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

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Abstract

Infection of plants by viruses is a complex process that involves several steps: inoculation into plant cells, replication in inoculated cells, cell-to-cell movement during leaf colonization and long-distance movement during systemic infection. The success of the different steps is conditioned by the effective viral population size (*Ne*) defined as the number of individuals that pass their genes to the *next generation*. During the infection cycle, the virus population will endure several bottlenecks leading to drastic reductions in *Ne* and to the random loss of some virus variants. If strong enough, these bottlenecks could act against selection by eliminating the fittest variants. Therefore, a better understanding of how plant affects *Ne* may contribute to the development of durable virus-resistant cultivars. We aimed to (i) identify plant genetic factors that control *Ne* at the inoculation step, (ii) understand the mechanisms used by the plant to control *Ne* and (iii) compare these genetic factors with other genes controlling virus life cycle and plant resistance durability.

The virus effective population size was measured in a segregating population of 152 doubled-haploid lines of *Capsicum annuum*. Plants were inoculated mechanically either with a *Potato virus Y* (PVY) construct expressing the green fluorescent protein (GFP), or a necrotic variant of *Cucumber mosaic virus* (CMV), the CMV-N strain of Fulton. *Ne* was assessed by counting the number of primary infection foci observed on inoculated cotyledons under UV light for PVY-GFP or the number of necrotic local lesions observed on inoculated leaves for CMV-N.

The numbers of primary infection foci and local lesions were correlated among the doubled-haploid lines (r=0.57) and showed a high heritability (h^2=0.93 and 0.98 for PVY and CMV, respectively). The effective population size of the two viruses was shown to be controlled by both common quantitative trait loci (QTLs) and virus-specific QTLs, indicating the contribution of both general and specific mechanisms. The PVY-specific QTL colocalizes with a QTL that had previously been shown to be involved in PVY accumulation and capacity to break a major-effect resistance gene down.

1. Introduction

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

In the plant, two well-known evolutionary forces act on the frequencies of the different variants composing 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. The two forces act jointly on the viral populations and can have opposite effects on its adaptation. Indeed, if genetic drift is stronger than selection, deleterious mutations may randomly be fixed or advantageous ones may be lost. The strength of genetic drift depends on a key parameter of virus evolution: the effective population size (*Ne*). *Ne* is defined as the number of individuals that pass their genes to the next generation [5] and the strength of the genetic drift is inversely proportional to *Ne*. Through the infection process, the viral population will endure several genetic bottlenecks that will strongly reduce *Ne*, and so, increase the genetic drift [6,7]. Bottlenecks can occur during all the infection steps like vector transmission, virus inoculation into plant cells, replication in infected cells, cell-to-cell or long-distance movements. Although estimation of bottleneck size and their effects on the genetic diversity of the viral population are well documented [8,9], the plant genetic determinants controlling bottleneck size are still unknown. However, studying how plant genetic factors affect *Ne* may contribute to the development of cultivars with durable virus resistance.

In this study, we focus on *Ne* during the inoculation step. We inoculated *Capsicum annuum* plants with two RNA viruses, a *Potato virus Y* (PVY) variant tagged with the green fluorescent protein (GFP) reporter gene and a necrotic variant of *Cucumber mosaic virus* (CMV), the CMV-N strain of Fulton. *Ne* was estimated by visualizing the number of primary infection foci under UV light and counting them for PVY-GFP and by counting the number of necrotic local lesions observed on inoculated leaves for CMV-N, which are two robust approaches to evaluate *Ne* [10,11].We aimed to (i) identify plant quantitative trait loci (QTLs) that control *Ne* at the inoculation step, (ii) understand the mechanisms used by the plant to control *Ne* and (iii) compare these genetic factors with other genes controlling virus life cycle and plant resistance durability.

2. Materials & Methods

2.1. Plant and virus material

A doubled-haploid (DH) population of *C. annuum* was obtained from the F_1 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 [12]. From this population, we phenotyped 152 *DH lines* carrying $pvr2^3$ and differing in their genetic background.

The DH lines were mechanically inoculated with two different viruses. The first one was a variant of the *Potato virus Y* (PVY; genus *Potyvirus*, family *Potyviridae*) isolate SON41p carrying the 115K substitution in the VPg cistron, allowing it to overcome the $pvr2^3$ resistance.

