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Reversion of a resistance-breaking mutation shows reversion costs and high virus diversity at necrotic local lesions

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Abstract

An instance of host range evolution relevant to plant virus disease control is resistance breaking. Resistance breaking can be hindered by across-host fitness trade-offs generated by negative effects of resistance-breaking mutations on the virus fitness in susceptible hosts. Different mutations in pepper mild mottle virus (PMMoV) coat protein result in the breaking in pepper plants of the resistance determined by the L^3 resistance allele. Of these, mutation M138N is widespread in PMMoV populations, despite associated fitness penalties in within-host multiplication and survival. The stability of mutation M138N was analysed by serial passaging in L^3 resistant plants. Appearance on passaging of necrotic local lesions (NLL), indicating an effective L^3 resistance, showed reversion to nonresistance-breaking phenotypes was common. Most revertant genotypes had the mutation N138K, which affects the properties of the virus particle, introducing a penalty of reversion. Hence, the costs of reversion may determine the evolution of resistance-breaking in addition to resistance-breaking costs. The genetic diversity of the virus population in NLL was much higher than in systemically infected tissues, and included mutations reported to break L^3 resistance other than M138N. Infectivity assays on pepper genotypes with different *L* alleles showed high phenotypic diversity in respect to *L* alleles in NLL, including phenotypes not reported in nature. Thus, high diversity at NLL may potentiate the appearance of genotypes that enable the colonization of new host genotypes or species. Collectively, the results of this study contribute to better understanding the evolutionary dynamics of resistance breaking and host-range expansions.

KEYWORDS
across-host trade-offs, costs of virulence, necrotic local lesions, pepper, tobamovirus, virus diversity

1 | INTRODUCTION

Breeding resistance into crop cultivars is a highly effective, target-specific, and environmentally friendly strategy for the control of viral diseases. However, deploying genetic resistance is not a sustainable strategy: most often, the protection conferred by resistance is not durable, as the appearance and increase in frequency in virus populations of resistance-breaking genotypes eventually renders

resistance inefficient (Brown, 2015; Fraile & García-Arenal, 2010; García-Arenal & McDonald, 2003). Efforts to manage resistance durability include understanding the evolution of resistance breaking, which is a major goal of plant pathology.

The evolution of plant–virus interactions can often be explained by the gene-for-gene (GFG) model, in which host proteins encoded in resistance genes (*R*) recognize viral proteins, which triggers a defence reaction that limits virus multiplication at the infection site (Moffett, 2016; Palukaitis & Yoon, 2020; de Ronde et al., 2014). The cessation of virus multiplication is shown by the development of necrotic local lesions (NLL) at infection sites. If mutations occur in the viral protein that impair its recognition by R proteins, resistance is not triggered and infection occurs, called resistance breaking. Resistance breaking confers on the virus the capacity to infect a larger set of host genotypes (Agrawal & Lively, 2002), thus the capacity for host-range expansion. If resistance-breaking mutations have negative pleiotropic effects on the virus protein function(s), known as resistance-breaking costs, across-host fitness trade-offs may be generated, and the resistance-breaking genotypes will be less fit than the nonresistance-breaking ones in susceptible hosts. Because resistance-breaking costs may determine virus evolution and resistance durability, much effort has been invested in their detection and in understanding the underlying mechanisms (García-Arenal & Fraile, 2013). There is abundant evidence of resistance-breaking costs in plant viruses, mostly showing a negative effect on within-host multiplication (e.g., Ayme et al., 2006; Fraile et al., 2011; Ishibashi et al., 2012; Janzac et al., 2010; Khatabi et al., 2013; Montarry et al., 2012; Poulicard et al., 2010, 2012), but also on other traits of the virus life history such as virulence, transmission, competition in coinfection, or survival in the environment (Bera et al., 2017; Moreno-Pérez et al., 2016).

Our group has contributed to understanding the role of resistance-breaking costs in virus evolution, and the mechanisms that generate them, by studying the interaction between virus species and genotypes in the genus *Tobamovirus* and pepper (*Capsicum annuum*) (Bera et al., 2017; Fraile et al., 2011, 2014; Moreno-Pérez et al., 2016). The interactions of tobamoviruses with pepper are according to the GFG model, being determined by the interaction between proteins encoded by alleles at the pepper resistance locus *L* (Boukema, 1980, 1984) and the virus coat protein (CP) (Berzal-Herranz et al., 1995; de la Cruz et al., 1997; Gilardi et al., 2004; Matsumoto et al., 2008). Recognition of the CP by *L* alleles elicits a hypersensitive resistance response, expressed by the development of NLL. According to the capacity to elicit resistance, or to infect pepper genotypes carrying the different resistance *L* alleles, tobamovirus species and genotypes are classified into pathotypes. 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/– plants are resistant only to pathotype P_0, L^2/– plants are resistant to pathotypes P_0 and P_1, L^3/– plants are resistant to pathotypes P_0, P_1, and $P_{1,2}$, and so on. Pathotype $P_{1,2,3,4}$ infects all known *L*-gene host genotypes. Isolates with $P_{1,2}$, $P_{1,2,3}$, and $P_{1,2,3,4}$ pathotypes are all genotypes of pepper mild mottle virus (PMMoV) (Antignus et al., 2008; Rast, 1988) (Table S1). Resistance-breaking mutations have appeared after deploying different *L* resistance alleles in pepper cultivars. The evolutionary dynamics of resistance breaking in tobamoviruses as a response to resistance deployment in pepper has been studied in detail in south-east Spain (Fraile et al., 2011). Different single and double mutations in the PMMoV CP have been reported as determinants of the conversion of pathotype $P_{1,2}$ to pathotypes $P_{1,2,3}$ and $P_{1,2,3,4}$ (Antignus et al., 2008; Berzal-Herranz et al., 1995; Genda et al., 2007; Hamada et al., 2007, 2008; Tsuda et al., 1998) (Table S1). It has been shown that these resistance-breaking mutations may have pleiotropic effects on different virus traits, including infectivity, within-host multiplication, virulence, competitive capacity, and survival in the environment (Bera et al., 2017; Moreno-Pérez et al., 2016). The sense and magnitude of pleiotropy, however, depends on the specific mutation, the genotype of the susceptible host, and on environmental conditions such as coinfection versus single infections (Bera et al., 2017; Moreno-Pérez et al., 2016). Most PMMoV isolates from pepper in the field are of a $P_{1,2}$ pathotype (Fraile et al., 2011; Moury & Verdin, 2012), in agreement with the lower within-host multiplication and survival of $P_{1,2,3}$ isolates (Fraile et al., 2011, 2014). CP mutants that break L^3 or L^4 resistance have always appeared in pepper cultivars heterozygous at the *L* locus, have been isolated only a few times, and have restricted geographical ranges, except mutant M138N, conferring a $P_{1,2,3}$ pathotype, which is widespread in the Mediterranean basin (Moury & Verdin, 2012). The effects of mutation M138N on virus fitness are complex. Mutation M138N increased virus infectivity (Bera et al., 2017) and, in a single infection, either increased the within-host fitness respective to the $P_{1,2}$ parental genotype or had no effect on this trait, depending on the genotype of the susceptible host; however, M138N mutants were outcompeted by the $P_{1,2}$ parental in coinfected plants (Moreno-Pérez et al., 2016). On the other hand, mutation M138N resulted in significantly less stable virus particles, affecting virus survival in the environment (Bera et al., 2017). Despite the complexity of the pleiotropic effects of mutation M138N on the virus fitness components, data on its frequency in virus populations suggest high fitness penalties under field conditions (Fraile et al., 2011).

