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Gene amplification and microsatellite polymorphism underlie a recent insect host shift

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Host plant shifts of herbivorous insects may be a first step toward sympatric speciation and can create new pests of agriculturally important crops; however, the molecular mechanisms that mediate this process are poorly understood. Certain races of the polyphagous aphid *Myzus persicae* have recently adapted to feed on tobacco (*Myzus persicae nicotianae*) and show a reduced sensitivity to the plant alkaloid nicotine and cross-resistance to neonicotinoids a class of synthetic insecticides widely used for control. Here we show constitutive overexpression of a cytochrome P450 (CYP6CY3) allows tobacco-adapted races of *M. persicae* to efficiently detoxify nicotine and has preadapted them to resist neonicotinoid insecticides. CYP6CY3, is highly overexpressed in *M. persicae nicotianae* clones from three continents compared with *M. persicae* s.s. and expression level is significantly correlated with tolerance to nicotine. CYP6CY3 is highly efficient (compared with the primary human nicotine-metabolizing P450) at metabolizing nicotine and neonicotinoids to less toxic metabolites in vitro and generation of transgenic *Drosophila* expressing CYP6CY3 demonstrate that it confers resistance to both compounds in vivo. Overexpression of CYP6CY3 results from the expansion of a dinucleotide microsatellite in the promoter region and a recent gene amplification, with some aphid clones carrying up to 100 copies. We conclude that the mutations leading to overexpression of CYP6CY3 were a prerequisite for the host shift of *M. persicae* to tobacco and that gene amplification and microsatellite polymorphism are evolutionary drivers in insect host adaptation.

Toxic secondary metabolites produced by many plant species present barriers to host range expansion by phytophagous insects, and the evolution of mechanisms to circumvent or detoxify these antiherbivore defenses is often a prerequisite for a successful host shift (1–3). Several studies have elucidated the molecular basis of insect adaptation to plant allelochemicals (4–7); however, most of these studies have examined adaptation that has occurred over a long evolutionary time scale and so have been unable to make direct comparisons with nonadapted conspecific races. As a result, identifying the initial genetic changes that enable a host shift has proved elusive. The recent host shift to tobacco (*Nicotiana tabacum*) by the peach–potato aphid, *Myzus persicae*, represents one of the few opportunities to study this evolutionary process in action (8). *M. persicae* is a globally distributed highly polyphagous aphid with a host range of >400 species, including many economically important crop plants (9). Tobacco-adapted races, *Myzus persicae nicotianae*, are morphologically and genetically differentiated from *M. persicae sensu stricto* (s.s.), although there are clear examples of recent gene flow between the two taxa (10). *M. persicae nicotianae* have evolved enhanced tolerance to the pyridine alkaloid nicotine (11), a potent natural insecticide produced by tobacco and other members of the Solanaceae family (12); however, the underlying mechanisms are unknown. Interestingly, tobacco-adapted forms also show reduced sensitivity to neonicotinoids, an important class of synthetic insecticides widely used for control that are chemically related to nicotine (11, 13).

One of the most important superfamilies of enzymes used by insect herbivores to detoxify xenobiotics, including the secondary metabolites of plants, is the cytochrome P450s (P450s) (14). Among the most well characterized examples of P450-mediated detoxification of plant allelochemicals is that of furanocoumarin metabolism within the genus *Papilio* (swallowtail butterflies) (15). Members of the P450 *CYP6B* gene subfamily are induced in several *Papilio* species by dietary furanocoumarin as a result of xanothotoxin-responsive elements in their promoters (4, 16, 17). The inducibility of individual P450s isolated from different species and their activity (substrate specificity for, and turn-over of, different furanocoumarins) display a strong correlation with the ecology of the different species (i.e., frequency of exposure to furanocoumarins) (18, 19). In the case of other insect species that have adapted to nicotine-producing plants, P450s have also been implicated in detoxification in the case of the tobacco hornworm, *Manduca sexta* (20). This tobacco specialist employs a putative alkaloid pump at the blood–brain barrier coupled with detoxifying enzymes to provide a metabolic blood–brain barrier to nicotine. Although there is evidence that the detoxification enzymes involved in this process are P450s, the specific enzyme(s) involved has never been identified.

In the present study, we used a range of molecular, biochemical, and transgenic approaches to (*i*) identify the metabolic process which *M. persicae nicotianae* has evolved to detoxify nicotine and so allow colonization of tobacco, (*ii*) determine the genetic changes (mutations) underpinning this adaptation, and (*iii*) determine whether the development of the mechanism to detoxify nicotine has preadapted these races to resist neonicotinoid insecticides.

Significance

Insect host shifts may lead to sympatric speciation and can create new crop pests, however identifying the genetic changes involved has proved elusive. We studied a subspecies of the aphid *Myzus persicae* that has recently host shifted to tobacco and are resistant to the plant alkaloid nicotine. We found these races overexpress a cytochrome P450 enzyme (CYP6CY3) that allows them to detoxify nicotine and also certain synthetic insecticides. Overexpression of CYP6CY3 is caused by gene amplification (up to 100 copies) and expansion of a dinucleotide microsatellite in the promoter. Our findings provide insights into the molecular drivers of insect host shifts.

