Detection of single-base substitution in an esterase gene and its linkage to malathion resistance in the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae)

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Abstract: *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) is an important parasitoid of stored-grain insect pests. Partial cDNA sequences of an esterase-like enzyme have been obtained from a malathion-resistant (R) strain and a susceptible (S) strain of this wasp. A single-base substitution in the R strain has been confirmed by using PCR amplification of specific allele (PASA) to amplify genomic DNA extracted from individual resistant and susceptible parents, F_1 hybrids from double reciprocal crosses, and progeny from backcrosses. The R allele appeared to be inherited in a strict Mendelian fashion in both diploid female and haploid male progeny. The esterase fragment co-segregated with resistance in these crosses and backcrosses. Female wasps in a mixed population of *A calandrae* that survived a malathion screen carried the R allele for the esterase-like enzyme, while those wasps that died did not have the R allele. The single base-pair mutation, guanine in the R strain and thymine in the S strain, presumably results in a tryptophan-to-glycine amino acid substitution in the encoded protein. We do not know how these amino acid substitutions may relate to functional differences in the enzyme. However, this esterase gene or another linked esterase gene may encode the resistance-associated malathion detoxifying activity in the R strain.

Keywords: *Anisopteromalus calandrae;* parasitoid; PCR; DNA; PASA; single-base substitution; insecticide resistance

1 INTRODUCTION

Resistance to organophosphate insecticides in insects is often associated with increased levels of hydrolase activity.[1–3] In the case of resistance to malathion, an organophosphorodithioate insecticide, resistance in many insect species is characterized by the presence or increased expression of a malathion-specific carboxylesterase rather than an increase in general esterase activity.[4–10] We have also found significantly higher levels of a malathion-specific carboxylesterase in a malathion-resistant strain, relative to that of a susceptible strain, of a solitary pteromalid parasitoid *Anisopteromalus calandrae* (Howard).[11] This increased activity of a malathion-degrading enzyme may be a major factor involved in the resistance. The malathion resistance in *A calandrae* is inherited as an incompletely dominant trait controlled by a single gene.[12] Because *A calandrae* is one of the few parasitoids to develop significant levels of naturally occurring resistance to an organophosphate insecticide,[13] we are continuing our analysis of the biochemical and molecular bases for this resistance.

Several polymerase chain reaction (PCR)-based techniques can be used for detecting and monitoring molecular polymorphisms between insect strains. These include PCR and restriction enzyme digest (PCR/REN), single-stranded confirmational polymorphism (SSCP), and PCR amplification of specific alleles (PASA). Advantages and disadvantages of each technique have been summarized by ffrench-Constant *et al.*[14,15] PASA was chosen for the present study because it is a very sensitive method for detecting known single-base changes as well as small deletions and insertions on genomic DNA. PASA was successfully used to help characterize insecticide resistance mechanisms in the Colorado potato beetle *Leptinotarsa decemlineata* (Say),[16,17] the fruit fly *Drosophila* spp,[18] the red flour beetle *Tribolium castaneum* Herbst,[19] and the coffee berry borer *Stephanoderes hampei* (Ferr).[14] In addition to being rapid, reproducible, inexpensive, non-isotopic and amenable to automation,[20] advantages of using PASA to investigate insect population genetics also include detection of specific genes of interest and

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(*Received 8 May 1998; revised version received 10 September 1998; accepted 17 November 1998*)

 Pestic Sci 0031-613X/99/$17.50

efficient characterization of genotypes of individuals without knowledge of their phenotype.

In the study reported herein, we attempted to determine if a structural change in a gene is involved in the resistance in *A calandrae*. cDNA libraries of both susceptible and resistant strains were constructed and fragments of carboxylesterase-like cDNA were amplified and sequenced from susceptible and resistant strains. We designed two PASA primers based on a point mutation that was found in the cDNA fragments to create the preferential match and mismatch between esterase alleles and primers.

2 MATERIALS AND METHODS

2.1 *Anisopteromalus calandrae* strains

A susceptible laboratory strain of *A calandrae* has been maintained in culture for more than 20 years. A malathion-resistant strain was collected from a farm storage near Bamberg, SC, in 1992 and subsequently maintained in the laboratory without selection pressure. Both strains are reared on larvae of the rice weevil, *Sitophilus oryzae* (L), infesting wheat at 27°C and 55–65% RH.

