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A single gene (*yes*) controls pigmentation of eyes and scales in *Heliothis virescens*.

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Abstract

A yellow-eyed mutant was discovered in a strain of *Heliothis virescens*, the tobacco budworm, that already exhibited a mutation for yellow scale, *y*. We investigated the inheritance of these visible mutations as candidate markers for transgenesis. Yellow eye was controlled by a single, recessive, autosomal factor, the same type of inheritance previously known for *y*. Presence of the recombinant mutants with yellow scales and wild type eyes in test crosses indicated independent segregation of genes for these traits. The recombinant class with wild type scales and yellow eyes was completely absent and there was a corresponding increase of the double mutant parental class having yellow scales and yellow eyes. These results indicated that a single factor for yellow eye also controlled yellow scales independently of *y*. This gene was named *yes*, for *yellow eye and scale.* We hypothesize that *yes* controls both eye and scale color through a deficiency in transport of pigment precursors in both the ommochrome and melanin pathways. The unlinked gene *y* likely controls an enzyme affecting the melanin pathway only. Both *y* and *yes* segregated independently of *AceIn*, acetylcholinesterase insensitivity, and sodium channel *hscp*, which are genes related to insecticide resistance.

Keywords: pigmentation, melanin, ommochrome, pteridine, pleiotropy, hypomorphic mutation, loss of function, development of compound eye, scale color, genetic linkage analysis, ATP-binding cassette transporter

Abbreviation:

hscp	a gene coding for the *Heliothis* sodium ion channel protein
AceIn	a gene controlling decreased sensitivity of acetylcholinesterase to inhibition by propoxur or methyl paraoxon
y	a gene conferring yellow scale color
ye	a gene conferring yellow eye color and yellow scale color
Yyes	a strain of *Heliothis virescens* with yellow scales later selected for yellow eyes and scales.
Dalzell98	a strain of *Heliothis virescens* collected in Dalzell, South Carolina in 1998
Ace-R	a strain of *Heliothis virescens* fixed for *AceIn*

Introduction

Genetic transformation of insects may lead to control or reduced pest status of species destructive of food and fiber (DeVault *et al.*, 1996). A transgenic system for *Heliothis virescens* is needed to explore genetic control or to unravel the complexities of insecticide resistance, a phenomenon for which this pest is among the most notorious in agriculture (Brown, 1996). Organophosphorus insecticide resistance in this species has been associated with the gene *AceIn* (Brown and Bryson, 1992) which was genetically linked to methyl parathion resistance (Gilbert *et al.*, 1996) and is located on chromosome 2 (Heckel *et al.*, 1998). Pyrethroid insecticide resistance appear to be associated with the gene *hscp* (Taylor *et al.*, 1993) in which there are several point mutations (Park *et al.*, 1998; Lee *et al.*, 1999).

While genetic transformation of dipterans has become routine, it was not until very recently that technology was developed to express a foreign gene in non-dipteran insects (Handler, 2000). Lepidopterans were transformed using *piggyBac* transposon as a vector for the transgenic marker green fluorescent protein (Peloquin *et al.*, 2000; Tamura *et al.*, 2000). This promising vector has not been tested in *H. virescens* or in other noctuids. Transformation of dipterans was developed by exploiting recessive eye color mutants beginning with *rosy* of *Drosophila melanogaster* (Rubin and Spradling, 1982). Transformants were identified by expression of the transgenic wild type alleles such as *w+* (Komori *et al.*, 1993), *v+* (White *et al.*, 1996) or *cn+* (Jasinskiene, 1998) that restored color in the mutant background. Recessive eye color mutants for

lepidopterans have been reported (Dittrich and Luetkemeier, 1980; Marec and Shvedov, 1990), but are not understood biochemically. Recently, the gene *Bmwh3*, similar in sequence and inferred protein structure to *D. melanogaster w*, was cloned from *Bombyx mori* and its expression was linked to *w3* and *w3ol* mutantions (Abraham *et al.*, 2000).

We describe a new visible marker in *H. virescens, yes*, for *yellow eye and yellow scale* that arose spontaneously in our strain that also carried a visible autosomal recessive marker, *y,* for yellow scale (Mitchell and Leach, 1994). We report the independent assortment of *y*, *yes*, *AceIn* and *hscp*, the latter two of which confer resistance to insecticides. We present a hypothesis to explain the control of pigmentation by *y* and *yes* with a view toward future transgenic technology. Our results characterize the most convenient and visible genetic landmarks, those of visible mutants which are rare in the species. We intend to find molecular markers linked to each visible marker in order to locate the genes controlling pigment deficiencies in a library of bacterial artificial chromosomes.

