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19. Fully degenerate oligonucleotide primers were prepared on the basis of the amino acid sequences KFREFL (sense orientation) and NENVLKP (antisense orientation). "Touchdown" PCR (*17*) was carried out using rat liver cDNA, and the 0.5-kb product was radiolabeled and used as a probe to screen a rat liver Uni-Zap cDNA library (Stratagene). The Blue-Script vector from positive clones was prepared after in vivo excision, and inserts were sequenced by the dideoxy chain termination method with an Applied Biosystems automated sequencer and "walking" primers.
20. PCR was carried out with rat genomic DNA using the following sense-antisense primer combinations: (i) 288 to 305 and 397 to 418 and (ii) 398 to 419 and 486 to 509 (numbering as in Fig. 1A). The exon-intron structure was determined by comparing the sequences of the products with that found in cDNA clone Z6.
21. Coupled in vitro transcription and translation was carried out with the TNT system (Promega) according to the manufacturer's instructions.
22. Truncated proteins (as well as clone Z5) were expressed in *E. coli* under the control of the T7 promoter (*18*). Different fragments from the coding region of clone Z6 were generated by PCR catalyzed by the Vent DNA polymerase (New England Biolabs) with primers containing sites for Nco I (sense orientation) or Bam HI (antisense orientation). The fragments were cloned into the pKM260 vector. Proteins were expressed in λDE3 lysogens of strain BL21 harboring pLysS by induction for 2.5 hours at 37°C in the presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside. Recombinant proteins were exclusively localized in inclusion bodies, which were purified by washing with 1% each of sodium cholate and NP-40 and were extracted with 5 M guanidine hydrochloride in 0.1 M phosphate buffer (pH 8). The guanidine extracts were diluted 15-fold with 25 mM MOPS (pH 7.5), which contained 0.1% hydrogenated Triton X-100. Insoluble material was removed by centrifugation, and 2-μl portions of the supernatant and serial dilutions thereof were assayed for protein concentration and GAP activity. Similar results were obtained when inclusion bodies were extracted with the detergent laurylsarcosine in place of guanidine.
23. Antibodies were raised by injecting rabbits with recombinant GAP (residues 1 to 257) in the form of purified inclusion bodies in complete Freund's adjuvant. For booster injections, the inclusion bodies were solubilized at a concentration of 12 mg/ml in 4 M guanidine-HCl, which contained 0.6% hydrogenated Triton X-100, and dialyzed overnight against phosphate-buffered saline. Serum was prepared after four injections in incomplete Freund's adjuvant at 3-week intervals. Antibodies were affinity-purified by adsorption to recombinant protein that had been resolved by SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose paper, and by elution with 0.1 M triethylamine (pH 11.4).
24. We thank K. Leuther for providing the pKM260 vector, and D. Ron and G. Neufeld for critical review of the manuscript. Supported by grants from the Israel Academy of Sciences and from the Fund for the Promotion of Research at the Technion.

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Gene Transfer into the Medfly, *Ceratitis capitata*, with a *Drosophila hydei* Transposable Element

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Exogenous functional DNA was introduced into the germline chromosomes of the Mediterranean fruit fly (medfly) *Ceratitis capitata* with a germline transformation system based on the transposable element *Minos* from *Drosophila hydei*. Transformants were identified as phenotypic revertants of a white-eyed mutation carried by the recipient strain. Clusters of transformants were detected among the progeny of 390 individuals screened for germline transformation. Five independent and phenotypically active integration events were identified, in each of which a single copy of the transposon was inserted into a different site of the medfly genome. Molecular analysis indicates that they represent transposase-mediated insertions of the transposon into medfly chromosomes.

The absence of methodology for introduction of exogenous DNA into the genome of insects other than *Drosophila* has been a serious obstacle to progress in the study of the molecular genetics of multiple insect species. In *Drosophila melanogaster* and related species, the *P* element has been used as the basis of a transformation system (*1*). This system does not, however, function in non-*Drosophila* species. Attempts to introduce *P* in *Anopheles* and *Aedes* mosquitoes have yielded only rare germline transformation events that represent random integrations of DNA segments including plasmid sequences, instead of transposase-mediated insertions of the transposable element alone (*2*).