The positive and negative pleiotropic effects of resistance-breaking mutation M138N in different fitness components makes predicting its fate in the virus population and, by extension, modelling host range expansions difficult. Thus, in this study we analyse the genetic stability of this mutation, testing the hypothesis that revertants to a $P_{1,2}$ pathotype could be selected as a result of the joint effects of its phenotypic consequences on different traits. Results of experimental evolution show that reversion to a $P_{1,2}$ pathotype occurs, but most revertants differ from the parental $P_{1,2}$ genotype and have associated fitness penalties. In addition, monitoring the fate of revertant genotypes yielded data on the genetic structure of the virus population in systemically infected host tissues and in NLL, which suggests that NLL may be a source of virus diversity for colonization of new host genotypes.

2 | RESULTS

2.1 | Stability of the M138N mutation

Host-specific epistatic interactions of resistance-breaking mutations may hinder the evolution of resistance breaking (Bedhomme et al., 2015). To test if mutation M138N resulted in selection on other genome positions due to epistasis, PMMoV TS-M138N (pathotype $P_{1,2,3}$; Moreno-Pérez et al., 2016) was passaged 10 times in the susceptible host *Capsicum annuum* 'Dulce Italiano' (L^+/L^+) and in the resistant host *C. annuum* 'Ferrari' (L^3/L^+). Five replicated lines were passaged in parallel, with two replicate plants (A and B) per line. For passage 1, 400 ng of virions was inoculated in 20-day-old plants and at 22 days postinoculation (dpi) c.2.5 g of leaf tissue was harvested from the upper two systemically infected leaves, which were ground in inoculation buffer to initiate passage 2 by inoculating the two first wholly expanded leaves of new plants. This process was repeated at 22-day intervals to complete 10 passages. All the inoculated plants of both hosts were systemically infected and showed symptoms of severe mosaic and leaf distortion. The nucleotide sequence of the complete genome was determined by Sanger sequencing for the initial inoculum and for each of the five lines at passage 10 in RNA extracts from pooled systemically infected leaves of plant A and B of each line. No mutation relative to the original genome sequence was detected in any of the five passaged lines in Dulce Italiano. In Ferrari three nucleotide substitutions were detected in different lines: in line 1 nonsynonymous substitution G3490A results in the amino acid change V1141I in the 183 K protein, in line 2 synonymous C633U substitution in the 126 K protein gene, and in line 4 synonymous U5981G substitution in the CP gene. These results do not support the hypothesis of selection on any genomic region due to epistatic effects of mutation M138N.

In the lines passaged in Ferrari, NLL were observed in inoculated and systemically infected leaves from passage 5 onwards (Table 1), and more frequently in inoculated than in systemically infected leaves ($t = 2.64$, $p = 0.010$). The number of NLL did not increase with time, if values from plant B of line 7 are excluded. NLL suggest the appearance of revertants to pathotype $P_{1,2}$ or to a lower pathotype after passage 5. None of the mutations detected by Sanger sequencing of the passaged genomes are candidates to explain the induction of NLL in Ferrari plants with L^3 resistance.

When NLL from the passage experiment were ground in inoculation buffer and used to inoculate three plants of *C. chinense* 'PI 159236' (L^3/L^3) or *C. annuum* 'Ferrari' (L^3/L^+), the symptoms were reproduced: NLL were induced in all the inoculated leaves, and all the plants were infected systemically and developed a severe mosaic (Figure 1a,b). Also, NLL were used to inoculate three plants of *C. annuum* 'Yolo Wonder' (L^1/L^1) or *C. frutescens* 'Tabasco' (L^2/L^2), which all showed mild systemic symptoms (Figure 1d,f), while two plants of *C. frutescens* 'Tabasco' (L^2/L^2) and one plant of *C. annuum* 'Yolo Wonder' (L^1/L^1) developed NLL in the inoculated leaves at low numbers, one or two per leaf (Figure 1c,e). These results indicate the virus population at NLL from the passage experiment is heterogeneous, including genotypes of $P_{1,2,3}$ and lower pathotypes.

TABLE 1 Number of necrotic local lesions in inoculated and systemically infected leaves of *C. annuum* 'Ferrari' ($L^3/-$)

	Line[b]				
Passage[a]	1[b]	2	3	4	5
5A	2/0				1/0
5B	1/0				
6A	2/0	1/0	1/0	2/1	3/2
6B	1/0	1/0		1/0	
7A	2/1		1/0	4/0	2/0
7B	48/14			6/6	2/0
8A	3/0	1/0			1/0
8B	1/2	1/0			
9A	2/1	1/0	1/0	4/1	
9B		1/0	0/0		
10A	2/0	6/3	4 /0	0/3	1/1
10B	2/0	2/1	0/3	1/2	2/1

[a]A and B indicate two infected plants per line for passages 5 to 10 in a serial passage experiment.

