcripts of genomic sequence instead.

We also sequenced the transcriptome of the strongly resistant parental strain, QRD569 (homozygous for both *rph1* and *rph2*) (Schlipalius *et al.* 2008; Mau *et al.* 2012b). We then narrowed down the list of candidate resistance transcripts from the introgressed line by retaining only those that had variants derived from the resistant parental strain (QRD569). The filtered candidate transcript list of 129 sequences was then compared for homology by tBLASTx against the *T. castaneum* official gene set and the *D. melanogaster* official gene set. The candidate transcript list is given in Table S1 in File S1. The ortholog of the *Cyt-b5-r* gene was found in the candidate gene list with an *E*-value = 5.51×10^{-150} (65% identity) against the *T. castaneum* official gene set and *E*-value = 8.79×10^{-93} (52% identity) against the *D. melanogaster* gene list.

The QRD_introgressed strain is derived from the QRD569 strain that has resistance alleles at both the *rph1* and *rph2* loci (Schlipalius *et al.* 2008). To confirm the validity of the approach, we searched our candidate gene list for the known *rph2* resistance gene (*dld*), as we expected to see transcripts for both *rph1* and *rph2* loci in the candidate gene list. We saw two fragments of the transcript from the *R. dominica dld* gene. We also saw several fragments of the *SF3b1* transcript, a gene adjacent to the *dld* gene in *R. dominica* (Schlipalius *et al.* 2012). Identification of these sequences in the candidate transcript list demonstrated the validity of this technique for detecting regions containing candidate resistance genes and also confirmed the partial fragmentation of the assembled transcriptome sequences.

Confirmation of linkage by marker analysis

We then tested the *Cyt-b5-r* transcript for genetic linkage to the *rph1* locus in *R. dominica*. To do this, we employed a 14-bp deletion variant of the *Cyt-b5-r* gene sequence in the QRD369 (*rph1* only) resistant strain that eliminates a restriction enzyme recognition site.

Thus, a DNA fragment across the region will only be cut by the enzyme if it originates from the susceptible parent that does not carry the deletion and therefore retains the restriction enzyme recognition site. We selected resistant progeny of a QRD14×QRD369 single-pair intercross that had been maintained in the laboratory for > 60 generations (Schlipalius *et al.* 2008) and genotyped them for the variant. Approximately 1000 insects were selected at 0.6 mg/liter for 20 hr and only 24 survived. It was found that 23 of the 24 survivors were homozygous for the restriction site variant of the resistant parent, with one individual homozygous for the alternative variant. The unexpected individual was a surprise, but the linkage of the *Cyt-b5-r* homolog to the resistance trait was very tight ($\chi^2 = 10.08$, d.f. = 1, $P = 7.1 \times 10^{-6}$). The resolution of the mapping was < 0.07 cM, which given the *R. dominica* recombination rate of ~1.2 Mbp/map unit (Schlipalius *et al.* 2002) would equate to less than ~80 kbp, a region of DNA that could contain several genes. It is possible that the one insect that was homozygous for the allele from the sensitive parent survived due to the high number of individuals selected and the known variability of the phenotype. However, at this stage we could not exclude the possibility that the deletion itself might not be the resistance allele and that a recombination event occurred during the preceding 60 generations between the marker that was used for mapping and the actual resistance allele.

S. oryzae candidate gene identification

S. oryzae also does not have genomic resources publicly available. Therefore, we took a similar approach to that used for

R. dominica. We assembled a transcriptome derived from next-generation RNAseq of a phosphine-susceptible strain (LS2) using CLC Genomics Workbench. Before using the transcript sequence list for further analysis, we noted that the *Cyt-b5-r* and *dld* genes were in the assembled transcriptome. The final assembled transcriptome had a total of 28,808 sequences covering a total of 31.7 Mbp with an average length of 1102 bp. This was comparable to the results obtained for *R. dominica*.

