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RESCUING STERILITY and LETHALITY PHENOTYPES CAUSED BY P[GAL4]C682 TRANSPOSONE

P[GAL4]c682 Transposon Mutasyonu Sonucu Ortaya Çıkan Sterilite ve Letalite Fenotiplerinin Kurtarılması

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SUMMARY

D. melanogaster is well-used experimental organism in analysis of developmental processes in eukaryotes. The enhancer trap technique gives oppurtunity to examine these processes at tissue level and facilitates cloning of the target gene responsible for this expression.

This article purposes to examine a homozygous male sterility caused by P[GAL4]c682 at the genetic analysis. The P-element insert was first excised to prove that the insert is the cause of sterility and 80 % of the excision events returned to fertility. In the remaining 20 %, imprecise excision produced 17 steriles and 6 lethals. Genetically, both excision events were mapped to the 26B region on the left arm of second chromosome as *kisir*. Genetical crossing showed that the lethals established a new independent lethal phenotype group. The rescue of sterility and lethality phenotypes by transgene of *kisir* without heat-shocking proved that *kisir* involves in viability and spermatogenesis processes of *D. melanogaster*.

Key Words: *D. melanogaster*, P[GAL4]c682, kisir, hs-kisir

ÖZET

D. melanogaster (sirke sineği) bir canlının gelişim evrelerini açıklamada yaygın olarak kullanılan ökaryotik deneysel bir organizmadır. İnsansır tırap tekniği bu evreleri doku düzeyinde tespit etme olanağını verdiği gibi, bu ekspresyondan sorumlu hedef genin klonlanmasını da kolaylaştırır.

Bu araştırmada homozigot steriliteye yol açan inhansır tırap P[GAL4]c682 transposununun inhansır tekniği ile genetik olarak analizi amaçlanmıştır. Bu amaçla mutasyona yolaçan P[GAL4]c682 transposunu eksizyon işlemi ile genomdan çıkarıldı ve bu işlemin yapıldığı sineklerin % 80'nin döllü hale döndüğü gözlendi. Geriye kalan % 20'lik gruptaki sineklerin 17'si steril ve 6'sı letal olarak belirlendi. Yapılan eksizyon işlemi sonucunda elde edilen steril ve letal fenotiplerinde kisir gibi ikinci kromozomun sol kolundaki 26B bölgesine yerleşmiş olduğu tespit edildi. Yapılan genetiksel eşlemeler sonucu letallerin yeni ve bağımsız bir fenotip grubunu oluşturduğu anlaşıldı. Sterilite ve ileri dönem embriyonik letalite fenotipleri transjenik *kisir* ile ısı şokuna tabi tutulmaksızın normal hale döndüğü, böylelikle *kisir*'in canlılık ve eşey hücrelerinin gelişim evresinde rol aldığı ispatlanmıştır.

Anahtar Sözcükler: Sirke sineği, P[GAL4]c682, kisir, hs-kisir.

INTRODUCTION

The fruitfly, *Drosophila* melanogaster, provides an excellent system in which to analyse behaviour by forward genetics. As a result of the short life cycle (10 days from fertilization to adult eclosion at 25 °C) and small genome size (165Mb or 1/20 of a typical mammalian genome) of the fruitfly, it is possible systematically screen for all genes, mutations of which may produce a given phenotype (1,2).

The standard approach to studying development in *D. melanogaster*, has been to identify mutations that disturb the process of interest, and then characterize the gene thus identified. Extensive classical genetical screens have been used to identify many genes involved in early developmental processes (3,4). Other approaches have been devised to isolate developmental genes in Drosophila. In recent years, a family of mobile DNA, P-elements, have been used extensively. In the last two decades, particular attention has been given to the P-elements family (5) which has been the subject of intensive research as a molecular tool. Complete P-elements are 2907-bp in length and feature a perfect 31-bp short inverted terminal repeat and 11-bp subterminal inverted repeat (6). The elements encode a well characterized, germ -line limited transposase and regulate their own copy number and activity in wild type strains.