[b]*i*/*j* indicate the number of necrotic local lesions (NLL) in inoculated and systemically infected leaves, respectively. Blank cells indicate no NLL were observed.

2.2 | High-throughput sequencing of the CP gene reveals mutations at sites that determine a $P_{1,2,3}$ genotype

As it could be that the NLL observed from passage 5 onwards were due to a minor component of the viral population, the genetic structure at the CP gene in NLL lesions was analysed by Illumina deep sequencing. For this, disks of 4 mm in diameter were cut that included NLL in leaves of one plant (either plant A or plant B) of each of the five lines of passage 7, except for line 2, which came from passage 8. As controls, 4-mm diameter disks were cut from systemically infected leaves of the same plants that did not show NLL. From these disks total RNA was extracted, the CP gene was reverse transcription (RT)-PCR amplified and amplicons were deep sequenced.

Table 2 shows that across the 10 high-throughput sequenced libraries nonsynonymous nucleotide substitutions occurred in 73 of the 473 nucleotide (nt) positions of the CP gene. Table 2 shows the nature of the substitution in respect to the bases in the parental virus genome TS-M138N, the resulting amino acid change, and the relative frequency of each mutation for each library. No nucleotide polymorphisms were found in the libraries from systemically infected tissues of lines 2 and 3, which are not shown in Table 2. The genetic diversity of each population was estimated as mean observed heterozygosity (Table 3). The frequency of each allele at each polymorphic position was calculated (see procedures below)

FIGURE 1 Symptoms induced by necrotic lesion transfer in different pepper species and genotypes. Necrotic local lesions that appeared in *Capsicum annuum* 'Ferrari' (L^3/L^+) during a serial passage experiment were used to inoculate *C. chinense* 'PI 159236' (L^3/L^3) (a, b), *C. frutescens* 'Tabasco' (L^2/L^2) (c, d) and *C. annuum* 'Yolo Wonder' (L^1/L^1) (e, f). Necrotic local lesions in inoculated leaves are shown in (a), (c), and (e), and systemic infection symptoms are shown in (b), (d), and (f)

and used to compute per-site heterozygosity (Cuevas et al., 2015; Li, 1997) for each population. The mean observed heterozygosity (Li, 1997) was calculated with a genome length (*L*) of 420 nt, the difference between the minimum and maximum positions in the CP where variants were called. The value of *L* is typically the length of the genome, and the CP gene region of PMMoV is approximately 470 nt. Data show that the virus populations at NLL were on average five times as diverse as those in systemically infected tissues, this difference being significant (Wilcoxon rank sum test $W = 23$, $p = 0.036$).

Among the observed mutations, some could affect the virus pathotype, for instance mutations L13F and G66V, which occurred in NLL of passage lines 1, 2, 3, and 4. It has been reported that when these two mutations occur together they determine a $P_{1,2,3}$ pathotype (Moreno-Pérez et al., 2016), which in line 2 would occur at the non-negligible frequency of 12.64%, as estimated from the frequency of each mutation. The N at position 138 of the parental TS-M138N virus is encoded by an AAU codon. Two mutations affected the amino acid at position 138, the substitutions A416T and T417G, which by themselves result in the amino acid changes N138I and N138K, respectively. An N at position 138 of the CP is necessary and sufficient to overcome L^3 resistance in the parental virus TS-M138N (Berzal-Herranz et al., 1995; Moreno-Pérez et al., 2016). The mutation A416T was found only in NLL of line 2, while the mutation T417G occurred in the virus populations at NLL of all five passage lines and in systemically infected tissues of lines 1 and 5. Read analyses showed that in line 2, mutations A416T and T417G occurred together with a frequency of 93.75%, which would lead to the reversion N138M. In addition, mutations N138I and N138K are candidate determinants for reversion to the $P_{1,2}$ pathotype and induction of

TABLE 2 Mutations detected in the coat protein gene on PMMoV TS-M138N after passage in *Capsicum annumm* 'Ferrari' (L^3/L^+) and their relative frequency in the virus population[a]

Mutation			Frequency (%)							
Pos[b]		AA[c]	NLL_1	Sys_1	NLL_2	NLL_3	NLL_4	Sys_4	NLL_5	Sys_5
42	A→T	L14F	3.63	–	35.93	1.42	2.26	–	–	–
152	A→G	D51G	1.41	–	11.47	–	–	–	–	–
200	G→T	G67V	3.88	–	35.21	1.38	2.30	–	–	–
269	C→A	T90N	–	–	–	–	7.33	–	14.69	–
276	C→A	N92K	–	–	–	–	6.28	–	–	8.05
281	T→G	I94R	–	–	–	–	–	–	17.92	8.28
284	T→G	I95R	–	–	–	–	–	–	17.82	–
290	T→G	V97G	–	–	–	–	10.62	–	12.21	–
291	T→G	V97V	–	9.46	–	–	8.97	–	13.02	–
297	T→G	N99K	9.26	–	–	–	–	–	–	–
298	C→A	P100D	–	–	–	–	5.67	–	19.05	7.82
299	C→A	P100Q	7.94	–	–	–	5.74	–	17.68	8.00
301	C→A	Q101K	–	–	–	9.96	6.00	–	13.90	–
306	T→G	N102K	–		–	9.03	–		–	–
307	C→A	P103T	–	–	–	11.63	–	–	16.67	–
308	C→A	P103H	–	–	–	–	–	–	16.11	–
309	T→G	P103P	8.88	–	–	13.37	–	–	–	–
311	C→A	T104K	–	–	–	9.50	–	–	13.1	–
314	C→A	T105N	–	–	–	9.22	5.53	–	12.28	–
315	T→G	T105T	–	16.91	–	–	–	–	15.29	9.74
317	C→A	A106D	–	–	–	9.65	6.67	–	13.22	–
318	C→A	A106A	–	–	–	10.19	6.15	–	12.78	–
323	C→A	T108K	–	–	–	9.30	–	–	11.86	–
325	C→A	L109I	–	–	–	7.76	–	–	10.84	–
326	T→G	L109R	–	8.75	–	–	7.91	–	10.06	–
327	T→G	L109L	11.11	–	–	16.52	–	–	–	–
330	T→G	D110E	16.85	–	–	21.65	–	–	7.78	–
332	C→A	A111A	–	–	–	9.01	6.72	–	14.36	8.19
335	C→A	T112K	–	–	–	8.73	5.81	–	14.36	–
340	C→A	R114R	–	–	–	9.09	–	–	10.93	–
344	T→G	V115G	21.21	17.81	–	29.36	19.12	10.2	12.78	10.37
348	C→A	D116D	–	–	43.44	7.83	6.33	–	11.86	–
351	T→G	D117E	11.18	9.72	–	11.21	11.11	–	–	–
353	C→A	A118E	–	–	–	8.97	6.28	–	11.24	–
356	C→A	T119K	–	–	–	8.22	5.71	–	10.87	–
359	T→G	V120G	8.48	–	–	–	7.2	–	–	–
362	C→A	A121D	–	–	–	7.41	6.61	–	11.86	–
363	C→A	A121A	–	–	–	8.68	–	–	9.78	–
365	T→G	I122S	–	–	–	–	5.56	–	–	–
371	C→A	A124D	–	–	–	–	–	–	8.25	–
372	C→A	A124A	–	–	–	6.82	–	–	9.69	–
377	T→G	I126R	8.24	–	–	15.38	–	–	7.88	–
381	T→G	S127R	–	–	–	7.86	–	–	–	–