To generate a candidate list of transcripts linked to phosphine resistance for *S. oryzae*, we used a single-pair intercross between susceptible (LS2) × weakly resistant (QSO335) strains. The QSO335 strain was expected to have a resistance allele only at the *rph1* locus, but not the *rph2* locus (Daglish *et al.* 2014; Nguyen *et al.* 2015, 2016). The crossing and selection scheme is outlined in Figure S2. Progeny were selected for resistance at the F_6 generation at a dose of 0.1 mg/liter phosphine for 48 hr, and the RNAseq sequence reads of selected and unselected progeny were mapped to the *de novo* reference transcriptome. The percentage of mapped reads was 90% in each case, which gave us confidence that we had ~90% or greater coverage of the adult *S. oryzae* transcriptome. We subsequently performed SNP analysis on the mapped reads and identified transcripts that were homozygous for variants from the resistant parent across the entire length of the transcript in phosphine-resistant F_6 progeny and heterozygous in sibling progeny that had not been exposed to phosphine. The subsequent candidate transcript list contained 206 sequences. The identity of these sequences was then determined by sequence homology using tBLASTx against the *T. castaneum* official gene set as well as the *D. melanogaster* official gene set. The candidate transcript list is given in Table S2 in File S1. We found the *Cyt-b5-r* homolog in the candidate gene list, indicating linkage to the *rph1* locus. As we expected, the *dld* gene that had previously been located and mapped to the *rph2* locus in this species was not found to be linked to phosphine resistance in the weakly resistant strain (QF37) that we used in this analysis as it only contains a resistance allele at the *rph1* locus.

C. ferrugineus candidate gene identification

C. ferrugineus also does not have genomic resources publicly available and so the approach used was very similar to that used for *S. oryzae*. We performed RNAseq on a susceptible strain (CF31) and used the reads to assemble a susceptible reference transcriptome, with the *dld* and *Cyt-b5-r* homologs manually assembled and annotated using CLC Genomics Workbench software. The final reference transcriptome for *C. ferrugineus* had 47,937 transcripts totaling 28.4 Mbp with an average length of 592 bp, which was less than the length of the average assembled transcript in either *R. dominica* or *S. oryzae*, but the total length of transcriptome sequence was comparable.

For finding transcripts that were linked to *rph1* resistance, a single-pair intercross of susceptible (CF31) × weakly resistant (CF37) strains was developed as per the crossing scheme outlined in Figure S2 (Jagadeesan *et al.* 2016). Insects of the F_4 generation were selected for resistance to phosphine using a dose of 0.04 mg/liter for 48 hr, as this dose discriminates between susceptible and weakly resistant individuals. RNAseq was performed on selected and unselected progeny and the reads were mapped against the *de novo* reference transcriptome derived from the susceptible parental strain. Subsequent SNP analysis was performed similarly to that of *S. oryzae* by filtering the variant list for transcripts that were homozygous for SNPs across the entire length of the transcript in the selected F_4 progeny, but heterozygous in the unselected progeny. The subsequent candidate transcript list contained 645 sequences that were then compared for homology by tBLASTx against the *T. castaneum* official gene set and the *D. melanogaster* official gene set. The candidate transcript list is given in Table S3 in File S1.

The *C. ferrugineus Cyt-b5-r* homolog showed homozygosity for a 7-bp deletion in the selected F_4 progeny, which was heterozygous in the unselected progeny (Figure 2). This confirmed both linkage to *rph1* and a likely full disruption of protein activity due to a frameshift variant. The ortholog of the *dld* gene did not show linkage to resistance, but the weak resistance phenotype in the CF37 strain is likely due to homozygosity for a resistance allele at *rph1* and not an *rph2* variant, similar to other resistant species.

Comparative homology analysis

BLAST analysis between all the candidate gene sequences from the three *de novo* transcriptomes, the *T. castaneum* official gene set, and the *D. melanogaster* reference gene set showed that there was only one gene that had significant homology between all three candidate gene sets and the candidate genes that had previously been mapped in *T. castaneum*, TC006231 (Figure 1).

In the *T. castaneum* genome, another Cyt-b5 desaturase homolog (TC006232) also occurs adjacent to *Cyt-b5-r* (TC006231) and was identified in the *R. dominica* and *S. oryzae* candidate gene sets, but was excluded from the candidate gene region in *T. castaneum* by genetic linkage analysis (Jagadeesan *et al.* 2013a).