2.2 Isolation of genomic DNA

DNA was extracted from adult wasps by using a method similar to the protocol of Dowdy and McGaughey.[21] Adults were placed individually in 0.5-ml microcentrifuge tubes and homogenized in 50 µl of isolation buffer: Tris-HCl (0.1 M; pH 9.1), containing NaCl (0.1 M), sucrose (0.2 M), EDTA (0.05 M), and sodium dodecyl sulfate (SDS; 10 g litre^{-1}). The homogenate was incubated for 30 min at 65°C. After addition of potassium acetate (8 M; 10 µl), the tube containing homogenate was incubated on ice for 30 min, and centrifuged for 15 min at 10 000 *g*. The supernatant was transferred to a new tube, and treated with RNase A. The DNA solution was extracted with phenol + chloroform + isoamyl alcohol (25 + 24 + 1 by volume), precipitated in ethanol overnight at −20°C, pelleted by centrifugation, and resuspended in 200 µl distilled and deionized water.

2.3 Cloning and sequencing of esterase cDNA fragment

Double stranded cDNA was synthesized using 5 µg of poly(A) RNA as a template (ZAP-cDNA synthesis kit, Stratagene, La Jolla, CA), directionally cloned into a UniZAP XR vector phage (Stratagene), and packaged using the ZAP-cDNA Gigapack II Gold cloning system (Stratagene). Esterase cDNA fragments were amplified using a T3 forward primer complementary to the promotor in the vector and a reverse degenerate primer designed from the conserved regions (FGESAGG) of carboxylesterases from mosquitoes and rats.[22,23] PCR products were separated on a low melting point gel. DNA fragments of the expected size (~700 bp) were sliced from the gel and extracted using a DNA purification system (Promega, Madison, WI). The isolated DNA fragments were ligated into a pGEM-T vector (Promega, Madison, WI) overnight at 14°C. *Escherichia coli* Cast & Chalm cells were transformed and plated on LB ampicillin/IPTG/X-Gal medium. White colonies were subjected to PCR amplification to confirm the presence of inserts and the expected sizes of fragments. A PCR-confirmed colony was inoculated to a 50-ml LB/ampicillin culture. Plasmid DNA was extracted using protocols described by Sambrook *et al*.[24] The DNA inserts were sequenced using a thermal cycling and silver staining sequencing kit (Promega, Madison, WI) and an automated sequencer (Applied Biosystem Model 393A) located at the College of Veterinary Medicine, Kansas State University, Manhattan, KS.

2.4 PCR amplification of specific allele (PASA)

Partial esterase cDNA sequences were obtained both from susceptible and resistant strains. A single-base substitution (or 'difference') was observed on cDNA cloned (Fig 1A) from resistant strain of *A calandrae*. To confirm that this base substitution is present in the esterase gene, and is strain-specific, PCR amplification of specific allele (PASA) was performed to amplify genomic DNA extracted from individual wasps. Two pairs of primers (Fig 1B) were designed from cDNA sequences. The base change is a transversion of T (susceptible) to G (resistant). To amplify the S-associated allele selectively, we used a forward primer WCF1 for both strains and a selective reverse primer SWRA with a nucleotide A at the 3′ terminus. It has been shown that a 3′ terminal mismatch in the primer/template decreases PCR amplification yield dramatically.[25] To amplify the R-associated allele selectively, we used reverse primer WCR3 and forward primer RWFG with a 3′ terminal G. PCR reactions contained Tris-HCl (10 mM; pH 9), $MgCl_2$ (1.5 mM), 1 µM of each primer, KCl (50 mM), 0.1 mM of each dNTP, Taq DNA polymerase (0.05 unit µl^{-1}), and DNA template (2 µl). The DNA was initially denatured for 3 min at 94°C, and the PCR amplification included 30 cycles of 30 s denaturing at 94°C, 30 s annealing at 60°C, and 1 min extension at 72°C in a PTC-100 thermocycler (MJ Research, Inc, Watertown, MA). PCR products were separated on 1% agarose gels, stained with ethidium bromide (0.5 µg ml^{-1}), and photographed under UV light. Number and length of PCR-amplified DNA fragments were predicted based on cDNA as shown in Fig 1C.

