

Quantitative trait loci in pepper control the effective population size of two RNA viruses at inoculation

Lucie Tamisier,^{1,2,*†} Elsa Rousseau,^{2,3,4†} Sebastien Barraillé,² Ghislaine Nemouchi,¹ Marion Szadkowski,¹ Ludovic Mailleret,^{3,4} Frederic Groggnard,³ Frederic Fabre,⁵ Benoit Moury^{2,*} and Alain Palloix¹

Abstract

Infection of plants by viruses is a complex process involving several steps: inoculation into plant cells, replication in inoculated cells and plant colonization. The success of the different steps depends, in part, on the viral effective population size (N_e), defined as the number of individuals passing their genes to the next generation. During infection, the virus population will undergo bottlenecks, leading to drastic reductions in N_e and, potentially, to the loss of the fittest variants. Therefore, it is crucial to better understand how plants affect N_e . We aimed to (i) identify the plant genetic factors controlling N_e during inoculation, (ii) understand the mechanisms used by the plant to control N_e and (iii) compare these genetic factors with the genes controlling plant resistance to viruses. N_e was measured in a doubled-haploid population of *Capsicum annuum*. Plants were inoculated with either a *Potato virus Y* (PVY) construct expressing the green fluorescent protein or a necrotic variant of *Cucumber mosaic virus* (CMV). N_e was assessed by counting the number of primary infection foci on cotyledons for PVY or the number of necrotic local lesions on leaves for CMV. The number of foci and lesions was correlated ($r=0.57$) and showed a high heritability ($h^2=0.93$ for PVY and $h^2=0.98$ for CMV). The N_e of the two viruses was controlled by both common quantitative trait loci (QTLs) and virus-specific QTLs, indicating the contribution of general and specific mechanisms. The PVY-specific QTL colocalizes with a QTL that reduces PVY accumulation and the capacity to break down a major-effect resistance gene.

INTRODUCTION

During the plant infection process, RNA viruses are generally able to evolve quickly and adapt to their host thanks to their high mutation rate and short generation time [1]. As a result, breakdown of plant resistance by the emergence of new virus variants may occur and cause important losses for agricultural production and quality [2, 3]. A better understanding of the evolutionary processes that shape viral populations and the extent to which we can control them is therefore required for the sustainable management of crop disease [4].

In plants, two well-known evolutionary forces act on the frequencies of the different variants comprising virus populations: natural selection and genetic drift. Natural selection is a deterministic force that increases the frequency of the fittest variants at each generation. In contrast, genetic drift is a

stochastic force that randomly changes the frequencies of the virus variants from generation to generation [5]. The two forces act jointly on viral populations and can have opposite effects on their adaptation. Indeed, if genetic drift is strong, deleterious mutations may be randomly fixed or advantageous ones may be lost. The strength of genetic drift depends on a key parameter of virus evolution: the effective population size (N_e). N_e can be defined as the number of individuals that pass their genes to the next generation [6], and the strength of genetic drift is inversely proportional to this number. N_e is generally much smaller than the census population size N [7]. Through the infection process, the viral population will endure several bottlenecks that will strongly reduce N_e and consequently increase genetic drift [8, 9]. These bottlenecks can occur during all the infection steps, like vector transmission [10, 11], virus inoculation into plant cells [12], replication in infected cells [13] and cell-to-cell or long-distance movements

Received 31 January 2017; Accepted 12 May 2017

Author affiliations: ¹INRA, UR1052 GAFL, Unité de Génétique et Amélioration des Fruits et Légumes, Domaine St Maurice - 67 Allée des Chênes, CS 60094, F-84143 Montfavet Cedex, France; ²INRA, UR407 PV, Unité de Pathologie Végétale, Domaine St Maurice - 67 Allée des Chênes, CS 60094, F-84143 Montfavet Cedex, France; ³INRIA, Biocore Team, F-06902 Sophia Antipolis, France; ⁴INRA, Université Nice Sophia Antipolis, CNRS, UMR 1355-7254 Institut Sophia Agrobiotech, Sophia Antipolis, France; ⁵INRA, UMR 1065 Santé et Agroécologie du Vignoble, BP 81, 33883 Villenave d'Ornon cedex, France.

*Correspondence: Lucie Tamisier, lucie.tamisier@inra.fr; Benoit Moury, benoit.moury@inra.fr

Keywords: effective population size; bottlenecks; quantitative trait loci; *Capsicum annuum*; virus infection; *Potato virus Y*; *Cucumber mosaic virus*.

Abbreviations: CMV, *Cucumber mosaic virus*; DH, doubled-haploid; h^2 , broad-sense heritability; LOD, logarithm of odds; N_e , effective population size; PVY, *Potato virus Y*; QTL, quantitative trait locus; ToMV, *Tomato mosaic virus*; VA, virus accumulation.

†These authors contributed equally to this work.

One supplementary table and three supplementary figures are available with the online Supplementary Material.

in the infected plant [14]. Although estimation of bottleneck size and its effects on the genetic diversity of the viral population is well documented [15, 16], the plant genetic determinants controlling bottleneck size have been little studied to date. Previous studies have found quantitative trait loci (QTLs) involved in the reduction of the viral accumulation, which corresponds to the census population size N [17–19], but no study has searched for genomic regions causing direct changes in the N_e of viruses. However, since N_e could be a more relevant parameter than N for virus evolution, understanding how plant genetic factors may affect it could contribute to the development of cultivars slowing down pathogen evolution and increasing resistance durability [20].

