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High efficiency of a double-screening method on single *P*-element insertion lines to identify quantitative trait mutants in *Drosophila melanogaster*

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Abstract

Enhancer trap *P*-element insertion has become a common method for generating new mutations in *Drosophila melanogaster*. When this method is used to isolate mutants for quantitative traits, an appropriate control must be established to define normal and mutant phenotypes. Considering that enhancer-trap lines are generated by crossing several strains, usually with no homogeneous genetic background, no clear control strain can be selected. Previous reports tried to overcome this problem by homogenizing the genetic background of the original lines. However, this is not the most common scenario, especially when functional phenotypes are studied in previously generated lines. Without such caution, is it possible to identify functional mutants among *P*-element insertion lines? We tested this for olfactory preference, a quantitative trait. Using as control measurement the average phenotype of 30 simultaneously generated *P*-element insertion lines with preferential reporter-gene expression in olfactory reception organs, we found that 25 of the lines exhibited mutant phenotypes in response to one or several of 5 tested odorants. Additional tests showed that the efficiency of the method for detecting olfactory mutations exceeded 60% even for such a small number of tested odorants. According to these results this approach greatly facilitates the identification of putative abnormal phenotypes, which must be extensively confirmed afterwards.

Introduction

Single *P*-element insertion in *Drosophila melanogaster* has been used for the last fifteen years as a common procedure for generating structural as well as functional mutations (Cooley, Kelley & Spradling, 1988; Spradling et al., 1995) but little attention has been paid to quantify the efficiency of the method. The reporter gene expression of enhancer-trap lines has revealed particular expression patterns in certain tissues (Bier et al., 1989) as well as the time course of gene expression (Rogina & Helfand, 1996), thus providing information on genes needed for the development of body structures. Functional tests in enhancer-trap lines with restricted expression pattern of the reporter gene to certain organs were expected to identify genes involved in the proper function of such organs (e.g. Scott et al., 2001). The olfactory system of *Drosophila* has been analyzed extensively using these methods: its development and organization (Riesgo-Escovar et al., 1992; Yang et al., 1995; Tissot et al., 1997), the temporal patterns of gene expression in the antenna of the adult *Drosophila melanogaster* (Helfand et al., 1995) and even the effects of *P*-element insertion on olfactory behavior

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(Anholt, Lyman & Mackay, 1996; Fedorowicz et al., 1998) have been described. These previous studies support the use of single *P*-insertion lines for genetically dissecting some olfactory pathway processes, such as the olfactory reception.

According to the combinatorial model proposed for olfactory coding in vertebrates, a single receptor molecule can be excited by different odorants and vice versa, a single odorant can couple to different receptor molecules (see review by Malnic et al., 1999). In *Drosophila* the first characterized receptor revealed a rather stringent specificity for benzaldehyde and closely related chemical structures (Stoertkuhl & Kettler, 2001). Other gene products related to olfactory reception, such as transduction proteins or odorant binding proteins, mediate olfactory information of some odorant subgroups but not others (see review by Schild & Restrepo, 1998). Mutations in genes related to all these processes are therefore likely to provoke quantitative defects instead of anosmia. Therefore, a functional description of olfactory reception mechanisms requires the use of special tests for quantifying olfactory sensitivity (Stoertkuhl & Kettler, 2001).

The definition of an appropriate control strain is an essential step for determining whether a certain *P*-insertion line has a mutant phenotype in a quantitative trait such as olfactory preference. Considering that *P*-insertion lines are generated by crossing several strains, usually with no homogeneous genetic background, no clear control strain can be selected. Previous reports have tried to overcome this problem by homogenizing the genetic background of the original lines (Anholt, Lyman & Mackay, 1996; Fedorowicz et al., 1998, Norga et al., 2003). However, this is not the most common scenario; for example, in large-scale projects like the Berkeley Drosophila Genome Project (BDGP) that intend to establish a gene disruption library affecting the complete *Drosophila* genome using *P*-element insertions. A recent report of BDGP (Bellen et al., 2004) described 7140 lines associated with 40% of *Drosophila* genes and only a 6.75% of these lines were generated in an isogenic background. Thus, finding a method to evaluate mutant phenotypes for quantitative traits in *P*-insertion lines would reinforce the utility of such formerly generated stocks.

In this report we studied 30 enhancer-trap lines with *Gal-4* reporter gene expression in the olfactory organs of *Drosophila* (antennae and maxillary palps) that were selected from 2000 lines generated previously by the same *P*-mutagenesis program (Hovemann et al., unpublished results). The single *P*-insertion has been located on the second chromosome in 21 lines, on the third chromosome in 3 lines and on the X chromosome in 6 lines. These 30 lines were analyzed for behavioral response to 5 odorants using a Y-maze, which allows quantitative measurement of olfactory preference. Normal phenotypes for each odorant were defined by the average response of all the lines, considering that they share basically the same genetic background since they were generated from the same crossing program. This kind of analysis has been usually applied to natural population studies estimating that the variation present in a natural population could be accurately represented by a high number of isofemale lines (approximately 50) (Parsons, 1979). Since the crossing scheme for generating *P*-insertion lines involves only a few original lines (4 in most cases) and maintains some complete chromosomes over several generations for producing homozygous lines, the level of initial variability should be much lower than that of a natural population and consequently the number of lines we studied should be sufficient.

