

Mutations That Determine Resistance Breaking in a Plant RNA Virus Have Pleiotropic Effects on Its Fitness That Depend on the Host Environment and on the Type, Single or Mixed, of Infection

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ABSTRACT

Overcoming host resistance in gene-for-gene host-virus interactions is an important instance of host range expansion, which can be hindered by across-host fitness trade-offs. Trade-offs are generated by negative effects of host range mutations on the virus fitness in the original host, i.e., by antagonistic pleiotropy. It has been reported that different mutations in *Pepper mild mottle virus* (PMMoV) coat protein result in overcoming *L*-gene resistance in pepper. To analyze if resistance-breaking mutations in PMMoV result in antagonistic pleiotropy, all reported mutations determining the overcoming of *L*³ and *L*⁴ alleles were introduced in biologically active cDNA clones. Then, the parental and mutant virus genotypes were assayed in susceptible pepper genotypes with an *L*⁺, *L*¹, or *L*² allele, in single and in mixed infections. Resistance-breaking mutations had pleiotropic effects on the virus fitness that, according to the specific mutation, the host genotype, and the type of infection, single or mixed with other virus genotypes, were antagonistic or positive. Thus, resistance-breaking mutations can generate fitness trade-offs both across hosts and across types of infection, and the frequency of host range mutants will depend on the genetic structure of the host population and on the frequency of mixed infections by different virus genotypes. Also, resistance-breaking mutations variously affected virulence, which may further influence the evolution of host range expansion.

IMPORTANCE

A major cause of virus emergence is host range expansion, which may be hindered by across-host fitness trade-offs caused by negative pleiotropy of host range mutations. An important instance of host range expansion is overcoming host resistance in gene-for-gene plant-virus interactions. We analyze here if mutations in the coat protein of *Pepper mild mottle virus* determining *L*-gene resistance-breaking in pepper have associated fitness penalties in susceptible host genotypes. Results show that pleiotropic effects of resistance-breaking mutations on virus fitness depend on the specific mutation, the susceptible host genotype, and the type of infection, single or mixed, with other virus genotypes. Accordingly, resistance-breaking mutations can have negative, positive, or no pleiotropic effects on virus fitness. These results underscore the complexity of host range expansion evolution and, specifically, the difficulty of predicting the overcoming of resistance factors in crops.

Changes in virus host range affect virus ecology and epidemiology, condition virus emergence, and can compromise the success of strategies for the control of viral diseases (1–4). The acquisition of new hosts, that is, host range expansion, would provide a virus with more opportunities for transmission and survival. However, differential host-associated selection may result in across-host fitness trade-offs so that increasing the virus fitness in a new host will decrease its fitness in the original one, which will hinder host range expansion (4–6). The simplest mechanism generating across-hosts fitness trade-offs is antagonistic pleiotropy, in which mutations that increase fitness in one host are deleterious in another one (7). Evidence indicates that antagonistic pleiotropy is common in RNA viruses as a result of their small genomes, which are compacted with genetic information and encode few, multi-functional proteins (5, 8).

Host range evolution is particularly relevant for the sustainable use of genetic resistance to control viral diseases of crops. Disease control based on resistant cultivars is target specific and highly efficient but is not durable due to the appearance of new virus genotypes able to infect otherwise resistant host genotypes. The increase in frequency of resistance-breaking virus genotypes eventually renders resistance inefficient (9–11). Plant-virus interac-

tions can often be explained by the gene-for-gene (GFG) model, under which direct or indirect recognition of viral proteins by those encoded by plant resistance genes (*R* genes) triggers plant defenses that limit virus multiplication to infection sites, thus triggering a hypersensitive response (HR) (12, 13). Mutations in the viral proteins that impair their recognition by *R* proteins result in resistance breaking so that the virus can infect a larger set of host genotypes, expanding its host range. Indeed, a key feature of GFG systems is that the capacity of pathogens to infect all host genotypes will evolve as a result of subsequent resistance-breaking

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events (14). The evolution of resistance breaking may be hindered by across-host fitness trade-offs if the resistance-breaking genotypes are less fit than the non-resistance-breaking ones in susceptible hosts, that is, if there are resistance-breaking costs. The relevance of resistance-breaking costs in plant-virus coevolution and as modulators of the durability of crop resistance has led to efforts for their detection and quantification. Analyses of virus multiplication in susceptible hosts have provided evidence for resistance-breaking costs in various plant-virus systems (15–21).

Our group has documented resistance-breaking costs in the tobamoviruses that infect pepper (*Capsicum annuum* L.) genotypes carrying different resistance alleles at the *L* locus. Tobamoviruses have single-stranded, messenger sense, monopartite RNA genomes encapsidated into rod-shaped particles and are transmitted in pepper crops through plant-to-plant contact or vertically through the seed (22). After plant harvest or death, infectious particles can survive for several months in the soil, a major source of inoculum for epidemics (23, 24). Four resistance alleles at the pepper *L* locus confer resistance to tobamoviruses (25, 26) according to the GFG model, and tobamovirus species and genotypes are classified into pathotypes according to their capacity to infect pepper genotypes carrying the various *L* alleles (27–29). Plants homozygous for allele L^+ are susceptible to all described pathotypes (P_0 , P_1 , $P_{1,2}$, $P_{1,2,3}$, and $P_{1,2,3,4}$), L^1/L^- plants are resistant only to pathotype P_0 ; L^2/L^- plants are resistant to pathotypes P_0 and P_1 ; L^3/L^- plants are resistant to pathotypes P_0 , P_1 , and $P_{1,2}$, and so on. Pathotype $P_{1,2,3,4}$ is able to infect pepper genotypes with each of the four known resistance alleles. *L*-gene resistance is elicited by the virus coat protein (CP), and the resulting HR is expressed by the appearance in inoculated leaves of necrotic local lesions at infection sites (30–33). The capacity to establish an infection or to elicit an HR depends on the alleles at the virus CP and the pepper *L* protein and is little affected by the host genetic background, except in the temporal rate of development of the hypersensitive reaction (25–33). Conclusive evidence for resistance-breaking costs in field isolates of pepper-infecting tobamoviruses was derived from long-term comparison of changes in pathotype frequency and in the fraction of the crop carrying the various resistance alleles in southeast (SE) Spain and by quantifying virus multiplication in the L^+/L^+ universally susceptible host, both in single infection and in competition (17). In the L^+/L^+ host, isolates of pathotype $P_{1,2,3}$ were outcompeted by isolates of pathotype $P_{1,2}$, and both were outcompeted by isolates of pathotype P_0 (17). Only isolates of pathotypes P_0 , $P_{1,2}$, and $P_{1,2,3}$ were present infecting pepper in SE Spain; P_0 isolates were *Tobacco mild green mosaic virus* (TMGMV) isolates, while $P_{1,2}$ and $P_{1,2,3}$ isolates were *Pepper mild mottle virus* (PMMoV) (17). Studies by Fraile et al. (17) were performed with field virus isolates that differed in genomic regions other than the CP gene, which precluded the analysis of the mechanisms generating the observed resistance-breaking costs.

