Virus Evolution, 2017, 3(2): vex033

doi: 10.1093/ve/vex033
Research article

Virulence evolution of a sterilizing plant virus: Tuning multiplication and resource exploitation

Viji Vijayan,† Silvia López-González,† Flora Sánchez, Fernando Ponz, and Israel Pagán*,‡

Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Autopista M-40, km 38, Campus Montegancedo, Pozuelo de Alarcón, 28223 Madrid, Spain

*Corresponding author: E-mail: jesusisrael.pagan@upm.es

†These authors contributed equally to this work.

‡http://orcid.org/0000-0001-8876-1194

Abstract

Virulence evolution may have far-reaching consequences for virus epidemiology and emergence, and virologists have devoted increasing effort to understand the modulators of this process. However, still little is known on the mechanisms and determinants of virulence evolution in sterilizing viruses that, as they prevent host reproduction, may have devastating effects on host populations. Theory predicts that sterilizing parasites, including viruses, would evolve towards lower virulence and absolute host sterilization to optimize the exploitation of host resources and maximize fitness. However, this hypothesis has seldom been analyzed experimentally. We investigated the evolution of virulence of the sterilizing plant virus *Turnip mosaic virus* (TuMV) in its natural host *Arabidopsis thaliana* by serial passage experiments. After passaging, we quantified virus accumulation and infectivity, the effect of infection on plant growth and development, and virulence of the ancestral and passaged viral genotypes in *A. thaliana*. Results indicated that serial passaging increased the proportion of infected plants showing absolute sterility, reduced TuMV virulence, and increased virus multiplication and infectivity. Genomic comparison of the ancestral and passaged TuMV genotypes identified significant mutation clustering in the P1, P3, and 6K2 proteins, suggesting a role of these viral proteins in the observed phenotypic changes. Our results support theoretical predictions on the evolution of virulence of sterilizing parasites and contribute to better understand the phenotypic and genetic changes associated with this process.

Key words: virulence evolution; Turnip mosaic virus; *Arabidopsis thaliana*; host resource exploitation.

1. Introduction

Virulence is an intrinsic property of parasites, defined as the deleterious effects of infection on the host fitness (Read 1994). Changes in virulence have been associated with modifications of parasite epidemiology and host–parasite co-evolutionary dynamics, with biodiversity loss and local extinction of host populations, and disease emergence and re-emergence (Bull 1994; Malmstrom et al. 2006; Little et al. 2010; Berngruber et al. 2013). Thus, explaining virulence evolution is fundamental to understand the life history of parasites and may be of socio-economic relevance given the significant impact of parasites on human, animal, and plant welfare (Dieckman et al. 2002). Most theoretical elaborations on the evolution of virulence derive from the *trade-off* hypothesis. According to this hypothesis, maximal parasite fitness would result from optimizing the within-host multiplication and the between-host transmission

components of parasite fitness (Anderson and May 1982; Ebert and Bull 2003; Cressler et al. 2016). Two key assumptions underlie the *trade-off* hypothesis: greater parasite load in an infected host increases the probability of transmission to a susceptible host; and parasite load is positively correlated with virulence measured as increased host mortality. A trade-off occurs because higher virulence, by reducing host longevity, reduces the infectious period and the probability of transmission. Thus, the *trade-off* hypothesis predicts that parasites would evolve to intermediate levels of virulence (Anderson and May 1982; Ebert and Bull 2003; Cressler et al. 2016). However, not every parasite may necessarily play with these rules. This is the case of sterilizing parasites, defined as those whose infection results in draining of host reproductive resources, eventually leading to castration (Lafferty and Kuris 2009). Reproduction draws energy away from survival, so by lessening host reproduction parasites can keep their host alive longer. This reduces parasite fitness costs associated with increased host mortality (Obrebski 1975) and allows the storage of sterilization-liberated resources into host growth until the parasite can exploit them (Ebert et al. 2004). Thus, modifications of the *trade-off* hypothesis to accommodate sterilizing parasites predict that they would maximize fitness at absolute sterilization (maximum amount of host resources dedicated to host survival), and at lower virulence (reduced mortality), provided that they are not vertically transmitted (Jaenike 1996; O'Keefe and Antonovics 2002; Ebert et al. 2004; Hall et al. 2007).

Sterilizing parasites have been described in a wide range of animal (Lafferty and Kuris 2009) and plant (Clay 1991; Clay and Kover 1996) hosts. As they prevent host reproduction, the effect of this type of parasites in host populations can be enormous. For instance, at high prevalence they may impose strong selection pressures leading to selection for resistant host genotypes (Lafferty and Kuris 2009). In addition, various mathematical models predict that, in the absence of resistance, higher prevalence of sterilizing parasites would reduce host population density (Antonovics 2002; Negovetich and Esch 2008; Lafferty and Kuris 2009). Moreover, if infection affects the recruitment capacity of the host population, preventing migration of host individuals from other populations with lower or no parasite burden, sterilizing parasites may even cause the local extinction of the host populations. These predictions are supported by several empirical analyses (reviewed by Lafferty and Kuris 2009). Despite the great potential of sterilizing parasites to affect the population dynamics of their hosts, experimental analyses on the evolution of virulence of such parasites are scarce. Evidence supporting predictions of the *trade-off* hypothesis (reduced mortality) comes from a handful of studies in parasite–invertebrate interactions (Jaenike 1996; Sorensen and Minchella 2001; Ebert et al. 2004; Jensen et al. 2006). Equivalent studies in plants are even scarcer and limited to fungal parasites (Burdon 1991; Kover 2000; Sloan et al. 2008). Remarkably, the predictions of the *trade-off* hypothesis for sterilizing parasites have not been tested for intracellular parasites such as viruses.

We analyzed the evolution of virulence of a sterilizing virus utilizing *Turnip mosaic virus* (TuMV) as a model. TuMV is a member of the *Potyviridae*, the largest family of plant viruses, and an economically important pathogen of *Brassica* species and other crops worldwide. TuMV is transmitted by around 89 aphid species and has a wide host range that includes more than 300 species of twenty dicotyledonous plant families (Walsh and Jenner 2002). One such host is *Arabidopsis thaliana* (Brassicaceae), whose natural populations are commonly infected by TuMV (Pagán et al. 2010). TuMV infection in *A. thaliana* affects many plant developmental traits, including flower and silique viability, which may severely affect plant fertility and eventually lead to sterility (Walsh and Jenner 2002; Sánchez et al. 2015). Thus, the interaction TuMV–*A. thaliana* represents a suitable system to analyze the evolution of virulence of a sterilizing virus. TuMV has a tubular flexuous capsid, which encapsidates a monopartite single-stranded RNA genome of approximately 10,000 nucleotides. The genome is characterized by a single major open reading frame (ORF) encoding a large polyprotein that is processed into ten functional proteins: the first protein (P1), helper component protease (HC-Pro), third protein (P3), 6K1, cylindrical inclusion protein (CI), 6K2, viral protein genome-linked (VPg), small nuclear inclusion protein (NIa), large nuclear inclusion protein (NIb), and coat protein (CP). Two additional proteins, P3N-PIPO and P3N-ALT, are originated through frameshifts in the P3 cistron (Chung et al. 2008; Hagiwara-Komoda et al. 2016). The small size of TuMV genome facilitates mapping mutations arising during virus evolution.

