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Methods paper

Accord insertion in the 5′ flanking region of *CYP6G1* confers nicotine resistance in *Drosophila melanogaster*

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ABSTRACT

What has driven the sweep of the *Accord* retrotransposon insertion allele of *CYP6G1* in the natural populations of *Drosophila melanogaster* is unknown. Previous studies on the DDT selection hypothesis produced conflicting data. To reexamine the DDT selection hypothesis and search for alternative explanations, we conducted a series of correlation and genetic linkage experiments with eight *D. melanogaster* natural populations collected from California (CM1, CM2, CM3, and CM7) and Africa (AM2, AM3, AM4, AM7). Diagnostic PCR showed that CM1, CM2, CM7, and AM3 have the *Accord* insertion in the *CYP6G1* locus, whereas the other four strains do not. RT-PCR analysis exhibits a 100% correlation between *Accord* insertion and *CYP6G1* overexpression. However, among the four strains with *Accord*-mediated *CYP6G1* overexpression only CM1 and CM7 are resistant to DDT, and the other two strains (CM2 and AM3), like the four *Accord*-free strains, are susceptible to DDT. By contrast, all the four strains with *Accord*-mediated *CYP6G1* overexpression are resistant to nicotine, a plant allelochemical. Genetic crosses between DDT resistant and susceptible *Accord*-insertion strains, as well as crosses between *Accord*-insertion and *Accord*-free strains demonstrated that *Accord* insertion and *CYP6G1* overexpression are genetically linked to nicotine resistance rather than DDT resistance. These results suggest that naturally-occurring allelochemicals such as nicotine are the initial driving force for the worldwide prevalence of the *Accord* insertion allele of *CYP6G1* in *D. melanogaster* natural populations.

1. Introduction

Transposable elements (TEs) are pieces of DNA capable of jumping out from their original locations and inserting into new loci throughout a genome. The genomic loci that TEs insert are often random, but their survival – retention or exclusion from the new loci – are non-random, subject to the resultant mutational effects on the fitness of the host organisms and natural selection. Natural selection tends to prevent TE accumulations within or around essential housekeeping genes but tolerate or even select for TE enrichment in environmental response genes (Chen and Li, 2007). Analyses of genomic distribution of TEs in the complete vertebrate genomes (Grover et al., 2003; Medstrand et al., 2005; Simons et al., 2006; van de Lagemaat et al., 2003; Wagner et al., 2003) support this notion. A biased TE enrichment provides environmental response genes with higher spontaneous mutation rate and genetic/genomic plasticity to cope with the ever-changing environment (Chen and Li, 2007; Kidwell and Lisch, 2001).

Cytochrome P450 monooxygenases (P450s) are membrane-bound hemoproteins that are involved in the detoxification of naturally occurring and synthetic xenobiotics and thus play an important role for adaptation to the changing environment (Feyereisen, 2005; Li et al., 2007). Various TEs are frequently found within or in close proximity to xenobiotic-metabolizing P450 genes (Chen and Li, 2007; ffrench-Constant et al., 2006; Li et al., 2007). Some TE-associated P450 alleles such as the *Accord* LTR or *Doc* non-LTR insertion alleles of *CYP6G1* (Catania et al., 2004; Daborn et al., 2002; Schlenke and Begun, 2004) and the *Bari-1* insertion allele of *CYP12A4* (Bogwitz et al., 2005; Caggese et al., 1995; Marsano et al., 2005) have swept to high frequency or fixation in the natural populations of *Drosophila melanogaster* or *D. simulans*. The fixation of the three P450 alleles indicates that the parallel insertions of *Accord* LTR or *Doc* non-LTR retrotransposon into the 5′-regulatory region of *CYP6G1* in *D. melanogaster* or *D. simulans* and the insertion of *Bari-1* Tc1-like element into the 3′ UTR of *CYP12A4* confer an adaptive phenotype to their hosts and thus are favored by natural selection.

Abbreviations: AM, *D. melanogaster* populations from Africa; CM, *D. melanogaster* populations from California; DDT, Dichloro-Diphenyl-Trichloroethane; LTR, long terminal repeat; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; P450, cytochrome P450 monooxygenase; TE, transposable element; UTR, untranslated region.