In this study, we focused on N_e during the inoculation of pepper (*Capsicum annuum*) plants with two RNA viruses, *Potato virus Y* (PVY; genus *Potyvirus*, family *Potyviridae*) and *Cucumber mosaic virus* (CMV; genus *Cucumovirus*, family *Bromoviridae*). We aimed to (i) identify the plant QTLs that control N_e at the inoculation step, (ii) understand the mechanisms used by the plant to control N_e and (iii) compare these genetic factors with others factors controlling the virus accumulation and plant resistance durability.

RESULTS

Very few primary infection foci are initiated by two PVY variants simultaneously

To measure N_e , we inoculated *C. annuum* plants with either a PVY construct expressing the green fluorescent protein (GFP), the PVY-GFP, or a necrotic variant of CMV, the CMV-N strain of Fulton. We then quantified the number of primary infection foci under a specific light wavelength for PVY-GFP, or the number of necrotic local lesions for CMV-N observed on the inoculated organs. We hypothesized that one primary infection focus or one local lesion was caused by only one viral particle or one ‘infectious unit’ composed of several particles containing different genome components in the case of a multipartite virus like CMV. Therefore, counting the number of foci or lesions on the inoculated organs would be a direct assessment of N_e at inoculation.

To validate the N_e estimation method, we conducted a control experiment using co-infections by two PVY constructs expressing different fluorescences (GFP and mCherry), similar to Zwart *et al.* [21]. Among a population of 152 *C. annuum* doubled-haploid (DH) lines issued from the F_1 hybrid between the PVY-resistant parent Perennial and the susceptible parent Yolo Wonder, we selected 13 DH lines that showed contrasting numbers of foci when inoculated by PVY-GFP alone (Fig. S1, available with the online Supplementary Material). A 1:1 mixture of the PVY variants carrying either the GFP or the mCherry fluorescent markers was inoculated on the first true leaf of the plants. We kept only the leaves showing at least 1 infection focus of each colour, and ended up with a total of 49 leaves and 1 to 8 leaves per DH line (Table S1).

For 75.5% of the inoculated leaves, no infection foci with dual fluorescences were observed. Overall, the mean frequency of foci showing both red and green fluorescences was 0.64% among the DH lines, with a maximum frequency per plant of 5.3%. The mean number of viral particles initiating a focus was assessed using the model proposed by Sacristán *et al.* [7] [equations (1) and (2)]. On average, 1.01 to 1.02 viral particles initiated an infection focus, with a maximum number per DH line of 1.06 (Table S1), which validated our hypothesis.

The numbers of primary infection foci and local lesions are highly heritable traits

The DH population comprising 152 lines of *C. annuum* was inoculated with either the PVY-GFP variant on the cotyledons or the CMV-N strain of Fulton on one leaf, which induced fluorescent infection foci or necrotic local lesions, respectively (Figs 1 and S2). The number of primary infection foci on the 2 cotyledons or local lesions on 1 leaf were then quantified on 10 plants per DH line. For PVY, the mean number of primary infection foci ranged from 1.15 to 44.05 among the different DH lines, with an overall mean number of 15.56 ± 9.47 (mean \pm standard deviation) (Fig. 2a). For CMV, the overall mean number of local lesions varied from 0.20 to 95.00, with a mean number of 24.59 ± 21.75 (Fig. 2b). The two variables were significantly correlated among the DH lines (Pearson $r=0.57$, $P=8.97 \times 10^{-15}$). They also both showed a high heritability, with $h^2=0.93$ for the foci induced by PVY and $h^2=20.98$ for the lesions induced by CMV. Furthermore, the numbers of foci and lesions of the F_1 hybrid (Yolo Wonder \times Perennial) were intermediate between those of the two parental lines. Since they were closer to Perennial (the parent showing the least number of foci or lesions) than to Yolo Wonder (the susceptible parent) in both cases (Fig. 2), we concluded that the numbers of foci and lesions have either a dominant or codominant inheritance.

Detection of QTLs controlling the numbers of primary infection foci and local lesions for PVY and CMV

Three QTLs were detected for each virus (Table 1 and Fig. 3). They were named PVY-6, PVY-7, PVY-12 and CMV-6, CMV-7, CMV-12 according to the virus used for the

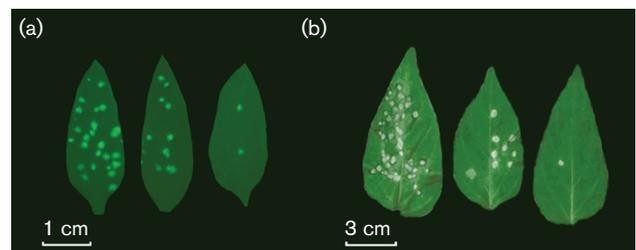


Fig. 1. Primary infection foci or necrotic local lesions on pepper cotyledons or leaves inoculated with PVY-GFP (a) or CMV-N (b).

