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SODIUM ION CHANNEL ALLELES IN WILD AND MUTANT TOBACCO BUDWORM (*Heliothis virescens* F.)

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Abstract

A cross of two strains of *Heliothis virescens* (tobacco budworm), each breeding pure for a different yellow-eyed mutant (wild type is grey-eyed) gave 6 black-eyed progeny among 500 progeny. From the black-eyed progeny were descended many lines in which several other mutant phenotypes were observed. Exposure to 5μg cypermethrin per vial killed 46.1% in the seventh generation descendants of that cross, with lines D38, D41 and D63 resistant with less than 17% mortality. For the domain *Hpy,* we found two new *Hpy* alleles (*Hpy51* and *Hpy52*) in eye mutant lines. Frequency of new alleles was 0.85. There are no new *Hpy* alleles in D8, D28, and, D29 lines which are susceptible type to cypermethrin. For the domain *IIS6* region in recent field tobacco budworm samples (Wadmalaw2000), the frequencies of homozygotes (*L/L*), heterozygotes (*L/H*), and homozygotes (*H/H*) were 0.44, 0, and 0.56 respectively without new *Hpy* alleles.

Introduction

A new mutation, black eye was observed in five sibling F1 progeny from a cross of strain Yyes and Ye for the two different recessive genes, *yes* conferring mutant yellow eye (Brown et al., 2001) and *ye* conferring mutant greenish yellow eye (Hasty & Payne, 1999). When two black-eyed hybrid females were mated to their wild type hybrid brother, two additional new phenotypes for eye pigmentation, golden eye and striped eye were observed along with black eye, yellow eye and wild type eye in the F2 generation. The mutations of eye pigmentation continued in the next generation. We were able to maintain new eye mutant colonies over 12 generations. Pyrethroid insecticide resistance in *H. virescens* has been linked to the sodium ion channel (*hscp* locus) (Park, 1998). Fifty different alleles have been found for the *Hpy* region which occurs within *hscp* (Taylor et al., 1996). A survey of genetic variation for *hscp-Hpy* in our spontaneous multiple mutant lineage suggested that two additional *Hpy* markers were possibly linked to pyrethroid resistance. In this report, we present the frequencies of new *Hpy* alleles in pyrethroid susceptible eye mutant lines and a pyrethroid resistant eye mutant line.

Material and Methods

All *Heliothis virescens* strains were reared in the laboratory or stored in -80 ºC for extracting genomic DNA. Wadmalaw 2000 colony was a pyrethroid-resistance strain. All eye mutant strains were propagated by single pair mating and were stored in freezer (-80 ºC) for further examination. All stages of all strains were maintained under similar, controlled environmental and rearing conditions (Ross & Brown, 1982). To test susceptibility**,** adults were exposed at 22°C to the insecticide coated 20 ml glass vial (Campanhola & Plapp, 1989). Each vial held one adult; vials were plugged with cotton wool. Adults were also exposed to acetone treated vials as control. The criterion for death was inability of the adult to cling to the side of the vial when the vial was rotated quickly. DNA was isolated from adults using a modification of a mammalian DNA isolation procedure (Ausubel et al., 1992). Polymerase chain reaction was performed for *hscp-IIS6* as published (Brown et al., 2001), and modified for *hscp-IS6* by using forward primer 5'-CTTGTCAGCTTTCCGCCTCAT-3' and reverse primer 5'-CTGTTCCTCTTCTTCGGCTT-3' at 53 ºC etc. The procedure for *hscp-Hpy* was from another publication (Park, 1998) modified by Cho. All sodium channel primers for *H. virescens* were designed using the published sodium channel gene sequence data as a guide (Park et al., 1998). The PCR products were separated gel electrophoresis on a 1.5% agarose gel to which 100 volts was applied for approximately 1 hour. The agarose gel was made with and run in 1X TAE buffer (40 mM Tris acetate and 2 mM EDTA in water). After electrophoresis, the gel was stained for 30 min in 0.01% SYBR™ Green I nucleic acid gel stain (FMC Bioproducts, Rockland, ME). Reactions that produced a single, bright band when viewed on a Spectroline® Model TR-302 302nm ultraviolet transilluminator were used for subsequent sequencing. Sequence was determined using a Dye Terminator Cycle Sequencing Ready Reaction™ (Perkin-Elmer, Applied Biosystems Division, Foster City, CA). Electrophoresis of the fluorescent products was performed on a 4.75% acrylamide gel by the Multiuser Biotechnology Facility for DNA sequencing at Clemson University.

