***Drosophila* resistance genes to parasitoids: chromosomal location and linkage analysis**

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Insect hosts can survive infection by parasitoids using the encapsulation phenomenon. In *Drosophila melanogaster* the abilities to encapsulate the wasp species *Leptopilina boulardi* and *Asobara tabida* each involve one major gene. Both resistance genes have been precisely localized on the second chromosome, 35 centimorgans apart. This result clearly demonstrates the involvement of at least two separate genetic systems in *Drosophila* resistance to parasitoid wasps. The resistance genes to *L. boulardi* and *A. tabida* are not clustered as opposed to many plant resistance genes to pathogens cloned to date.

Keywords: *Drosophila melanogaster*; resistance genes; parasitoid wasps; encapsulation; host–parasite specificity

1. INTRODUCTION

In recent years, increasing attention has been directed towards genetic and evolutionary aspects of the relationships between hosts and parasites. Some of these inter-specific genetic interactions have been illustrated by the 'gene-for-gene' model established by Flor (1956), and molecularly demonstrated in plant–pathogen associations: a series of alleles controlling resistance in the host corresponds to a series of alleles controlling avirulence in the pathogen. The study of the genetics of virulence–resistance interactions in parasitoid–insect systems is still in its infancy, but data are accumulating on the *Drosophila*–wasp models.

The genetics and mechanisms of parasitoid virulence are better understood than those of *Drosophila* resistance. For instance, some parasitoid species carry polyDNA-virus particles (PDV) that are injected into the host and regulate its physiology, notably by blocking the immune system (Beckage 1998). It is now established that PDV sequences are integrated in the wasp genome and transmitted vertically with the chromosomes (Stoltz 1990; Savary *et al.* 1997). The virus-like particles found in *Leptopilina boulardi*, a *Drosophila melanogaster* parasitoid species, also demonstrate a Mendelian transmission and can be considered as a 'single gene' (Dupas *et al.* 1996, 1998).

Physiological aspects of *Drosophila* resistance to wasps are well documented (review in Carton & Nappi 1997), but comparatively little is known about its genetic support and mode of transmission. The detection and localization of genes involved in resistance to parasitoids is, however, essential for the understanding of their immunological function and role in host specificity and, more specifically, in resistance–virulence interactions. It must be pointed out that it is only by the acquisition of highly selected virulent and avirulent lines of parasitic wasps, as well as susceptible and resistant lines of hosts, that the host–cellular immune reaction can be genetically analysed and quantitatively estimated (Henter & Via 1995; Carton & Nappi 1997; Orr & Irving 1997).

Strains of parasitoid wasps demonstrated to be non-immunosuppressive (avirulent) against *D. melanogaster* have been used to select highly homozygous host strains with predictable responses to a parasitic infection. The basis of resistance to parasitism has been investigated using these strains. Results strongly indicate a simple genetic determinism in the case of parasitism by *L. boulardi* (Carton *et al.* 1992) and *Asobara tabida* (Benassi *et al.* 1998). The major genes are inherited autosomally, with the resistant phenotype showing complete dominance over the susceptible one. The susceptible phenotypes do not represent 'sick' phenotypes (Carton & Nappi 1997) as suggested by several authors (Orr & Irving 1997), since both resistant and susceptible lines to *L. boulardi* are immune competent against avirulent strains of *A. tabida*. Using well-defined lines of parasitoids and hosts concerning virulence and resistance, we have analysed more precisely *D. melanogaster* resistance genes to *L. boulardi* and *A. tabida* by specifying their chromosomal and genetic location.

2. MATERIAL AND METHODS

(a) Origin of strains

D. melanogaster strains of African origin, strain 445 resistant to *L. boulardi* and strain 22 susceptible to *L. boulardi*, are described in Carton *et al.* (1992). The resistant strain 940 corresponds to a selected isofemale line originating from the S₁R₂S₃ stock (table 1). This stock carries chromosome 2 from the resistant strain 445 in the genetic background of strain 22. The observed level of encapsulation against *L. boulardi* was 92.9% (227 eggs tested) for strain 940, and 2.9% (187 eggs tested) for strain 22. Finally, a strain completely homozygous for chromosome 2 (strain 1088) was obtained from strain 940. Its level of encapsulation was 97.3% (675 eggs tested) against *L. boulardi* and 100% (34 eggs tested) against *A. tabida*. Strain 249 has a Canton S strain origin and was demonstrated to be susceptible to *A. tabida* (Benassi *et al.* 1998) and *L. boulardi* (level of encapsulation 7.2%, 251 eggs tested).

The *D. melanogaster* loci that confer resistance to *L. boulardi* and *A. tabida* have been termed *Rlb* and *Rat* (Carton & Nappi 1997; Benassi *et al.* 1998), *Rlb*⁺ and *Rat*⁺ corresponding to the resistant alleles, *Rlb*[−] and *Rat*[−] to the susceptible ones.

