The *Anopheles gambiae* detoxification chip: A highly specific microarray to study metabolic-based insecticide resistance in malaria vectors

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Metabolic pathways play an important role in insecticide resistance, but the full spectra of the genes involved in resistance has not been established. We constructed a microarray containing unique fragments from 230 *Anopheles gambiae* genes putatively involved in insecticide metabolism [cytochrome P450s (P450s), GSTs, and carboxylesterases and redox genes, partners of the P450 oxidative metabolic complex, and various controls]. We used this detox chip to monitor the expression of the detoxifying genes in insecticide resistant and susceptible *An. gambiae* laboratory strains. Five genes were strongly up-regulated in the dichlorodiphenyltrichloroethane-resistant strain *ZAN/U*. These genes included the GST *GSTE2*, which has previously been implicated in dichlorodiphenyltrichloroethane resistance, two P450s, and two peroxidase genes. *GSTE2* was also elevated in the pyrethroid-resistant *RSP* strain. In addition, the P450 *CYP325A3*, belonging to a class not previously associated with insecticide resistance, was expressed at statistically higher levels in this strain. The applications of this detox chip and its potential contribution to malaria vector insecticide resistance management programs are discussed.

mosquito | cytochrome P450 | GST | carboxylesterase

Metabolic-based resistance mechanisms are important in conferring insecticide resistance. Biochemical analysis has identified three enzyme families that are involved in insecticide metabolism: the cytochrome P450s (P450s), the GSTs, and the carboxylesterases (COEs). However, each of these enzyme families is encoded by supergene families, and in the majority of cases, the identity of the individual genes that are up-regulated or amplified in insecticide resistant individuals have yet to be determined. This is particularly true for the mosquito *Anopheles gambiae.* Only one gene in this malaria vector has been indisputably associated with metabolic resistance to insecticides. This gene, *GSTE2*, is overexpressed in dichlorodiphenyltrichloroethane (DDT)-resistant mosquitoes and encodes an enzyme that is very efficient at catalyzing the dehydrochlorination of this insecticide (1, 2). This resistance mechanism was identified in a DDT-selected laboratory strain of *An. gambiae* and its expression levels in field populations have yet to be established. Given the genetic redundancy present in superfamilies of genes involved in insecticide metabolism, it is possible that alternative routes of detoxification may have been selected for in different mosquito populations. Little is known about the genes responsible for the increased rates of pyrethroid detoxification that have been reported in several *Anopheles* populations (3–5), and yet, countries throughout Africa are relying increasingly on this insecticide class for malaria control (6).

Monitoring the spread of insecticide-resistance alleles is an important aspect of any sustainable vector control activity. PCR-based assays to detect resistant alleles of insecticide target sites are routinely used by many research programs attached to malaria control activities in Africa (e.g., refs. 7 and 8). Before equivalent assays can be developed for metabolic resistance mechanisms, candidate genes must be identified, and their role in insecticide metabolism must be verified. As a first step in this process, we developed a simple microarray for simultaneously examining the transcription profile of the superfamilies of genes involved in insecticide detoxification. Several large-scale microarrays have already been developed for *An. gambiae* (ref. 9 and www.malaria.mr4.org), but none of these microarrays are fully representative of the gene superfamilies of interest in the present study. There are several reasons for the deficiencies in these first generation *An. gambiae* arrays. For example, arrays constructed by using sequence data from the first draft of the automatic annotation of the *An. gambiae* genome inevitably contain a number of incorrect gene assemblies. The alternative type of *An. gambiae* array, generated by spotting cDNAs from an EST library avoids this problem, but such arrays can be biased toward genes expressed in the particular tissue or cell type used to generate the libraries (for example, the EST clone collection generated from *An. gambiae* hemocyte-like cell lines is deficient in some key members of all of the detoxification gene superfamilies). We therefore developed and produced a small-scale array containing unique fragments from 230 putative detoxification genes in *An. gambiae.* The sequence database used to design the probes was manually curated, and in many cases, experimentally verified, before probe design. We have named the resulting array the detox chip. Here, we describe the use of this detox chip to identify genes differentially expressed in laboratory insecticide resistant and susceptible strains of *An. gambiae.*

Materials and Methods

Mosquito Strains and Sample Collection. Three different *An. gambiae* strains were used. The *Kisumu* strain (from Kisumu, Western Kenya; ref. 4), susceptible to all insecticides was used as the reference. The reduced susceptibility to permethrin (*RSP*) strain (4) was colonized from the same geographical area of Kenya, but has a low level of permethrin resistance. The *ZAN/U* strain is DDT-resistant and was colonized in 1982 from a field population from Zanzibar, Tanzania. Both the *RSP* and *ZAN/U* strains have been maintained under regular selection pressure by exposure to filter papers impregnated with 0.75% permethrin or 4% DDT,

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Abbreviations: DDT, dichlorodiphenyltrichloroethane; P450, cytochrome P450; COE, carboxylesterase; cmRNA, copy messenger RNA.