Materials and Methods

Criteria for scoring traits

Yellow eyes (wild type eyes are grey) and yellow scales (wild type scales on wings and body are green) in the adult were scored by visual comparison of color with photographs of type specimens (Figure 1). Yellow scale was controlled by a single, recessive, autosomal gene (Mitchell and Leach, 1994) here termed *y*.

AceIn, a co-dominant gene for acetylcholinesterase insensitivity, was scored for genotype using an assay of enzyme inhibition previously described (Brown and Bryson, 1992; Gilbert *et al.*, 1996) using a Vmax® microtiter plate reader with Softmax® for automated data acquisition (Molecular Devices, Palo Alto, CA). Heads of individual moths frozen at -70oC were homogenized in a ground-glass tissue grinder (Kontes, Model #20, Vineland, NJ) in 0.5ml of MOPS pH7.5 buffer. The homogenate was centrifuged for one minute and the supernatant was used as the source for the acetylcholinesterase. Acetylcholinesterase activity was monitored spectrophotometrically for 15 or 30 min during exposure to insecticidal inhibitors. On the basis of activity remaining after inhibition, genotypes of *AceIn AceIn, AceIn AceIn*+, or *AceIn*+ *AceIn*+ were assigned from scatter plots. Assignments were confirmed by inspecting the original plots of change in optical density per minute. Enzyme preparations from homozygous methyl parathion-resistant strains (*AceIn AceIn*) were resistant to propoxur and susceptible to monocrotophos. Enzyme preparations from homozygous methyl parathion-susceptible strains (*AceIn*+ *AceIn*+) were susceptible to propoxur and resistant to monocrotophos. Enzyme preparations from hybrids *(AceIn AceIn+)* were intermediately resistant to both inhibitors (Brown and Bryson, 1992). This method was applied previously to isolate the two alleles from a mixed culture by pair matings to produce lines homozygous for each allele; a cross of these lines produced only the intermediate phenotype expected of a heterozygote (Gilbert *et al.* 1996). In those experiments, no progeny were susceptible to both inhibitors nor were any resistant to both inhibitors, and the three phenotypes were clustered with no overlap. The resistant allele cosegregated with resistance to methyl parathion.

Figure 1. Mutants of scale color and eye color in *Heliothis virescens;* Upper left: wild type scale color (green); Upper right: mutant scale color (yellow); Lower left: wild type eye color (grey); Lower right: mutant eye color (yellow).

The genotype of *hscp,* a gene encoding the *Heliothis sodium channel protein,* was scored for the polymorphism *CTT* or *CAT* in the codon of amino acid 1029 that results in L or H, respectively (Park *et al.*, 1998). Genomic DNA was isolated from *H.virescens* adults by conventional methods (Taylor *et al.*, 1995).

The locus *hscp L1029H* was amplified from DNA of unknowns by PCR using primers IIS6f (5'-GATGTCTCTTGTATACC-3') and IIS6r (5'-TTGTTGGTRTCCTGATC-3') based on previously determined sequences of the region (Park, 1999). The primers used were purchased (Research Genetics, Huntsville, AL). All amplifications were executed in a Model 480 Perkin-Elmer thermal cycler using reagents purchased from Perkin-Elmer (Norwalk, CT). A total of 35 cycles were used to amplify the DNA template. A five-minute denaturation step of 93oC proceeded 30 cycles of 93oC for 35 s, 53oC for 1 min, and 72oC for 30 s. The final five cycles were run after the initial 30 cycles, using 93oC for 35 s, 53oC for 1 min, and 72oC for 2 min. Amplified products were separated by gel electrophoresis on 1.5% agarose gels at 100 v for approximately 1 h using a 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%

SYBRTM Green I nucleic acid gel stain (FMC Bioproducts, Rockland, ME).

Sequences of amplified products were determined for both strands using an ABI automated sequencer following instructions of the manufacturer (Tracy and Mulcahy, 1991) using the amplified products as templates and the same primers as those used for amplification. In a computerized reconstruction of the electropherogram used to read the sequence, the heterozygous codon*, hscp L(CTT)1029H(CAT),* appeared as two overlapping peaks, one red for thymine and one green for adenosine (see arrow in Figure 2). The genotype of each sample was scored by visual inspection of this computerized reconstruction.

Description of strains

The wild type of *H. virescens* possessed a green body with dark stripes on forewings and grey compound eyes (Figure 1). The single mutant strain Yel (yellow scale) (Mitchell and Leach, 1994) was reared in our laboratory since May 1995. This strain was fixed for *y* and moths displayed yellow scales on the body and stripes on the wings were very faint. Compound eyes were wild type grey.

The double mutant strain Yyes (yellow with yellow eyes and scales) was founded in October 1998 when 3 female and 4 male moths with yellow eyes appeared spontaneously in the Yel strain (Figure 1). They were mated *en masse*. Subsequently, five families breeding true for moths with yellow scales and yellow eyes were selected to continue strain Yyes.

