Characterisation of knockdown resistance to pyrethroid insecticides in *Plutella xylostella*

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Abstract

A combination of toxicological, electrophysiological and molecular studies confirmed target site insensitivity (often termed knockdown resistance or *kdr*) to be at least partly responsible for high and stable pyrethroid resistance in a Taiwanese strain of the diamondback moth, *Plutella xylostella*. Non-synergizable cross-resistance to a range of pyrethroids and DDT, as well as incompletely recessive autosomal inheritance of the resistance trait, provided indirect evidence for the presence of *kdr* in this strain. A larval neuromuscular preparation was used to assess spontaneous miniature excitatory post-synaptic potentials (mEPSP) and evoked EPSP's in response to varying concentrations of the type II pyrethroid deltamethrin. Intracellular recordings revealed a pyrethroid-induced increase in mEPSP activity and a decline in the EPSP amplitude, responses which were induced at considerably higher concentrations in resistant larvae when compared to larvae of a susceptible standard strain. These findings were supported by the detection of an amino acid substitution in the voltage-sensitive sodium channel (the primary target site of pyrethroids) of the resistant strain, which has previously been shown to correlate with *kdr* in the housefly, *Musca domestica*.

Key words: *Plutella xylostella*, pyrethroids, knockdown resistance (*kdr*), neurophysiology, sodium channel gene

Introduction

The ability of the diamondback moth, *Plutella xylostella* (L.), (Lepidoptera: Yponomeutidae) to develop high levels of resistance to pyrethroid insecticides is well documented (Talekar, 1992). Detoxification by mixed function oxidases (mfo) has been considered to be the major mechanism involved in this type of resistance (Sun, 1992) although indirect evidence has also accumulated for the widespread occurrence of reduced nerve sensitivity to pyrethroids (Cheng, 1988; Liu *et al.*, 1981, 1982a, 1982b; Miyata *et al.*, 1992). This type of resistance was originally described for houseflies (*Musca domestica* L.) under the name knockdown resistance (*kdr*) (Busvine, 1951; Milani, 1954). *Kdr* and a second more potent type of nerve insensitivity, termed *super-kdr*, are thought to result from structural changes in the voltage-gated sodium channel, the primary target site for pyrethroids and DDT in the insect nervous system (Bloomquist, 1996). Recently, two mutations in the *para*-type sodium channel gene of the housefly have been linked to the occurrence of *kdr* and *super-kdr* resistance in this insect (Williamson *et al.*, 1996). Moreover, one of these mutations has also been reported in a *kdr* strain of the German cockroach (*Blattella germanica* L.) (Miyazaki *et al.*, 1996). Although nerve insensitivity to pyrethroids has been investigated in other insects (reviewed in Soderlund and Bloomquist, 1990), housefly and German cockroach remain the only species where this type of resistance has been characterised at the molecular level.

The first direct evidence for the presence of *kdr*-type resistance in the diamondback moth was provided by Hama *et al.* (1987) who demonstrated electrophysiologically a reduced sensitivity of the central nerve cord in larvae of pyrethroid resistant Japanese strains. We now report evidence for the presence of *kdr*-type resistance in a pyrethroid resistant strain of the diamondback moth from Taiwan based on results obtained with toxicological, electrophysiological and molecular techniques.

Toxicological studies

Pyrethroids and DDT in acetone were applied topically to fourth instar larvae of two diamondback moth strains. The susceptible strain (Rothamsted) had been maintained in laboratory culture for over 30 years without insecticide selection. The pyrethroid resistant strain, FEN (formerly FP), was obtained from C. N. Sun in 1995. FEN was collected in Taiwan in 1983 and subsequently selected with fenvalerate (Chen and Sun, 1986). Topical bioassays at Rothamsted with a range of pyrethroids confirmed a high level of pyrethroid resistance previously reported in this strain (Chen and Sun, 1986; Yac *et al.*, 1988). Resistance to pyrethroids possessing an α-cyano-3-phenoxybenzyl alcohol moiety, such as fenvalerate and deltamethrin, was impossible to quantify with resistance factors exceeding 10,000 *(Table 1)*. Although mortality was

Table 1. Susceptibility to pyrethroids and DDT of larvae of a susceptible (Rothamsted) and a resistant (FEN) diamondback moth strain

Compound	Strain		RF[b]
	Rothamsted LD_{50} (µg/larva)[a]	FEN LD_{50} (µg/larva)	
Fenvalerate	0.003	100µg=0%	>33 000
Deltamethrin	0.001	10µg=6%	>10 000
Bioresmethrin	0.006	c. 10	1 700
Cismethrin	0.004	c. 10	5 000
DDT	0.92	10µg=0%	>10

[a]Topical application to fourth instar larvae, assessment of mortality five days after treatment.
[b]Resistance factor = LD_{50} of FEN/LD_{50} of Rothamsted strain

still observed with pyrethroids based on a 5-benzyl-3-furylmethyl alcohol, such as bioresmethrin and cismethrin, the resistance factors were still extremely high (1700–5000) *(Table 1)*.