Sequence characterization of the Cyt-b5-r gene from multiple species

We identified sequence variants by comparing RNA sequences from either cDNA (Sanger sequencing) or next-generation RNAseq for the *Cyt-b5-r* orthologs from phosphine-resistant strains of each species. These strains were derived from several countries including India, Vietnam, China, and Australia. Each species showed multiple SNPs in resistant strains that caused either frameshifts in the open reading frame or were calculated by Sorting Intolerant From Tolerant (SIFT) analysis (Kumar *et al.* 2009) to cause nontolerated amino acid substitutions that were likely to affect protein function. A diagram is shown in Figure 2 that represents the alignment of predicted protein sequences, in which the mutations identified for each resistant strain are highlighted.

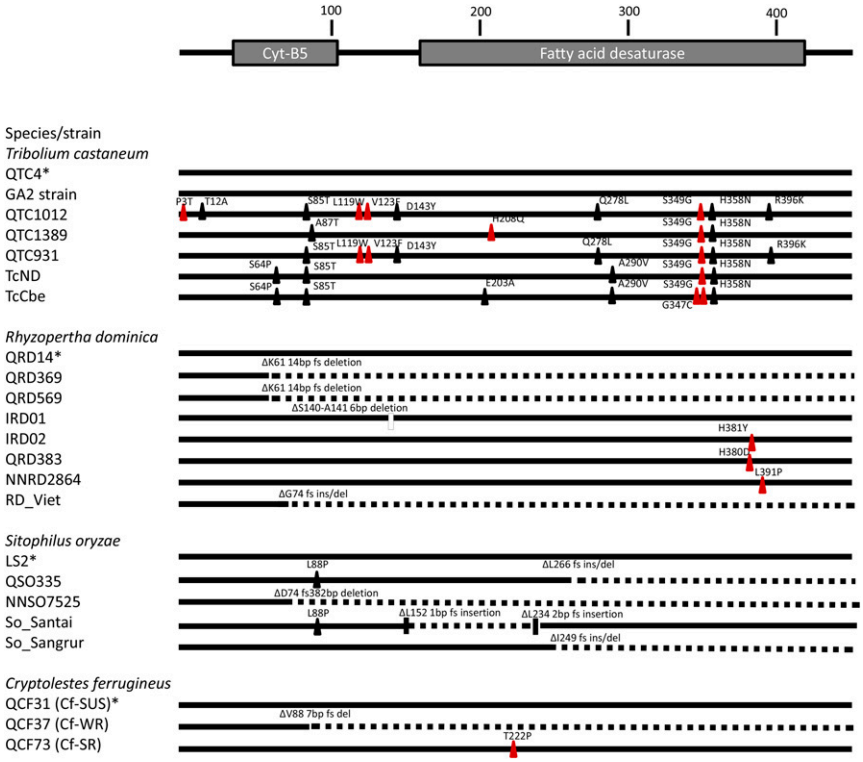

Figure 2 Aligned predicted protein models for phosphine-resistant strains of the four species studied. The susceptible reference strains used for the analysis are denoted by an asterisk. SNPs that changed amino acids compared to the respective susceptible reference strains are denoted by triangles, with those that are likely ($P < 0.05$) to cause gene disruption by SIFT analysis highlighted in red. Dotted lines indicate a translation frameshift.

Ka/Ks ratio analysis

We evaluated whether *Cyt-b5-r* has evolved under strong functional constraints by using the Ka/Ks approach of calculating rates of molecular evolution across genes. We aligned the wild-type protein-coding sequence of *Cyt-b5-r* from each of the four species—*T. castaneum*, *R. dominica*, *S. oryzae*, and *C. ferrugineus*—and used the Pamillo-Blanchi-Li approach (Li *et al.* 1985; Pamilo and Bianchi 1993). We used the implementation by Liberles (2001) in the online calculator provided by the Computational Biology Unit at the University of Bergen (http://services.cbu.uib.no/tools/kaks). Briefly, this method estimates unequal rates of substitution by taking into account transitional and transversional substitution rates as well as synonymous and nonsynonymous substitution rates. The Ka/Ks ratio of the comparisons between all four species was consistently and significantly < 1, with a range of 0.26–0.3395, suggesting that the gene was under strong purifying selection prior to the widespread use of phosphine (Table S6 in File S1).

Discussion

The challenges involved in identifying the molecular genetic basis of traits in organisms that are relatively uncharacterized and have little to no genomic information available can be readily overcome by combining classical genetic methods with next-generation sequencing.