TABLE 2 (Continued)

Mutation			Frequency (%)							
Pos[b]		AA[c]	NLL_1	Sys_1	NLL_2	NLL_3	NLL_4	Sys_4	NLL_5	Sys_5
384	C→A	N128K	–	–	–	6.67	–	–	14.66	–
385	C→A	L129I	–	–	–	6.19	–	–	10.71	–
386	T→G	L129R	6.01	–	–	12.89	6.01	–	10.33	–
389	T→G	M130R	9.39	–	–	16.30	–	–	8.16	–
393	T→G	N131K	14.69	10.06	–	26.24	12.25	–	12.44	10.44
397	T→G	L133V	18.44	16.77	11.45	27.88	16.28	9.55	10.61	12.22
398	T→G	L133*	14.69	–	–	23.64	7.66	–	13.76	–
401	T→G	V134G	14.67	11.39	11.28	20.26	13.26	9.36	10.4	11.22
402	T→G	V134V	8.33	–	–	16.37	–	–	–	–
405	T→G	R135R	11.89	13.94	–	14.54	12.82	8.19	7.80	9.19
410	C→A	T137K	–	–	–	–	–	–	6.83	–
416	A→T	N139I	–	–	97.32	–	–	–	–	–
417	T→G	N139K	20.74	9.43	98.40	26.42	13.78	–	9.38	8.79
418	T→G	Y140D	14.06	9.58	–	18.26	10.04	–	9.13	9.33
423	T→G	N141K	15.87	14.81	–	26.01	13.62	–	8.96	9.44
424	C→A	Q142K	–	–	–	8.89	–	–	9.71	–
428	C→A	A143D j	–	–	–	20.11	–	–	11.23	–
429	T→G	A143A	14.86	–	–	14.52	6.87	10.13	7.73	–
430	C→A	L144M	–	–	–		–	–	12.43	–
431	T→G	L144R	9.55	–	–	13.37	–	–	–	–
433	T→G	F145V k	17.88	10.42	–	24.74	10.25	12.66	10.16	–
434	T→G	F145C k	13.74	–	–	15.79	6.75	11.04	8.74	–
435	C→A	F145G l	–	–	–	8.15	–	–	14.21	–
441	C→A	S147R	–	–	–	10.23	–	–	10.80	–
443	C→A	A148E	11.9	–	–	12.64	6.31	11.81	18.82	–
447	T→G	S149R	16.36	–	–	28.82	6.91	–	11.88	–
452	T→G	L151R	13.99	12.40	–	27.10	8.82	–	–	–
457	T→G	W153G	–	–	–	11.63	–	–	–	–
462	T→G	A154A	15.27	–	–	21.67	–	–	–	–

[a]Populations correspond to necrotic local lesions (NLL) or systemically infected tissues (Sys) of passage lines 1 to 5. No polymorphisms were detected in systemically infected tissues of lines 2 and 3, which are not shown in the table.

[b]Nucleotide position of the coat protein gene and nucleotide substitution respective to the parental virus sequence are numbered as in PMMoV-TS, accession no. NC_003630.

[c]Amino acid change with indication of amino acid position in the coat protein respective to the parental virus sequence. Note than amino acid numbering considers as position 1 the N-terminal M residue that is not present in the mature virion CP. In column AA, * indicates a stop codon if the indicated mutations at nucleotide positions 397 and 398 occur together; j indicates an A143E change if mutations at nucleotide positions 428 and 429 occur together; k indicates an F145G change if two or three of mutations at nucleotide positions 433, 434 and 435 occur together.

NLL, but mutation N138K would better explain the distribution of NLL that appeared in all lines during the serial passage experiment in *C. annuum* 'Ferrari' plants.

2.3 | Characterization of mutant N138K

The mutation N138K was introduced by site-directed mutagenesis in pTS-M138N. Capped transcripts of the resulting clone pTS-M138K were infectious, inducing NLL in *Nicotiana tabacum* 'Xanthi-nc' plants and a mild mosaic in *C. annuum* 'Dulce Italiano' plants (Figure 2a). To determine its pathotype, the mutant was also inoculated in *C. annuum* 'Yolo Wonder' (L^1/L^1), *C. frutescens* 'Tabasco' (L^2/L^2), *C. chinense* 'PI 159236' (L^3/L^3), *C. annuum* 'Ferrari' (L^3/L^+), and *C. chacoense* 'PI 260429' (L^4/L^4). Inoculations resulted in the development of mild (in *C. annuum* 'Yolo Wonder') or severe (in *C. frutescens* 'Tabasco', Figure 2b) systemic mosaics in plants of genotypes L^1/L^1 and L^2/L^2. When sap from the upper leaves of these plants was used to inoculate fully expanded leaves of *N. tabacum* 'Xanthi-nc', abundant NLL developed in 3–4 days (Figure 2c), which indicated

systemic infection of the pepper plants. On the contrary, inoculation of *C. chinense* 'PI 159236' (L^3/L^3), *C. annuum* 'Ferrari' (L^3/L^+) and *C. chacoense* 'PI 260429' (L^4/L^4) resulted in the development of NLL in the inoculated leaves (Figure 2d) and not in systemic infection. These results show that TS-M138K is of a $P_{1,2}$ pathotype.