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It is not known what has driven the sweep of the three P450 alleles. Since no detectable DDT or lufenuron resistance resulted from the *Bari-1* insertion even though it does cause 10-fold overexpression of *CYP12A4* (Bogwitz et al., 2005; Marsano et al., 2005), the resultant adaptive phenotype and hypothetic selection agent remain unknown for the *Bari-1* insertion allele of *CYP12A4*. For the *Accord* LTR insertion allele of *CYP6G1*, three worldwide surveys confirmed an exact correlation of the *Accord* insertion with DDT resistance and with *CYP6G1* upregulation (Catania et al., 2004; Daborn et al., 2002; Schmidt et al., 2010). Moreover, overexpression of *CYP6G1* transgene in a susceptible strain could confer resistance to a low dose (10 μg) of DDT (Daborn et al., 2002). These data suggest that the synthetic insecticide DDT may have driven the sweep of the *Accord* insertion allele of *CYP6G1*. Two studies, however, contradict with this notion. Examination of natural populations of *D. melanogaster* or *D. simulans* collected from California and Africa revealed that some populations showing *Accord*- or *Doc*-mediated overexpression of *CYP6G1* were DDT-susceptible (Schlenke and Begun, 2004). Similarly, two laboratory strains of *D. melanogaster*, Canton-SH and Hikone-RH, were found to be DDT-susceptible although they carry the *Accord* insertion allele of *CYP6G1* and exhibit high *CYP6G1* expression (Festucci-Buselli et al., 2005; Kuruganti et al., 2007). The susceptible phenotype of Canton-SH and Hikone-RH strains is not due to mutation that may inactivate the *CYP6G1* protein (Kuruganti et al., 2007).

Conflicting or sparse evidence for the notion that the widespread application of DDT has driven the sweep of the TE-mediated overexpressing alleles of *CYP12A4* and *CYP6G1* promoted us to search for alternative selection agents. Because P450s play important roles in conferring protection against both naturally occurring phytochemicals and man-made insecticides (Li et al., 2007), and since *Drosophila* has a long history of exposure to various toxic plant allelochemicals, it is logical to look for alternative agents from plant allelochemicals. The data obtained demonstrate that *Accord* insertion and *CYP6G1* overexpression are genetically linked to nicotine resistance rather than DDT resistance, and suggest that the spreading and fixation of the *Accord* insertion allele of *CYP6G1* have been driven by nicotine and/or other allelochemicals.

2. Materials and methods

2.1. Drosophila melanogaster strains and stocks

Eight populations (CM1, CM2, CM3, CM7, AM2, AM3, AM4, and AM7) of *D. melanogaster* were generously provided by Dr. Todd Schlenke (Department of Biology, Emory University, 1510 Clifton Road NE, Atlanta, GA 30322, U.S.A.). CM1, CM2, CM3, and CM7 were originally collected from California, USA, while AM2, AM3, AM4, and AM7 were from Africa (Schlenke and Begun, 2004). Stock cultures were maintained at 17 °C and working cultures were maintained at 25 °C with a photoperiod of 16 h light: 8 h dark. All *Drosophila* stocks were maintained in plastic vials (94 mm × 25 mm) of standard cornmeal media surfaced with yeast and were transferred to new vials every three weeks.

2.2. Accord diagnosis

For every strain or sample, genomic DNA was extracted from individual female adults, using the procedure described in Chen and Li (2007). The diagnostic PCR for the absence or presence of the *Accord* insertion at the 5′ flanking region of *CYP6G1* was performed as described in Catania et al. (2004) using the *Accord* flanking sequence-specific primers *Accord* F (5′-GAAAGCCGGTTGTGTTTAAATTAT-3′) and *Accord* R (5′-CTTTTTGTGTGCTATGGTTTAGTTAG-3′) (Fig. 1A). A standard 10 μl PCR reaction mixture contained 100 ng template DNA, 2 μM of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs and 0.5 U *Taq* polymerase. PCR conditions were as follows: 3 min at 94 °C, 32 cycles of 50 s at 94 °C, 50 s at 52 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were run on a 1.5% agarose gel in 1× TAE buffer to determine the genotypes of individual flies. Flies homozygous for *Accord* insertion (AA) had a single band of 604 bp, whereas flies homozygous for no *Accord* insertion (aa) had a single band of 114 bp (Fig. 1B).