Results and Discussion

M. persicae nicotianae Is Resistant to Nicotine. Five tobacco-adapted clones of *M. persicae nicotianae* (5410R, T25, JR, 926B, and 5191A) and three unadapted clones of *M. persicae* s.s. (4106A, NS, and 4255A) of diverse geographic origin were used in this study (*Materials and Methods*). Several of the *M. persicae nicotianae* clones have been shown to be resistant to nicotine and/or neonicotinoids previously (11, 21–23). We used an artificial feeding bioassay to confirm that all *M. persicae nicotianae* clones are able to survive for 144 h on a diet containing 30 ppm nicotine. In contrast, all *M. persicae* s.s. clones showed 100% mortality at this time point on this diet (Fig. 1*A*). At higher nicotine concentrations (200 ppm), variability in the level of nicotine tolerance of the five *M. persicae nicotianae* clones was observed, with JR the most sensitive (100% mortality at 144 h) and 5410R the most resistant (7% mortality) (Fig. 1*A*).

CYP6CY3 Is Overexpressed in _M. persicae nicotianae_ Compared with _M. persicae_ s.s. A previous study on the molecular basis of neonicotinoid resistance in *M. persicae sensu lato* compared global gene expression levels in the *M. persicae nicotianae* clone 5191A with the *M. persicae* s.s. clone 4106A using custom microarrays (23). A single P450 gene (*CYP6CY3*) was found to be highly and constitutively overexpressed (~22-fold) in the 5191A clone. In the present study, we used quantitative PCR to show that *CYP6CY3* is highly overexpressed (10- to 75-fold) in all five *M. persicae nicotianae* clones compared with the *M. persicae* s.s. clones (Fig. 1*B*). Furthermore, *CYP6CY3* expression levels were significantly correlated with observed mortality after feeding on 200 ppm nicotine ($R^2 = 0.91$, $P = 0.0003$). Interestingly, *CYP6CY3* was overexpressed in two *M. persicae nicotianae* clones, JR and 926B, that have been shown previously to be resistant to neonicotinoid insecticides (21, 22). Significantly, both of these clones were collected and established before the introduction of neonicotinoids in 1991 (21, 22), indicating that *CYP6CY3* overexpression has not evolved as a result of selection from this insecticide class but rather may have evolved to allow enhanced detoxification of nicotine during adaptation to tobacco. To investigate this possibility further, we examined the ability of
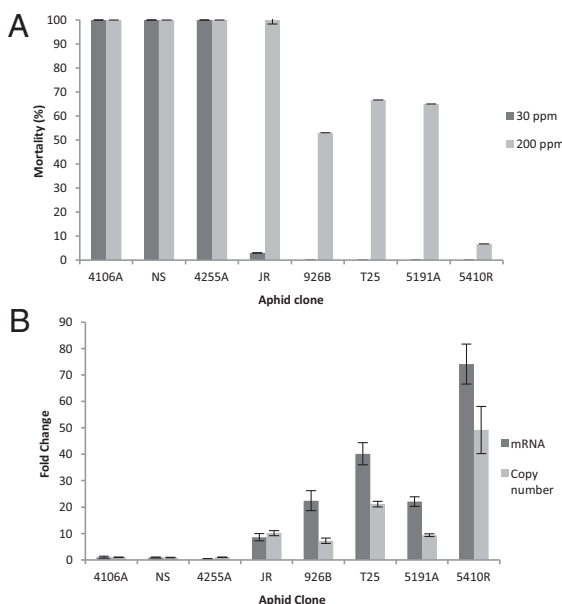

Fig. 1. Response to nicotine and changes in copy number and mRNA expression of *CYP6CY3* in *M. persicae nicotianae* and *M. persicae* s.s. clones. (*A*) Mean mortality ± SE (*n* = 3) after feeding on nicotine (30 and 200 ppm) for 144 h. (*B*) Fold change in *CYP6CY3* mRNA expression and copy number. Error bars indicate 95% confidence intervals (*n* = 3).

heterologously expressed CYP6CY3 to metabolize nicotine and neonicotinoids.