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Table 1. *Cellular immune response (encapsulation rate) of parental and chromosome-substituted D. melanogaster strains against L. bouhardi*

	chromosome genotype ^a	total number of larvae tested (number of repetitions)	encapsulation rate (%) s.e.m.
parental lines	R ₁ R ₂ R ₃ (strain 445)	188 (14)	82.6 ± 3.8
	S ₁ S ₂ S ₃ (strain 22)	139 (14)	2.9 ± 1.9
reconstructed stocks	R ₁ R ₂ S ₃	73 (4)	90.6 ± 2.3
	R ₁ S ₂ S ₃	71 (6)	0
	S ₁ R ₂ R ₃	63 (4)	81.4 ± 4.4
	S ₁ S ₂ R ₃	104 (4)	2.5 ± 1.2
	S ₁ R ₂ S ₃	112 (3)	85.8 ± 4.2
	R ₁ S ₂ R ₃	64 (4)	0

^a R and S denote the stock origin: R for the resistant line (strain 445) and S for the susceptible line (strain 22); the subscripts denote the chromosomes.

Consequently, the genotypes of the strains used are (numbers correspond to Gif stocks):

- strain 445: *Rlb*⁺/*Rlb*⁺, not determined for *Rat*
- strain 940: *Rlb*⁺/*Rlb*⁺, *Rat*⁺/*Rat*⁺
- strain 1088: *Rlb*⁺/*Rlb*⁺, *Rat*⁺/*Rat*⁺ (isogenic for chromosome 2)
- strain 22: *Rlb*[−]/*Rlb*[−], *Rat*⁺/*Rat*⁺
- strain 249: *Rlb*[−]/*Rlb*[−], *Rat*[−]/*Rat*[−]

The origins of the avirulent strains of *L. bouhardi* (Gif stock number 486) and *A. tabida* (Gif stock number 490, Leiden stock WOV) are described elsewhere (Dupas *et al.* 1998; Benassi *et al.* 1998). Encapsulation occurs only in the case of parasitism of resistant *D. melanogaster* strains by the avirulent parasitoid strains.

(b) *Bioassay procedure*

Determination of the encapsulation ability of *D. melanogaster* strains was as described in Carton *et al.* (1992). The encapsulation rate was calculated as the ratio of the number of encapsulated eggs to the total number of eggs recovered. Superparasitized larvae were included in the counts since there is no correlation between the degree of superparasitism and the encapsulation rate under our experimental conditions (Carton & Kitano 1981) and the total number of tested larvae was high. Encapsulation rates did not vary whether calculated with monoparasitized or superparasitized larvae.

(c) *Chromosome contribution to resistance*

To estimate the contribution of the three major chromosome pairs to the resistance phenomenon, new strains were constructed using a laboratory strain with the second and third chromosomes balanced lethal. Chromosomes 2 were SM-1 carrying the dominant marker *Curly* (*Cy*), and the standard brown-dominant (*bw*rd) chromosome. Chromosomes 3 were TM-3 carrying *Beaded-Serrate* (*Bd*^S) and the other *Stubble* (*Sb*). All these markers have been described by Lindsley & Zimm (1992). Using appropriate crosses (Peyronnet *et al.* 1994), we obtained the six homozygous combinations with the parental stocks 445 and 22 and three homozygous combinations with the parental stocks 940 and 249.

(d) *Recombination mapping of Rlb*

The autosomal mutant markers *lightoid* (*ltd* 2-59.0) and *brown* (*bw* 2-104.5), located on chromosome 2, were used to perform this analysis. Their phenotype can be determined in the third

instar larvae, at the same time as the encapsulation rate parameter. These markers both modify the Malpighian tubule coloration of the larvae (Ashburner 1989) but they seem not to interact with the phenoloxidase cascade (Wright 1987). The mutant strains were obtained from (Bloomington *Drosophila* Stock Center, Bloomington, IN, USA) and shown to be susceptible to *L. bouhardi*: the encapsulation rates were 5.5% (339 eggs tested) and 4.4% (193 eggs tested), respectively, for the *ltd* and *bw* strains. Females of strain 940 were mated to males of the *ltd* and *bw* strains and the F₁ females backcrossed with mutant males. The progenies were infested and scored two days later (third instar larvae) for their *ltd* (or *bw*) and *Rlb* (encapsulation rate) phenotypes. Recombination distances (centimorgans, cM) were calculated from the frequency of parental and recombinant genotypes.