The goal of the present work is the analysis of the mechanisms generating resistance-breaking fitness costs in pepper-infecting tobamoviruses. Since TMGMV and PMMoV differ in $\approx 30\%$ of amino acid positions at the CP, the analysis was restricted to PMMoV. Most reported PMMoV isolates are of pathotype $P_{1,2}$ (22), and different mutations at the CP have been reported as determinants of pathotypes $P_{1,2,3}$ and $P_{1,2,3,4}$ (27, 30, 34–36). We addressed the following specific questions: (i) if resistance-breaking mutations in PMMoV CP have associated fitness costs in susceptible hosts, (ii) if pleiotropic effects of resistance-breaking mu-

tations vary according to the genotype of the susceptible host, and (iii) if pleiotropic effects of resistance-breaking mutations vary according to the type, single or mixed, of infection. In addition, we characterized the relationship between infectivity, that is, the capacity to infect a host (14), and virulence, that is, the negative effect of infection on the host fitness (2), which is an understudied topic (37). Results show pleiotropic effects of resistance-breaking mutations both on virus fitness and on virulence. However, the magnitude and sense of pleiotropy, antagonistic or positive, depended on the genotype of the susceptible host and on the type, single or mixed with other virus genotypes, of infection. Thus, across-host fitness trade-offs depend on the interactions of virus genotype versus host genotype and on the interactions of virus genotype versus type of infection, an observation which underscores the complexity of mechanisms underlying host range expansion in RNA viruses.

MATERIALS AND METHODS

Plants, inoculations, virus, and virulence quantification. Species and cultivars of *Capsicum* plants differing in the alleles at the *L* resistance locus were used for characterizing the pathotypes of the different PMMoV genotypes and for assays of virus multiplication and virulence: *C. annuum* cv. Dolce Italiano (L^+/L^+), *C. annuum* cv. Yolo Wonder (L^1/L^1), *C. frutescens* cv. Tabasco (L^2/L^2), *C. chinense* PI 159236 (L^3/L^3), *C. annuum* cv. Ferrari (L^3/L^+), *C. chacoense* PI260429 (L^4/L^4), and *C. annuum* cv. Imperio (L^4/L^+). Note that we used the original *Capsicum* sp. genotype donors of the resistance alleles or widely grown cultivars; hence, the assayed hosts differed in their genetic makeup in addition to the differences at the *L* locus. However, for convenience, the different hosts will be identified by their genotypes at the *L* locus rather than by the full species or cultivar name. Plants were grown in 15-cm-diameter, 1.5-liter pots at 23 to 25°C and with a 16-h light photoperiod in a P2-level biological containment greenhouse. Plants were inoculated in the first two true leaves with 400 ng of freshly purified virus particles suspended in 0.1 M phosphate buffer, pH 7.2. Infection status was checked 10 days postinoculation (dpi) by reverse transcription-PCR (RT-PCR) amplification of the coat protein gene (primer sequences are available upon request).

PMMoV particles were purified as described in Bruening et al. (38), and virion RNA was extracted with phenol-chloroform after virus particles were disrupted in 0.1 M Tris-HCl (pH 9.0)–5% SDS.

Virus multiplication was estimated at 21 dpi as viral RNA accumulation in all systemically infected leaves. Leaf RNA was extracted using TRIzol reagent (Life Technologies), and viral RNA accumulation was determined by quantitative real-time RT-PCR, as described in Fraile et al. (39), using different sets of primers for each viral genotype (available upon request). Levels of viral RNA were deduced from comparison with standard curves produced using a set of serial dilutions of purified virion RNA. Virus fitness was estimated from virus RNA accumulation as described in Lalic et al. (40) using the Malthusian parameter m , which represents the population exponential growth rate at a time t after inoculation, calculated as $m = (1/t) \ln(Q)$, where Q is virus accumulation. Fitness, W , is then computed as $W = e^m$ (41).

Virulence (V) was quantified as the effect of infection on the above-ground host plant biomass, calculated as $V = 1 - (P_i/P_m)$, where P_i is the dry weight of each infected plant and P_m is the mean dry weight of mock-inoculated plants (42).

Virus isolates and mutants. Inoculations were done with transcripts from full-length infectious cDNA clones derived from two PMMoV field isolates of the $P_{1,2}$ pathotype. Thus, plasmid pTS, generating TS transcripts, was derived from isolate PMMoV-S and has been described previously (30). Plasmid pMG, generating MG transcripts, was obtained in this work, derived from isolate P84/8 (17) using primers complementary and identical to the 3' and 5' ends of the genomic RNA (available upon request); the 5' primer included the sequence of the T7 transcription

TABLE 1 Description of PMMoV genotypes used in this work

Genotype	CP gene profile		Capacity to infect the indicated host ^c					Pathotype ^d
	Amino acid substitution(s) ^a	Nucleotide substitution(s) ^b	L ¹ /L ¹	L ² /L ²	L ³ /L ³	L ⁴ /L ⁺	L ⁴ /L ⁴	
TS			+	+	NLL	NLL	NLL	P _{1,2}
TS-(M138N)	M138N	T433A + G434T	+	+	+	NLL	NLL	P _{1,2,3}
TS-(T43K+D50G)	T43K + D50G	C131A + A152G	+	+	+	+	NLL	P _{1,2,3}
TS-(L13F+G66V)	L13F + G66V	A42T + G200T	+	+	+	NLL	NLL	P _{1,2,3}
MG			+	+	NLL	NLL	NLL	P _{1,2}
MG-(M138N)	M138N	T433A + G434T	+	+	+	NLL	NLL	P _{1,2,3}
MG-(T43K+D50G)	T43K + D50G	C131A + A152G	+	+	+	+	NLL	P _{1,2,3}
MG-(A87G)	A87G	G260C	+	+	+	+	+	P _{1,2,3,4}

^a Change of amino acid in the CP from parental TS or MG genotype (pathotype P_{1,2}).

