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A single sex-linked dominant gene does not fully explain the codling moth's resistance to granulovirus

Marie Berling,[a] Benoît Sauphanor,[b] Antoine Bonhomme,[c] Myriam Siegwart[b]* and Miguel Lopez-Ferber[a]

Abstract

BACKGROUND: In 2004, resistance to a commercial formulation of the *Cydia pomonella* granulovirus (CpGV) was identified in a field population of *Cydia pomonella* from an organic orchard in southern France. The genetic inheritance of this resistance was analysed in the resistant laboratory strain RGV. This strain was obtained using successive crosses between the resistant field population and a susceptible laboratory strain, SV, with selection for CpGV resistance at each generation.

RESULTS: After eight generations of introgression of the resistant trait into SV, the RGV-8 strain exhibited 7000-fold higher resistance than SV. Mass-crossing experiments showed that resistance to CpGV is strongly dominant, sex dependent and under the control of a single major gene. However, the contribution of other genes is required to explain all of the data obtained in this study. These additional genes do not follow the laws of classical Mendelian transmission.

CONCLUSION: Transmission of granulovirus resistance in the RGV-8 strain of *C. pomonella* cannot be fully explained by the effect of a locus located on the Z chromosome. The action of other factors needs to be considered.

Keywords: codling moth; *Cydia pomonella* granulovirus; CpGV; resistance; inheritance; sex linkage

1 INTRODUCTION

Over the past 20 years, the *Cydia pomonella* granulovirus (CpGV, *Baculoviridae*, *Betabaculovirus*) has shown great promise for codling moth control. The efficiency of CpGV has been proven in organic orchards and for integrated pest management strategies to reduce the environmental impact of chemical treatments and to delay selection of resistance to pesticides.[1] The use of this biological product in codling moth control has increased in all areas where apples are cultivated. Since 2003, resistance to CpGV has been detected in *C. pomonella* populations in organic orchards in Germany, France and Italy.[2–4]

Development of resistance to chemical insecticides has become increasingly more common in many arthropod pests.[5] Additionally, resistance to some biological products has also been described. The best-known case of resistance to a biological control product is against *Bacillus thuringiensis* Berliner (*Bt*). The first record of this phenomenon was established in 1965 when resistance to *Bt* was induced in a laboratory strain of the housefly *Musca domestica*.[6] After this, many cases of resistance to *Bt* were reported that originated from both laboratory selection and field populations.[7–10] Other studies have been carried out for entomopathogenic viruses. Resistance to a nucleopolyhedrovirus was first obtained through laboratory selection of a strain of the light-brown apple moth *Epiphyas postvittana* (Walker).[11] In this instance, it was shown that the resistance was genetically determined. However, until very recently, the resistance ratio of insects to viruses has not exceeded tenfold.[12] In contrast, the 60 000-fold resistance of *C. pomonella* to CpGV[13] raises concerns for the sustainability of microbiological control of pests when applied continuously.

In *C. pomonella*, as for all Lepidoptera, the female is the heterogametic sex, with a WZ sex chromosome pair, and the male is homogametic with a double Z chromosome pair.[14–16] Very few genes on the W chromosome are known, whereas far more genes on the Z chromosome have been described.[17,18] The majority of genes carried on the sex chromosome are thus present in only one copy in the female.

The mode of inheritance of resistance to chemical pesticides has been described for many pest species, but few cases of sex-linked resistance have been recorded. Sex-linked resistance has been described for the nematode *Haemonchus contortus* (Rudolphi),[19] the oriental fruit moth *Grapholita molesta* (Busck)[15] and the coffee berry borer *Hypothenemus hampei* (Ferrari).[20]

The first investigations into the resistance of *C. pomonella* to CpGV were carried out in Germany, using backcross

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experiments.[2] These researchers first suggested an autosomal and incomplete dominant inheritance. Further experiments on the same populations using single-pair crosses clearly indicated monogenic and sex-linked resistance.[21]

Resistance to CpGV occurred simultaneously in distant populations of different European countries, suggesting distinct selection events rather than a single selection followed by dispersal of the resistant populations.[3,4] The heterogeneity or the genetic background of the field populations may interfere with the resistance genes, which presumably occurred for the first time in German populations.[2] Therefore, in the present work, inheritance of the CpGV resistance mechanism introduced to a susceptible laboratory colony was characterised by analysing the dose–response relationship to CpGV in the SV and RGV-8 colonies, which have similar genetic backgrounds obtained by introgression and by reciprocal crosses and backcrosses.