(e) *Recombination distance between Rlb and Rat*

Two *D. melanogaster* strains showing opposite immune capacities against *L. bouhardi* and *A. tabida* parasitoids (Benassi *et al.* 1998) were crossed: the double resistant strain 1088 (genotype *Rlb*⁺/*Rlb*⁺, *Rat*⁺/*Rat*⁺) (Vass *et al.* 1993) and the double susceptible strain 249 (genotype *Rlb*[−]/*Rlb*[−], *Rat*[−]/*Rat*[−]). F₁ hybrids were backcrossed with the susceptible 249 strain and the genotypes were scored after a double infestation by the two parasitic wasps. The wasp origin of the capsule formed around the eggs was easily recognized: the capsules surrounding *L. bouhardi* eggs showed a black-sausage elongated shape with a short tail (Carton & Nappi 1997) while the capsules formed around *A. tabida* eggs were spherical (Kraaijeveld & Van Alphen 1994).

(f) *Detection of potential chromosome aberrations*

The estimation of genetic distances using recombination rates is accurate only in the absence of chromosomal aberrations on the studied chromosomes. We have looked for such aberrations in the 940 resistant strain using Canton S as a reference for standard polytene chromosome structure. Polytene chromosomes were prepared from (Canton S × 940) F₁ larvae, as described in Engels *et al.* (1986), and potential aberrations were checked under a microscope after coloration.

3. RESULTS

(a) *Chromosomal analysis of resistance to L. bouhardi and A. tabida*

Resistant (445) and susceptible (22) strains were used to determine the localization of the *Rlb* resistance gene to

Table 2. Cellular immune response (encapsulation rate) of parental and chromosome-substituted *D. melanogaster* strains against *A. tabida*

	chromosome genotype ^a	number of larvae tested	encapsulation rate (%)
parental lines	R ₁ R ₂ R ₃ (strain 940)	34	100
	S ₁ S ₂ S ₃ (strain 249)	177	0.91
reconstructed stocks	R ₁ S ₂ S ₃	116	0.75
	S ₁ R ₂ S ₃	32	83.8
	S ₁ S ₂ R ₃	48	0.0

^a R and S denote the stock origin: R for the resistant line (strain 940) and S for the susceptible line (strain 249); the subscripts denote the chromosomes.

L. bouhardi. Chromosome transfers were performed on six generations, using the classical method with balanced chromosomes. As demonstrated in table 1, the susceptible strain only gained a resistant phenotype (encapsulation rate of 85.9%) when chromosome 2 from the resistant strain was transferred. The new strain, of genotype S₁R₂S₃, corresponds to the initially susceptible strain 22 that was made resistant by the exchange of chromosome 2 from strain 445. A total absence of encapsulation (0%) was demonstrated in the R₁S₂R₃ stock, obtained by replacing chromosome 2 of the resistant strain by its homologue from the susceptible strain. The encapsulation levels recorded in the other constructed strains (R₁R₂S₃, R₁S₂S₃, S₁S₂R₃ and S₁R₂R₃) confirmed that substitution of the first or third chromosome has no effect on the encapsulation ability. The localization of the resistance gene *Rlb* was thus restricted to the second chromosome.

A similar method using balanced chromosomes was used to localize the resistance gene to *A. tabida*, *Rat*, starting from the two parental strains 940 and 249. In this case, only the exchange of chromosomes from the resistant strain to the susceptible one was performed. As recorded in table 2, the transfer of chromosome 2 provides a clear resistant phenotype to the susceptible strain: the constructed stock S₁R₂S₃ showed an encapsulation rate of 83.8%. As the other stocks (R₁S₂S₃ and S₁S₂R₃) proved completely susceptible, it was concluded that *Rat* is also located on chromosome 2.

(b) Genetic localization of *Rlb*

Having demonstrated that *Rlb* is located on chromosome 2, we had to find phenotypic markers as close as possible to this gene for mapping experiments. Furthermore, their phenotype had to be detectable in third instar larvae, since no susceptible adult fly can be recovered after infestation by the parasitoid. The larval mutant marker *bw* was the first to be used because of its localization at the right end of chromosome 2 (2-104.5). From classical recombination results, *Rlb* appeared to be located at *ca.* 20.4 ± 3.1 cM from *bw* (34 recombinants out of 167 F₂ larvae). This gene is thus on the right arm of chromosome 2, to the left of *bw*. The accuracy of these data were further confirmed by the absence of chromosomal aberrations on chromosome 2R of the resistant strain.

In order to map the precise position of *Rlb*, other recombination studies were undertaken using the larval marker *ltd* (2-59.0). Genetic results demonstrated that *Rlb* maps at

ca. 27.5 ± 1.5 cM from *ltd* (253 recombinants out of 919 F₂ larvae). Considering that the mapping with *ltd* is more accurate than the mapping with *bw* (919 larvae studied compared to 167), we concluded that *Rlb* maps to the right of *ltd* at a genetic location of *ca.* 2-86.7 (i.e. 2-59.0 + 27.5 cM).

