14. Assuming a hypercolumn spacing of 1 mm, it follows that two cells separated maximally in our tetrode recordings [~130 μm (*8*)] should exhibit an orientation preference difference no larger than 23°, with the majority yielding correspondingly smaller values. Yet in iso-orientation domains, there was a substantial number of cell pairs (28%) whose orientation preference differed by more than 23°.
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Genetic Feminization of Pheromones and Its Behavioral Consequences in *Drosophila* Males

Jean-François Ferveur,* Fabrice Savarit, Cahir J. O'Kane, Gilles Sureau, Ralph J. Greenspan,† Jean-Marc Jallon

Pheromones are intraspecific chemical signals important for mate attraction and discrimination. In the fruit fly *Drosophila melanogaster*, hydrocarbons on the cuticular surface of the animal are sexually dimorphic in both their occurrence and their effects: Female-specific molecules stimulate male sexual excitation, whereas the predominant male-specific molecule tends to inhibit male excitation. Complete feminization of the pheromone mixture produced by males was induced by targeted expression of the *transformer* gene in adult oenocytes (subcuticular abdominal cells) or by ubiquitous expression during early imaginal life. The resulting flies generally exhibited male heterosexual orientation but elicited homosexual courtship from other males.

In many animal species, sex- and species-specific bouquets of odors elicit subtle changes in potential sexual partners, which in turn may respond by appropriate behavior (*1*). In the fruit fly *Drosophila*, the stereotyped courtship behavior exhibited by male flies is induced largely by chemical cues, or pheromones, produced by his mate (*2*). These pheromones—the most abundant hydrocarbon molecules present on the fly cuticle (*3*)—are sensed principally by contact and are thought to play a crucial role in sexual isolation, tending to prevent interspecific mating (*4*, *5*).

In *D. melanogaster*, pheromones are strikingly sexually dimorphic (*6*) and have very different effects on male courtship behavior (*7*, *8*) (Table 1). Female flies produce dienes (two double bonds) with 27 and 29 carbons [*cis*,*cis*-7,11-heptacosadiene (7,11HD) and *cis*,*cis*-7,11-nonacosadiene (7,11ND)]. A few tens of nanograms of both dienes together can elicit vigorous male precopulatory behavior (*7*, *8*). Male flies synthesize monoenes (one double bond) with 23 and 25 carbons [*cis*-7-tricosene (7-T) and *cis*-7-pentacosene (7-P)]. 7-T can inhibit dose-dependent male excitation (*8*, *9*), whereas 7-P stimulates males of some strains (*4*, *7*, *8*).

One of the few genetic factors known to control the production of sex pheromones in *D. melanogaster* (*10*, *11*) is the gene *transformer* (*tra*), which controls the sexual dimorphism of pheromones (*8*, *12*) as part of its larger influence on somatic sex determination. When the feminizing transgene *UAS-tra*, made with the female cDNA of the *tra* gene, is expressed in certain regions of the male brain, the male exhibits a bisexual orientation (*13*, *14*). The *tra* gene also affects downstream sex-determination genes like *fruitless* and *doublesex*, which in turn control the sex pheromones or the male sexual orientation (*15*). Here, we expressed the *UAS-tra* transgene at different stages of development and in a particular group of abdominal cells, with the aim of producing a male fly with an unaltered sexual orientation, but with a female pheromonal profile.

To assess the critical period during which the *tra* gene product regulates pheromone expression, we transiently expressed *UAS-tra* throughout the organism at different developmental stages by crossing it to a line in which *GAL4* is fused to a *heat shock 70* promoter (*16*). The *tra* gene, fused to a promoter containing a *GAL4*-dependent upstream activation sequence (*UAS*), was therefore expressed with the same temporal