The wild type pigmented strain Ace-R (acetylcholinesterase resistant) was derived from a methyl parathion-resistant strain, Woodrow83 (Brown and Bryson, 1992), originally obtained from cotton fields in Woodrow, South Carolina in 1983. Woodrow83 was interbred with susceptible strain, Florence87 (Brown, 1991), originally obtained from tobacco fields in Florence, South Carolina in 1987. After nterbreeding, families homozygous for *AceIn* were selected to establish strain Ace-R (Gilbert *et al.*, 1996).

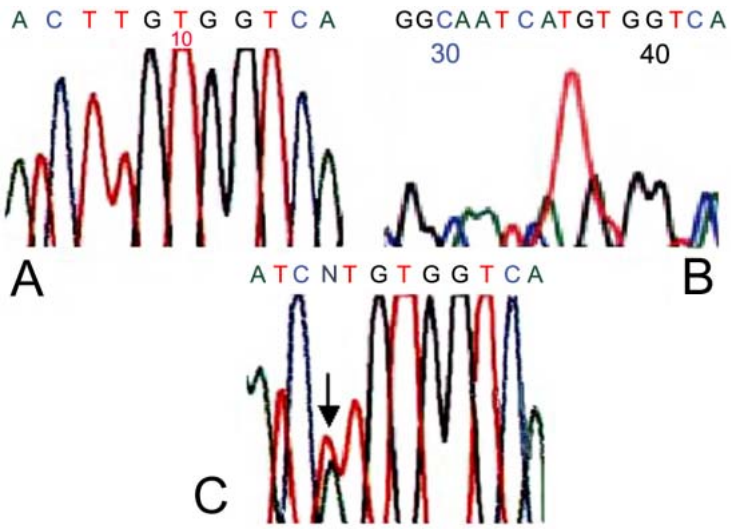

Figure 2. Partial nucleic acid sequence of the IIS6 segment of the *Heliothis virescens sodium* channel. The region indicated by the arrow is where the L1029H mutation occurs. Leucine is encoded by CTT and Histidine is encoded by CAT. Examples of the three genotypes are shown; A) Luecine homozygote (CTT/CTT). B) Histidine homozygote (CAT/CAT), and C) Leu/His heterozygote (CTT/CAT).

The wild type pigmented strain Pyr-R (pyrethroid-resistant) was originally collected from several states and selected for high larval resistance to permethrin (Payne *et al.*, 1988). Resistance in this strain was synergized by propynyl ethers (Brown *et al.*, 1996a), accompanied by susceptibility to chlorfenapyr (Pimprale *et al.*, 1997), and correlated with the sodium channel mutation *hscp V421M* (Lee *et al.*, 1999). The wild type pigmented strain Dalzell98 was collected from cotton in Dalzell, SC in the summer of 1998 and the adults were found to be resistant to cypermethrin (data not shown).

Voucher specimens have been placed in the Clemson University Arthropod Collection, Clemson, South Carolina.

Test Crosses

All crosses were written according to convention by describing the female first, then the male. When hybrids from reciprocal mating were employed, they are written with a "/" rather than an "x".

To test for independent assortment of yellow scale from yellow eye, an experiment was begun in 1999 in which moths from the double mutant strain Yyes were mated to wild type strain Dalzell98 in single pairs. Eight pairs were fertile and produced 240 wild type moths. These dihybrid moths were mated in the backcross Yyes/Dalzell98 x Yyes from which seven pairs were fertile and 516 backcross progeny were scored (see Table 1). In addition, dihybrid moths were mated in the intercross Yyes/Dalzell98 x Yyes/Dalzell98 from which three pairs were fertile and 153 F2 progeny were scored (see Table 2).

This experiment was repeated in 2000, when a new lot of dihybrid moths were mated in the backcross Yyes/Dalzell98 x Yyes from which six pairs were fertile and 417 backcross progeny were scored (see Table 1). Again some dihybrid moths were mated in the intercross Yyes/Dalzell98 x Yyes/Dalzell98 from which four pairs were fertile and 363 F2 progeny were scored (see Table 2).

To test for independent assortment of yellow scale or yellow eye from *AceIn* or *hscp*, genotypes were determined in parents of productive matings. One of seven fertile pairs from the backross Yyes/Dalzell98 x Yyes conducted in 1999 was informative for both *AceIn* and *hscp* in addition to scale and eye pigmentation (see Table 3).