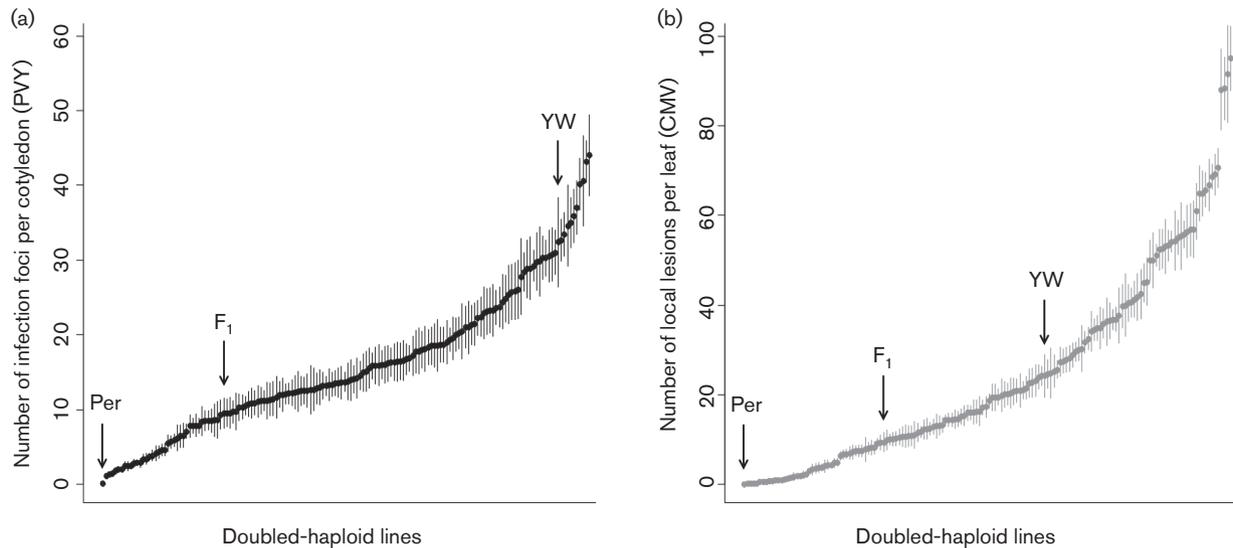


Fig. 2. The number of primary infection foci induced by PVY-GFP (a) and the number of local lesions induced by CMV-N (b) among the DH lines. Error bars represent the standard error of the mean. The positions of the parental lines, Perennial (Per) and Yolo Wonder (YW), and the F_1 hybrid are indicated.

inoculation and the chromosome number. The QTLs PVY-6, PVY-7 and PVY-12 explained respectively 6.28, 34.73 and 26.22 % of the variation of the primary infection foci numbers for PVY. Similarly, QTLs CMV-6, CMV-7 and CMV-12 explained respectively 11.18, 31.53 and 21.67 % of the variation of the local lesion numbers for CMV. For both viruses, analysis revealed a significant epistatic interaction between the QTLs on chromosomes 7 and 12. This epistatic interaction explained 11.46 and 9.38 % of the primary infection foci numbers and the local lesion numbers for PVY and CMV, respectively. More precisely, for the DH lines with the Perennial allele at both QTLs, the number of foci/lesions was on average lower than expected if there was no epistasis (i.e. it is a case of synergistic epistasis; Fig. 4). Regarding all the QTLs independently, the Perennial allele always decreased the trait value, except in the case of CMV-6. Finally, the model combining the additive and epistatic effects of the three QTLs explained 57.82 and 50.88 % of the trait variation for PVY and CMV, respectively.

The number of infection foci induced by PVY correlates with the number of local lesions induced by ToMV

Eight DH lines were selected according to the QTLs they carried. We chose one DH line without QTL, one DH line with one QTL (PVY-6), two DH lines with two QTLs (PVY/CMV-7 and PVY-6), two others DH lines with two QTLs (PVY/CMV-7 and PVY/CMV-12) and two DH lines with three QTLs (PVY/CMV-7, PVY/CMV-12 and CMV-6). The eight DH lines were mechanically inoculated with *Tomato mosaic virus* (ToMV; genus *Tobamovirus*, family *Virgaviridae*). At 5 days post-inoculation (p.i.), the number of local lesions observed on leaves was quantified. The mean number of lesions ranged from 3.13 to 26.15 among the DH lines, with a

mean number of 10.66 ± 8.34 . The variable was significantly correlated with the number of foci caused by PVY (Pearson's $r=0.86$, $P=5.60 \times 10^{-3}$, Fig. 5a), but was not significantly correlated with the number of lesions caused by CMV (Pearson's $r=0.56$, $P=0.15$, Fig. 5b).

DISCUSSION

One key parameter of virus evolution is N_e , which corresponds to the number of virus individuals that pass their genes to the next generation [6]. As a viral population infects a plant, it will experience several bottlenecks that will reduce N_e . A low value of N_e will generally have a negative impact on the fitness of a viral population, even if some exceptions exist [15]. A low N_e will increase genetic drift, reduce genetic diversity and possibly lead to fitness decline by losing the most adapted variants. The repeated bottleneck events can also be conducive to Muller's ratchet processes. In this situation, the population will accumulate slightly deleterious mutations at each generation. The individuals without deleterious mutations are definitively lost by genetic drift and the final consequence can be the extinction of the population if the deleterious mutations continue to accumulate [22]. Therefore, it is of primary importance to better understand the evolutionary constraints imposed by the plant that reduce the N_e of the pathogen.

