

Transcripts of the nicotinic acetylcholine receptor subunit gene *Pxyl α 6* with premature stop codons are associated with spinosad resistance in diamondback moth, *Plutella xylostella*

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Received: 16 February 2010 / Accepted: 27 April 2010 / Published online: 25 May 2010
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Abstract The cDNA sequence of the $\alpha 6$ nicotinic acetylcholine receptor subunit of diamondback moth (*Plutella xylostella*) was cloned and sequenced. Transcripts were similar between the spinosad-susceptible G88 and Wapio strains. All transcripts from the spinosad-resistant Pearl-Sel strain contained premature stop codons, and most transcripts have not been previously reported. None of these truncated transcripts were seen in the spinosad-susceptible strains. Proteins made from these transcripts would likely have no, or greatly altered, receptor function. An F_2 backcross and spinosad bioassay showed that all spinosad bioassay survivors produced truncated $\alpha 6$ transcripts. Thus, it appears that spinosad resistance in diamondback moth is due to a mutation(s) that results in no functional *Pxyl α 6* being produced.

Keywords Nicotinic acetylcholine receptor · Alternative splicing · Insecticide resistance · *Plutella xylostella* · A-to-I RNA editing

Introduction

Nicotinic acetylcholine receptors (nAChRs) form cationic-selective, ligand-gated ion channels that are involved in

cholinergic transmission in the insect central nervous system. nAChRs can be homopentamers of α subunits (Fayyazuddin et al. 2006) or heteropentamers of both α and β subunits (Thany et al. 2006). Sequencing insect genomes has revealed 10–12 subunit genes in insects (Sattelle et al. 2005; Jones et al. 2006; Shao et al. 2007). Insect nAChR subunit genes utilize alternative exons, A-to-I RNA editing, and other post-transcriptional modifications to generate a diverse pool of transcripts from the 10 to 12 subunit genes in each of their genomes (Sattelle et al. 2005; Rinkevich and Scott 2009). Transcripts with premature stop codons, which either have some unknown function or are targets for nonsense-mediated decay (Chang et al. 2007), have been noted in *Drosophila melanogaster* (Grauso et al. 2002), *Apis mellifera* (Jones et al. 2006), *Musca domestica* (Gao et al. 2007b) and *Tribolium castaneum* (Rinkevich and Scott 2009).

The spinosyns, which include spinosad, are a modern and expanding class of insecticides (Crouse et al. 2001; Salgado and Sparks 2005). They are highly regarded for their effectiveness against insect pests, minimal environmental impact and low vertebrate toxicity (Casida and Quistad 2004; Salgado and Sparks 2005). Although spinosyns exert their toxic effects through interactions with nAChRs (Salgado 1997), they bind to receptors with unique subunit composition (Salgado and Saar 2004) or at locations on the receptor outside of agonist-binding domains (Orr et al. 2009). Studies on *D. melanogaster* have found that loss of *D α 6* results in a strain resistant to spinosad (>1,000-fold) (Perry et al. 2007; Watson et al. 2010).

Diamondback moth (*Plutella xylostella*) is the most important insect pest worldwide of cruciferous vegetables (e.g. cabbage, cauliflower and broccoli) and field crops (e.g. canola), and control of diamondback moth is estimated to exceed \$1,000,000,000 annually (Talekar and Shelton 1993). It is particularly a troublesome pest because

Electronic supplementary material The online version of this article (doi:10.1007/s10158-010-0102-1) contains supplementary material, which is available to authorized users.

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of its ability to evolve resistance to insecticides, including organophosphates (Sayyed et al. 2005), pyrethroids (Kwon et al. 2004), neonicotinoids (Ninsin et al. 2000) and spinosad (Zhao et al. 2006). The first case of spinosad resistance was the Pearl-Sel strain of diamondback moth which was collected from Pearl City, Hawaii in October 2000 from a field where repeated applications of spinosad failed to provide control. Following further spinosad selection in the laboratory, the Pearl-Sel strain developed >13,000-fold resistance. Resistance was found to be recessive, autosomal, monofactorial and could not be overcome with metabolic inhibitors piperonyl butoxide or *S,S,S*-tributyl phosphorotrithionate, suggesting resistance was due to an altered target site (Zhao et al. 2002). A similar pattern of inheritance was seen in the field collected CH₁ strain from the Cameron Highlands of Malaysia with >20,000 fold resistance to spinosad (Sayyed et al. 2004).

Spinosad resistance has also been documented in the tobacco budworm (*Heliothis virescens*) (Wyss et al. 2003) and house fly (*Musca domestica*) (Shono and Scott 2003). In both studies, the patterns of inheritance of spinosad resistance were similar to those in the Pearl-Sel and CH₁ strains of *P. xylostella*.

Herein, we describe the full-length cDNA of the *Pxylα6* subunit from the spinosad-susceptible Geneva 88 and Wapio strains and the spinosad-resistant Pearl-Sel strain of diamondback moth. We describe the differences in cDNA sequences between diamondback moth strains that may act as the resistance mechanism. An F₂ backcross and bioassay were performed to demonstrate genetic association of transcript differences with spinosad resistance.