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In addition to the study of their interesting properties, P-elements have been developed as an important tool of genetics and molecular biology in *D. melanogaster*. They have been used in numerous applications such as insertional mutagenesis (7-9), transposon tagging of genes for cloning (10), P-element-mediated transformation (11,12), site-specific recombination (13-15), generating flanking deletions (16,17).

The availability of P-elements for genomic manipulation in *Drosophila* facilitates genetic analysis of behaviour following mutagenesis, transformation rescue, and molecular cloning and expression analysis of the tagged genes using reporter gene, *ß-galactosidase*. The enhancer trap line P[GAL4)c682 subjected to this article contains yeast activator protein, GAL4 and is a kind gift of Dr. D. Shepherd from Southampton University. The line was reported to have an expression in the giant fibre circuit of the fly. This nerve circuit is well used in the examination of behavioural mutant phenotypes such as *Shaking-B* and *bendless* (18,19). This study determined the line P[GAL4)c682 as homozygous male sterile. The aim in this project is to prove that the insertion of P[GAL4)c682 into the fly genome causes homozygous male sterility. If the insertion is the cause of male sterility, to rescue the mutant phenotype (s) by generating transgenic animals.

MATERIALS and METHODS

Fly Stocks

Flies were generally maintained on standard cornmeal, agar, molasses and yeast medium. Fly stock were standard laboratory stocks (except where stated) and were cultured at either 18 °C or 25 °C in plastic vials (Regina Industries Ltd) or glass bottles of food. White canton S was used as a wild type in all the genetical and molecular analysis and is shown with apostrophe, 'wild type'. Mutant and balancer chromosomes are listed in Lindsley and Zimm (20). Some other fly strains were provided from Bloomington Stock center or by personal communication and they are shown below. *Deficiency chromosomes*; Df(2L)E110 (25F3-26A1; 26D3-D11), Df(2L)cl-h3 (25D2-D3; 26B2-B5). P-element lethals; (1(2) 10424[k06801]/CyO, EiF4a[k14518]/CyO, 1(2)10424[10424]/CyO. The donor for these lethals was the Berkely Drosophila Genome Project.

Examination of fertility with mating and dissection

One male was crossed to three females which were confirmed as virgin by keeping at 25 °C for 3 days. The cross was left at 25 °C for 3 days and in the third day it was searched for larvae under microscope in the vial. The vial was kept five days more at 25 °C to see pupae, unless we could see larvae. The male genitalia was then dissected in 1X PBS and then mounted with 50 % PBS-glyceral.

Cuticle preparations

Flies laid eggs on grape juice agar plates and cuticle preparations were performed (21).

RESULTS

The phenotypic description of the male sterility

The enhancer-trap line had been reported as homozygous male sterile, homozygous flies were analysed in different aspects. 100 homozygous male flies for P[GAL4)c682 aged 4-10 days were collected. They were then first examined for fertility and the male reproductive system of these were dissected to examine under Nomarski optics after mounting with 15 % glycerol in PBS. No hatched larva were observed after incubation at 25 °C as described. This proved that they were homozygous sterile. 20 'wild type' flies were also examined in a similar way, all produced larva. The male reproductive system of the mutant flies were examined under the microscope and found that they had an agametic appearance with reduced and shrivelled testes (Figure 1A-B). All the other parts of the reproductive system appeared to be normal, including the paragonial gland which is a tissue producing secretory part of the seminal fluid. The testes seemed to lack any spermatogenic steps and appeared devoid of germinal content. The mutant flies were analysed in relation to the male reproductive system of 'wild type' flies. The mutant phenotype will be named hereafter as *kisir*.

From a stock population of, P[GAL4)c682, 410 flies, only 32 were found to be homozygous. This represent only 8% from the

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expected 25 %, thus male and female homozygous flies for the insert show reduced viability.