Link between the number of primary infection foci or local lesions and the effective population size

The inoculation step consists of a particularly narrow bottleneck for viruses: only a few individuals from the inoculum source succeed in initiating infection of new plants [10, 15]. Previous studies have reported very low values of N_e at this step. For example, depending on the virion dose, N_e of

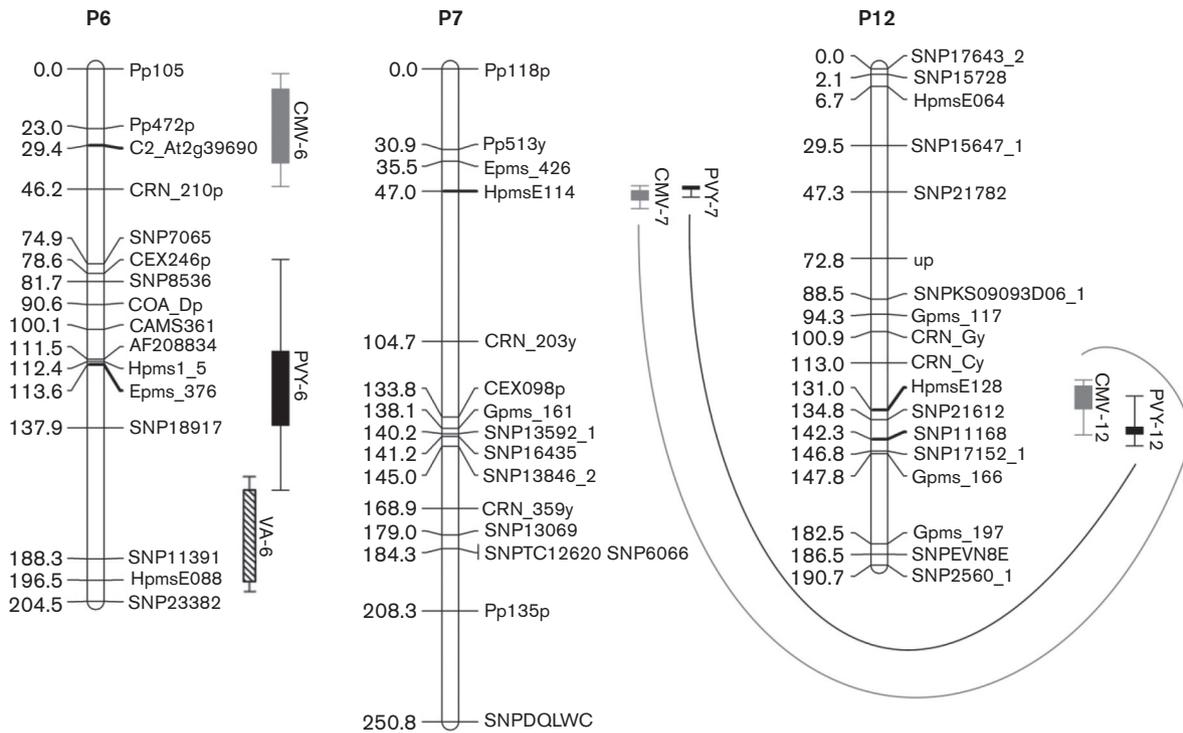


Fig. 3. QTL map for the effective population size at inoculation for PVY (black) and CMV (grey). A previously detected QTL (VA-6) controlling PVY accumulation is also mapped (hatched). For each QTL, confidence intervals obtained using the 1-LOD drop and 2-LOD drop methods are indicated. The lines represent epistatic effects between loci.

Tobacco etch virus (TEV) was estimated to have between 1 and 50 viral particles following mechanical inoculation in *Nicotiana tabacum* [21]. Considering aphid transmission, the number of virus particles transmitted was estimated to be between 0.53 and 3.24 for PVY [10] and between 0.5 and 35.8 for CMV [11]. In our study, the number of infection foci (for PVY) or local lesions (for CMV) are the minimum values for N_e at the inoculation step, since they are initiated by at least one virus particle. They would correspond to exact N_e values if and only if each infection focus/local lesion was initiated by exactly one virus particle. As proposed by Zwart *et al.* [21], we tested this hypothesis by co-inoculating *C. annuum* leaves with PVY expressing two different reporter proteins showing either green or red fluorescence, respectively. We found that the frequency of infection foci showing both green and a red fluorescence was very low, with a mean frequency of 0.64 % per DH line, and we estimated that the mean number of viral particles initiating a focus ranged from 1.01 to 1.02. Thus, we could conclude that the huge majority of foci were initiated by a single viral particle and that the number of primary infection foci was a precise estimate of N_e at the inoculation step.

The same approach could not be undertaken for CMV or ToMV. However, the quantification of local lesions has been widely used to investigate the relationship between the virus inoculum dose and the number of lesions [23, 24].

Combining models and experimental data, it has been shown that most of the local lesions are initiated by a single virus particle when considering low to moderate inoculum concentration [25, 26]. More recently, Sacristán *et al.* [12] provided an estimate of the number of founders that start an infection by contact transmission. They measured the number of necrotic local lesions induced by two *Tobacco mosaic virus* (TMV; genus *Tobamovirus*) genotypes on tobacco plants and found that, on average, one viral particle initiated each local lesion. These results suggest that the number of local lesions is also an accurate estimate of N_e for CMV and ToMV. Therefore, in the next sections of the paper, we will treat N_e at inoculation as being synonymous with the number of primary infection foci or local lesions.