Materials and methods

Insects

Three strains of diamondback moth were used in this experiment. The Geneva 88 (G88) strain is insecticide susceptible (Zhao et al. 2002). The Pearl-Sel strain is >18,000-fold resistant to spinosad (Zhao et al. 2002). The Wapio strain is resistant to indoxacarb and permethrin, but not spinosad (Chen and Zhao, unpublished). Colonies were reared on an artificial diet and environmental conditions as previously described (Shelton et al. 1991). Five individual fourth instar larvae were collected in a 1.5-ml centrifuge tube with 200 μl of absolute ethanol and stored at –80°C. Prior to collection, the Pearl-Sel strain was treated with a discriminating dose (10 ppm) of spinosad (Zhao et al. 2002), and survivors were collected and stored in ethanol at –80°C. Larvae were rehydrated by replacing the ethanol with decreasing concentrations of ethanol (95, 70, 35 and 0%) every 2 min before RNA extraction.

Reverse transcription, PCR and cloning

Total RNA was extracted from five larvae using 1 ml of TRIzol as described by the manufacturer (Invitrogen, Carlsbad, CA). cDNA was synthesized with 5 μg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's directions, except the reverse transcription step was extended to 3 h.

Degenerate primers were designed based on conserved amino acid residues in the α6 subunits from an alignment of Agamα6 (AY705401), Amelα6 (NM_001080095), Bmα6 (NM_001098372), Dα6 (NM_164874) and Tcasα6 (EF526088). MaF2 and MaR1 were also used (Gao et al. 2007b). The *PxylActinF/R* primer pair amplified a 500-bp fragment of actin cDNA (AB282645) that was used as a positive control in every PCR. Primer sequences are shown in Table S1. PCR was performed using the following iCycler thermocycler program (Bio-Rad, Hercules, CA): initial denaturation 95°C for 2 min; 35 cycles of 95°C for 30 s, 46°C for 30 s and 72°C for 90 s; final extension of 72°C for 10 min. PCR products were separated on a 1% agarose gel stained with ethidium bromide (10 μg/ml) and visualized under UV light. The PCR products resulting from the primer pair *Pxylα6ProF8/MaR1* was purified using Wizard PCR Purification kit (Promega, Madison, WI). Purified PCR products were cloned into pGEM-T (Promega, Madison WI) according to the manufacturer's directions with an overnight ligation at 4°C. JM109 competent cells were transformed with 2 μl of the ligation reaction according to the manufacturer's directions. Individual colonies were spotted to a fresh plate and then screened for positive inserts using the colony directly in a 15-μl PCR with GoTaq (Promega, Madison, WI) and T7 and SP6 primers (Table S1). Thermocycler conditions were as follows: initial denaturation 95°C for 2 min; 35 cycles of 95°C for 30 s, 42°C for 30 s and 72°C for 60 s; final extension of 72°C for 10 min. PCR products were visualized on an agarose gel as described above. Colonies with the correct insert were grown in 3 ml of Luria-Bertani medium supplemented with ampicillin at a final concentration of 50 μg/ml. Plasmids were purified using PureYield Miniprep kit (Promega, Madison WI). Plasmids were sequenced in both directions using T7 and SP6 primers at Cornell University's Biotechnology Resource Center. Sequences were aligned using MegAlign (Lasergene, Madison, WI).

Gene-specific primers *Pxyla6A*, *Pxyla6AR*, *Pxyla6B* and *Pxyla6BR* were designed based on the sequence obtained from the above protocol (Table S1). 5' and 3' RACE was performed using FirstChoice RLM-RACE (Ambion, Austin, TX). 5' RACE was performed using *Pxyla6BR* and the kit provided 5' RACE-outer primer in the first round of PCR (initial denaturation 95°C for 2 min;

35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 3.5 min; final extension of 72°C for 10 min) in 100- μ l PCRs using GoTaq. Five microliters of the first-round product was used in a second-round 100- μ l PCR using Pxyla6AR and the kit provided 5' RACE-Inner primer using the same thermocycler conditions as above. PCR products were visualized, purified, cloned and sequenced as described above.

3' RACE reverse transcription was performed using FirstChoice RLM-RACE according to the manufacturer's directions. Two microliter of the reverse transcription product was used in a 50- μ l first-round PCR with the Pxyla6A primer and the kit provided 3' RACE-outer primer. Thermocycler conditions were as follows: initial denaturation 95°C for 2 min; 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1.5 min; final extension of 72°C for 10 min. A second-round reaction was performed using 5 μ l of the first-round reaction and the Pxyla6B and the kit provided 3' RACE-inner primers. Thermocycler conditions were as described above. PCR products were visualized, purified, cloned and sequenced as described above.

Alignments of the 5' and 3' RACE products yielded the tentative full-length cDNA. Primers were designed based on the ends of the tentative full-length cDNA (PxylA65UTRF, PxylA65UTRF2, PxylA63UTRR and PxylA63UTRR2). First-round PCR was performed in 50- μ l GoTaq reactions using 2 μ l of cDNA with the primer combination of PxylA65UTRF and PxylA63UTRR and the following thermocycler conditions: initial denaturation 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2 min; final extension of 72°C for 10 min. Second-round PCR was performed in 100- μ l GoTaq reactions using 5 μ l of first-round PCR product with the primer combination of PxylA65UTRF2 and PxylA63UTRR2 and the following thermocycler conditions: initial denaturation 95°C for 2 min; 35 cycles of 95°C for 30 s, 47°C for 30 s and 72°C for 2 min; final extension of 72°C for 10 min. PCR products were visualized, purified, cloned and sequenced as described above. Cloning of full-length cDNAs was performed on at least two independent batches of five larvae for each strain. At least five clones from each batch were sequenced for each strain. Sequences were aligned using MegAlign to identify alternative exon use, putative A-to-I RNA editing, alleles and other transcript modifications. Putative phosphorylation and *N*-linked glycosylation sites were identified using NetPhos 2.0 (Blom et al. 1999) and NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>), respectively.