^b Change of nucleotide in the CP gene from parental TS or MG genotype (pathotype P_{1,2}).

^c NLL, necrotic local lesions; +, infection present.

^d Pathotype of the parental and mutant genotypes.

promoter, and a MluI restriction site was added to the 5' of the 3' primer. The full-length sequence of the insert of plasmid pMG was also determined (primer sequences are available upon request). Plasmids pTS and pMG and derived mutants were multiplied in *Escherichia coli* XL2-Blue MRF' (Agilent Technologies).

Mutations were introduced in the coat protein gene of plasmids pTS and pMG by site-directed mutagenesis, generating the following set of genotypes of P_{1,2,3} pathotype: TS-(M138N) and MG-(M138N) in which the substitution M138N was introduced through the mutations T416A and G417T in pTS and pMG, respectively, using primers M138N-F and M138N-R; TS-(T43K+D50G) and MG-(T43K+D50G), in which the substitutions T43K and D50G were introduced through the mutations C131A and A152G in pTS and pMG, respectively, using primers T43KD50G-F and T43KD50G-R; TS-(L13F+G66V), in which the substitutions L13F and G66V were introduced through the mutations A42T and G200T in plasmid pTS using the primer pair L13F-F and L13F-R and the pair G66V-F and G66V-R, respectively.

The genotype MG-(A87G) of the P_{1,2,3,4} pathotype was also obtained, in which the substitution A87G was introduced through the mutation C260G using primers A87G-F and A87G-R. All primer sequences are available upon request. Nucleotide and amino acids mentioned above are numbered according to their positions in the CP gene or protein.

The pathotypes of all parental and mutant genotypes were checked by their inoculation in the cultivars and genotypes of *Capsicum* homozygous for the various *L*-gene alleles, described above (Table 1).

Statistical analyses. The distribution of data of all analyzed variables was tested against the null hypothesis of normality using a Shapiro-Wilk test, and homogeneity of variances was analyzed by Levene's test. The distribution of data was normal for the fitness of 5 out of 7 virus genotypes in single infection, but distributions were not normal for fitness in mixed infection, for virus accumulation in single or mixed infection, or for virulence. Also, no data distribution was homoscedastic. Thus, differences in virus accumulation, relative fitness, and virulence were analyzed using generalized linear models (GzLM) as this method is robust with respect to data distribution and allows for unbalanced experimental designs. The significance of the differences among classes within a factor was tested using the least significant difference (LSD) test. The relationship between virus accumulation and virulence was analyzed by bivariate Spearman correlation. All statistical analyses were implemented in SPSS, version 17.0 (SPSS, Inc.), and are described in Sokal and Rohlf (43).

Accession number(s). The complete genome sequence of the PMMoV isolate pMG was deposited in the GenBank under accession number [KX063611](https://doi.org/10.1093/nar/kxw061).

RESULTS

Characterization of parental and mutant virus genotypes. The sequence of the genomes of PMMoV isolates inserted in the full-

length infectious clones pTS and pMG were determined (GenBank accession numbers [NC_003630](https://doi.org/10.1093/nar/kxw061) and [KX063611](https://doi.org/10.1093/nar/kxw061), respectively), with 6,357 nucleotides (nt) for TS and 6,361 nt for MG. TS and MG sequences differed at 19 positions: two were in the 5' untranslated region (UTR), eight were in the 126-kDa protein open reading frame (ORF), three were in the read-through portion of the 183-kDa protein ORF, three were in the movement protein ORF, one was in the CP ORF, and two were in the 3' UTR. All nucleotide substitutions within ORFs were silent.

The pathotypes of the parental genotypes TS and MG and of the derived CP mutants were determined by inoculation of plants of the indicator hosts *C. annuum* cv. Dulce Italiano (L⁺/L⁺), *C. annuum* cv. Yolo Wonder (L¹/L¹), *C. frutescens* cv. Tabasco (L²/L²), *C. chinense* PI 159236 (L³/L³), *C. annuum* cv. Ferrari (L³/L⁺), *C. chacoense* PI260429 (L⁴/L⁴), and *C. annuum* cv. Imperio, (L⁴/L⁺). Results are shown in Table 1; all genotypes had the expected pathotypes. However, it was found that TS-(T43K+D50G) and MG-(T43K+D50G) were able to systemically infect *C. annuum* cv. Imperio (L⁴/L⁺) plants but not *C. chacoense* PI260429 (L⁴/L⁴), thus partially overcoming allele L⁴ in addition to fully overcoming allele L³.

Fitness of PMMoV genotypes in single infection of susceptible host genotypes. To analyze if the CP mutations that determine resistance breaking of alleles L³ and L⁴ are associated with fitness penalties in susceptible hosts, the parental genotypes TS and MG with the P_{1,2} pathotype, the mutant genotypes TS-(M138N), TS-(T43K+D50G), TS-(L13F+G66V), and MG-(M138N) with the P_{1,2,3} pathotype, and MG-(A87G) with the P_{1,2,3,4} pathotype were assayed in *C. annuum* cv. Dulce Italiano (L⁺/L⁺), *C. annuum* cv. Yolo Wonder (L¹/L¹), *C. frutescens* cv. Tabasco (L²/L²), *C. chinense* PI 159236 (L³/L³), and *C. annuum* cv. Ferrari (L³/L⁺). The assays followed a random block design with 35 virus genotype treatments versus host genotype treatments, plus the five mock-inoculated controls, with 10 replicated plants per treatment/control. Plants were harvested at 22 dpi, virus RNA was quantified in systemically infected leaves (data not shown), and fitness was estimated as described in Materials and Methods. Data are shown in Fig. 1.