2 MATERIALS AND METHODS

2.1 Insect colonies

Two *C. pomonella* laboratory strains were used in this analysis: a susceptible laboratory inbred strain (SV) as the reference, and the CpGV-resistant laboratory strain (RGV). The RGV strain was derived from the original CpGV-resistant population isolated in the field (St-Andiol).[13]

The St-Andiol population was collected during the autumn of 2004 using cardboard traps in an organic orchard in the south-east of France (St-Andiol, Bouches du Rhône), where the only protection against *C. pomonella* has been CpGV, which has been increasingly applied (up to 14 treatments associated with mating disruption in 2004) for more than 10 years. The RGV resistant strain was derived from this field population and was obtained through eight generations of introgression of the resistance trait into the SV-associated susceptible genetic background. Resistance was selected for at each generation by treating with CpGV-M at a discriminating concentration.[13]

The *C. pomonella* larvae were reared on an artificial diet.[22] The rearing chamber was maintained under standard laboratory conditions (25 ± 1 °C, 16:8 h photoperiod). Adults were sexed just after their emergence to isolate virgin females. They were then placed in cylindrical cages for mating (Ø = 10 cm; h = 8 cm), supplied with smooth paper for oviposition and given sucrose solution in distilled water (15%), which was provided in plastic cups with cotton wicks for feeding. Resistant males and females were separated and crossed with the same number of susceptible moths. Each cage received 6–8 adult pairs. Eggs were collected twice a week and incubated in the rearing chamber. The larvae were used for bioassays or for rearing.

2.2 Virus isolates

The CpGV isolate used in the bioassays was the commercial formulation Carpovirusine® (NPP; Arysta LifeScience, France), which was derived from the CpGV-M isolate.[23] The concentration of occlusion bodies (OBs) was determined using the dark field optics of a light microscope and a Petroff–Hauser counting chamber (0.01 mm depth).

2.3 Genetic crosses of insects

Three types of cross were made (Fig. 1): (i) parental crosses with the susceptible strain (SV) and the resistant population (St-Andiol) or the resistant strain (RGV-8); (ii) reciprocal parental crosses

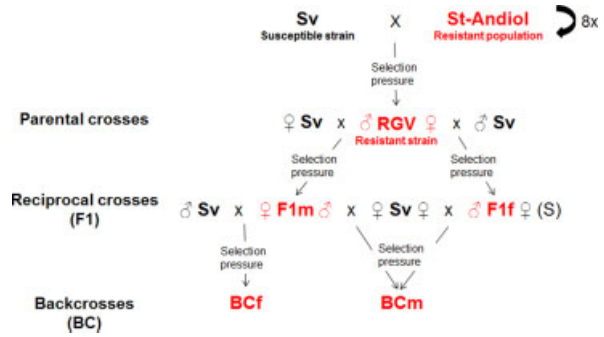

Figure 1. Blueprint of the different crosses performed in this study.

(F1) between resistant (RGV-8) and susceptible moths (SV); (iii) backcrosses of heterozygous resistant F1 (RS) with susceptible moths. The F1 generation was obtained by mass crossing the RGV-8 strain with SV.

The two complementary crosses ♂RGV × ♀SV (F1m) and ♀RGV × ♂SV (F1f) were carried out, and the resistance of the progeny to CpGV-M was assessed. The backcross (BC) was carried out by crossing SV with F1 resistant individuals that had survived treatment with a discriminating dose of CpGV. The two backcross types (BCm from an F1 R male and BCf from an F1 R female) were analysed separately.