F₂ backcross and bioassay

The spinosad resistance status of each strain was confirmed before crosses were initialized (Zhao et al. 2006). Pearl-Sel

males were mass crossed with G88 females. Female F₁s were backcrossed with Pearl-Sel males. The resulting offspring were treated with a diagnostic dose of spinosad as previously described (Zhao et al. 2006). A small subset (c.a. 100) of larvae at each generation and treatment were collected and stored in ethanol at -80°C. RNA extraction, reverse transcription, PCR, cloning and sequencing were performed as described above.

Results

Pxyl α 6 from spinosad-susceptible strains

The consensus full-length cDNA and features of the *Pxyl α 6*-deduced amino acid sequence from the G88 and Wapio strains are shown in Fig. 1 (GenBank Accession GQ247883). The 5' and 3' UTRs are 114 and 97 bp, respectively. There was no variation in the length or sequence of both the 5' and 3' UTRs between strains. The amino acid sequence of the protein (deduced from the nucleotide sequence of *Pxyl α 6*, Isoform I) shares highest identity with *D α 6* and *Bmo α 6* (82.4 and 95.4%, respectively) (Table 1), and groups with α 6 orthologs from other species in an alignment of insect nAChRs (Fig. 2). There are potentially eight serine, six threonine and three tyrosine phosphorylation sites and three *N*-linked glycosylation sites on the protein. The length of the open reading frame is variable, due a splice site variation, as discussed below. Neither cassette exons, retained introns, nor premature stop codons were observed in the *Pxyl α 6* transcripts in the G88 and Wapio strains, although these were observed in subunit transcripts from *A. mellifera* (Jones et al. 2006), *D. melanogaster* (Grauso et al. 2002), *M. domestica* (Gao et al. 2007b) and *T. castaneum* (Rinkevich and Scott 2009).

Alternative exons were only observed for exon 3 (3a and 3b). Only isoforms I and III were observed (Fig. 3A and E). A summary of the percent identity of the nucleotide and amino acid sequence of *Pxyl α 6* and the frequency of exon use in each strain is shown in Table 2. The nucleotide sequence of *Pxyl α 6* exon 3a is 66.7% and 88.9% identical compared to *D α 6* exon 3a and *Bmo α 6* exon 3a, respectively. The translated amino acids coded by *Pxyl α 6* exon 3a are 93% and 100% identical to both *D α 6* exon 3a and *Bmo α 6* exon 3a, respectively. The frequency of exon 3a usage was 0.09 in G88 and 1.0 in Wapio. The nucleotide sequence of *Pxyl α 6* exon 3b is 82.2 and 95.6% identical compared to *D α 6* exon 3b and *Bmo α 6* exon 3b, respectively. The translated amino acids coded by *Pxyl α 6* exon 3b are 93% and 100% identical to both *D α 6* exon 3b and *Bmo α 6* exon 3b, respectively. Exon 3b contains a serine phosphorylation site at amino acid position 76 that is