Genetical mapping of the enhancer-trap insert with the deficiencies and with known male steriles

The insert facilitates cloning of the target gene due to that the P[GAL4]c682 insert contains multiple cloning sites in the Bluescript vector. Molecularly, the chromosomal position of the insert was determined to be as 26B region (data not shown). The mapping of this new locus was also proved by genetical mapping. To do tihs, *Drosophila* chromosomal deficiencies were used. A flybase search showed that there were two deficiencies chromosomes which uncovered the region 26B to which P[GAL4]c682 mapped. Deficiencies, Df(2L) E110 and Df(2L) cl-h3, were crossed to the insert. Twenty Cy^+ male progeny from each cross were aged and then analyzed for fertility. The male reproductive system of 20 flies were dissected and they were all found to be sterile (data not shown). This result also genetically proved that the insert maps to 26B region on the left arm of second chromosome which was uncovered by the two deficiencies around the region.

A single male sterile allele of *chickadee* and *adenosine* 2 (chi^{11} and $ade2^{10}$) were crossed to P[GAL4]c682 and 20 progeny from both crosses were tested for fertility and dissected. The fertility test and dissections demonstrated that both *ademosine2* and *chickadee* complemented P[GAL4]c682 and therefore the insert represent a new locus required for fertility.

Does the P[GAL4]c682 insert cause of sterility

To prove the P[GAL4]c682 insertion disrupts a locus required for fertility, the enhancer-trap line was subjected to an excision screen. The enhancer-trap line, P[GAL4]c682, was brought together with a transposase source on the third chromosome (The crossing scheme and schematization for the insert P[GAL4]c682 out of genome are shown in Figure 2). 165 crosses were set up and 108 excision events were produced (Table 1). The nomenclature for excisions described using vial number and sex of fly for example; Δ69M1: Δ depicts deletion, 69th excision event as male from the vial number 1. The excisions were examined for fertility with mating experiments. This analysis showed that 80% (85) excisions were restored to fertility, but 17 still remained sterile. In addition, possible imprecise excision of P transposon insert produced 6 homozygous lethals.

Table 1. The results for the excision of P [GAL4]c682 out of genome
Tablo 1. P[GAL4]c682'nin total genomdan çıkarılması ile elde edilen sonuçlar.

	The number of excisions
Fertile	85
Sterile	17
Lethal	6

Steriles are alleles of the insert

During the excision of P[GAL4)c682 from the genome, in a number of excision events (17/108), fertility could not be restored. These still remained sterile as confirmed by dissection (data not shown). Three possibilities existed, either they were internal deletion of the P-element or contained genomic deletions, or represented different sterile mutations. These sterile excisions were then crossed to the original insert to test whether they are allelic. All the male progeny carrying one copy of P [GAL4)c682 and the different excision event were found to be sterile, therefore they are allelic to the original insert (data not shown).

Characterization of the Excision Lethals

6 homozygous lethals; Δ4F4, Δ58M2, Δ58M4, Δ69M1 , Δ69F2, Δ78F1 balanced over CyO were isolated in the excision of P [GAL4]c682 (Table 1). Over 1000 flies were counted from each stock and these six excisions were all confirmed to be homozygous lethal. To determine if they were allelic to the original insert, they were crossed to P[GAL4]c682 and the male progeny carrying both c682 and the excision lethal chromosome were tested for fertility. All the lethals were found to be sterile, and therefore allelic to the *kisir* insert. This possibly indicated that the *kisir* insert is required for both viability and fertility. To confirm that all

the lethals represented a single complementation group, they were intercrossed to each other (data not shown).

Given that the stock was not isogenised before the screen it was important to map the lethal mutations to the 26B region on the chromosome. To map the lethals to the kisir genomic region, the lethals were crossed to deficiency chromosomes, Df(2L)cl-h3 and Df(2L)E110 which uncovers the kisir insert. Δ69M1, Δ69F2, and Δ78F1 were found to be alleles of both deficiencies, but Δ58M2 and Δ58M4. The last two were somewhere from the genome, but from the kisir region. The lethals were also crossed to the known P-lethals from the region; l(2)10424^{k06801} (26B1-B2), Eif4a^{k14518} (26B1-B2), l(2)10424^{10424} (26A8-A9) balanced over CyO. Complementation analysis was performed with these three lethals and they were recognised as an independent lethal group (data not shown).