Materials and methods

Fly stocks

One set of 2000 Gal-4 enhancer-trap lines with single insertions of the engineered P{GawB} transposable element was generated by the usual crossing methods (Brand & Perrimon, 1993). Preliminary β-galactosidase staining was performed according to previous reports (Riesgo-Escovar et al., 1992). Analysis of adult hybrids of these lines with a UAS-lacZ strain detected 30 Gal-4 lines with preferential β-galactosidase expression in the olfactory receptor organs: antennae and maxillary palps (generation and analysis of enhancer trap lines were performed by Hovemann et al., unpublished results). Strains Df(2R)PC4 (55A;55F), Df(2R)42 (42C3-8; 42D-2-3), Df(2R)ST1 (42B3-5; 43E15-18), Df(2R)Stan1 (46 D7-9; 47F15-16), Df (2R)en-B (47E3-48A4) for deficiency mapping were obtained from the Bloomington Stock Center.

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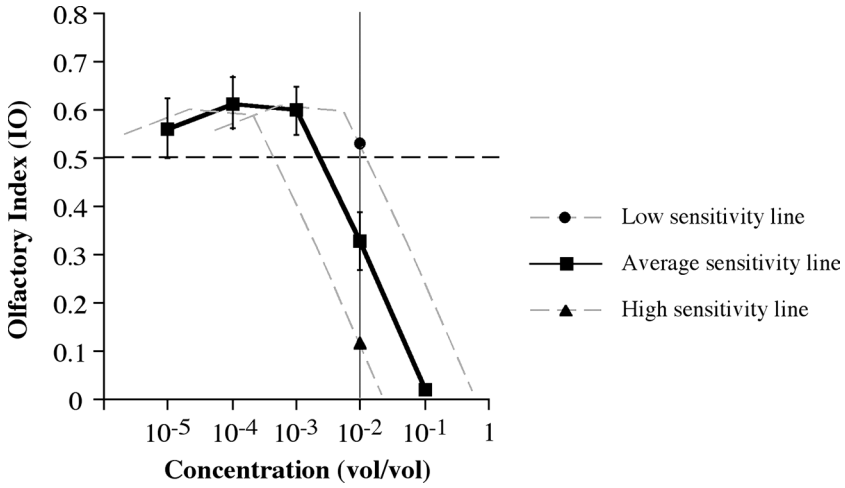

Figure 1. Typical dose-response curve in the Y-maze. ■ Responses of Canton-S flies to ethyl acetate. Single concentration at the intermediate repellent region of the curve used to detect lines with different olfactory sensitivity to the stimulus. ■ Average, ● low and ▲ high sensitivity lines, respectively.

Stocks *w; UAS-TNT-G* (UAS-TNT insert on chromosome II), *w; UAS-TNT-E* (insert on chromosome II) and *w; UAS-TNT-K* (insert on chromosome III) were crossed to a sample of 10 Gal-4 lines to direct tetanus toxin light chain expression at the corresponding cell subgroups to study olfactory behavior changes (Sweeney et al., 1995). For crossing to each Gal-4 line we selected the UAS-TNT stock that produced the strongest expression of toxin among viable offspring (G and K gave strong expression, but E produces a weaker expression). As control flies, hybrids were generated between the same Gal-4 lines and *w; UAS-IMPTNT-V1-A* (insert at chromosome II) or *w; UAS-IMPTNT-V1-B* (insert at chromosome III), producing the inactive toxin. The tetanus-toxin stocks were donated by Dr C.J. O'Kane (University of Cambridge, Great Britain).

Behavioral tests

A Y-maze (Alcorta & Rubio, 1988) was used to measure olfactory preference for different odorant concentrations (for details on chemosensory behavior studies see the review of Devaud, 2003). Briefly, forty individuals introduced into the initial tube (I) chose, during 30 min, between a control tube (C) containing a piece of filter paper soaked in 0.5 ml of solvent and a stimulus tube (S) containing 0.5 ml of a certain odorant concentration, alternating S and C in the left or right side to avoid laterality effects. The maze was assembled immediately before the test started. Olfactory preference was measured by an olfactory index (IO) calculated as the number of flies choosing the stimulus side of the Y-maze compared to the total number of moving flies that arrived either at the control or the stimulus side. The use of an index that considered only the flies moving towards the end of the maze prevented us of classifying mobility mutants as olfactory ones. However, it diminished the number of individuals whose response was tested from the 40 flies introduced in the initial tube. In our measurement conditions more than 40% of the flies reached the end of the maze as an average, but when less than 5 individuals move to the end of the maze the test was discarded (for details on the mobility behavior in a Y-maze see Alcorta & Rubio, 1989).

IO = No. Flies at S/ (No. Flies at S + No. Flies at C). Attraction values ranged from 1 to 0.5, and values between 0.5 and 0 indicated repellent responses. The value 0.5 may correspond to two different cases: (i) no detection of odorant or, (ii) an intermediate response between attraction and repellency. For this behavioral assay a continuous scale of indifferent, attractant and repellent responses was previously reported (Alcorta & Rubio, 1989) with increasing odorant concentration for different odorants (as an example see the dose-response curve to ethyl acetate of wild type Canton-S flies, Figure 1). A similar level of IO

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standard error was found for all kind of responses, excluding additional effects due to group measurement.

