

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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- The *w* mutation was recovered as a spontaneous event in a laboratory strain and has a "bleached white" eye phenotype in the homozygous form. In addition, *w/w* larvae have white malpighian tubules. Extensive breeding of the *w* strain has shown that it is stable; no phenotypic revertants were recovered from over 10⁶ flies. (I. Livadaras, M. Vasilaki, K. Panethymitakis, C. Savakis, unpublished data).
- 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.
- 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 (7).
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The restriction patterns of three G1s from cage 1 were identical to those of the 3.2-3.6 event. Evidently, a G0 male present in cage 3 had mated with a G0 female of cage 1 before the G0 flies were sorted into cages.

- 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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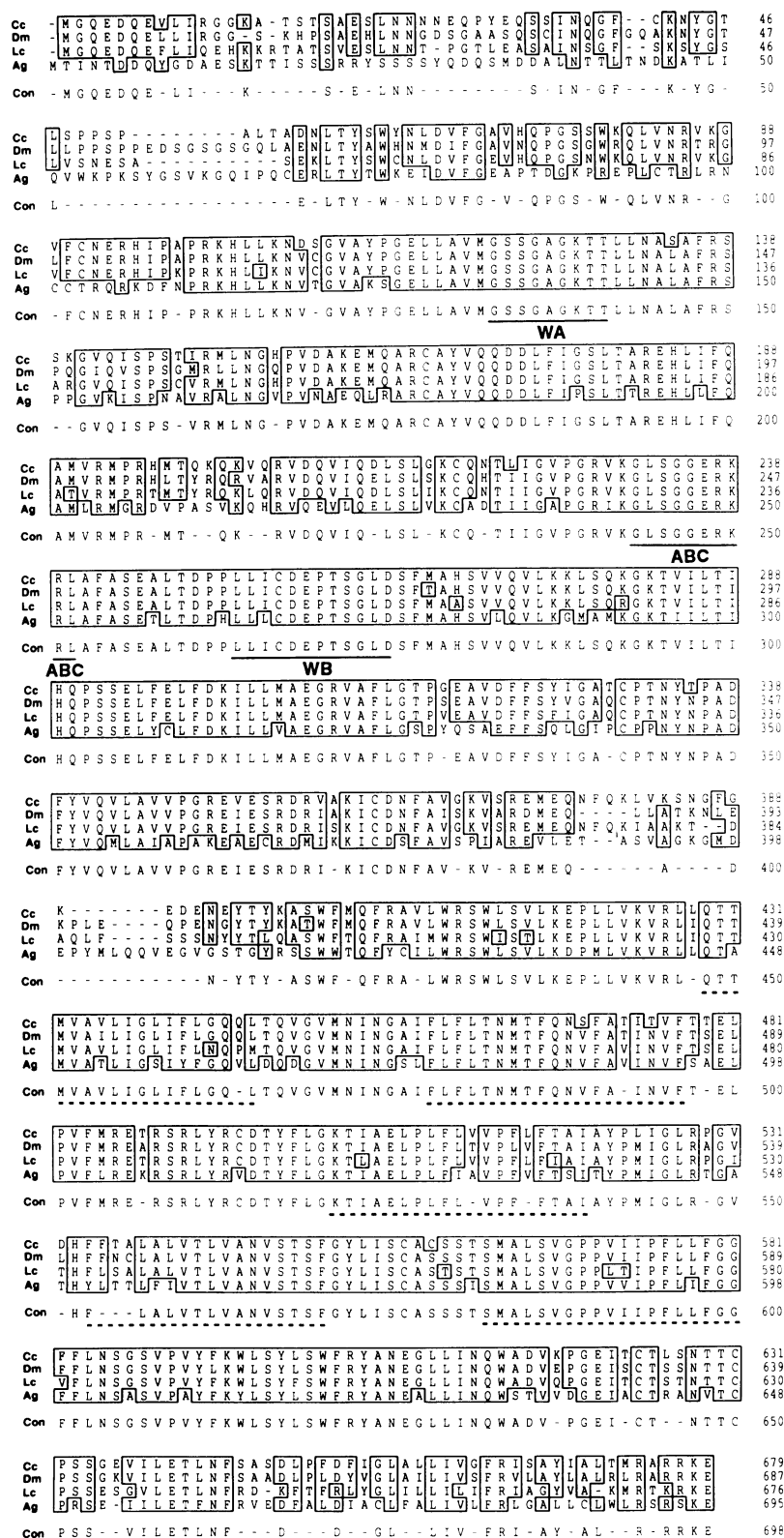