Recombinant CYP6CY3 Metabolizes Nicotine and Neonicotinoids in Vitro to Less-Toxic Metabolites. CYP6CY3 was coexpressed with the *Drosophila melanogaster* cytochrome P450 reductase in Sf9 insect cells with 100 pmol P450/mg protein obtained from isolated membranes. Incubation of microsomal preparations containing P450/cytochrome P450 reductase (CPR) with nicotine demonstrated that CYP6CY3 is highly effective at metabolizing nicotine with 90% of a 10 μM solution metabolized in 60 min (Fig. 2*A*). We were unable to source reference standards for all known nicotine metabolites, however, based on molecular mass (161.1073 Da) and the metabolic fate of nicotine in humans (Fig. S1) the primary metabolite produced was predicted to be $\Delta^{1'(5')}$ or $\Delta^{1'(2')}$ iminium ion (theoretical mass of 161.1079 Da) (Fig. 2*C*). As shown in Fig. S1, these intermediates would be further processed in vivo to cotinine and aminoketone, respectively. Neither of these was found to cause any mortality to either *M. persicae* subspecies in bioassays using 2,000-ppm solutions of either compound, clearly demonstrating that CYP6CY3 detoxifies nicotine. When honeydew collected from *M. persicae nicotianae* fed on nicotine was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), aminoketone was present at a fourfold higher amount than cotinine (Fig. 2*D*), suggesting CYP6CY3 primarily metabolizes nicotine to $\Delta^{1'(2')}$ iminium ion. As this is one of the P450-mediated pathways of nicotine metabolism in humans (24), the efficiency of nicotine metabolism by CYP6CY3/insect CPR was compared with CYP2A6/human CPR the main high-affinity metabolizer of nicotine in humans (24). Based on substrate depletion, CYP6CY3 metabolizes nicotine more efficiently than CYP2A6 (Fig. 2*B*). The ability of both CYP6CY3 and CYP2A6 to metabolize two neonicotinoids, imidacloprid and clothianidin, was also examined. CYP6CY3 metabolizes imidacloprid to 5-hydroxy-imidacloprid (0.82 ± 0.04 pmol metabolite/min/pmol P450) and clothianidin to the n-desmethylated form thiazolylnitroguanidine (TZNG; 1.33 ± 0.13 pmol metabolite/min/pmol P450). Both of these metabolites have been shown to be less toxic to aphids than the parent insecticide (25). Interestingly, human CYP2A6 also metabolizes both neonicotinoids but with lower efficiency (0.29 ± 0.03 pmol 5-hydroxy-imidacloprid/min/pmol P450 and 0.07 ± 0.01 pmol TZNG/min/pmol P450). These results clearly demonstrate that the active site of both CYP6CY3 and CYP2A6 can accommodate and metabolize both plant- and man-made antiherbivory compounds, albeit with different efficiency. The capacity of polyphagous insects and mites to evolve metabolic resistance to synthetic insecticides/acaricides has been suggested to result from the recruitment of detoxification mechanisms that have evolved to process plant allelochemicals (26). Our findings now provide clear evidence that this is true for the aphid *M. persicae nicotianae*.

Transgenic Expression of _CYP6CY3_ in _Drosophila melanogaster_ Confers Resistance to Nicotine and Neonicotinoids in Vivo. To confirm that CYP6CY3 can confer resistance to nicotine and a neonicotinoid in vivo we generated a transgenic *D. melanogaster* strain containing an intact copy of the aphid *CYP6CY3* gene on chromosome 2. This transgene was confirmed by quantitative PCR to be constitutively expressed in adult flies with male flies expressing higher levels of *CYP6CY3* than female flies (Fig. S2). For this reason, the ability of male flies expressing the transgene to resist nicotine and a neonicotinoid (clothianidin) was examined in oral bioassays. These flies displayed significant resistance to both compounds (Fig. 3) compared with control progeny (flies of the same genetic background without the *CYP6CY3* transgene), demonstrating unequivocally that transcription of *CYP6CY3* alone is sufficient to give a resistant phenotype.

CYP6CY3 Is Overexpressed in _M. persicae nicotianae_ as a Result of Gene Amplification and Is Enhanced by a Polymorphic Dinucleotide Microsatellite. To identify the mutations that underlie *CYP6CY3* overexpression in *M. persicae nicotianae* we used quantitative
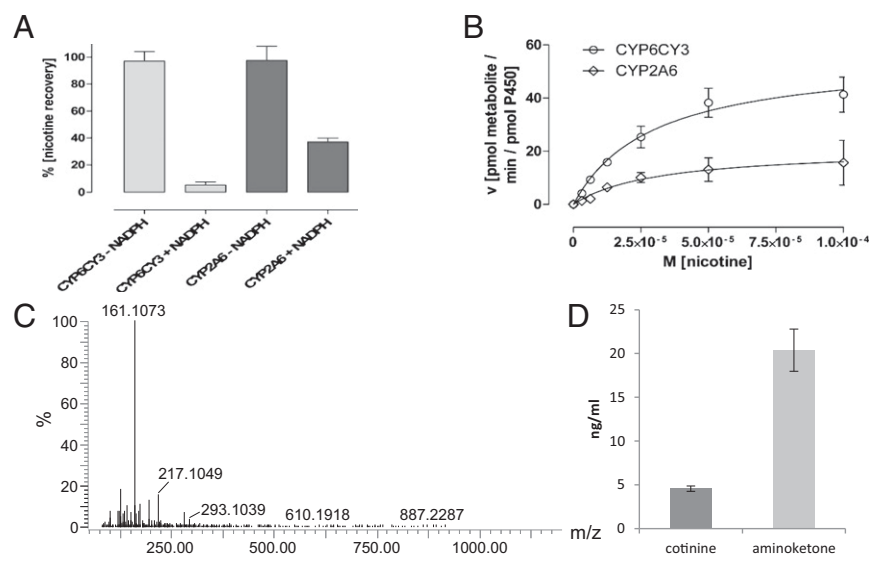
Fig. 2. Metabolism of nicotine by recombinantly expressed aphid (CYP6CY3) and human (CYP2A6) P450s. (*A*) The recovery of nicotine after 10 µM nicotine was incubated with 50 pmol P450+reductase in the presence or absence of NADPH for 60 min. Mean data ± SD ($n = 6$). (*B*) Rate of nicotine metabolism with increasing nicotine concentration. The apparent k_{cat} and K_m values were calculated as k_{cat} of 56.41 ± SEM ($n = 3$) 6.18 pmol metabolite/min/pmol P450 and K_m of 30.00 ± 8.06 µM for CYP6CY3, and k_{cat} of 21.03 ± 4.11 and K_m of 31.25 ± 10.77 µM for CYP2A6. (*C*) High-resolution mass spectrum of the major metabolite [the peak at *m/z* 161.1079 corresponds to the theoretical mass of nicotine-$\Delta^{1'(5'2')}$-iminium ion]. (*D*) Amount of cotinine and aminoketone in aphid honeydew after feeding on nicotine-treated leaf discs for 48 h.

