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ScienceDirect
Insect Biochemistry and Molecular Biology 37 (2007) 184–188
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

Short communication

A *Dα6* knockout strain of *Drosophila melanogaster* confers a high level of resistance to spinosad

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Received 24 October 2006; received in revised form 21 November 2006; accepted 21 November 2006

Abstract

A null mutation of the nicotinic acetylcholine receptor (nAChR) subunit *Dα6*, in *Drosophila melanogaster*, confers 1181-fold resistance to a new and increasingly important biopesticide, spinosad. This study's molecular characterisation of a spinosad resistance mechanism identifies *Dα6* as a major spinosad target in *D. melanogaster*. Although *D. melanogaster* is not a major field pest, target site resistances found in this species are often conserved in pest species. This, combined with the high degree of evolutionary conservation of nAChR subunits, suggests that mutations in *Dα6* orthologues may underpin the spinosad resistance identified in several economically important field pests.

Keywords: Spinosad; Nicotinic acetylcholine receptor; Insecticide; *CyO*

1. Introduction

The evolution of resistance to different insecticides has made effective control of pests increasingly dependent on the use of new classes of chemicals. One such group, the spinosyns, derive from fermentation products of *Saccharopolyspora spinosa* which have varying degrees of insecticidal activity (Sparks et al., 1995; Bret et al., 1997). *S. spinosa* produces a large number of these secondary metabolites. Two of these form the commercialised insecticide formula, spinosad (85% spinosyn A :15% spinosyn D) (marketed as Success[1]). Efficacy has been reported against *Diptera*, *Lepidoptera* and *Thysanoptera* as well as many other insects (Bret et al., 1997; Thompson et al., 1995).

The targets of spinosyn A and D are thought to be the nicotinic acetylcholine receptors (nAChRs) with some evidence to suggest additional targeting to the GABA receptor (Salgado, 1997; Watson, 2001). Involuntary contractions and neuron over-excitation are initial responses to spinosad with paralysis and death following prolonged exposure. This is due to exhaustion of the firing capability of the neurons and not through damage to the nervous system (Salgado, 1998). While both spinosyns and the neonicotinoid class insecticides target nAChRs, spinosyns do not displace a neonicotinoid (imidacloprid) from receptors. This suggests that these insecticides bind to non-overlapping sites (Salgado, 1997) and/or may bind to separate classes of nAChR's (Salgado and Saar, 2004).

High-level resistance to spinosad has been documented in field populations of several pest species including *Spodoptera exigua* (Beet armyworm, >345-fold) (Wang et al., 2006), *Plutella xylostella* (Diamondback moth, 20,600-fold) (Sayyed et al., 2004; Zhao et al., 2002), *Heliothis virescens* (Tobacco budworm, 669-fold) (Wyss et al., 2003), and *Musca domestica* (Housefly, >150-fold) (Shono and Scott, 2003). This study characterises a recessive spinosad resistance (1181-fold) in a strain of *Drosophila melanogaster*. This resistance is caused by a loss of function mutation in an nAChR subunit gene, *Dα6*. Loss of function mutations in *Dα6* orthologues may lead to spinosad resistance in field populations of insect pests.

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doi:10.1016/j.ibmb.2006.11.009

2. Materials and methods

D. melanogaster strains used in this study were: the control strain, *y*; *cn bw sp*, deficiencies, {*w*; Df(2L)s1402, P{w^{+mC} = lacW}s1402/*CyO*} and Df(2L)Exel6025/*CyO* which were sourced from the Bloomington Stock Centre, USA. They were maintained at 25 °C and reared on standard semolina media.

The insecticide formulation, spinosad (120 g/l) was added to media after appropriate dilutions in water for screening. Survival to adult emergence was recorded for a range of spinosad concentrations from 0 to 100 ppm. Three replicates of 50 first instar larvae were placed in vials of the food and kept in the dark at 25 °C. Analysis was performed using the PriProbit program (v1.63) (Sakuma, 1998) to determine lethal concentration values. For the molecular analysis genomic DNA was purified using a Qiagen gDNA kit as per manufacturers instructions. Primer sequences are presented in Supplementary Table 1 and were synthesised by Invitrogen. PCR reactions were carried out using Taq DNA polymerase (Promega), as per manufacturers instructions with thermocycler conditions of an initial cycle of 95 °C 3 min, followed by 35 cycles of 95 °C 30 s, 60 °C 30 s, 72 °C 3 min and a final step at 72 °C for 10 min. Long PCR was performed using the Jumpstart Accutaq LA DNA polymerase (Sigma) as per manufacturers instructions with thermocycling conditions of 94 °C 3 min, then 35 cycles of 94 °C 30 s, 60 °C 30 s, 68 °C 12 min followed by 68 °C for 20 min. Agarose gels (1% w/v) were run for 1 h at 100 V and imaged through exposure to UV light and a digital camera (Kodak EDAS 290). Big Dye v3.1 (Applied Biosystems) was used for sequencing reactions and the sample was analysed at the Australian Genome Research Facility (Parkville). The sequence output was analysed using Sequencher v4.6 (Genecodes corporation).