2.3. RNA extraction and RT-PCR

Total RNA was extracted from 50 adult female flies for each strain using the guanidinium–HCl procedure (Sambrook et al., 1989) and resuspended in 40 μl DEPC-treated water. First strand cDNA was synthesized at 42 °C for 1.5 h in a 20 μl reaction containing 1 μg total RNA, 8 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.4), 75 mM KCl, 5 μM DTT, 1 mM dNTP, 40 U recombinant RNasin ribonclease inhibitor (Invitrogen), 15 U AMV Transcriptase (Invitrogen), and 2 μM Oligo $(dT)_{18}$ primer. The resultant cDNA were used as templates to PCR-amplify *CYP6G1* and *RP49* transcripts using the *CYP6G1*-specific primers *CYP6G1* F (5′-AACCATGCTACTGGCAACG-3′) and *CYP6G1* R (5′-TGCAATCGTGGCTATGCT-3′) and the *RP49*-specific primers *RP49* F (5′-ATCCGCCCAGCATACAG-3′) and *RP49* R (5′-TCCGACCAGGTTACAAGAA-3′), respectively. PCR amplifications of *CYP6G1* or *RP49* transcripts in each cDNA sample were conducted in a 50 μl reactions containing 5 μl 10× PCR reaction buffer [50 mM KCl, 20 mM Tris–HCl (pH 8.4)], 1.5 mM $MgCl_2$, 200 μM dNTP mix, 0.2 μM each primer, 2 U *Taq* DNA polymerase, and 2 μl first-strand cDNA. The amplification conditions for *CYP6G1* and *RP49* transcripts were denaturation for 5 min at 94 °C, followed by 36 cycles of 94 °C denaturation for 50 s, 55 °C (*CYP6G1*) or 52 °C (*RP49*) annealing for 50 s, 72 °C extension for 1 min, and a final extension of 10 min at 72 °C. For comparison of *CYP6G1* expression among the eight *Drosophila* strains, 20 μl of PCR products from each amplification reaction was run in parallel on 1.5% agarose gel and then visualized by ethidium bromide staining.

2.4. DDT resistance bioassay

D. melanogaster strains were tested for resistance to DDT using a contact assay (Daborn et al., 2002; Schlenke and Begun, 2004). Glass scintillation vials (20 ml) were coated with acetone (control) or DDT by rolling 200 μl of acetone or 200 μl of acetone containing 15 μg of DDT inside the vials until the acetone evaporated. The vials were plugged with cotton soaked in 5% sucrose. For each of three replicates of each strain, 20 female flies 2–8 days posteclosion were placed in an acetone- (control) or DDT-coated vial. Mortality was assessed after 24 h DDT exposure.

2.5. Nicotine sulfate resistance bioassay

The susceptibility of each strain to nicotine (1-methyl-2-(3-pyridyl) pyrrolidine) sulfate was determined by placing 30 female flies 2–8 days posteclosion in a plastic vial (94 mm × 25 mm) containing cornmeal media with nicotine sulfate at the concentration of 0 (control), 0.5, 1, 2, or 4 mg/g. The nicotine-containing food was used within 4 days of preparation. Mortality (dead flies and flies incapable of moving or standing up) was recorded 24 h after initiation. Three replicates (each of 30 female flies) were used for each concentration and control. Dose–response regressions were estimated by probit analysis in PriProbit Version 1.63.

2.6. Linkage analysis

Two genetic linkage analyses each with two trials were conducted to determine the linkage relationship between *Accord* insertion and DDT or nicotine resistance. For the first trial of linkage analysis 1, a female adult from the *Accord*-homozygous and nicotine-resistant CM2 strain was crossed to a male fly from the *Accord*-free homozygous