PCR and genome walking to look for gene copy number variation and *cis*-acting regulatory mutations in the 5′ putative promoter region. Amplification of *CYP6CY3* has been associated previously with resistance to neonicotinoids in the clone 5191A (23). In the present study, we found *CYP6CY3* overexpression in all *M. persicae nicotianae* clones, is due, at least in part, to gene amplification, with tobacco-adapted clones showing a 7- to 49-fold increase in gene copy number (from 2 copies in the diploid *M. persicae* s.s. clones to 14–100 copies in the *M. persicae nicotianae* clones) (Fig. 1*B*). Sequencing of *CYP6CY3* coding sequence revealed very little sequence variation either between or within any of the *M. persicae* s.l. clones, suggesting the amplification has occurred recently. In terms of interaphid clonal variation two SNPs are present in the eight aphid clones, a synonymous SNP at nucleotide position 642 and a nonsynonymous SNP at position 74 causing a threonine to isoleucine substitution in the region encoding the transmembrane anchor of the P450 protein which would not be predicted to affect catalytic activity (Fig. S3). In terms of intraaphid clonal variation, two alleles are present in all three *M. persicae* s.s. clones distinguished by a single G/A SNP resulting in either a glutamic acid or lysine amino acid at this position (Fig. S3). For all *M. persicae nicotianae* clones, only a single *CYP6CY3* coding sequence allele was detected (Fig. S3). The identical *CYP6CY3* nucleotide sequence in five *M. persicae nicotianae* clones from three continents supports a single origin and global spread of the amplification event.

Beyond insects, the best documented examples of P450 gene amplification come from human P450s, specifically CYP2A6 and CYP2D6. Remarkably, both of these are involved in nicotine metabolism. CYP2A6 is duplicated in certain humans who show increased levels of nicotine metabolites in blood plasma and modified smoking behavior (27). CYP2D6 plays a more minor role in nicotine metabolism, but humans with up to 13 active copies, known as "ultrarapid metabolizers," can metabolize nicotine and a range of other xenobiotics (including many therapeutic drugs) more rapidly than humans of normal copy number (28). The evolutionary parallels between human CYP2D6 and aphid CYP6CY3 continue further as amplification of both may have evolved as a mechanism to overcome plant secondary metabolites that defend against human and/or insect herbivory. CYP2D6 has a very high affinity for plant alkaloids and amplification of this P450 has been suggested to have arisen in North East Africa (where a high frequency of the population have the gene amplification) to allow previously toxic plants to be exploited during periods of famine (28).

For all but one of the *M. persicae nicotianae* clones (JR), the increase in *CYP6CY3* gene copy number is approximately half

the increase in the level of mRNA expression (Fig. 1*B*), suggesting that additional factor(s) underlie the change in expression level in four of the five clones. To identify additional regulatory mutations, a ~1.5-kb region of the putative 5′ promoter region of *CYP6CY3* in all *M. persicae* s.s. and *M. persicae nicotianae* clones was PCR amplified by genome walking, cloned, and sequenced. Only two main sequence differences were observed between the *M. persicae* s.s. clones and the majority of the *M. persicae nicotianae* clones: a single A/G SNP 138 bp upstream of the translational start codon and a significant change in length of a $AC_{(n)}$ dinucleotide repeat (198 bp upstream of the start codon) from 15 repeat units in *M. persicae* s.s. clones to 48 repeat

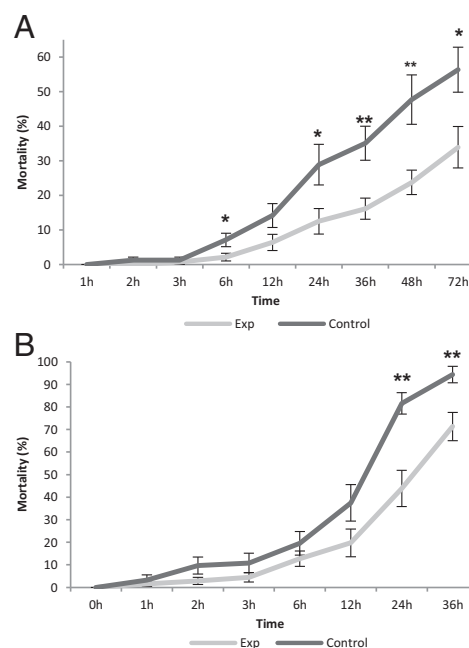**Fig. 3.** Bioassays with transgenic *Drosophila* expressing CYP6CY3. (*A* and *B*) Mortality of transgenic CYP6CY3 male flies (Exp) to nicotine (*A*) and clothianidin (*B*) compared with controls (Control), which do not have the P450 transgene. Error bars indicate SE ($n = 6$–9). Asterisks indicate a significant difference in mortality between experimental and control lines. *$P < 0.05$, **$P < 0.01$.