Three transposable elements, unrelated to *P*, have been used for germline transformation of *D. melanogaster*: *hobo* from *D. melanogaster*, *Mariner* from *D. mauritiana*, and *Minos* from *D. hydei* (*3*, *4*). Moreover, mobilization of *hobo* transposons from plasmids injected into preblastoderm embryos has been documented in non-*Drosophila* species (*5*). These results, in combination with evidence that horizontal transmission of mobile elements across insect orders may be widespread (*6*), suggest that transposons other than *P* may be useful for germline transformation of non-*Drosophila* species.

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Minos, a member of the *Tc1* family of transposable elements, was isolated from *D. hydei* and is absent from *D. melanogaster* (*7*) and *Ceratitis capitata* (*8*). Like *P*, *hobo*, and *Mariner*, it belongs to a phylogenetically heterogeneous class of mobile elements that have terminal inverted repeats and transpose by means of a DNA intermediate (*9*). The transposase encoded by *Minos* has been expressed in *D. melanogaster* (*10*) and can catalyze precise insertion of a *Minos* transposon into *D. melanogaster* chromosomes in a manner that can be used for germline transformation (*11*).

The Mediterranean fruit fly *C. capitata* (Diptera: Tephritidae) is a widespread agricultural pest of many fruit species. The medfly has been introduced recently into the New World and is spreading rapidly, threatening fruit production in North America (*12*). The sterile insect technique (SIT) has been used successfully for medfly eradication or control. This method relies on the decrease in or collapse of fly populations caused by the release of large numbers of sterile insects (*13*). The ability to produce transgenic medflies is needed to improve the SIT (*14*) and also to advance knowledge about the molecular genetics of this insect pest.

In addition to a vector, a second requirement for transforming the medfly is an effective genetic marker for transformation. Transformation of the medfly with *Minos* and other transposable element vectors by means of a neomycin resistance marker was unsuccessful (*15*). In *D. melanogaster*, dominant eye color genetic markers have been effective in monitoring transformation. The medfly homolog of the *white* gene of *Dro-*

sophila has been cloned and functions as a transformation marker in transgenic *Drosophila* (*16*). Moreover, this *w* gene hybridizes to 5L on the polytene chromosomes, a site that corresponds to the cytogenetic locus of a white-eyed recessive medfly mutation, which was designated *w* (*17*). The wild-type medfly *w* complementary DNA (cDNA) was incorporated into a *Minos* transformation vector in the hope that it would function as a dominant eye color marker when introduced in the mutant medfly strain, as it does in *Drosophila* (*16*).

Two *Minos*-based plasmids were injected into medfly embryos. The transposase-producing plasmid pPHSS6hsMi2 contains a modified *Minos* element in which the left-hand inverted repeat has been replaced by a 456–base pair (bp) fragment containing the promoter of the *D. melanogaster Hsp70*(87C) gene and has been used for transformation of *D. melanogaster* (*11*). The marked transposon pMihsCcw is derived from a *Minos* element [vector pMiNot (*18*)], in which the central part of the transposase gene has been replaced by the wild-type *white* cDNA of *C. capitata*, flanked by promoter and terminator sequences of the *D. melanogaster Hsp70* gene (*16*).

DNA was introduced into preblastoderm medfly *w* embryos by a microinjection procedure essentially identical to that used for *Drosophila* (*19*). Modifications were unnecessary, because the eggs of the two species are similar in morphology and in resistance to desiccation. A total of 3998 embryos were injected; after injection, they were left to hatch under halocarbon oil, and first-instar larvae were transferred to petri dishes containing standard larval food (*20*). The 390 adults (G0 generation) resulting from injected embryos were collected within 12 hours after eclosion and back-crossed to *w* flies in small groups consisting of either 5 G0 males and 10 virgin *w* females, or 10 G0 females and 5 *w* males. Fifty-nine such G0 groups were reared in small plastic cages, and the G1 progeny were collected and handled separately for each group. To induce expression of the *w* minigene from the *Hsp70* promoter, G1 pupae were exposed daily to a 39°C heat shock for 1 hour. The 62,510 G1 flies that were produced were screened for the presence of nonwhite eye phenotypes. A total of 72 flies with colored eyes were recovered from four different cages (Fig. 1).