For particle purification, viruses were multiplied in plants of *Nicotiana clevelandii* that were inoculated with RNA transcripts from plasmid pTS-M138K. Particles of PMMoV TS-M138N were purified in parallel after multiplication in the same host. Purification of PMMoV TS-M138K particles was inefficient by the standard procedure used for PMMoV and other tobamoviruses, in which plant tissues are homogenized in 40 mM EDTA, 36 mM NaOH, pH 7.0, 0.1% mercaptoethanol (Bruening et al., 1976). Monitoring the presence of encapsidated viral RNA along the purification procedure showed that at odds with TS-M138N, most encapsidated viral RNA was found in the first low-speed centrifugation pellet, indicating particle aggregation and sedimentation with plant tissue debris (Figure 3). Assay of other procedures of particle purification showed efficient purification of TS-M138K when plant tissues were homogenized in 0.5 M Na_2HPO_4 pH 7.0, 0.1% mercaptoethanol. Using this procedure virion RNA was weakly detected in the first low-speed centrifugation pellet, at odds with that of TS-M138N (Figure 3) that, as most tobamoviruses, are not efficiently purified by this procedure (Bruening et al., 1976). The yield of particles from *N. clevelandii* leaves after purification with

TABLE 3 Genetic diversity[a] at the coat protein gene in virus populations in necrotic local lesions (NLL) and systemically infected tissues (Sys) in different passaged lines

Line	NLL	Sys
1	0.015	0.007
2	0.005	0.000
3	0.029	0.000
4	0.013	0.004
5	0.027	0.006
Mean	0.018	0.003
SD	0.010	0.003

[a]Genetic diversity quantified as mean observed heterozygosity.

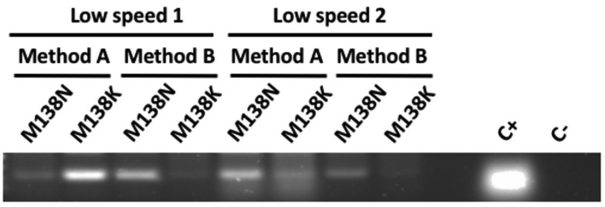

FIGURE 3 Monitoring of encapsidated virus RNA along the process of particle purification. Virus particles of either TS-M138N (M138N) or TS-M138K (M138K) were purified from systemically infected leaves of *Nicotiana clevelandii* by tissue homogenization in either 40 mM EDTA, 36 mM NaOH, 0.1% mercaptoethanol (method A) or 0.5 M Na_2HPO_4 pH 7.0, 0.1% mercaptoethanol (method B). Encapsidated virus RNA was detected by reverse transcription (RT)-PCR in either the first (low speed 1) or the second (low speed 2) low-speed centrifugation pellet. C+ and C− indicate RT-PCR on virion RNA of TS-M138N or water, respectively

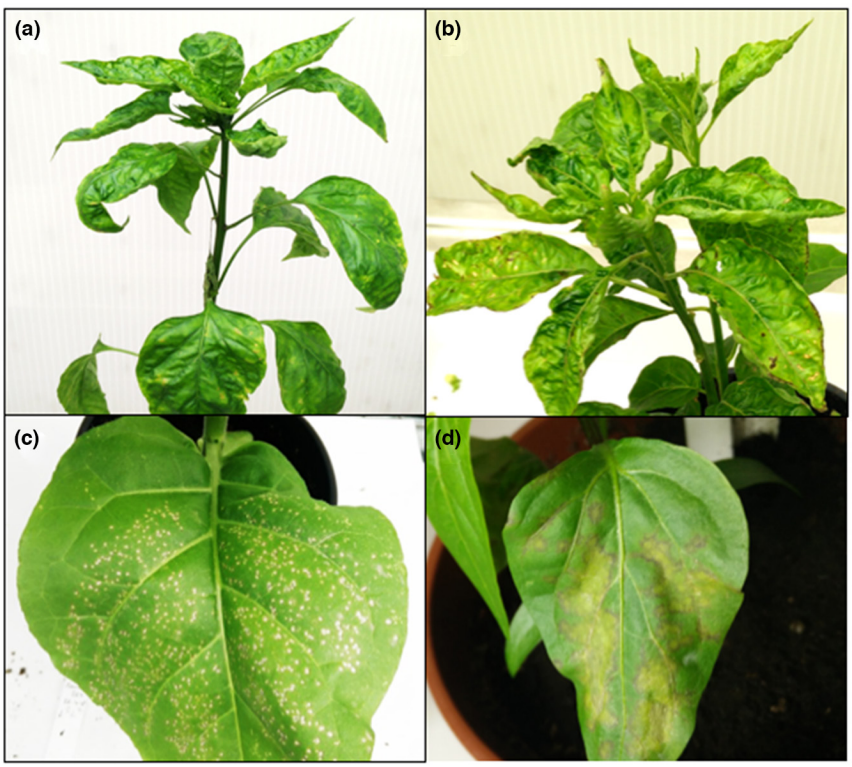

FIGURE 2 Pathotype characterization of PMMoV TS-M138K. PMMoV TS-M138K infection induced systemic mild mosaic in *Capsicum annuum* 'Dulce Italiano' plants (L^+/L^+) (a) or systemic severe mosaic in *C. frutescens* 'Tabasco' (L^2/L^2) plants (b). Inoculation of *Nicotiana tabacum* 'Xanthi-nc' leaves with sap from upper noninoculated leaves of *C. frutescens* 'Tabasco' plants induced the appearance of abundant necrotic local lesions (c). Inoculation of *C. chacoense* 'PI 260429' (L^4/L^4) plants resulted in the development of necrotic local lesions in the inoculated leaves (d)