units in *M. persicae nicotianae* clones (Fig. 4*A* and Fig. S4). Interestingly, the exception to this was the *M. persicae nicotianae* clone JR, which like the *M. persicae* s.s. clones lacks the microsatellite expansion. This is the only *M. persicae nicotianae* clone where the fold-change increase in *CYP6CY3* gene copy number is equal to the increase seen in the level of mRNA expression (Fig. 1*B*), suggesting that the microsatellite polymorphism or the A/G SNP may play a functional role in further enhancing *CYP6CY3* expression. To explore this possibility, the ~1.5-kb region upstream of *CYP6CY3* was PCR-amplified from the 5191A and 4106A clones and cloned into the reporter gene vector pGL3. Reporter gene assays showed that the *M. persicae nicotianae* promoter insert drove around twofold greater reporter gene expression than the *M. persicae* s.s. insert (Fig. 4*B*). To examine whether the increase in $AC_{(n)}$ length or the single A/G SNP was responsible, site-directed mutagenesis was used to change the adenine at position −138 bp present in the *M. persicae nicotianae* promoter to a guanine, mimicking the situation in *M. persicae* s.s. When the activity of this mutated insert was compared with the *M. persicae nicotianae* promoter insert, no reduction in reporter gene expression was observed (Fig. 4*B*), indicating that it is the increase in length of the $AC_{(n)}$ repeat that is exclusively responsible for the expression difference. To investigate this possibility further, a series of synthetic versions of the *M. persicae* s.s. promoter were synthesized with $AC_{(n)}$ repeat lengths of $AC_{(15)}$, $AC_{(30)}$, $AC_{(45)}$, and $AC_{(60)}$. When these inserts were used in reporter gene assays, enhanced expression was only observed for the $AC_{(45)}$ and $AC_{(60)}$ constructs (Fig. 4*C*). Furthermore, no significant differences in expression were observed between the two longest or the two shortest constructs, suggesting a threshold effect. This finding also suggests that the $AC_{(48)}$ repeat seen in most of the *M. persicae nicotianae* clones is the optimal length for enhancing the expression of *CYP6CY3*. We note that these findings are somewhat in contrast to previous studies where increases in the length of dinucleotide repeats show a more linear relationship with gene expression (29).

Microsatellites including $AC_{(n)}$ repeats have been historically considered nonfunctional neutral markers or "junk DNA." However, there is now mounting evidence that they may be important *cis*-regulatory DNA sequences that have direct influence on both gene expression and phenotypic variation (30). Studies on humans where polymorphic $AC_{(n)}$ microsatellites are overrepresented in the genome have suggested they may influence transcription by binding transcription factors or by affecting DNA conformation (30). In yeast, polymorphic tandem repeats in promoters may facilitate gene expression by affecting local chromatin structure consistent with a role as "evolutionary tuning knobs" that mediate rapid evolution of gene expression (29). Our findings demonstrate that dinucleotide microsatellite variation is also a driver of adaptive change in gene expression during insect evolution.

Analysis of intraaphid clone sequence variation within the ~1.5-kb region of the putative 5′ promoter region revealed two variant promoter sequences in all *M. persicae* s.s. clones (distinguished by a G/A SNP at position −545, Fig. S4), both of which lack the microsatellite expansion. Two alleles were also detected in clone JR (distinguished by a G/A SNP at position −138 bp, Fig. S4), both of which lack the microsatellite expansion. For all other *M. persicae nicotianae* clones, only a single promoter allele with the tandem repeat expansion was observed (Fig. S4). The finding that, for four of the *M. persicae nicotianae* clones, all amplified copies of *CYP6CY3* appear to share the microsatellite expansion suggests that this mutation predates the gene amplification event. Similar time courses of *cis*-acting mutation followed by duplication have been described for P450s associated with insecticide resistance in fruit flies and mosquitoes (31, 32).