Figure 2 shows the phenotypes of some of the putative transformants. The *w* minigene gave partial reversion of the pheno-

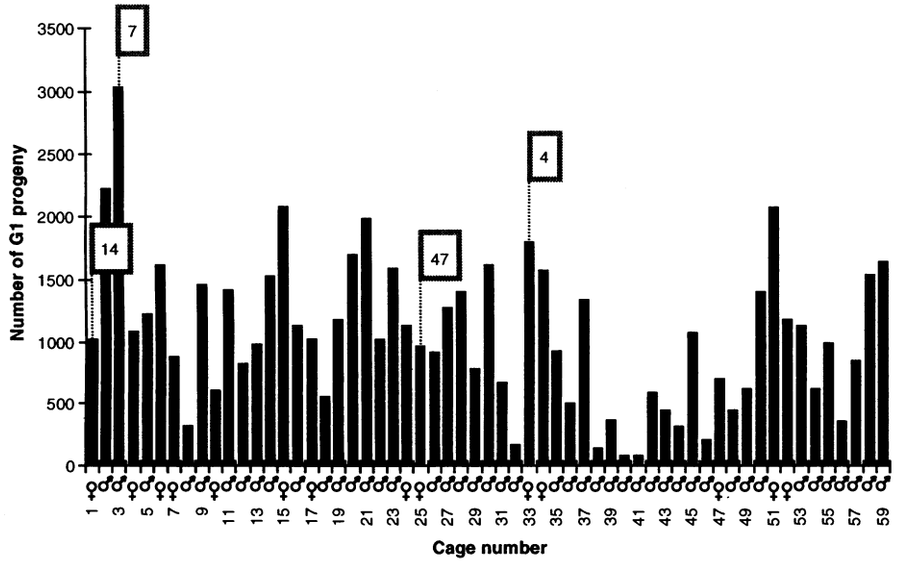

Fig. 1. Frequencies of transformants among G1 progeny. Small groups of flies (5 males or 10 females) that resulted from injected embryos (G0 flies) were backcrossed to *w* flies in small cages. The G1 progeny from individual cages were grown and screened for the nonwhite eye phenotype separately. Bars indicate the numbers of G1 flies from the individual cages. The sex of the G0 flies in each cage is indicated. The numbers above cages 1, 3, 25, and 33 indicate the number of w^+ flies that were recovered from these cages.

Table 1. Phenotypes of progeny from individual G1s backcrossed to *w* flies. ND, not determined.

G1	Eye color of heterozygotes	With heat shock		Without heat shock		Eye color of homozygotes
		Nonwhite eyes	White eyes	Nonwhite eyes	White eyes	
1.1	Pale yellow	46	53	0	59	Apricot
1.8	Pale yellow	220	274	0	77	Apricot
1.12	Pale yellow	94	69	0	8	Apricot
3.1	Yellow	267	237	110	97	Yellow
3.3	Yellow	225	214	53	49	Yellow
3.2	Pale yellow	132	118	0	76	Apricot
3.6	Pale yellow	70	81	0	81	Apricot
25.7	Pale apricot	119	156	116*	91	Apricot
25.8	Pink	24	18	0	27	Peach
25.9	Pink	30	34	0	9	Peach
33.2	Pale orange	42	50	ND	ND	Orange
33.3	Pale orange	29	31	ND	ND	Orange
33.4	Pale orange	16	15	ND	ND	Orange

*Eye color much weaker than with heat shock.