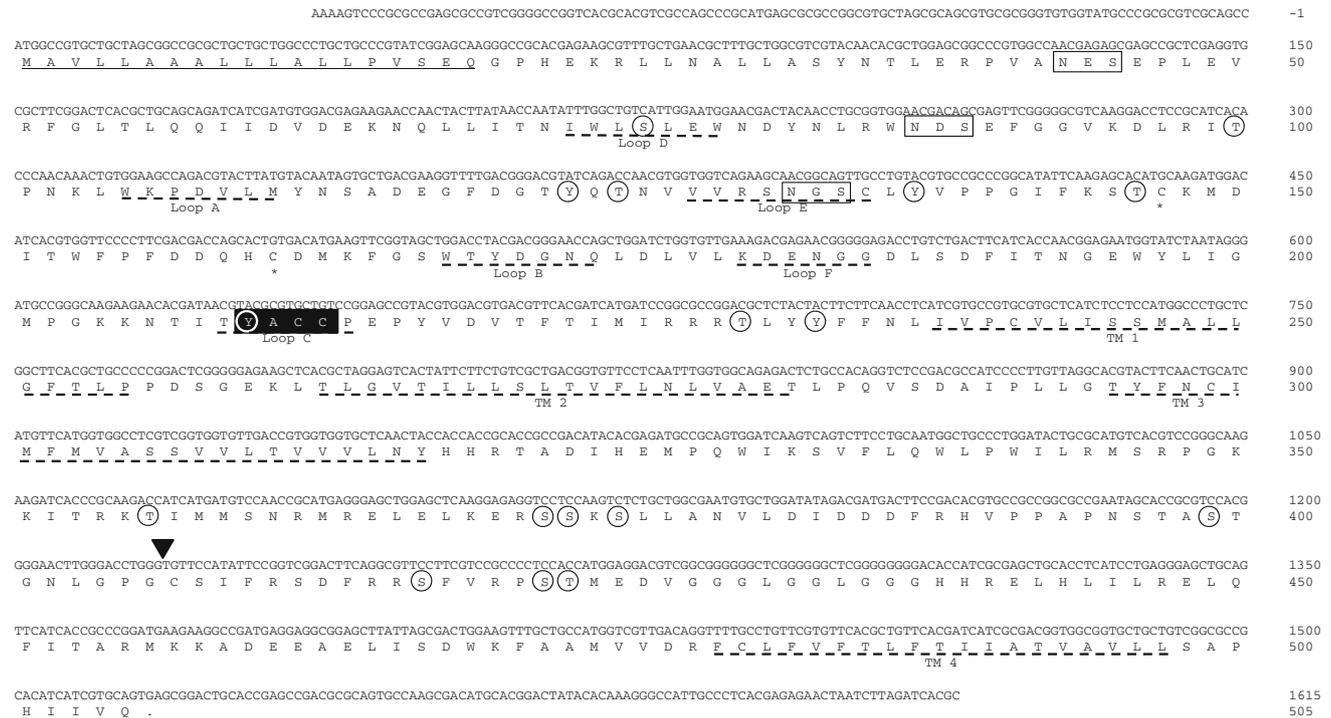


Fig. 1 Nucleotide and deduced amino acid sequence of *PxyIα6* (GenBank Accession GQ228552). Numbers on the right indicate the nucleotide (upper) and amino acid (lower) position. The N-terminal signal peptide is underlined with a solid line. Putative N-glycosylation and phosphorylation sites are boxed and circled, respectively. Ligand-

binding loops and transmembrane domains are underlined with dashed lines. The YxCC motif is shaded and the formation of Cys loop residues are marked with asterisks. The location of the intron 10 splice site variation that adds 10 amino acids to the protein is indicated by an inverted triangle

Table 1 Comparison of the percent identity of the deduced amino acid sequence of *PxyIα6* to *D. melanogaster* and *B.mori* nAChR subunits

	<i>PxyIα6</i>	<i>Bma1</i>	<i>Bma2</i>	<i>Bma3</i>	<i>Bma4</i>	<i>Bma5</i>	<i>Bma6</i>	<i>Bma7</i>	<i>Bma8</i>	<i>Bma9</i>	<i>Bmβ1</i>	<i>Bmβ2</i>	<i>Bmβ3</i>	
<i>PxyIα6</i>		36.4	35.6	36.6	33.6	39.8	95.4	67.4	35.0	17.7	35.8	17.9	13.4	<i>PxyIα6</i>
<i>Dα1</i>	36.8		54.4	56.9	53.3	38.9	37.2	36.9	56.4	19.1	40.1	16.6	14.6	<i>Bma1</i>
<i>Dα2</i>	36.6	52.4		52.0	47.5	34.7	35.7	36.6	50.8	18.4	39.5	16.6	13.1	<i>Bma2</i>
<i>Dα3</i>	37.2	54.9	47.6		69.3	38.0	36.9	39.2	57.3	19.6	41.6	15.3	14.1	<i>Bma3</i>
<i>Dα4</i>	36.2	52.9	44.9	62.9		36.5	36.7	37.0	53.6	18.7	41.5	16.8	14.1	<i>Bma4</i>
<i>Dα5</i>	65.2	34.0	34.2	24.7	33.1		38.6	40.1	36.8	18.5	35.6	16.4	13.1	<i>Bma5</i>
<i>Dα6</i>	82.4	38.1	34.8	36.4	37.4	67.6		66.4	35.7	17.9	35.9	17.1	12.6	<i>Bma6</i>
<i>Dα7</i>	66.4	35.6	37.1	37.3	36.2	74.4	66.6		36.2	17.5	35.0	14.0	13.1	<i>Bma7</i>
<i>Dβ1</i>	36.0	41.2	39.7	43.3	41.6	34.1	35.4	33.7		19.1	39.3	15.3	14.1	<i>Bma8</i>
<i>Dβ2</i>	35.0	54.8	51.7	56.3	54.0	31.5	36.2	35.2	39.0		18.4	24.9	21.9	<i>Bma9</i>
<i>Dβ3</i>	18.1	17.6	19.7	15.6	18.8	16.5	17.6	15.8	17.4	19.9		16.1	14.9	<i>Bmβ1</i>
													23.6	<i>Bmβ2</i>
	<i>PxyIα6</i>	<i>Dα1</i>	<i>Dα2</i>	<i>Dα3</i>	<i>Dα4</i>	<i>Dα5</i>	<i>Dα6</i>	<i>Dα7</i>	<i>Dβ1</i>	<i>Dβ2</i>	<i>Dβ3</i>			<i>Bmβ3</i>

The numbers to the upper right portion of the diagonal line of black boxes indicate the % identity to *B.mori* nAChR subunits. The numbers to the lower left portion of the diagonal line of black boxes indicate the % identity to *D. melanogaster* nAChR subunits

absent in exon 3a. The frequency of exon 3b usage was 0.91 in G88 and 0.00 in Wapio.

There was only one exon 8 (8b) observed. The nucleotide sequence shares 75.9 and 90.8% identity with *Dα6* exon 8b and *Bmoα6* exon 8b, respectively. The translated amino acids coded by *PxyIα6* exon 8b are 100% identical to both *Dα6* and *Bmoα6* exon 8b.

A splice site variation of intron 10 (spliced out 30 bp upstream) was observed at a frequency of 0.13 in G88 and 0.00 in Wapio. This splice site variation added an

additional 10 amino acids to the protein in the middle of the intracellular linker between TM3 and TM4. These amino acids add two putative serine phosphorylation sites. This insertion was seen in isoforms Ia and IIIa (Fig. 3B, F).