To determine if pathotype and/or host genotype affected virus fitness, data were analyzed by a GzLM considering host genotype and virus pathotype fixed factors, and virus genotype nested to pathotype was considered a random factor in a full factorial model. Because genotypes of the P_{1,2} pathotype do not infect

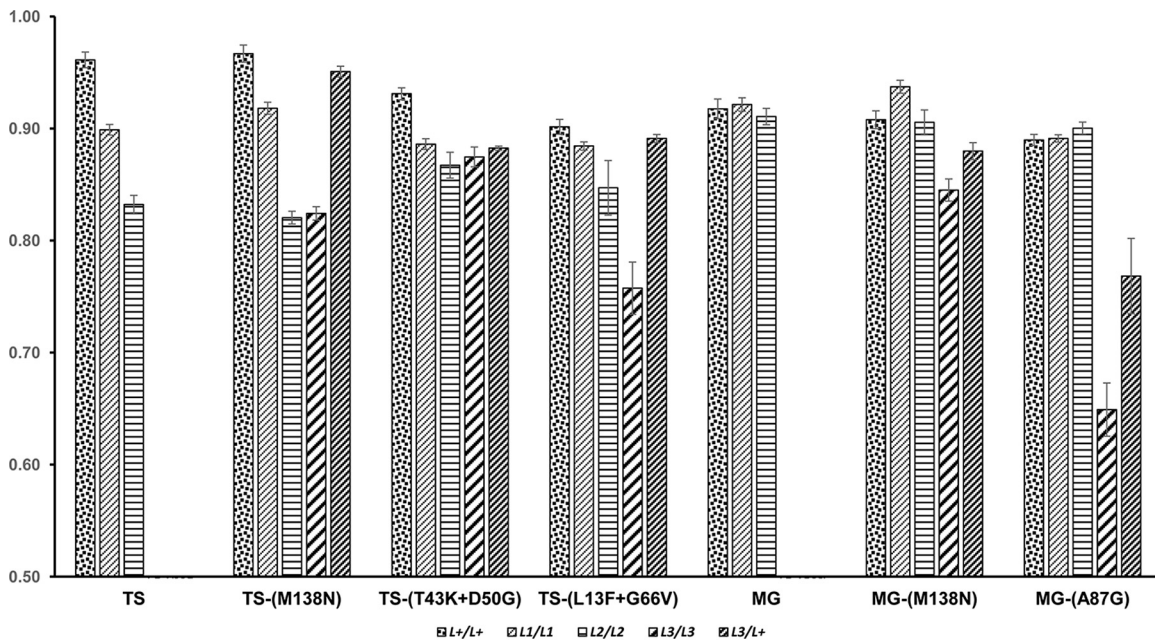


FIG 1 Fitness of seven PMMoV genotypes in single infection in five *Capsicum* host genotypes differing at the *L* locus. Data are means \pm standard errors from 10 replicated plants.

plants of L^3/L^- genotypes, L^3/L^- plants were excluded from the analysis. Virus fitness depended on pathotype, virus genotype nested to pathotype, and host genotype [$\chi^2_{W(2)} = 6.37$, $P = 0.041$; $\chi^2_{W(4)} = 46.31$, $P = 1 \times 10^{-4}$; $\chi^2_{W(2)} = 99.92$, $P = 1 \times 10^{-4}$, respectively), and the interaction of pathotype versus host genotype and the interaction of virus genotype nested to pathotype versus host genotype were also significant ($P = 1 \times 10^{-4}$). To determine if the resistance-breaking mutations had an effect on virus fitness, a second GzLM analysis was done in which virus genotype and host genotype were considered fixed factors, which showed that virus genotype, host genotype, and their interaction had significant effects on virus fitness [$\chi^2_{W(6)} = 52.48$, $P = 1 \times 10^{-4}$; $\chi^2_{W(2)} = 166.72$, $P = 1 \times 10^{-4}$; $\chi^2_{W(12)} = 183.91$, $P = 1 \times 10^{-4}$, respectively]. LSD analyses showed that, over hosts, the fitness of the three pathotypes ranked $P_{1,2} > P_{1,2,3} = P_{1,2,3,4}$ ($P < 0.023$), and the fitness of the pooled set of virus genotypes significantly differed according to the host genotype ($P = 1 \times 10^{-4}$) in the order $L^+/L^+ > L^1/L^1 > L^2/L^2$. Over host genotypes, fitness levels of MG and MG-(M138N) were similar ($P > 0.140$) and higher than the fitness levels of TS, TS-(M138N), TS-(T43K+D50G), and MG-(A87G), which were also similar; TS-(L13F+G66V) was the least fit genotype ($P < 0.026$).

Then, the fitness levels of the $P_{1,2,3}$ and $P_{1,2,3,4}$ virus genotypes were analyzed over all host genotypes. A GzLM analysis was done in which host genotype and virus pathotype were considered fixed factors and virus genotype nested to pathotype was considered a random factor in a full factorial model. Again, virus fitness depended on pathotype, virus genotype nested to pathotype, and host genotype [$\chi^2_{W(1)} = 108.97$, $P = 1 \times 10^{-4}$; $\chi^2_{W(3)} = 36.23$, $P = 1 \times 10^{-4}$; $\chi^2_{W(4)} = 437.66$, $P = 1 \times 10^{-4}$, respectively], and the interactions of pathotype versus host genotype and of virus genotype nested to pathotype versus host genotype were also significant ($P = 1 \times 10^{-4}$). LSD analyses showed that the pooled set of virus genotypes, with the exclusion of TS and MG, significantly

differed in fitness according to the host genotypes in the order $L^+/L^+ > L^1/L^1 > L^2/L^2 = L^3/L^+ > L^3/L^3$, and the fitness of $P_{1,2,3}$ genotypes was higher than that of the $P_{1,2,3,4}$ genotype MG-(A87G) ($P < 0.025$).

The fitness of each virus genotype in each host genotype was compared in new GzLM analyses considering host genotype a fixed factor. For all virus genotypes except MG, host genotype had a significant effect ($P < 0.016$) on virus fitness. LSD analyses ranked virus fitness over hosts differently according to virus genotype, as presented in Fig. 1, showing that the variation of fitness according to host genotype was specific for each virus genotype.

The significance of the interactions of virus genotype versus host genotype in all analyses above indicates host genotype-dependent pleiotropic effects of resistance-breaking mutations on the virus fitness. To quantify the magnitude of these pleiotropic effects, the difference between the fitness of each mutant (W_m) and that of the parental genotype (W_p) was computed in each host genotype, and its significance was analyzed by GzLM and LSD. Table 2 shows these results for host genotypes L^+/L^+ , L^1/L^1 , and L^2/L^2 as the parental genotypes TS and MG with the $P_{1,2}$ pathotype did not infect plants with an L^3/L^- genotype. In the genetic context of the TS genotype, the mutation M138N increased fitness in L^1/L^1 and had no effect on L^+/L^+ or L^2/L^2 ; the double mutation T43K+D50G decreased fitness in L^+/L^+ , had no effect on L^1/L^1 , and increased fitness in L^2/L^2 ; and the double mutation L13F+G66V decreased fitness in L^+/L^+ and L^1/L^1 and had no effect on L^2/L^2 . In the genetic context of the MG genotype, the mutation M138N increased TS fitness in L^1/L^1 and had no effect on L^+/L^+ or L^2/L^2 as in the TS context, and the mutation A87G decreased fitness in L^+/L^+ and L^1/L^1 and had no effect on L^2/L^2 . Thus, the magnitude of pleiotropy is significantly different from zero in 8/15 interactions of virus genotype versus host genotype, with five antagonistic and three positive interactions. Interestingly, the sense of pleiotropy was always antagonistic in plants of