2.4 Bioassays

A quantity of 150 μL of soybean instant diet (Stonefly Industries, Bryan, TX) prepared in an aqueous solution with 0.2% acetic acid and 0.1% formaldehyde was distributed into each well of a 96-well microplate. Virus suspension (6 μL) was deposited on the air–liquid interface (well surface 28 mm^2). One *C. pomonella* neonate larva (0–12 h old) was then placed on each well. Bioassays were performed using five concentrations of CpGV for the parental strains (SV and RGV-8), nine concentrations for the F1 progeny and twelve concentrations for the backcross (BC). The concentrations varied according to the resistance level of the progeny and ranged from 4 to 2500 OBs μL^{-1} for the SV strain and from 2500 to 1.5625 × 10^6 OBs μL^{-1} for the RGV-8 strain. At least three replicates of 16, 24 or 32 larvae were deposited for each concentration, according to the number of available larvae and the number of concentrations tested. Each row of eight wells was covered with a strip of Parafilm®, and the larvae were incubated in the rearing chamber (25 ± 1 °C, 16:8 h photoperiod) for 7 days. The larvae that died on the first day after the deposit were excluded from the test. Mortality was recorded on the seventh day, and a larva was considered to be dead if it did not respond to probing with forceps.

2.5 Data analysis

Mortality data were corrected for natural control mortality using Abott's formula[24] and were subjected to probit analysis.

Analysis of reciprocal crosses between susceptible and resistant colonies provides information on maternal effects and sex linkage, the degree of dominance and the number of genes involved in resistance.[25] The method used to assess the mode of inheritance is based on the mortality of the offspring from a backcross between F1 progeny and the parental strain that differs the most from the F1, which is SV in this study.

The degree of dominance (D) of the resistance trait was determined using the short description method.[26] Because there was evidence for sex linkage (see Section 3), only the data for F1

progeny descending from resistant males (RGV-8_m × SV_f) were used to calculate D, by means of the formula

$$D = (2X_2 - X_1 - X_3) / (X_1 - X_3)$$

where $X_1 = \log_{10}(LC_{50})$ of the resistant strain, $X_2 = \log_{10}(LC_{50})$ of the F1 and $X_3 = \log_{10}(LC_{50})$ of the susceptible strain. $D = 1$ indicates complete dominance, $0 < D < 1$ incomplete dominance, $-1 < D < 0$ incomplete recessiveness and $D = -1$ complete recessiveness.

The hypothesis of monogenic resistance was tested using the mortality data of backcrossed progeny compared with the theoretical expectations using the χ^2 test.[25] The null hypothesis tested in the standard backcross method is that resistance is controlled by one locus with two alleles (S and R). If so, the parental R strain is 100% RR and the F1 offspring are 100% RS. Further, the RS × SS will produce 50% RS and 50% SS offspring. If the null hypothesis is true, the Y expected mortality in the RS × SS backcross at dose X is calculated as

$$Y = 0.50\,(W_{RS} + W_{SS})$$

where W_{RS} and W_{SS} are the mortalities of the presumed RS (F1) and SS (parental line) at dose X.

3 RESULTS

3.1 Reference mortality data for susceptible and resistant insects

The results of bioassays for the susceptible and resistant colonies of *C. pomonella* with the CpGV-M formulation are presented in Table 1. In 2005, using the bioassay procedure, the SV strain presented a median lethal concentration (LC_{50}) value of 47.2 OBs μL^{-1}, while the offspring of the diapausing population of St-Andiol had an LC_{50} of 5.74×10^5 OBs μL^{-1}, exhibiting a close to 12 000-fold greater resistance than SV. The concentration–mortality data for the St-Andiol population were well adjusted to a two-step line. Between 100 and 12 500 OBs μL^{-1}, a constant mortality level (ca 15%) is observed. For higher concentrations, a good fit is obtained with a straight line. This response indicates that the field population was not homogeneous for the CpGV resistance trait, and that the population contained a few individuals (~15%) that were susceptible to the virus (Fig. 2a). Both the presence of susceptible individuals at a low frequency and the linearity of the dose–response relationship of the St-Andiol population to the virus suggested strong dominance of the resistance trait. For a more detailed analysis of the resistance, a homogeneous genetic background was preferable. To obtain this population, introgression of the resistance trait of the St-Andiol population was performed in the susceptible strain (SV) in 2006.

In 2007, the concentration–mortality data of the new resistant strain (RGV-8) exposed to CpGV no longer showed any susceptible larvae at low concentrations (Fig. 2b). At the highest concentration, 1.5625×10^6 OBs μL^{-1}, the mortality of the RGV-8 strain reached only 54.8%. The calculated LC_{50} value was 1.41×10^6 OBs μL^{-1}, which was twofold higher than the previous value for the St-Andiol field population. Under the same conditions, the LC_{50} value for the SV strain in 2007 was fourfold higher than 2 years before (Table 1). The origin of this variation has not been further explored, but is likely linked to small variations in the rearing conditions. The resistance factor between SV and RGV-8, which have almost the same genetic background, thus reached 7000-fold.

