Insect Biochemistry and Molecular Biology 66 (2015) 96–102

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

A single nucleotide change in a core promoter is involved in the progressive overexpression of the duplicated *CYP9M10* haplotype lineage in *Culex quinquefasciatus*

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ARTICLE INFO

Article history:
Received 13 July 2015
Received in revised form
8 October 2015
Accepted 13 October 2015
Available online 19 October 2015

Keywords:
Insecticide resistance
Core promoter
TATA-box
Regulatory mutation
Cytochrome P450

ABSTRACT

Although the importance of *cis*-acting mutations on detoxification enzyme genes for insecticide resistance is widely accepted, only a few of them have been determined as concrete mutations present in genomic DNA till date. The overexpression of a cytochrome P450 gene, *CYP9M10*, is associated with pyrethroid resistance in the southern house mosquito *Culex quinquefasciatus*. The haplotypes of *CYP9M10* exhibiting overexpression (resistant haplotypes) belong to one specific phylogenetic lineage that shares high nucleotide sequence homology and the same insertion of a transposable element. Among the resistant haplotypes, allelic progression involving an additional *cis*-acting mutation and gene duplication evolved a *CYP9M10* haplotype associated with extremely high transcription and strong pyrethroid resistance. Here we show that a single nucleotide substitution G−27A, which is located near the transcription start site of *CYP9M10*, is involved in the progression of the duplicated haplotype lineage. The deletion of a 7-bp AT-rich sequence that includes nucleotide −27 inhibited the initiation of transcription from the original transcriptional initiation site. The mutation was suspected to reside within a core promoter, TATA-box, of *CYP9M10*.

1. Introduction

The application of insecticides selects for mutations that confer insecticide-resistance. Once such mutations are present at a high frequency in the population, human health, and food production face serious problems because pest control is no longer effective using such insecticides. Insecticide resistance is most often acquired through metabolic degradation or alteration in target site of insecticides. Metabolic degradation is typically related to an increase in the activities of detoxification enzymes, which degrade active insecticide ingredients into nontoxic forms. Well-known enzyme classes responsible for the metabolism of insecticides in animals are cytochromes P450, glutathione S-transferases, and carboxylesterases (Hemingway et al., 2004; Li et al., 2007). Mutations that enhance the production of these enzymes or nonsynonymous point mutations that alter the kinetic properties of an enzyme can increase the metabolic rate of insecticide degradation and, hence, the resistant phenotype.

Regulatory mutations are one of the important classes of mutations, conferring resistance via the overproduction of the mRNA encoding a detoxifying enzyme. Mutations of the promoter of the gene (*cis*-acting mutation) or a regulatory factor encoded in another genetic locus (*trans*-acting mutation) alter the expression level of an mRNA. Further, gene copy number changes such as duplication or amplification is often observed in the overexpression of detoxification enzyme genes. Although *cis*-acting mutations are often implicated in resistance phenotypes, only a few studies have identified them as *bona fide* mutations on nucleotide sequence to date (Feyereisen et al., 2015). For example, a haplotype of the cytochrome P450 gene *Cyp6g1* of *Drosophila melanogaster* confers overexpression due to an insertion of a long terminal repeat of a retrotransposon *Accord* in the upstream region, thus conferring dichlorodiphenyltrichloroethane (DDT) resistance (Daborn et al., 2002; Chung et al., 2009). The change in the number of a dinucleotide repeat in a microsatellite located upstream of *CYP6CY3* in the genome of the peach-potato aphid alters its expression level, which confers resistance to neonicotinoid insecticides and the

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http://dx.doi.org/10.1016/j.ibmb.2015.10.006

natural plant alkaloid nicotine (Bass et al., 2013).

