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Age-specific expression of a P450 monooxygenase (*CYP6CM1*) correlates with neonicotinoid resistance in *Bemisia tabaci*

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ABSTRACT

The whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), is an invasive insect pest of agriculture and horticulture worldwide. The neonicotinoid insecticides, which target nicotinic acetylcholine receptors in post-synaptic nerve membranes, play a major role in controlling *B. tabaci* although the increasing incidence of resistance to these compounds is now undermining their effectiveness. Neonicotinoid resistance has been shown to be caused by up-regulation of a cytochrome P450-dependent monooxygenase gene, *CYP6CM1*, involved in insecticide detoxification. The main aim of this study was to further investigate the finding that expression of resistance to the neonicotinoid imidacloprid is largely restricted to adults of *B. tabaci*, whereas immature stages retain susceptibility. Imidacloprid resistance was first characterised in four strains of *B. tabaci* (B- and Q-biotypes) using bioassays, biochemical and molecular-based techniques. A relative gene expression assay was then developed to measure mRNA levels of *CYP6CM1* in whitefly adults, pupae and nymphs. We show that *CYP6CM1* expression correlated with age-specific imidacloprid resistance in adults and nymphs from each strain. Furthermore, in adult whiteflies, the up-regulation of this gene was correlated with imidacloprid metabolism into a hydroxy metabolite as determined by liquid chromatography–mass spectrometry (LC–MS). These findings strengthen the involvement of *CYP6CM1* imidacloprid detoxification and suggest that transcriptional changes in *CYP6CM1*, possibly constrained by the role of P450 enzymes in insect development, are the cause of an age-specific resistance phenotype in *B. tabaci*.

1. Introduction

The development of neonicotinoid insecticides represents a significant milestone in crop protection. Neonicotinoids act agonistically at post-synaptic nicotinic acetylcholine receptors (nAChRs) in the insect nervous system [57]. They target a range of sucking insect pests including whiteflies, aphids and planthoppers (order Hemiptera) as well as economically-important members of the orders Coleoptera (beetles), Thysanoptera (thrips) and Lepidoptera (moths) [16]. Their unique mode of action coupled with their versatility and selectivity is largely responsible for their current predominance in the crop protection market [42].

Insect resistance to neonicotinoids has been surprisingly, and fortunately, slow to develop compared with other insecticide groups [41]. Amongst crop pests, the best-documented cases of neonicotinoid resistance in the field relate to the cotton whitefly, (*Bemisia tabaci* Gennadius) [24,41], the glasshouse whitefly (*Trialeurodes vaporarium* Westwood) [19], the Colorado potato beetle (*Leptinotarsa decemlineata* Say) [60], the brown planthopper (*Nilaparvata lugens* Stål) [20,59] and the peach potato aphid (*Myzus persicae* Sulzer) [47].

Bemisia tabaci is a primary and problematic pest of agriculture and horticulture. Until recently, it was considered a cryptic group of several morphologically indistinguishable populations differing in biological characteristics such as host-plant preference, geographical range and capacity to transmit plant viruses [2,35]. These so-called 'biotypes' have been characterised through various biochemical and molecular techniques [3,5,6,25,28,56]. There is now growing evidence that some biotypes should be reclassified as distinct species based on the limited gene flow and extensive genetic diversity that exists between them [2,4,10,14,15,31]. Two biotypes in particular, the B and Q biotypes, have attracted attention due to their invasiveness, ability to develop insecticide resistance and the economic damage they inflict upon cropping systems.

The first report of imidacloprid resistance came from Q biotype *B. tabaci* originating from the Almeria region of southern Spain in 1994 [9]. Resistance in this area of intensive horticultural production

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increased gradually to over 100-fold by the year 2000 following repeated systemic and foliar applications [40]. The first documented case of imidacloprid resistance in field collected B biotype populations was from Guatemala [7]. Continued reliance on neonicotinoids for *B. tabaci* control has led to widespread reports of resistance affecting most compounds in this class [23,41,34,51,58]. Resistance management tactics have been implemented in some countries as part of integrated pest management (IPM) programmes in order to preserve the efficacy of neonicotinoids against whiteflies [22,46].

A target-site resistance mutation in nAChR subunits has been reported from a laboratory selected neonicotinoid resistant strain of *N. lugens* [32]. However, this mutation has not been found in resistant strains collected from the field [48]. On recent evidence, it is clear that neonicotinoid resistance is at present based largely on P450-dependent monooxygenases (P450s). An increase in overall P450 activity was demonstrated along with the presence of the 5-hydroxy imidacloprid metabolite (IMI-5-OH) in resistant whitefly strains [40,50]. Furthermore, in ligand binding experiments using [^{3}H]imidacloprid, the lack of any difference between resistant Q biotypes and a susceptible strain of *B. tabaci* suggested an absence of any altered nAChRs [50]. Karunker et al. [26] isolated several P450 cDNAs from *B. tabaci* representing the *CYP4* and *CYP6* families and characterised their expression in resistant and susceptible insects. This demonstrated a strong correlation between mRNA levels of one of these genes, *CYP6CM1*, and neonicotinoid resistance in B and Q biotypes. Moreover, in the same study, three SNPs in the first intron of the Q biotype *CYP6CM1* allele (*CYP6CM1-Q*) were associated with reduced susceptibility to imidacloprid. Subsequently, the Q biotype *CYP6CM1* allele (*CYP6 CM1-Q*) was heterologously expressed *in vitro* and catalysed a more rapid conversion of imidacloprid to IMI-5-OH [27]. The authors also identified the key amino acids and hydroxylation site within *CYP6CM1-Q*.