Piperonyl butoxide (PB) and a range of other mfo inhibitors, including PBX, Niagara 16824, TCPB and *m*-nitro propargyl ether, were tested with fenvalerate. When applied topically to larvae 30–60 min prior to the insecicides, synergism was unexpectedly low. PB was the most effective synergist but only resulted in up to 59% mortality at a dose of 100 µg fenvalerate per larvae (compared to a LD_{50} of 0.003 µg for susceptible larvae). In contrast, Chen and Sun (1986) had been able to reduce the LD_{50} of FEN for fenvalerate (spray application) from >100 mg/ml to 5.5 mg/ml (synergism ratio of >18) by pretreatment with PB. At present it remains unclear why mfo inhibitors were ineffective in synergising pyrethroids in our study. DDT was also ineffective against FEN *(Table 1)*. Resistance to DDT was not synergisable by PB or FDMC, an inhibitor of DDT-dehydrochlorinase.

Virgin adults of the two strains were crossed and larvae of the F_1 generation tested by topical application of insecticides. LD_{50} values were drastically lower than for the FEN strain, with resistance factors of 20 and <5 for fenvalerate and bioresmethrin, respectively. There was no significant difference between reciprocal crosses, leading to the conclusion that pyrethroid resistance in FEN was a largely recessive autosomal trait, confirming previous results with other diamondback moth strains (Hama *et al.*, 1987; Liu *et al.*, 1981; Kim *et al.*, 1991, Miyata *et al.*, 1992; Motoyama *et al.*, 1992). No selection pressure was applied to the FEN strain after its arrival in the UK in 1995, and no decrease in resistance has since been observed over 25 generations, indicating a high level of homozygosity of the resistance genes.

Electrophysiological assay for nerve insensitivity

The central nerve cord and the ventral internal lateral (VIL) muscles of decapitated fourth instar diamondback moth larvae were exposed by dissection under saline. Stimulation of the segmental ganglion using a suction electrode evoked excitatory post-synaptic potentials (EPSP) which were recorded intracellularly in the adjacent segmental muscle.

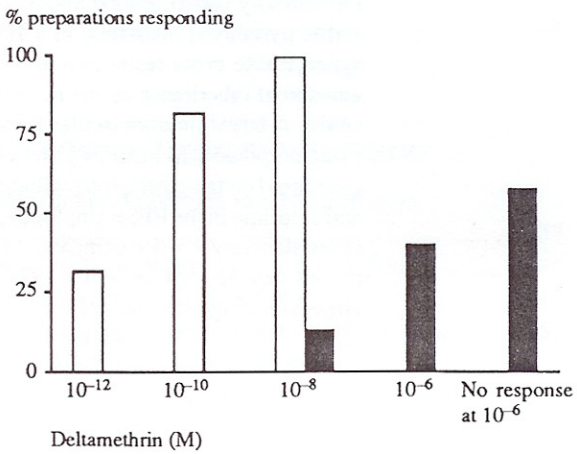

Figure 1. Distribution of neuronal responses of fourth instar diamondback moth larva nerve muscle preparations treated with increasing doses of deltamethrin (❑ Rothamsted strain, ■ FEN strain, n=22)

Preparations were perfused with concentrations of deltamethrin ranging from 10^{-12}M to 10^{-6}M for 10 min at each concentration. Recordings revealed that deltamethrin induced a decline in the EPSP amplitude and an increase in miniature EPSP activity. Over 80% of susceptible larvae responded at a concentration of 10^{-10}M deltamethrin or lower *(Figure 1)*. Much higher concentrations were necessary to elicit a response in FEN larvae. Only 14% of FEN larvae reacted at 10^{-8}M deltamethrin rising to only 41% at the highest dose (10^{-6}M). Sixty percent of FEN larvae did not respond to the highest deltamethrin dose. EC_{50} values were estimated at 10^{-11}M and 10^{-6}M for the susceptible and the FEN strains, respectively, a resistance ratio of over 300,000-fold. The electrophysiological assay thus demonstrated a high level of nerve insensitivity in the FEN strain.