Finally, since N_e is dose-dependent [21], the inoculum dose used in our study is also an important parameter in the estimation of N_e . Our goal was to use an inoculum dose that maximizes the differences in the number of foci/lesions between the DH lines in order to perform an efficient QTL detection. This objective was achieved, since the number of foci per cotyledon for PVY-GFP ranged from 1.15 to 44.05 for the DH lines and was 0.10 for the Perennial parent (Fig. 2a). The result was similar for CMV-N, with between 0.20 and 95.00 lesions/leaf (Fig. 2b). For the DH lines showing the highest numbers of foci, higher inoculum doses would have produced too many foci for them to be

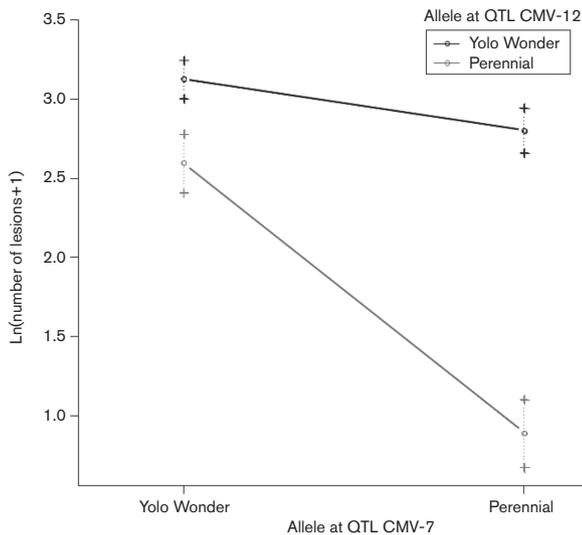


Fig. 4. Epistasis effect between QTLs CMV-7 and CMV-12. The average number of lesions induced by CMV after $\ln +1$ transformation is plotted as a function of the parental alleles (Yolo Wonder or Perennial) at QTL CMV-7 and QTL CMV-12. Error bars represent the standard error of the mean. Similar results (i.e. synergistic epistasis) were obtained for PVY.

visualized without ambiguity, i.e. we would be at a ‘technical saturation’ point of the inoculum concentration density of the infection foci curve. Clearly, we do not encounter this type of saturation in our work. First, the curves representing the number of foci/lesions among the DH lines increase continuously and do not reach a plateau, both for PVY and CMV, suggesting that these numbers are not the highest possible for the vast majority of the lines (Fig. 2a, b). Since there are only small differences between the cotyledon surface areas of the different DH lines, the curve representing

the foci density also increases continuously among the lines and does not reach a plateau (Fig. S3). This demonstrates that the number of foci per cotyledon does not reach the maximum value which is technically achievable given the cotyledon size. The reason could be either that the inoculum dose is not saturating or that the maximum number of virus entry sites is very low for some of the DH lines.

Common and virus-specific QTLs control the effective population size of PVY and CMV at inoculation

For both PVY and CMV, we identified three QTLs controlling N_e at inoculation and localized on chromosomes 6, 7 and 12 (Table 1 and Fig. 3). The QTLs on chromosome 7, PVY-7 and CMV-7, were detected at the same location in the genome (the strongest association was with marker HpmsE114), and the QTLs on chromosome 12, PVY-12 and CMV-12, were identified at very close positions (139.1 and 125.7 cM). On each chromosome, the confidence intervals of the two QTLs overlapped to a large extent. Moreover, the phenotypic variation explained by the QTLs was similar, with PVY-7 and CMV-7 explaining 34.73 and 31.53 % of the trait variation, and PVY-12 and CMV-12 explaining 26.22 and 21.67 % of the trait variation. We also found that for both viruses there was an epistasis between the QTLs on chromosomes 7 and 12, and that the epistasis had a similar explanatory power for both viruses (11.46 % for PVY and 9.38 % for CMV). Therefore, the same QTLs on chromosomes 7 and 12 control N_e for PVY and CMV, and the same genetic factor(s) may be responsible for this dual effect. Note that in a previous study, Caranta *et al.* [27] detected three pepper QTLs controlling the number of necrotic local lesions caused by the CMV-N strain of Fulton. At the time, the pepper linkage map coverage was incomplete, making the comparison difficult. However, CMV-12 seems to correspond to one of the three QTLs, as they are located in the same genomic region.

Table 1. QTLs detected for the effective population size N_e of PVY and CMV at inoculation

QTL*	Chr†	Position (cM)	Closest marker	LOD score	2-LOD support interval	Variation explained (%)		Estimated effect of Perennial QTL allele‡	h^2
						QTL	Model		
PVY-7	7	45.6	HpmsE114	20.71	45.1–49.3	34.73	57.82	–0.803	0.93
PVY-12	12	139.1	SNP11168	15.30	125.7–144.8	26.22		–0.652	
PVY-6	6	123.8	Epms_376	4.93	73.2–161.9	6.28		–0.320	
PVY-7 × PVY-12	7/12	–	HpmsE114/SNP11168	8.48	–	11.46		–0.906	
CMV-7	7	48.4	HpmsE114	16.36	44.9–53.7	31.53	50.88	–1.200	0.98
CMV-12	12	125.7	HpmsE128	12.06	119.5–140.7	21.67		–1.091	
CMV-6	6	31.5	C2_At2g39690	6.77	1.8–45.2	11.18		0.712	
CMV-7 × CMV-12	7/12	–	HpmsE114/HpmsE128	5.77	–	9.38		–1.397	

*QTL name includes the virus used for plant inoculation followed by the number of the chromosome carrying the QTL.

