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INDEPENDENT ASSORTMENT OF YELLOW EYE (*ye*) FROM OTHER GENES IN TOBACCO BUDWORM (*Heliothis virescens*)

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Abstract

A single autosomal gene for greenish yellow eyes, *ye* (Hasty & Payne, 1999) was combined with a single autosomal gene for yellow scales, *y* in the absence of *yes* which controlled color of both eyes and scales (Brown et al., 2001). Independent assortment of *ye* from *y* was observed in a test cross. SNPs (single nucleotide polymorphisms) were discovered in *CYP6B10*, *hscp* (*heliothis sodium channel protein*)), *JHE (juvenile hormone esterase)* and *Cry1A toxin receptor* genes in a backcross family. Size differences of PCR products were also observed in *CYP9* and *ryanodine receptor* genes in a backcross family. The genetic linkage of *CYP6B10* and *hscp* was found in backcross families (Park & Brown, 2002). The sex linkage of *JHE* gene was discovered in two backcross families.

Keywords; SNPs (single nucleotide polymorphisms); *hscp* (*heliothis sodium channel protein*); *CYP6B10*; independent assortment.

Introduction

A genomic approach is useful in the studies of mechanisms of insecticide resistance. Linkage mapping is one of the methods to analyze multiple resistance mechanisms to insecticide in the tobacco budworm moth, *H. virescens*, an important economic pest of cotton (Brown, 1990) (McCaffery, 1998). Linkage groups are identified by specific marker loci. *H. virescens* has 31 chromosomes and chromosome one is the sex chromosome, which is the largest (Heckel et al., 1998). *CYP9A1* was mapped on linkage group seven by restriction fragment length polymorphism studies (Rose et al., 1997). *AceIn* (*acetylchoinesterase insensitivity*) was genetically linked to *isocitrate dehydrogenase-2* (*IDH-2*) placing it on chromosome two (Heckel et al., 1998). It was also linked to methyl parathion resistance (Gilbert et al., 1996).

In previous study, we found independent assortment of *y*, *yes*, *AceIn***,** and *hscp***,** the latter two of which are genes related to insecticide resistance (Brown et al., 2001). We also observed co-segregation of *hscp* and *CYP6B10* (GeneBank Accession AF140279) which controls detoxication of xenobiotics, such as insecticide (Park & Brown, 2002). However, those linked genes segregated from *y*, *ye* and sex. In a linkage analysis, visible linkage markers, SNPs, and size polymorphism of PCR products were used to test for linkage of various genes in backcross families.

Methods

Strains

The wild type of *H.virescens* individuals (NC, green scales with grey eyes) was provided by R.M. Roe, North Carolina State University in 2000 and reared in our laboratory. The double mutant strain YYE (yellow scales with greenish yellow eyes) was constructed by the intercross Y (yellow scales with grey eyes) with YE (green scales with greenish yellow eyes) provided by G.T. Payne, State University of West Georgia followed by the dihybrid cross and reared in our laboratory since August 2000 (Brown et al., 2001) (Hasty & Payne, 1999). For linkage study, we made a hybrid of NC with YYE and female hybrids were backcrossed with male YYE. Most lepidopterans have heterogametic females and crossing over occurs only in males (Heckel, 1993).

PCR Amplification and Sequence Analysis

Genomic DNA was isolated from each moth thorax, precipitated with 100 % ethanol and dissolved in 100 µL of 0.1 mM EDTA/10 mM Tris, pH 8.0 buffer. Primers were synthesized (Pimprale, 1998) (Park & Taylor, 1997) (Taylor et al., 1996) (Rose et al., 1997) (Oltean et al., 1999) (Puente et al., 2000) by Research Genetics (Huntsville, AL) (Table 1). Amplification was performed in a reaction volume of 50 µL in glass capillary tube in the following manner: 5 µL genomic DNA were added to 45 µL reaction solution (38 µL master mix, 1.35 µL forward primer, 1.35 µL reverse primer and 8.1 µL water). Master mix was 11.5 µL enzyme dilution buffer, 12.5 µL of 30 mM $MgCl_2$, 12.5 µL cresol red, 12.5 µL of 2 mM dNTP, 32.5 µL water and 1 µL of AmpliTaq DNA polymerase (5.0 U/ µL stock, Perkin Elmer, Foster City, CA). Amplification was

performed in a Rapid Cycler (Idaho Tech, Salt Lake City, UT) with denaturization for 0 sec at 94 °C, annealing for 0 sec at 45 °C ~55 °C and extension for 15 sec at 72 °C for 35 cycles. Sequence was obtained with an Applied Biosystems Model 373 DNA analyzer.