The repellent region of these dose-response curves showed a linear relationship between odorant concentration and olfactory index, increasing repellency by increasing odorant concentration. Repellent responses were therefore selected for our behavioral tests and the concentration used for testing olfactory preference for each odorant was such that it evoked an average response of intermediate repellency for all 30 lines. We expected different sensitivities between lines for detecting such a concentration: for example, if the IO for all the 30 lines has an average value of 0.3, the more sensitive lines may show repellent values as high as IO = 0, and the less sensitive lines would give preference values closer to indifference, 0.5, or even higher, in the attractant region.

Odorants and concentrations:

Five odorants were selected for behavioral studies: ethyl acetate, acetone, acetic acid, propionaldehyde and ethanol. Most of these are usually produced by the fermentation of fruits, the natural substrate of *Drosophila melanogaster*. Moreover, previous studies showed the ability of these compounds to trigger behavioral responses in fruit flies in a Y-maze (Alcorta & Rubio, 1988, 1989). These odorants were also selected for having different chemical groups and a similar short chain size. Both criteria have been proposed as the information detected by olfactory receptors.

The following odorant concentrations, expressed as vol/vol dilutions, were tested. Ethyl acetate 10^{-2} and propionaldehyde 10^{-2} were diluted in paraffin oil (non-smelling solvent), whereas ethanol $10^{-0.5}$, acetone $10^{-1.25}$ and acetic acid 10^{-1} were diluted in distilled water, since they do not mix with paraffin oil. As a rule, 20 replicate tests were carried out for each line and odorant. For those odorants where a lower number of replicate tests or lines were performed, the pertinent indication is included in the results section. In experiments with the Gal-4/UAS-TNT hybrids, only three of these five odorants were tested and 15 replicate tests were performed for each line and odorant.

Statistical analysis

The Olfactory Index (IO) was defined as the ratio of flies choosing the stimulus tube divided by the total number of flies that get to the end of the maze. This type of measurement approximately followed the normal distribution, though compressed at both ends. This deviation from the normal distribution can be solved by applying the Arcsin transformation:

$$Y = \arcsin\sqrt{\mathrm{IO}}$$

The Olfactory Index obtained for each line in response to each particular odorant was converted by the arcsin transformation and compared to the average value of all 30 lines, considered as the reference population, using a t-test for comparing means:

$$t = (X_{\mathrm{line}} - X_{\mathrm{population}}) / \sqrt{(\mathrm{Variance}_{\mathrm{whithin}}/n_{\mathrm{population}}) + (\mathrm{Variance}_{\mathrm{whithin}}/n_{\mathrm{Line}})}$$

X_{line} and $X_{\mathrm{population}}$ corresponded respectively to the average olfactory preference value for a particular line or for all 30 lines. The $\mathrm{Variance}_{\mathrm{within}}$ was estimated from all the replicated tests performed by all the 30 lines. Since the IO standard error for each odorant was homogeneous among lines, the $\mathrm{Variance}_{\mathrm{whithin}}/n_{\mathrm{Line}}$ component was considered the most accurate variation measurement within line in all cases.

$n_{\mathrm{population}}$ was the total number of replicate tests and n_{Line} the number of replicate tests for each particular line.

Since all 30 lines were generated using the same crossing program, we assume that the average IO value represented the average response that

Table 1. Ethyl acetate 10^{-2} (vol/vol)

Line	*n*	IO ± error (arcsin scale)	IO	*t*	P_B
Line 272	20	41.38 ± 2.48	0.437	5.774	3.82E-07***
Line 181a	17	34.77 ± 4.03	0.325	3.482	0.0142*
Line 7a	16	9.73 ± 3.32	0.029	3.451	0.0152*
Line 101a	19	33.5 ± 4.05	0.305	3.297	0.0252*
Line 522L	19	11.84 ± 2.57	0.042	3.124	0.0436*
Line 254	20	31.74 ± 2.61	0.277	2.845	0.1021 ns
Line 375L	13	11.88 ± 4.36	0.042	2.590	0.2081 ns
Line 211a	19	14.89 ± 2.45	0.066	2.220	0.5382 ns
Line 179a	20	29.63 ± 3.08	0.244	2.204	0.5328 ns
Line 170	18	28.70 ± 4.02	0.231	1.826	1.2329 ns
Line 385	17	15.93 ± 4.22	0.075	1.812	1.2015 ns
Line 212a	19	17.52 ± 2.90	0.091	1.440	2.4077 ns
Line 75	19	17.52 ± 2.45	0.091	1.440	2.2573 ns
Line 345	19	17.80 ± 4.66	0.093	1.357	2.4553 ns
Line 208a	19	17.86 ± 3.05	0.094	1.339	2.3542 ns
Line 36a	20	19.52 ± 2.17	0.112	0.868	4.6272 ns
Line 168a	17	24.63 ± 2.76	0.174	0.633	5.7995 ns
Line 457	19	20.27 ± 3.31	0.120	0.625	5.3231 ns
Line 588	19	20.27 ± 3.41	0.120	0.625	4.7908 ns
Line 555	18	20.27 ± 3.68	0.120	0.609	4.3433 ns
Line 525	17	24.14 ± 4.03	0.167	0.495	4.3456 ns
Line 250	20	23.61 ± 2.60	0.160	0.374	4.2499 ns
Line 148a	19	21.18 ± 3.61	0.131	0.355	3.6131 ns
Line 131a	17	23.50 ± 3.76	0.159	0.315	3.0109 ns
Line 274	14	21.20 ± 3.38	0.131	0.301	2.2901 ns
Line 462	20	22.82 ± 4.34	0.150	0.134	1.7865 ns
Line 565	19	22.10 ± 2.96	0.142	0.082	0.9343 ns
	$n_P = 493$	$X_P = 22.38$	$IO_P = 0.145$	$Variance_{within} = 208.17$	

***$p < 0.001$; *$p < 0.05$.

corresponds to the genetic background before *P*-element insertion. Moreover, as the possible effects in the Olfactory Index due to transposon insertion should affect only a few lines, it would not significantly affect the average IO value.