Data deposition: The array detox chip has been deposited in the EMBL ArrayExpress database, www.ebi.ac.uk/arrayexpress (accession no. A-MEXP-137).

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respectively, according to standard World Health Organization procedures (10). For each biological replicate, 500 mosquitoes from each strain were simultaneously reared in distilled water and fed with finely grounded fish food (Tetra). To minimize gene expression variations because of differential development rates, rearing conditions were standardized. For each strain comparison, four batches of five 1-day-old adult females from both strains were simultaneously collected and immediately used for total RNA extraction and copy messenger RNA (cmRNA) amplification. Each biological replicate consisted of mosquitoes from distinct generations to take into account stochastic variations.

Microarray Construction. A microarray containing fragments of 230 *An. gambiae* genes from families associated with metabolic-based insecticide resistance was constructed. These gene fragments included 103 P450s, 31 COEs, 35 GSTs, 41 Red/Ox genes, 5 ATP-binding-cassette transporters, tissue-specific genes and housekeeping genes (see Table 1, which is published as supporting information on the PNAS web site, for details). To evaluate the quality of the microarray experiments, 23 artificial spike-in control genes (Universal Lucidea Scorecard, Amersham Pharmacia) were also spotted on the array. Each gene represented on the microarray was either obtained by PCR amplification or artificially synthesized as a 70-mer antisense oligo (Qiagen, Crawley, U.K.). To keep cross hybridization between closely related genes to a minimum, gene-specific segments were selected by using PRIMEGENS software (ref. 11 and http://compbio.ornl.gov/structure/primegens). By using software default cutoff values, fragments toward the 3′ end of the genes between 70 and 300 bp in length were selected as probes for the microarray, provided they matched the criteria of <75% similarity to all other genes in the *An. gambiae* genome. For genes having >75% similarity to another gene, 3′ UTRs were used for the probe design. Gene-specific fragments were obtained by PCR amplification from cDNA, cloned into pGEM T-easy vector (Promega), and sequenced. Subsequent PCR amplifications were performed with vector-specific primers, and the products were purified by using the QIAquick PCR purification kit (Qiagen). The 70-mer oligos and artificial spike-in controls were resuspended in nuclease-free water, and both quality and quantity checked on agarose gels before spotting. Arrays were spotted in duplicate onto gamma-amino-propyl-silane-coated glass slides (UltraGaps, Corning) by using a Biorobotics MicroGrid II printer (BioRobotics, Cambridge, U.K.). All genes were spotted in 50% DMSO (Sigma) at concentrations of 50, 200, and 1,000 ng/μl for spike-in controls, PCR products and 70-mer oligos, respectively. Four replicates of each PCR product and 70-mer oligo were spotted, as were eight replicates of spike-in controls. Printed slides were stored for 24 h at room temperature in a desiccator before DNA fixation by using a UV auto crosslinker (power 600mJ, Stratagene).