It is generally assumed that resistance to insecticides is expressed consistently throughout the life cycle of an insect. Recently, Nauen et al. [43] demonstrated that laboratory susceptible nymphs of *B. tabaci* were 4–10 times more sensitive to imidacloprid compared with their adult counterparts. In the same study, resistance levels were significantly reduced in second/third instar nymphs (N2/N3) from resistant field strains, to levels close to those of susceptible adults [43]. Here, we explore this age-dependency further by quantifying expression of *CYP6CM1* across different life-stages in two Q biotype and two B biotype strains of *B. tabaci* using a specific gene expression assay. In addition, adult whiteflies from each strain were subject to imidacloprid treatment and the hydroxy metabolite was detected and quantified using liquid chromatography–mass spectrometry (LC–MS).

2. Materials and methods

2.1. Whitefly strains and insecticides

The Q biotype strains used throughout the study were ALM07 (Spain 2007) and CRT-1 (Crete 2006) and the B biotype strains were GRB/MEX (USA/Mexico 2006) and PIRGOS (Cyprus 2003). Insects were reared at Rothamsted Research on cotton plants (*Gossypium hirsutum* cv. Linda) at 26 ± 2 °C under a 16 h photoperiod. The biotype status of each strain was determined using the B/Q TaqMan® allelic discrimination assay described by Jones et al. [25]. Formulated imidacloprid ('Confidor', 25% SL, Bayer CropScience, Monheim) was used in all leaf-dip bioassays. Serial dilutions were made in 0.01% of the non-ionic wetter Agral® (Syngenta).

2.2. Leaf-dip bioassays

Resistance of adult *B. tabaci* to imidacloprid was measured using a leaf-dip bioassay [8]. Leaf discs (40 mm wide) were cut from intact cotton (*G. hirsutum* cv. Linda) leaves and immersed for 10 s in solutions of imidacloprid diluted to the required concentration. Control discs were dipped in the diluent only. Leaf discs were placed adaxial surface down onto a bed of agar held in a small, plastic Petri dish and allowed to dry. Approximately 20 adult female whiteflies were aspirated onto each disc and all concentrations were assayed in triplicate. Bioassays were incubated at 28 °C and mortality scored after 72 h.

Resistance in nymphs was characterised using a bioassay described by Nauen et al. [43]. Leaves on 15-day old cotton plants were cut into rectangles approximately 40 × 50 mm and 30–50 female *B. tabaci* were left to oviposit on each leaf for 24 h. Adults were removed and eggs left to hatch and develop to the N2 stage for 9 days. Leaves plus nymphs were then dipped into imidacloprid diluted to the required concentration in water plus Agral®. Control leaves were dipped in the diluents only. The number of nymphs per leaf varied between 20 and 200 due to differences in fecundity between strains and the number of ovipositing females. Each bioassay was done in triplicate and incubated at 28 °C. Mortality was scored 20 days following oviposition with individuals failing to emerge as adults scored as 'dead'.

2.3. RNA extraction

Total RNA for gene expression was extracted from 150 adults (mixed sex), pupae or N1/N2 nymphs from each strain using TRIzol® reagent (Molecular Research Inc.) following the suppliers guidelines. Live insects were snap-frozen in liquid nitrogen and ground using a homogeniser in a 1.5 ml sterile microcentrifuge tube. Adults and pupae were extracted at one-half scale of the recommended protocol whereas N1/N2 nymphs were extracted at one-tenth scale. RNA was re-suspended in 15 μl of diethylpyrocarbonate (DEPC)-treated water, treated with 1 μl of DNase I (Promega) to remove any contaminating gDNA and the samples phenol–chloroform extracted. RNA samples were quantified using the ND-1000 spectrophotometer (NanoDrop Technologies Inc.) and the integrity confirmed using a denaturing agarose gel.

2.4. cDNA synthesis

First strand cDNA was synthesised from total RNA using Superscript II Reverse Transcriptase (Invitrogen) following the suppliers' guidelines. For amplification of *CYP6CM1* and *actin*, 1 μl of oligo(dT)$_{20}$ (500 μg/ml) was added to each reaction. To improve homogeneity between biological replicates for relative gene expression the concentration of input RNA was standardised (1 μg). RNaseOUT® (1 μl, Invitrogen UK) was added to each reaction to protect mRNA from RNase activity and improve cDNA yield. Superscript II RT enzyme (1 μl) was then added to a final volume of 20 μl and each cDNA synthesis reaction was performed at 42 °C for 60 min. The cDNA was stored at −20 °C and diluted to the required concentration for gene expression in nuclease-free sterile distilled water.

2.5. Relative gene expression

Here, the comparative C_t method (also known as the $\Delta\Delta C_t$ or $2^{-\Delta\Delta C_t}$ method) was used to quantify *CYP6CM1* expression in each of the four strains in adults, pupae and nymphs. $2^{-\Delta\Delta C_t}$ uses an arithmetic formula to calculate the relative changes in gene expression based on the threshold cycle (C_t) of the real-time PCR reactions [33]. A constitutively expressed endogenous control gene is required to normalise target quantities and as a means of correcting data that may be affected by differences in cDNA input. Following an assessment of both *actin* and *18S rRNA* as potential