Summary

The genetic structure of global *M. persicae* s.l. populations suggests that adaptation to tobacco arose as a recent, single evolutionary event, originating in East Asia, where tobacco-adapted forms were first reported (33); our findings are consistent with this. We conclude that the mutations leading to overexpression
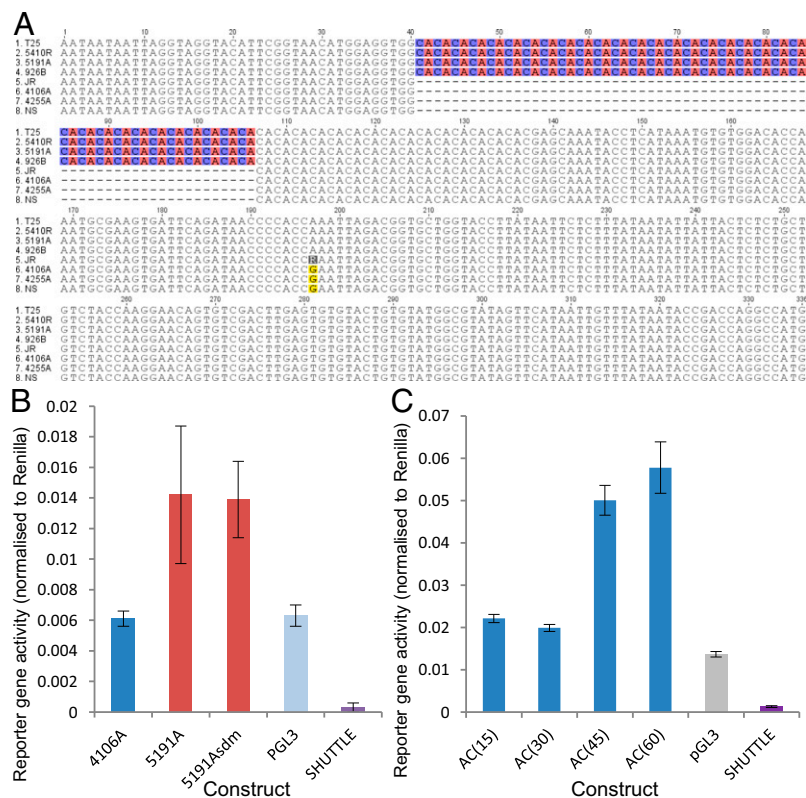

Fig. 4. *CYP6CY3* promoter analysis in *M. persicae nicotianae* and *M. persicae* s.s. (*A*) Alignment of a region of the 5′ putative promoter region immediately upstream of the translation start codon of *CYP6CY3* highlighting two polymorphisms, an A/G SNP at position −138 bp and a change in length of an $AC_{(n)}$ dinucleotide repeat at position −198 bp. (*B*) Reporter gene activity (normalized to renilla fluorescence) of the 5′ putative promoter regions of *CYP6CY3* amplified from the *M. persicae* clone 4106A and the *M. persicae nicotinae* clone 5191A. The activity of a mutated version of the 5191A promoter (5191Asdm), which has an A/G SNP present in the 5191A promoter corrected is also shown along with pGL3 and shuttle constructs (both controls). (*C*) Reporter gene activity of synthetic promoter constructs with different lengths of the $AC_{(n)}$ dinucleotide repeat. For *B* and *C*, mean data ± SD (n = 4) is shown.

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of *CYP6CY3* were a prerequisite for the host shift of *M. persicae* to tobacco. Furthermore, the recruitment of this detoxification mechanism preadapted this subspecies to resist neonicotinoids, a globally important class of insecticide. We suggest that mutation of a microsatellite was the initial step in this process and that subsequent gene amplification greatly enhanced expression of this gene. These results provide fundamental insights into the evolutionary processes that underlie the genotypic and phenotypic changes that are involved in insect host shifts and we envisage that both gene amplification and dinucleotide microsatellites may be an important source of genetic variation for adaptive evolution in other insect species.

Materials and Methods

Aphid Clones. Clones 4106A, NS, and 4255A are clones of *M. persicae* s.s. collected from potatoes in Scotland in 2000, from an unrecorded plant in Germany (Bonn) in 1967, and from oilseed rape in the United Kingdom in circa 2000, respectively. Clones 926B, 5191A, and T25 are clones of *M. persicae nicotianae* collected in Greece from tobacco in 1990, 2007, and 2007; 5410R is a clone of *M. persicae nicotianae* collected from tobacco in Zimbabwe in 2010. Clone JR was collected from eggplants (a member of the Solanacae that contains relatively high levels of nicotine; ref. 34) in Toyoda, Japan, in 1983 but was found to readily accept tobacco (22). Japanese populations of *M. persicae nicotianae* are difficult to distinguish from *M. persicae* s.s., but morphological examination suggested JR is "a tobacco feeding form of *M. persicae* closely related to the tobacco aphid *M. persicae nicotianae*" (22). We refer to it in this study as *M. persicae nicotianae*. JR was previously found to be 10-fold resistant to nicotine compared with the *M. persicae* strain NS in an oral feeding assay and 7-fold resistant to the neonicotinoid imidacloprid in a leaf-dip bioassay (22). Clone 926B showed fivefold resistance to nicotine in fumigation bioassays and 11-fold resistance to imidacloprid rising to 100-fold resistance to the neonicotinoid clothianidin compared with the *M. persicae* clone 4106A (11, 21). Clone 5191A was recently shown to be 30- to 40-fold resistant to several neonicotinoids compared with 4106A (23).