The determination of lethal phase of the lethals

To determine the time at which the lethality of the P[GAL4]c682 occurs, the lethals were first placed into a wild type background to remove the CyO balancer chromosome. This was achieved by crossing the 'wild type' male flies to virgin female of the P[GAL4]c682 lethals. The white excision lethals were selected. The same cross was performed for each lethal excision. The siblings of w, Cy+produce 25 % homozygous excision mutant flies for analysis. The flies (w, the excision lethals/Cy+) were kept in laying cages to collect eggs on grape juice agar plates. 500 eggs were collected and laid on another grape juice agar plate for further incubation at 25 °C. Eggs were incubated at 25 °C for 24 hours until they hatched and unhatched or immobilised embryos were counted for each lethal excisionas (Table 2). In all cases each group produced approximately 25 % dead or immobolised embryo. The majority of embryos failed to hatch and could be seen to be full developed.

The lethality of the excision lethal were also examined during larval and pupal stages. As an example the scores of Δ69M1 are given shown. 500 larvae of w, Δ69M1/+ were transferred into vials and 92 % (460) formed pupae and 89 % (445) emerged as adult. As control, 200 larvae of WC-S were transferred into vial and 95 % (190) formed pupa, 183 out 195 (91.5 %) emerged as adult. The ratio of larva and pupa in the excision lethal in relation to the wild type remained almost the same. Therefore, the only lethal phase for the excision lethals was determined as late embryonic-first instar larva border.

Phenotypic characterization of the lethals

For further examination of the lethality, the unhatched embryos for the lethals, were used for cuticle preparation (21). The cuticles were removed, cleared and mounted prior to examination in dark field microscopy. The samples were analysed using a number of key markers such as denticle belts, mouthparts, filzkörper and anal pads which are found on the embryonic cuticle. The mutant embryos were compared to the cuticle of 'wild type' embryos and did not show any defect at the morphological level (data not shown).

Table 2. The examination of lethal phase at embryonic stage
Tablo 2. Embiryonik aşamada letal fazın incelenmesi.

Number of egg laying	Number of egg laying	Number of unhatched	Number of immobilised	The % between unhatched immobilised and total
Δ4F4	500	73	32	21 %
Δ69M1	500	81	43	24.8 %
Δ69F2	500	90	37	25.4 %
Δ78F1	500	83	53	27.2 %
WC-S	500	6	0	1.2 %

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Making Transgenic flies of kisir with heat shock vector

Sterility was the first identified phenotype and proved to be the insertion of P[GAL4]c682, but there was not any molecular proof for which gene mutation resulted in sterility. To make this, the gene of interest was cloned into pCaSpeR-hs (12) which is a P-element vector with a white selectable marker for expressing open reading frames under heat shock promoter control. Technical aspects of the method have been described elsewhere (24,25).

The cDNAs of kisir all mapped within the 2Kb PstI genomic fragment (data not shown) and it was therefore the best candidate for rescuing the related sterility and lethality phenotypes of the enhancer-trap. The fragment was cloned blunt-ended into the StuI site of pCaSpeR-hs. Ligation and transformation reaction were performed (27).

To produce transgenic flies of kisir, 10 µg DNA of pCaSpeR-hs+kisir-1 was co-precipitated with 625 ng DNA of transposase-making "helper plasmid", pπwchsΔ2-3 (25). The precipitate was resuspended in 10 µl of distilled water The transformation was performed as described in Sambrook (26). Blastoderm stage of 'wild type' embryos were used in injection of heat shock-kisir construct-1 (hs-kisir-1). The construct was injected into 1400 embryos and 142 of them survived (10 %). 35 out of 142 were identified to be Go. Of these, 8 were found to be transformants (white+) containing the hs-kisir construct and they formed three independent transformants group; hs-kisir-1/6, hs-kisir-1/3 (5 identical transformants), hs-kisir-1/8 (2 identical transformants).