3. Results

Deficiency strains hemizygous for known nAChR subunit genes in *D. melanogaster* were screened with spinosad (not shown). The Df(2L)s1402/*CyO* strain was identified as being resistant. Dosage mortality analysis showed that the Df(2L)s1402/*CyO* strain was 1181-fold resistant to spinosad compared to a susceptible strain, *y*;*cn bw sp* (Table 1). The *Dα6* nAChR subunit gene is located in the region deleted on the deficiency chromosome. Investigation of resistance status of heterozygotes revealed that the trait was incompletely recessive ($D = -0.83$ calculated using Stone's formula—Stone, 1968). Both the *CyO* and deficiency chromosomes from the Df(2L)s1402/*CyO* strain were slightly less than two-fold resistant in heterozygous combinations with chromosome 2 from the susceptible *y*;*cn bw sp* strain (Table 1). This suggested that the *CyO* chromosome also carries a mutation at the spinosad resistance locus. A second strain, Df(2L)Exel6025/*CyO*, deleted for *Dα6* and 11 other genes, also exhibited recessive resistance (data not shown). Given the recessive inheritance and published evidence that it targets nAChRs, *Dα6* was a strong candidate for the resistance gene. This hypothesis was tested by looking for a mutation in the *Dα6* allele on the *CyO* chromosome.

Table 1
Spinosad LC99 (ppm) (with 95% confidence interval) and fold resistance for Df(2L)s1402/*CyO*, *y*;*cn bw sp* and their F1 heterozygotes

	LC99	95% CI	Fold resistance
y;*cn bw sp*	0.08	0.06–0.10	1.0
y;*cn bw sp*/*CyO*	0.16	0.12–0.23	2.0
y;*cn bw sp*/Df(2L)s1402	0.13	0.10–0.19	1.7
Df(2L)s1402/*CyO*	90.76	66.16–137.40	1181

Sequencing of the *Dα6* coding region from *CyO* did not reveal any amino acid substitutions or nonsense mutations as compared to the published *D. melanogaster* genome sequence. The structure of the *Dα6* gene was then examined with long PCR. Primers were designed to allow the amplification of introns (Supplementary Table 1) but the region between exons 8a and 8b (for nomenclature see Sattelle et al., 2005) failed to amplify (Fig. 1).

Inverse PCR of this region using gDNA digested with EcoRI (Promega) and primers nar30d10AF and nar30d8R (Supplementary Table 1) showed that one of the inversions on the *CyO* chromosome fused *Dα6* from exon 8b (cytologically located at 30D) to the 5′ end of *Prosap*, (a gene at cytological location 50C) (Fig. 2). There was also an internally deleted remnant of a *pogoR11* transposable element on the *Dα6* side of the inversion.

This fusion was confirmed using primers designed to *Dα6* and *Prosap* (Supplementary Table 1) which successfully amplified gDNA from *CyO*, but not from the wild-type strain, *y*;*cn bw sp* (Fig. 3). This result agrees with previously reported polythene chromosome analysis of the *CyO*

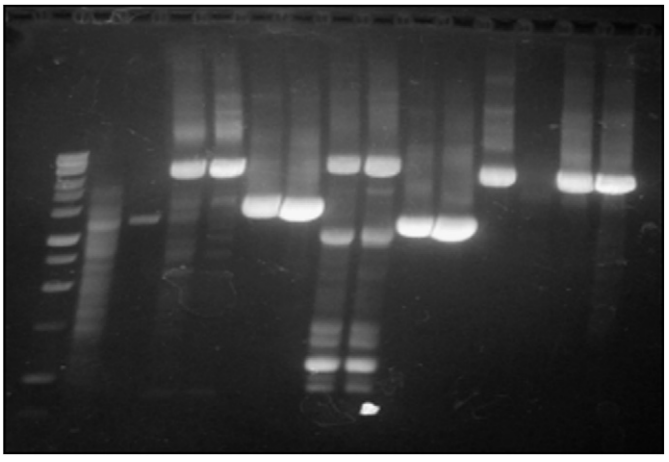

Fig. 1. Molecular analysis of the *CyO Dα6* locus. Long PCR analysis of *Dα6*, detected a non-amplifying fragment of DNA between exons 8b and 8c (lane 13) in the resistant Df(2L)s1402/*CyO* strain. Lane 1—1 kb ladder (Promega), Lanes 2,4,6,8,10,12,14—*y*;*cn bw sp*, Lanes 3,5,7,9,11,13,15—Df(2L)s1402/*CyO*. Primer sequences for each fragment can be found in Supplementary Table 1.