Aphid Bioassays. Nicotine (Sigma) was dissolved directly into an aphid artificial diet (35) and filter sterilized. A total of 0.5 mL was pipetted onto the end of a plastic cylinder (size: 25 mm deep, 25 mm internal diameter) that had been sealed with stretched Parafilm. A second layer of Parafilm was then stretched on top of this to form a sachet. Ten adult apterous aphids were transferred into the cylinder and the remaining opening sealed with Parafilm. Three replicates were used for each concentration and mortality was assessed after 144 h at 18 °C under a 16:8 h light:dark regime. Mortality was controlled for by feeding aphids on artificial diet without nicotine under the same conditions.

Quantitative RT-PCR. Quantitative PCR was used to examine the expression of *CYP6CY3* and changes in gene copy number using the primers CYP6F and CYP6R (Table S1) as described (23). Data were analyzed according to the $\Delta\Delta C_T$ method (36), using the geometric mean of two housekeeping genes (actin and *para*, which encodes the voltage-gated sodium channel) for normalization according to the strategy described previously (37).

CYP6CY3 Coding Sequence Analysis. The coding sequence of CYP6CY3 was amplified from cDNA (prepared as described above) using primers CY3CDSF and CY3full R2 (Table S1). PCR reactions (20 μL) consisted of 1 unit of Kappa high-fidelity polymerase (Kappa Biosystems), 4 μL of PCR buffer containing 10 mM magnesium chloride, 1 μL of 10 mM dNTPs, 1 μL of each primer (10 mM), and 2 μL of cDNA. Temperature cycling conditions were 95 °C for 1 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, followed by a final extension of 72 °C for 10 min. Amplified fragments were visualized on 1% agarose gels, purified using the Wizard SV Gel kit and PCR Clean-Up System (Promega), and either "direct sequenced" or cloned (using the Stratalone PCR Cloning kit; Agilent Technologies) and sequenced using primers CY3seqF and CY3seqR (Table S1).

Genome Walking and Promoter Analysis of CYP6CY3. The GenomeWalker Universal Kit (Clontech) was used to PCR amplify the genomic DNA sequence upstream of *CYP6CY3*. DNA was extracted from a pool of five adult aphids of the 5191A clone using the DNeasy Plant DNA Mini Kit (Qiagen) and used for genome walking following the manufacturer's protocol with the primers shown in table S1. PCR products were recovered from agarose gels, cloned using the Stratalone PCR Cloning kit (Agilent Technologies) and sequenced. Specific primers were then designed (Table S1) and used to characterize the

~1.5-kb putative promoter region immediately upstream of the translation start codon of *CYP6CY3* of all of the aphid clones used in this study. PCR products were sequenced directly or cloned and sequenced as described for the characterization of *CYP6CY3* coding sequence. For reporter gene assays, the same 1.5 kb of promoter sequence was amplified from DNA extracted from aphid clones 5191A and 4106A in PCR using a high fidelity taq (Advantage 2 polymerase, Clontech), the conditions detailed above and primers CY3hindIIIR and CY3sac1F (Table S1). Following excision with HindIII and SacI these were ligated into pGL3-Basic (Promega) and transformed into XL1-Blue (Agilent). Plasmids were extracted with the GeneJet plasmid miniprep kit (Fermentas), sequenced and then adjusted to 300 ng/μL for use in dual luciferase assays using the *An. gambiae* cell line Sua5.1 maintained in Schneider's *Drosophila* medium supplemented with 10% FCS and 1% penicillin/streptomycin. Approximately 5×10^5 cells per well were plated into 24-well plates 1 d before transfection and allowed to reach 60–70% confluency. Qiagen effectene transfection reagent was used for transfection of constructs, and the Dual-Luciferase Reporter Assay (Promega) used for promoter activity measurements. Reporter constructs (600 ng, CYP6CY3 upstream sequences in pGL3-Basic) and pGL3 without insert or 600 ng of shuttle construct pSLfa1180fa as controls (http://flybase.org/reports/FBmc0002761.html) were cotransfected with 1 ng of actin-Renilla internal control in 60 μL of DNA condensation buffer, 4.8 μL of enhancer and 6 μL of effectene in triplicate. Following incubation at 27 °C for 48 h and washing of cells with PBS, cells were harvested in 100 μL passive lysis buffer (Promega) and luciferase activity measured on a luminometer (EG&G Berthold). Construct luciferase activity was normalized to Renilla luciferase activity. The Quik-Change II Site-Directed Mutagenesis kit (Agilent) was used with primers CY3SDMF and CY3SDMR (Table S1) to change an adenine nucleotide at position −198 bp in the *M. persicae nicotianae* promoter to a guanine. The plasmids obtained were sequenced to identify promoter constructs containing the nucleotide change. Finally, a series of synthetic versions of ~1.5 kb of the *M. persicae* 4106A promoter were synthesized (GeneArt, Life Technologies) where the $AC_{(n)}$ repeat length present ~200 bp upstream of the translational start codon was changed in length to, $AC_{(15)}$, $AC_{(30)}$, $AC_{(45)}$, and $AC_{(60)}$. These were then cloned into pGL3-Basic and used in reporter assays as described above.