A-to-I RNA editing was investigated at 16 previously identified RNA-editing sites (Tian et al. 2008). Putative A-to-I RNA editing is summarized in Table 3. In the G88 strain, editing sites 1, 7, 9, 10, 11, 13, 14 and 15 were A in all samples, even though editing sites 1, 11 and 15 were edited in *Bmα6* (Jin et al. 2007). Editing sites 2, 3, 4b and 8

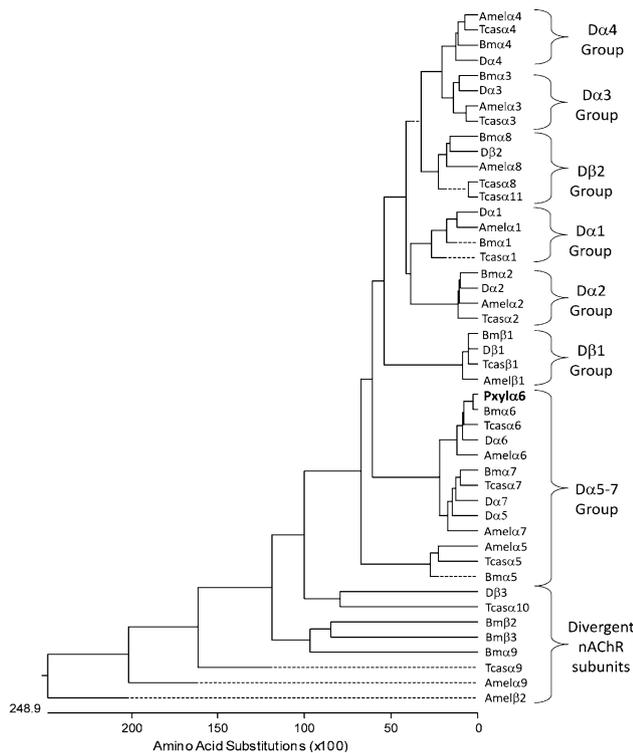


Fig. 2 Phylogeny based on deduced amino acid sequence of the all nAChRs from *B. mori*, *T. castaneum*, *D. melanogaster* and *A. mellifera*. The $\alpha 6$ subunit from *P. xylostella* (*Pxyλα6*) is located in the D $\alpha 5$ –7 Group near its *B. mori* ortholog (*Bmα6*)

had G in all clones. It is unknown whether these are the result of high-frequency RNA editing or encoded G genomically as in the case of *Bmox6*. Highly conserved editing sites 5, 6 and 12 were G at frequencies of 0.25, 0.79 and 0.80, respectively. These sites are edited at a high frequency in *Bmox6* (Jin et al. 2007). Editing site 16, which is edited only in *Amelα6* (Jones et al. 2006), was a G in 1 clone. A new putative editing site, arbitrarily named 4a, at position 391 was G at a frequency of 0.58, resulting in an S131G substitution. In the Wapio strain, only editing sites 4b and 12 were G (in all clones). No RNA editing was observed on exon 4 of *Pxyλα6*, although it was found in *Bmox6* (Jin et al. 2007). These putative editing sites need to be confirmed with genomic sequence information.

Pxyλα6 from the spinosad-resistant Pearl-Sel strain

All transcripts from the spinosad-resistant Pearl-Sel strain coded for apparently non-functional *Pxyλα6* (Fig. 3C, D, G–I, O, P, and S). Furthermore, all transcripts contained premature stop codons, except isoform XXX. Multiple isoforms were found. Isoforms Ib and IIIb have an intron 9 donor splice site variant that adds 40 additional bases (Fig. 3C and G). These variants result in a premature stop codon at amino acid 341, which would produce a protein