The virus was also tagged with a green fluorescent marker, the green fluorescent protein (GFP) reporter gene. The PVY-GFP was constructed by duplicating the NIa protease cleavage site at the C-terminus of the NIb cistron and inserting the GFP gene between the two sites, allowing the NIa to cleave the GFP. The second one was the CMV-N strain of Fulton, a necrotic variant of *Cucumber mosaic virus* (CMV; genus *Cucumovirus*, family *Bromoviridae*). Finally, 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.

2.2. Counting the primary infection foci and local lesion numbers for PVY and CMV

The effective population size (*Ne*) of PVY and CMV during the inoculation step was estimated by counting the number of primary infection foci on the inoculated cotyledons or leaves. For the PVY variant, we determined this number thanks to the fluorescence of the GFP under UV light. For CMV, an intrinsic property of the Fulton strain is to cause necrotic local lesions on the leaves which correspond to primary infection foci.

The PVY-GFP cDNA clone was first inoculated in *Nicotiana clevelandii* plants by DNA-coated tungsten particle bombardments. In order to obtain the inoculum, extracts of these plants were then used to propagate the virus in *Nicotiana tabacum cv.* Xanthi plants. Finally, ten pepper plants per DH line were mechanically inoculated on their two cotyledons three weeks after sowing. At six days post inoculation (dpi), the number of primary infection foci on each inoculated cotyledon was counted under UV light (450-490 nm) (Figure 1A). All the plants were grown under greenhouse conditions.

The CMV-N strain of Fulton was propagated on *Vinca rosea* plants. From extracts of these plants, ten pepper plants per DH line were mechanically inoculated on their two first leaves three weeks after sowing. At five dpi, the number of necrotic local lesions per inoculated leaf was counted (Figure 1B). The experiment was realized in a climate-controlled room (20–22 °C, 12-h light/day).

We realized an additional experiment to study the link between the number of foci and *Ne*. A 1:1 mixture of PVY-GFP and PVY-mCherry was inoculated in the first leaf of plants belonging to 16 DH lines. The number of foci showing green and/or red fluorescence was then estimated.

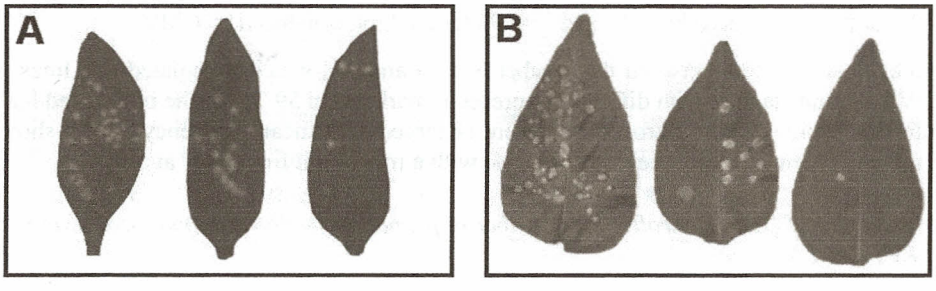

Figure 1:
Illustration of the symptoms obtained after inoculation.
A: Foci of primary infection due to PVY-GFP. B: Local lesions due to CMV-N.

2.3. *Statistical analyses*

The statistical analyses were performed using the R software (http://www.r-project.org/). For the two phenotypic traits, narrow-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 to the environment variance and n to the number of replicates (n=10). An ln(x+1) transformation was applied to the two traits to approximate a normal distribution.

2.4. *QTL analysis*

Quantitative trait loci (QTLs) detection was performed with the R/qtl software package [13]. A preliminary analysis was realized by using a standard interval mapping approach. In addition, a two-dimensional genome scan was done 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 10000 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 a 1-LOD and 2-LOD drop-off method. *The graphical* representation of the QTLs was generated using MAPCHART version 2.3 [14].

3. Results

3.1. *Measure of the primary infection foci and local lesion numbers*

Two populations of 152 DH lines of *C. annuum* were inoculated with two different RNA viruses: the PVY-GFP and the CMV-N strain of Fulton. The effective population size at the inoculation step was assessed by quantifying the number of primary infection foci and local lesions on cotyledons or leaves respectively inoculated by PVY and CMV. After applying log transformation to the data, the number of primary infection foci ranged from 0.77 to 3.8 with a mean number of 2.64±0.67 (mean±sd) (Figure 2A). The number of local lesions varied from 0.14 to 4.54 with a mean number of 2.61±1.14 (Figure 2B). The two variables were well correlated among the doubled-haploid lines (Pearson r=0.57, *p-value* < 2.2e-16). They also both shown a high heritability with h^2=0.93 for the foci induced by PVY and h^2=0.98 for the lesions induced by CMV.