Target Preparation and Microarray Hybridizations. RNA extractions, cmRNA synthesis, and labeling reactions were performed independently for each replicate to take into account technical variation. Total RNA was extracted from batches of five females by using Tri Reagent (Sigma) according to the manufacturer's instructions. Total RNA pellets were then resuspended in diethylpyrocarbonate-treated water and purified by using the RNeasy minikit (Qiagen). Total RNA quantity and quality were assessed by using a Nanodrop spectrophotometer (Nanodrop Technologies, Oxfordshire, U.K.). An RNA amplification step was performed before the labeling reaction by using the SuperScript Choice system (Invitrogen) and the Ambion MEGAscript T7 RNA synthesis kit. Amplified cmRNAs were purified by using RNeasy mini kit (Qiagen) and resuspended in diethylpyrocarbonate-treated water. cmRNA quantity and quality were checked by using both the Nanodrop spectrophotometer and agarose gel electrophoresis. Each strain comparison was repeated three times with different biological replicates by using the *Kisumu* strain as a reference. For each biological replicate, four hybridizations were performed. The Cy3 and Cy5 labels were swapped between hybridizations to provide technical replicates. For each sample, 8 μg of cmRNAs and 0.5 μl of the corresponding mRNA spike mix (Lucidea Universal Scorecard, Amersham Pharmacia) were reverse-transcribed into Cy-labeled cDNAs by using random hexamers (Life Technologies), Cy3 or Cy5-dUTPs (Amersham Pharmacia), and Superscript III (Invitrogen) for 2.5 h at 50°C. Unlabeled RNA was removed by adding 1 μl of 1M NaOH, 20 mM EDTA, and incubating for 5 min at 70°C. Then, Cy3- and Cy5-labeled cDNAs were pooled together, purified by using a QIAquick PCR purification kit (Qiagen), and eluted in nuclease-free water. Cy-dye nucleotide incorporation efficiency was checked, and 2.5 μg of polyA DNA (Sigma) were added to the labeled cDNAs. The labeled cDNA mix was vacuum-dried and resuspended in 15 μl of formamide-based hybridization buffer (Corning). Hybridizations were performed by using the Universal Hybridization kit (Corning). Slides were presoaked for 20 min at 42°C, prehybridized for 15 min at 42°C, and then washed according to manufacturer's recommendations. Cy-labeled cDNAs were hybridized on the array by using a 22 × 22-mm coverslip (Hybrislip, Sigma) for 16 h at 42°C. After hybridization, slides were washed according to manufacturer's instructions and immediately scanned at a 5-μm definition by using a Genepix 4100A microarray scanner (Axon Instruments) where laser photo-multiplier tubes were adjusted to obtain the best dynamic intensity range between all spots.

Microarray Data Analysis. Spot finding, signal quantification, and spot superimposition for both dye channels were performed using GENEPIX 5.1 software (Axon Instruments). For each data set, any spot satisfying one of the following conditions for any channel was removed from the analysis: (*i*) intensity values of <300 or >65,000, (*ii*) signal-to-noise ratio of <3, and (*iii*) <60% of pixel intensity superior to the median of the local background ± 2. Data files were then loaded into GENESPRING 6.1 (Silicon Genetics) for normalization and statistic analysis. For each array, the spot replicates of each gene were merged and expressed as median ratios ± SD. Data from dye swap experiments were then reversed and ratios were log-transformed. Ratio values of <0.01 were set to 0.01. Data were then normalized using the local intensity-dependent algorithm LOWESS (12) with 20% of data used for smoothing. For each strain comparison, only genes detected in at least 50% of all hybridizations were used for further statistical analysis. Mean expression ratios were then submitted to a one-sample Student *t* test against the baseline value of 1 (equal gene expression in both samples) with a multiple testing correction (Benjamini and Hochberg false-discovery rate; ref. 13). Genes showing both *t* test *P* values <0.001 and an expression ratio of >1.5-fold in both directions were considered differentially expressed for pairwise comparisons. These over- and underexpression thresholds were chosen according to the maximum and minimum expression ratios obtained during two technical replicate experiments using the reference sample only (see Fig. 2). Genes significantly differentially expressed between either *RSP* or *ZAN/U* strains, and the reference strain *Kisumu* were then further investigated for differential expression between the two insecticide resistant strains by clustering analysis based on expression ratio changes across all experiments.

Results and Discussion

Detox Chip Quality Assessment and Overall Analysis. A microarray containing 230 genes potentially involved in insecticide resistance in the mosquito *An. gambiae* was constructed and then

used to identify transcripts differentially expressed among (*i*) the insecticide susceptible *Kisumu* strain (used as a reference), (*ii*) the permethrin-resistant strain *RSP*, and (*iii*) the DDT-resistant strain *ZAN/U*. The detox chip contains 940 DNA spots of 235 different probes representing 230 different genes. The use of artificial control genes (spike-in controls) allowed us to investigate the detection threshold of the microarray. Controls with concentrations of >3 pg mRNA per microgram of target RNA were detected in >90% of hybridizations. The range of mRNA concentrations detected for the detoxifying genes was estimated as between 1 pg and 1 ng of mRNA per microgram of target RNA, representing a 1- to 1,000-fold relative variation in mRNA quantity. Transcripts of three COEs (*COEJHE4F*, *COEAE6O*, and *COEAE1A*), and one Δ class GST (*GSTD5*), were never detected in 1-day-old mosquito females. Although the majority of detoxification genes were expressed at medium or low levels, four genes (*GSTS1–2*, *CYP325C2*, *CYP4H25*, and *SOD3B*) showed strong expression signals equivalent to those obtained for housekeeping genes, indicating their high expression in adult female mosquitoes. Among the 231 detected probes, 36 and 42 probes gave nonsignificant signal values in >50% hybridizations for *RSP* versus *Kisumu* and *ZAN/U* versus *Kisumu* strain comparisons, respectively. Because the expressions of these genes were too low to be reliable, those probes were then not considered for any further statistical analysis. Technical error evaluation across two control experiments revealed maximum and minimum mean expression differences of 1.23-fold overexpression and 1.48-fold underexpression (see Fig. 2 for more details), allowing us to choose 1.5-fold as arbitrary expression thresholds for over- and underexpression.

