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

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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 parathion
y a gene conferring yellow scale color
yes 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 mutations (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*, *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 -70°C 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 for automated data acquisition (Molecular Devices, Palo Alto, CA). Enzyme preparations from homozygous methyl parathion-susceptible strains (*AceIn+/AceIn+, 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 immediately 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.

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'-TTGTGTTGTRTCCTGATC-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 93°C proceeded 30 cycles of 93°C for 35 s, 53°C for 1 min, and 72°C for 30 s. The final five cycles were run after the initial 30 cycles, using 93°C for 35 s, 53°C for 1 min, and 72°C 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 0.1%
SYBR 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 interbreeding, families homozygous for Aceln were selected to establish strain Ace-R (Gilbert et al., 1996).

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 “×”.

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 × 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 × 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 × 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 × 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 Aceln or hscp, genotypes were determined in parents of productive matings. One of seven fertile pairs from the backcross Yyes/Dalzell98 × Yyes conducted in 1999 was informative for both Aceln and hscp in addition to scale and eye pigmentation (see Table 3).

To test for independent assortment of yellow scale from Aceln, an experiment was conducted in October 1995 in which twelve pairs of Ace-R × Yel and twelve pairs of Yel × Ace-R were mated as single pairs. Progeny survived from two of the Ace-R × Yel matings and from seven of the Yel × 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 (Aceln Aceln) and that all of the yellow Yel parents (y y) were homozygous susceptible (Aceln+ Aceln+).

Twenty-five of the female hybrid progeny (Ace-R/Yel; y+ y, Aceln Aceln+) were backcrossed to males of the Yel strain (y y, Aceln+ Aceln+). 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 unexpectedly. The backcross parents of the surviving progeny were assayed, confirming that the Ace-R/Yel hybrid mothers (y+y+) were Aceln Aceln+, and that the Yel fathers (y y) were Aceln+ Aceln+.

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.

<table>
<thead>
<tr>
<th>Parental Phenotype Recombinant</th>
<th>Yellow scale</th>
<th>Green scale</th>
<th>Yellow eye</th>
<th>Green eye</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>455</td>
<td>229</td>
<td>249</td>
<td>0</td>
<td>78**</td>
</tr>
<tr>
<td>Unlinked(^3)</td>
<td>233.25</td>
<td>233.25</td>
<td>233.25</td>
<td>233.25</td>
<td>466**(^2)</td>
</tr>
<tr>
<td>Linked(^4)</td>
<td>466.5</td>
<td>466.5</td>
<td>0</td>
<td>0</td>
<td>121**</td>
</tr>
<tr>
<td>Unlinked with lethal(^5)</td>
<td>233.25</td>
<td>233.25</td>
<td>233.25</td>
<td>0</td>
<td>213**</td>
</tr>
<tr>
<td>Unlinked y, yes(^6)</td>
<td>466.5</td>
<td>233.25</td>
<td>233.25</td>
<td>0</td>
<td>1.43</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Parental Phenotype Recombinant</th>
<th>Yellow scale</th>
<th>Green scale</th>
<th>Yellow eye</th>
<th>Green eye</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>122</td>
<td>298</td>
<td>96</td>
<td>0</td>
<td>347**(^2)</td>
</tr>
<tr>
<td>Unlinked(^3)</td>
<td>32.25</td>
<td>290.25</td>
<td>96.75</td>
<td>96.75</td>
<td>78**</td>
</tr>
<tr>
<td>Linked(^4)</td>
<td>258</td>
<td>258</td>
<td>0</td>
<td>0</td>
<td>250**</td>
</tr>
<tr>
<td>Unlinked with lethal(^5)</td>
<td>32.25</td>
<td>290.25</td>
<td>96.75</td>
<td>0</td>
<td>0.611</td>
</tr>
<tr>
<td>Unlinked y, yes(^6)</td>
<td>129</td>
<td>290.25</td>
<td>96.75</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Mutant phenotype</th>
<th>Wild phenotype</th>
<th>Number of adults</th>
<th>Scale Color Ratio</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td></td>
<td></td>
<td>Yellow $^1$</td>
<td>704</td>
</tr>
<tr>
<td>Unlinked to eye color $^4$</td>
<td></td>
<td></td>
<td>Green $^2$</td>
<td>466.5</td>
</tr>
<tr>
<td>Unlinked to eye color with lethal $^6$</td>
<td></td>
<td></td>
<td>Green $^2$</td>
<td>466.5</td>
</tr>
<tr>
<td>Unlinked $y$, yes $^7$</td>
<td></td>
<td></td>
<td>Yellow $^1$</td>
<td>699.75</td>
</tr>
</tbody>
</table>

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.