Results

Independent assortment of *ye* and *y* was observed in a test cross (Table 2). All crosses between the double mutant strain YYE and NC produced all wild type progeny. It indicated yellow scales and yellow eyes have recessive inheritance in which *y* controls yellow-scale color and *ye* controls yellow-eye color. Among 523 backcross progeny, we observed 132 progeny with the maternal phenotype (green scales and grey eyes), 129 with the paternal phenotype (yellow scales and yellow eyes), 126 with the recombinant phenotype of yellow scales with grey eyes, and 136 progeny with the recombinant phenotype of green scales with yellow eyes. Expected results for a model in which there are independent assortment of *y* controlling yellow scales from *ye* controlling yellow eyes would be 131 in each of four phenotype. There was no significant difference from expected values. The total χ^2 value was 0.396 and the probability was 0.936.

Single nucleotide polymorphisms were discovered in *CYP6B10, hscp*, *JHE,* and *Cry1A toxin receptor* genes in backcross families. We observed three SNPs in *CYP6B10* in exon region. The first mutation altered the codon glutamine to glutamic acid. We also found one mutation in *IIS6* of *hscp* in intron. In *Hpy* of *hscp*, we observed two mutations in intron and two synonymous SNPs in coding region. The genetic linkage of *CYP6B10* and *hscp* was found in two backcross families (Park & Brown, 2002). We observed both parental genotypes and no recombinant genotypes among 24 progeny. It was not significantly different from a model in which two genes occupy the same chromosome (Table 3).

We assumed *JHE* was sex-linked based on the result of the SNP study in two backcross families. Within an intron region of the *JHE*, we found two heterozygous C/T and A/T in the mother [… CTT TCA ATC AAA (C/T)GG TCA ACA ATT TTA ……CAA TAT GCG TTA (A/T)AA ACT AAT TAA ATG …] and two homozygous C and A in the father (Table 4). Heterozygous C/T and A/T were observed all male progeny and homozygous C and A were observed all female progeny. In a coding region of the *Cry1A toxin receptor*, we observed heterozygotes T/C and T/C in the mother [… TCT TAC GCC TTA (T/C)TT CAC TGG (T/C)AC TAC …] and homozygotes T and T in the father of two backcross families. However, neither mutation altered amino acids (Table 5).

Size differences of PCR products were discovered in *CYP9* and *ryanodine receptor* genes in a backcross family BNP10. In an intron region of the *CYP9* gene, we observed two bands by agarose gel electrophoresis in the mother and one band in the father. The lower band had no insert and the upper band had an insert of approximately 300 bp. Among 12 backcross progeny, we found eight progeny with maternal type and four with paternal type. However, this gene was not linked with any other genes (Table 6). In an intron region of the *ryanodine receptor* gene, two bands were also found in the mother and one band was found in the father. There was no insert in the lower band and the upper band had approximately 50 bp insert. Among eight backcross progeny, three progeny exhibited the maternal type and five the paternal type; however, we did not find any linkage to the *ryanodine receptor* gene (Table 6).

Discussion

Independent assortment *ye* from *y*, *JHE*, *Cry1A toxin receptor*, *hscp*, *CYP6*, *CYP9*, *ryanodine receptor* and sex was observed in *H. virescens*. Segregation *ye* and *y* was found in 16 backcross families. Observed values were not significantly different from expected values of a model in which there existed two unlinked genes with *y* controlling yellow scales and *ye* controlling yellow eyes. SNPs were discovered in *CYP6B10*, *hscp*, *JHE* and *Cry1A toxin receptor* genes. *CYP6B10*, which controls detoxication, was linked to *hscp*, which is known to encode with several point mutations for pyrethroid resistance. This result suggests the possibility of a resistance cassette in this species and it could have serious implication for management of this pest (Park & Brown, 2002).