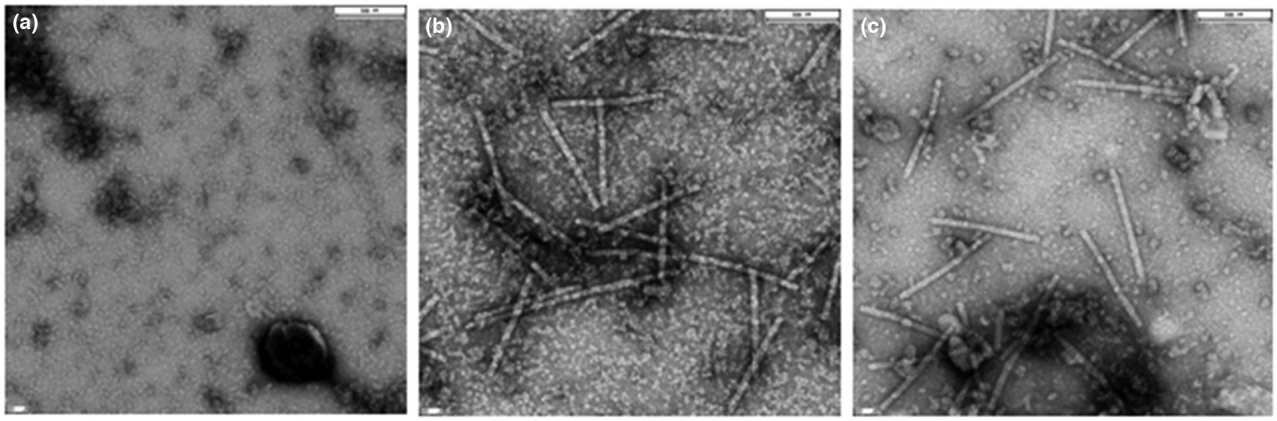

FIGURE 4 Electron micrography of virus particles. Virus particles were visualized after uranyl acetate staining of clarified homogenates of upper noninoculated leaves of mock-inoculated plants (a) or of plants infected by TS-M138N (b) or by TS-M138K (c)

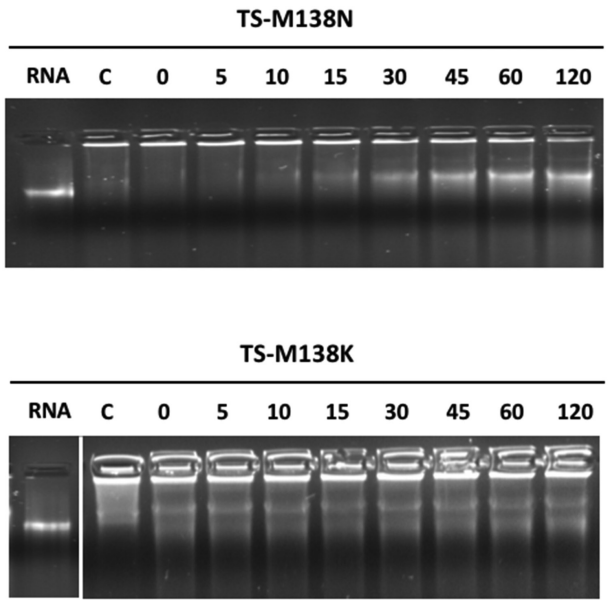

FIGURE 5 Kinetics of virus particle disassembly. Particles of either TS-M138N (upper panel) or TS-M138k (lower panel) were incubated in 0.1 M Tris-HCl pH 8.75 for the indicated times (min). RNA, electrophoretic mobility of genomic RNA; C, untreated purified virion. Note that nonencapsidated viral RNA is detected in particle preparations before incubation

the optimal method was about half for TS-M138K compared to TS-M138N (0.42 vs. 0.80 mg/g fresh leaf). Differences in the optimal procedure for particle purification suggest significant differences between TS-M138N and TS-M138K particles. However, electron microscopy of the leaf sap of infected plants showed no differences between the particles of both virus genotypes, which had the typical structure and dimensions of tobamovirus particles (Figure 4). The stability of TS-M138K and TS-M138N particles was compared by monitoring the kinetics of disassembly at basic pH. Figure 5 shows that disassembly of TS-M138N particles was evident after 10 min and was complete after 45 min at pH 8.75, while TS-M138K particles did not disassemble after 120 min, demonstrating their higher stability. Note also that TS-M138K particles hardly entered the agarose gel, suggesting particle aggregation in Tris-HCl pH 8.75 and/or the electrophoresis buffer, Tris-borate-EDTA pH 8.4.

3 | DISCUSSION

The acquisition of new hosts provides a virus with more opportunities for transmission and survival, thus affecting its ecology and epidemiology, and the effectiveness of strategies for its control. Host range expansion may be hindered by across-host fitness trade-offs so that increasing the virus fitness in a new host will decrease its fitness in the original one (Elena et al., 2014; McLeish et al., 2018). Across-host fitness trade-offs due to the pleiotropic effects of host range mutations have been widely documented for RNA viruses, including plant viruses (Bedhomme et al., 2015; Elena & Sanjuán, 2007). A case of host range expansion much studied by plant virologists is the capacity to infect previously resistant crop genotypes, that is, resistance breaking. Evidence for the costs of resistance breaking has been reported for various plant–virus systems (Goulden et al., 1993; Ishibashi et al., 2012; Janzac et al., 2010; Jenner et al., 2002; Wang & Hajimorad, 2016), including the pepper–PMMoV system here analysed (Bera et al., 2017; Fraile et al., 2011, 2014; Moreno-Pérez et al., 2016).