†Chromosome number.

‡Estimated effect of the Perennial allele on the value of the trait studied. For the pairwise epistasis effects, it corresponds to the difference in the effects at the first QTL considering the two alleles at the other QTL separately.

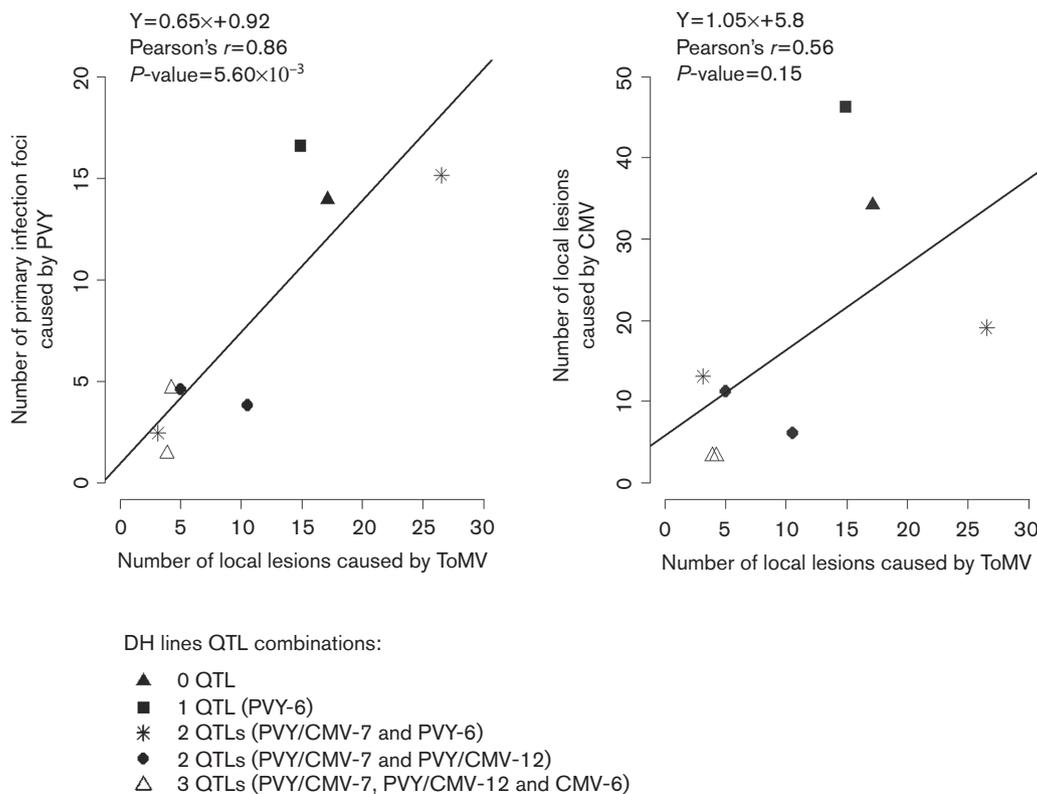


Fig. 5. Correlation between the number of lesions induced after inoculation by ToMV and PVY (a) or ToMV and CMV (b) on eight DH lines carrying different QTL combinations. The linear regression equations, Spearman's correlation coefficients and P -values of the correlations are indicated.

In contrast to the QTLs PVY/CMV-7 and PVY/CMV-12, the two QTLs detected on chromosome 6, PVY-6 and CMV-6, differed according to the virus. PVY-6 was localized at 123.8 cM and associated with the marker Epms_376, whereas CMV-6 was positioned at 31.5 cM and associated with the marker C2_At2g39690. Further, the QTL effects showed opposite directions, since the Perennial allele decreased the value of the trait for PVY-6 and increased its value for CMV-6. Even if the two viruses are quite different, our study highlights that two common QTLs control the effective population size of both viruses, indicating that general mechanisms underlie this trait. However, we also found one specific QTL for each virus, demonstrating that virus-specific mechanisms also act.

Hypothesis on the mechanisms of action of the QTLs

We have identified two QTLs that are shared between PVY and CMV, and two virus-specific QTLs that control N_e . Leaves were chosen to inoculate CMV because the number of CMV lesions in cotyledons is usually very low and the necrotic lesions are quite large at the moment of their detection. In contrast, cotyledons were chosen to inoculate PVY-GFP because the number of fluorescent infection foci on leaves is usually very high. Consequently, since the

inoculations have not been performed on the same plant organs for the two viruses, we do not know if the QTLs detected for a given virus will still act in another plant organ. To answer this question, we inoculated 10 pepper lines on either one leaf or one cotyledon, both for PVY and CMV. The number of foci induced after PVY inoculation was significantly correlated between the leaves and the cotyledons (Pearson's $r=0.93$, $P=9.72\times 10^{-5}$, data not shown), as was the number of lesions caused by CMV (Pearson's $r=0.75$, $P=0.01$, data not shown). Therefore, as the N_e at inoculation is significantly correlated between leaves and cotyledons for both PVY and CMV, we can reasonably assume that the mechanisms controlling N_e at inoculation are not organ-specific.