Results

In our previous results, at least three *Hpy* marker alleles, *Hpy1*, *Hpy3,* and *Hpy3'* were linked to IS6 and IIS6 point mutations in the sodium channel (Table 1). The *Hpy1* allele was associated with the *IS6* mutant type (*M/M*) in Woodrow83 samples and Dalzell98 samples respectively. The reported pyrethroid-resistant haplotype carrying *V421M*, which it is known as *421M-*

1029wt-Hpy1, was found in Woodrow83 samples and Dalzell98 samples as formerly reported by others (Table 1). The haplotype *421wt-1029H-Hpy3* was found in Woodrow83 and Dalzell98 population samples (Table 1). We observed that another haplotype occur in these strains; *421wt-1029H-Hpy3'*. This variation of the *Hpy*3 allele occurred in one individual from the Woodrow83 strain and the Dalzell98 strain. The new *Hpy3* sequence was slightly different from *Hpy*3 known in YA17 and LA3-35.35. This new *Hpy3'* allele also was linked to a point mutation of *T* to *A* changing *L 1029* to *H* in the amino acid sequence in domain *IIS6* region. This individual haplotype was denoted as *421wt-1029H-Hpy3'*.

Exposure to 5 *u*g cypermethrin per vial killed 46.1% of all the seventh generation descendants of that cross, with lines D38, D41 and D63 resistant with less than 17% mortality. We found two additional new *Hpy* alleles in eye mutant D63 line. These PCR products of *Hpy* were less mobile in agarose gel electrophoresis. Analysis of DNA sequence revealed that these two new *Hpy* marker alleles have a 32-nucleotide insertion and a 319-nucleotide insertion at two different insertion points near each other in the *hscp-Hpy* intron. Among 26 samples examined in D63 mutant line which was resistant to cypermethrin, the frequencies of wild type allele, 319-nucleotide insert allele (*Hpy 51*), and 32-nucleotide insert allele (*Hpy 52*) were 0.17, 0.26, and 0.57 respectively (Table 2). Thus, the total ratio of two new alleles of *Hpy* in D63 eye mutant line was 0.83. However, no new *Hpy* alleles were found in D8, D28, and D29 eye mutant lines that were susceptible to cypermethrin (Table 2). In Wadmalaw2000 samples, genotypic frequencies of *IIS6* region were 0.44 homozygote leucine (*L/L)*, 0 heterozygote (*L/H*), and 0.56 homozygote histidine (*M/M*) (Table 3). However, no new *Hpy* alleles were found in these samples (Table 3). This individual haplotype was denoted as *V421-1029H-no insert Hpy*.

Discussion

Pyrethroid resistance in the tobacco budworm (*Heliothis virescens* F.), a major cotton pest, is associated with mutations in the locus encoding a voltage-gated sodium channel *hscp*, homologous to the *para* locus of *Drosophila melanogaster* (Taylor et al., 1995). A total of 50 alleles for *Hpy* which lies 5kb downstream from the domain *IIS6* in the sodium channel gene was examined by denaturing gradient gel electrophoresis for gel-mobility differences (Taylor et al., 1996). The *Hpy* allelic diversity of samples were significantly correlated with knockdown resistance of pesticides. The *Hpy1* and *Hpy3* alleles are usually linked to pyrethroid insecticide resistance with a sodium channel gene *IS6* region point mutation and sodium channel gene *IIS6* region point mutation (Park et al., 1997).