2.5 Inheritance of esterase fragment

Crossing experiments to investigate inheritance of the dimorphism were conducted as described by Baker *et al*.[12] F_1 hybrid wasps were obtained from double reciprocal crosses. Backcross progeny were obtained by backcrossing hybrid females from the cross (R♀ × S♂) with susceptible males, and by

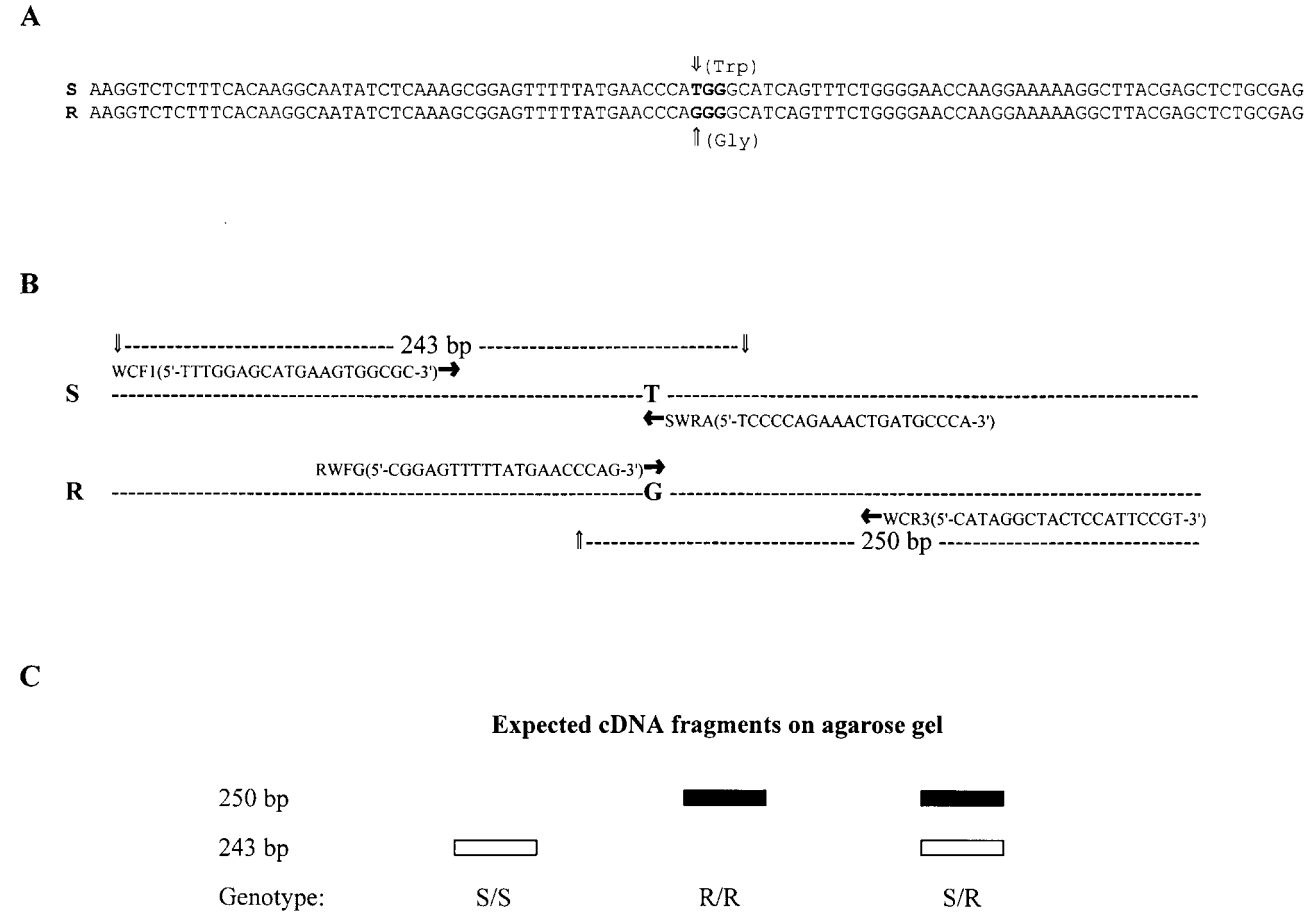

Figure 1. A. Partial cDNA sequences cloned from S and R strains of *Anisopteromalus calandrae*. Codon TGG codes tryptophan and codon GGG codes glycine. B. Primer design strategy to create a DNA template/primer match and mismatch: primer WCF1 paired with SWRA, designated as primers that match the susceptible allele and mismatch the resistant allele, primer RWFG paired with WCR3, designated as primers that match the resistant allele and mismatch the susceptible allele. C. Expected cDNA fragment numbers and size for each *A calandrae* genotype. S = susceptible, R = resistant, G = guanine, A = adenine, C = cystosine, T = thymine.

backcrossing hybrid females from the cross (S♀ × R♂) with resistant male wasps. Genomic DNA was isolated from five individual wasp progeny per sex that emerged from each cross. PASA was performed using primer pairs WCF1 plus SWRA and RWFG plus WCR3.

2.6 Malathion screen

A mixed culture of *A calandrae* was prepared by adding R males to a culture of the S strain for several generations. Females were bioassayed against a discrimination concentration (10 µg per vial) of malathion[12] and genomic DNA was extracted from six individual females that survived the treatment and from six individual females that died. PASA was conducted using primer pairs WCF1 plus SWRA and RWFG plus WCR3.

3 RESULTS

3.1 Partial esterase cDNA sequences and single-base mutation

Fragments were amplified and sequenced from cDNA libraries both of susceptible and resistant strains of *A calandrae*. Alignment of resistant cDNA with susceptible cDNA showed that the fragments from R and S strains were identical except for a single-base substitution. A nucleotide thymine in the susceptible strain is replaced by guanine in the resistant strain (Fig 1A).