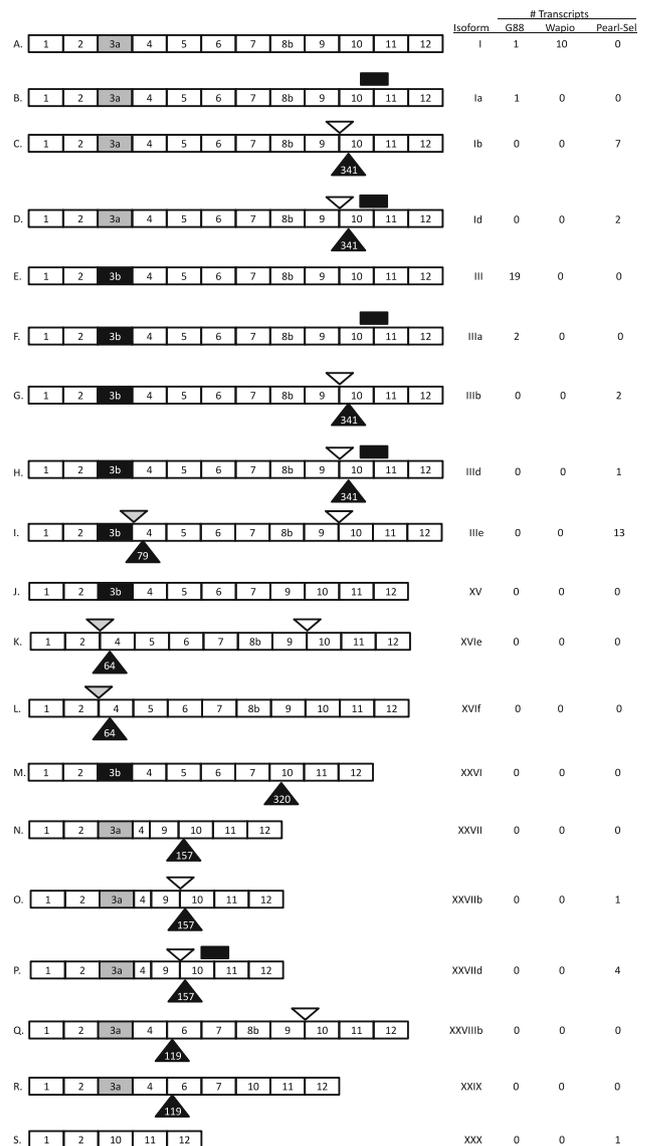


Fig. 3 Schematic diagrams of *Pxyλα6* transcripts from susceptible (G88 and Wapio) and spinosad-resistant (Pearl-Sel) strains of diamondback moth. Numbered boxes represent the exons of *Pxyλα6*. The black triangles indicate the approximate location of the premature stop codons. The inverted white triangles indicate the location of the intron 9 splice variant that adds 40 bp (ATGAGTACCAACATTTACATCA-CAAAATTCTAATTCATAG). The inverted shaded triangle indicates the location of the intron 3 splice variant that adds 7 bp (CAATAAA). The black bar indicates the location of the intron 10 splice variant that adds 30 bp (CCTAACTAACGTGAGTGTATCGGGCCCAG). The number of times each type of transcript was found in the G88 ($n = 23$), Wapio ($n = 10$) and Pearl-Sel ($n = 31$) strains is shown to the right of each diagram. Isoforms not seen in the G88, Wapio or Pearl-Sel strains as indicated in the figure were seen in clones from the F2 backcross experiment (Table 4). Isoforms were numbered based on prior conventions (Grauso et al. 2002; Rinkevich and Scott 2009) (isoforms I, III, XV, XVI) or arbitrarily (XXVI–XXX)

that is truncated shortly after TM3. Isoform XXVII lacks the intervening sequence between the first 20 bases of exon 4 through the first 16 bases of exon 9 (Fig. 3N). This

Table 2 Comparison of percent identity of the nucleotide and amino acid sequences and frequency of exons 3a, 3b and 8b of the $\alpha 6$ subunit orthologs between *P. xylostella*, *B. mori* and *D. melanogaster*

Exon		<i>Pxylα6</i> versus		Frequency		
		<i>Dα6</i> (%)	<i>Bmα6</i> (%)	G88	Wapio	Pearl-Sel ^a
3a	nt	66.7	88.9	0.09	1.00	0.47
	aa	93	100			
3b	nt	82.2	95.6	0.91	0.00	0.53
	aa	93	100			
8b	nt	75.9	90.8	1.00	1.00	1.00
	aa	100	100			

^a The calculation of the frequency of 3a and 3b in Pearl-Sel does not include isoform XXX (Fig. 3S) because isoform XXX does not possess exon 3

Table 3 Putative A-to-I RNA-editing sites in *Pxylα6*

Site	Position	Strain			<i>Bmα6</i> Counterpart
		G88	Wapio	Pearl-Sel	
1	377	A	A	A	Edited
2	379	G	G	G	Genomic G
3	384	G	G	G	Genomic G
4a ^a	391	G (14/24)	A	A	Not edited
4b	392	G	G	G	Edited
5	394	G (6/24)	A	G (19/27)	Edited
6	395	G (19/24)	A	G (19/27)	Edited
7 ^b	400	A	A	A	Not edited
8 ^b	401	G	G	G	Genomic G
9 ^b	430	A	A	A	Not edited
10 ^b	431	A	A	A	Not edited
11	443	A	A	A	Edited
12	447	G (20/24)	G	G	Edited
13 ^b	449	A	A	G (5/27)	Not edited
14	451	A	A	A	Not edited
15	454	A	A	A	Edited
16 ^b	470	G (1/24)	A	A	Not edited

Editing site number corresponds to the convention set forth by Tian et al. (2008). Sites 4a and 4b are designated arbitrarily. The position is numbered based on *Pxylα6* nucleotide numbering of the open reading frame. The ratio in parenthesis indicates the proportion of clones that were putatively edited at that site

^a New putative editing site

^b Edited in *A. mellifera* only

deletion results in a premature stop codon at amino acid 157 and would code for a protein with only loops D, A and E. Isoform XXVIIIb is missing the intervening sequence between the first 20 bases of exon 4 through the first 16 bases of exon 9 and has the intron 9 donor splice site variant that add 40 bases (Fig. 3O). Isoform XXX remains in frame but is missing exons 3 through 9 (Fig. 3S). This

transcript would produce a protein without extracellular loops or TM1-3. Transcripts with premature stop codons or large gaps in critical sequences were not seen in the spinosad-susceptible G88 or Wapio strains. The 3' splice site of intron 10 that was spliced out 30 bp upstream and added 10 additional amino acids to the protein was seen in the Pearl-Sel strain at a 0.22 frequency (isoforms Id, IIIId and XXVIIId).

RNA editing was generally similar between the Pearl-Sel and spinosad-susceptible strains, although there were some differences. Editing sites 5 and 6 were both G at a frequency of 0.70, while editing site 12 was G in all samples. In the Pearl-Sel strain, editing site 13 was G at a 0.19 frequency. The same site was unedited in the G88 strain and only edited in *Amelα6* (Tian et al. 2008). Editing sites 4a and 16 were A in all Pearl-Sel samples.