Heterologous Expression of CYP6CY3. *CYP6CY3* (GenBank accession no. HM009309) and the *D. melanogaster* (DROME) NADPH-dependent CPR (GenBank accession no. Q27597) were obtained by gene synthesis (Geneart) and inserted into the pDEST8 expression vector (Invitrogen). The PFastbac1 vector with no inserted DNA was used to produce a control virus. The recombinant baculovirus DNA was constructed and transfected into Sf9 insect cells (Gibco) using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions. The titer of the recombinant virus was determined following standard protocols of the supplier. Sf9 cells were maintained in suspension culture under serum-free conditions (SF-900 II SFM, Gibco) at 27 °C containing 25 μg/mL^{-1} gentamycin (Gibco). Insect cells grown to a density of 2×10^6 cells per mL^{-1} were coinfected with recombinant baculoviruses containing CYP6CY3 and CPR with various multiplicity of infection (MOI) ratios to identify the best conditions. Control cells were coinfected with the baculovirus containing vector with no insert (ctrl-virus) and the recombinant baculovirus expressing CPR using the same MOI ratios. Ferric citrate and δ-aminolevulinic acid hydrochloride were added to a final concentration of 0.1 mM at the time of infection and 24 h after infection. After 60 h, cells were harvested and washed with PBS, and microsomes of the membrane fraction were prepared according to standard procedures and stored at −80 °C (38). P450 expression and functionality was estimated by measuring CO-difference spectra in reduced samples (38). The protein content of samples was determined using Bradford reagent (Sigma) and BSA as a reference.

Metabolism Assays and Ultra-Performance LC-MS/MS Analysis. Metabolism of nicotine and neonicotinoids (imidacloprid and clothianidin) was assayed by incubating recombinant CYP6CY3/CPR (2 pmol P450 per assay) or ctrl-virus/CPR microsomes in 0.1 M potassium phosphate buffer with an NADPH-regenerating system (Promega; 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl$_2$, 0.4 U/mL^{-1} glucose-6-phosphate dehydrogenase) and substrate (12.5 μM; 0.8–100 μM for enzyme kinetics) at 27 °C for 1 h. The total assay volume was 200 μL using three replicates for each data point. Microsomes incubated without NADPH served as a control. The assay was quenched by the addition of ice-cold acetonitrile (to 80% final concentration) and centrifuged for 10 min at 3,000 × *g*, and the supernatant subsequently analyzed by tandem mass spectrometry as described (39). For the chromatography, acetonitrile/water/0.1% formic acid was used as eluent in

gradient mode. For detection and quantification, the multiple reaction monitoring transitions 163 > 117 (nicotine); 177 > 80 (cotinine); 179 > 106 (aminoketone); 256 > 175 (imidacloprid); 250 > 132 (clothianidin) were monitored. Recovery rates of parent compounds using microsomal fractions without NADPH were normally close to 100%. Substrate turnover from two independent reactions were plotted vs. controls. Michaelis–Menten kinetics were determined using GraphPad Prism version 5 (GraphPad Software).

Analysis of Aphid Honeydew. Leaf discs were dipped in aqueous nicotine (2,000 ppm) containing 0.1% (wt/vol) Triton X-100, allowed to dry for 30 min, and then placed on a bed of agar in a small vented Petri dish. Ten aphids of clone JR were placed on the leaf disk, the lid of the Petri dish was sealed, and the dish was stored inverted for 48 h. Five replicates were prepared in this way. Aphid honeydew that had collected on the lid of the Petri dish was washed off in 500 μL of water and analyzed for the presence of aminoketone and cotinine by ultra-performance LC-MS/MS analysis as detailed above.

Transgenic Expression of CYP6CY3 in *D. melanogaster*. CYP6CY3 was PCR amplified using primers CY3Xba1F and CY3HindIIIR (Table S1) following the conditions described above. Following digestion with HindIII and XbaI, the PCR amplicon was ligated into a modified pattB vector that had been digested with the same enzymes and transformed into XL1-Blue (Agilent). Using the PhiC31 system, clones were transformed into the germ line of a *D. melanogaster* strain carrying the attP docking site on chromosome 2 ["y^{1}w^{67c23}; P attP", "1;2"] (40). The transgenic line was obtained and

balanced, and the integration of the intact gene was confirmed by PCR and sequencing as described above. Bioassays were used to assess the susceptibility of adult male flies to nicotine and the neonicotinoid insecticide clothianidin. Compounds were dissolved in molten 0.5% agarose containing 5% (wt/vol) sugar, and 2 mL was allowed to set in the bottom of 15-mL falcon tubes. Adult flies (15–20, 2–5 d post eclosion) were then added to the tubes, and the mortality by oral ingestion was assessed at several time points spanning 1–74 h. Mortality was compared with control progeny (flies of the same genetic background but without CYP6CY3). Six to nine replicates were carried out for each concentration. Mortality due to factors other than insecticide was controlled using tubes containing agarose/sucrose minus insecticide. Unpaired Student *t* tests were used to compare the mortality of each experimental group with each control group for each time point. To confirm the expression of CYP6CY3 in the experimental group and the absence of expression in the control group, total RNA was extracted from three pools of five flies of each, and cDNA was synthesized as described above. Quantitative PCR was performed using the synthesized cDNA as template as described above but using the *Drosophila* RPL11 housekeeping gene for normalization (Table S1).

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