To evaluate the link between the number of foci and *Ne*, we co-inoculated DH lines with two PVY variants tagged with different fluorescent markers. In 59.7% of the inoculated leaves, no infection foci with dual fluroescences were observed. The mean frequency of foci showing both red and green fluorescences was of 0.9%, with a maximum frequency at 5.3%.

3.2. *Detection of QTLs controlling the number of primary infection foci and local lesions for PVY and CMV*

MQM was performed and three QTLs were detected for each virus (Table 1, Figure 3). They were named PVY-6, PVY-7, PVY-12 and CMV-6, CMV-7, CMV-12 according to the virus used for the inoculation and the chromosome location. 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, the analyses revealed a significant epistatic interaction between the QTLs on

chromosomes 7 and 12. The Perennial allele decreased the trait value for all QTLs, except 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.

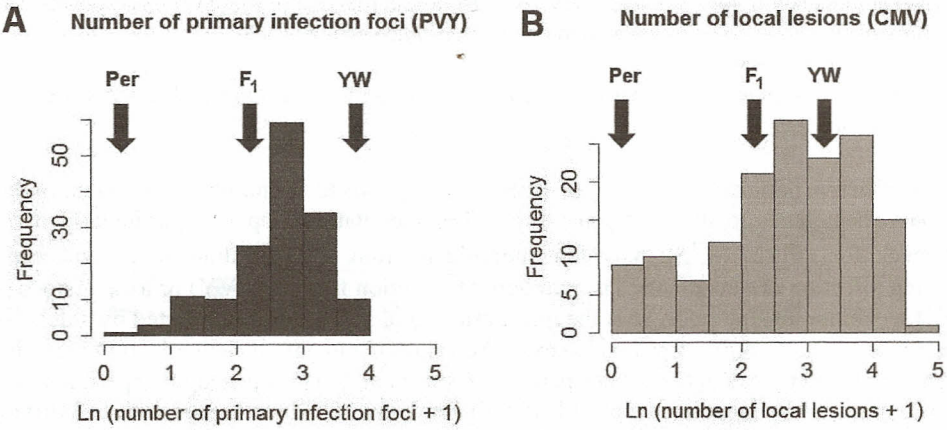

Figure 2:
Histograms of the frequency distribution of the DH lines for (A) the number of primary infection foci caused by PVY-GFP and (B) the number of local lesions caused by CMV-N. The position of the parents and the F_1 are indicated. Per: Perennial and YW: Yolo Wonder.

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 x 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 x CMV-12	7/12	-	HpmsE114/ HpmsE128	5.77	-	9.38		-1.397	

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

4. Discussion

The breakdown of plant resistance genes is a major threat to the genetic control of crop diseases. Since the durability of the resistance relies on the evolution of the pathogen, a better

understanding of the evolutionary constraints imposed by the plant to the pathogen and of the underlying genetic factors can improve our management of crop diseases. In this study, we identified and mapped pepper QTLs controlling a key parameter of virus evolution, the effective population size (*Ne*) at inoculation, for PVY and CMV. We also found that one of these QTLs colocates with a QTL previously shown to be implicated in pepper resistance against PVY.

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

The effective population size (*Ne*) of viruses corresponds to the number of virus individuals that pass their genes to the next generations. The inoculation step is a particularly narrow bottleneck for viruses [8,15]: only few individuals from the inoculum source succeed in initiating infection of new plants. The numbers of infection foci (for PVY) or local lesions (for CMV) are minimum value for *Ne* at the inoculation step, since they are initiated by at least one virus particle. They would correspond to exact *Ne* values if and only if each infection focus/local lesion is initiated by exactly one virus particle. Zwart et al. [11] proposed an experimental test of this hypothesis by inoculated a plant leaf with a mixture of viruses tagged with two different fluorescent proteins, GFP and mCherry, that can be visualized by green and red fluorescence, respectively. Infection foci showing both a green and a red fluorescence were initiated by the two virus variants.

We performed a similar experiment with our pathosystem by co-inoculating PVY-GFP and PVY-mCherry in plants corresponding to 16 DH lines. We found that the frequency of foci showing both green and red fluorescence was very low, with a mean number of 0.9%. Thus, we could conclude that the huge majority of foci were initiated by a single virus particle and that the number of primary infection foci was a highly precise estimation of *Ne*. The same approach could not be undertaken for CMV. However, the timing and development of CMV local lesions are similar to those of PVY infection foci and certainly correspond to the same processes of infection initiation followed by cell-to-cell movement. The only difference is the elicitation of plant defenses by CMV leading to necrosis of the infection foci. This suggests that the number of local lesions is also a precise estimation of *Ne*.