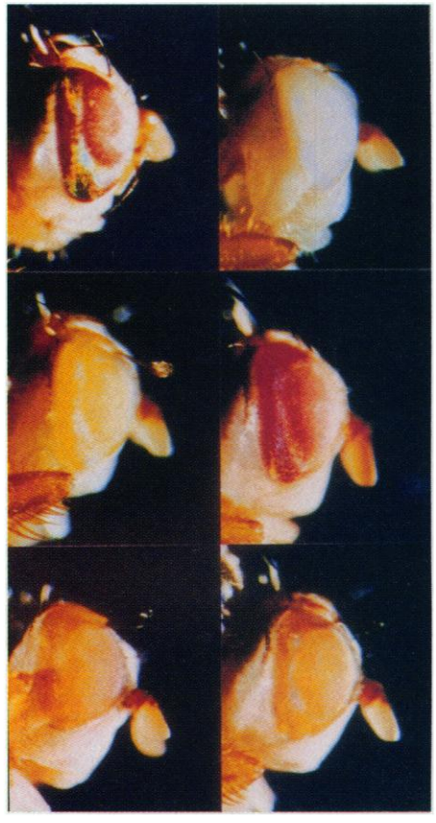

Fig. 2. Eye color phenotypes of transformants. All transformants are homozygous for insertions, except 3.1 and 33.2, which are heterozygous G3 flies. From left to right, (**top**) wild-type (Benakeion) strain and *w* strain; (**middle**) transformants 3.1 and 25.9; (**bottom**) transformants 3.2 and 33.2. Flies were reared at 24°C and received a 1-hour 39°C heat shock every day during pupal development.

type. The eye color varied among different transformants, and the phenotype was dosage-dependent; homozygotes had stronger colors than did heterozygotes. These characteristics of *w* markers have been observed previously in *Drosophila* (*11*, *16*) and are the result of low levels of expression combined with chromosomal position effects.

To establish transformed lines, individual G1s were backcrossed to *w* flies. Single pairs of transformed G2 progeny were then mated, and their homozygous G3 progeny, recognized by their stronger w^+ phenotypes, were used to construct homozygous lines. Table 1 shows results from the G1 backcrosses. In these crosses, the nonwhite eye (w^+) phenotype was inherited as a single dominant trait. To determine the effect of temperature on the expression of the *w* minigene, a number of G2 pupae were not subjected to the heat shock treatment. When compared with the heat-shocked cohort, G2 flies that were not heat shocked as pupae showed either paler eye color or no eye color at all; the only exception was lines 3.1 and 3.3, which exhibited an invariant strong yellow eye phenotype. The heat shock dependence clearly showed that the flies (perhaps with the exception of 3.1 and 3.3) were true transformants, rather than revertants of the *w* mutation. In cages 3 and 25, differences in the eye color phenotypes of individual G1s from the same cage were detected and bred true, which suggests that independent transformation events had occurred in the same cage (Table 1).