All the Go were then crossed to w; CyO/Sp; TM6B/rf10 and five transformants originating from the hs-kisir-1/3 Go mapped to the third chromosome. The transformants were named as hs-kisir-1/3. 1, 2, 3, 4, 5 (the data for mapping the transformants is not shown).

Rescuing the sterility phenotype caused by the enhancer-trap

Excision of the P[GAL4]c682 showed that the insert disrupts a locus involved in fertility of the fly. To answer this question, hs-kisir-1/3. 1 and 3.2 transformants were crossed with the P[GAL4]c682 background. The crosses were kept at 25 °C and dissections were performed to the male genitalia of homozygous for kisir under hs-kisir-1/3.1 and 3.2 control without applying any heat treatment (Table 3). The male genitalia dissections are presented in figure 3. Restoring fertility in c682 mutants proved that sterility is the phenotype of kisir and can be recoverable in the majority of transgenic flies without heat-shocking.

Rescuing the lethality of kisir mutation

A number of lethals were isolated from the P[GAL4]c682 genomic region via P-element excisions. To prove that the lethality phenotype was due to disruption of kisir, hs-kisir-1/3.1 transgenic flies were crossed to lethal Δ69M1 from the excision screen. The cross was kept continuously at 25 °C and homozygous flies for the lethals under hs-kisir control were first counted without heat shocking. The score for each genotype are presented in a table (Table 4). The results of table shown that homozygous lethality of excision event Δ69M1 is recovered by hs-kisir without inducing a heat shock.

Table 3. Rescuing the sterility phenotype by transgene of kisir.
Tablo 3. Kisir transgeni ile steril fenotiplerin kurtarılması.

The genotype of homozygous male flies	Fertile	Sterile	%
w ; c682 ; hs-kisir-1/3.1 / w/Y c682 hs-kisir-1/3.1/+	95	5	95
w ; c682 ; hs-kisir-1/3.2 / w/Y c682 hs-kisir-1/3.2/+	71	15	80

Table 4. Rescuing the lethality phenotype by transgene of kisir.
Tablo 4. Kisir transgeni ile letal fenotiplerin kurtarılması.

Parents	The genotypes of progeny	Phenotype	NOF
w ; Δ69M1 ; hs-kisir-1/3.1 w/Y CyO +	w ; Δ69M1 ; hs-kisir-1/3.1 w/Y CyO hs-kisir-1/3.1/+	w+, Cy	86
	w ; Δ69M1 ; + w/Y CyO +	w, Cy	32
	w ; Δ69M1 ; hs-kisir-1/3.1 w/Y Δ69M1 hs-kisir-1/3.1/+	w+, Cy+	22
	w ; Δ69M1 ; + w/Y Δ69M1 +	w, Cy+	0

DISCUSSION

The regulation of fertility in D. melanogaster males is a complex process. The magnitude of the genetic control is best demonstrated by the number of loci when mutated will cause sterility. In general, male-sterile mutations fall into three broad class; a) weak alleles of genes involved in also metabolic function, b) special alleles of genes required for vital functions, c) genes required only during spermatogenesis. A number of male-sterile mutants are undoubtedly weak alleles of genes involved in general or metabolic processes where gametogenesis is especially sensitive. In fact, many temperature-sensitive lethal mutations that result in male sterility, also affect female fertility (27). This could be a reflection of the sensivity of the developing germ cells to the changes in basic cellular functions. Some autosomal genes are expressed exclusively in the male germline during spermatogenesis as well as other tissues, like B2t which encodes spermatogenesis specific isoform. This could also signify that many gene products required spermatogenesis are also needed for other developmental process.

Several of the more recent screens have utilized controlled mobilization of a marked P transposable element, a single P element insertional mutagenesis (28), instead of chemical mutagenesis, to conducts a genetic analysis of spermatogenesis (24). Castrillion and his coworkers (30) isolated and phenotypically characterized 83 male-sterile autosomal mutants. Spermatogenesis can be divided according to the phenotypes of sterile mutants: proliferation defects causing early interrupted spermatogenesis and testes with reduced germinal contens, growth phase defects affecting spermatocyte growth, meiotic entry and meiosis defects, post-meiotic differentiation, behavioural defect, sperm transfer defect (22,23).