Here, we analyzed TuMV virulence evolution in *A. thaliana* through serial passages of virus horizontal transmission in this host. In particular, we compared virus multiplication, infectivity, and the effect of infection on plant growth, reproduction, development, and mortality of the passaged and ancestral virus genotypes. We also explored which viral genes may potentially be associated with the observed phenotypic changes by analyzing the spatial distribution of mutations arisen during serial passages.

2. Materials and methods

2.1 Viral genotypes and host species

Genotype JPN1–TuMV (from here on 'JPN1'; genomic sequence: Acc. N. KM094174; Sánchez et al. 2015) and JPN1A–TuMV (from here on 'JPN1A'; genomic sequence: Acc. N. KX377967; this work) were used. The viral genotype JPN1, obtained from a field-infected plant of Radish (*Raphanus sativus*, Brassicaceae), was subjected to sixty passages of horizontal transmission in *A. thaliana* accession Col-0, with three replicated lines/lineages per passage. For serial passaging, JPN1-infected Radish tissue was used for mechanical inoculation of three batches of ten Arabidopsis Col-0 plants that had five to six rosette leaves (6-day-old plants), such that each batch of plants initiated a virus lineage. Fifteen days post-inoculation (i.e. 20-day-old plants), leaf tissue from plants of each batch of JPN1-infected Arabidopsis plants was collected, pooled, and used for mechanical inoculation of three new batches of Arabidopsis Col-0 plants. This means that tissue was collected close to the end of the Arabidopsis reproductive period, which range between 22 and 25 days (Supplementary Table S1). The procedure was repeated until the sixty passages were completed, thus generating three serially passaged JPN1A lineages. In the initial passage, four to five plants per lineage did not survive to the next serial passage event. In the following 10 passages, between two and five plants per lineage (average = 3) were lost. Afterwards, all plants reached the 20 days of age at which samples were collected.

Arabidopsis thaliana Col-0 (from here on 'Arabidopsis') and Indian mustard (*Brassica juncea*, from here on 'Mustard') were selected as hosts. Arabidopsis was selected as the host in which JPN1 was serially passaged. Radish, the host from which JPN1 was isolated, has a high degree of self-incompatibility (Kitashiba and Nasrallah 2014). This prevented accurately monitoring Radish reproductive traits and lifespan in our greenhouse conditions, such that a meaningful measure of TuMV virulence could not be obtained. Thus, we selected Mustard as a reference

host because: (1) it is the closest relative of Radish [both plant species belong to the same monophyletic group defining the Brassiceae tribe (Bailey et al. 2006)], which is self-compatible (Kitashiba and Nasrallah 2014), (2) TuMV infection has not been reported to cause sterility in Radish nor in Mustard and induces similar symptoms in both hosts (Supplementary Fig. S1), and (3) JPN1 and JPN1A multiplication and infectivity are similar in Radish and Mustard (Supplementary Fig. S1). Plant seeds were stratified for 7 days at 4 °C in pots of 15 cm of diameter, 0.43 l volume for Arabidopsis and in pots of 15 cm of diameter, containing 3:1 peat:vermiculite mix. Afterwards, pots were moved for seed germination and plant growth to a greenhouse at 22 °C, under 120–150 μmol $m^{-2}s^{-1}$ light intensity, and long day conditions (16 h light) with 65–70 per cent relative humidity. Seed germination percentages were of 95–100 per cent for both plant species.

2.2 Experimental design

All plants were mechanically inoculated by rubbing 200 μl of a preparation containing 0.2 g of TuMV-infected plant tissue ground in 2 ml of 0.1 M Na_2HPO_4+0.5 M NaH_2PO_4+0.02 per cent DIECA, or by rubbing 200 μl of inoculation buffer in the case of mock-inoculated plants, onto the surface of three leaves per plant. Carborundum was used as abrasive. Inoculations were done when plants had five to six rosette leaves. After inoculation all individuals were randomized in the same greenhouse. Two batches of simultaneously inoculated plants were utilized. Batch 1 was used to analyze TuMV multiplication and virulence, effect of virus infection on plant growth and reproduction, and effect on plant development and consisted in sixty Arabidopsis and sixty Mustard plants. For each host species, twenty plants were inoculated with JPN1, twenty with JPN1A, and twenty plants were mock inoculated. At least six plants per JPN1A lineage were inoculated. Batch 2 was used to analyze the kinetics of TuMV multiplication during the course of infection and consisted of 100 Arabidopsis and 100 Mustard plants. Half of the plants of each host were inoculated with JPN1 and the other half with JPN1A. Every 2 days between 2 and 20 dpi, five plants of each treatment were sampled for quantification of viral RNA accumulation. Raw data of all the measures recorded in both plant batches is provided in the Supplementary dataset.

2.3 Quantification of TuMV multiplication

TuMV multiplication was quantified as viral RNA accumulation via Quantitative real-time-PCR (qRT-PCR). For each plant, four leaf disks of 4 mm in diameter from four randomly chosen systemically infected leaves were collected. Total RNA extracts were obtained using TRIzol® reagent (Life Technologies, Carlsbad, CA) followed by a phenol–chloroform extraction, and 0.32 ng of total RNA were added to the Brilliant III Ultra-Fast SYBR Green qRT-PCR Master Mix (Agilent Technologies, Santa Clara, CA) according to manufacturer's recommendations. For both TuMV genotypes, the TuMV CP primers described by Lunello et al. (2007) were used to amplify a 70 nucleotides fragment of the viral CP gene. Thermal parameter for qRT-PCR amplifications were programmed as: RT at 65 °C for 3′, 50 °C for 10′, 95 °C for 5′′, and forty PCR cycles at 95 °C for 10′′, 60 °C for 20′′, 60 °C for 30′′, 95 °C for 30′′, 60 °C for 1′′. Each plant sample was assayed by triplicate on a Light Cycler 480 II real-time PCR system (Roche, Indianapolis, IN). Absolute viral RNA accumulation was quantified as ng of viral RNA/μg of total RNA utilizing internal standards consisting in a 10-fold dilution series of RNA transcripts of the same 70 nucleotides TuMV CP fragment amplified in the qRT-PCR (from 2×10^{-3} to 2×10^{-7} ng). RNA transcripts were derived from a plasmid containing the 70 nucleotides fragment from the JPN1–TuMV isolate. Viral RNA was detected in every inoculated plant, indicating that the JPN1 and JPN1A inocula did not shown differences in infectivity.