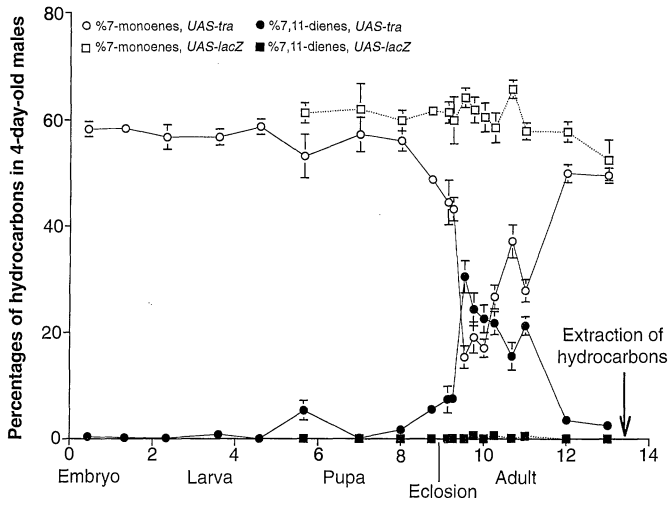

Fig. 1. Production of sex pheromones in 4-day-old male flies as a function of temporal activation of *UAS-tra* or of *UAS-lacZ*. A single pulse of heat shock (37°C) was applied for 2 hours, at various times (or 6 hours before pupariation). Each data point represents the mean percentage (±SE) of 7-monoenes (%7-T + %7-P) and of 7,11 dienes (%7,11-HD + %7,11-ND) for 20 *hsp-GAL4 UAS-tra* individuals and for 10 *hsp-GAL4 UAS-lacZ* individuals. Control, non–heat-shocked *hsp-GAL4 UAS-tra* and *hsp-GAL4 UAS-lacZ* males yielded 52.8 ± 1.5 and 57.5 ± 2.3% 7-monoenes, and 0.9 ± 0.5 and 0% 7,11 dienes, respectively. Values were measured as in (*21*).

J.-F. Ferveur, F. Savarit, G. Sureau, J.-M. Jallon, Mécanismes de communication, Unité de Recherche Associée–CNRS 1491, Bâtiment 446, Université Paris-Sud, 91405, Orsay-Cedex, France.
C. J. O'Kane, Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK.
R. J. Greenspan, Department of Biology and Center for Neural Science, New York University, 1009 Main Building, Washington Square, New York, NY 10003, USA.

*To whom correspondence should be addressed. E-mail: ferveur@ext.jussieu.fr
†Present address: The Neurosciences Institute, 10640 John Jay Hopkins Drive, San Diego, CA 92121, USA.

Table 1. Effects of *UAS-tra* expression on 4-day-old males. For the production of pheromones, the percentage of 7-tricosene (%7-T), 7-pentacosene (%7-P), and 7,11-dienes (%7,11-heptacosadiene and %7,11-nonacosadiene pooled) were calculated from the total quantities of cuticular hydrocarbons (ΣHc). 7,11-Dienes were pooled because their respective contributions were approximately the same in all strains. Percentages (mean ± SE) were obtained by gas chromatography of extracts from 20 individual flies (*21*). Amounts of *cis*-vaccenyl acetate (cVA) were estimated from ΣHc: (++) male-like, (+) reduced, (0) absence. For behavioral tests, *PGAL4 UAS-tra* males were examined both as objects with courting males of the *55B-GAL4* strain and as subjects with Canton-S (Cs) male or with shibire (shi) female objects (*34*). All flies were 4 days old, and the target flies were decapitated before the 10-min experiment. Decapitation prevents reciprocal courtship and allows measurement of unidirectional behavior. The percentage of courting males only includes males that courted for more than 20 s. The courtship index is the mean fraction of time (±SE in parentheses) spent actively courting by all males (wing vibration, licking, and attempt to copulate) (*8*), with at least 20 trials per strain.