3.2 Single-base mutation on genomic DNA

With the point mutation placed at the 3′ end of the primer, genomic DNA was selectively amplified from susceptible or resistant strains by using PASA primers. A ~470 bp DNA fragment (Fig 2) was amplified from the susceptible strain using primers WCF1 and SWRA. A ~380 bp DNA fragment was amplified from the resistant strain using primers RWFG and WCR3. Both DNA fragments contain untranslatable introns because the fragments are larger than those cDNA fragments by approximately 240 bp on the 5′ upstream side of the point mutation and by ~ 130 bp on the 3′ downstream side of the point mutation. Genomic DNA of ~470 bp was amplified only from susceptible wasps (three females and three males, lanes 1–6, Fig 2) with primers WCF1 and SWRA perfectly matching the cDNA sequence associated with the S strain. The ~380 bp DNA fragment was amplified only from resistant

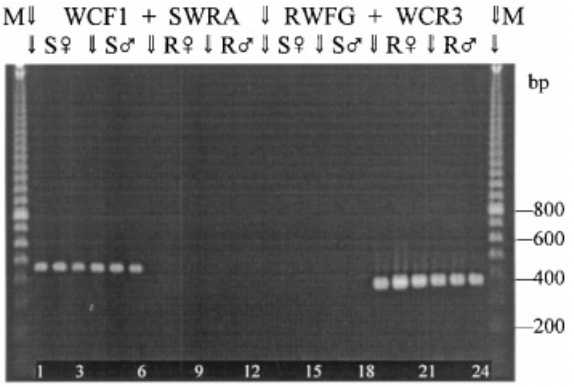

Figure 2. PASA amplification of individual wasp genomic DNA extracted from parent populations of malathion-susceptible and -resistant strains of *Anisopteromalus calandrae*. Six individuals (three females and three males) from each strain were used for each pair of primers. S = susceptible, R = resistant, M = 100 bp DNA ladder from Pharmacia.

wasps (three females and three males, lanes 19–24, Fig 2) using primers RWFG and WCR3 which perfectly matched the cDNA sequence associated with the R strain DNA sequence. No ~470 bp fragment was amplified from resistant wasps using primers WCF1 and SWRA (lanes 7–12, Fig 2), and no ~380 bp fragment was amplified from susceptible wasps using primers RWFG and WCR3 (lanes 13–18, Fig 2). These results indicated that the difference between the S and R strain-derived cDNAs resulted from neither a rare allele in one strain nor a PCR artifact.