2.4 Estimation of the effect of TuMV infection on plant growth and reproduction

Above ground plant structures were harvested at complete senescence and dry weight was determined after maintaining plants at 65 °C until constant weight. The weights of the rosette (*RW*), inflorescence (*IW*), and seeds (*SW*) were obtained separately. Importantly, the viability of seeds from infected plants, estimated as per cent germination, did not significantly differ between mock-inoculated (98.5%) and TuMV-infected (95.5%) plants ($\chi^2 \leq 0.81$, $P \geq 0.445$). In addition, TuMV infection did not affect the weight of a single seed ($F \leq 1.03$, $P \geq 0.387$). Thus, *SW* similarly reflects the number of viable seeds in both mock-inoculated and infected plants. Following Thompson and Stewart (1981), rosette weight was used as an estimate of plant resources dedicated to growth, and inflorescence and seed weights were taken as an estimate of plant resources dedicated to reproduction. In addition, other traits related to plant growth and reproduction were recorded: rosette diameter in cm (*RD*) and number of rosette leaves (*RL*) were measured every 2 days from inoculation to the beginning of flower production. Using data on *RD*, rosette relative growth (*RRG*) was measured as the average difference in *RD* between every two consecutive time points until maximum *RD*. Also, inflorescence size (*IS*) as height in cm and ramification degree (*RA*), as the presence of secondary, tertiary, and quaternary branches in the inflorescence, were measured every 2 days from plant bolting to senescence. To quantify the effect of TuMV infection on all these traits, infected to mock-inoculated plants ratios were obtained for each of them by dividing the value of each infected plant by the mean value of the mock-inoculated plants of the same host ($Trait_i/Trait_m$, i and m denoting infected and mock-inoculated plants). For comparison purposes between Arabidopsis and Mustard, values of trait ratios are generally presented in the text. Average values of the raw variables can be found in Supplementary Table S1.

2.5 Estimation of the effect of infection on plant development and of TuMV virulence

To analyze the effect of infection on plant development, two host life-history traits were recorded: growth period (*GP*), as the time in days from inoculation to the opening of the first flower; and reproductive period (*RP*), as the time in days from the opening of the first flower to the shattering of the first silique. Importantly, in Arabidopsis the opening of the first flower co-occurs with the end of the rosette growth, and the shattering of the first silique co-occurs with the end of flower production (Boyes et al. 2001). TuMV virulence was measured as the effect of virus infection on plant mortality. For that, plant life period (*LP*), as the time from inoculation to plant senescence, was recorded. Again, the effect of TuMV infection on growth period, reproductive period, and life period was quantified as infected to mock-inoculated plants ratios. Supplementary Table S1 summarizes the duration of each developmental stage in mock-inoculated and infected plants.

2.6 Quantification of TuMV infectivity

Initial JPN1 and JPN1A RNA concentrations of 20 ng/µl were diluted at 1/10, 1/50, 1/100, 1/300, 1/600, and 1/1,000. Twenty microliters of each dilution were inoculated in three rosette leaves of Arabidopsis and Mustards plants. Twenty-five replicated inoculations were done per treatment in a fully randomized design. At 15 dpi, total plant RNA from each plant was extracted and TuMV presence was detected by qRT-PCR as described above.

2.7 Statistical analysis

Analyzed traits and their ratios were normally distributed and homoscedastic (variances were homogeneous), and therefore differences between virus genotypes and hosts were analyzed using General Linear Models (GLMs). Exceptions were virus RNA accumulation and raw values and infected to mock-inoculated plants ratios of the number of rosette leaves (*RL*) in the two utilized hosts, and differences in these traits between TuMV isolates and/or between hosts were analyzed by Generalized Linear Models (GzLMs). Given that these variables were positively skewed, we fitted GzLMs to a Gamma distribution. Similar analyses using the raw variables led to the same conclusions (Supplementary Table S2). The three lineages of JPN1A yielded similar values for all the analyzed traits (Supplementary Table S3), and lineage was not considered as a factor in our analyses. Infectivity to virus concentration curves were compared using analysis of variance to test the equality of slopes and intercepts. Differences between classes were analyzed via Least Significant Difference tests. Differences in the percentage of sterile plants were analyzed using Fisher exact tests, with Yates's correction when necessary. Statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL).

2.8 Sequence of JPN1A

The complete nucleotide sequence of the genomic RNA was determined by Immunocapture-RT-PCR (IC-RT-PCR) using a proofreading DNA polymerase (Pfu; Biotools M&B Labs SA, Madrid, Spain). At least two clones of each IC-RT-PCR fragment were sequenced. Primer pairs were designed to produce ten fragments such that adjacent fragments overlapped by at least 70 nucleotides. Primer sequences and their locations in the JPN1A genome are summarized in Supplementary Table S4. The amplified fragments were cloned in the Zero Blunt® vector with the TOPO® PCR Cloning Kit (ThermoFisher Scientific, Waltham, MA) and sequenced. These sequences were assembled eliminating the amplification primers using Sequencher v5.4.1 (Gene Codes Corp., Ann Arbor, MI), revealing 99–100 per cent nucleotide identity in all cases. Similar nucleotide identity was found in overlapping regions of adjacent fragments. Only mutations present in all lineages were considered in genomic analyses.

2.9 Genomic analyses

The JPN1 and JPN1A genomic sequences were aligned using MUSCLE 3.8 (Edgar 2004) and were compared between them using MEGA 7 (Kumar et al. 2016). To explore which of the viral genes could be associated with the phenotypic changes observed after serial passaging, we analyzed which of these genes were under positive selection through analyses of mutation clusters in the viral genome. Given that we only had the JPN1 and the JPN1A genomes for our comparisons, we chose this approach as it allowed estimating which genes were under positive selection using only a pair of sequences (Wagner 2007). We used a bootstrap method to infer whether mutations were clustered spatially across the TuMV genome compared with a null model, which assumed random mutation placement following a Poisson distribution. Bootstrap distributions and null distributions were calculated for the index of dispersion statistic and then compared using a Mann–Whitney *U* test (R Development Core Team 2011) as described by Simmons et al. (2012).

3. Results

3.1 Effect of TuMV infection on plant growth and reproduction

We quantified the effect of TuMV infection on Arabidopsis and Mustard growth as infected to mock-inoculated plants ratios of rosette weight (RW_i/RW_m), and the effect of virus infection on plant reproduction as inflorescence and seed weight ratios (IW_i/IW_m and SW_i/SW_m, respectively). The effects of virus infection on other traits related to rosette growth (rosette diameter and number of leaves, and relative rosette growth) and to inflorescence growth (inflorescence size and degree of ramification) were also analyzed.

