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Allele-specific PCR reveals that *CYP6D1* is on chromosome 1 in the house fly, *Musca domestica*

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Abstract. A cytochrome P450, termed $P450_{lpr}$, is the major P450 responsible for pyrethroid resistance in the Learn-PyR (LPR) strain of house fly. Recently, the putative gene (*CYP6D1*) coding for $P450_{lpr}$ has been sequenced from the LPR and aabys strains of house fly. Allele-specific polymerase chain reaction (ASPCR) was used for linkage group analysis with backcross progeny from the wild type LPR strain and a multiple marker strain (aabys). We found that *CYP6D1* is linked to chromosome 1. The possible role of regulatory or modifying genes responsible for elevated $P450_{lpr}$ expression is discussed in relation to the chromosomal linkage of *CYP6D1*.
Key words. Cytochrome P450 monooxygenase; Insecta; insecticide; resistance.

The microsomal cytochrome P450 monooxygenases are important in the metabolism of endogenous compounds and xenobiotics[1]. In insects, cytochrome P450 monooxygenases are also a major mechanism by which insects become resistant to insecticides[2–4]. In the house fly, *Musca domestica*, P450-mediated detoxication has been linked to chromosomes 1, 3 (ref. 5), 2 and 5 (refs. 6, 7). However, questions of whether changes in the enzyme (i.e. structural gene) and/or in the amount of enzyme (i.e. regulatory genes) are responsible for increased detoxication are unresolved. To understand more fully the molecular basis of P450-mediated resistance several pieces of information are needed, including the chromosomal linkage of the P450 structural genes involved in resistance.

Cytochrome $P450_{lpr}$ is a developmentally regulated house fly P450 that detoxifies pyrethroids[8]. $P450_{lpr}$ is expressed at high levels in Learn-PyR (LPR) house flies and is responsible for monooxygenase-mediated pyrethroid resistance in this strain[8]. The gene (*CYP6D1*) coding for $P450_{lpr}$ has been sequenced from LPR[20] and four pyrethroid susceptible strains of house fly (Scott et al., unpublished).

To determine the chromosomal linkage of *CYP6D1*, an oligonucleotide primer was designed from a polymorphic site in the *CYP6D1* sequence (Scott et al., unpublished), and allele specific polymerase chain reaction (ASPCR) was applied to the backcross progeny of LPR and the morphologically marked aabys strains (i.e. aabys × F_1(aabys × LPR)).

Materials and methods

Two parental strains were used: LPR, a strain collected from a dairy near Horseheads, New York State, USA in 1980 and selected in the laboratory with permethrin, attaining a 6000-fold level of resistance and appearing homozygous for pyrethroid resistance[9]; aabys, a strain with the recessive morphological markers ali-curve (*ac*), aristapedia (*ar*), brown body (*bwb*), yellow eyes (*ye*) and snipped wings (*snp*) on autosomes 1, 2, 3, 4 and 5, respectively, was obtained from Dr. T. Hiroyoshi (Osaka University). Flies were reared as described by Wheelock and Scott[10].

To determine chromosomal linkage of *CYP6D1*, a backcross experiment was done as described by Georghiou[11]. Virgin females were collected within 8 h after emergence. LPR males were crossed with aabys females. F_1 males were backcrossed to aabys females and 32 combinations of phenotypes were produced in the backcross (BC_1) generation. Since crossing over does not occur in male house flies[6], the absence of a recessive morphological marker in BC_1 flies indicates that the corresponding chromosome was derived from the parental LPR strain. The following ten phenotypes (in parentheses) were isolated from the BC_1 and named according to the autosomes bearing wild type markers (i.e. from the LPR strain): R1 (+; *ar*; *bwb*; *ye*; *snp*), R2 (*ac*; +; *bwb*; *ye*; *snp*), R3 (*ac*; *ar*; +; *ye*; *snp*), R4 (*ac*; *ar*; *bwb*; +, *snp*), R5 (*ac*; *ar*; *bwb*; *ye*; +), R2345 (*ac*; +; +; +; +), R1345 (+; *ar*; +; +; +), R1245 (+; +; *bwb*; +; +), R1235 (+; +; +; *ye*; +), and R1234 (+; +; +; +; *snp*). Twenty male flies each from the F_1, and selected BC_1 phenotypes were collected in liquid nitrogen and stored at −80 °C.

Allele specific PCR was our method of choice for determining the chromosomal linkage of *CYP6D1* because

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this technique offers several advantages over standard genetic methods[11] (i.e. isolating lines and conducting insecticide bioassays on each). The PCR method requires fewer animals to be tested and allows for testing of BC_1 individuals as opposed to the laborious methods needed for strain isolation by standard methods. The PCR method is also highly specific for the gene of interest (i.e. *CYP6D1*) compared to insecticide bioassays which cannot always discriminate between different resistance mechanisms (i.e. genes). Furthermore, the PCR method requires no a priori assumption about the role of the *CYP6D1* per se in resistance. For example, it is possible that the structural gene may not contribute to resistance (i.e. catalytically similar enzymes in susceptible and resistant strains) and, therefore, could not be mapped by standard methods.