While linkage mapping is powerful, it can require a lot of complex analysis; comparative methods can both decrease the requirement for such complexities and extend linkage methods in multiple species, and help to confirm the genetic basis of shared traits.

We have combined next-generation sequencing and transcriptome assembly with bulk segregant analysis of three species of economic importance for which little genome information was available. Our analysis of the genetics of phosphine resistance in these species was combined with results of genetic mapping in *T. castaneum*, which has a well-characterized genome. The comparative transcriptome variant analyses and genetic analysis of the four species identified a fatty acid desaturase (*Cyt-b5-r*) ortholog with a fused cytochrome b-5 domain in all four species. The *Cyt-b5-r* gene was genetically confirmed to cosegregate with the previously described *rph1* resistance to phosphine locus in two of the four species studied. Desaturases are responsible for introducing double bonds into long-chain fatty acids; however, the exact species of fatty acids that *Cyt-b5-r* affects is not currently known. Previous studies in *D. melanogaster* have demonstrated mitochondrial localization (Kula and Rozek 2000) by immunostaining, and have shown that this gene is expressed in muscle tissue (Levin *et al.* 1989) and is developmentally regulated (Graveley *et al.* 2011; Guan *et al.* 2013). Interestingly, in *D. melanogaster*, the gene is most strongly expressed during the larval and adult phases of development (Graveley *et al.* 2011; Guan *et al.* 2013) and most weakly during egg and pupal stages. These stages correspond to the most susceptible and tolerant life stages determined in grain pests, respectively (Hole *et al.* 1976; Kaur *et al.* 2012).

The predicted desaturase activity of the resistance gene strongly suggests that polyunsaturated lipids are intimately

associated with the toxicity of phosphine, possibly by making specific lipid species more susceptible to peroxidation. It remains to be elucidated exactly which lipid species the *Cyt-b5-r* enzyme desaturates and the affected molecular pathways.

Nature of the polymorphisms within the gene

Sequencing of the gene from multiple strains within each species revealed that all the resistant strains have SNPs that cause amino acid missense mutations and/or large insertion/deletion polymorphisms that cause frameshift mutations. This indicates that there is either total disruption or a high level of disruption of protein activity associated with resistance. However, only point mutations have been observed in the *T. castaneum* TC006231 gene. The level of resistance caused by *rph1* variants in *T. castaneum*-resistant strains (3.2×) (Jagadeesan *et al.* 2012) is much lower than that seen in weakly resistant strains of the other species (12×–30×) when assessed under similar conditions (Schlipalius *et al.* 2002; Daglish *et al.* 2014). This is possibly due to only partial loss-of-function effected by single amino acid changes, in contrast to the deletion mutations observed in the other species.

In insects, the *Cyt-b5-r* gene is commonly found in a locus physically adjacent to a close homolog, also a cytochrome b5 desaturase (*e.g.*, *T. castaneum* TC006232); however, phosphine resistance appears to be specific to *Cyt-b5-r*. This implies that the *Cyt-b5-r* gene and its close paralog are not redundant, but rather have different functional activities. Both of these desaturase genes usually reside either adjacent to or within 500 kb of a myosin heavy chain (*Mhc*) gene homolog. This is the case in diverse orders of insects whose genomes have been sequenced including *D. melanogaster*, *T. castaneum*, *Apis mellifera*, *Bombus terrestris*, *Atta cephalotes*, *Camponotus floridanus*, *Acromyrmex echinator*, and *Plutella xylostella*. This evidence of microsynteny implies that these genes have been physically associated across a long evolutionary time span and may constitute a functional locus within insects. The expression of the *Cyt-b5-r* gene also appears to be developmentally correlated with the *Mhc* gene in *D. melanogaster* muscle tissues (Levin *et al.* 1989).