Upon TuMV infection, reduction of rosette weight depended on the viral genotype ($F_{1,71} = 3.16$; $P = 0.046$), the host species ($F_{1,71}$=9.94; $P = 2\text{x}10^{-3}$), and the interaction between both factors ($F_{1,71} = 3.34$; $P = 0.044$). The effect of TuMV infection on rosette weight (RW_i/RW_m) was greater in Mustard than in Arabidopsis (Table 1). In addition, rosette weight was smaller in JPN1- than in JPN1A-infected plants due to differences in Arabidopsis: the effect of infection was significantly higher in JPN1- than in JPN1A-infected Arabidopsis ($F_{1,36} = 3.61$; $P = 0.053$, Table 1), the rosette weight of JPN1A-infected plants being similar to that of mock-inoculated ones ($F_{2,37} = 0.02$; $P = 0.893$, Supplementary Tables S1 and S2); whereas such difference was not observed in Mustard ($F_{1,35} = 0.01$; $P = 0.930$, Table 1). The effect of virus infection on the number of rosette leaves depended on the host species ($\chi^2_{w(1,71)} = 42.88$; $P < 1 \times 10^{-3}$) and the viral genotype ($\chi^2_{w(1,71)} = 9.84$; $P = 2 \times 10^{-3}$), but not on the interaction between factors ($\chi^2_{w(1,71)} = 0.12$; $P = 0.725$). The effect of TuMV infection on the number of rosette leaves was higher in Arabidopsis than in Mustard plants (Table 1). Differences between viral genotypes were again due to the effect of infection in Arabidopsis. The effect of infection on the number of rosette leaves was higher in JPN1- than in JPN1A-infected plants (Table 1), the number of rosette leaves in infected plants being always smaller than in mock-inoculated ones ($\chi^2_{w(2,37)} = 7.17$; $P = 7 \times 10^{-3}$, Supplementary Tables S1 and S2). The opposite was observed in Mustard, which showed higher effect of infection in the number of rosette leaves for JPN1A- than for JPN1-infected plants ($\chi^2_{w(1,35)} = 4.10$; $P = 0.043$, Table 1), with JPN1-infected Mustard having similar number of rosette leaves than mock-inoculated ones ($\chi^2_{w(2,35)} = 1.35$; $P = 0.245$, Supplementary Tables S1 and S2). Finally, the effect of virus infection on the rosette diameter and the relative rosette growth depended on the host species ($F_{1,71} \geq 4.81$; $P \leq 0.032$), such that it was higher in Arabidopsis than in Mustard (Table 1), but not on the viral genotype ($F_{1,71} \leq 3.56$; $P \geq 0.063$) or on the interaction between factors ($F_{1,71} \leq 0.23$; $P \geq 0.632$). Indeed, no significant differences in both traits between JPN1- and JPN1A-infected plants were found in any host ($F \leq 3.30$; $P \geq 0.078$) (Table 1). In Arabidopsis, infected plants showed shorter rosette diameters and slower rosette growth rates than mock-inoculated ones ($F_{2,55} \geq 4.11$; $P \leq 0.022$,

Table 1. Effect of JPN1 and JPN1A infection on growth and reproduction, development and mortality of Arabidopsis and Mustard.[a]

Category	Trait[b]	Arabidopsis		Mustard	
		JPN1	JPN1A	JPN1	JPN1A
Plant growth	*RW*	0.66±0.11	1.02±0.16	0.53±0.04	0.52±0.04
	RL	0.60±0.02	0.70±0.03	0.93±0.05	0.81±0.03
	RD	0.79±0.03	0.77±0.02	0.93±0.03	0.90±0.03
	RRG	0.75±0.07	0.86±0.06	0.84±0.06	1.02±0.08
Plant reproduction	*IW*	0.13±0.05	0.07±0.02	0.68±0.08	0.64±0.07
	IS	0.20±0.03	0.18±0.04	1.02±0.11	0.91±0.14
	RA	0.67±0.04	0.69±0.05	0.80±0.09	0.80±0.08
	SW	0.14±0.04	0.01±0.00	0.46±0.05	0.47±0.06
	(*IW*+*SW*)	0.14±0.05	0.05±0.02	0.55±0.05	0.57±0.05
Plant development	*GP*	0.90±0.06	1.31±0.11	0.97±0.07	0.98±0.05
	RP	0.80±0.07	0.73±0.17	0.70±0.09	0.72±0.09
Virulence	*LP*	0.55±0.05	0.68±0.04	0.90±0.02	0.88±0.03

[a]For each host–virus combination, data are the mean of the ratios of the trait value for twenty infected plants divided by the mean value of twenty repetitions of the mock-inoculated controls.

[b]*RW*, rosette weight; *RD*, rosette diameter; *RL*, number of rosette leaves; *RRG*, relative rosette growth; *IW*, inflorescence weight; *IS*, inflorescence size; *RA*, ramification degree; *SW*, seed weight; *GP*, growth period; *RP*, reproductive period; *LP*, life period.

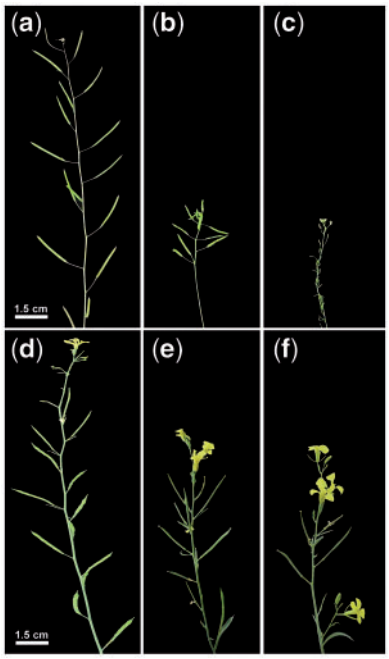

Figure 1. Effect of TuMV infection in Arabidopsis and Mustard silique formation. Inflorescences from Arabidopsis mock-inoculated plants (a), JPN1- and JPN1A-infected plants that produced seeds (b), and sterilized JPN1- and JPN1A-infected plants (c), plus Mustard inflorescences of mock-inoculated (d), JPN1- (e), and JPN1A-infected (f) plants are shown.

Supplementary Tables S1 and S2). In Mustard, this was so only for rosette diameter ($F_{2,54}=3.61$; $P=0.034$), being the relative rosette growth of infected plants similar to that of mock-inoculated ones ($F_{2,54}=3.61$; $P=0.137$, Supplementary Tables S1 and S2). Thus, the differential effect of the TuMV genotypes on Arabidopsis rosette weight was associated with a differential reduction in the number of rosette leaves but not in rosette diameter or relative rosette growth.

The effect of TuMV infection on inflorescence weight (IW_i/IW_m) depended on the host species ($F_{1,71}=96.30$; $P<1\times10^{-4}$), but not on the viral genotype ($F_{1,71}=0.28$; $P=0.601$) or on the host–virus interaction ($F_{1,71}=0.66$; $P=0.419$). The reduction of inflorescence weight in TuMV-infected plants was higher in Arabidopsis than in Mustard (Table 1), and within the same host both viral genotypes similarly reduced inflorescence weight as compared with mock-inoculated plants ($F\geq8.90$; $P<1\times10^{-4}$, Supplementary Tables S1 and S2). Similar results were obtained for inflorescence size (IS_i/IS_m) ($F_{1,71}=64.75$; $P<1\times10^{-3}$ and $F_{1,71}=0.29$; $P=0.592$, for host species and viral genotype, respectively) (Table 1), except that there was no effect of TuMV infection on Mustard inflorescence size ($F_{2,54}=0.46$; $P=0.635$, Supplementary Tables S1 and S2). Neither host species nor viral genotype affected inflorescence ramification degree ($F_{1,71}\leq3.19$; $P\geq0.078$) (Table 1). In both hosts, ramification degree was smaller in TuMV-infected than in mock-inoculated plants ($F\geq3.94$; $P\leq0.025$, Supplementary Tables S1 and S2). Although all Arabidopsis plants produced inflorescences and siliques, in many infected plants all siliques aborted such that they did not produce seeds (Fig. 1 and Supplementary Table S1) and these plants were sterile. The percentage of sterile plants was higher in JPN1A- than in JPN1-infected Arabidopsis (10/20 *vs.* 18/20, respectively, $\chi^2_{1,71}=5.83$; $P=0.016$). On the other hand, all Mustard plants were fertile (Supplementary Table S1). The effect of TuMV infection on seed weight (SW_i/SW_m) varied between hosts species ($F_{1,71}=84.65$; $P<1\times10^{-4}$), being higher in Arabidopsis than in Mustard plants (Table 1). In both hosts, infected plants always produced less seeds than mock-inoculated ones ($F\geq15.61$; $P<1\times10^{-4}$) (Supplementary Tables S1 and S2). Differences on SW_i/SW_m according to virus genotype ($F_{1,\ 71}=4.36$; $P=0.029$) were also found. These differences were due to the effect of TuMV infection on Arabidopsis, for which the reduction of seed weight was higher in JPN1A- than in JPN1-infected plants ($F_{1,\ 36}=9.85$; $P=0.003$), whereas such difference was not observed in Mustard plants ($F_{1,\ 35}=0.01$; $P=0.923$) (Table 1). Thus, JPN1A-infected Arabidopsis had higher sterilization rates and produced less seeds than JPN1-infected ones.