In the southern house mosquito *Culex quinquefasciatus,* overexpression of a cytochrome P450 gene *CYP9M10* is related to pyrethroid resistance (Hardstone et al., 2010; Itokawa et al., 2013, 2010; Komagata et al., 2010; Liu et al., 2011; Wilding et al., 2012). A specific phylogenetic lineage of *CYP9M10* haplotypes that overexpress *CYP9M10* mRNA is associated with strong pyrethroid resistance. The haplotypes of this haplogroup (referred to as "resistant haplotypes") are characterized by an insertion of the transposable element *CuRE1* (*Culex* repetitive element 1), which belongs to the miniature interspersed transposable element family, located 0.2 kb upstream from the transcriptional start site (TSS) (Itokawa et al., 2010). The resistant haplotypes show a small number of polymorphic nucleotides in the upstream noncoding region regardless of the geographical region from which the insects were sampled (Itokawa et al., 2013; Wilding et al., 2012), indicating an ongoing selective sweep and a rapid spread of resistant haplotypes worldwide. Because a reporter assay conducted using *Anopheles* cell culture did not show the effect of *CuRE1* on *CYP9M10* promoter activity (Wilding et al., 2012), it is not certain that *CuRE1* itself is the *cis*-acting mutation mediating overexpression. Nevertheless, the *CuRE1* insertion can serve as a useful genetic marker to diagnose resistance, because the insertion is considered to be in linkage disequilibrium with a true, yet uncharacterized, *cis*-acting mutation (Itokawa et al., 2013; Wilding et al., 2012).

Variation in expression level is observed even among *CYP9M10* resistant haplotypes (Itokawa et al., 2011, 2013). It is known that the most highly overexpressed haplotypes, which are associated with the strongest resistance, contain identical tandem duplications of a 100-kb sequence containing the *CYP9M10* locus (Itokawa et al., 2010, 2013). Because the nucleotide sequences of the two gene copies in the duplicated haplotypes are identical (Itokawa et al., 2013), the duplication likely has occurred relatively recently. Although the gene duplication explains, in part, the genetic variance among the resistant haplotypes, the difference in copy number was insufficient to explain the difference in the expression levels between the duplicated haplotype and one resistant haplotype without duplication (Itokawa et al., 2011), indicating that extreme overexpression of the duplicated haplotype was caused not only by the duplication but also by an additional (secondary) *cis*-acting mutation.

Indeed, we recently identified two unduplicated resistant haplotypes exhibiting higher expression level than other previously known unduplicated resistant haplotypes in *C. quinquefasciatus* collected from Singapore and Kenya (Itokawa et al., 2013). Among the resistant haplotypes investigated so far, there were only three segregating nucleotides within the region 2.2-kb upstream from the TSS, −2000T/G, −1176A/G, and −27G/A (defining the TSS as +1), which generate three haplotypic states as follows: T-A-G, T-G-G, and G-G-A (nts −2000, −1176, and −27, respectively) (Itokawa et al., 2013). The divergent haplotypes outside the resistant haplogroup had T-A-G at the corresponding sites without exception, suggesting those are ancient states. The upstream copies of all duplicated haplotypes were G-G-A, indicating that the duplication occurred after those three substitutions. Interestingly, the two unduplicated resistant haplotypes from Singapore and Kenya also had G-G-A at those sites. Both haplotypes were expressed at higher levels than other unduplicated resistant haplotypes with the T-A-G and T-G-G nucleotides (Itokawa et al., 2013). Thus, allelic progression of the duplicated haplotype is considered to involve at least two *cis*-acting mutations, an "early" mutation, which is likely in complete linkage disequilibrium with the *CuRE1* insertion, and a "secondary" mutation, which remains polymorphic among the resistant haplotypes before the duplication (Fig. S1A) (Itokawa et al., 2013). In the present study, we asked whether either T−2000G or G−27A mutations or both were responsible for the difference in expression levels of *CYP9M10*. In luciferase reporter assays, we found that a mutation, G−27A, altered *CYP9M10* promoter activity approximately two-fold. The mutation is immediately downstream from an AT-rich region. The elimination of the AT-track, including nucleotide (nt) −27 from the reporter plasmid, completely inhibited transcription initiation from the authentic transcription start site (+1).