(c) Recombination distance between the *Rlb* and *Rat* genes

As both resistance genes are located on chromosome 2, it could be questioned whether the same gene encodes both resistant phenotypes. The genetic distance between *Rlb* and *Rat* was determined by estimating the recombination rates. We obtained 45 recombinants out of 127 larvae, which corresponds to a genetic distance of 35.4 ± 4.2 cM. *Rat* would then be localized at 2-51.3, near the centromere.

4. DISCUSSION

D. melanogaster resistance against the parasitic wasps *L. bouhardi* and *A. tabida* is under the control of major genes (Carton *et al.* 1992; Orr & Irving 1997; Benassi *et al.* 1998). These genes, *Rlb* (resistance to *L. bouhardi*) and *Rat* (resistance to *A. tabida*), have been localized on chromosome 2 using chromosome-transfer experiments. Recombination studies between *Rlb* and *Rat* demonstrated that they lie 35.5 cM apart, showing that two different loci at least are involved in *D. melanogaster* resistance to hymenopteran wasps. Using phenotypic larval markers, *Rlb* was more precisely localized on the right arm of chromosome 2, at 2-85.6. *Rat* is located on the same arm, near the centromere, and may thus correspond to the major encapsulation factor(s) involved in resistance to *A. tabida*, described by Orr & Irving (1997).

Recent work from Fellowes *et al.* (1998) has demonstrated that selection for resistance to *L. bouhardi* leads to an increased resistance to *A. tabida* and that lines selected for resistance to *A. tabida* show a very slight increase in their ability to encapsulate *L. bouhardi*. In this case, improved resistance in the selected lines would have a non-specific component, more or less effective against both wasps, and a specific component required for encapsulation of *L. bouhardi*. This interpretation is consistent with the fact that these selected lines demonstrate superior resistance to another wasp species, *Leptopilina heterotoma*, as compared to control lines (Fellowes *et al.* 1998). However, we recently demonstrated that our strain

1088, resistant to *L. bouhardi* and *A. tabida*, is completely susceptible to the strain of *L. heterotoma* used by Fellowes *et al.* (0% encapsulation, unpublished results). Furthermore, from our linkage analysis, it appears clearly that the *Rlb* and *Rat* genes are able to confer complete resistance to *L. bouhardi* and *A. tabida*, respectively. These results are in agreement with the fact that in *D. melanogaster* natural populations the encapsulation abilities against *L. bouhardi* and *A. tabida* are not correlated (Kraaijeveld & Van Alphen 1995), and confirm the occurrence of specific mechanisms in insect immune response to parasitism.

Selection by Kraaijeveld & Godfray (1997) and Fellowes *et al.* (1998) was done with parasitoid strains of mid-virulence status whereas our work was developed with totally avirulent strains. The specific and non-specific components of host resistance could have been selected differently according to the parasitoid strain retained. It is also possible that the host lines obtained by Fellowes *et al.* (1998) and Kraaijeveld & Godfray (1997) differ in the number of resistance genes selected or even in the nature of these genes, from the resistant strains used in our experiments. The host populations submitted to selection pressures are of different geographic origin, the parasitoid strains used are also of different origin and virulence status, and the experimental processes of selection and the final levels of resistance are different (60% and 45% as compared to 95%). The different outcomes of laboratory-selection experiments and gene isolation following field selection have been extensively discussed in the area of insecticide resistance (Roush 1987). In our case, this hypothesis could also explain why resistance to *A. tabida* appears of intermediate dominance in the selected lines of Kraaijeveld & Godfray (1997), but proves completely dominant in our strain 1088.

Parasitoid–host relationships can be superficially described by the ‘gene-for-gene’ model proposed for plant–pathogen associations even though the molecular bases of the interactions are probably different. In plants, two genetic models of arrangement have been observed among resistance loci: single loci and, more frequently, compound loci forming R-gene clusters (Dangl & Holub 1997). These clusters usually contain resistance genes to related pathogen species, but resistance genes to taxonomically diverse pathogens have also been co-localized (Ashfield *et al.* 1998; Van der Voort *et al.* 1999). It has been proposed that frequent sequence exchanges between the tandemly repeated genes can create novel resistance-gene specificity (Parniske *et al.* 1997). The *D. melanogaster* *Rlb* and *Rat* resistance genes to parasitoids are not tightly linked. There is therefore no indication that specificity could originate from genic duplication and subsequent diversification.

The localization of *Rat* and *Rlb* is the first step toward their molecular characterization. Hopefully, the cloning of these genes will aid a better understanding of the mechanisms of resistance to parasitism and the specificity of parasitoid–host interactions.

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