To test whether TuMV infection differentially affected plant growth (rosette weight) and reproduction (inflorescence and seed weights), a GLM analysis including host species, virus genotype, and plant part (rosette or inflorescence + seeds) as factors, and the effect of infection in these plant parts ($Trait_i/Trait_m$) as a response variable was done. Results indicated that the effect of infection on plant growth was smaller than on plant reproduction [RW_i/RW_m: 0.69 ± 0.05 *vs.* $(IW+SW)_i/(IW+SW)_m$: 0.32 ± 0.03; $F_{1,142}=43.47$; $P<1\times10^{-4}$], with this differential effect depending on the host × plant part interaction ($F_{1,142}=52.00$; $P<1\times10^{-4}$). Indeed, in Arabidopsis both virus genotypes caused a greater reduction in reproduction than in growth ($F_{1,36}\geq21.23$; $P<1\times10^{-4}$), whereas no such difference was observed in Mustard ($F_{1,36}\leq0.52$; $P\geq0.478$) (Table 1). These analyses suggested that TuMV-infected Arabidopsis, but not Mustard, plants tended to devote higher proportion of resources to growth than to reproduction. To confirm this observation, we calculated the balance between resources dedicated to growth and reproduction in mock-inoculated, JPN1- and JPN1A-infected plants, measured as the $(IW+SW)/RW$ ratio (Fig. 2). In Arabidopsis, this ratio was significantly higher in mock-inoculated than in JPN1-infected plants (12.55 ± 1.83 *vs.* 2.84 ± 1.03, $F_{1,39}=20.81$; $P<1\times10^{-4}$), and in JPN1- than in JPN1A-infected plants (2.84 ± 1.03 *vs.* 0.71 ± 0.21, $F_{1,39}=4.12$; $P=0.050$) (Fig. 2a). This indicated that mock-inoculated plants devoted the majority of resources to reproduction, whereas infection by

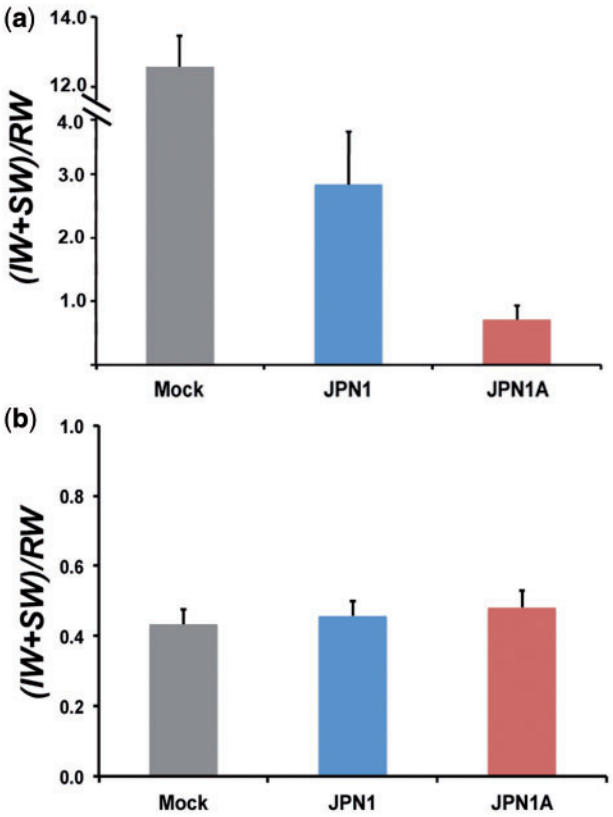

Figure 2. Effect of TuMV infection on plant resource allocation. Distribution of Arabidopsis (a) and Mustard (b) resources between growth (as rosette weight, *RW*) and reproduction (as inflorescence + seeds weights, *IW* + *SW*) in mock-inoculated (grey), JPN1 (blue), and JPN1A (red) infected plants.

JPN1 tended to equilibrate the resources allocation, and JPN1A-infected plants devoted more resources to growth than to reproduction. These differences were not observed in Mustard (*IW*/*RW*: 0.43 ± 0.04, 0.46 ± 0.04 and 0.48 ± 0.05, for mock-inoculated, JPN1- and JPN1A-infected plants, respectively; $F_{1,\ 54}=0.28$; $P=0.759$) (Fig. 2b).

3.2 Effect of TuMV infection on plant development and TuMV virulence

The effect of TuMV infection on plant development was quantified as the effect of infection on growth and reproductive periods (GP_i/GP_m and RP_i/RP_m, respectively). The effect of infection on growth period was affected by the host species ($F_{1,\ 71}=4.23$; $P=0.043$), being higher in Mustard than in Arabidopsis (Table 1). Such effect also depended on the viral genotype ($F_{1,\ 71}=11.30$; $P=0.001$) and on the host × virus interaction ($F_{1,\ 71}=10.90$; $P=0.002$). In Arabidopsis, the growth period was significantly shorter in JPN1- than in JPN1A-infected plants ($F_{1,\ 36}=18.57$; $P<1\times10^{-4}$) (Supplementary Tables S1 and S2). Moreover, JPN1A-infected plants had significantly larger growth period than mock-inoculated plants ($F_{1,\ 37}=11.61$; $P=0.002$), and the opposite was observed in JPN1-infected individuals ($F_{1,\ 37}=1.35$; $P=0.051$) (Supplementary Tables S1 and S2). In Mustard, none of the viral genotypes had significant effect on growth period ($F_{1,\ 37}=0.02$; $P=0.963$) (Table 1). In addition, TuMV-infected plants had shorter reproductive periods than mock-inoculated ones in both hosts ($F_{1,\ 46}\geq12.72$; $P<1\times10^{-4}$) (Supplementary Tables S1 and S2), such effect not depending on the host species ($F_{1,\ 45}=0.19$; $P=0.667$), the virus genotype ($F_{1,\ 45}=0.04$; $P=0.853$), or the interaction between both factors ($F_{1,\ 45}=0.12$; $P=0.737$) (Table 1).

The effect of TuMV infection on the plant life period (LP_i/LP_m) was used as a measure of virus effect on plant mortality, an optimal proxy of virulence: the shorter the life period, the higher the virulence/mortality (Day 2002). The effect of virus infection on life period depended on the host species ($F_{1,\ 71}=73.85$; $P<1\times10^{-4}$), the viral genotype ($F_{1,\ 71}=5.18$; $P=0.026$), and the interaction between both factors ($F_{1,\ 71}=12.61$; $P=0.001$) (Table 1). In Arabidopsis, reduction of the life period upon TuMV infection was smaller in JPN1A- than in JPN1-infected plants ($F_{1,\ 55}=69.73$; $P<1\times10^{-4}$), whereas in Mustard no differences were observed ($F_{1,\ 54}=1.08$; $P=0.305$) (Table 1). Hence, in Arabidopsis virulence was lower in the passaged than in the ancestral TuMV genotype, due to an extension of the growth period, but such differences were not observed in the reference host.