To avoid false significant differences due to the high number of performed comparisons a very conservative method, the sequential Bonferroni correction (Rice, 1989), was applied and only high-level differences were declared statistically significant (Sokal & Rohlf, 1995). In our case, after sorting olfactory response values from lower to higher deviation from the average value, the probability (PL) of the *t*-Student for each line was substituted by a corrected probability (PB) calculated as the product PL*K; K being the ordinal position of a particular line's value in the complete population. Statistical significance is achieved if $PB < a$ ($a = 0.05$, 0.01 or 0.001 for *, ** or ***, respectively).

Results

Olfactory responses to different odorants

Table 1 describes the olfactory responses displayed by 27 homozygous Gal-4 lines to ethyl acetate 10^{-2} (although 30 lines were studied, problems with 3 lines at the time of measuring responses to ethyl acetate reduced to 27 the number of lines tested for this odorant). '*n*' Represents the number of replicate tests performed for each line. Statistical

analysis including *t*-test and average value calculations used IO values in the arcsin scale, which followed the normal distribution (see the material and method section). The next IO column presents the same olfactory preference values in the original scale for understanding purposes (for example an IO value of 0.437 means that 43.7% of the flies that move to the end of the maze preferred the stimulus tube).

The IO mean of all the lines was close to 0.14, an intermediate repellent response that allows deviations in the direction of increasing or decreasing repellency that coincided with increasing or decreasing sensitivity, respectively. Five lines appeared significantly different from the global population: line 272 at the 0.001 and the other 4 lines at the 0.05 probability level. Three of the five lines showed deviations in the direction of decreasing and the other two in the direction of significantly increasing olfactory sensitivity.

Olfactory responses to Acetic Acid 10^{-1} are shown in Table 2. For this odorant and concentration, the olfactory response was repellent as an average (IO = 0.11), although it allowed deviations of olfactory preference to be detected in both directions. Statistically significant differences from the general population were observed in eight lines, in five cases decreasing sensitivity and increasing sensitivity in the other three lines.

The highest level of variability among the tested odorants and concentrations was found in response to ethanol $10^{-0.5}$. Table 3 shows statistically

Table 2. Acetic acid 10^{-1} (vol/vol)

Line	*n*	IO ± error (arcsin scale)	IO	*t*	P_B
Line 269L	18	0.00 ± 0.00	0.000	4.715	0.00009***
Line 208a	18	38.41 ± 4.61	0.386	4.662	0.00012***
Line 159	16	37.90 ± 4.54	0.377	4.287	0.0006***
Line 385	17	37.20 ± 4.03	0.366	4.248	0.0007***
Line 457	19	3.00 ± 1.66	0.003	4.088	0.0013**
Line 7a	20	4.79 ± 2.05	0.007	3.730	0.0053**
Line 272	20	33.47 ± 5.08	0.304	3.637	0.0073**
Line 192a	19	32.81 ± 5.60	0.294	3.382	0.0178*
	$n_P = 544$	$X_P = 19.35$	$IO_P = 0.110$	$Variance_{within} = 292.34$	

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Table 3. Ethanol $10^{-0.5}$ (vol/vol)

Line	*n*	IO ± error (arcsin scale)	IO	*t*	P_B
Line 345	19	59.31 ± 5.74	0.739	7.229	5.30E-11***
Line 179a	20	56.09 ± 3.72	0.689	6.668	1.95E-09***
Line 211a	18	2.45 ± 1.39	0.002	5.410	2.70E-06***
Line 250	20	5.81 ± 2.45	0.010	4.919	3.17E-05***
Line 148a	20	47.21 ± 4.71	0.539	4.623	1.24E-04***
Line 131a	19	47.05 ± 5.18	0.536	4.474	2.37E-04***
Line 7a	17	8.80 ± 3.68	0.023	3.910	2.52E-03**
Line 212a	15	7.79 ± 2.86	0.018	3.881	2.70E-03**
Line 36a	20	11.03 ± 3.27	0.037	3.714	4.98E-03**
Line 254	19	43.46 ± 4.96	0.473	3.665	5.73E-03**
Line 555	20	12.09 ± 3.46	0.044	3.472	1.12E-02*
Line 385	19	11.86 ± 3.64	0.042	3.438	1.20E-02*
	$n_P = 543$	$X_P = 27.15$	$IO_P = 0.208$	$Variance_{within} = 363.24$	

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

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Table 4. Acetone $10^{-1.25}$ (vol/vol)

Line	n	IO ± error (arcsin scale)	IO	t	P_B
Line 345	17	50.87 ± 4.96	0.602	5.731	4.992E-07***
Line 522L	20	46.59 ± 4.38	0.528	5.065	1.71E-05***
Line 269L	17	46.69 ± 6.19	0.530	4.707	9.30E-05***
Line 36a	20	13.19 ± 3.08	0.052	3.785	0.0047**
Line 375L	16	43.31 ± 5.93	0.470	3.765	0.0049**
Line 588	19	13.04 ± 2.55	0.051	3.731	0.0054**
Line 211a	18	13.82 ± 3.63	0.057	3.437	0.0154*
	$n_P = 568$	$X_P = 27.43$	$IO_P = 0.212$	$Variance_{within} = 275.21$	