Genes Differentially Expressed in the Permethrin-Resistant Strain *RSP*. A one-sample Student *t* test (Benjamini and Hochberg multiple testing correction, with a false discovery rate of 0.001) revealed 34 differentially expressed genes between the *RSP* and *Kisumu* strain although only six of these ratios exceeded the 1.5-fold threshold described above (Fig. 1*A*). Two genes, *GSTE2* and *CYP325A3*, were overexpressed 2.36- and 1.72-fold, respectively, in the permethrin-resistant strain. Although *GSTE2* has been previously shown to be involved in DDT resistance in *An. gambiae* (1, 14), it has not previously been implicated in resistance to pyrethroids. Nevertheless, elevated GST expression has been associated with pyrethroid resistance in other insect species, either by acting as a pyrethroid-binding protein (15) and sequestering the insecticide (16), or by protecting against oxidative stress and lipid peroxidation induced by pyrethroid exposure (17). The role of *GSTE2* in permethrin resistance in *An. gambiae* warrants further investigation.

Many studies have implicated P450s in metabolic resistance to pyrethroids in insects (18, 19), and the gene *CYP6Z1* has been shown to be significantly overexpressed in the permethrin-resistant *RSP* strain of *An. gambiae* with a quantitative RT-PCR ratio of 3.5-fold (20). The present study also found higher expression of *CYP6Z1* in the *RSP* strain, but the 1.31-fold overexpression was below our previously defined cutoff value of 1.5-fold. This result may be partially explained by the well known underestimation of gene expression ratios by microarrays compared with quantitative RT-PCR experiments (21). The present study also revealed a strong and significant overexpression of the gene *CYP325A3* (1.72-fold). The *CYP325* P450 gene family has not been studied previously in relation to insecticide resistance. There are no direct orthologues of the 14 *A. gambiae CYP325* genes in *Drosophila*. The P450s in this genus with the greatest amino acid similarity to CYP325 are the *Drosophila melanogaster* CYP4D1 and the *Drosophila mettleri* CYP4D10, which are involved in plant alkaloid detoxification in *Drosophila* (22).

Enhanced esterase activity has also been reported in permethrin-resistant *An. gambiae* (23), *Anopheles albimanus* (24),