3.3 Kinetics of TuMV within-host multiplication and virus infectivity

We also analyzed the kinetics of within-host virus RNA accumulation in JPN1- and JPN1A-infected plants. For that, we quantified TuMV RNA systemic accumulation in rosette leaves every 2 days between 2 and 20 dpi, such that we monitored the kinetics of virus infection for a time span that included all the developmental stages of the Arabidopsis rosette (Fig. 3a) and about half of the plant life span (Supplementary Table S1). As plant life-history traits and kinetics of TuMV infection were quantified in different plant batches (see Section 2), we confirmed that these were comparable by analyzing accumulation of the two viral genotypes at 14 dpi in both plant batches. As expected, values did not differ between batches in any of the hosts ($\chi^2_w\leq2.88$; $P\geq0.090$).

JPN1 and JPN1A RNA accumulation reached a maximum earlier in Mustard (10/12 dpi) than in Arabidopsis (18/20 dpi) (Fig. 3a and b), such maximum being higher in JPN1A than in JPN1 in both hosts ($\chi^2_w\geq14.78$; $P<1\times10^{-4}$). However, the kinetics of viral RNA accumulation depended on the host species and on the viral genotype. In Arabidopsis, JPN1 accumulated to higher levels than JPN1A until 6 dpi ($\chi^2_w\geq8.73$; $P\leq0.003$), no differences in virus accumulation were observed between 8 and 12 dpi ($\chi^2_w\leq2.56$; $P\geq0.110$); and from that point to the end of the monitored period, JPN1A accumulated to higher levels than JPN1 ($\chi^2_w\geq6.36$; $P\leq0.012$) (Fig. 3a). On the other hand, accumulation of both viral genotypes in Mustard was similar until 4 dpi ($\chi^2_{w(1,\ 9)}\leq1.12$; $P\geq0.290$), and afterwards JPN1A accumulated to higher levels than JPN1 ($\chi^2_w\geq5.35$; $P\leq0.021$) (Fig. 3b).

We also analyzed whether serial passages resulted in changes of TuMV infectivity. To do so, we calculated infectivity curves of JPN1 and JPN1A in Arabidopsis and Mustard. TuMV infectivity curves indicated a faster decrease of the percentage of infected Arabidopsis plants with reduced inoculum dose in JPN1 than in JPN1A (Fig. 3c) ($F_{1,\ 13}=18.04$; $P=0.002$), whereas in Mustard the opposite trend was observed ($F_{1,\ 13}=32.95$; $P=2\times10^{-4}$) (Fig. 3d). Thus, serial passaging of TuMV in Arabidopsis resulted in increased infectivity in this host, but not in Mustard.

3.4 Genetic changes in the TuMV genome

We obtained the complete genomic sequence of JPN1A. The JPN1A genome consisted of 9,835 nucleotides, including a

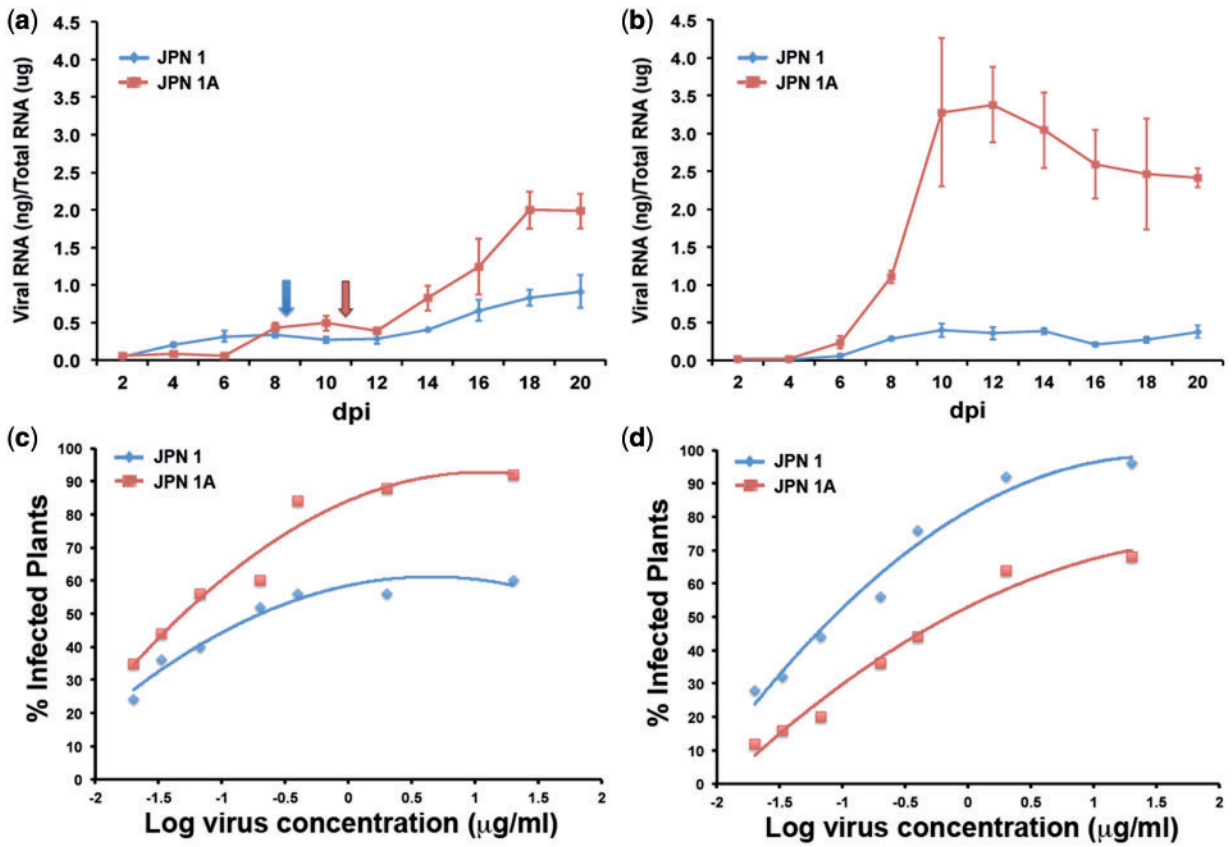

Figure 3. JPN1 and JPN1A multiplication kinetics and infectivity curves in Arabidopsis and Mustard. JPN1 (blue) and JPN1A (red) RNA accumulation in Arabidopsis (a) and Mustard (b) between 0 and 20 days post-inoculation (dpi). Values at each time point are mean ± SE of five replicates. Blue and red arrows indicate bolting time of JPN1- and JPN1A-infected plants, respectively. Infectivity–dilution curves of the two TuMV genotypes in Arabidopsis (c) and Mustard (d) are also represented.

typical potyvirus polyprotein-encoding ORF of 9,495 nucleotides. Comparison of the JPN1 and JPN1A ORFs revealed a nucleotide sequence homology of 99 per cent, with only 38 nucleotide substitutions. The deduced amino acid sequence of the putative polyprotein indicated that 19/38 substitutions resulted in amino acid changes located in the P1 (5), the NIb (4), the HC-Pro (1), the P3 (3, none in the PIPO or ALT ORFs), the CI (2), the 6K2 (2), and the CP (2) (Fig. 4). Viral genes involved in the observed changes of TuMV virulence and multiplication are expected be under positive selection (Holmes 2009) and accumulate more non-synonymous mutations than expected by chance (Wagner 2007). Thus, to gather more information on the TuMV genes that could be associated with the phenotypic changes described above, we analyzed the spatial distribution of the nineteen non-synonymous mutations in the viral polyprotein. Using a X^2 goodness-of-fit test for a Poisson distribution, we determined whether the number of non-synonymous mutations per gene was greater than would be expected by chance. Such spatial clustering of mutations was detected in only three regions of the TuMV genome: the P1, the P3, and the 6K2 genes ($P < 1 \times 10^{-4}$; Fig. 4).