Molecular study

Molecular cloning studies of the *para*-type sodium channel gene in the housefly (Williamson *et al.*, 1996) and German cockroach (Miyazaki *et al.*, 1996) have identified two amino acid changes in the channel sequence that correlate with *kdr* resistance phenotypes. Both changes are located in the domain II region of the channel and involve: 1) a leucine to phenylalanine (Leu to Phe) substitution in the hydrophobic IIS6

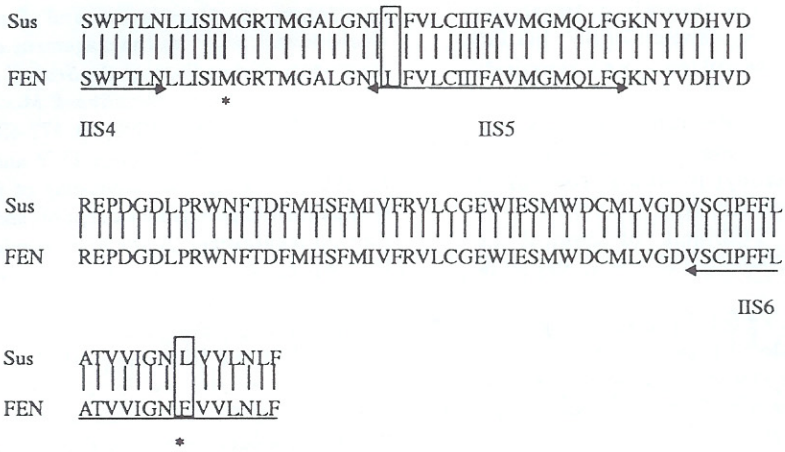

Figure 2. Alignment of amino acid sequences of the IIS4–IIS6 sodium channel region of the Rothamsted (Sus) and the pyrethroid resistant FEN strain of the diamondback moth. Mutations in the diamondback moth are highlighted in boxes whereas mutations associated with kdr resistance in the housefly are indicated by stars.

transmembrane segment (found in *kdr* and *super-kdr* houseflies and *kdr* cockroaches), and 2) a methionine to threonine (Met to Thr) substitution within the intracellular loop between IIS4 and IIS5 (only found in *super-kdr* houseflies). We therefore examined this region of the sodium channel in the Rothamsted and FEN strains of diamondback moth to determine whether either or both of these changes were also associated with resistance in this species.

Degenerate PCR primers were based on conserved sequences of vertebrate and invertebrate sodium channel, enabling selective amplification of the IIS4–IIS6 region of the channel from any insect species. Total RNA was extracted from 4th instar larvae of Rothamsted and FEN strains and cDNA synthesised as the template for PCR. cDNA was used in preference to genomic DNA because the *para* gene contains several introns in this region (Loughney *et al.*, 1989), one of which disrupts the 3' primer site. Following two rounds of PCR with the degenerate primers, discrete fragments of the expected size (350bp) were obtained for both strains which were later cloned and sequenced. The fragments encoded amino acid sequences with close identity to the corresponding region of housefly (94%) and cockroach (89%) *para* sodium channels. An alignment of the Rothamsted and FEN sequences *(Figure 2)* revealed two amino acid differences in this region. One was the same Leu (Rothamsted) to Phe (FEN) substitution in IIS6 channel sequence as has been previously reported for housefly and cockroach *kdr* strains *(Figure 2)*. The second change, however, was different to that in *super-kdr* housefly strains, revealing a threonine (Thr) (Rothamsted) to isoleucine (Ile) (FEN) substitution at the beginnning of the IIS5 segment *(Figure 2)*.

The identification of the same Leu to Phe substitution in the sodium channel sequence of FEN insects further consolidates the association of this mutation with *kdr*-type resistance and indicates an important role in conferring the nerve insensitive phenotype of this strain. The significance of the second mutation (Thr to Ile) is still unclear since this has not been reported previously and may simply represent a polymorphism with no relevance to the pyrethroid resistance. However, its close proximity to the housefly Met to Thr substitution, and the high degree of conservation of the Thr residue in other sodium channel sequences (vertebrate and invertebrate), may indicate a more direct role for this mutation in contributing to the strong resistance of the FEN strain.

Conclusion

The high resistance of the FEN strain to pyrethroids is at least partially based on a marked decrease in nerve sensitivity to these compounds. Knockdown resistance in this strain is characterised by cross resistance to pyrethroids and DDT, little or no synergism of pyrethroid and DDT resistance, incompletely recessive autosomal inheritance, over 300 000-fold reduced nerve sensitivity in an electrophysiological assay, and the presence of the housefly *kdr*-type mutation in the otherwise highly conserved sodium channel gene. The low level of synergism of pyrethroids by mfo inhibitors, although in line with the presence of knockdown resistance, does not conform with previous work on the FEN strain and needs further investigation. This work presents the first report of the housefly-*kdr* mutation (Leu to Phe) in a crop pest species. We are presently investigating the distribution of this mutation in the diamondback moth by analysing strains from different parts of the world. This will also help to determine whether the second mutation (Thr to Ile) correlates with the *kdr* resistance phenotype in this insect.

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