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Table 5. Propionaldehyde 10^{-2} (vol/vol)

Line	n	IO ± error (arcsin scale)	IO	t	P_B
Line 101a	20	57.66 + 3.84	0.714	5.816	3.06E-07***
Line 565	20	56.39 + 3.50	0.694	5.537	1.38E-06***
Line 159	18	52.74 + 6.24	0.633	4.502	0.0002***
Line 208a	20	12.15 + 3.38	0.044	4.147	0.0011**
Line 525	20	15.33 + 3.63	0.070	3.451	0.0156*
Line 588	20	45.71 + 4.92	0.512	3.198	0.0365*
	$n_P = 584$	$X_P = 31.10$	$IO_P = 0.267$	$Variance_{within} = 403.49$	

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

significant differences for 12 lines out of 30. The average response value was around 0.20, in the intermediate repellent region of the dose-response curve, but the olfactory index of some lines oscillated from 0.002, extremely repellent, to 0.739, highly attractant. Seven lines deviated in the direction of increased sensitivity and another five deviated in the opposite direction.

The analysis of olfactory responses to acetone $10^{-1.25}$ is shown in Table 4. Once again, highly significant differences from the responses of the general population were found for seven lines. Three were in the direction of increasing repellent responses and four showing decreased sensitivity from an intermediate repellent average response of 0.21.

Olfactory responses to Propionaldehyde 10^{-2} are presented in Table 5. For this odorant and concentration, average IO responses were around 0.26, intermediate repellent responses. Six lines deviated significantly from the average responses, four in the direction of decreasing sensitivity and two in the opposite direction. Responses ranged from extremely repellent, 0.044 to highly attractant, 0.714.

Table 6 presents the summary of abnormal responses to the five tested odorants for the 25 lines with significant deviation from the population average response. A few lines were not tested in response to ethyl acetate and these appeared marked with the NO sign. Deviation was observed in both directions – increasing or decreasing sensitivity – for the five tested odorants. Some lines showed differences only in response to a single odorant; this was most frequent with respect to ethanol. In other cases, a particular line displayed abnormal responses to two or even three of the tested odorants. When differences appeared for a particular line in response to several odorants, these occurred most often (in 8 cases out of 12) in the same direction for all the odorants, either increasing sensitivity or decreasing sensitivity for all of them. The four cases with opposite deviations for different

Table 6. Abnormal olfactory responses

Line	Ethyl Acetate	Ethanol	Acetone	Acetic acid	Propionaldehyde
Line 7a	a*	a**		a**	
Line 36a		a**	a**		
Line 101a	b*				b***
Line 131a		b***			
Line 148a		b***			
Line 159	N/A			b***	b***
Line 170					
Line 179a		b***			
Line 181a	b*				
Line 192a	N/A			b*	
Line 208a				b***	a**
Line 211a		a***	a*		
Line 212a		a **			
Line 250		a ***			
Line 254		b **			
Line 269L	N/A		b ***	a ***	
Line 272	b ***			b **	
Line 345		b ***	b ***		
Line 375L			b **		
Line 385		a *		b ***	
Line 457				a **	
Line 522L	a*		b ***		
Line 525					a*
Line 555		a *			
Line 565					b ***
Line 588			a **		b*

a = increased sensitivity (increased repellency).
b = decreased sensitivity (decreased repellency).
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
N/A = due to problems with the line they were not tested for response to ethyl acetate.

odorants each involved a distinct combination of odorants. It has been reported previously that some olfactory receptor neurons in *Drosophila* give opposing responses depending on the odorant (de Bruyne et al., 2001).

Evaluation of the P-insertion as the cause of the olfactory mutant phenotype

Since the *P*-insertion lines were generated by crossing several non isogenic strains, it cannot be previously excluded that producing homozygosis in genes already present in the genetic background of these strains may result in abnormal olfactory behavior phenotypes independent of the transposon insertion.

Several attempts have been made to test if the *P*-insertion was indeed the cause of the abnormal behavioral phenotype in the Gal-4 lines.

The use of deficiency mapping was again hindered by genetic background differences, not only among Gal-4 lines but also among the classical deficiency stocks. For example, heterozygous flies of standard stocks and deficiency lines displayed significantly different olfactory indexes depending on the parental stocks. Two-way analysis of variance of the responses to pentyl acetate $10^{-1.5}$ of six different heterozygous stocks (Oregon-R/Def 1547, Oregon-R/Def 1142, Oregon-R/1888 and Canton-S/ Def 1547, Canton-S/Def 1142, Canton-S/Def 1888) showed significant olfactory differences depending on the parental standard stock

(Oregon-R or Canton-S, $F=42{,}73$, df = 1, 90, $p=0.0001$) and the deficiency line (1547: 55A-55F, 1142: 47E3-48A4 or 1888: 42B3-43E18, $F=4.94$, df = 2, 90 , $p=0.0092$). No significant interaction was found between both factors ($p=0.4143$), excluding the presence of specific alleles in particular regions of Oregon-R or Canton-S as the cause of olfactory differences.

Attempts to use other deficiency stock collections generated in the same genetic background, like the Exelixis deficiency collection (Parks et al., 2004), were prevented by the lack of the appropriate deficiency stocks.