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).

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 $y^+$, 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, $y^+$, yellow eyed moths would express yellow scales due to the same gene that conferred yellow eye. The expected recombinant $y^+$, yellow eyed moths would actually occupy the parental class with yellow scales and yellow eyes.

The observed data fit a model in which 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

![Figure 3](https://bioone.org/journals/Journal-of-Insect-Science on 07 Aug 2019)

**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.
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).

<table>
<thead>
<tr>
<th>MEMBER</th>
<th>ACEIN</th>
<th>GENOTYPE</th>
<th>SCALE</th>
<th>EYE</th>
<th>HSCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grandmother</td>
<td>0.889</td>
<td>AceIn AceIn</td>
<td>Green</td>
<td>Grey</td>
<td>CAT/CAT</td>
</tr>
<tr>
<td>Grandfather</td>
<td>0.161</td>
<td>AceIn AceIn</td>
<td>Yellow</td>
<td>Yellow</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Mother</td>
<td>0.582</td>
<td>AceIn AceIn</td>
<td>Green</td>
<td>Grey</td>
<td>CAT/CAT</td>
</tr>
<tr>
<td>Father</td>
<td>0.174</td>
<td>AceIn AceIn</td>
<td>Yellow</td>
<td>Yellow</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 17</td>
<td>0.143</td>
<td>AceIn AceIn</td>
<td>Yellow</td>
<td>Grey</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 16</td>
<td>0.181</td>
<td>AceIn AceIn</td>
<td>Yellow</td>
<td>Yellow</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 15</td>
<td>0.164</td>
<td>AceIn AceIn</td>
<td>Yellow</td>
<td>Grey</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 20</td>
<td>0.463</td>
<td>AceIn AceIn</td>
<td>Yellow</td>
<td>Yellow</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 11</td>
<td>0.561</td>
<td>AceIn AceIn</td>
<td>Green</td>
<td>Grey</td>
<td>CAT/CAT</td>
</tr>
<tr>
<td>Progeny 25</td>
<td>0.569</td>
<td>AceIn AceIn</td>
<td>Green</td>
<td>Yellow</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 8</td>
<td>0.586</td>
<td>AceIn AceIn</td>
<td>Green</td>
<td>Grey</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 13</td>
<td>0.601</td>
<td>AceIn AceIn</td>
<td>Yellow</td>
<td>Yellow</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 14</td>
<td>0.692</td>
<td>AceIn AceIn</td>
<td>Green</td>
<td>Grey</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 19</td>
<td>0.793</td>
<td>AceIn AceIn</td>
<td>Green</td>
<td>Grey</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 28</td>
<td>1.154</td>
<td>AceIn AceIn</td>
<td>Yellow</td>
<td>Yellow</td>
<td>CTT/CTT</td>
</tr>
<tr>
<td>Progeny 15</td>
<td>nd</td>
<td>AceIn AceIn</td>
<td>Yellow</td>
<td>Grey</td>
<td>CAT/CAT</td>
</tr>
</tbody>
</table>

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).

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.

Genotype for acetylcholinesterase insensitivity was assigned based on 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.

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

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 ATP-binding 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 $ye$ mutant might be analogous to the yellow eye mutant of Euphestia 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 $ye$ and half the expected parental wild type class $y^+$ $ye$, $ye$ $yes$ 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 $y$ $ye$, $yes$ $yes+$ moths. The hypothesis predicts that one-half parental double marker class progeny are actually $y$ $y$, $yes$ $yes$ rather than $y$ $ye$, $yes$ $yes$ 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 $y$ $ye$, $yes$ $yes+$ 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 $Yes$ 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 $y$, $yes$, Aceln 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. Aceln 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, Aceln 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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