4. Discussion

Increasing evidence suggests that parasitic sterilization is not uncommon in animal– and plant–parasite interactions (Hall et al. 2007; Lafferty and Kuris 2009). In some cases, sterilization may be the by-product of the infection by highly virulent parasites that kill the host before maturity (Bonds 2006). Also, temporal sterility has been described as a host response in order to divert resources from reproduction into defense to cope with parasite infection (Hurd 2001; Day and Burns 2003). Finally, sterilization may be an infectious strategy of the parasite to optimize its fitness through elimination of host reproduction as the primary means of acquiring resources (Lafferty and Kuris 2009). Parasites included in this last category are called castrators. Two conditions need to be met for a parasite to be considered as a castrator. First, the effect of infection mainly targets host reproductive structures, blocking offspring production; and second, host sterility occurs when the host matures (Lafferty and Kuris 2009). To date, only fungal parasites have been described as plant castrators (Clay 1991; Kover 2000). In our experimental system, TuMV infection caused a reduction of 0–34 per cent of the rosette weight (plant growth structures), whereas reduction of inflorescence/seed weights (plant reproductive structures) was never smaller than 85 per cent, leading to nearly complete sterility. In addition, most of the TuMV-infected plants reached the bolting stage, which is considered as indicative of plant maturity (Boyes et al. 2001), and produced siliques although these were not fertile. Hence, our results provide for the first time evidence compatible with the role of a plant virus as a castrating parasite. Models on the evolution of virulence of castrating parasites predict that they would maximize fitness at absolute host sterilization (maximum amount of host resources dedicated to survival) and at lower virulence (reduced mortality) (Jaenike 1996; O'Keefe and Antonovics 2002; Ebert et al. 2004; Hall et al. 2007). Comparison of the effect of infection of JPN1 (ancestral) and JPN1A (passaged) in Arabidopsis indicated that

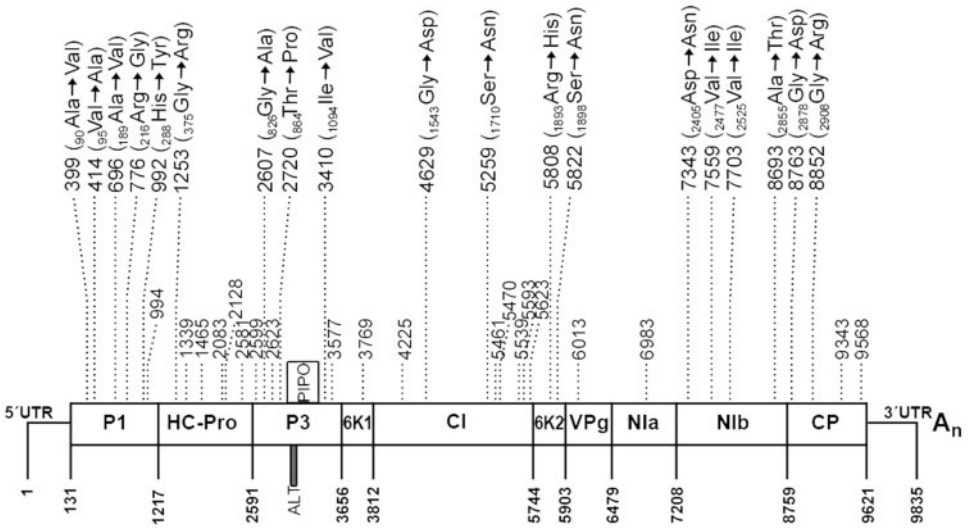

Figure 4. Location of nucleotide differences between the JPN1 and JPN1A polyproteins. White boxes indicate the ten proteins encoded in the TuMV genome, and PIPO (light grey) and ALT (dark grey) proteins. Amino acid changes for non-synonymous nucleotide substitutions are shown, whereas for synonymous substitutions only the nucleotide positions are shown.

JPN1A induced sterilization in a larger proportion of plants and showed reduced plant mortality. These modifications in plant life-history traits were accompanied by an increase in the maximum level of multiplication of JPN1A as compared to JPN1. Our results therefore support theoretical models and are also in agreement with experimental analyses in other plant– and invertebrate–parasite interactions (Jaenike 1996; Kover 2000; Sorensen and Minchella 2001; Ebert et al. 2004; Jensen et al. 2006). Reduced mortality after serial passages resulted in an increase of 12 days (25%) in plant life span, which elongates the TuMV infectious period. Although this increase may seem modest as compared with the lifespan of a perennial plant, it may have relevant consequences for virus epidemiology. In wild populations, Arabidopsis plants do not germinate synchronously, but in winter and spring cohorts. The first cohort germinates in autumn, vegetates during the winter, and flowers in early spring; on the other hand, the spring cohort plants germinate, vegetate and flower in spring, and die in early summer (Picó 2012). Thus, elongation of the life span in winter cohort plants would facilitate TuMV transmission to the spring cohort, allowing the virus to remain in the host population across seasons. Similarly, elongating host life span would increase the chances for virus transmission to younger individuals of the same cohort or to alternative host species.

It should be noted that our results are also compatible with TuMV adaptation to Arabidopsis. Indeed, the observed phenotypic changes would optimize virus fitness: higher infectious periods, virus titer, and infectivity have been shown to increase between-host transmission rates in a wide range of plant–virus interactions, including potyviruses (reviewed by Froissart et al. 2010). In addition, after TuMV serial passages in Arabidopsis we observed modifications of other infection traits that would also increase virus fitness. For instance, JPN1A-infected Arabidopsis developed a more intense yellow mosaic than JPN1 plants (Supplementary Fig. S2), which has been shown to increase plant attractiveness to vectors, and therefore between-host transmission (Fereres and Moreno 2009; Mauck et al. 2012; Salvaudon et al. 2013). Such changes were not observed in Mustard. Further experimental analyses on the effect of serial passages on the rate of TuMV between-host transmission would contribute to elucidate whether the observed changes in virulence are indeed involved in host adaptation.