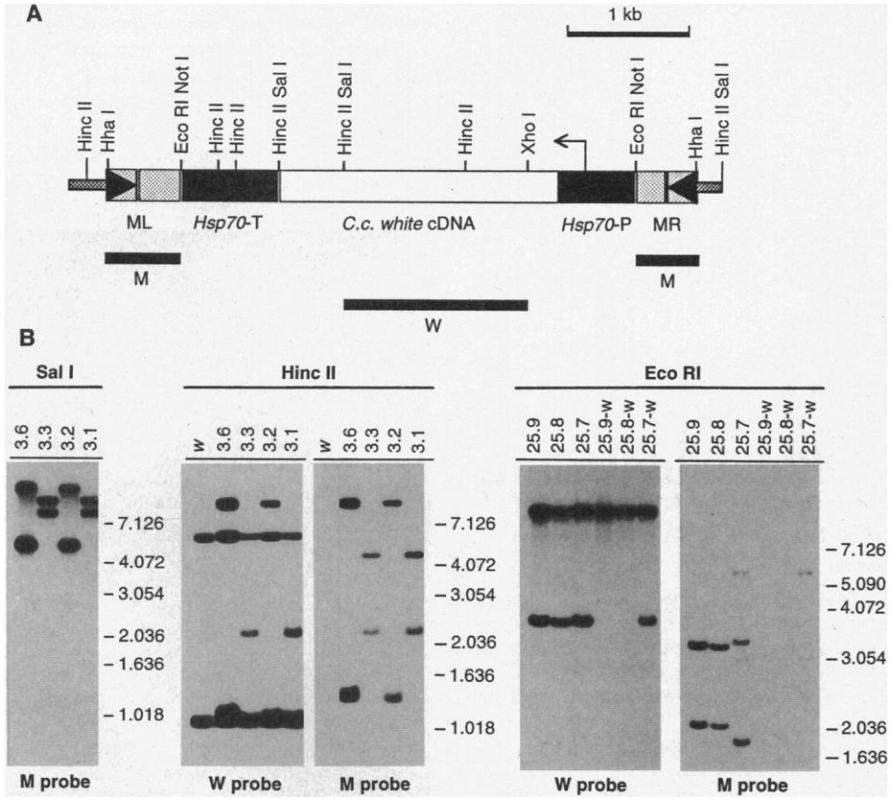

Fig. 3. (**A**) Insert of the transposon plasmid pMihsCcw. The pMihsCcw vector was constructed by insertion of an approximately 3.7-kb Not I fragment containing the *Hsp70-white* cDNA fusion (*16*) into the Not I site of the *Minos* vector pMiNot (*18*). Medfly *w* sequences are shown in white, the *Hsp70* promoter (P) and terminator (T) are in black, and the *D. hydei* sequences flanking the *Minos* element are indicated by stippled bars. ML and MR signify the left- and right-end parts of *Minos*, respectively. Only the restriction sites mentioned in the text or used in the analysis of transformants are indicated. The original *Escherichia coli* vector used (*21*) was pTZ18R (Pharmacia). Black bars below the map indicate the *Minos* (M) and medfly *white* (W) sequences that were used as probes for the analysis of transformants. (**B**) Southern (DNA) blot analysis of transformants. Adult genomic DNA (approximately 10 μg per lane) was digested with a restriction endonuclease, subjected to agarose gel electrophoresis, blotted onto nitrocellulose membrane filters, and hybridized with ^{32}P-labeled probes (*25*). Sal I panel: DNA from lines 3.1, 3.2, 3.3, and 3.6 was cut with Sal I and hybridized with a 1-kb Hha I fragment containing *Minos* sequences present in pMiNot (M probe). Hinc II panel: DNA from the recipient *w* strain and from lines 3.1, 3.2, 3.3, and 3.6 was cut with Hinc II and probed with a Sal I–Xho I fragment containing 1.5 kb of medfly *w* cDNA sequences (W probe) and then probed with the M probe. Between the two hybridizations, the filter was dehybridized by being washed with boiling 0.5% SDS solution for 2 min. Eco RI panel: DNA from strains 25.7, 25.8, and 25.9 was cut with Eco RI and hybridized with the W and M probe sequentially. In addition to the transformants showing nonwhite eye phenotypes, white-eyed siblings (25.9-w, 25.8-w, and 25.7-w) were included in this analysis. Lengths of size markers are in base pairs.

To determine the nature of the integration events, DNA from transformants was analyzed by blot hybridizations with several restriction enzymes and two probes (Fig. 3A), one (M) containing the *Minos* sequences at the ends of the transposon (which are not present in the nontransformed medfly), and another (W) containing an internal fragment of the *w* cDNA sequences (which is present in the endogenous *w* gene). In *Drosophila*, insertions of elements such as *Minos* can occur at many different chromosomal sites and are characterized by precise integration extending through the terminal inverted repeats of the element without transposition of any flanking plasmid DNA (*11*). The results of M-hybridized Sal I digests indicate that the events in the medfly are of the same nature (Fig. 3B). The transposon inserted in variable host DNA sites, without (>0.2 kb) flanking plasmid DNA to the right of the transposon, because this would have generated a 2.9-kb band (Fig. 3A). The results confirm that two independent events occurred in cage 3, one represented by lines 3.1 and 3.3 and the other by lines 3.2 and 3.6 (Table 1). These conclusions were also confirmed with Hinc II digests. Similarly, blots of Hinc II digests hybridized with the W probe showed the two endogenous *w* gene bands, plus a third novel band that is characteristic of the insertion event (3.1-3.3 or 3.2-3.6). The shortest band is longer than the 1.9-kb band that would have been expected if the Hinc II site 0.2 kb to the right of the *Minos* end had been present. The same Hinc II blot hybridized with the M probe showed that the shortest band is longer than the 1.1-kb band that would have been expected if plasmid sequences to the left of the transposon were present. These results were confirmed with W-hybridized Sal I digests (*21*).