To test for independent assortment of yellow scale from *AceIn*, an experiment was conducted in October 1995 in which twelve pairs of Ace-R x Yel and twelve pairs of Yel x Ace-R were mated as single pairs. Progeny survived from two of the Ace-R x Yel matings and from seven of the Yel x Ace-R matings. The parents of these nine families were scored for genotype indicating that all green Ace-R parents *(y+ y+)* were homozygous resistant *(AceIn AceIn)* and that all of the yellow Yel parents *(y y)* were homozygous susceptible *(AceIn+ AceIn+).*

Twenty-five of the female hybrid progeny (Ace-R/Yel; *y+ y, AceIn AceIn+)* were backcrossed to males of the Yel strain (*y y, AceIn+ AceIn+).* Of these, progeny survived from eight families, but only three of these families were assayed for their genotype because all pupae of some families entered diapause unexpectantly. The backcross parents of the surviving progeny ere assayed, confirming that the Ace-R/Yel hybrid mothers *(y+ y)* were *AceIn AceIn+,* and that the Yel fathers *(y y)* were *AceIn+ AceIn+.*

The test for independent assortment of yellow scale from

Table 1. Test crosses for segregation of scale and eye color genes in female diheterozygous *Heliothis virescens*; combined results of 13 pair matings of Yyes/Dalzell98 x Yyes.

	Parental Phenotype		Recombinant		
	Yellow scale Yellow eye	Green scale Green eye	Yellow scale Green eye	Green scale Yellow eye	χ^2
Observed	455[1]	229	249	0	
Unlinked[3]	233.25	233.25	233.25	233.25	466**[2]
Linked[4]	466.5	466.5	0	0	121**
Unlinked with lethal[5]	233.25	233.25	233.25	0	213**
Unlinked *y, yes*[6]	466.5	233.25	233.25	0	1.43

1 = Number of adults
2 **=Significant @ p<0.001
3 Expected results for a model in which there are two independently assorting genes, one controlling yellow scales and another gene controlling yellow eye.
4 Expected results for a model in which two genes, one controlling yellow scales and another controlling yellow eye, occupy the same chromosome. The special case of lack of recombination in female lepidopterans is discussed in the text.
5 Expected results for a model in which there are two independently assorting genes, one gene controlling yellow scales and another gene controlling yellow eye. Zygotes in one recombinant class (green scale with yellow eye) die.
6 There are two independently assorting genes, *y* confers yellow scales only and *yes* confers both yellow eye and yellow scale.

Table 2. Segregation of scale and eye color in a dihybrid cross of *Heliothis virescens;* combined results of 7 pair matings of Yyes/Dalzell98 x Yyes/ Dalzell98.

	Parental Phenotype		Recombinant		
	Yellow scale Yellow eye	Green scale Green eye	Yellow scale Green eye	Green scale Yellow eye	χ^2
Observed	122[1]	298	96	0	
Unlinked[3]	32.25	290.25	96.75	96.75	347**[2]
Linked[4]	258	258	0	0	78**
Unlinked with lethal[5]	32.25	290.25	96.75	0	250**
Unlinked *y, yes*[6]	129	290.25	96.75	0	0.611

1 = Number of adults
2 **=Significant @ p<0.001
3 Expected results for a model in which there are two independently assorting genes, one controlling yellow scales and another gene controlling yellow eye.
4 Expected results for a model in which two genes, one controlling yellow scales and another controlling yellow eye, occupy the same chromosome. The special case of lack of recombination in female lepidopterans is discussed in the text.
5 Expected results for a model in which there are two independently assorting genes, one gene controlling yellow scales and another gene controlling yellow eye. Zygotes in one recombinant class (green scale with yellow eye) die.
6 There are two independently assorting genes; *y* confers yellow scales only and *yes* confers both yellow eye and yellow scale.

AceIn was replicated in May 1995 using the Pyr-R strain. Three crosses of Pyr-R x Yel and one cross of Yel x Pyr-R were performed to produce hybrid progeny. All green Pyr-R parents *(y+ y+)* were homozygous resistant *(AceIn AceIn)* and all of the yellow Yel parents *(y y)* were homozygous susceptible *(AceIn+ AceIn+).* Female hybrid progeny from these families were backcrossed to males of the Yel strain. Six backcross families were obtained, but only two of these were analyzed for *AceIn.*

A control experiment was done by establishing five single pairs of Ace-R females *(y+ y+, AceIn AceIn)* with Ace-R x Yel hybrid males (*y+ y, AceIn AceIn+).* This control experiment was performed to determine whether the Ace-R strain was fixed for *y+* and *AceIn* and to test the recessive inheritance of *y.*

Experimental design and analysis

Linkage analysis in *H. virescens* was designed to exploit the fact that recombination has never been reported in females of the Lepidoptera, although recombination of linked genes has been observed in males (Heckel, 1993; Shimada *et al.*, 1994). Test crosses were designed so that the mother was heterozygous for the traits of interest while the father was homozygous. In this orientation, the presence of a single recombinant offspring proved the lack of linkage of the genes of interest.