3.3 Inheritance of point mutation

Female progeny obtained from crossing susceptible females with resistant males have the genotype S/R, and PASA demonstrated that these females were also heterozygous for esterase alleles. PASA yielded a ~470 bp fragment (lanes 1–3, Fig 3) amplified from the susceptible allele matched by primers WCF1 and SWRA, and a ~380 bp fragment (lanes 13–15, Fig 3)

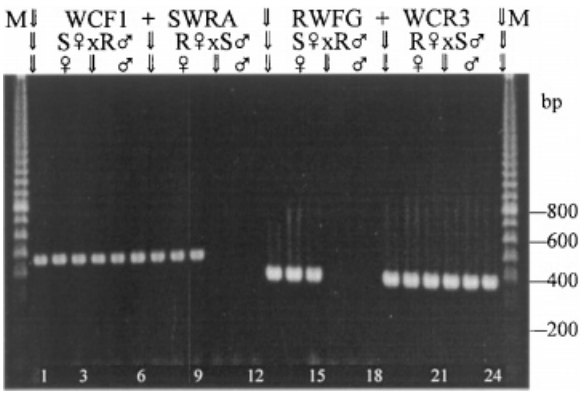

Figure 3. PASA amplification of individual wasp DNA extracted from F_1 hybrids of *Anisopteromalus calandrae* obtained from crossing susceptible females with resistant males, and from crossing resistant females with susceptible males. Six individuals (three females and three males) from each hybridization were used for each pair of primers. S = susceptible, R = resistant, M = 100 bp DNA ladder from Pharmacia.

amplified from the resistant allele matched by primers RWFG and WCR3. Male progeny obtained from crossing susceptible females (S/S) with resistant males (R/−) have the haploid genotype S/−, inherited exclusively from the susceptible mother. PASA yielded a ~470 bp fragment (lanes 4–6, Fig 3) amplified from the susceptible allele matched by primers WCF1 and SWRA. There was no detectable ~380 bp fragment (lanes 16–18, Fig 3) because the wasps have no resistant allele to match primers RWFG and WCR3.

Female progeny obtained from crossing resistant females (R/R) with susceptible males (S/−) have the diploid heterozygous genotype R/S. PASA amplification yielded a ~470 bp fragment (lanes 7–9, Fig 3) amplified from the susceptible allele matched by primers WCF1 and SWRA, and a ~380 bp fragment (lanes 19–21, Fig 3) amplified from the resistant allele matched by primers RWFG and WCR3. Male progeny obtained from crossing resistant females (R/R) with susceptible males (S/−) have the haploid genotype R/−, inherited exclusively from the resistant mother. PASA yielded a ~380 bp fragment (lanes 22–24, Fig 3) amplified from the resistant allele matched by primers RWFG and WCR3. There was no detectable ~470 bp fragment (lanes 10–12, Fig 3) because these haploid males have no susceptible allele to match primers WCF1 and SWRA.

Half of the female progeny obtained from backcrossing heterozygous females (R/S) from the cross (R♀ × S♂) with susceptible males (S/−) were expected to have the R/S genotype and half should have the S/S genotype. PASA amplification of DNA from individual backcross females showed that a ~470 bp fragment matched by primers WCF1 and SWRA was found in all 10 females (lanes 1–10, Fig 4A). A ~380 bp fragment matched by primers

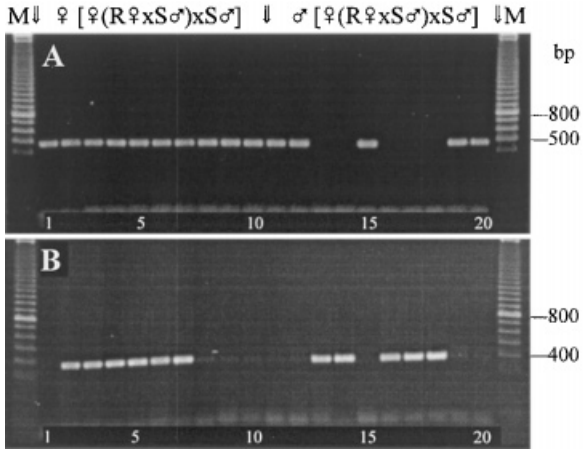

Figure 4. PASA amplification of individual wasp DNA extracted from F_1 progeny of *Anisopteromalus calandrae* from the backcross of females from the cross (R♀ × S♂) with susceptible males. Twenty individuals (10 females and 10 males) were used for each pair of primers. A: amplification using primers WCF1 and SWRA. B: amplification using primers RWFG and WCR3. S = susceptible, R = resistant, M = 100 bp DNA ladder from Pharmacia.