The SNP study demonstrated that the *JHE* gene is inherited on sex chromosome. We also found the sex linkage of *TPI* (*triosephosphate isomerase*) in one test cross family BYE2 based on SNP study (data not shown). Size differences of PCR products were discovered in *CYP9* and *ryanodine receptor* genes. We have two additional linkage markers, *ye*s controlling yellow eyes and yellow scales and *b* (black eye) segregated independently from *y* and *yes*. We could not find informative families for *DDC* (*dopa decarboxylase*) and we failed to analyze *XDH* (*xanthine dehydrogenase*) due to multiple amplification products. Gene *ye*s was isolated from *y* in a strain, breeding true for yellow eyes and yellow scales. An outcross confirmed recessive inheritance and was followed by a backcross confirming previous results of a hybrid to hybrid test cross that a single gene controlled both yellow eyes and yellow scales. We will continue to search for informative families and linkage markers to complete a linkage map in *H. virescens*.

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Table 1. Lists of primers of linkage markers.

Name	Primer sequence
CYP6F1	TGAAGGCTCTTGAAATTGTAG
CYP6R1	GTTCGTATATCAAATAGGCCA
IIS6F	GATGTCTCTTGTATAACC
IIS6R	TTGTTGGTRTCCTGATC
Hpy F	CTGATCTTCGCCATCATGGG
Hpy R	GCGGTCGTTCATGATCTGTATCCA
JHE1F	GACCTACACGGACCAGAATA
JHE1R	GGGATTTTTGTTGTGTTCAT
CYP9F1	AGTTGTTGTTTCAAAACACC
CYP9R1	TGTCTTATGACTTCTGACGAG
CrylR 1F	ACTACAGAGCGCCCTCTCCTT
CrylR 1R	ATTCACATTCGGGCTTGCA
RyanodineR 1F	CTTCGTGTTCAACCTGTACAAGG
RyanodineR 1R	ACTGGTCACGCAGCTCTCCGA

Table 2. Independent assortment of genes conferring the two traits yellow scales for body color (YB) and yellow eyes (YE).

Phenotype[a]	Expected[b]	Observed	Chi^2[c]
YBYE	130.75	129	0.002
GBGE	130.75	132	0.011
YBGE	130.75	126	0.172
GBYE	130.75	136	0.211
Total	523	523	0.396

Probability; 0.936.

[a] YBYE; yellow scale, yellow eye.
GBGE; green scale, grey eye.
YBGE; yellow scale, grey eye.
GBYE; green scale, y*e*llow eye.

[b] Model of two unlinked genes in which *y* controlled yellow scales and *ye* controlled yellow eyes.

[c] Observed values were not significantly different from those expected.

Table 3. Statistical analysis for linkage of *CYP6B10* and *hscp* in two test cross families.

	Parental Genotype		Recombinant Genotype		
	Hetero/Hetero[a]	Homo/Homo	Hetero/Homo	Homo/Hetero	χ^2
Observed	12 [b]	12	0	0	
Unlinked	6 [c]	6	6	6	24 [d]
Linked	12 [e]	12	0	0	0.0

[a] Genotype zygosity for *CYP6B10*/*IIS6*, respectively.

[b] Number of adults.

[c] Expected value for a model in which two genes assort independently.

[d] Significant at $p < 0.001$.

[e] Expected value for a model in which two genes occupy the same chromosome.

Table 4. Linkage of *JHE* and sex in backcross families BNP9 and BNP10.