The hypothesis tested was that any two genes of interest were not linked and would segregate independently according to the Mendelian model. In a backcross of *y+ y, AceIn AceIn+* (mother) x *y y, AceIn+ AceIn+,* it would be expected that progeny would be distributed 50% among the two parental types and 50% among the two recombinant types (1:1:1:1 ratio). A statistically significant departure from this model would be required to reject independent assortment and to conclude linkage. Applying the assumption of no recombination in female lepidopterans, the special expectation would be that, upon linkage, only parental types would be observed. A single recombinant individual would be sufficient to prove

Table 3. Independent analysis for Mendelian inheritance of each of two traits, scale color and eye color, in *Heliothis virescens* using data from test crosses; combined results of 13 pair matings of Yyes/Dalzell98 x Yyes.

	Scale Color		Ratio	χ^2
	Yellow[1]	Green[2]		
Observed	704[3]	229	3.1:1	
Unlinked to eye color[4]	466.5	466.5	1:1	242**[5]
Unlinked to eye color with lethal[6]	466.5	233.25	2:1	121**
Unlinked *y*, *yes*[7]	699.75	233.25	3:1	0.103

	Scale Color		Ratio	χ^2
	Yellow[1]	Green[2]		
Observed	455	478	0.98:1	
Unlinked to eye color[4]	466.5	466.5	1:1	0.567
Unlinked to eye color with lethal[6]	233.25	466.5	1:2	121**
Unlinked *y*, *yes*[7]	466.5	466.5	1:1	0.567

1 Mutant phenotype.
2 Wild phenotype.
3 Number of adults.
4 Expected value for a model in which scale color and eye color assort independently.
5 Significant at $p<0.001$.
6 Expected value for a model in which independent assortment is accompanied by a lethal interaction of mutant genes killing the recombinant class green scales with yellow eyes (see Table 1).
7 Expected value for a model in which one gene (*y*) controls scale color and a second, unlinked gene (*yes*) controls both eye color and scale color as explained in the text.

independent assortment.

Results

All crosses between double mutant Yyes and Dalzell98 produced all wild type progeny indicating recessive inheritance of the trait yellow eye and confirming the recessive inheritance of yellow scale (Mitchell and Leach, 1994). We assumed a single gene controlled each trait. Average emergence of adults from pupae was 89 ±12%. There was no significant difference in results when separated by sex, therefore, pooled results are presented.

Independent assortment between *y* and yellow eye was proven by the presence of progeny of the recombinant class with yellow scales and grey eyes among backcross progeny (Table 1), however, neither the expected model for independent assortment, nor the expected model for linkage fit our observations (Tables 1 & 2). The recombinant class with yellow scales and grey eyes was found in the expected proportion of 0.25 in addition to the two parental classes. Had *y* been linked to a single gene for yellow eye, this recombination would not be expected (Experimental design and analysis). Confirmation of independent assortment was found in the observation of this recombinant class in the expected proportion in the dihybrid cross (Table 2).

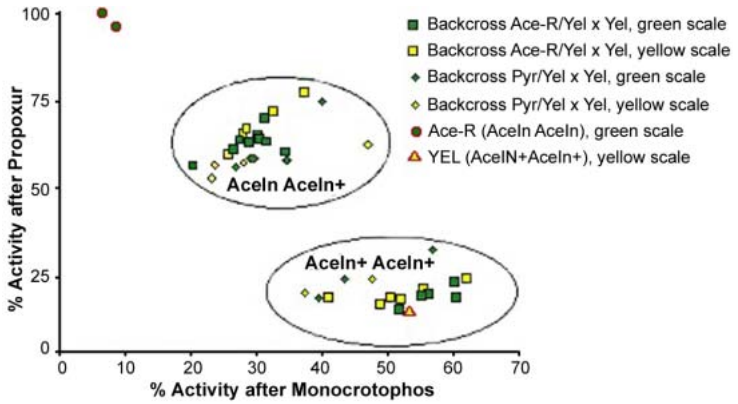

Figure 3. Independent assortment of the genes for yellow scale, *y*, and for acetylcholineesterase insensitivity *AceIn*, of *Heliothis virescens* in two backcross experiments. Strains and scoring are described in Materials and Methods.

Absent in both the test cross and dihybrid cross was the second expected recombinant class with green scales and yellow eyes (Tables 1 & 2). Also, the proportion of the parental class with yellow scales and yellow eyes was increased beyond the expected proportion by an amount equal to the missing class with green scales and yellow eyes. Observations did not fit a model in which *y*+ *y,* yellow eyed was lethal.

The observed data fit a model in which a single gene, independent of *y,* conferred both yellow eye and yellow scale, suggesting that two unlinked genes conferred yellow scale while one of those genes also controlled yellow eye (Tables 1 & 2). According to this model, *y*+ *y,* yellow eyed moths would express yellow scales due to the same gene that conferred yellow eye. The expected recombinant *y*+ *y,* yellow eyed moths would actually occupy the parental class with yellow scales and yellow eyes.