To investigate the spectrum of action of the QTLs more deeply, we used a third virus, a strain of ToMV, which also induces local lesions on the leaves. The plant L^1 allele required for the expression of hypersensitive necrotic local lesions upon ToMV inoculation was only present in half of the DH lines. Consequently, this small number of DH lines was insufficient to conduct QTL mapping with ToMV. Therefore, we inoculated ToMV to a representative subset of eight DH lines carrying different QTL combinations. We then counted the local lesions on the leaves and compared

the results to those of PVY and CMV. We found that the values of N_e at inoculation were significantly correlated between ToMV and PVY (Fig. 5a). In contrast, no significant correlation was observed between ToMV and CMV (Fig. 5b). These findings support our previous results that found that some of the QTLs have a broad spectrum of action, including ToMV and PVY in this case. It also confirms the inference that some QTLs have a narrower spectrum of action, potentially explaining the differences between ToMV and CMV. We can hypothesize that the broad-spectrum QTLs are generalists because they act during the primary steps of the infection, before the establishment of narrow molecular interactions between the virus and intra-cellular plant components. These broad-spectrum QTLs could affect penetration of the virus in the cotyledon or leaf cells by controlling traits such as tissue thickness or sensitivity to wounding. The narrow-spectrum QTLs could act slightly later during the infection, playing on the probability for a given virus to successfully initiate the infection focus once it has penetrated into the first cells. These two mechanisms would have contrasting effects according to the virus inoculation mode, i.e. manual or with aphids. QTLs acting early on virus penetration may have little effect on aphid inoculation, since the aphid stylet will penetrate more deeply into the leaf tissues and will bypass the action of these QTLs. In contrast, QTLs acting later, once the virus is within the cell, are expected to have a similar effect in mechanical and aphid inoculations.

These findings are also interesting from an agronomic viewpoint because ToMV is a contact-transmitted virus. In this situation, plants that reduce N_e as soon as the inoculation step occurs could be particularly helpful for reducing or stopping plant-to-plant transmission and epidemics in the field. In contrast, PVY and CMV are naturally transmitted by aphids. Thus, further experiments are required to determine whether our observations of N_e variation between DH lines after mechanical inoculation can be extended to aphid transmission.

Relationship between QTLs of effective population size and plant resistance

The results from previous studies revealing the phenotypic and genetic factors involved in pepper resistance to PVY and CMV can be compared to our results. With the same DH population, Quenouille *et al.* [19] identified the pepper QTLs controlling the durability of the major PVY resistance gene *pvr2*³ and PVY accumulation. In theory, we could expect that a lower N_e at inoculation would delay plant infection and therefore decrease virus accumulation. We could also expect that a lower N_e at inoculation would help to lose well-adapted virus variants by genetic drift, therefore increasing resistance durability. However, the genetic evidence for links between N_e at inoculation, virus accumulation and resistance durability is weak in our case. The only co-localization with the QTLs controlling the variation of N_e at inoculation would concern a QTL on chromosome 6, named VA-6, which affects PVY accumulation (Fig. 3).

QTL VA-6 showed an epistatic interaction with RB-3, a QTL on chromosome 3 controlling the virus capacity to break down *pvr2*³ resistance. The Perennial allele at QTL PVY-6 decreases N_e , while at QTL VA-6 it increases resistance durability and decreases within-host virus accumulation, which is consistent with our expectations. However, even if the confidence interval of VA-6 includes a part of the confidence interval of PVY-6, the two QTLs are not localized at exactly the same position. A lack of markers in this genomic region does not allow precise QTL mapping and this hypothesis should be evaluated further by adding new molecular markers.

Regarding CMV, a breeding programme has been developed in order to increase CMV resistance in pepper. The reduction of the number of local lesions at inoculation has been used as a resistance trait that was introgressed into pepper breeding lines also expressing resistance to CMV systemic movement and accumulation within the plant. The final pepper lines were close to virus immunity, which was not the case for pepper lines that only carry resistance to CMV systemic movement and/or accumulation [28]. This suggests that reducing N_e at inoculation increases plant resistance substantially, at least when combined with resistance to systemic movement and accumulation.

In this study, we showed that N_e at inoculation is a well-diversified and highly heritable trait among a DH population of *C. annuum*. We then identified and mapped the pepper QTLs controlling N_e for both PVY and CMV. Finally, comparison with previous results showed that one of these QTLs may also decrease virus adaptation to a major-effect resistance gene. From an agricultural point of view, the use of these QTLs could be even more beneficial, because some of them could act against several viruses belonging to distant groups. Therefore, our results suggest that these plant genetic factors could correspond to general mechanisms and be efficient against multiple pathogens.

METHODS

Plant and virus material

A DH population of *C. annuum* was obtained from the F₁ hybrid between Yolo Wonder, a line susceptible to PVY isolates, and Perennial, a cultivar carrying the PVY resistance allele *pvr2*³. A genetic map comprising 190 molecular markers was previously built for this progeny [19]. From this population, we phenotyped 152 DH lines carrying *pvr2*³ and differing in their genetic background.