			Nucleotide	
Name	**Sex**	**Phenotype[a]**	***JHE***	
BNP9 mother	Female	GBGE	C/T	A/T
BNP9 father	Male	YBYE	C	A
BNP9 progeny 2	Male	GBYE	C/T	A/T
BNP9 progeny 4	Male	GBYE	C/T	A/T
BNP9 progeny 6	Female	GBGE	C	A
BNP9 progeny 7	Male	YBGE	C/T	A/T
BNP10 mother	Female	GBGE	C/T	A/T
BNP10 father	Male	YBYE	C	A
BNP10 progeny 1	Male	GBGE	C/T	A/T
BNP10 progeny 2	Male	GBYE	C/T	A/T
BNP10 progeny 3	Male	YBGE	C/T	A/T
BNP10 progeny 4	Male	YBYE	C/T	A/T
BNP10 progeny 5	Female	GBGE	C	A
BNP10 progeny 6	Female	GBYE	C	A
BNP10 progeny 7	Female	YBGE	C	A
BNP10 progeny 8	Female	YBYE	C	A
BNP10 progeny 15	Male	YBGE	C/T	A/T
BNP10 progeny 16	Male	YBYE	C/T	A/T
BNP10 progeny 17	Female	GBYE	C	A
BNP10 progeny 18	Female	YBYE	C	A

[a] See table 2.

Table 5. Linkage analysis of *Cry1A toxin receptor* in backcross families, BNP9 and BNP10.

			Nucleotide	
Name	**Sex**	**Phenotype[a]**	***Cry1A toxin receptor***	
BNP9 mother	Female	GBGE	T/C	T/C
BNP9 father	Male	YBYE	T	T
BNP9 progeny 2	Male	GBYE	T/C	T/C
BNP9 progeny 4	Male	GBYE	T	T
BNP9 progeny 5	Male	YBYE	T	T
BNP9 progeny 6	Female	GBGE	T	T
BNP9 progeny 7	Male	YBGE	T/C	T/C
BNP9 progeny 8	Female	YBYE	T	T
BNP10 mother	Female	GBGE	T/C	T/C
BNP10 father	Male	YBYE	T	T
BNP10 progeny 1	Male	GBGE	T	T
BNP10 progeny 2	Male	GBYE	T/C	T/C
BNP10 progeny 3	Male	YBGE	T	T
BNP10 progeny 4	Male	YBYE	T	T
BNP10 progeny 5	Female	GBGE	T/C	T/C
BNP10 progeny 6	Female	GBYE	T	T
BNP10 progeny 7	Female	YBGE	T	T
BNP10 progeny 8	Female	YBYE	T/C	T/C

[a] See table 2.

Table 6. Linkage analysis of *CYP9* and *ryanodine receptor* in a backcross family BNP10.

Name	Sex	Phenotype [a]	Nucleotide	
			CYP9	***ryanodine receptor***
Mother	Female	GBGE	In^+/In [b]	In^+/In
Father	Male	YBYE	In/In	In/In
Progeny 1	Male	GBGE	In^+/In	In/In
Progeny 2	Male	GBYE	In/In	In^+/In
Progeny 3	Male	YBGE	In^+/In	In^+/In
Progeny 4	Male	YBYE	In^+/In	In/In
Progeny 5	Female	GBGE	In^+/In	In/In
Progeny 6	Female	GBYE	In^+/In	In/In
Progeny 7	Female	YBGE	In/In	In^+/In
Progeny 8	Female	YBYE	In^+/In	In/In
Progeny 15	Male	YBGE	In/In	NA
Progeny 16	Male	YBYE	In/In	NA
Progeny 17	Female	GBYE	In^+/In	NA
Progeny 18	Female	YBYE	In^+/In	NA

a See table 2.

b " In^+ " indicates it has no insert and " In " indicates it has an insert.

	ye	***y***	***CYP6***	***hscp***	***JHE***	***Cry1A toxin R***	***CYP9***	***ryano-dine R***	***Sex***
ye		-	-	-	-	-	-	-	-
y	-		-	-	-	-	-	-	-
CYP6	-	-		+	-	-	-	-	-
hscp	-	-	+		-	-	-	-	-
JHE	-	-	-	-		-	-	-	+
Cry1A toxin R	-	-	-	-	-		-	-	-
CYP9	-	-	-	-	-	-		-	-
ryano-dine R	-	-	-	-	-	-	-		-
Sex	-	-	-	-	+	-	-	-	

Figure 1. Genetic linkage relationships in *Heliothis virescens*; " + " indicates linkage and " - " indicates independent segregation was observed.