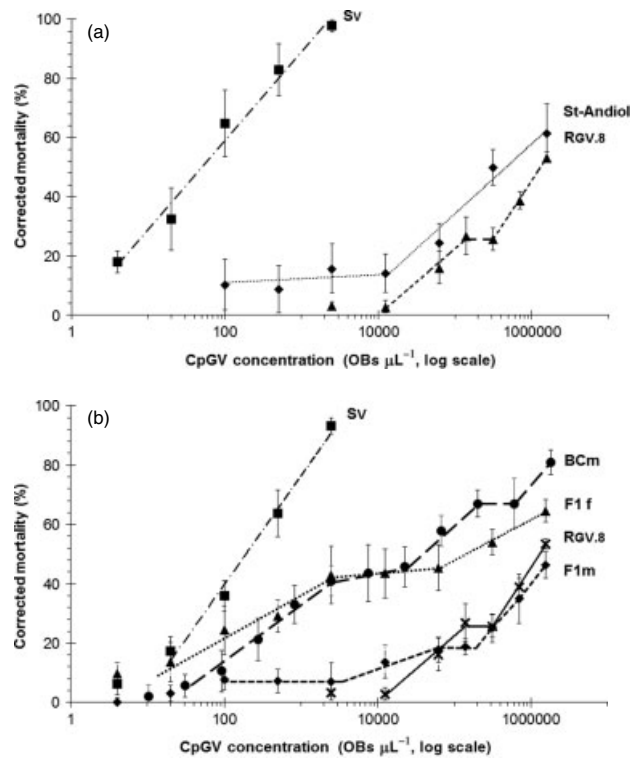

Figure 2. Concentration–mortality response (± SD, $n = 5$) for the progeny of (a) a susceptible strain (SV) and resistant colonies (St-Andiol and RGV-8) and (b) reciprocal crosses between RGV-8 and SV (F1m and F1f) and backcrosses between F1m and SV (BCm) of *C. pomonella* in 7 day bioassays with CpGV deposited on the surface of the media.

3.2 Mode of inheritance of CpGV resistance

3.2.1 Sex linkage

The maternal effects on CpGV resistance were examined by comparing the LC_{50} values of progeny derived from reciprocal parental crosses. The F1 progeny resulting from the cross of SV males with resistant RGV-8 females (F1f) presented a concentration–mortality line lying between those of the two parental strains ($LC_{50} = 6.73 \times 10^4$ OBs μL^{-1}), whereas the resistant male F1 cross progeny (F1m) were more resistant ($LC_{50} = 2.71 \times 10^6$ OBs μL^{-1}) (Fig. 2b). The concentration–mortality responses of these reciprocal parental crosses (F1) differed significantly from each other according to the sex of the resistant parent ($\chi^2 = 55.2$; df = 3; $P < 0.001$). Moreover, the sex ratio of insects surviving the virus treatment also differed. The resistant progeny from F1m crosses was composed of 50% of each sex, whereas the resistant progeny from F1f crosses was only composed of resistant males.

3.2.2 Evaluation of dominance

Owing to the sex linkage of this trait, the degree of dominance was calculated using only data for the F1m progeny. The concentration–mortality line for F1m was closer to that of the RGV-8 strain than to that of the SV strain (Fig. 2b). Comparison of the concentration–mortality lines at concentrations exceeding 12 500 OBs μL^{-1} for RGV-8 and F1m showed a similar resistance level (χ^2 =3.61; df = 3; $P = 0.306$). Stone's method, which is based on the LC_{50}, indicated a dominance level $D > 1$. As it was difficult to obtain more than 50% mortality in RGV-8 and F1m, the LC_{50} values obtained from the probit analysis lack accuracy. However, according to the concentration–mortality comparison, the degree