Genomic DNA was extracted from individual adult males using a modified version of the protocol described in Takada and Konami[12]. The abdomens of frozen flies were separated on dry ice and individually homogenized in 500 µl of ice cold homogenization buffer (10 mM Tris-HCl (pH 7.5), 60 mM NaCl and 10 mM EDTA (pH 8.0)) in a 1.5 ml microcentrifuge tube with a pestle (Kontes, Vineland, New Jersey, USA). SDS and proteinase K were added to final concentrations of 1% and 200 µg/ml, respectively, and samples were incubated at 50 °C for 4 h. The buffer was extracted three times with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) and once with an equal volume of chloroform: isoamyl alcohol (24:1) by gentle mixing for 20 min, followed by centrifugation for 1 min at 14,000 rpm. RNase (50 µg/ml) was added to the aqueous phase and the samples were incubated at 37 °C for 1 h followed by another 2 h incubation at 50 °C with addition of proteinase K (100 µg/ml). The mixture was again extracted with phenol: chloroform: isoamyl alcohol (25:24:1). The aqueous phase was collected and NaCl added to a final concentration of 0.1 M. DNA was precipitated with 2 volumes of 100% ethanol and pelleted by centrifugation at 14,000 rpm for 10 min. The pellet was washed with 400 µl of 70% ethanol and dissolved in 20 µl of TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)).

Oligonucleotide primers were synthesized by the Analytical and Synthesis Facility of the Cornell Biotechnology Institute using an Applied Biosystems (Foster City, California, USA) instrument. Three primers were designed from the transcribed region of *CYP6D1* in the LPR strain: S21, 5'-CACAAAATGACCGGCAACTA-3'; AS14, 5'-ACATTGTCGACTTCTTTGGG-3'; S17, 5'-ACGGCCATTTGGCCTGGTTA-3'. The sequences from which they were chosen are shown in figure 1. PCR solutions contained 1 µg of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 µM of each primer pair, 0.2 mM of each dNTP (Perkin Elmer Cetus) and 2.5 units of *Taq* polymerase (Gibco BRL, Grand Island, New York, USA) in a final volume of 100 µl incubated in a thermal cycler (Omnigene, National Labnet Co., Woodbridge, New Jersey, USA). Two rounds of PCR were conducted. For the first PCR, solution with 1 µg of DNA template and the allele-independent primer set (S21 and AS14) was heated to 95 °C for 2 min, then put through 30 cycles of reaction: 95 °C × 45 s, 55 °C × 45 s, 67 °C × 45 s, 72 °C × 45 s, followed by a final extension of 72 °C × 7 min. The second PCR was done with solution containing 1–3 µl of the first round PCR reaction solution (volumes adjusted to standardize the amount of template between strains) plus an allele-specific primer set (S17 and AS14) under the same conditions described above except that 15 cycles were used.

PCR products were analyzed by agarose gel electrophoresis with 5×10^{-5}% ethidium bromide (3% NuSieve GTG/1% SeaPlaque, FMC, Rockland, Maine, USA) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

Results and discussion

Sequence comparison of the *CYP6D1* alleles between the LPR and aabys strains revealed a polymorphism (A to T) at cDNA position 270 (Scott et al., unpublished). Thus, a LPR strain allele specific *CYP6D1* primer set was designed by placing the specific single mismatched base at the 3' end of one oligonucleotide (S17) to permit preferential amplification of one allele over another[13,14]. In this study, genomic DNA was extracted from parental, F_1 and BC_1 male flies. In order to increase priming specificity and avoid false-negative results, two rounds of PCR amplification were performed. In the

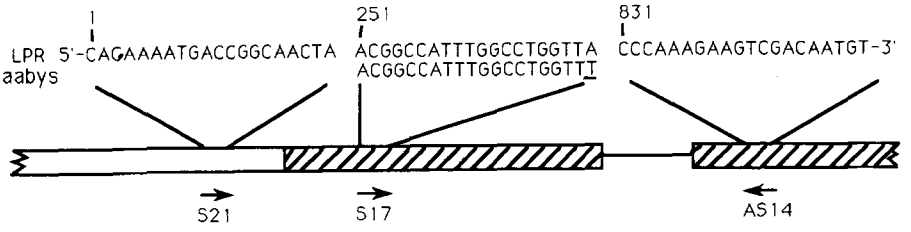

Figure 1. Graphic representation of *CYP6D1* showing sequences and locations of the allele-specific and allele-independent primers used for this study.