Although evolution of TuMV virulence would optimize virus between-host transmission, it results in absolute Arabidopsis castration, which can result in unstable disease dynamics: TuMV-induced castration probably exerts strong selection for resistant host genotypes, which in turn may eventually lead to TuMV extinction and, in the absence of resistance, may drastically reduce host population size, such that susceptible hosts become unavailable. However, the strength of the selection imposed by the parasite on the host population depends on its prevalence (Haldane 1949; Clay and Kover 1996; Lafferty and Kuris 2009). Average TuMV prevalence in Arabidopsis wild populations is around 20 per cent (Pagán et al. 2010). At this prevalence level, 80 per cent of susceptible hosts produce progeny, a sufficiently large proportion to prevent resistant genotypes to take over the host population, and to maintain host population size. Also, in nature not all plants are infected at the same developmental stage, and the outcome of infection may depend on the host age at infection. Indeed, previous analyses using the same Arabidopsis and TuMV genotypes, but in plants inoculated at a later developmental stage, showed a milder effect of infection (Sánchez et al. 2015). Thus, even at high prevalence, not every TuMV infection may necessarily induce castration, which would provide another mechanism by which castrating TuMV genotypes may be maintained in host populations. Finally, host populations are not isolated demes but they are connected with other populations of the same or different plant species. Thus, TuMV would persist in Arabidopsis wild populations as long as these recruit individuals from other populations with lower or no virus burden, and/or alternative hosts coexist with Arabidopsis.

Parasitic castration can be achieved by various mechanisms (Lafferty and Kuris 2009). The most obvious is draining host resources from reproduction into host growth, making them available for parasite consumption (Jaenike 1996; Kover 2000; O'Keefe and Antonovics 2002; Bonds 2006; Hall et al. 2007). In TuMV-infected Arabidopsis, this resource reallocation is apparent from the comparison of the inflorescence + seed to rosette weight ratios. While mock-inoculated plants dedicated the

majority of resources to reproduction (ratio ≈ 12), JPN1 infection highly reduced this ratio (ratio ≈ 2). Moreover, serial passaging resulted in the imbalance of resources towards plant growth, as the ratio was smaller than 1 in JPN1A-infected Arabidopsis. To optimize the usage of host resources, theoretical elaborations propose that castrating parasites would elongate host growth period, such that sterilization-liberated resources would be stored into host growth until the parasite exploits them to produce its offspring (Jaenike 1996; O'Keefe and Antonovics 2002; Hall et al. 2007). Our results could be explained on the basis of this theory. Lower mortality of JPN1A-infected as compared to JPN1-infected plants was associated with a larger growth period. The lower levels of JPN1A accumulation at early times of infection would favor this elongation of growth period. Afterwards, at plant bolting when the maximum of host resources dedicated to growth has been reached (Boyes et al. 2001) usage of such resources by JPN1A would explain the increase of virus accumulation at later times of infection. Thus, changes in the plant developmental patterns would allow a more efficient exploitation of host resources. Altogether, our results strongly suggest that the increase of virus multiplication in Arabidopsis after serial passages could be attributed to the fine-tuning of resource exploitation and virulence level.

Phenotypic changes during virus evolution are often linked to genetic variation (Holmes 2009). The spatial distribution of mutations in the JPN1A genome suggests that potyviral proteins P1, P3, and 6K2 might be involved in the observed phenotypic changes of JPN1 after serial passaging in Arabidopsis. The P1 protein is the first protease encoded in potyvirus genomes. Recently, it has been elegantly demonstrated that the N-terminal region of P1, in which three JPN1A-specific substitutions were located, controls early replication of potyviruses (Pasin et al. 2014). Pasin et al. proposed that such regulatory function evolved to keep viral multiplication below host detrimental levels and maintain higher long-term replicative capacity. This would be compatible with our observation of JPN1A reduced accumulation at earlier, but not at later, infection times as compared with JPN1. Like P1, P3 has also been proposed to be involved in virus replication (Cui et al. 2010) and, together with 6K2, in virus movement and symptom development (Spetz and Valkonen 2004). Thus, the known functions of P1, P3, and 6K2 are coherent with the phenotypic changes observed after TuMV passage in Arabidopsis. Obviously, further molecular analyses are required to test the role of the identified genes/mutations in TuMV virulence evolution, and this would be an avenue of future research.

Some cautionary comments on our results are, however, needed. First, serial passages of TuMV in Arabidopsis involved mechanical inoculation and not aphid transmission, which is the mode of virus dispersion in nature. Hence, our inoculum dose was higher than during aphid transmission. Several studies using species of the genus *Potyvirus* have shown that virus phenotypes observed after serial passages through mechanical inoculation reflect the virus behavior in natural conditions (Hajimorad et al. 2011) or show the same but faster evolutionary trends than after serial passages of aphid transmission (Wallis et al. 2007). Thus, mechanical transmission would reduce the effects of genetic drift, likely accelerating but not changing the outcome of virus evolution. Second, during serial passages we imposed a selection for isolates that can be transmitted after 15 dpi. Thus, the TuMV phenotypic/genetic changes described here might be the result of adaptation to our transmission conditions, rather than reflecting virus evolution in nature. However, this time was not randomly selected. Arabidopsis wild plants rarely sustain aphid colonies, and transmission occurs mainly through winged aphids. In a large proportion of the Arabidopsis distribution area, winged aphids generally appear and their density peaks in mid-spring (Nebreda et al. 2005; Mondal et al. 2016); that is, about half way of the infected Arabidopsis life span (see Supplementary Table S1). Thus, transmission at 15 dpi (plants of 20 days of age) reflects the beginning of the period in which transmission is most likely to occur. Finally, we only used a single host and virus genotype in our analyses, such that the observed virulence evolution may be specific of this particular host–virus interaction. Very little is known on the effect of parasite infection on plant fertility, and we can only speculate on how general is TuMV castration. Here, plant sterilization was observed in Arabidopsis but not in Mustard plants. This could be due to the fact that plant developmental schedule differs between both species. In Mustard, transmission at 15 dpi occurs within the rosette growth period, making evolution towards longer life span unnecessary. Serial TuMV passages in Mustard at the same developmental stage could also result in evolution towards plant castration. Indeed, flower bud malformations and reduction of seed viability associated with TuMV infection have reported in plant species closely related to Mustard (Walsh and Tomlinson 1985; Lockhart 2012). This suggest that, in certain conditions, TuMV infection may cause sterility in hosts other than Arabidopsis. Nevertheless, analyses in other TuMV–host pairs are needed to clarify this point.

In summary, our analyses in a plant–virus interaction support the predictions of the *trade-off* hypothesis for the evolution of virulence of castrating parasites. *In silico* genomic analyses identified clusters of mutations in genes with functions that are coherent with the observed phenotypic changes. Altogether, our results contribute to better understand the process of virus virulence evolution.

Acknowledgements

We thank Prof. Fernando García-Arenal and Dr Aurora Fraile for providing necessary facilities for developing this work and for extremely useful discussion of the results and various versions of the manuscript. Antolín López and Lucía Zurita provided excellent technical assistance. This work was supported by a Ramón y Cajal grant from the Ministerio de Economía y Competitividad de España (RYC-2011-08574). V.V. was supported by an Erasmus Mundus BRAVE Scholarship of the EU (2013-2536/001-001).

Data availability

All data generated or analyzed during this study are included in this published article and its Supplementary information files.

Supplementary data

Supplementary data are available at *Virus Evolution* online.

Conflict of interest: None declared.

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