The D63 eye mutant line possessed new haplotype due to new *Hpy* alleles. Our two new two *Hpy* alleles were observed at highest frequencies in mutant lines which have pyrethroid resistance. This evidence suggests that the new *Hpy* alleles may be associated with pyrethroid resistance. We must examine the *IS6* and *IIS6* regions of this line. If we cannot find any mutations of *IS6* region and *IIS6* region, this line may have other resistance mechanisms or other regions of sodium channel are subjected to mutation by mobile DNA similar to *Hpy* region. Then, this mutation will be tested for resistance to pyrethroid insecticides. Our results suggest that two new *Hpy* alleles might be linked to pyrethroid resistance. However, no such correlation was observed for eye mutant phenotypes. Only 10 *Hpy* alleles among 50 *Hpy* alleles have been sequenced and only one 7-nucleotide insert has been observed in previously known alleles of *hscp-Hpy9* (Park, 1998).

We concluded that these insertions may be associated with the action of mobile DNA or an active transposon. Transposable elements have several common characteristics. 1) autonomous transposable elements carry a transposase gene which encodes a polypeptide that catalyzes the transposition process (Kempken & Windhofer, 2001) 2) terminal inverted repeats in transposable elements are bound by transposase and combine with the subterminal direct repeat for the excision of the target site (Kempken & Windhofer, 2001) 3) target site duplication, generated upon insertion, serves to distinguish transposon superfamilies (Le et al., 2000). Also, transposable elements often leave a footprint in the form of a target site duplication of DNA. In *hscp-Hpy*-in32, this insert includes one perfect subterminal repeat of "atattgtgt". Subterminal repeats have been observed in several transposons (Fowler & Mitton, 2000). In *hscp-Hpy*-in319, the terminal sequence of the insert, "atattgtgt" is identical to the preceding sequence of the intron, suggesting a target site duplication (TSD) of a transposon. Although we have not traced the new allele to its ultimate origin, we conclude that these long *Hpy* alleles descended from G0 parents (Yyes and YyeP) because both insertions occurred from G0 grandparents.

The haplotypes of Wadmalaw2000 samples and D63 eye mutant line (highly resistance type) were *421wt-1029H-No insert* and *IS6?-IIS6?-new Hpy51 and 52* respectively; however, *Hpy51* and *Hpy52* have not been observed in D8, D28, and D29 which were susceptible to pyrethroid insecticides. The *Hpy51* and *Hpy52* might be linked to pyrethroid resistance.

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Table 1. Haplotypes for *hscp* sodium channel polymorphisms *V421*, *L1029H*, and *Hpy* in strains of tobacco budworm.

Samples	**Phenotypes ***	***V421M***	***L1029H***	***Hpy***	**References**
hscp (wild type)	S	*Val*	*Leu*	*Hpy12*	Park, 1997
RR strain	R	*Met/Met*	*Leu/Leu*	*Hpy1*	Park, 1997
YA17	R	*Met/Met*	*Leu/Leu*	*Hpy1*	Park, 1997
LA3-35.35	R	*Val/Val*	*His/His*	*Hpy3*	Park, 1997
Dalzell98	R	*Met/Met*	*Leu/Leu*	*Hpy1*	Cho
		Val/Val	*His/His*	*Hpy3*	
		Val/Val	*His/His*	*Hpy3'*	
Woodrow83	S	*Met/Met*	*Leu/Leu*	*Hpy1*	Cho
		Val/Val	*His/His*	*Hpy3*	
		Val/Val	*His/His*	*Hpy3'*	

* S indicates scored as susceptible, R resistant to pyrethroids.

Table 2. Frequencies of 32-nucleotide and 319-nucleotide insertions in eye mutant lines.

		Domain *Hpy*			
Lines	**Phenotypes ***	**Wild type allele**	**319in allele**	**32in allele**	**Frequency of insertion allele**
D63 (26)	R	0.17	0.26	0.57	0.83
D8 (1)	S	1	0	0	0
D28 (1)	S	1	0	0	0
D29 (2)	S	1	0	0	0

* S indicates scored as susceptible, R resistant to pyrethroids.

( ) indicates examined number.

Table 3. Genotype frequency for hscp sodium channel polymorphisms *L1029H* and *Hpy* in 2001 field strain, SC.

	Domain *IIS6*			
Strain	**Homozygote (*Leu/Leu*)**	**Heterozygote (*Leu/His*)**	**Homozygote (*His/His*)**	**Domain *Hpy***
Wadmalaw 2000* (9)	0.44 (4)	0	0.56 (5)	No insertion

( ) indicates examined number.

* indicates location and year collected.