Nonetheless some partial results were obtained by using one of the Gal-4 stocks that did not display abnormal behavior to any of the tested odorants (line 274) as the control Gal-4 line. Deficiency mapping was able to localize the abnormal behavioral response of line 101a to ethyl acetate 10^{-2} between the 55A and 55F positions in heterozygous flies of the 101a and the Df(2R)PC4 strains (Figure 2a). This result was in good agreement with the cytological position of the *P*-insertion in 101a that was mapped to 55C (Figure 3).

However, deficiency mapping could not be extensively considered as a reliable method for olfactory behavior mapping since the genetic background of the different deficiency stocks affects the behavioral phenotype. Even line 274 displayed less repellent responses to ethyl acetate (EA) and benzaldehyde (BZ), respectively, in heterozygosis to deficiency (2R) Stan1 (46 D7-9; 47F15-16) and deficiency (2R)42 (42C3-8; 42D-2-3) but gave normal responses to both odorants in heterozygosis to deficiency (2R) PC4 (55A; 55F) (Figure 2b).

An alternative method to test if the Gal-4 insertion was responsible for the abnormal behavioral phenotype consisted in inducing a similar mutant phenotype using the Gal-4 insert to drive the expression of other genes by the Gal4/UAS method (Brand & Perrimon, 1993). Moreover, for this experiment we benefit from a suitable control.

Obtaining abnormal phenotypes in hybrids of each Gal-4 line and an experimental UAS strain, Gal-4/UAS-E, compared to hybrids with a control UAS strain (with the same genetic background that the experimental UAS stock), Gal-4/UAS-C, would also argue against a background effect, either dominant or recessive, as the cause of the behavioral phenotype. A dominant background effect in the original Gal-4 line should appear in experimental hybrids as well as in the control ones.

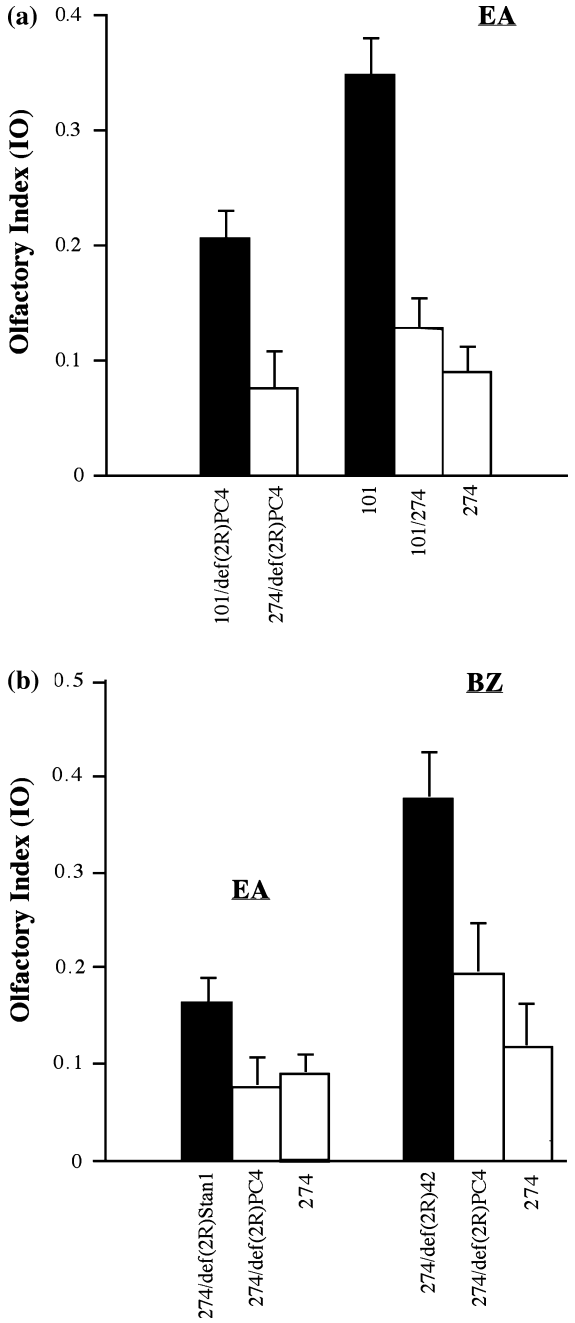

Figure 2. Deficiency mapping for the (a) olfactory response to ethyl acetate (EA) of the 101a Gal-4 line compared to the control 274 Gal-4 strain. (b) Olfactory response to ethyl acetate (EA) and benzaldehyde (BZ) of hybrid flies of the 274 line and 3 different deficiency stocks. Black color indicates an abnormal phenotype.

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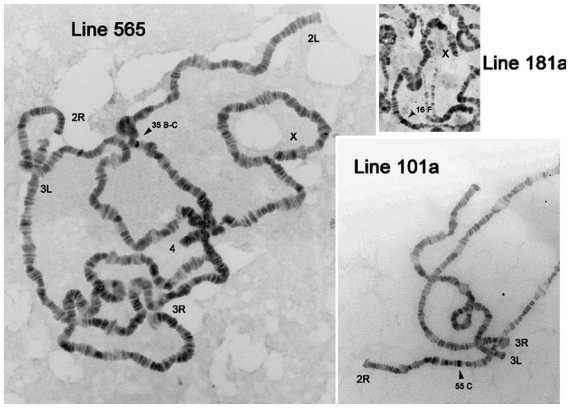

Figure 3. '*In situ*' hybridization showing *P*-element insertion in the polytenic chromosomes of lines 565, 181a and 101a. Control hybridization appears at the *w* locus.