Strain	Sex pheromones					Induce courtship of control males (*55B-GAL4*)			Court	
									Males (Cs)	Females (shi)
	7-T (%)	7-P (%)	7,11-dienes (%)	ΣHc (ng)	cVA (level)	(*n*)	%	Index	Index	
				Control						
Wild-type female (Cs strain)	3.3 (0.3)	7.0 (0.6)	40.6 (2.1)	1788 (119)	0	(20)	95	0.54 (0.04)		
Wild-type male (Cs strain)	41.4 (1.6)	11.9 (0.8)	0	1423 (92)	(++)	(25)	4	0.02 (0.01)	0.02 (0.01)	0.54 (0.03)
UAS-tra male	16.6 (1.2)	28.8 (1.2)	0	1491 (70)	(++)	(25)	12	0.04 (0.03)	0.02 (0.01)	0.51 (0.07)
				Oenocyte-expressed GAL4-tra *males*						
A-*tra*	2.8 (0.5)	6.4 (1.4)	44.0 (2.3)	1863 (186)	0	(20)	85	0.61 (0.08)	0.31 (0.03)	0.57 (0.03)
B-*tra*	7.6 (1.0)	9.6 (1.3)	25.6 (1.8)	1328 (69)	0	(25)	96	0.48 (0.05)	0.19 (0.05)	0.40 (0.06)
C-*tra*	4.3 (0.3)	6.4 (0.7)	40.5 (1.8)	1468 (94)	(+)	(20)	80	0.42 (0.06)	0.04 (0.01)	0.54 (0.07)
D-*tra*	3.8 (0.4)	5.5 (0.3)	40.5 (1.2)	1463 (80)	(+)	(20)	60	0.28 (0.04)	0.01 (0.01)	0.42 (0.06)
E-*tra*	4.5 (0.5)	4.7 (0.4)	37.2 (1.2)	1402 (46)	(++)	(20)	40	0.25 (0.03)	0.01 (0.01)	0.63 (0.04)
				Nonoenocyte-expressed GAL4-tra *males*						
F-*tra*	29.1 (1.9)	33.1 (2.1)	0	1974 (156)	(++)	(25)	12	0.05 (0.03)	0.01 (0.01)	0.62 (0.06)
G-*tra*	31.2 (1.4)	30.8 (0.9)	0	2206 (230)	(+)	(20)	20	0.12 (0.04)	0.04 (0.02)	0.47 (0.06)

pattern as *GAL4* (*17*). Heat shock induced ubiquitous *tra* expression at different developmental stages from embryo to 4-day-old adults (Fig. 1). The extent of feminization of pheromone production (the replacement of 7-monoenes by 7,11-dienes) reached a peak when *UAS-tra* expression was induced by a single heat shock between 12 and 48 hours of adult life. No pheromonal feminization was observed with control males expressing *UAS-lacZ* under the same heat shock conditions. This result suggests that the gene product or products being synthesized in these flies, after a 2-hour heat shock, have a sufficiently long-lasting effect to enable the production of female pheromones up to 4 days later and confirms that early imaginal life is the critical period during which sexually dimorphic hydrocarbons replace immature hydrocarbons on the fly cuticle (*18*).

Mosaic studies have localized the origin of pheromonal sexual dimorphism in the fly abdomen (*19*). To precisely map the cells that control the production of sex pheromones, we generated *PGAL4 UAS-tra* strains in which males show different patterns of regional feminization in their abdomen. The *PGAL4* system uses enhancer detection to express the *GAL4* transcriptional activator in different cellular patterns (*16*, *20*). The feminizing *UAS-tra* gene is therefore expressed with the same tissue specificity as *GAL4* (*14*).

Out of 50 *PGAL4 UAS-tra* lines originally screened, we identified five lines (A through E) in which male flies exhibited a female pattern of pheromones (Table 1). These regionally feminized flies, chromosomally XY, produced high amounts of female dienes (7,11HD and 7,11ND) and low amounts of male monoenes (7-T and 7-P). The *UAS-tra* expression was responsible for the feminization of sex pheromones because neither *PGAL4 UAS-lacZ* males nor *PGAL4 UAS-tra* females from these five *PGAL4* strains showed any substantial variation of their male or female pheromonal pattern (*21*).

We examined the pattern of *GAL4* expression in the five feminized strains (A through E) to seek a relation between their expression patterns and pheromonal feminization. The *GAL4* expression patterns were revealed by a cross to a *UAS-lacZ* reporter strain (Fig. 2). The adult expression patterns were of varying complexity, but they overlapped in two cell types: the oenocytes and the midgut (*22*). Oenocytes are subcuticular abdominal cells found in segmentally repeated rows that form crescent-shaped strands on the tergites and small clusters on the sternites (*23*). Oenocytes were the only cells able to change the production of pheromones because males of the other *PGAL4 UAS-tra* strains that were not feminized for their pheromones (Table 1) often showed strong expression in the midgut but not in the adult oenocytes (Fig. 2, F and G). A correlation between oenocyte expression and pheromone feminization was confirmed by analysis of a larger number of *PGAL4* lines (*24*). Together with previous studies of such unrelated insects as the desert locust (*25*) and the mosquito *Culicoïdes nubeculosus* (*26*), this result suggests that pheromones may be synthesized in the oenocytes of many insect species (*27*).