This study aimed to investigate the phenotypic changes in male and female flies resulting from the insertion of the enhancer-trap, P[GAL4]c682. Homozygous male flies for the insert were found to be sterile and the testes were shrivelled and significantly smaller than those of 'wild type' males. Close up examination of the testes indicated that the testes from 4-10 days old flies are agametic and do not contain any trace of spermatogenesis (Figure 1B). The reduced size and shrivelled-shape of the testes in kisir flies are similar to the rudimentary germline-less organs from male progeny of oskar 301/oskar301 females. *Multi sex combs* (mxcG43/Y), *diaphanous* (dia) and *Chickadee* (chi). castrillion and his colleagues (29) proposed that altered testes morphology as a screening criteria for mutations that cause either degeneration after developmental arrest, or underproliferation of the germline as described in kisir.

Kisir is a new sterility locus

Dissections and fertility tests of male flies when homozygous for the enhancer-trap line had a sterile phenotype. 108 excisions were screened for the reversion of fertility via fertility test and dissection, 85 (80%) returned to fertility. The majority of excision events

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showed that the sterility was derived from the P [GAL4]c682 insert. Excision screening for the insert also generated some predicted imprecise excisions having a sterile or lethal phenotype. Different chromosomal aberrations in Drosophila facilitates the determination of the locus by complementation analysis. To define P [GAL4]c682 as a new locus on the genome, the insert was firstly crossed to deficiency chromosomes, Df(2L)cl-h3 and Df(2L)E110 from the 26B region where the original P-element mapped. Siblings of these crosses were analysed for fertility and found to be sterile. This defines the position of P[GAL4]c682 insert as 26B on the left arm of second choromosome. Known male steriles for this region, *chickadee*^{11} and *ademosine*2^{10}, were first tested for complementation. Both were found to complement, therefore the P[GAL4]c682 insert defined a new sterility locus, kisir.

Rescuing the late embryonic lethality and sterility phenotype of the insert P[GAL4]c682

The disruption or deletion of sequence from the region causes sterility and lethality. Even though, northern and western analysis showed a low level of expression in mutant flies for kisir (data not shown), there was not enough molecular proof to consider this gene responsible for these phenotypes.

To answer this question, the 2Kb PtsI genomic fragment was used to gererate transgenic flies using heat shock vector. Transformants were used to rescue both the sterility and lethality phenotypes. The homozygous male flies for kisir under ks-kisir-1/3.1 and 3.2 were directly dissected without heat shock regime, and the majority of them returned to fertile. This proved that kisir is the responsible for sterility phenotype. The transgenic flies for kisir were also used to rescue homozygous lethality for the excision lethal Δ69M1. The homozygous flies for the lethal under hs-kisir scored in the right ratio. The assessment of this score proved that lethal phenotype of these excision was also recovered without heat shock regime the same as rescuing sterility. This also implied that lethality is the null phenotype of kisir deletion.