Table 1. Concentration–mortality results for the progeny of susceptible crosses (SV), resistant crosses (St-Andiol and RGV-8), reciprocal crosses between SV and RGV-8 (F1) and reciprocal backcrosses (BC) between resistant F1 and SV of *C. pomonella*. Bioassays were carried out at 25 ± 1 °C with a 16:8 h photoperiod by the virus surface deposit method on an artificial diet containing 0.1% formaldehyde. Mortality was recorded 7 days post-infection. Numbers in square brackets indicate the 95% CI

Crosses	*n*	LC_{50} (OBs μL^{-1})	[min–max]	Slope ± SE	χ^2	RR[a]
Parents in 2005						
SV	1169	47.2	[24.4–77.2]	1.03 ± 0.08	3.90	1
St-Andiol	456	5.74×10^5	[7.90×10^4–2.11×10^6]	0.68 ± 0.14	4.01	12 161
Parents in 2007						
SV	1256	197.2	[131.3–272.5]	1.35 ± 0.13	1.87	1
RGV-8	1393	1.41×10^6	[8.10×10^5–2.49×10^6]	0.86 ± 0.14	3.66	7150
Reciprocal crosses F1						
F1m (RGV-8♂ × SV♀)	1180	2.71×10^6	[9.51×10^5–9.80×10^6]	0.63 ± 0.14	2.53	15 357
F1f (RGV-8♀ × SV♂)	1109	6.73×10^4	[4.23×10^4–3.87×10^5]	0.28 ± 0.04	5.11	345
Backcross						
BCm (F1♂ × SV♀)	1826	2.13×10^4	[6.68×10^3–5.01×10^4]	0.43 ± 0.03	9.21	132
BCf (F1♀ × SV♂)	1259	1.39×10^4	[2.27×10^4–4.75×10^5]	0.37 ± 0.03	12.39	86

[a] Resistant ratio based on the susceptible LC_{50}.

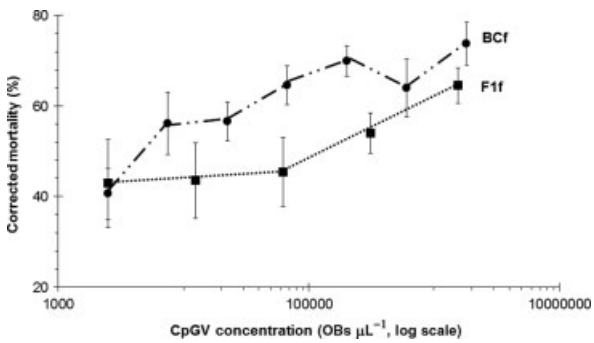

Figure 3. Concentration–mortality response (± SD, $n = 5$) for the progeny of reciprocal crosses between ♀RGV-8 and ♂SV (F1f) and backcrosses between ♀F1m and ♂SV (BCm) of *C. pomonella* in 7 day bioassays with CpGV deposited on the surface of the media.

of dominance obtained from male resistant parents was very close to 1.

3.2.3 *Number of genes involved in resistance*

To test the hypothesis that a single locus accounts for resistance to CpGV, the concentration–mortality responses of the F1f crosses and BCm backcrosses were compared with the mortality expected from a monogenic inheritance model (Fig. 3). The chi-square (χ^2) test was used for each virus concentration to determine the statistical significance of the differences between observed and expected data.

For BCm, only one concentration data point differed significantly from the monogenic model (92.6 OBs μL^{-1}: $\chi^2 = 4.66$; df = 1; $P = 0.031$) (Fig. 4b). For all of the other concentrations, the data are in agreement with the expected values, including the plateau between 2500 and 2.25×10^4 OBs μL^{-1} characterising the monogenic inheritance model of resistance. The second plateau observed for RGV-8 was also present in the BCm for very similar concentrations of the virus. This second plateau occurred at 67% mortality and separated the resistant insects into two distinct phenotypes. With a global χ^2 of 17.64 for the 12 concentrations, the monogenic inheritance model fitted the observed data well (df = 11, $P = 0.090$).