Oenocytes have multiple endocrine functions, including the regulation of ecdysteroids (*28*), one of which, 20-OH-ecdysone, controls an elongase required for the synthesis of 23 and 27 C hydrocarbons in

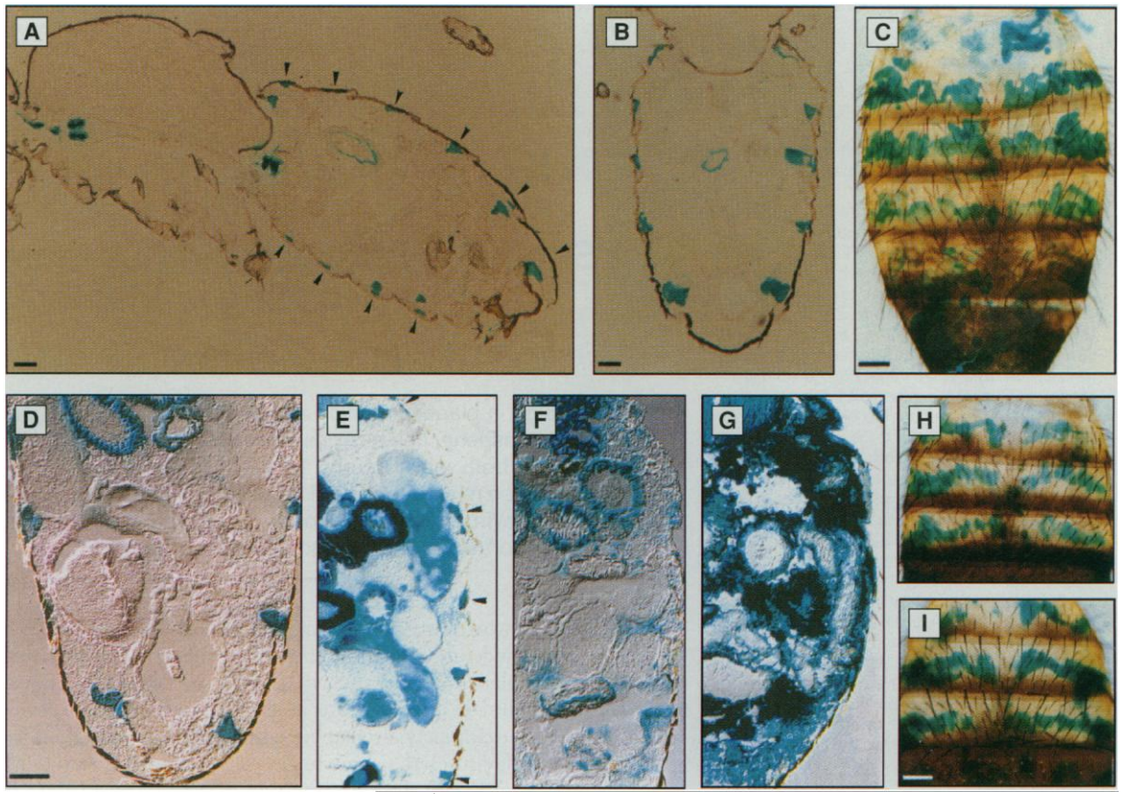

Fig. 2. Photomicrographs showing *lacZ* expression pattern (blue) in the abdominal oenocytes of various *PGAL4* strains. All flies are 4-day-old males. (**A**) Sagittal frozen section of the thorax and abdomen, and (**B** and **D** to **G**) horizontal frozen sections (10 μm) in the abdomen of *PGAL4 UAS-lacZ* males. (**C**, **H**, and **I**) Dorsal views of the abdominal cuticule of *PGAL4 UAS-lacZ* males (C and H) and *PGAL4 UAS-tra; UAS-lacZ* males (I). (A to C) Strain C; (D) strain B; (E) strain E; (F) strain F; (G) strain G; and (H and I) strain D. Arrowheads (A and E) indicate the oenocytes. Bars, 50 μm (D, E, F, and G; H and I have the same magnification).

Musca domestica (*29*). In *D. melanogaster*, according to the biosynthetic scheme proposed by Jallon (*6*), an elongase, perhaps coupled with a desaturase, would be sufficient to replace 7-monoenes by 7,11-dienes. In the mutant *Drosophila ecdysoneless 1*^{ts} females, 7,11-dienes are to a large extent replaced by 7-monoenes (*30*).