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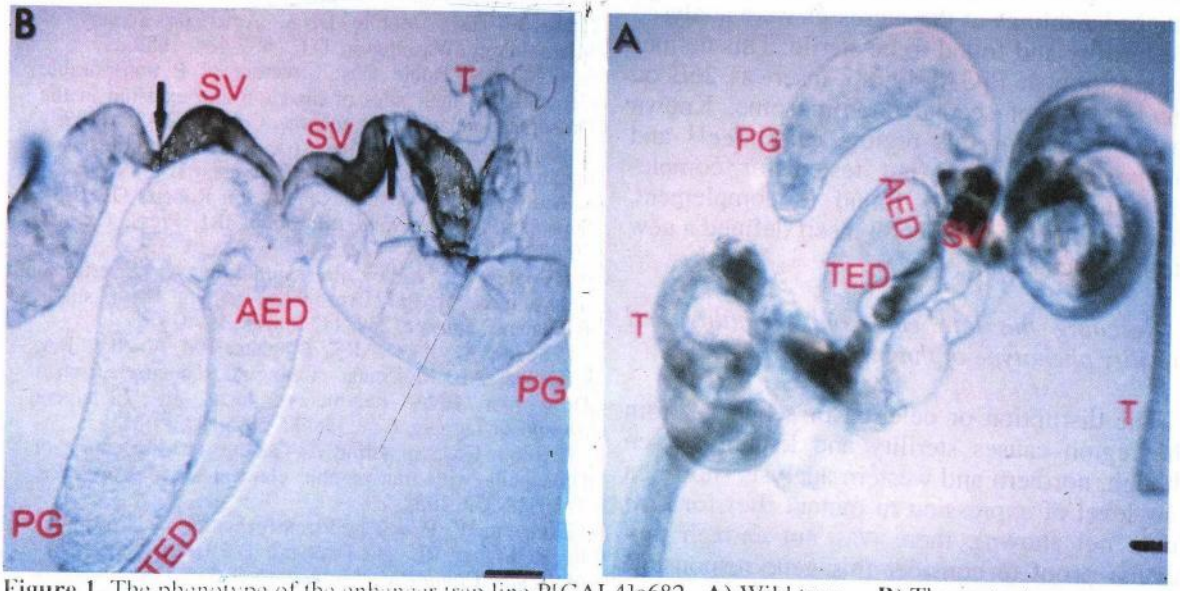

Figure 1. The phenotype of the enhancer trap line P[GAL4]c682, **A)** Wild type **B)** The mutant
Abbreviations; **T:** Testes, **PG:** Paragonial gland, **SV:** Seminal Vesicalis, **AED:** Anterior Ejaculatory Duct, **TED:** Tubular Ejaculatory Duct, Scale bars; 50 $_{u}$m
Resim 1. İnhansır trap P[GAL4]c682'nin fenotipi, **A)** Normal görünüm **B)** Mutant gürünüm.
T: Testisler, PG: Paragonal bezler, SV: Vesicula seminalis, AED: Ön ejakulator kanal, TED: Tübüler ejakulatör kanal, Bar: 50$_{u}$m

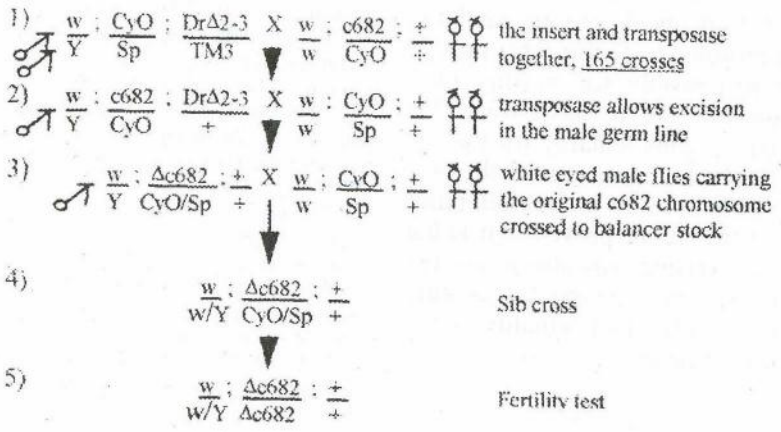

Figure 2. The crossing schema for the excision of P[GAL4]c682.
Resim2. P[GAL4]c682'nin çıkarılması için yapılan kroslamaların şematik görünümü.