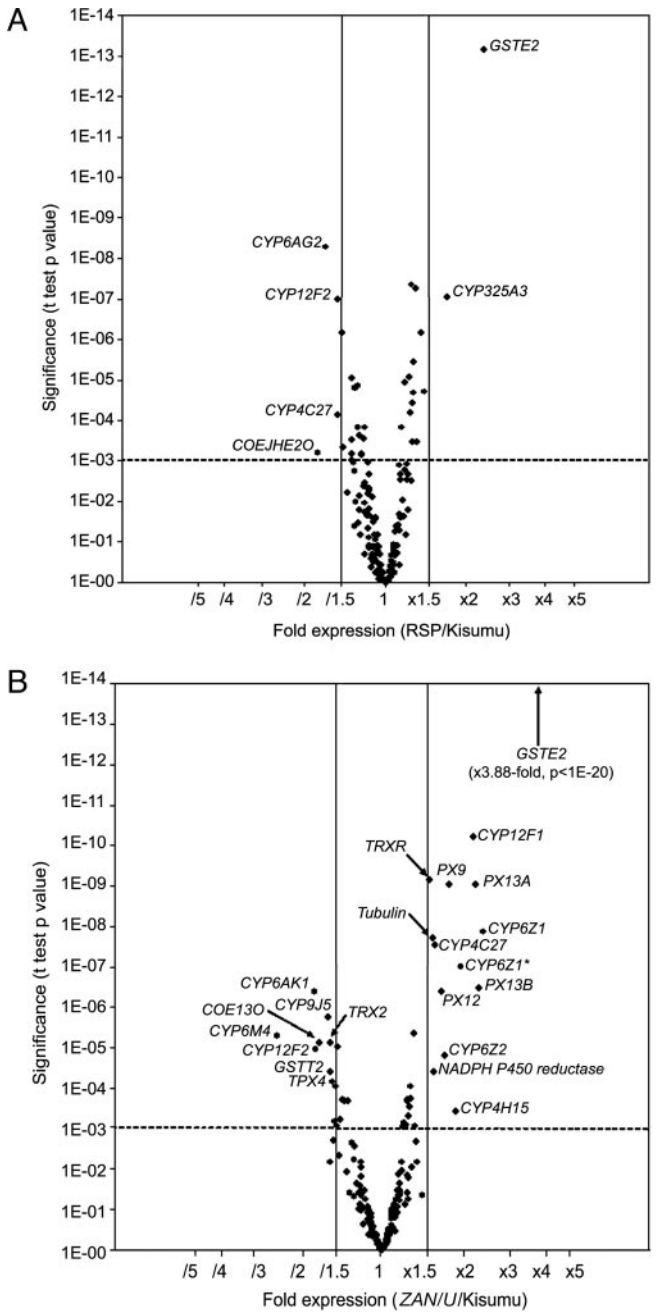

Fig. 1. Expression profile of detoxification genes across analyzed mosquito strains. (*A*) Differential expression of *An. gambiae* genes between the insecticide-susceptible strain *Kisumu* and the permethrin-resistant strain *RSP*. Differences in gene expression are indicated as a function of both expression ratio (*RSP*/*Kisumu*) and significance (Student's *t* test *P* values) for the 195 genes showing consistent data in at least 6 of 12 hybridizations. Vertical lines indicate 1.5-fold expression difference in either direction. Horizontal line indicates significance threshold ($P < 0.001$) adopted for the one-sample Student *t* test (Benjamini and Hochberg multiple testing correction). Genes showing both >1.5-fold differential expression and a significant *P* value are named. (*B*) Differential expression of *An. gambiae* genes between the insecticid-susceptible strain *Kisumu* and the DDT-resistant strain *ZAN/U*. Differences in gene expression are indicated as a function of both expression ratio (*ZAN/U*/*Kisumu*) and significance (Student's *t* test *P* values) for the 182 genes showing consistent data in at least 6 of 12 hybridizations. Vertical lines indicate 1.5-fold expression difference in either direction. Horizontal line indicates significance threshold ($P < 0.001$) adopted for the one-sample Student *t* test (Benjamini and Hochberg multiple testing correction). Genes showing both >1.5-fold differential expression and a significant *P* value are named. When both cDNA and 70-mer oligonucleotides probes are present, the asterisk indicates the oligonucleotide.

and the dengue vector *Aedes aegypti* (25), but esterase gene expression was not up-regulated in the permethrin-resistant strain analyzed in this study. Finally, one esterase and three P450 genes (*COEJHE2O*, *CYP6AG2*, *CYP12F2*, and *CYP4C27*) were >1.5-fold underexpressed in the permethrin-resistant strain *RSP*. Constitutive repression of the expression of some detoxifying enzymes may be contributing to the resistant phenotype (26). Studies in *Drosophila* have found that, whereas expression of a large number of P450 and GST genes are induced by stress, a similar number are underexpressed under the same conditions (27).

Genes Differentially Expressed in the DDT-Resistant Strain *ZAN/U*. A one-sample Student *t* test (Benjamini and Hochberg multiple testing correction, with a false-discovery rate of 0.001) identified 42 genes differentially expressed between the DDT-resistant strain *ZAN/U* and the susceptible strain *Kisumu*. Twenty-one of these genes had expression differences of >1.5-fold, ranging from 3.88-fold overexpression to 2.40-fold underexpression (Fig. 1*B*). Among these genes, 13 were >1.5-fold overexpressed in the DDT-resistant strain, including 5 genes (*GSTE2*, *CYP6Z1*, *PX13A*, *PX13B*, and *CYP12F1*) that were >2-fold overexpressed. The strong overexpression of *GSTE2* (3.88-fold) reported here is consistent with previous quantitative PCR experiments performed by using *ZAN/U* and *Kisumu* strains that showed >5-fold overexpression of this gene in the *ZAN/U* strain (14). The physical location of *GSTE2* within the boundaries of a DDT-resistance quantitative trait locus (28) and its high DDT dehydrochlorinase activity (2) both strongly support a role for the gene in DDT resistance in *An. gambiae*.