Drosophila adult oenocytes show a slight sexual dimorphism (*23*, *31*), but this does not seem to underlie the pheromonal difference between the sexes. To visualize directly whether the ectopic feminization of the oenocytes by the *tra* gene could have changed their sex-specific pattern, we simultaneously expressed both *UAS-tra* and *UAS-lacZ* transgenes (*32*). Resulting XY flies (*PGAL4 UAS-tra UAS-lacZ*) did not differ in their segmental pattern of *lacZ* expression, as compared with *PGAL4 UAS-lacZ* males (Fig. 2), nor in their production of sex pheromone, as compared with XY *PGAL4 UAS-tra* flies (*33*).

The sex pheromones produced by the feminized XY flies from the five strains (A through E) functioned as female pheromones and elicited a more vigorous courtship response in control males than in males from F-*tra*, G-*tra*, and control strains (*34*). The variation in these male courtship responses may reflect variability in controlling signals other than female pheromones such as the chemicals 7-T, 7-P, and *cis*-vaccenyl-acetate (cVA) (*35*) and visual cues like the abdominal and genital morphology of target *PGAL4 UAS-tra* males, the phenotypes of which seem to be independent of oenocyte feminization (*36*).

When tested as subjects against control male and female flies, feminized males from C-*tra*, D-*tra*, and E-*tra* strains retained a strong and typical male heterosexual behavior (Table 1), suggesting no relation between the feminization of their hydrocarbons and their sexual orientation. However, A- and B-*tra* males exhibited some bisexual behavior, possibly because they were feminized in the calyces of their mushroom bodies (strain A) and in a dorso-medial subset of their antennal lobes (strains A and B). However, these two brain structures, which function in mate recognition (*13*, *14*), were not feminized in the other GAL4-*tra* strains (strains C through G), showing that they are not required for feminization of the pheromonal profile.

Our analysis shows that in *D. melanogaster*, two aspects of individual sexual identity—the perception of others and the presentation of self to others—are under separate genetic and anatomical control. Homosexual courtship may take place either because of factors in the courter's brain (*13*, *14*) or because of factors in the courted fly's pheromonal profile. The interactive aspect of courtship and the complex nature of sexual identity in an animal as relatively simple as the fruitfly indicate that simplistic explanations of the genetic bases of sexuality are unlikely to be true.

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21. Cuticular hydrocarbons were analyzed by gas chromatography [J.-F. Ferveur, *Genetics*, **128**, 293 (1991)]. Mean percentages (each based on 10 individuals and expressed relative to total extracted hydrocarbons) for *PGAL4 UAS-lacZ* male flies: 29.7 to 35.6% 7-T, 10.7 to 18.9% 7-P, and 0 to 0.4% 7,11-dienes; and for *PGAL4 UAS-tra* females: 2.5 to 6.5% 7-T, 3.1 to 12.2% 7-P, and 27.2 to 46.4% 7,11-dienes. In *D. melanogaster* males, the production of 7-T and of 7-P are under the control of autosomal factors (*10*).
22. The patterns of β-galactosidase expression were determined on whole flies and on frozen sections from 3- to 5-day-old male and female flies of *PGAL4 UAS-lacZ* strains, with at least 10 flies per genotype. Abdominal structures that show a reproducible *GAL4* expression in male adult flies are the midgut (all strains); the oenocytes (strains A through E); the testis, the anterior ejaculatory duct, or the male accessory glands (strains A, B, D, and F; E and G showed both); the crop (C, E, F, and G); the Malpighian tubules (A, E, and G); the fat body (A and G); and the nephrocytes (D). There is also some variable and nonoverlapping *GAL4* expression in thoracic muscles and in neurons in the thorax and the head. LacZ expression was also detected in the salivary glands of all strains, including very weak expression in the control *UAS-lacZ* strain.
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33. F. Savarit and J.-F. Ferveur, unpublished data.
34. Flies from the three control strains [*55B-GAL4* as subject males; Canton-S males and shibire (shi) females as objects] were chosen for their clear behavioral phenotype (*8*). With the *PGAL4 UAS-tra* males (of strains A through E), more than 60% of the *55B-GAL4* males showed sustained wing vibration (40% with strain E), more than 50% showed licking (30% with strain E), and 20 to 50% attempted copulation (10% with strain D). With males of the four control strains, 4 to 12% of *55B-GAL4* males yielded wing vibration (20% with G-*tra*), less than 5% showed licking, and 0% attempted copulation. *55B-GAL4* males showed 95%, 80%, and 65% of these behaviors with target shi females.
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37. We thank A. Brand, N. Perrimon, R. F. Stocker, and G. Technau for the *PGAL4* strains (A = 29B, B = 1407, C = OK72, D = 323-CyO, E = OK376, F = 55B, and G = 24B); J. Connolly, J. Keane, K. Moffat, and S. Sweeney for generating strains; J. M. Belote for antibodies to *tra*; G. J. Blomquist, F. M. Butterworth, J. A. Coyne, and F. Romer for comments; and M. Cobb for help with the manuscript. Supported in part by the Human Frontiers Science Program grant RG 93/94 (J.-F.F., F.S., and R.J.G.), by a fellowship from the Ministry of Education and Research (G.S.), and by grants from the Wellcome Trust (034320/Z/91/Z) and the European Union (ERBSC1*CT920790) (C.J.O'K.).