Fig. 1. Amino acid sequence alignment of Dipteran *w* gene products. The predicted sequences of the *w* products of the medfly (*Cc*; this study) (EMBL accession number X89933), *D. melanogaster* [*Dm* (8)], *L. cuprina* [*Lc* (9)], and *A. gambiae* [*Ag* (17)] were aligned with the use of PILEUP (15). Nucleotide binding (Walker A and B) and ABC transporter signature motifs in the NH₂-terminal region are indicated by solid underlines; α -helical transmembrane segments in the COOH-terminal region are marked by dashed underlines. Dashes in sequences indicate either gaps in alignment or areas where no consensus is possible. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

mutant, whose phenotype (2) resembles the *white* (*w*) mutant of *D. melanogaster*, encouraged us to clone the medfly *w* gene as a potential transformation marker.

To clone medfly *w*, we designed degenerate oligonucleotide primers (7) based on sequence conservation between the protein products of the *w* genes of *D. melanogaster* (8) and the blowfly *Lucilia cuprina* (9), together with data on medfly codon usage (10). With the use of medfly genomic DNA as a template, specific polymerase chain reaction (PCR) products of the expected size were generated, cloned, and identified by DNA sequence as homologous to *w* by BLAST analysis (11). One of these PCR products was used as a probe to obtain a phage clone from a genomic library prepared from wild-type medfly DNA (12). This clone was partially sequenced, facilitating the design and synthesis of nondegenerate *w*-specific primers for a PCR-based screen (13) of a medfly third-instar larval complementary DNA (cDNA) library (14). This yielded a 2252-base pair (bp) poly(A)₂₅-terminated cDNA with an open reading frame encoding a predicted protein of 676 amino acid residues with a molecular mass of 75.1 kD; Fig. 1 shows an alignment with the products of *w* genes from other Diptera. Quantitative comparison (15) over the en-

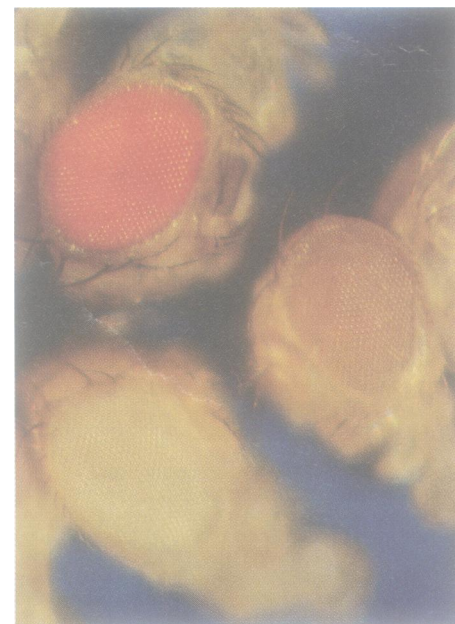


Fig. 2. Partial rescue of the white-eye phenotype in adult *D. melanogaster*. A heat shock-inducible expression cassette encompassing the medfly *w* cDNA was introduced into the germ line of *ry⁵⁰⁶* flies (25) and crossed into the *w* genetic background. After daily heat shock (28), adult male transformants (right) display partial pigmentation. Untransformed reference stocks are shown, including the unpigmented *yw* parental strain (bottom left) and the fully pigmented wild-type Canton S strain (top left).

tire protein length indicates that the medfly sequence is closely related to the *w* gene products of *D. melanogaster* (8) and *L. cuprina* (9) (85 and 82% identical, respectively), whereas the corresponding sequence of the mosquito *Anopheles gambiae* (16) is somewhat more divergent (62% identical). The putative medfly *w* gene product is less similar to insect pigment transporters encoded by the *topaz* gene of *L. cuprina* (9) (37% identical) and by the *scarlet* (17) and *brown* (18) genes of *D. melanogaster* (35 and 31% identical, respectively).

The high sequence similarity to the *w* gene products of other insects also involves identity in characteristic functional motifs (Fig. 1). In the NH₂-terminal region, a 248-residue segment of invariant length contains three motifs representing the adenosine triphosphate-binding cassette of the ABC active transporter superfamily of membrane-associated proteins (19). These motifs include the "Walker A" motif (20), which is thought to be the site of phosphate group-mediated nucleotide binding (21); a less-characterized site known as the "Walker B" motif (19); and a generally defined signature consensus for this class of proteins, located between the two Walker motifs. All three motifs are identical in the products of the putative *w* medfly sequence and of the known *Drosophila* and *Lucilia* *w* genes, whereas that of the *A. gambiae* *w* gene differs by a single conservative replacement in the Walker B motif. An additional conserved feature is a predicted transmembrane domain in the COOH-terminal region consisting of six α -helical segments, five of which are within a conserved 246-residue segment that is invariable in length (Fig. 1).