The first virus used was derived from a cDNA clone of PVY isolate SON41p. It carried the 115K substitution in the VPg, which allows it to overcome the *pvr2*³ resistance [29]. The virus was also tagged with the GFP reporter gene. The PVY-GFP was constructed by duplicating the NIa protease cleavage site at the C-terminus of the N1b cistron and inserting the GFP gene between the two sites,

allowing the virus NIa protease to cleave the GFP. For the purpose of a control experiment, the same PVY infectious clone carrying the mCherry reporter gene (expressing a red fluorescent marker) instead of the GFP gene was used. The second virus was the CMV-N strain of Fulton, a variant of CMV causing necrotic local lesions on the leaves. A third virus, ToMV (isolate Vi76), was used for the inoculation of eight representative DH lines. ToMV induces necrotic local lesions on the leaves of pepper plants carrying the *L*¹ resistance allele, harboured by the Yolo Wonder parent.

Evaluation of the N_e estimation method

A control experiment similar to the one developed by Zwart *et al.* was performed to assess the reliability of the N_e estimation method [21]. We used a mixture of the PVY variants carrying either the GFP or the mCherry fluorescent marker, which had been multiplied separately in *Nicotiana glauca* plants beforehand. Twenty-eight days after sowing, the 1 : 1 mixture was inoculated on the first true leaf of 13 DH lines selected for showing contrasting numbers of foci induced by the PVY-GFP variant alone (Fig. S1). The number of foci showing green and/or red fluorescence was estimated under a specific light wavelength (450–490 nm) at 4 and 6 days p.i. Finally, the surface area of the cotyledons was measured using ImageJ software, with 10 cotyledons for each DH line.

Counting the primary infection foci and local lesion numbers induced by PVY, CMV and ToMV in a DH population

All of the DH lines were mechanically inoculated with either PVY or CMV for the QTL detection. Based on the results of the QTL detection, a subset of eight DH lines was selected and inoculated with ToMV in order to compare the previous results with a third virus.

The PVY-GFP cDNA clone was first inoculated in *N. glauca* plants by DNA-coated tungsten particle bombardment. In order to obtain the inoculum, extracts of these plants were then used to propagate the virus in *Nicotiana tabacum* cv. Xanthi plants. Leaves from infected Xanthi plants were ground in phosphate buffer (0.03 M Na₂HPO₄, 0.2 % sodium diethyldithiocarbamate; 4 ml buffer per gramme of leaves) supplemented with active charcoal (90 mg ml⁻¹) and carborundum (90 mg ml⁻¹) to obtain the final inoculum. A single inoculum was used to mechanically inoculate 10 plants on their two cotyledons for each pepper DH line, 3 weeks after sowing. At 6 days p.i., the number of primary infection foci on each inoculated cotyledon was counted under a specific light wavelength (450–490 nm) (Fig. 1a). All the plants were grown under greenhouse conditions.

The CMV-N strain of Fulton was propagated on *Catharanthus roseus* plants. From extracts of these plants, 10 pepper plants per DH line were mechanically inoculated on their first leaf, 4 weeks after sowing. At 5 days p.i., the number of necrotic local lesions per inoculated leaf was counted

(Fig. 1b). The experiment was realized in a climate-controlled room (20–22 °C, 12 h light day⁻¹).

The ToMV test was performed in a detached leaf assay to avoid contamination, since ToMV is readily transmitted by contact. Five weeks after sowing, the first leaf from 12 plants per DH line was excised, mechanically inoculated and kept in a humid plastic box. At 5 days p.i., the number of necrotic local lesions per inoculated leaf was counted.

Statistical analyses

The statistical analyses were performed using R software (<http://www.r-project.org/>). For the two phenotypic traits (number of infection foci and local lesions initiated by PVY or CMV), broad-sense heritability was estimated using the formula $h^2 = \sigma^2_G / (\sigma^2_G + \sigma^2_E/n)$, where σ^2_G corresponds to the genotypic variance, σ^2_E corresponds to the environment variance and n corresponds to the number of replicates ($n=20$ for PVY and 10 for CMV). An ln(x+1) transformation was applied to the two traits to approximate a normal distribution.

QTL analysis

QTLs detection was performed with the R/qtl software package [30]. A preliminary analysis was realized by using a standard interval mapping approach. In addition, a two-dimensional genome scan was performed to identify potential interactions between QTLs. Multiple QTL mapping (MQM) was then performed, using the markers previously identified as the initial set of cofactors. Finally, the positions and the effects of the QTLs were refined in the context of a multiple QTL model. The significance LOD threshold was calculated by performing a permutation test with 10 000 replicates. The LOD threshold was set at 3.79 for the foci induced by PVY and 3.18 for the lesions induced by CMV ($P=0.05$). The confidence intervals for the location of each QTL were determined by using the 1-LOD and 2-LOD drop-off methods. The graphical representation of the QTLs was generated using MapChart version 2.3 [31].

Funding information

L.T.'s PhD is supported by the BAP (Biologie et Amélioration des Plantes) department and the SMaCH (Sustainable Management of Crop Health) metaprogramme of INRA, and by the Région Provence-Alpes-Côte d'Azur (PACA). The experimental work was supported by the SMaCH metaprogramme.

Acknowledgements

We thank the greenhouse staff for their support with plant experimentation.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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