Previous studies had shown that CP mutation M138N in PMMoV, which determines a $P_{1,2,3}$ pathotype, had no negative effects on virus multiplication in single infection of susceptible hosts (Moreno-Pérez et al., 2016), but had negative effects on the virus competitive ability with the $P_{1,2}$ parental and on survival in the environment (Bera et al., 2017; Moreno-Pérez et al., 2016). To better understand the widespread geographical occurrence of mutation M138N and its persistence over time in PMMoV populations (Fraile et al., 2011; Moury & Verdin, 2012), we analyse here other mechanisms that may result in, or decrease, costs. The complete nucleotide sequences of the genomes of PMMoV TS-M138N serially passaged in a susceptible or a resistant host suggest that mutation M138N does not

establish strong epistatic interactions with other sites in the PMMoV genome. This result is relevant, as epistatic interactions among host range mutations and other loci are a major cause of across host fitness trade-offs, in addition to antagonistic pleiotropy (Bedhomme et al., 2015).

The serial passage experiment in the resistant host *C. annuum* 'Ferrari' (L^3/L^+), allowed detection of revertants of a lower pathotype by the appearance of NLL (Table 1). The number of NLL did not increase over passaging, as expected from the inability of the virus genotype(s) inducing them to multiply and colonize systemically plants with L^3 resistance. Neither did the number of NLL decrease on passaging, which suggests a constant rate of reversion. The higher frequency of NLL in inoculated than in systemically infected leaves can be explained by the higher probability of a revertant genotype to establish an infection in the inoculated leaf, where infection foci occur at discrete sites (García-Arenal & Fraile, 2011), thus decreasing competition with M138N, than in systemically infected leaves. These results indicate that reversion of TS-M138N to a lower pathotype occurs and is common, which suggest costs of resistance breaking additional to those identified so far. When NLL from the serial passage experiment were used to inoculate *C. annuum* 'Yolo Wonder' (L^1/L^1), *C. frutescens* 'Tabasco' (L^2/L^2), *C. chinense* 'PI 159236' (L^3/L^3) or *C. annuum* 'Ferrari' (L^3/L^+), the appearance of a systemic mosaic in all hosts showed the presence of genotypes of a $P_{1,2,3}$ pathotype in the NLL virus population, which coexisted with genotypes of pathotyes $P_{1,2}$, P_1 and P_0 that induced NLL in L^3, L^2 and L^1 plants, respectively (Figure 1). These results show that the virus population at NLL is highly heterogeneous in terms of resistance-breaking phenotypes. PMMoV isolates of P_1 and P_0 pathotypes have never been reported from nature, which suggest that mutations towards lower, not only higher, pathotype may have important fitness costs, so that PMMoV genotypes of P_1 and P_0 pathotypes would be outcompeted by P_0 and P_1 genotypes of other tobamovirus species commonly found in pepper crops (Fraile et al., 2011; Moury & Verdin, 2012; Vélez-Olmedo et al., 2021).

High-throughput sequencing of the CP gene confirmed the high genetic heterogeneity of the virus population at NLL, which was much higher than in systemically infected tissues. To our knowledge, this is the first analysis of the genetic structure of a virus population in NLL, that is, in resistant tissues. Despite the high mutation rates of RNA viruses (Sanjuán, 2012), in systemically infected tissues genetic diversity may be low, as the fittest genotype will prevail, with few mutants occurring at low frequency, as in lines 1, 4, and 5, or even becoming fixed, as in lines 2 and 3 (Table 2). This genetic structure has been shown often in tissues of susceptible plants (Cuevas et al., 2015; Fabre et al., 2012; García-Arenal et al., 2001). On the other hand, in NLL cells undergoing defence reactions effective towards a wide range of virus genotypes, mutants newly generated during virus replication would explore a broad phenotypic space that includes the escape of plant defences: new phenotypes will be produced that may have relevant traits, including overcoming, or not, specific resistances. In support of this hypothesis, the data in Table 2 show the appearance of $P_{1,2,3}$ pathotype determinants other than M138N, such as mutation D50G, which together with T43K (not detected) determines a $P_{1,2,3}$ pathotype and L13F and G66V in four of five passaged lines, which if occurring together would restore a $P_{1,2,3}$ pathotype (Hamada et al., 2007; Moreno-Pérez et al., 2016). Reported $P_{1,2,3}$ isolates determined by L13F + G66V or T43K + D50G belong to different phylogenetic clusters than those with a M138N determination (Moury & Verdin, 2012), which underscores the potential for generation in NLL of new resistance-breaking genotypes. The large number of CP amino acid mutations in the NLL virus population uncovered by high-throughput sequencing incites speculation on the occurrence at NLL of genotypes with other, unidentified, determinants of $P_{1,2,3}$ pathotype, or with determinants of the P_1 and P_0 pathotypes unveiled by NLL transfer to different pepper genotypes. The study of these possibilities is out of the scope of this work.

Reversion of the mutation M138N requires at least two nucleotide substitutions at codon 139 of the CP gene (note that according to the literature, the numbering of positions in the CP does not count the initial methionine residue, which is eliminated from the mature CP) to change the N codon in TS-M138N (AAU) to the M codon in TS (AUG). This reversion, sensu stricto, was detected in line 2, where the substitutions A416U and U417G occur together with a 93.75% frequency in the virus population (Table 2 and not shown). Reversion to a $P_{1,2}$ pathotype seems to have been more frequent via a simpler mutational pathway, the substitution U417G that results in the N138K mutation, which occurred in all five passaged lines (Table 2). The consistent occurrence of this mutation across lines and its high frequency prompted us to engineer a TS-M138K genotype, which when assayed in different pepper genotypes showed a $P_{1,2}$ pathotype, as expected. Additionally, the data in Table 2 show that a low frequency, of about 9%, of this mutant in the virus population is sufficient for recognition by *L* to induce a defence reaction and the development of NLL.