Functional homologs of the *Cyt-b5-r* gene appear in most insects; however, some have only one cytochrome b5 desaturase gene. Moreover, the *Cyt-b5-r* gene responsible for phosphine resistance does not appear to be essential. The beetle strains that we have sequenced that have nonfunctional copies of *Cyt-b5-r* do not appear to have major fitness deficits under laboratory conditions, such as adverse effects on development or reproduction (Jagadeesan *et al.* 2012; Kaur *et al.* 2012, 2013; Daglish *et al.* 2014). Consistent with this idea, a global survey conducted in the 1970s (Champ and Dyte 1977) found that weak phosphine resistance was widespread and occurred in every country that used the fumigant. This implies that *rph1* alleles were possibly already preexisting and rapidly became widespread in the field after extensive phosphine exposure. However, the C*yt-b5-r* gene in susceptible beetle strains appears to be under strong purifying selection, and therefore any deletions (like the ones conferring resistance) should be costly in the wild. It may be that compensatory effects of other genes mediate the spread of the resistance allele in areas where phosphine is used.

Proposed mechanism of action

It is well established that exposure to phosphine induces oxyradical and hydrogen peroxide production (Bolter and Chefurka 1990; Liu *et al.* 2015). Lipid peroxidation is one of the most consistent effects unambiguously linked to phosphine exposure and has been shown to occur in all organisms; nematodes (Valmas *et al.* 2008), insects (Chaudhry and Price 1992; Liu *et al.* 2015), rats (Hsu *et al.* 2000, 2002a,b), plants (Niu *et al.* 2013; Mi *et al.* 2014), and cell tissue cultures (Hsu *et al.* 1998). Furthermore, a study of lipid peroxidation in phosphine- resistant and -susceptible *R. dominica* found that it was significantly lower in resistant insects (Chaudhry and Price 1992). At that time, only the weak resistance phenotype was observed, making it likely that the resistant strain that was investigated carried a resistance allele at *rph1*. Long-chain polyunsaturated fatty acids are highly susceptible to peroxidation (Hulbert 2005) and produce highly toxic compounds including 4-hydroxynonenal (4-HNE) and malondialdehyde, both of which are detected by the thiobarbituric acid reactive substances (TBARS) assay that has been used to monitor oxidative stress following phosphine exposure. TBARS are produced upon phosphine exposure in insects (Chaudhry and Price 1992). Lipid peroxidation products such as 4-HNE are likely to be major mediators of phosphine toxicity, as 4-HNE has been shown to bind most strongly to sulfhydryl moieties, such as those found in cysteine (Carini *et al.* 2004). 4-HNE thus causes widespread inhibition of multiple enzyme complexes, including complex IV of the oxidative phosphorylation pathway (Kaplan *et al.* 2007), and depresses respiration. It has also been shown to bind to other reduced sulfhydryl moieties, including dihydrolipoamide (Humphries and Szweda 1998a,b; Stofan *et al.* 2002; Hardas *et al.* 2013), which is a cofactor of the E2 subunit in enzyme complexes containing DLD (*rph2*), such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched chain amino acid dehydrogenase, and the glycine cleavage system. Binding of 4-HNE to the dihydrolipoamide cofactor would inhibit electron transfer to the active site of DLD, a situation that promotes a side reaction of DLD, which is the production of reactive oxygen species (ROS). Increasing the production of ROS would likely stimulate further lipid peroxidation and 4-HNE production. The result would be a synergistic increase in the toxicity of phosphine over time. This model is consistent with a puzzling observation regarding phosphine toxicity. While phosphine is comparatively more toxic than most other gases, it requires more time to be effective and requires the presence of oxygen (Chaudhry 1997). If any of the components of this synergistic mechanism of toxicity were to be suppressed or removed, more time would be required for similar levels of toxicity. We therefore propose that the apparent synergistic resistance mechanism is likely due to disruption of synergistic toxicity.

Conclusion and future directions

We currently do not know the substrates or products of the *Cyt-b5-r* enzyme, nor do we know the functional consequences of loss of this lipid desaturase. However, we do observe a strong functional interaction between the two (seemingly) independent pathways of lipid synthesis/desaturation via *Cyt-b5-r* and energy metabolism mediated by DLD, which has major toxicological consequences. A study of the *Cyt-b5-r* pathway and its role in lipid peroxidation and metabolism will further our understanding of the relationship between lipids, mitochondria, and oxidative stress, and their respective roles in the toxic response.

At a practical level, the knowledge that most insects carry functional orthologs of the *Cyt-b5-r* gene also implies that many insects may be able to achieve some measure of phosphine resistance if exposed at low enough doses to allow the survival of weakly resistant insects. This has practical consequences for all industries that use phosphine as a disinfestant to manage insect pests.

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