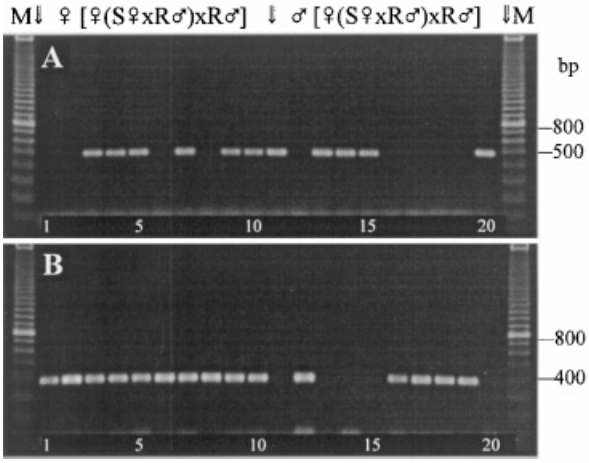

Figure 5. PASA amplification of individual wasp DNA extracted from F_1 progeny of *Anisopteromalus calandrae* from the backcross of females from the cross (S♀ × R♂) with resistant males. Twenty individuals (10 females and 10 males) were used for each pair of primers. A: amplification using primers WCF1 and SWRA. B: amplification using primers RWFG and WCR3. S = susceptible, R = resistant, M = 100 bp DNA ladder from Pharmacia.

RWFG and WCR3 was produced from six females (lanes 2–7, Fig 4B). Results indicated that the ratio of S/S (females No 1 and Nos 8–10) to S/R (females Nos 2–7) is 4 : 6 in this small sample of backcross progeny. Haploid male progeny obtained from backcrossing hybrid females (R/S) from the cross (R♀ × S♂) with susceptible males (S/−) inherited genetic material from the heterozygous mother only. Five males contained the susceptible allele matched by primers WCF1 and SWRA (lanes 11–12, 15, 19–20, Fig 4A), and five males contained the resistant allele matched by primers RWFG and WCR3 (lanes 13–14, 16–18, Fig 4B).

Half of the female progeny obtained from backcrossing heterozygous females (S/R) from the cross (S♀ × R♂) with resistant males (R/−) were expected to have the S/R genotype and half should have the R/R genotype. PASA amplification of DNA from

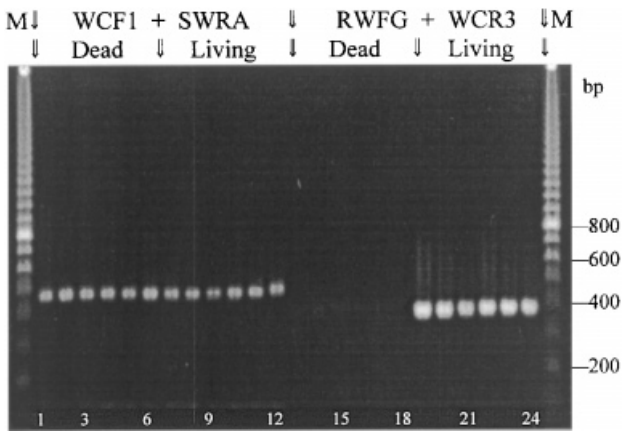

Figure 6. PASA amplification of individual wasp DNA extracted from *Anisopteromalus calandrae* in a mixed population selected with 10 μg per vial malathion. Twelve individual females (six dead and six living) were used for each pair of primers. M = 100 bp DNA ladder from Pharmacia.