We conclude that a single gene, *yes,* confers not only yellow eye, but also yellow scale. It does so via a mechanism that cannot be overcome by one allele of *y*+ background; *i. e.*, both genes *y* and *yes* confer yellow scales. While this result could also be due to two linked genes in the backcrosses, there were no recombinants with green scales and yellow eyes observed among 516 progeny in the

Table 4. Pedigree and test cross for independent assortment of yellow scale *(y),* yellow scale plus yellow eye *(yes),* acetylcholinesterase insensitivity *(AceIn)* and *Heliothis sodium channel protein* (codon *hscp L1029H).*

MEMBER[1]	ACEIN[2]	GENOTYPE[3]	SCALE	EYE	*HSCP*[4]
Grandmother	0.989	AceIn AceIn	Green	Grey	CAT/CAT
Grandfater	0.161	AceIn$^+$ AceIn$^+$	Yellow	Yellow	CTT/CTT
Mother	0.582	AceIn AceIn$^+$	Green	Grey	CTT/CAT
Father	0.174	AceIn$^+$ AceIn$^+$	Yellow	Yellow	CTT/CTT
Progeny 17	0.143	AceIn$^+$ AceIn$^+$	Yellow	Grey	
Progeny 16	0.161	AceIn$^+$ AceIn$^+$	Yellow	Yellow	CTT/CTT
Progeny 20	0.164	AceIn$^+$ AceIn$^+$	Yellow	Grey	
Progeny 12	0.463	AceIn AceIn$^+$	Yellow	Yellow	CTT/CTT
Progeny 11	0.561	AceIn AceIn$^+$	Green	Grey	CTT/CAT
Progeny 25	0.569	AceIn AceIn$^+$	Yellow	Yellow	CTT/CAT
Progeny 8	0.586	AceIn AceIn$^+$	Green	Grey	CTT/CTT
Progeny 13	0.601	AceIn AceIn$^+$	Yellow	Yellow	CTT/CAT
Progeny 14	0.692	AceIn AceIn$^+$	Green	Grey	CTT/CTT
Progeny 19	0.793	AceIn AceIn$^+$	Green	Grey	CTT/CTT
Progeny 28	1.154	AceIn AceIn$^+$	Yellow	Yellow	CTT/CTT
Progeny 15	nd	AceIn AceIn$^+$	Yellow	Grey	CTT/CAT

1 Members of a pedigree including the maternal grandparents, the parents and 12 offspring. The grandmother was from Dalzell98; the grandfather and the father were from Yyes (strains described in text).

2 Acetylcholinesterase insensitivity is represented by the proportion of inhibition when exposed to propoxur for 15 min; i.e. the quotient of the optical density following propoxur exposure corrected for blank divided by the optical density following acetone control exposure corrected for blank. Progeny 15 was not determined (nd) due to a failure of the control.

3 Genotype for acetylcholinesterase insensitivity was assigned based upon plots of inhibition as described in the text. Progeny 28 was scored heterozygous based on relative rates of inhibition by propoxur and monocrotophos due to partial failure of the control. Progeny 15 was scored heterozygous based on relative rates of inhibition due to complete failure of the control.

4 Genotype for sodium ion channel codon polymorphism for amino acid 1029 as described in the text.

intercross experiments in which recombination was possible in heterozygous fathers (Table 2).

When considered independently from yellow eye, yellow scale was found at nearly 3:1 over green scale (Table 3), which is predicted by our model in which *yes* confers not only yellow eye, but also yellow scale. Considered independently from *y*, yellow eye was found at a ratio of 0.98: 1 with the wild type eye indicating that eye color was controlled by the single, recessive gene, *yes* (Table 3). This was confirmed in dihybrid crosses in which the observed count of *yes yes* to *yes+ yes* was 122 to 394 (expected ratio 129: 378).

No genetic linkage was observed between *y* and *AceIn,* as indicated by the presence of 23 parental phenotypes and 19 recombinant phenotypes in two backcross experiments involving strain Yel (Figure 3). A model for independent assortment of two genes predicted a 1:1:1:1 ratio in the four possible phenotypes: yellow scale, *AceIn+ AceIn+;* green scale, *AceIn AceIn+;* yellow scale, *AceIn AceIn+;* and green scale, *AceIn+ AceIn+*. In the backcross (Ace-R x Yel) x Yel the observed count of 6; 8; 7; 5 moths among the phenotypes, respectively, did not differ from the model of independent assortment (?2 = 1.5768, p<.05, df = 3). In the backcross (Pyr-R x Yel) x Yel the observed count of 4; 5; 4; 3 moths among the phenotypes, respectively, did not differ from the model of independent assortment (?2 = 0.5, p<.05, df = 3).