F₂ backcross and bioassay

The genotypes of the backcross and bioassayed insects are summarized in Table 4. The parental genotypes were confirmed in that the G88 strain produced only full-length transcripts and Pearl-Sel produced truncated transcripts. The F₁s had close to the expected frequency of susceptible ($f = 0.375$ (isoforms I and III)) and resistant ($f = 0.625$ (isoforms IIIb, XVIf, XXVII and XXVIIb)) transcripts (Fig. 3G, L–O). The bioassay survivors produced only resistant transcripts (isoforms IIIb and XXVIIb), while those killed in the bioassay produced both susceptible (I and III) and resistant (IIIb, XV, XXVI, XXVIIb, XXIX and XXX) isoforms (Fig. 3G, J, M, O, R and S). This association of only truncated transcripts with the resistant phenotype strongly suggests that *Pxylα6* is involved in spinosad resistance in the Pearl-Sel strain of diamondback moth.

Discussion

The full-length cDNA of *Pxylα6* from spinosad-susceptible strains codes for a typical nAChR subunit with an extracellular ACh-binding domain and four transmembrane segments. The pattern of alternative exon use was similar to *Bmα6* (Jin et al. 2007) but was very different from *Dα6* (Grauso et al. 2002) and *Tcasα6* (Rinkevich and Scott 2009). The presence of only two full-length isoforms (I and III) is the lowest number observed in any insect thus far, although *Bmoα6* has only I, III, IV (also named Type III in (Jin et al. 2007)) and XVI (Shao et al. 2007). Only one exon 8 (8b) was found for *Pxylα6*, consistent with results in *Bmoα6* (Jin et al. 2007). The lack of exon 8a and 8c in *Pxylα6* transcripts supports the observation that exon 8a was lost and exon 8c is not transcribed in the Lepidoptera

Table 4 Genotypes of diamondback moths used in the F₂ backcross-bioassay procedure. Isoforms I and III are full-length or susceptible transcripts, while the remaining isoforms are truncated or resistant transcripts

Sample	n ^a	Isoform							
		Susceptible		Resistant					
		I	III	IIIb	XVIf	XXVII	XXVIIIb	Other	
Pearl ♂ Parental	10 (1)	0	0	10	0	0	0	0	0
G88 ♀ Parental	14 (2)	6	8	0	0	0	0	0	0
F ₁	40 (3)	7	9	8	6	2	8	0	0
BC ₁ Survivors	15 (3)	0	0	7	0	0	8	0	0
BC ₁ Dead	28 (2)	0	10	7	0	0	7	4 ^b	

^a Number in parentheses indicates how many independent batches of larvae were used

^b Other isoforms include one each of isoforms XV, XXVI, XXVIIIb and XXIX

lineage (Jin et al. 2007; Baxter et al. 2010). No cassette exon use was observed in *Pxylα6* transcripts from susceptible strains. Thus, it appears that Lepidoptera produce a relatively small number of splicing isoforms.

Receptor function may be affected by the additional 10 amino acids coded by the intron 10 splice site variant that adds two putative phosphorylation sites and four additional hydrophobic residues (GenBank Accession GQ247883). Alanine-scanning and chimera construction showed that hydrophobic residues in the TM3–TM4 intracellular domain are important for subunit expression, surface manifestation and toxin binding (Ren et al. 2005; Kracun et al. 2008). These amino acids are added after the early stretch of highly conserved amino acids immediately following the TM3 segment. Residues following the conserved sequence tend to be highly variable across subunits and species (Jones and Sattelle 2007). Phosphorylation at the intracellular linker between TM3 and 4 is important for receptor desensitization and conductance (Huganir et al. 1986; Thany et al. 2006). Interestingly, a similar 3' splice site variant was seen in *Amelα4* where two phosphorylation sites were added to the intracellular linker between TM3 and TM4 (Jones et al. 2006). This similarity between divergent organisms indicates 3' splice site variation that introduces post-translational modification sites may be important for generating pharmacologically unique proteins. Given that this splice variant was found in G88 and Pearl-Sel indicates it is not associated with spinosad resistance. This splice variant was not found in Pearl-Sel in a previous study (Baxter et al. 2010).

Putative A-to-I RNA editing occurs at five sites on exon 5 of *Pxylα6*. We observed a putative novel editing

site (4a, resulting in a S131G substitution), consistent with a previous report (Baxter et al. 2010). Substituting serine with glycine at this position may affect protein folding or receptor function as it occurs in loop E, which is important for agonist binding (Amiri et al. 2008). This putative editing site was only seen in G88, but not Wapio or Pearl-Sel, suggesting it is not involved in spinosad resistance.

None of the *Pxylα6* transcripts from the Pearl-Sel strain code for complete proteins. We detected many novel isoforms (IIIe, XVIe, XVIf, XXVII, XXVIIIb, XXVIIIb, XXIX and XXX) that have not been previously reported. We did not detect isoforms I/IIIc or XXV that were previously reported (Baxter et al. 2010). The proteins coded for by transcripts with premature stop codons are illustrated in Fig. 4. These novel isoforms were overlooked in a previous report because the primers used in PCR were for exons 7–12 (Baxter et al. 2010). Thus, this technique was unable to record the skipping of exon 3 (isoforms XVIe and XVIf; Fig. 3K, L) or exon 5 (isoforms XXVIIIb and XXIX; Fig. 3Q, R), the large missing regions between exons 4 through 9 (isoforms XXVII and XXVIIIb; Fig. 3N, O), or the 7-bp addition at the beginning of exon 4 (isoforms IIIe, XVIe, and XVIf; Fig. 3I, K, L). We feel identification of these isoforms is of significant importance because in Pearl-Sel isoforms IIIId and XXVIIIb (Fig. 3I, O) account for a majority of transcripts. More than half of the transcripts in bioassay survivors are isoform XXVIIIb (Table 4). These isoforms would have earlier stop codons than those generated by isoforms Ib and IIIb. In the case of isoforms IIIe, XVIe and XVIf, transcripts would produce a protein with only loop D, which, most likely, is a non-functional protein at any level. The proteins created by transcripts with premature stop codons are displayed in Fig. 4.