TABLE 2 Magnitude and sense of fitness differences between mutant and parental genotypes

Host genotype	Virus genotype	W_m^a	W_p^b	$W_m - W_p$	P^c
L^+/L^+	TS-(M138N)	0.967	0.961	0.006	0.552
L^+/L^+	TS-(T43K+D50G)	0.931	0.961	-0.030	0.002*
L^+/L^+	TS-(L13F+G66V)	0.902	0.961	-0.059	0.000*
L^+/L^+	MG-(M138N)	0.908	0.918	-0.010	0.367
L^+/L^+	MG-(A87G)	0.890	0.918	-0.028	0.010*
L^1/L^1	TS-(M138N)	0.918	0.899	0.019	0.007*
L^1/L^1	TS-(T43K+D50G)	0.886	0.899	-0.013	0.068
L^1/L^1	TS-(L13F+G66V)	0.884	0.899	-0.015	0.040*
L^1/L^1	MG-(M138N)	0.937	0.921	0.016	0.025*
L^1/L^1	MG-(A87G)	0.891	0.921	-0.030	<10 ⁻³ *
L^2/L^2	TS-(M138N)	0.820	0.832	-0.012	0.505
L^2/L^2	TS-(T43K+D50G)	0.867	0.832	0.035	0.048*
L^2/L^2	TS-(L13F+G66V)	0.847	0.832	0.015	0.393
L^2/L^2	MG-(M138N)	0.905	0.910	-0.005	0.773
L^2/L^2	MG-(A87G)	0.900	0.910	-0.010	0.577

^a W_m is the average fitness of each mutant in each host at 22 dpi.

^b W_p is the average fitness of each parental genotype.

^c The asterisk indicates significance at a P value of ≤ 0.05 for fitness differences according to an LSD analysis.

the L^+/L^+ genotype and antagonistic or positive in L^1/L^1 plants, and there was no pleiotropy or it was positive in L^2/L^2 plants.

Thus, results show that resistance-breaking mutations have a pleiotropic effect on virus fitness that depends on the host genotype, and this pleiotropy may be an important cause of the observed fitness in the interaction of virus genotype versus host genotype.

Virulence of PMMoV resistance-breaking genotypes in single infection of susceptible host genotypes. In the above-described experiment it was observed that the symptoms developed by infected plants differed according to virus genotype so that in general those induced by the $P_{1,2,3,4}$ genotype were more severe than those induced by the $P_{1,2,3}$ genotypes, which were more severe than those of the $P_{1,2}$ genotype, suggesting differences in virulence. To further analyze the possible effect of resistance-breaking mutations on virulence, virulence was quantified as the effect

of infection on plant biomass (data not shown), and the derived virulence values are shown in Fig. 2.

The possible effect of pathotype on virulence was analyzed by GzLM considering host genotype and virus pathotype fixed factors and virus genotype nested to pathotype a random factor in a full factorial model. Virulence depended on pathotype, virus genotype nested to pathotype, and host genotype [$\chi^2_{W(2)} = 8.38$, $P = 0.015$; $\chi^2_{W(4)} = 22.47$, $P = 1 \times 10^{-4}$; $\chi^2_{W(4)} = 143.156$, $P = 1 \times 10^{-4}$, respectively], and the interactions of pathotype versus host genotype and of virus genotype nested to pathotype versus host genotype were also significant ($P < 0.004$). To determine if the resistance-breaking mutations had an effect on virulence, GzLM analyses were done considering host and virus genotypes fixed factors in a full factorial model. Both factors and their interaction had an effect on virulence [$\chi^2_{W(4)} = 150.23$, $P = 1 \times 10^{-4}$; $\chi^2_{W(6)} = 32.36$, $P = 1 \times 10^{-4}$; $\chi^2_{W(24)} = 83.65$, $P = 1 \times 10^{-4}$]. LSD

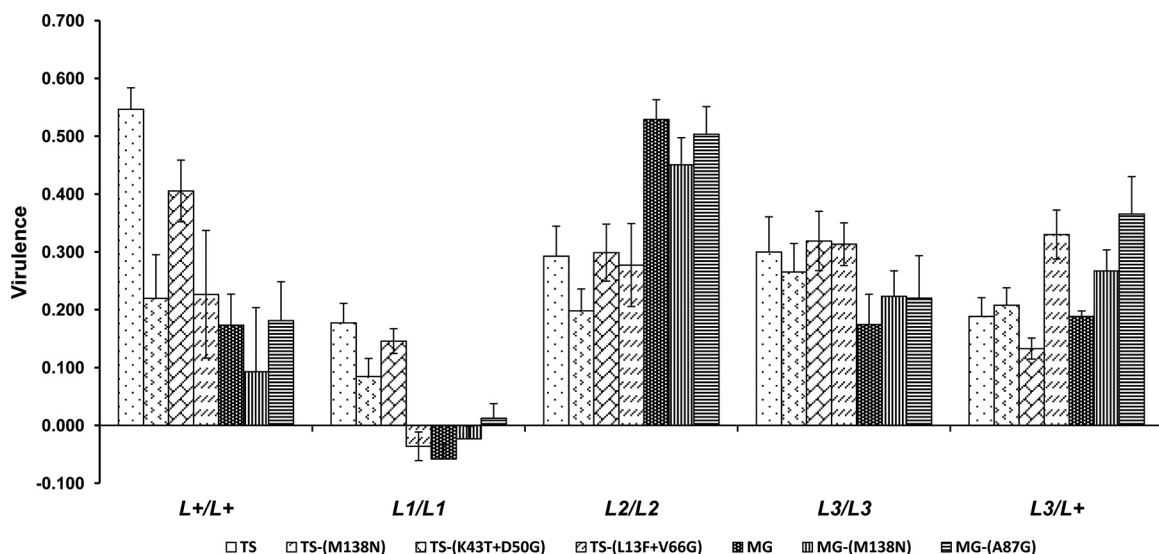


FIG 2 Virulence of seven PMMoV genotypes in single infection in five *Capsicum* host genotypes differing at the L locus. Data are means \pm standard errors from 10 replicated plants.