Cytological studies support the hypothesis that the putative *w* gene homolog is impaired in the spontaneous white-eye medfly mutation (2): This recessive mutation is uncovered by a small deletion that spans the 65C subdivision of chromosome arm 5L, including the site of in situ hybridization of the putative *w* clone (22). This site is consistent with previous molecular and genetic evidence (23) for persistent synteny of X-linked genes and identification of the medfly 5L chromosome with the X of *D. melanogaster* (where the *D. melanogaster* *w* locus resides).

Definitive proof that the cloned medfly cDNA is *w* and that it can function across an evolutionary gap of 100 to 120 million years (24) was obtained by heterologous transformation. This cDNA can restore pigmentation in the white-eye genetic background of *D. melanogaster* if placed in a P-element vector under heat shock control. For this purpose we used 5' and 3' flanking *hsp70* sequences (25), which when combined with *D. melanogaster* *w* cDNAs in

conspecific transformants have been shown to rescue the white-eye phenotype by restoring partial and variable eye color (26). Adult *Drosophila* transformants carrying one copy of the medfly *w* insertion showed a similar dark peach eye color if heat-shocked, as compared with the unpigmented *yw* parental strain (Fig. 2). Therefore, we predicted that this cloned gene may also be used as a dominant transformation marker in the medfly to effectively detect germline transformants in the medfly *w* genetic background. This prediction is confirmed in an accompanying report (2).

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13. PCR-based screening of a third-instar larval cDNA library (14) was performed as described [D. I. Israel, *Nucleic Acids Res.* **21**, 2627 (1993)]. Initially, a cDNA phage lysate containing approximately 3×10^6 phages was divided into 64 wells of a microtiter plate for five rounds of PCR screening to yield a clonal lysate for plaque purification that used pCcW1 as a probe. In vivo excision (Stratagene Cloning Systems) of this purified phage yielded phagemid containing the 2.3-kb cDNA insert denoted pCcW2.
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25. A 2.3-kb fragment containing the medfly *w* cDNA was prepared from the original clone by complete Pst I and partial Kpn I digestions. After creation of blunt ends (27), this fragment was subcloned into the Xba I (blunted) site of the shuttle plasmid pHSS6hspt (2), which contained 450 bp of the 5' and 900 bp of the 3' flanking sequences from the *hsp70* gene of *D. melanogaster*. The orientation of the coding region relative to the *hsp70* promoter was verified by PCR, and the entire expression cassette was subcloned into the Not I site of the P-element vector pDM30 [D. Mismar and G. M. Rubin, *Genetics* **116**, 565 (1987)] and introduced into the germ line of γ^{506} flies according to standard protocols [G. M. Rubin and A. C. Spradling, *Science* **218**, 348 (1982)]. Resultant transformants were selected for ry^+ eye color, and two independent lines, carrying the insertion on chromosomes 2 and 3, respectively, were established by segregation with appropriate balancer stocks.
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29. We thank S. Brogna for a medfly third-instar cDNA library, Y. Livadaras for *Drosophila* microinjection and handling, T. Loukeris for pHSS6/hspt plasmid, X. Gouzzi for chromosome preparations, and the

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Identification of a Member of the MAPKKK Family as a Potential Mediator of TGF- β Signal Transduction

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The mitogen-activated protein kinase (MAPK) pathway is a conserved eukaryotic signaling module that converts receptor signals into various outputs. MAPK is activated through phosphorylation by MAPK kinase (MAPKK), which is first activated by MAPKK kinase (MAPKKK). A genetic selection based on a MAPK pathway in yeast was used to identify a mouse protein kinase (TAK1) distinct from other members of the MAPKKK family. TAK1 was shown to participate in regulation of transcription by transforming growth factor- β (TGF- β). Furthermore, kinase activity of TAK1 was stimulated in response to TGF- β and bone morphogenetic protein. These results suggest that TAK1 functions as a mediator in the signaling pathway of TGF- β superfamily members.

Activation of MAPKs after ligand binding to various receptors has been correlated with numerous cellular responses, including proliferation, differentiation, and regulation of specific metabolic pathways in differentiated cell types. The MAPK signal transduction pathways include three protein kinases, MAPKKK, MAPKK, and MAPK; MAPKKK phosphorylates and activates MAPKK, which in turn phosphorylates and activates MAPK (1). Thus, MAPK cascades constitute functional units that couple upstream input signals to a variety of outputs. Several MAPK cascades have been identified and characterized in organisms as diverse as yeasts and mammals (1). In the budding yeast *Saccharomyces cerevisiae*, at least six MAPK pathways have been identified and individual MAPK cascades regulate distinct responses (2). This marked reiteration in yeast suggests that a similar reiteration of signal transduction modules may exist in mammalian cells to mediate responses to different extracellular stimuli.