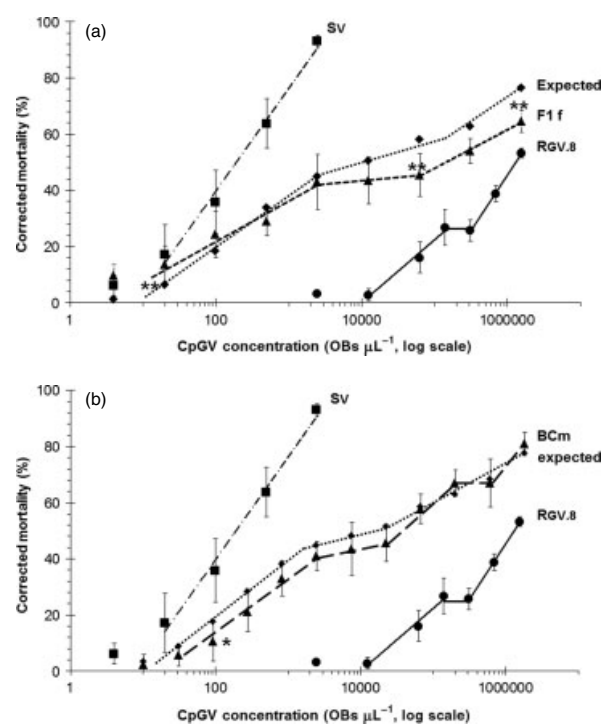

Figure 4. Comparison of observed and expected concentration–mortality responses (± SD, $n = 5$) for (a) F1f and (b) BCm progeny of *C. pomonella* in the case of a standard monogenic inheritance model. Asterisks indicate the points that significantly differ from the expected point: * significant, ** highly significant.

For F1f, the concentration–mortality line likewise shows a plateau at concentrations between 2500 and 2.25×10^4 OBs μL^{-1}. However, the mortality rates of three of the nine tested concentrations deviated significantly from the expected values (20, 6.55×10^4 and 1.56×10^6 OBs μL^{-1}) (Fig. 4a). The second part of the concentration–mortality line is composed of RS males only, and the mortality observed for these resistant insects is lower than expected. With a global χ^2 of 35.88, the monogenic inheritance model does not match with the results (df = 7; $P < 0.001$). The

mortality responses for the resistant progeny (second part of the line) differ significantly between both F1f and BCm ($\chi^2 = 10.04$; df = 3; $P = 0.018$).

4 DISCUSSION

A first genetic analysis of CpGV resistance in *C. pomonella* RGV-8 strain using mass crossing experiments verified the presence of a major resistance gene, which is dominant and carried on the Z chromosome. Female insects (WsZr) have only one resistant allele, as opposed to male insects which can have one (ZsZr) or two (ZrZr) copies of the resistant allele. These observations differ from the first mass crossing experiments carried out on a CpGV-resistant strain (CpR) in Germany,[2] which suggested autosomal, incompletely dominant inheritance of CpGV resistance. However, the results for RGV-8 were in agreement with those obtained later using the CpRR1 strain with single-pair crosses,[21] which also led to the conclusion that CpGV resistance is carried by a gene on the Z sex chromosome. The high level of resistance detected in European populations thus seems mainly to be due to the effect of the same major resistance locus. The differences detected in the levels of resistance could be attributed to differences in the genetic background of *C. pomonella* populations with different geographic origins.

However, a second detailed genetic analysis suggests that another gene is likely involved in codling moth resistance to CpGV. This analysis was made performed by constructing inbred insect strains that differed only in their resistance to CpGV-M. With this approach, results highlighted some divergences from a single gene transmission hypothesis for CpGV-M resistance in the RGV-8 strain.

The two F1 crosses performed did not result in similar resistance levels. The F1m progeny is theoretically composed exclusively of resistant insects of both sexes. However, two mortality plateaus are observed, one close to 6% mortality for the concentrations ranging from 100 to 2500 OBs μL^{-1}, and the second at 25% mortality, indicating that these progeny are not phenotypically homogeneous. The RGV-8 concentration–mortality response revealed the presence of some remaining susceptible individuals (dying after exposure to concentrations between 100 and 2500 OB μL^{-1}). As the main gene is dominant, a residual level of susceptible alleles will be maintained in the population, even under selection pressure for resistance. If it is assumed that 6% of individuals in F1m are homozygous for the susceptible allele, this could result in 12% heterozygous males (RS) in the RGV-8 strain. In addition, the concentration–mortality response of RGV-8 is not linear. A plateau consistently appears at approximately 25% mortality, suggesting the presence of at least two different resistant phenotypes with two distinct resistance levels. As the maximum mortality does not reach 100% for RGV-8, the presence of additional plateaus (that is, additional resistance levels) cannot be observed.