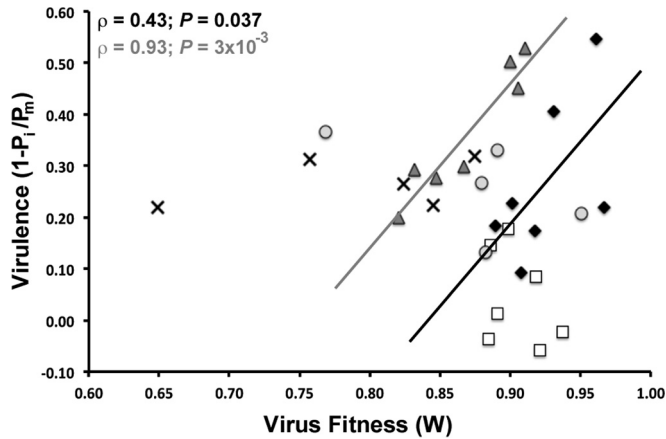


FIG 3 Relationship between fitness and virulence in five susceptible hosts. Hosts are identified by their genotype at the *L* locus as L^+/L^+ (black diamonds), L^1/L^1 (empty squares), L^2/L^2 (gray triangles), L^3/L^+ (crosses), or L^3/L^3 (gray circles). Significant positive correlations between fitness and virulence are shown for L^+/L^+ (black) and L^2/L^2 (gray).

analyses showed that virulence ranked according to host genotype as $L^1/L^1 < L^+/L^+ = L^3/L^+ = L^3/L^3 < L^2/L^2$ ($P \leq 0.001$ for differences). Over hosts the virulence of the three pathotypes ranked as $P_{1,2} = P_{1,2,3,4} > P_{1,2,3}$ ($P = 0.001$). Virus genotypes ranked according to virulence as $TS = TS-(T43K+D50G) > MG-(A87G) = TS-(L13F+G66V) = TS-(M138N) = MG-(M138N) = MG$ ($P \leq 0.012$ for differences). The effect of the interaction of host genotype versus virus genotype on virulence is further shown in Fig. 2. When the virulence of the resistance-breaking genotypes was compared with that of their parental genotypes, it was found that $TS-(M138N)$ and $TS-(L13F+G66V)$ were less virulent than TS in L^+/L^+ and L^1/L^1 plants; all other comparisons were not significant (data not shown).

The fitness of the various virus genotypes was correlated with their virulence only in hosts L^+/L^+ and L^2/L^2 ($\rho > 0.45$, $P = 1 \times 10^{-4}$; Spearman test) (Fig. 3).

Fitness of PMMoV resistance-breaking genotypes in mixed infection of the universal susceptible host genotype L^+/L^+ . The fitness of the virus genotypes above was also analyzed in mixed infections, in which two different genotypes compete for the host resources. For this assay, the universally susceptible host genotype L^+/L^+ was chosen, and all the virus genotypes of the previous assay were included with the exception of $TS-(L13F+G66V)$ as we were unable to develop a specific RT-quantitative PCR (RT-

qPCR) for quantification of its accumulation in mixed infection. On the other hand, the double mutant $MG-(T43K+D50G)$, previously unavailable, was included. In this experiment each virus genotype was assayed in single and in mixed infections so that each genotype of a $P_{1,2,3}$ pathotype competed with its $P_{1,2}$ parental genotype, and the genotype of the $P_{1,2,3,4}$ pathotype competed with both $P_{1,2}$ genotypes and with the $P_{1,2,3}$ genotypes derived from the same parental genotype (Table 3). The assay followed a random block design with 23 virus infection treatments, plus the mock-inoculated controls, with 10 replicated plants per treatment/control. Plants were harvested at 22 dpi, virus RNA was quantified in systemically infected leaves, and fitness was estimated.

Table 3 shows the fitness values of each virus genotype in single and in mixed infection. To determine if the infection type, single or mixed, had an effect on viral fitness according to the virus pathotype, data were analyzed by a GzLM in which infection type and pathotype were considered fixed factors and virus genotype was considered a random factor nested to pathotype in a full factorial model. Infection type, pathotype, and virus genotype nested to pathotype had significant effects on fitness [$\chi^2_{W(1)} = 21.42$, $P = 1 \times 10^{-4}$; $\chi^2_{W(2)} = 36.72$, $P = 1 \times 10^{-4}$, $\chi^2_{W(4)} = 11.92$, $P = 0.018$, respectively], as well as the interaction of pathotype versus infection type [$\chi^2_{W(2)} = 36.60$, $P = 1 \times 10^{-4}$]. LSD analyses showed that fitness of pathotypes $P_{1,2}$ and $P_{1,2,3}$ ($P < 0.007$) but not $P_{1,2,3,4}$ ($P = 0.098$) was higher in single than in mixed infection and that in mixed infection the fitness of the three pathotypes ranked $P_{1,2,3,4} > P_{1,2} > P_{1,2,3}$ ($P \leq 0.005$).

To determine if the resistance-breaking mutations had an effect on the virus fitness in mixed infection, a GzLM analysis was performed in which infection type and virus genotype were considered fixed factors in a full factorial model. Infection type, virus genotype, and their interaction had significant effects on fitness [$\chi^2_{W(1)} = 59.49$, $P = 1 \times 10^{-4}$; $\chi^2_{W(6)} = 47.51$, $P = 1 \times 10^{-4}$; $\chi^2_{W(6)} = 44.00$, $P = 1 \times 10^{-4}$, respectively]. Similar analyses were done considering only the set of genotypes that competed with TS or those that competed with MG . In both cases the interaction of infection type versus genotype was significant ($P \leq 0.050$), and results showed that the fitness of genotypes of then $P_{1,2}$ (TS and MG) or $P_{1,2,3,4}$ [$MG-(A87G)$] pathotype did not depend on the type of infection, while that of genotypes of the $P_{1,2,3}$ pathotype [$TS-(M138N)$, $TS-(T43K+D50G)$, $MG-(M138N)$, and $MG-(T43K+D50G)$] was lower in mixed than in single infection ($P < 0.003$). Also, the fitness of genotypes of the $P_{1,2,3}$ pathotype in mixed infection was lower than that of their $P_{1,2}$ parental genotypes ($P < 0.036$). Last, fitness levels of the $P_{1,2,3}$ pathotype geno-