One of the MAPK pathways in *S. cerevisiae* controls the response to mating phero-

mone (2). This signaling cascade consists of the Ste11p, Ste7p, and Fus3p or Kss1p kinases, which correspond to MAPKKK, MAPKK, and MAPK, respectively. These yeast proteins act sequentially to transmit a signal to the transcription factor Ste12p, which activates transcription of mating-specific genes such as *FUS1* (Fig. 1A) (2). We developed a genetic approach for the assay of mammalian MAPKKK activity that relies on

a *STE7^{P368}* mutation in the yeast pheromone-induced MAPK pathway (3); an activated form of mammalian Raf (Raf Δ N) or MEKK1 (MEKK1 Δ N) can substitute for Ste11p activity in a Ste7p^{P368}-dependent manner as monitored by the histidine phenotype (His) conferred by the mating pathway-responsive reporter gene *FUS1p::HIS3* (Fig. 1B). We used this approach to screen a complementary DNA (cDNA) expression library (4) from a murine cell line, BAF-B03, for MAPKKKs that might suppress the transcriptional defect of *ste11 Δ STE7^{P368}* cells. One cDNA clone was isolated that activated the *FUS1p::HIS3* reporter gene in a Ste7p^{P368}-dependent manner (Fig. 1B). This cDNA encodes a protein kinase, which we designated TAK1 for TGF- β -activated kinase.

To obtain a full-length clone, we screened the same cDNA library with the TAK1 cDNA insert as a probe and five clones were identified. Four clones contained cDNA corresponding to an additional ~230 base pairs of sequence in the 5' region (5). The full-length TAK1 cDNA encodes a protein of 579 amino acids (Fig. 2A). The primary sequence of the TAK1 protein contains a putative NH₂-terminal protein kinase catalytic domain and a 300-residue COOH-terminal domain (Fig. 2B). The catalytic domain contains consensus sequences that correspond to protein kinase

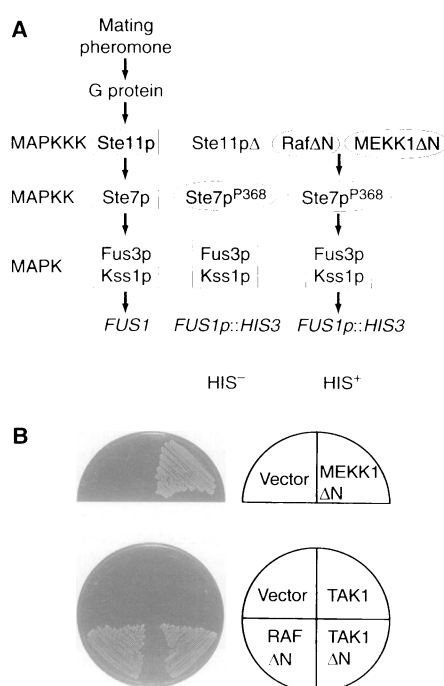


Fig. 1. Screening for mammalian MAPKKK family members in yeast. **(A)** Model for the yeast pheromone-stimulated MAPK pathway. The pheromone-stimulated MAPK pathway induces transcription of mating-specific genes such as *FUS1*. The *FUS1p::HIS3* reporter gene comprises the *FUS1* upstream activation sequence joined to the *HIS3* open reading frame, and allows signal activity in a *his3 Δ FUS1p::HIS3* strain to be monitored by the ability of cells to grow on medium lacking exogenous histidine (His phenotype). A *his3 Δ ste11 Δ FUS1p::HIS3 STE7^{P368}* (proline substitution at serine-368) strain has a His⁻ phenotype because the activity of Ste7p^{P368} is dependent on the presence of the upstream Ste11p MAPKKK (3). Expression of a mammalian MAPKKK such as Raf Δ N or MEKK1 Δ N in this strain confers a His⁺ phenotype (27). **(B)** Suppression of the *ste11 Δ* mutation by mammalian genes. Strain SY1984-P (*his3 Δ ste11 Δ FUS1p::HIS3 STE7^{P368}*) was transformed with various plasmids (27), and each transformant was streaked onto SC-His plates and incubated at 30°C. Plasmids were as follows: (upper panel) YCplac22 (vector) and pRS314PGKMEKKAT (MEKK1 Δ N); (lower panel) pNV11 (vector), pNV11-HU11F (TAK1), pADU-Raf Δ N (Raf Δ N), and pNV11-HU11 (TAK1 Δ N).

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