To assess the integrity of the internal part of the transposon, restriction analysis with Eco RI was performed in three lines derived from cage 25. The results of the hybridization with the W probe indicate that the entire 3.7-kb fragment containing the *Hsp70-w* marker fusion is present in the w^+ transformants (Fig. 3). Hybridization of the same filter with the M probe, which detects chimeric end fragments, showed that lines 25.8 and 25.9 contain the same single insertion of the transposon. The pattern in 25.7 is consistent with the presence of two insertions, neither of which is identical to the 25.8-25.9 event. One insertion, defined by the ~3-kb and ~5.5-kb bands, is also present in the white-eyed siblings of the 25.7 flies. This, presumably, represents a "silent" insertion that does not express the phenotype either because of an undetected lesion in the transposon or because the transposon has integrated into a silent (per-

haps heterochromatic) genomic region.

In situ hybridization to salivary gland polytene chromosomes (*22*) confirmed that the insertions are localized to different chromosomal positions; insertion 3.1-3.3 is at 51A (4L), 3.2-3.6 is at 94A (6R), and 25.8-25.9 is at 51C (4L). The two insertions in line 25.7 are localized to different chromosomes: at 65C (5L) and at the X chromosome [heterochromatic network (*23*)].

In summary, two independent transformants were represented among the G1 progeny of cage 3, two in cage 25, and one in cage 33 (*24*). Only one of these five transformants (25.7) had a second (phenotypically silent) event in the same germ line. We cannot determine whether the different transformants from the same cages are derived from single or multiple G0 parents. The overall frequency of phenotypically detectable transformation events (5/390 G0 adults) is sufficient for producing several transformants from a single experiment, because thousands of embryos can be injected and hundreds of G0 adults can be obtained within a week with the use of a relatively simple experimental setup. Because of the simplicity and safety in handling and delivery inherent with DNA vectors, transposable elements with wide "host range," such as *Minos*, coupled with appropriate phenotypic markers, could be the vectors of choice for germline transformation of insects of agricultural and medical importance.

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18. Vector pMiNot was constructed by replacement of a 644-bp Msc I fragment that contains the 60-bp intron and part of exons 1 and 2 of the *Minos* transposase gene by a Not I linker.
19. For egg collecting, flies were mass-reared in population cages at 24°C. Eggs were collected at 24°C for 60 min and then were dechorionated, dessicated, and microinjected at 18°C with a mixture of helper plasmid (100 μg/ml) and transposon plasmid DNA (400 μg/ml) as described for *Drosophila* embryos (*1*).
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25. Membranes were prehybridized for 6 hours at 65°C in 7% SDS, 0.5 M phosphate buffer (pH 7.4), and 1 mM EDTA. Hybridization was for 12 to 14 hours at 65°C in 7% SDS, 0.5 M phosphate buffer (pH 7.4), and 1 mM EDTA. Excess probe was removed by two 10-min washes with 5% SDS, 40 mM phosphate buffer (pH 7.4), and 1 mM EDTA at 65°C, followed by a 20-min wash at room temperature with the same buffer prewarmed at 65°C.

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The *white* Gene of *Ceratitis capitata*: A Phenotypic Marker for Germline Transformation

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Reliable germline transformation is required for molecular studies and ultimately for genetic control of economically important insects, such as the Mediterranean fruit fly (medfly) *Ceratitis capitata*. A prerequisite for the establishment and maintenance of transformant lines is selectable or phenotypically dominant markers. To this end, a complementary DNA clone derived from the medfly *white* gene was isolated, which showed substantial similarity to *white* genes in *Drosophila melanogaster* and other Diptera. It is correlated with a spontaneous mutation causing white eyes in the medfly and can be used to restore partial eye color in transgenic *Drosophila* carrying a null mutation in the endogenous *white* gene.

In spite of promising leads (*1*), a reliable method for germline transformation in insects other than *Drosophila* has not been available until now (*2*). A substantial obstacle has been the lack of genetic markers that are suitable for establishing and maintaining putative transformants. Therefore, to develop transformation markers for a major agricultural pest, the medfly *C. capitata* (Diptera: Tephritidae) (*3*), we have focused on genes controlling adult eye color, which have proven their utility for *Drosophila* transformation (*4*).

The medfly displays a complex eye phenotype with reflective and metallic hues, but has only three basic eye pigments: xanthommatin (brown), sepiapterin (yellow), and tetrahydropterin (colorless) (*5*). Numerous mutant eye color phenotypes have been described (*6*), which by inference from *D. melanogaster* should include defects in the formation of eye pigments. The existence of a spontaneous white-eye medfly

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