P450s have also been implicated in DDT resistance in insects. By using a small-scale microarray representing all *Drosophila* P450 genes, Daborn *et al.* (29) showed that a single gene (*CYP6G1*) was overexpressed in DDT-resistant strains of *Drosophila*. Subsequently, by using a genome-wide microarray approach, Pedra *et al.* (30) demonstrated that multiple detoxifying genes (including three *CYP6*s, one *CYP12*, and one *GST*) were constitutively overexpressed in DDT-resistant strains and hypothesized that multiple genes could contribute to the DDT-resistant phenotype in *Drosophila*. Here, we show that multiple P450s are overexpressed in the *ZAN/U* strain, including members of the *CYP6Z*, *CYP4C*, *CYP4H*, and *CYP12F* subfamilies. The slight overexpression of the electron donor *NADPH P450 reductase* (1.57-fold), required for P450 activity (31), also supports a P450 monooxygenase-based resistance mechanism in *An. gambiae*.

Hence, this study suggests that both GSTs and P450s may be involved in DDT resistance in the *ZAN/U* strain of *An. gambiae.* In addition to their direct effect on the primary target sites, insecticides also cause toxicity by inducing a state of oxidative stress. A large range of enzymes help the insect to tolerate and/or eliminate reactive oxygen species, including superoxide dismutases (*SOD*), peroxidases (*PX*), catalases (*CAT*), thioredoxin reductases (*TRX*), and thioredoxin peroxidases (*TPX*) (32). In the present study, four peroxidase genes (*PX13A*, *PX13B*, *PX9*, and *PX12*) were significantly overexpressed in the *ZAN/U* strain. Among these genes, two that are closely related, *PX13A* and *PX13B*, situated contiguously in the genome, had the strongest overexpression with 2.25- and 2.31-fold, respectively. Pedra *et al.* (30) found 11 transcripts with oxidoreductase activity differentially expressed in the DDT-resistant strains of *Drosophila*, but none of these are orthologous to the redox genes overexpressed in *An. gambiae.* Finally, eight genes were >1.5-fold underexpressed in the *ZAN/U* strain, including the four P450s (*CYP6AK1*, *CYP9J5*, *CYP12F2*, and *CYP6M4*), the esterase *COE13O*, the GST *GSTT2*, and the thioredoxin peroxidase *TPX4*.

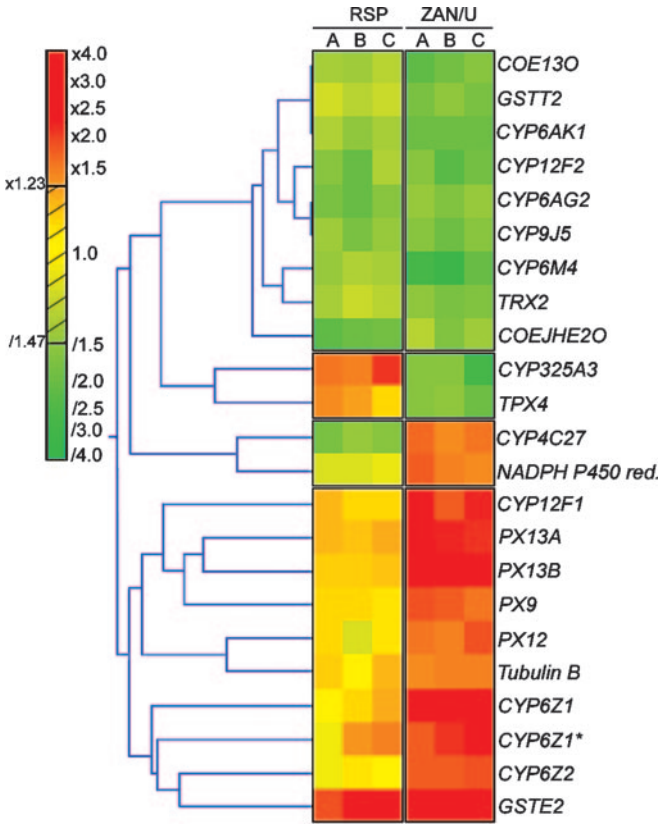

Fig. 2. Clustering analysis of gene expression patterns between the permethrin-resistant strain *RSP* and the DDT-resistant strain *ZAN/U*. Genes overexpressed in either insecticide-resistant strain are red and genes overexpressed in the reference *Kisumu* strain are green. Color intensity scale (top left) represents fold expression comparatively to the reference and the mean technical error range obtained from two control experiments (striped box). Each colored squared represents the mean expression ratio of one gene across four hybridizations, including two dye-swaps. A, B, and C represent the three biological replicates for each strain comparison. Only genes showing significant over- or underexpression between either *RSP* or *ZAN/U* strains versus the reference *Kisumu* strain (*P* values of <0.001 and expression ratios of >1.5-fold in both directions) were used for this analysis. The gene tree on the left was constructed by using the correlation of expression ratio changes between the two insecticide-resistant strains across all hybridizations. When both cDNA and 70-mer oligonucleotide probes are present, the asterisk indicates the 70-mer oligonucleotide.