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A Similarity Between Viral Defense and Gene Silencing in Plants

Frank Ratcliff, Bryan D. Harrison, David C. Baulcombe*

Gene silencing in plants, in which an endogenous gene is suppressed by introduction of a related transgene, has been used for crop improvement. Observations that viruses are potentially both initiators and targets of gene silencing suggested that this phenomenon may be related to natural defense against viruses. Supporting this idea, it was found that nepovirus infection of nontransgenic plants induces a resistance mechanism that is similar to transgene-induced gene silencing.

It has been shown that gene silencing (*1*) and virus resistance are related phenomena in transgenic plants. Transgenes that are derived from viral cDNA and are able to induce gene silencing may also suppress the accumulation of viruses that are similar in nucleotide sequence (*2*). In addition, nonviral transgenes are able to suppress virus infection if the virus is modified by insertion of the transgene sequence into the viral genome (*3*).

Viruses are also able to silence host genes. For example, in *Nicotiana benthamiana* inoculated with modified tobacco mosaic tobamovirus (TMV) (*4*) or potato X potexvirus (PVX) (*5*) that carried host-related inserts, there was suppression of genes homologous to the inserts. Viruses can also induce silencing of transgenes that are similar in sequence to the inoculated virus (*6*). Early in the course of infection, expression of the transgene was unaffected by the virus, and the normal viral symptoms were produced. However, later on, in the upper leaves that developed after the virus had spread systemically, gene silencing affected both the transgene and the homologous virus. Thus, leaves that developed later contained lower concentrations of the transgene RNA, were free of the virus, and were resistant to secondary infection by the virus. The plants exhibiting this response were said to have "recovered" (*6*).

This type of recovery from virus disease is not confined to transgenic plants. In nepovirus-infected *Nicotiana* sp., there are severe viral symptoms on the inoculated and first systemic leaves. However, the upper leaves that develop after systemic infection are symptom-free and contain a lower concentration of virus than do the symptomatic leaves (*7*). For example, *N. clevelandii* inoculated with tomato black ring nepovirus (strain W22) initially shows symptoms and later recovers (Fig. 1). After secondary reinoculation of W22 to the recovered leaves, there was no additional accumulation of W22 RNA above that resulting from the primary inoculation (Fig. 2) and the plants remained symptom-free. In contrast, plants previously unexposed to W22 produced a high concentration of W22 RNA (Fig. 2) and showed disease symptoms. The resistance of recovered leaves to subsequent viral challenge suggests the existence of a resistance mechanism that restricts or prevents infection by the challenge virus.

In similar experiments, the recovered leaves of W22-infected *N. clevelandii* were inoculated with viruses that were progressively less related to W22. These analyses confirmed that the resistance associated with recovery was specific to strains that were related in genomic sequence to the recovery-inducing virus (*8*). In upper leaves challenge-inoculated with the tomato black ring nepovirus (strain BUK) there was detectable accumulation of the BUK RNA but at a substantially lower concentration in the recovered plants than in plants that were initially mock-inoculated (Fig. 2). There was also partial protection from disease induction by secondary infection with BUK (*8*). However, primary infection with W22 provided no protection against secondary infection with tomato ringspot nepovirus or with the unrelated PVX (Fig. 2).

F. Ratcliff and D. C. Baulcombe, Sainsbury Laboratory, John Innes Centre, Colney, Norwich NR4 7UH, UK.
B. D. Harrison, Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK.
*To whom correspondence should be addressed. E-mail: baulcombe@bbsrc.ac.uk