The mechanism(s) responsible for generating transcripts with premature stop codons is unknown. The appearance of isoforms in the F₁s and backcross that were not observed in the parental strains suggests truncated transcripts are not produced in a simple Mendelian fashion. Based on the observed parental transcripts, the F₁s should have only produced isoforms I, III and IIIb. Unexpectedly, isoforms XXVII, XXVIIIb and XVIf were also seen in the F₁s. Isoforms XV, XXVI, XXIX and XXX were only seen in the BC dead sample. These results suggest that the truncated isoforms are generated by differential splicing through multiple resistance alleles, splicing is not tightly regulated in Pearl-Sel, or altered splicing machinery is responsible for the errant splicing. These truncated transcripts may also be tissue-specific splice variants. However, little is known about the distribution of nAChR expression in insects (Sattelle et al. 2005), let alone tissue-specific splice variation.

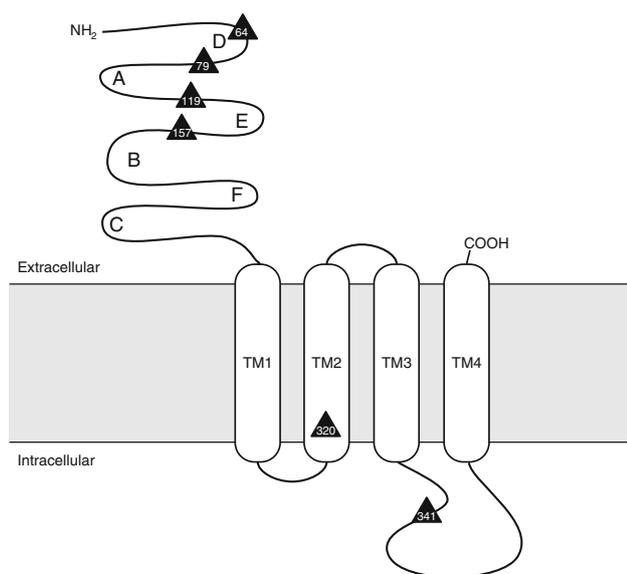


Fig. 4 Diagram of the topology of *Pxlα6*. Ligand-binding loops are designated by letters in the extracellular portion of the protein. Transmembrane domains (TM1–4) are designated by cylinders. The amino acid positions of stop codons created by alternative splicing are shown by black triangles. The numbers inside the black triangles indicate at which amino acid position the protein is truncated. The black triangles correspond to the position indicated by black triangles in Fig. 3

The recessive pattern of spinosad resistance in the Pearl-Sel strain (Zhao et al. 2002) is consistent with the observation of only truncated transcripts in bioassay survivors and both full length and truncated transcripts in insects killed in the bioassay. Transcripts of nAChRs with intron 3' splice site variations and transcripts with premature stop codons have been reported in other insects (Gao et al. 2007b; Rinkevich and Scott 2009). In *Amelα3*, a truncated transcript was seen that was similar to the *Pxlα6* transcript that possessed an intron 9 3' splice site variant in that they both code for proteins that terminate between TM3 and TM4 (Jones et al. 2006). Little information exists about the in vivo function of these truncated proteins. In vitro receptors formed by truncated *Dα1* transcripts do not produce currents when acetylcholine is bound (Schulz et al. 2000). Interestingly, truncating *Dβ1* transcripts via EMS mutagenesis reduces sensitivity to neonicotinoids (Perry et al. 2008). While this is an interesting prospect for a spinosad and/or imidacloprid resistance mechanism via truncated *Pxlα6* transcripts, neonicotinoids and spinosad interact with different areas and types of receptor complexes (Salgado and Saar 2004; Orr et al. 2009).

While the work in diamondback moth may serve as a foundation for future studies of spinosad resistance in other insects, truncated transcripts as a mechanism of spinosad resistance could be unique to some strains of diamondback

moth, and not a universal mechanism of resistance in all species. For example, the Spino-SEL strain of diamondback moth appears to have cytochrome-P450 monooxygenases and esterases as the major mechanisms of spinosad resistance (Sayyed et al. 2008), suggesting truncated *Pxlα6* transcripts may not be important in Spino-SEL. It would be of interest to study *Pxlα6* in the CH₁ strain of diamondback moth, as spinosad resistance in the CH₁ strain is similar to that found in the Pearl-Sel strain (Sayyed et al. 2004). Although spinosad resistance in the housefly is recessive and cannot be overcome with synergists (Shono and Scott 2003), spinosad resistance is not due to mis-spliced *Mdα6* (Gao et al. 2007b), *Mdα5* or *Mdβ3* (Gao et al. 2007a). Therefore, we should be cautious of extrapolating truncated transcripts of *α6* as a resistance mechanism to other species. It will be interesting to elucidate the expression of *α6* orthologs in spinosad-resistant tobacco budworm (Wyss et al. 2003) and western flower thrips (Bielza et al. 2007) in order to determine whether mis-spliced *α6* is the spinosad resistance mechanism in these species.

Acknowledgments We thank John Diaz for transferring samples between Geneva and Ithaca, and J.-Z. Zhao for his initial work on these resistant strains of diamondback moth. A grant from DowAgrosciences, a Sarkaria Fellowship and the Grace Griswold fund supported this research.

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