TABLE 3 Fitness of PMMoV genotypes in single and mixed infections in L^+/L^+ host plants

Genotype	Fitness in single infection ^a	Fitness in mixed infection by pathotype ^a						
		$P_{1,2}$		$P_{1,2,3}$				
		TS	MG	TS-(M138N)	TS-(T43K+D50G)	MG-(M138N)	MG-(T43K+D50G)	$P_{1,2,3,4}$ [MG-(A87G)]
TS	0.96 ± 0.03			0.98 ± 0.98	0.92 ± 0.02			0.93 ± 0.01
MG	0.99 ± 0.02					0.97 ± 0.01	0.97 ± 0.00	0.94 ± 0.02
TS-(M138N)	0.96 ± 0.03	0.84 ± 0.01						
TS-(T43K+D50G)	0.98 ± 0.01	0.84 ± 0.04						
MG-(M138N)	0.99 ± 0.01		0.83 ± 0.01					0.79 ± 0.01
MG-(T43K+D50G)	0.99 ± 0.01		0.90 ± 0.03					0.92 ± 0.01
MG-(A87G)	0.98 ± 0.03	1.00 ± 0.02	0.97 ± 0.01			0.99 ± 0.01	1.02 ± 0.004	

^a Data are average fitness (W) ± standard error for 10 replicated plants.

TABLE 4 Magnitude and sense of fitness differences between mutant and parental genotypes in competition in L^+/L^+ host plants

Parental genotype	Mutant genotype	W_m^a	W_p^b	$W_m - W_p$	P^c
TS	TS-(M138N)	0.845	0.986	-0.141	0.001*
TS	TS-(T43K+D50G)	0.848	0.926	-0.079	0.036*
MG	MG-(M138N)	0.835	0.970	-0.136	<10 ^{-3*}
MG	MG-(T43K+D50G)	0.900	0.980	-0.080	0.013*
MG	MG-(A87G)	0.966	0.945	0.021	0.506

^a W_m is the average fitness of the mutant.

^b W_p is the average fitness of the parental genotype.

^c The asterisk indicates a significant difference according to an LSD analysis.

types MG-(M138N) and MG-(T43K+D50G) were significantly lower in mixed infection with the $P_{1,2,3,4}$ genotype MG-(A87G) than in single infection and were lower than the fitness of MG-(A87G) ($P < 0.002$), while fitness levels did not differ in single infection. Thus, virus fitness depends on the interaction of genotype versus environment (G versus E), considering the type of infection, single or mixed, as the environment.

To quantify the magnitude of these pleiotropic effects, the difference between the fitness of each mutant and that of the parental genotype was computed, and its significance was analyzed by GzLM and LSD. Fitness differences were significant only for the magnitude of the pleiotropic effects of the mutations determining the $P_{1,2,3}$ pathotype (M138N and T43K+D50G), which showed antagonistic pleiotropy in mixed versus single infection (Table 4). The magnitude of the antagonistic pleiotropy was higher for the M138N mutation than for the T43K+D50G double mutation, and, interestingly, the magnitude was independent of the genetic context, TS or MG (Table 4).

Thus, regardless of the relative accumulation in single infection, in mixed infection the genotypes of $P_{1,2,3}$ pathotype were outcompeted by those of the $P_{1,2}$ or $P_{1,2,3,4}$ pathotype, showing that mutations determining the $P_{1,2,3}$ pathotype have a negative effect on virus fitness in the susceptible host. This is not the case for the mutation determining the $P_{1,2,3,4}$ pathotype.

DISCUSSION

It is often considered that RNA viruses have a high potential to adapt to new hosts because their high census numbers and the high error rates of RNA-dependent RNA polymerases will translate into a strong potential to generate host range mutants (1, 5, 44). However, RNA viruses have small, information-compact genomes encoding few multifunctional proteins, which will favor pleiotropic effects of mutations (5, 45). A consequence can be the generation of across-host fitness trade-offs so that mutants that perform well in one host will perform poorly in another one, limiting host range expansion and, more generally, virus emergence (45, 46). It is thought that the major cause of across-host fitness trade-offs in viruses is antagonistic pleiotropy, that is, the negative fitness effect in the original host of mutations that increase fitness in the new one (7), expressed as the interaction of the virus genotype versus the host environment (G versus E) (5, 40). The role of antagonistic pleiotropy in limiting host range expansion has been well documented in viruses infecting bacteria, animals, and plants, often from host adaptation experiments (e.g., 40, 47–54).

A particular case of host range expansion is the overcoming of host resistance in GFG host-pathogen interactions, which compromises the control of crop viral diseases. In GFG systems there is a hierarchy of infectivity alleles in the pathogen and of resistance

alleles in the host so that some pathogen infectivity alleles are intrinsically better than others, conferring the capacity to infect and multiply in a larger set of host genotypes, and, conversely, some host resistance alleles confer the capacity to resist a larger set of pathogen genotypes (14, 55). In GFG systems the pathogen is predicted to evolve by successive steps of resistance breaking until it infects and multiplies in all host genotypes (14). Host-pathogen coevolution under the GFG model has been extensively modeled; models assume that resistance-breaking mutations have fitness costs in the susceptible host (56, 57), thus generating across-host fitness trade-offs. Evidence for resistance-breaking fitness costs derives mostly from analyses of the multiplication of resistance-breaking and wild-type virus genotypes in a susceptible host genotype. Previous reports refer to different plant-virus systems (16, 18, 58–60), including the pepper-PMoV system analyzed here (17).

The interaction of PMMoV genotypes with pepper genotypes carrying different resistance alleles at the L locus is according to the GFG model (30–33). Most PMMoV field isolates are of a $P_{1,2}$ pathotype; i.e., they infect plants of the universally susceptible L^+/L^+ genotype and those carrying resistance alleles L^1 and L^2 , but $P_{1,2,3}$ isolates, which also infect L^3 plants, also occur in pepper crops. Analyses of field PMMoV isolates of $P_{1,2}$ and $P_{1,2,3}$ pathotypes have shown fitness costs associated with L^3 resistance breaking (17). To identify the mechanisms that generate such fitness costs, different mutations in the virus CP reported as determinants of resistance breaking of the L^3 or L^3 and L^4 alleles ($P_{1,2,3}$ or $P_{1,2,3,4}$ pathotype, respectively) (27, 30, 34, 36) were introduced into two PMMoV $P_{1,2}$ isolates by site-directed mutagenesis of infectious cDNA clones. Mutation M138N is present in all characterized $P_{1,2,3}$ isolates from Spain and the Mediterranean basin (17, 30), while mutations T43K+D50G and L13F+V66G have been reported only in the $P_{1,2,3}$ isolates PMMoV-Ij and PMMoV-Is, respectively, from Japan (34, 36). Mutation A87G has been reported as determining the $P_{1,2,3,4}$ pathotype in a PMMoV isolate from Israel, together with mutation L47Q (27). The parental genotype MG has a Q at position 47 of the CP, rendering the introduction of mutation L47Q unnecessary. All mutants had the expected pathotype, confirming that the introduced CP mutations are responsible for the viral pathotype, regardless of the genotype of the $P_{1,2}$ isolate in which they were introduced.