A recessive background effect will disappear in both types of hybrid flies.

To check whether the *P*-insertion was likely to produce the olfactory mutant phenotypes, a sample of 10 lines was randomly chosen among the 25 with olfactory defects: lines 345, 131a, 148a, 179a, 272, 212, 250, 457, 555 and 588. These Gal-4 enhancer trap lines were used for blocking synapses of the corresponding olfactory receptor neuron subsets by driving the expression of the tetanus toxin light chain in heterozygosis with a UAS-TNT line (Sweeney et al., 1995; Keller et al., 2002). Olfactory behavior was analyzed for each Gal-4 line in heterozygous Gal-4/UAS-TNT flies compared to their corresponding control Gal-4/UAS-IMPTNT flies, where only inactive toxin was produced.

Responses to three odorants were tested for hybrids with each Gal-4 line. At least one of the selected odorants evoked a mutant phenotype and another one a normal phenotype for each line in the previous study (Table 6). The rationale behind the experiment was that if the *P*-insertion was responsible for the mutant behavioral phenotype in response to a particular odorant because of its action in certain olfactory receptor neurons, blocking synaptic connection of these neurons should affect at least the response to that odorant. This approach presented two limitations, it only uncovers defects associated to genes expressing at neurons and, we expected that the synapsis blockage, acting at the cellular level, gave a broader spectrum of abnormal olfactory responses that the mutation acting at the molecular level. This will be the case for those odorants whose reception is mediated by the same neuron but is not affected by the mutation induced by *P*-element insertion.

Table 7 presents the odorant specificity profile deduced after synaptic blockage of the neurons expressing the Gal-4 gene in each case. Abnormal response to a certain odorant was displayed as a grey cell. Black rectangles, reflecting abnormal olfactory behavior in the original Gal-4 lines, appeared preferentially correlated to similar responses in the corresponding Gal-4/UAS-TNT hybrids. For 2 Gal-4 lines, 212 and 457, expression of the tetanus toxin light chain did not induce any behavioral changes, probably because the corresponding gene was not expressing at olfactory neurons. For the rest 8 tested strains, synaptic blockage affected significantly the response to the expected odorant, except for responses to acetic acid and to ethanol in two lines, 272 and 555, respectively. It could be explained if abnormal perception of an odorant was not due to the *P*-insertion but also if this odorant was not mediated by receptors and synaptic transmission, as probably occurred with the acetic acid. Nevertheless, for 6 out of 8 Gal-4 lines (75%) observed behavioral changes were completely compatible with the *P*-insertion being the cause of the mutant phenotype, and at least partially compatible for 7 out of 8 (87.5%) if we include line 272 that gave the ex-

Gal-4 LINE	ETHYL ACETATE	ACETONE	ETHANOL	
131a	■	■	■	
148a			■	
179a	■	■	■	
250	■	■	■	ACETIC ACID
272	■		N/A	■
345		■	■	
555		■	■	PROPION-ALDEHYDE
588		■	N/A	■

N/A : not-applicable

AFFECTED PROCESS:

■ MUTANT PHENOTYPE IN THE GAL-4 LINE

■ SYNAPTIC BLOCKAGE **(significant differences, P< 0.05, between each Gal-4/UAS-TNT hybrids and the corresponding Gal-4/UAS-IMPTNT flies)**

pected response with ethyl acetate, but not with acetic acid.

Using the Gal-4/UAS approach to overexpress olfactory transduction genes with the same 8 Gal-4 lines included in table 7 and a UAS-*dnc* stock with a genetic background non isogenic with the UAS-TNT lines background, gave also olfactory behavior defects completely consistent with the ones previously described (see complete results in Gomez-Diaz, Martin & Alcorta, 2004). Similar results were obtained by overexpressing the *IP3K1* gene in the same 8 Gal-4 lines (Gomez-Diaz et al., 3 in press).

All together, these data support the idea that the *P*-insertion was indeed the main cause of the described abnormal behavior in the original Gal-4 lines. Of the initial 30 Gal-4 lines that were studied for behavioral changes, 25 were considered mutants (83.33%). If, in the worst case, only 75% of the relevant mutations are due to the *P*-element insertion, we still have an overall efficiency of 62.50% functional mutants from those lines screened for olfactory behavior changes.

Finally, *P*-element insertion was mapped systematically to the polytenic chromosomes of each Gal-4 line (Figure 3). Molecular characterization of the putative genes responsible for the olfactory phenotypes based on mRNA analysis yielded to the *GstE9* gene for the line 101a (Kim, 1996; Schwaerzel and Hovemann, unpublished results) and the *ari-1* gene for the 181a line (Kim, 1996). The *GstE9* gene encodes for a Glutathion S-transferase, an enzyme involved in chemical detoxification, probably concerning odorant clearance. Previous reports suggested a role of chemical detoxification genes in olfactory function (Hovemann, Sehlmeyer & Malz, 1997). The *ari-1* gene, which is located next to the insertion site of line MSK181 at position 16F7 on the X-chromosome, has been described as a gene involved in nervous system development of *Drosophila melanogaster* (Aguilera et al., 2000) and has been related to axon guidance and synapse maturation (Baas & Luo, 2001).