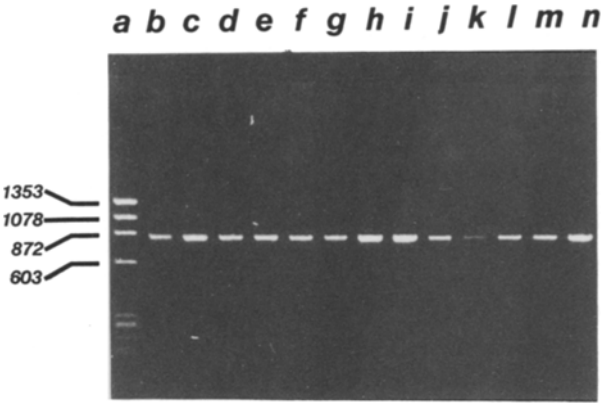

Figure 2. PCR using the *CYP6D1* allele-independent primer set. DNA templates used were from the following flies: LPR (*b*), aabys (*c*), R2345 (*d*), R1345 (*e*), R1245 (*f*), R1235 (*g*), R1234 (*h*), R1 (*i*), R2 (*j*), R3 (*k*), R4 (*l*), R5 (*m*) and F1 (*n*). Lane *a*: ∅X174 RF DNA/*Hae* III size markers in units of bp.

first PCR, a *CYP6D1* allele independent primer set was used, and the expected size (850 bp) products were obtained (fig. 2). The second PCR was performed with an allele-specific primer set using the first PCR product as template. The LPR strain allele-specific primer amplified a DNA fragment of the expected size (580 bp) only in flies having the chromosome 1 wild type marker (i.e., ac^+) (fig. 3), demonstrating that *CYP6D1* is located on chromosome 1. This experiment was repeated 4 times with the same results.

It has been suggested that monooxygenase-mediated resistance could arise due to a change in catalytic activity of the P450 (i.e. the structural gene) and/or to a change in the amount of enzyme[3,15]. Monooxygenase-mediated pyrethroid resistance in the LPR strain has been linked to chromosomes 1, 2, 3 and 5[16]. Given that the level of $P450_{lpr}$ protein is 40-fold elevated in the LPR strain compared to susceptible stains[17] and that

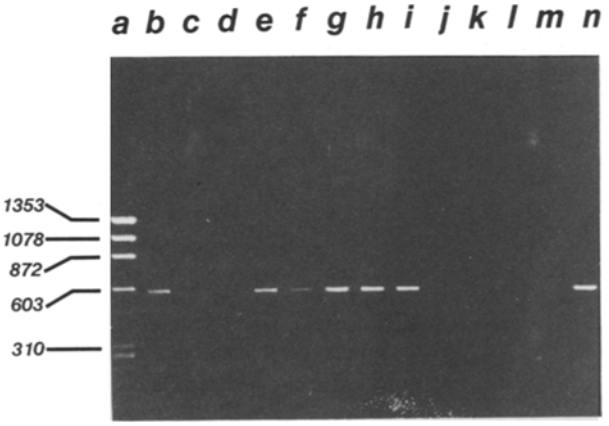

Figure 3. PCR using the *CYP6D1* allele-specific primer set. DNA templates from the first round PCR were from the following flies: LPR (*b*), aabys (*c*), R2345 (*d*), R1345 (*e*), R1245 (*f*), R1235 (*g*), R1234 (*h*), R1 (*i*), R2 (*j*), R3 (*k*), R4 (*l*), R5 (*m*) and F1 (*n*). Lane *a*: ∅X174 RF DNA/*Hae* III size markers in units of bp.

$P450_{lpr}$ is responsible for monooxygenase-mediated resistance in the LPR strain[8], it appears likely that at least part of the high level expression of $P450_{lpr}$ is *trans*-regulated in the LPR strain. Understanding the role of *CYP6D1* regulatory genes on chromosome 1, and the role (if any) of differences in the CYP6D1 protein between resistant and susceptible strains in pyrethroid resistance will require further study.

Monooxygenase-mediated resistance to organophosphate, carbamate and organochlorine insecticides has been most frequently associated with chromosomes 2 and 5 (ref. 6). A major regulatory gene on chromosome 2 responsible for resistance to these compounds has been postulated[7]. CYP6D1, a P450 that is expressed at higher levels (mRNA) in the organophosphate resistant Rutgers and multiresistant LPR strains of house flies, compared to susceptible strains[18,19], was recently mapped to chromosome 5 (R. Feyereisen, pers. commun.) and its expression appears to be *trans*-regulated by a genetic factor(s) on chromosome 2 (refs. 18, 19). P450-mediated resistance in LPR may also involve genetic factors on chromosomes 2 and 5 (ref. 16). Examination of the regulatory genes involved in monooxygenase-mediated resistance to different classes of insecticides will provide a new understanding of how this type of resistance develops and will offer new insights into the differential expression of cytochrome P450s among insecticide resistant and susceptible strains.

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