individual wasps showed that a ~470 bp fragment was obtained from six females that had the allele matched by primers WCF1 and SWRA (lanes 3–5, 7 and 9–10, Fig 5A). A ~380 bp fragment was found in all females. These females contained the resistant allele matched by primers RWFG and WCR3 (lanes 1–10, Fig 5B). The ratio of S/R genotypes (females Nos 3–5, 7 and 9–10) to R/R genotypes (females Nos 1–2, 6 and 8) is 6 : 4 in this sample of backcross progeny. Haploid male progeny obtained from backcrossing hybrid females (S/R) from the cross (S♀ × R♂) with resistant males (R/−) inherited genetic materials from the heterozygous mother only. Five males contained the allele matched by primers WCF1 and SWRA (lanes 11, 13–15 and 20, Fig 5A), and five males contained the allele matched by primers RWFG and WCR3 (lanes 12 and 16–19, Fig 5B).

3.4 Malathion screen

Results showed that among the 12 female wasps analyzed, all had the allele matched by primers WCF1 and SWRA and that the ~470 bp fragment was amplified from each individual (lanes 1–12, Fig 6). No ~380 bp fragment was amplified in females that died, indicating that no resistance allele was present, (lanes 13–18, Fig 6). PASA produced a ~370 bp fragment from all six surviving wasps, indicating that these wasps had the resistant allele (lanes 19–24, Fig 6). PASA results suggested that dead wasps had the S/S genotype, and that surviving wasps were heterozygous (S/R).

4 DISCUSSION

Esterases are a large group of enzymes that metabolize a wide variety of substrates, including toxicants. The mechanism of resistance to these toxicants in many cases is based on elevated levels of esterases.[26–31] Esterase levels can be elevated by either gene amplification or altered gene expression. Esterases may also be mutated to produce structurally different enzymes which are able to metabolize insecticides more efficiently.[32] In the Australian sheep blow fly *Lucilia cuprina* (Weid) and the mosquito *Culex tarsalis* (Coquillett), a carboxylesterase appears to be structurally altered in resistant populations to produce high levels of resistance to malathion.[33,34] More recently, Newcomb *et al*[35] found that a single amino acid substitution, $gly^{137} \rightarrow asp$, within the active site of esterase E3 from resistant *L cuprina*, correlates with a loss of carboxylesterase activity and a corresponding increase in organophosphate hydrolysing activity.

In the current study, partial cDNA sequences of esterase-like enzymes were cloned and sequenced from both S and R strains of *A calandrae*. A GenBank search[36,37] indicated a 30–35% sequence identity between cDNAs from *A calandrae* and esterase-like enzymes of the peach-potato aphid

Myzus periscae (Sulzer),[27] the fruit fly *Drosophila melanogaster Meig*,[38] the mosquito *Culex quinquefasciatus* Say,[39] and the black rat *Rattus rattus* (L).[40] In addition to these sequence similarities, a single base-pair difference in cDNA between malathion-susceptible and resistant strains of *A calandrae* was found and evidence indicates that this difference is inherited in a Mendelian fashion and is linked to resistance. The single base-pair mutation in *A calandrae* presumably results in an amino acid substitution. Codon GGG encodes glycine in resistant wasps and TGG encodes tryptophan in the susceptible strain. How these single amino acid substitutions may relate to functional differences between esterases in susceptible and resistant strains of this wasp is not yet understood.

At this time we have no direct evidence that the esterase fragment cloned from *A calandrae* encodes the malathion-specific hydrolysing activity in this wasp. To specifically characterize the gene(s) responsible for resistance in *A calandrae*, and to determine mechanisms involved in expression of this enzyme, studies are underway to (1) obtain full cDNA sequences of this esterase gene, and (2) compare this sequence with clone(s) obtained from library screens based on a primer designed from the *N*-terminal sequence of the purified malathion-specific carboxylesterase from the R strain.

ACKNOWLEDGEMENTS

The authors would like to thank Drs Jeffery Stuart, Kun Yan Zhu and Srini Kambhampati for reviewing an earlier version of this manuscript. The authors are grateful to Joel Perez-Mendoza, Jeffrey Fabrick, and Michele Zuercher for their assistance in the laboratory.

NOTE

Mention of a proprietary product does not constitute an endorsement or recommendation by the USDA.

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