Differential Gene Expression Between the *RSP* and *ZAN/U* Strains. Overall strain comparison showed that more genes were differentially expressed in the *ZAN/U* strain than in the *RSP* strain (23% in *ZAN/U* versus 17% in *RSP*) compared with the *Kisumu* strain. This finding may reflect the different geographical origin of the *ZAN/U* strain (Zanzibar, off the coast of Tanzania) compared with the two other strains, which were both colonized from western Kenya. Genes previously identified as significantly differentially expressed in either resistant strain were further investigated for differential expression between the two insecticide-resistant strains by clustering analysis based on changes of expression ratios. Clustering analysis revealed four different gene clusters: (*i*) genes overexpressed in both resistant strains, (*ii*) genes underexpressed in both resistant strains, (*iii*) genes overexpressed in the *RSP* strain and underexpressed in the *ZAN/U* strain, and (*iv*) genes underexpressed in the *RSP* strain and overexpressed in the *ZAN/U* strain (Fig. 2). Nine genes were overexpressed in both resistant strains, all showing a stronger overexpression in the DDT-resistant strain. The stronger overexpression of four peroxidases (*PX13A*, *PX13B*, *PX12*, and *PX9*)

GENETICS

in the *ZAN/U* strain may also indicate the capability of this DDT-selected strain to respond to insecticide-induced oxidative stress. Among the nine genes underexpressed in both insecticide-resistant strains, the P450s *CYP6M4* and *CYP6AK1* and the esterase *COE13O* reveal stronger underexpression in the *ZAN/U* strain, whereas the esterase *COEJHE2O* is more underexpressed in the *RSP* strain. Considering the two clusters where gene expression is inverted in the two resistant strains, the overexpression of the P450 *CYP4C27* and the electron donor *NADPH P450* reductase in the *ZAN/U* strain supports the involvement of monooxygenase metabolic pathways in DDT resistance in *Anopheles* as previously demonstrated in *Drosophila* (29, 33). Finally, the overexpression of the P450 *CYP325A3* and the thioredoxin peroxidase gene *TPX4* in the permethrin-resistant strain *RSP* concomitantly with their underexpression in the *ZAN/U* strain may indicate the specific response of these genes to pyrethroid selection in *Anopheles* mosquitoes. Expression levels of these genes in the two resistant strains after DDT and permethrin exposure will need to be established to provide more information about their potential metabolic function in relation to insecticide resistance.

Conclusion and Future Perspectives. Microarray technology is a powerful tool for examining the relationship between global gene expression profiles and various physiological states. The use of whole-genome microarrays provides an efficient way to screen for new candidate genes associated with a particular trait, but difficulties can arise at the detailed experimental level and the data analysis steps. In this study, we combined microarray technology with our knowledge of insecticide resistance to develop a small-scale microarray containing all genes putatively involved in metabolically based insecticide resistance in *An. gambiae*. This detox chip is a highly specific, sensitive, flexible tool that will have many applications in the study of detoxification mechanisms in insects. Ultimately, the data generated from these experiments should lead to the identification of metabolic targets for new synergists to block insecticide resistance and to simple molecular tools to detect insecticide resistant alleles in field populations, an important requirement for effective insecticide resistance management strategies. In addition, the detox chip will help elucidate the endogenous functions of three large supergene families. Finally, several of these genes catalyze key steps in biosynthetic pathways in mosquitoes and may represent targets for novel intervention strategies.