The fitness of the mutants in single infection was compared to that of the parental genotypes in five hosts with different alleles at the L locus (Fig. 1). The virus fitness depended on the host genotype, the virus genotype, and the virus pathotype, but increasing infectivity is not correlated with increasing fitness penalties. This analysis also showed that virus fitness depended on the interaction between virus and host genotypes, i.e., on a genotype per environ-

ment (G versus E) interaction, which is evidence of pleiotropic effects of the resistance-breaking mutations (5, 40). When the sense and magnitude of the pleiotropy, whether negative (i.e., antagonistic) or positive, were analyzed for the various resistance-breaking mutants, they were found to depend on both the specific resistance-breaking mutation and on the host genotype. Most (3/5) resistance-breaking mutants showed antagonistic pleiotropy in the universally susceptible host L^+/L^+ . Interestingly, only 1/5 mutants showed antagonistic pleiotropy in the L^1/L^1 host, and none showed antagonistic pleiotropy in L^2/L^2 ; conversely, 2/5 mutants showed positive pleiotropy in L^1/L^1 , and the only detected pleiotropy in L^2/L^2 was positive. Hence, the negative effects of resistance-breaking mutations on virus fitness are reduced, or inverted, in the susceptible hosts carrying *L* alleles of a higher hierarchy (Table 3). It should be underscored that the genotype at the *L* locus of the assayed hosts is the major determinant of the infectivity of each virus genotype (25–33), but since the assayed hosts were different pepper cultivars or species that will differ in many other genes, host-dependent differences in fitness among virus genotypes cannot be attributed only to their interaction with the *L* alleles. Still, it could be speculated that the broader the *L* allele resistance, the lower the antagonistic pleiotropy effects of resistance-breaking mutations.

Antagonistic pleiotropy has been shown to explain resistance-breaking-associated fitness costs in several plant viruses (16, 17, 58, 59), as shown here for some interactions. Our results also provide evidence of resistance breaking without cost, as occasionally reported (48, 61–64). What is novel in our results and highly significant is the evidence of the host-dependent positive pleiotropy of resistance-breaking mutations, which may be costly or favorable in different susceptible hosts.

Mixed infection of hosts by different virus species or genotypes is frequent in nature (65–68) and may be relevant for virus evolution, including the evolution of resistance breaking. Comparison of virus fitness levels in mixed versus single infection in the susceptible L^+/L^+ host also showed interactions of G versus E, where E represents the type of infection, single or mixed. Thus, resistance-breaking mutations have pleiotropic effects on fitness that depend on the type of infection. Mutations determining a $P_{1,2,3}$ pathotype, including M138N, involve fitness penalties in mixed infection in the L^+/L^+ host, as shown by significant magnitudes of antagonistic pleiotropy (Table 4). Fitness costs due to pleiotropic effects of resistance-breaking mutations were best unraveled under competition with the parental genotype than in single infection, in agreement with reports from field isolates of PMMoV and with other systems (16, 17). Interestingly, the $P_{1,2,3,4}$ -determining A87G mutation had no fitness costs in mixed infection, which may increase the risks of breaking L^4 allele resistance, presently bred into most hybrid cultivars of bell pepper. It is relevant to stress that the interaction of G versus E according to the type of infection would result in trade-offs across infection types. Consequently, the frequency of mixed infections would be a further determinant of the evolution of host range expansion in virus populations, a topic that to our knowledge has not been analyzed previously.

Resistance-breaking mutations could also affect other components of the virus life history, such as virulence. We analyzed the virulence of the parental and the mutant genotypes in the five susceptible hosts in single infection. Plant biomass correlates with both fecundity and survival (37, 69, 70); thus, the effect of infection on biomass is a proxy to virulence. As was the case for fitness,

virulence depended on the interaction of G versus E, revealing a new pleiotropic effect of resistance-breaking mutations. Pleiotropic effects on virulence were mutation specific and were unrelated to those on fitness, and, overall, virulence and fitness did not correlate, as is often the case for plant viruses (71, 72). It is noteworthy that although pathotype has a significant effect on virulence, increased infectivity does not necessarily translate into virulence changes. This is an interesting result as the relationship between infectivity and virulence has rarely been analyzed either experimentally or theoretically (but see reference 73). Our data are consistent with the only other analysis we know of, in which it was shown that rice yellow mottle virus genotypes differing in infectivity on the *Rymv-2* resistance allele did not differ in virulence (74); that is, virulence and infectivity were not linked. Virulence may also be an important determinant of resistance-breaking evolution in the pepper-PMMoV system because growers eliminate infected plants when detected (22), and detection will be easier in plants infected by more virulent virus genotypes.

The present results thus significantly contribute to understanding the complexity of the mechanisms that determine the evolution of resistance breaking and host range expansion in RNA viruses. One novel result of this work is that pleiotropic effects on virus multiplication of host range mutations depend on the specific mutation and on the specific susceptible host. Host range mutations are not always pleiotropic on fitness, and when they are, pleiotropy can be positive or negative, favoring or hindering, respectively, host range expansion. Thus, the fate of host range mutations in the virus population will depend on not only the frequency of resistant versus susceptible hosts genotypes, as often considered (75), but also the frequency of the different susceptible host genotypes. A second novel result is that the pleiotropic effects on the virus fitness of host range mutations differ in single and in mixed infections so that trade-offs across infection types (single or mixed) are expected to occur, similarly to across-host effects. Hence, the evolution of host range expansion will also be determined by the frequency and type of mixed infections. Last, pleiotropic effects of host range mutations on other virus life history traits, such as survival (39) or virulence (this work), may also affect host range expansion. All of these factors should be considered in modeling the evolution of host range expansion and, specifically, the evolution of resistance breaking in crops.

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