2. Materials and methods

2.1. Plasmid construction

PrimeSTAR Max DNA Polymerase (Takara, Shiga, Japan) and Ligation-High Ver. 2 (Toyobo, Osaka, Japan) were used in every PCRs and ligation reaction. *Dpn*I (Takara) was used to eliminate template plasmid after PCR as required. The sequences of the oligonucleotides are shown in Table S1. A firefly luciferase vector, pGL3-Basic (Promega, Madison, WI, USA), was modified for compatibility with the Golden-Gate cloning method (Engler et al., 2008) as follows: the *Bsa*I recognition site in the Amp^R gene of the original pGL3-Basic plasmid was eliminated by replacing it with a synonymous mutation using a modified Quick Change method (Zheng et al., 2004) with primers pGL3-A3224G_F1 and pGL3-A3224G_R2. A DNA cassette with *Bsa*I recognition sites on both ends was formed by annealing oligonucleotides KpnI-Bsalx2-HindIII (+) and (−), which were inserted between the *Kpn*I and *Hind*III sites in the multiple cloning site of the modified pGL3-Basic vector. The plasmid is referred to as pGL3-GG.

The region from nts −2118 to +76 relative to the transcriptional start site (+1) (Itokawa et al., 2010) of the JPP *CYP9M10v1* (upstream copy) allele (GenBank accession number AB551111), the JNB allele (AB607193), and the corresponding region of the JNA allele (AB607192) were each amplified using the primers 9M10-BsaI-CCGG_F1 and 9M10-BsaI-AGAT_R2 from the genomic (g)DNAs of each strain, and were then cloned into pGL3-GG using the Golden-Gate cloning method as follows: Approximately 10 fmol of each DNA was reacted with 1 × Ligation High version 2 (Toyobo) and 5 U of *Bsa*I-HF (NEB, Ipswich, MA, USA) in 10 μL. The reaction was conducted using ten cycles at 37 °C for 5 min and 16 °C for 5 min, followed by single cycles at 50 °C for 5 min and 75 °C for 5 min. The plasmids cloned with the JPP (v1), JNB and JNA alleles were designated pGL3-CYP9M10-RH (resistant-high), pGL3-CYP9M10-RL (resistant-low), and pGL3-CYP9M10-JNA, respectively.

pGL3-CYP9M10-$RH^{\Delta CuRE1}$ was constructed by deleting *CuRE1* from pGL3-CYP9M10-RH as described below. Two fragments, upstream and downstream of *CuRE1*, were individually amplified from the gDNA of the JPP strain using primers 9M10-BsaI-CCGG_F1 and 9M10-CuRE1elim_R as well as primers 9M10-CuRE1elim_F and 9M10-BsaI-AGAT_R2, respectively. Those two fragments were overlapped to each other at the end by 16-bp that were included in both primer 9M10-CuRE1elim_R and −F. The fragments were mixed and fused by PCR-amplification using the primers 9M10-BsaI-CCGG_F1 and 9M10-BsaI-AGAT_R2. The recombinant plasmid was cloned into the pGL3-GG vector using the Golden Gate method.

Mutations were introduced at nts −27 of pGL3-CYP9M10-RH and pGL3-CYP9M10-RL (A-to-G and G-to-A, respectively) as follows: Each plasmid was amplified using the 9M10-BsaI-27G_F1 and 9M10-BsaI-27G_R2 primers and 9M10-BsaI-27A_F1 and 9M10-BsaI-27A_R2 primers, respectively, and the fragments were self-ligated using the Golden Gate reaction. These plasmids were designated pGL3-CYP9M10-RH^{A-27G} and pGL3-CYP9M10-RL^{G-27A}, respectively. The sequence TATTTAA (nts −33 to −27) was deleted from pGL3-9M10-RH by amplifying the plasmid using the 9M10-