Two hypotheses could explain these results: (i) RR and RS males and RW females have different resistance levels, or (ii) other genes influence the response to virus challenge. Asser-Kaiser *et al.*[21] suggested the first hypothesis in their analysis of the CpRR1 strain, concluding that RS males are more susceptible than R females. The present results using the F1f crosses are compatible with this hypothesis. However, this hypothesis is not compatible with the plateau observed at 67% mortality in the BCm crosses; the progeny of the backcrossed BCm is theoretically composed of 50% susceptible individuals (25% S females and 25% S males) and 50% resistant individuals (25% R females and 25% RS males). Thus, a single plateau would be at 50% for similar resistance between males and females. RGV-8 also presents a plateau at the same concentrations, confirming that the observation is not an artefact. In addition, the F1f progeny is theoretically composed of 50% susceptible females and 50% RS males, but the slope observed for the resistant insects (second part of the concentration–mortality line) is lower than would be expected were the RS males more susceptible to the virus, as observed in the CpRR1 strain in Germany.[21] In the present experiments, RS males seem to be the genotype exhibiting the highest resistance level in RGV-8. Moreover, the concentration–mortality response of RGV-8 is not linear. Some mechanisms of chromosome dosage compensation could be responsible for the difference in resistance level according to the virus concentration. Moreover, these results could be explained by a concentration-dependent dominance, as described in Germany.[21]

With a standard monogenic inheritance model, the mortality of F1f and BCf should be similar. The opposite was observed (Fig. 3): the backcrossed BCf showed a lower resistance level than F1f. Resistant females seem to give an additional resistance factor to their progeny in the first generation. These different results make the single gene on the Z chromosome hypothesis unlikely.

The alternative hypothesis would imply the involvement of other genes, although the levels of resistance conferred would not be as important, or a resistance mechanism that does not follow classical Mendelian transmission.

The exact number of genes involved in a resistance trait is difficult to determine by comparing the concentration–mortality response of backcrossed generations to parents over a range of concentrations.[25] Polygenically determined resistance is reflected by a straight line in the backcross generation. Small genetic differences between individual BC progeny result in a continuous phenotypic distribution over a range of insecticide concentrations.[27] If resistant phenotypes are determined by the segregation of alleles at a single genetic locus, two plateaus are observed. These cases do not match the present results. Mendelian inheritance governs most characteristics, but some do not follow this law. Non-Mendelian inheritance is based on extranuclear (cytoplasmic) inheritance of genetic information. This information is carried by cellular organelles, such as mitochondria,[28,29] chloroplasts[30,31] or parasites.[32] Alternatively, this type of inheritance can rely on the presence of nuclear gene products (mRNA or proteins) in the cytosol.[33] *Drosophila melanogaster* (Meigen) is an insect model widely used for genetic studies. In this species, different traits are inherited cytoplasmically.[34–37] For example, *D. melanogaster* lifespan is controlled by two major nuclear genes, one autosomal and the other linked to the X sex chromosome.[38,39] Yonemura *et al.*[40] showed that an additional cytoplasmic factor is involved in the expression of nuclear genes affecting insect lifespan. This cytoplasmic factor is strictly maternally inherited and could rely on mitochondrial DNA. Expression of a mitochondrial inherited trait is variable. This mode of inheritance could explain the difference between F1f and BCf, issuing from higher expression of resistance in the progeny of an R female of the first generation (F1f), while R females from the second generation (BCf) would be more susceptible. Through the different generations of introgression, some resistant females could have lost this potential extranuclear factor of resistance. The first part of the concentration–mortality line for the RGV-8 strain (25% of individuals) would be the consequence of progeny from females that do not carry the second extranuclear factor or the consequence of variable expressiveness.

In conclusion, the transmission of CpGV-M resistance in the RGV-8 strain of *C. pomonella* cannot be fully explained by the effect of a locus located on the Z chromosome. The action of other factors needs to be considered. It is likely that these other factors are variable between populations from different geographic origins. This resistance has a more complex mechanism than has been previously described. Results highlight the presence of variability in resistance at a population level, making this organism more adaptive to environmental conditions. These findings imply better local stability of resistance. In the future, the use of this virus in pest management must be locally monitored to maximise the efficiency of these bioinsecticides.

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