Discussion

Obtaining functional mutants in *Drosophila melanogaster* has been always a laborious task. The work applied to obtain morphological mutants that can be eye-selected needs to be supplemented in the case of functional mutants with additional tests to uncover abnormal performance. Moreover, when mutation does not induce an all-or-none effect, several replicate tests have to be carried out to define the phenotypic variation range corresponding to a certain line. Such studies applied to thousands of mutagenized lines have been carried out in the past with low efficiency and mainly for the screening of mutants of the X chromosome, where generating flies or lines showing mutant phenotypes becomes easier. Some attempts to systematically isolate olfactory behavior mutants of the X chromosome obtained 5 mutants from 913 lines screened after EMS (Ethylmethanosulfonate) mutagenesis (Woodard et al., 1989) and only 1 mutant from 227 lines after X-Ray mutagenesis or hybrid dysgenesis with previous enrichment procedures for olfactory mutants. In a similar study using a different behavioral paradigm (McKenna et al., 1989), 9 mutants were recovered from 1000 lines mutagenized with EMS and none from another 1000 lines established after mutagenesis by X-radiation or hybrid dysgenesis followed by an olfactory mutant enrichment protocol. These results corresponded in the best case, after EMS-mutagenesis, to efficiency values of 0.5% or 0.9%, respectively, of the total number of lines screened for olfactory behavior defects. Compared to these figures, the results we report here, 62.50–83.33% of olfactory reception mutants obtained from the total number of lines screened for behavioral defects, appear to be extremely high. Two steps probably contributed to increasing effectiveness. Mutagenesis by single *P*-element insertion in enhancer-trap lines has been proven successful for generating olfactory behavior mutants (Anholt, Lyman & Mackay, 1996), providing a 3.69% yield from the originally generated lines in the second and third chromosomes. Reporter gene analysis in these lines showed expression in the olfactory receptor organs, antennae and maxillary palps, for 10 of the 14 identified mutants (Anholt, Lyman & Mackay, 1996). This means that 2.64% of the lines generated by mutagenesis were most probably olfactory reception mutants. In the present report, where 25 lines were identified as reception mutants in response to some odorants, an initial number of 2000 lines were generated. Therefore,

only 1.25% of the initial lines turned out to be mutants according to the conservative statistical procedure applied, very much within the range of the other studies. In our case, however, proceeding from the reporter gene expression study (Hovemann et al., unpublished results) allowed us to limit behavioral analysis to just 30 lines, diminishing work most significantly and increasing efficiency to extremely high levels. Moreover, we expected a high percentage of the lines that preferentially expressed the reporter gene at olfactory receptor organs to be possibly related to olfactory reception, since the antennae and the maxillary palps are highly specialized organs (Stocker, 1994). In cases where no such functional selection to certain tissues can be previously applied, screening efficiency will probably be lower.

A final question concerning *P*-element insertion as the basis of the abnormal olfactory behavior phenotype has to be considered, because the strains used for the crossings that originate the Gal-4 lines of the present study were not isogenic. Therefore, some degree of variability was already present in the genetic background of these strains and might emerge by homozygosis of the chromosome containing the *P*-insertion when generating the enhancer trap lines. Different techniques could be applied for mapping behavioral mutants. Preliminary experiments to use deficiency mapping in some lines gave contradictory results, probably because deficiency lines do not share the same genetic background. The alternative approach we use to deduce the possible role of *P*-insertion as the cause of the abnormal behavioral phenotype gave good results. Using the same *P*-element that we believe to be responsible for the mutant phenotype to drive the expression of other genes under the activation of a UAS sequence, we tried to reproduce some of the mutant properties. If the olfactory phenotype were caused by a difference in the genetic background other than the *P*-insertion, it would not necessarily affect the same cells in which the reporter gene is expressed. If the affected cells were olfactory receptor neurons, blocking the synapses by means of the tetanus toxin in Gal-4 line/UAS-TNT hybrids would affect responses to at least those odorants that evoked abnormal phenotypes for each line. Correspondence was notably good in 6 out of the 8 tested lines. Similar results were obtained by directing expression of other transduction cascade genes, *dnc* (Gomez-Diaz, Martin & Alcorta, 2004) and *IP3K1* (Gomez-Diaz et al., in press) with the same 8 Gal-4 lines, and these findings cannot be explained neither by recessive nor dominant background effects of the original Gal-4 lines, as has been explained in the results section.

In summary, with our data we cannot conclude that *P*-insertion was the cause of the behavioral mutation for all the lines, but in the worst case it seemed at least responsible for 62.5% of the mutant phenotypes. These highly effective results speak in favor of the double-screening method by enhancer-trap reporter gene expression and behavioral assay to select olfactory mutants in *Drosophila melanogaster*. Extension of these methods to screening mutants for other phenotypes with similar efficiency may be limited only to the specialized nature of the affected tissues at the moment of selecting reporter gene expression in the corresponding cell subsets.

Since using quantitative methods to define a mutant phenotype statistically by comparison with the average populational phenotype appears to be a valid method, those lines already present in stock centers or those generated simultaneously in *P*-insertion programs of mutagenesis (i.e. Spradling, 1995, 1999) seem to be an appropriate material for screening for functional mutants affecting quantitative traits in *Drosophila melanogaster*. Alternatively, lines selected by expression pattern or map position can be tested for functional mutant phenotypes using for control measurements a sufficient number of lines simultaneously generated.

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