The ratio of green to yellow was 13:13 in the backcross (Ace- R x Yel) x Yel and 8:8 in the backcross (Pyr-R x Yel) x Yel confirming the hypothesis that a single gene encodes the yellow trait (Mitchell and Leach, 1994). Also, the ratios of 15 *AceIn AceIn+* : 11 *AceIn+ AceIn+* in the backcross (Ace-R x Yel) x Yel and 9 *AceIn AceIn+* : 7 *AceIn+ AceIn+* in the backcross (Pyr-R x Yel) x Yel confirmed that a single gene *AceIn* controlled acetylcholinesterase insensitivity to propoxur (Brown and Bryson, 1992).

Of the progeny from the control group, Ace-R x Ace-R/ YEL, 27 were green and one was yellow. The yellow moth in the control group was unexplained, but it was likely due to human error when pupae were sorted into containers for emergence. The other explanation was that there was a yellow allele in the Ace-R strain at a frequency of 0.04 which was very unlikely in that no yellow mutants had been observed in this lineage for over ten years. These observations confirmed the recessive inheritance of yellow scale as *y*.

AceIn was genetically linked to isocitrate dehydrogenase-2 (IDH-2) placing its locus on chromosome two (Heckel *et al.*, 1998). *AceIn* is linked to increased survivorship (resistance) of larvae exposed to methyl parathion and resistance is completely dominant, being fully expressed in heterozygotes that are *AceIn AceIn+* (Gilbert *et al.*, 1996). This linkage is consistent with the model of a structural mutation giving a resistant enzyme that confers complete resistance from one allele. That is, a half dose of the AceIn enzyme can function in the presence of methyl parathion to restore synaptic responsiveness. However, evidence for a structural gene mutation model is lacking until the acetylcholinesterase gene from tobacco budworm can be cloned and sequenced. Independent assortment of *yes* from *AceIn* and from *hscp* was observed in one family of the test cross Dalzell98/Yyes x Yyes (Table 4). This family was informative for genes *y, yes, AceIn* and *hscp* as seen in the pedigree.

The *AceIn* allele from the heterozygous mother, absent in the father, was found in five grey-eyed progeny of the parental type, but it was observed also in four yellow-eyed progeny which were of the recombinant type (Table 4). Because recombination was prohibited in this backcross (see Experimental Design), this result demonstrated independent assortment. Likewise, the *hscp* allele *CAT* present in the heterozygous mother, but absent in the father, was found in two grey-eyed progeny, but it was observed also in three yellow-eyed progeny proving the independent assortment of *yes* from *hscp* (Table 3). The *hscp* allele *CAT* was found to segregate independently of scale color; one greenscaled progeny inherited this allele from the mother, but three green-scaled progeny inherited the *CTT* allele from the mother and were thereby recombinant type (Table 4). The *hscp* allele *CAT* was found to segregate independently of *AceIn* as there were four recombinant progeny exhibiting the genotype *AceIn AceIn+, CTT/CTT* (Table 4). In this family, one recombinant type for pigmentation was observed (yellow scale with grey eye), but the other recombinant type was absent (green scale with yellow eye) (Table 4). These data were typical of the model described above for two genes controlling yellow scale, one of which also controlled yellow eye (Table 1 and 2).

Discussion

In *D. melanogaster,* some eye color mutations result from

deficiencies in specific enzymes of biosynthesis (Sarkar and Collins, 2000) while other mutations are due to deficient ATPbinding cassette (ABC) transporters of precursors of pigments, or in the transport of pigment granules (Lloyd *et al.*, 1998). In *H. virescens*, our double mutant adult was not devoid of color, but retained a brilliant yellow scale color and a greenish-yellow eye color versus the wild type in which scales were green with black bands and eyes are grey (Figure 1). The source of the yellow color could be the synthesized compound xanthopterin, a guanosine derivative known to impart yellow color to lepidopteran scales. Pterins are also found in compound eyes and they are cofactors in synthesis of another group of pigments, the ommochromes. Synthesized from tryptophan through kynurenine, xanthommatin is a very common red or brown pigment of insect compound eyes and deficiencies in this synthetic pathway are the basis of several eye color mutants of *D. melanogaster,* of several other dipterans, and of *B. mori.*

A black pigment synthesized in insects is melanin, a polymer of indole-5,6-quinone derived from tyrosine. This black pigment, when deposited with the background yellow pigment in scales, could result in the green scale color and black bands on wings observed in the wild type.

In *D. melanogaster,* some eye color mutations result from the loss of ATP-binding cassette (ABC) transporter proteins which appear to be responsible for the active transport and availability of precursors such as tryptophan or guanine (Ewart and Howells, 1998). These transporters are heterodimers so that the transporter of tryptophan is composed of the products of *w*+ and *st*+, while the transporter of guanine is composed of the products of *w*+ and *bw*+. White eye was displayed in homozygous *w* fruit flies or in homozygous *st bw* fruit flies (Sarkar and Collins, 2000).