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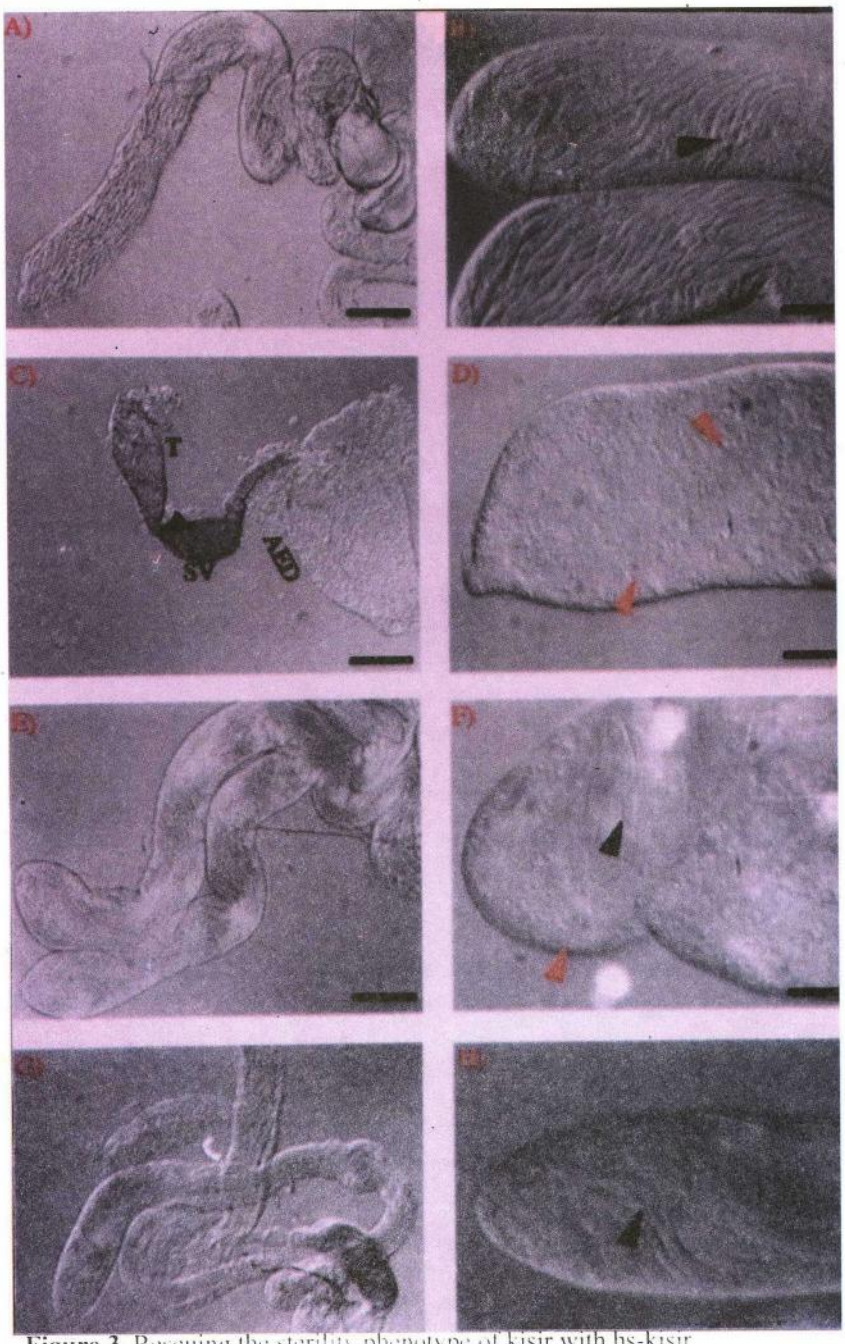

Figure 3. Rescuing the sterility phenotype of kisir with hs-kisir
A-B: The 'wild type', **C-D:** Homozygous mutant male for kisir, **E-F:** Homozygous mutant male for kisir in hs-kisir/3.1 background, **G-H:** Homozygous mutant male for kisir in hs-kisir/3.2 background.
Scale bars: 100 μm in A, C, E, G; 20 μm in B, H; 25 μm in D, F.
Resim 3. Kısıra ait siterilite fenotipinin hs-kısır ile kurtarılması.
A-B: Normal görünüm, **C-D:** Homozigot mutant kısırdan görünüm, **E-F:** Hs kısır/3.1 kontrolü altındaki homozigot mutant kisirdan görünüm, **G-H:** Hs-kisir/3.2 kontrolü altındaki homozigot mutant kısırdan görünüm.