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

For each virus, we identified three QTLs involved in *Ne* at inoculation and localized on chromosomes 6, 7 and 12 (Table 1, Figure 3). The QTLs on chromosome 7 (PVY-7 and CMV-7) were detected at the same location on the genome (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 2 QTLs overlap largely. 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 22.26% and 21.67% of the trait variation. We also found that, for both viruses, there was epistasis between the QTLs on chromosomes 7 and 12. Therefore, the same QTLs on chromosomes 7 and 12 control *Ne* for PVY and CMV and the same genetic factor may be responsible for this dual effect. In contrast, 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. Besides, QTL effects shown opposite directions since Perennial allele decreased the value of the trait for PVY-6 and increased its value for CMV-6.

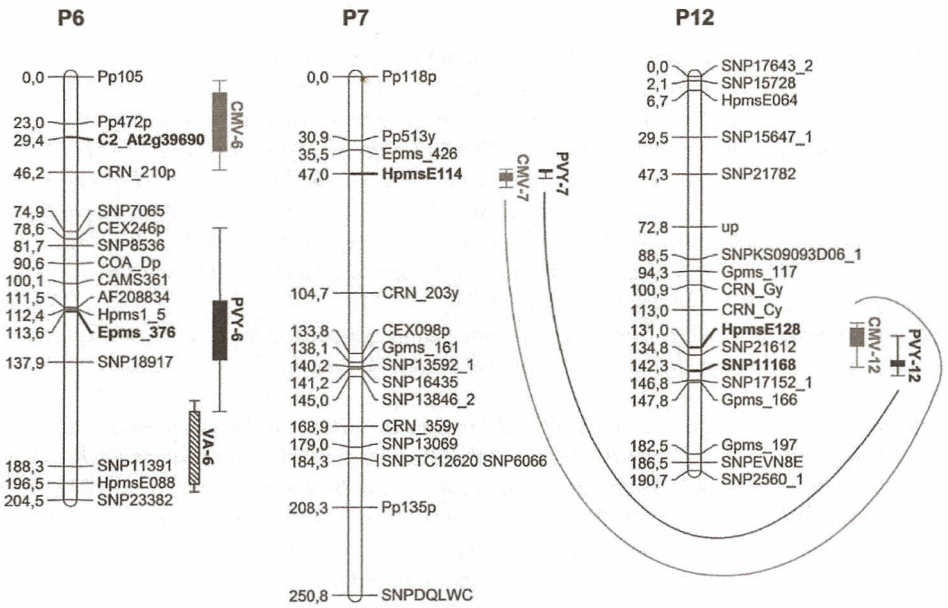

Figure 3

QTL map for the effective population size at the inoculation step for PVY (black) and CMV (gray). 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.

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 are under this trait. However, we also found one specific QTL for each virus, demonstrating that virus-specific mechanisms also act.

4.3. Relationship between QTLs of effective population size and the plant resistance

With the same DH population as the one we used, Quenouille et al. [12] identified QTLs controlling the durability of the major PVY resistance gene *pvr2*^{3} and PVY accumulation. They notably mapped a QTL on chromosome 6, named VA-6, which affects the PVY accumulation (Figure 3). The confidence interval of VA-6 includes a part of the confidence interval of PVY-6, although the two QTLs are not exactly localized at the same position. Interestingly, VA-6 shown epistatic interaction with RB-3, a QTL controlling the virus capacity to break the *pvr2*^{3} resistance down. We could make the hypothesis that VA-6 and PVY-6 are linked or belong to the same locus, and so that PVY-6 might contribute to increase the resistance durability. This hypothesis could be valid because the Perennial allele at QTL PVY-6 decreases *Ne* while at QTL VA-6 it increases resistance durability. Indeed, by reducing the effective population size, the allele could help to lose well-adaptated virus variants by genetic drift at inoculation, therefore increasing resistance durability.

Our study demonstrated the existence of plant genetic factors capable of controlling pathogen evolution and which could slow down their adaptation. From an agricultural point of

view, the use of these factors could be even more beneficial because our results suggest that they could induce general mechanisms and therefore act against multiple pathogens.

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