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1. Ranson, H., Rossiter, L., Ortelli, F., Jensen, B., Wang, X., Roth, C. W., Collins, F. H. & Hemingway, J. (2001) *Biochem. J.* **359,** 295–304.
2. Ortelli, F., Rossiter, L. C., Vontas, J., Ranson, H. & Hemingway, J. (2003) *Biochem. J.* **373,** 957–963.
3. Elissa, N., Mouchet, J., Riviere, F., Meunier, J. Y. & Yao, K. (1993) *Ann. Soc. Belg. Med. Trop.* **73,** 291–294.
4. Vulule, J. M., Beach, R. F., Atieli, F. K., Roberts, J. M., Mount, D. L. & Mwangi, R. W. (1994) *Med. Vet. Entomol.* **8,** 71–75.
5. Hargreaves, K., Koekemoer, L. L., Brooke, B. D., Hunt, R. H., Mthembu, J. & Coetzee, M. (2000) *Med. Vet. Entomol.* **14,** 181–189.
6. World Health Organization (2000) in *World Health Organisation Technical Report* (World Health Organization, WHO Press, Geneva), Vol. 892, pp. 37–42.
7. Stump, A. D., Atieli, F. K., Vulule, J. M. & Besansky, N. J. (2004) *Am. J. Trop. Med. Hyg.* **70,** 591–596.
8. Diabate, A., Baldet, T., Chandre, F., Dabire, K. R., Simard, F., Ouedraogo, J. B., Guillet, P. & Hougard, J. M. (2004) *J. Am. Mosq. Control Assoc.* **20,** 195–196.
9. Dimopoulos, G., Christophides, G. K., Meister, S., Schultz, J., White, K. P., Barillas-Mury, C. & Kafatos, F. C. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 8814–8819.
10. World Health Organization (1970) in *World Health Organisation Technical Report* (World Health Organization, WHO Press, Geneva), Vol. 443, pp. 47–56.
11. Xu, D., Li, G., Wu, L., Zhou, J. & Xu, Y. (2002) *Bioinformatics* **18,** 1432–1437.
12. Clevel, W. S. & Devlin, S. J. (1988) *J. Am. Stat. Assoc.* **83,** 596–610.
13. Benjamini, Y. & Hochberg, Y. (1995) *J. R. Stat. Soc. B* **57,** 289–300.
14. Ding, Y., Ortelli, F., Rossiter, L. C., Hemingway, J. & Ranson, H. (2003) *BMC Genomics* **4,** 35–51.
15. Grant, C. M., Perrone, G. & Dawes, I. W. (1998) *Biochem. Biophys. Res. Commun.* **253,** 893–898.
16. Kostaropoulos, I., Papadopoulos, A., Metaxakis, A., Boukouvala, E. & Papadopoulou-Mourkidou, E. (2001) *Pest Manag. Sci.* **57,** 501–508.
17. Vontas, J. G., Small, G. J. & Hemingway, J. (2001) *Biochem. J.* **357,** 65–72.
18. Kumar, S., Thomas, A., Sahgal, A., Verma, A., Samuel, T. & Pillai, M. K. (2002) *Arch. Insect Biochem. Physiol.* **50,** 1–8.
19. Kamiya, E., Yamakawa, M., Shono, T. & Kono, Y. (2001) *Appl. Entomol. Zool* **36,** 225–229.
20. Nikou, D., Ranson, H. & Hemingway, J. (2003) *Gene* **318,** 91–102.
21. Yuen, T., Wurmbach, E., Pfeffer, R. L., Ebersole, B. J. & Sealfon, S. C. (2002) *Nucleic Acids Res.* **30,** e48.
22. Danielson, P. B., Foster, J. L., McMahill, M. M., Smith, M. K. & Fogleman, J. C. (1998) *Mol. Gen. Genet.* **259,** 54–59.
23. Vulule, J. M., Beach, R. F., Atieli, F. K., McAllister, J. C., Brogdon, W. G., Roberts, J. M., Mwangi, R. W. & Hawley, W. A. (1999) *Med. Vet. Entomol.* **13,** 239–244.
24. Brogdon, W. G. & Barber, A. M. (1990) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **96,** 339–342.
25. Mourya, D. T., Hemingway, J. & Leake, C. J. (1993) *Med. Vet. Entomol.* **7,** 11–16.
26. Hatano, R., Scott, J. G. & Dennehy, T. J. (1992) *J. Econ. Entomol.* **85,** 1088–1091.
27. Girardot, F., Monnier, V. & Tricoire, H. (2004) *BMC Genomics* **5,** 74–90.
28. Ranson, H., Jensen, B., Wang, X., Prapanthadara, L., Hemingway, J. & Collins, F. H. (2000) *Insect Mol. Biol.* **9,** 499–507.
29. Daborn, P. J., Yen, J. L., Bogwitz, M. R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P., *et al.* (2002) *Science* **297,** 2253–2256.
30. Pedra, J. H., McIntyre, L. M., Scharf, M. E. & Pittendrigh, B. R. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 7034–7039.
31. Scott, J. G. & Wen, Z. (2001) *Pest Manag. Sci.* **57,** 958–967.
32. Zou, S., Meadow, S., Sharp, L., Jan, L. Y. & Jan, Y. N. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 13726–13731.
33. Le Goff, G., Boundy, S., Daborn, P. J., Yen, J. L., Sofer, L., Lind, R., Sabourault, C., Madi-Ravazzi, L. & ffrench-Constant, R. H. (2003) *Insect Biochem. Mol. Biol.* **33,** 701–708.