There are several ways to interpret these results including that a single gene coordinately regulates transcription of genes in both the melanin and ommochrome pathways, or that a single gene produces an inhibitor of enzymes in both the melanin and ommochrome pathways. We favor the simplest interpretation based on known mechanisms which is that an ABC transporter limits the precursors to both melanin and ommochrome. It is known that point mutations in the *white* gene of *D. melanogaster* can decrease both red and brown pigment putatively impairing both tryptophan and guanine transport (Mackenzie *et al.,* 1999).

In *H. virescens,* assuming that the background yellow color is due to xanthopterin, our *yes* mutant might be analogous to the yellow eye mutant of *Ephestia kuehniella* which complemented white eye and red eye (Marec and Shvedov, 1990), or to the *st* mutant of *D. melanogaster,* that has lost transport of tryptophan for ommochrome synthesis while retaining transport of guanine for pterin synthesis. The observed interaction in *H. virescens* in which green and black scales are not found in yellow-eyed progeny as should be expected from segregation of these genes, could be explained by the simultaneous loss of tyrosine transport and tryptophan transport so that both ommochrome and melanin synthesis were prevented, even in the presence of *y*+ alleles, leaving only xanthopterin yellow synthesis to proceed. On the other hand, presence of yellow scales does not prevent expression of the wild type eye color and this suggests that the mutation for yellow scale is specific for melanin and does not affect ommochrome synthesis.

Although it has been conventional to reject linkage and accept independent assortment upon the first observation of recombination in test cross progeny from diheterozygous females (Shimada *et al.*, 1994), this unusual case in which one recombinant class was observed in full proportion, but the other recombinant class was absent requires that the alternate interpretation be explored as follows: the lack of the recombinant class with green scales and yellow eyes could have been due to linkage of *y* to *yes* and half the expected parental wild type class *y*+ *y, yes*+ *yes* somehow expressed yellow scale to produce the then unexpected recombinant class with yellow scales and grey eyes. We cannot provide a logical biochemical mechanism for this alternative explanation of the test cross results. We conclude that there was no linkage between *y* and *yes* based on the presence of recombinant progeny.

We can test this hypothesis by repeating the test cross and mating progeny of the parental double marker class to *y y*, *yes*+ *yes*+ moths. The hypothesis predicts that one -half parental double marker class progeny are actually *y*+ *y*, *yes yes* rather than *y y*, *yes yes* as they appear; therefore, one-fourth the proposed matings would produce all wild type progeny. If the alternate hypothesis is correct, then no matings of this type would produce wild type progeny. We have purified a line of *y y, yes*+ *yes*+ to make this test possible.

Another yellow eye mutant has been observed independently in a strain cultured in Georgia (Hasty and Payne, 1999). This mutant was controlled by a single, autosomal, recessive gene and was found in moths having wild type scales. In Yyes and in the crosses described herein, we have not observed yellow eye in a moth with wild type scales. Our hypothesis predicts that this is a separate locus from *yes* or a different allele that does not simultaneously control yellow scale. Test crosses for complementation revealed complementation of *yes* and this second gene for yellow eye, *ye* (Cho *et al.,* unpublished observations). We have purified inbred lines of yellow scale, grey eye, constructed another strain of yellow scale with yellow eye from this mutant and are testing the segregation of the genes *y* from *ye*.

Development of selectable markers for transgenesis in this species should be directed to finding the wild type allele of an enzyme involved in pigment synthesis. This gene could then be exploited as a marker in vectors when transforming a visible mutant strain. Our results also suggest possible pitfalls in such research when visible marker genes interact to yield unexpected results. In particular, we suggest the possibility that restoration of an enzyme activity as likely conferred by an allele such as *y*+ might not yield the expected transgenic phenotype for visible selection in the presence of a deficiency in a transporter. To our knowledge, this is the first report of a single gene controlling two traits in this species and it is the first report of inheritance relationships of both visible mutations and biochemical markers in this species.

In conclusion, two genes conferring pigmentation and two genes conferring insecticide resistance assorted independently indicating that the genes *y, yes, AceIn* and *hscp* occupied four separate autosomes among the 31 chromosomes of *H. virescens.* These fo7ur genes now become convenient markers for future linkage mapping, *y* and *yes* because they are visible as homozygotes. *AceIn* and *hscp* are convenient due to their clear codominant expression in a simple biochemical assays as described herein. Although these visible mutants are not known from wild populations at this time, *AceIn* was present at an average frequency of 14%

(Brown *et al.*, 1996b) and *hscp1029H* was observed at approximately 20% (Park, 1998) in collections from agricultural fields. It should be easy to capture these alleles for future genetic linkage studies.

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