Reversion to a $P_{1,2}$ pathotype through mutation N138K may have fitness penalties, as it results in viral particles that aggregate under a range of conditions, such as in 40 mM EDTA, 36 mM NaOH, pH 7.3 during extraction by standard procedures for tobamoviruses, or in Tris-borate-EDTA pH 8.1. Modelling of the three-dimensional structure of PMMoV (Bera et al., 2017; Fraile et al., 2014) shows amino acid 138 located at a loop between two alpha helices perpendicular to the particle axis (Fraile et al., 2014), and the major change M138N results in a significant decrease of contacts and hydrogen bonds among CP subunits, and in changes to the electrostatic potential in the RNA-binding groove region that weakens the CP–RNA binding and destabilizes the particles (Bera et al., 2017). The change M138K results in highly stable particles that do not disassemble at basic pH. While higher particle stability will increase survival in the environment, particle aggregation would reduce the infectivity of the primary inoculum from the environment. Thus, the mutation M138K may involve significant fitness penalties and, accordingly, a K at position 138 has not been found in any tobamovirus isolate infecting pepper, an M at this position occurring regardless of species or pathotype, except for PMMoV isolates of pathotype $P_{1,2,3}$

(Fraile et al., 2014). Thus, the wide occurrence through space and time of mutation M138N in the PMMoV population despite the costs of within-host multiplication under coinfection and of survival, can be explained, at least in part, by limited mutational pathways for reversion that result in $P_{1,2}$ revertants of a lower fitness.

The results of this study contribute to understanding the complexity of pleiotropic effects of host range mutations that collectively modulate the evolution of host range expansions. Specifically, we show that in addition to the effects of host range mutations on different life history traits, pathways of reversion and fitness of revertant genotypes may play a role in determining the fate of host range mutations in virus populations. Another major contribution of this study is to show the high genetic diversity of the virus population in NLL and, possibly, other plant tissues undergoing defence reactions. Such high diversity may potentiate the appearance of genotypes that overcome host defences to colonize new host genotypes or species, thus contributing to virus emergence.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plants and virus inoculations

The following species and cultivars of *Capsicum* that differ in the alleles at the *L* resistance locus were used: *C. annuum* 'Dulce Italiano' (L^+/L^+), *C. annuum* 'Yolo Wonder' (L^1/L^1), *C. frutescens* 'Tabasco' (L^2/L^2), *C. chinense* 'PI 159236' (L^3/L^3), *C. annuum* 'Ferrari' (L^3/L^+) and *C. chacoense* 'PI 260429' (L^4/L^4). Plants were grown in 15-cm diameter, 1.5-L pots at 23–25 °C and under 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 inoculation buffer (0.1 M sodium phosphate, pH 7.2). For serial passage experiments, the inoculum consisted of the sap of young systemically infected leaves from the previous passage plants ground in inoculation buffer. *N. tabacum* 'Xanth-nc', which has the *N* gene of resistance to tobamoviruses, was used as a local lesion assay host, and *N. clevelandii* was used as a multiplication host.

4.2 | Virus isolates and mutants

Viral genotypes were multiplied in *N.clevelandii* after inoculation with transcripts from full-length cDNA clones. Clone pTS, which generates PMMoV-TS, of a $P_{1,2}$ pathotype, and its derived CP mutant pTS-M138N, which generates PMMoV TS-M138N, of $P_{1,2,3}$ pathotype, are described in Moreno-Pérez et al. (2016). In the CP gene of plasmid pTS-M138N, the mutation N138K was generated through introduction of the T417G substitution by site-directed mutagenesis as in Moreno-Pérez et al. (2016) using primers N138K fw (5′-CGTG GCACGGGAAAGTACAATCAAGCTCTG-3′) and N138K rv (5′-CAGA GCTTGATTGTACTTTCCCGTGCCACG-3′), which generate plasmid pTS-M138K. Plasmids were multiplied in *Escherichia coli* XL2-Blue MRF′ (Agilent Technologies).

PMMoV particles were purified as in Bruening et al. (1976), and their electrophoretic mobility was analysed in 1.2% agarose gels in Tris-borate-EDTA pH 8.1 and ethidium bromide staining as in Hogue and Asselin (1984). To monitor the fate of virus particles along the purification procedure, pellets of low- and high-speed centrifugation steps were treated with 5% SDS in 0.1 M Tris-HCl pH 9.0 for particle disruption, phenol extracted, and virus nucleic acid detected by RT-PCR using primers identical and complementary to positions 5853–5874 and 5937–5958 of PMMoV accession number NC_003630. The resulting 106 nt amplicons were resolved by electrophoresis in 1.2% agarose gels in Tris-HCl, Na acetate, EDTA, pH 8.2. The kinetics of particle disassembly at basic pH was assayed in 0.1 M Tris-HCl pH 8.75 as in Bera et al. (2017). For electron microscopy, 1 g of systemically infected leaf was ground in 300 µl of 0.01 M NaH_2PO_4 pH 7.2, and the supernatant was stained for 2 min in 2% uranyl acetate as in Lu et al. (1996).

4.3 | Nucleotide sequence determination and analyses

Leaf RNA was extracted using TRIzol reagent (Life Technologies). Nucleotide sequence determination by Sanger method was outsourced at Macrogen (Amsterdam, Netherlands).

For high-throughput sequencing, RNA was extracted from two pooled disks of either NLL or systemically infected tissues of the same plant, and the CP gene was RT-PCR amplified using primers complementary to nt positions 6142–6167 and identical to nt positions 5685–6158 (positions numbered as in PMMoV accession number M81413.1). After equimolar pooling of the 10 amplicons (five from NLL and five from systemically infected tissues) sequencing was done at Genoscreen, Lille, France, using a HiSeq 2500 – Illumina platform. Paired-end libraries with multiplex adapters were prepared along with an internal PhiX control. The pool of tagged samples was sequenced on MiSeq run in 2×250 bp paired-end reads. The average number of single reads per sample was 343,000, which yielded 86 megabases per sample, with a quality score of Q30 (associated error rate 0.1%). Sequencing data were converted to FASTQ format with FastQ Groomer v. 1 (Blankenberg et al., 2010), the trimming of paired-end was made with Trimmomatic (Bolger et al., 2014), with a required average quality Q20, minimum length of reads to be kept a240. Mapping was done against the reference genome of PMMoV-TS using Bowtie v. 2.1.0 (Langmead & Salzberg, 2012). Call variant detection and alignment were performed with Mpileup (Li, 2011) and Varscan (Koboldt et al., 2012) with minimum base quality at a position to count a read Q28, minimum variant allele frequency threshold 0.01.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available at NCBI, BioProject ID PRJNA838011 at https://www.ncbi.nlm.nih.gov/sra/PRJNA838011.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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