



An extremely narrow bottleneck limits cucumber mosaic virus RNA2 during phloem unloading within inoculated leaves

Shuhei Miyashita¹ · Sorachi Kimura¹ · Winnie Zhou^{1,2} · Hideki Takahashi¹

Received: 29 June 2025 / Accepted: 30 July 2025 / Published online: 9 August 2025
© The Author(s) 2025

Abstract

Previous studies have suggested that plant viruses experience genetic bottlenecks in phloem-mediated long-distance movement; however, the detailed mechanisms of these genetic bottlenecks have not been analyzed, and their size has not been estimated for segmented viruses. In this study, we used a tripartite RNA virus, cucumber mosaic virus, and showed that strong bottleneck events occurred at the phloem unloading step of RNA2 in *Nicotiana benthamiana*. Half-leaf inoculation method allowed us to estimate the genetic bottleneck size as 0.042 ± 0.021 (mean $\pm$ standard error), indicating that a strong genetic bottleneck occurs specifically at the phloem unloading step within inoculated leaves.

Keywords Adaptation · Evolution · Long-distance movement · Multiplicity of infection · Plant viruses

Abbreviations

CFP Cyan fluorescent protein
CMV Cucumber mosaic virus
YFP Yellow fluorescent protein

Plant viruses experience many genetic bottlenecks during their life cycles, including during single-cell or cell-to-cell infection, long-distance movement via phloem, and vector or mechanical plant–plant horizontal transmission. For example, the number of tomato yellow leaf curl virus genomes that initiated replications in an inoculated cell was estimated to be 2.20 ± 0.08 to 2.60 ± 0.05 , probably due to superinfection exclusion mechanisms (Ren et al. 2023). First and second cell-to-cell infections by the RNA2 of Japanese soil-borne wheat mosaic virus were shown to occur with 5.97 ± 0.22 and 5.02 ± 0.29 copies, respectively (Miyashita and Kishino 2010). Similarly, cell-to-cell infections by tomato mosaic virus (ToMV) and the RNA2 of cucumber mosaic virus (CMV) occur with 3.95 ± 0.27 and 5.72 ± 0.24 copies, respectively (Abebe et al. 2021; Miyashita et al. 2015). Inoculation

of tobacco protoplasts with ToMV by electroporation showed that introduction of ~5000 viral genome RNA molecules into a cell resulted in replication of ~4 genome molecules, suggesting that the probability of each viral genomic RNA molecule to initiate replication was less than 0.1% (Miyashita et al. 2015). The numbers of potato virus Y particle and CMV transmitted by an aphid vector were estimated to be 0.5–3.2 and 1–2, respectively (Betancourt et al. 2008; Moury et al. 2007). Cellular and tissue infections with such small numbers of viral genomes function as strong genetic bottlenecks that determine the structure of viral populations in each infected plant. Genetic bottlenecks generally enhance genetic drift, leading to “survival of the luckiest”, however, this is not always the case. We previously suggested that stochastic isolation of viral genomes by narrow genetic bottlenecks in cell-to-cell infections enhance selection on *trans*-acting genes or elements (i.e., the majority of coding sequences in viral genomes) by enhancing competition among intercellular populations formed after cell-to-cell infections, resulting in “survival of the fittest”. Our simulations suggested that narrow genetic bottlenecks are indispensable for the rapid adaptation of plant viruses (Miyashita and Kishino 2010; Qu et al. 2020). Furthermore, *R*-gene-mediated resistance against CMV has been shown to decrease the size of the genetic bottleneck in cell-to-cell infection of RNA2 (and presumably other segments as well), stopping the spread of viral infection (Abebe et al. 2021), and the extent of the decrease in bottleneck size determines the extent of viral spread (Abebe et al. 2021; Karino et al. 2023). Thus, the size of the genetic

✉ Shuhei Miyashita
shuhei.miyashita.d7@tohoku.ac.jp

¹ Laboratory of Plant Pathology, Graduate School of Agricultural Science, Tohoku University, Sendai 980-8572, Japan

² School of Biological Science, University of California San Diego, San Diego, CA, USA

bottleneck is both an essential factor in viral evolution and a point of strategic importance in the struggle between viruses and host plants.

Despite the importance of genetic bottlenecks in the plant virus life cycle, our knowledge of genetic bottlenecks in phloem-mediated movement remains limited. Previous studies have suggested the existence of genetic bottlenecks at the tissue infection level following observations of the stochastic separation of co-inoculated variants among the upper leaves of dicot plants or among new tillers of a monocot plant (Hall et al. 2001; Li and Roossinck 2004; Sacristán et al. 2003); some studies have reported the spatial separation of co-inoculated virus variants within the upper leaves (Hall et al. 2001; Takahashi et al. 2007), implying that genetic bottlenecking events also occur within infected leaves. Gutiérrez et al. (2015) showed that co-inoculation of turnip mosaic virus (TuMV) vectors carrying a GFP or an RFP gene to turnip resulted in rare co-infected sites in upper uninoculated leaves. They used the ratio of co-infected sites to singly infected sites to estimate the size of genetic bottlenecks in infection site formation in upper leaves as 0.072. However, it remained unclear whether genetic bottleneck events occur at phloem loading or unloading, and if segmented viruses

experience similarly narrow bottlenecks. In this study, we used CMV, a positive-strand tripartite RNA virus, to investigate the site and size of these genetic bottlenecks.

The CMV genome consists of three RNA segments called RNA1–3. We prepared wild-type RNA1 and RNA3, and the *YFP*- or *CFP*-labeled RNA2 variants RNA2-YFP and RNA2-CFP, by transcribing their cDNA constructs (pCY1, pCY3, and pCY2-YFP or pCY2-CFP) (Abebe et al. 2021; Suzuki et al. 1991; Takeshita et al. 2012) using the AmpliCap-Max T7 High Yield Message Maker Kit (CELLSCRIPT, Madison, WI, USA) (Fig. 1a, b). pCY2-YFP and pCY2-CFP were constructed by inserting *YFP*/*CFP* cDNA (Miyashita and Kishino 2010) to pC2-A1 (Takeshita et al. 2012) and the resultant RNA2-YFP and RNA2-CFP express *YFP* and *CFP* as fusion proteins to full-length 2b that retain (at least) partial activity of 2b to support systemic infection. *Nicotiana benthamiana* plants were grown for 4–5 weeks as described previously (Abebe et al. 2021), and an equal mixture of the four RNA segments (5 pmol each in 70 µl of 100 mM potassium phosphate buffer, pH 8.0) were inoculated to newly expanded leaves using carborundum as an abrasive. Infected sites in the inoculated leaves and upper uninoculated leaves were observed by fluorescent microscopy using an

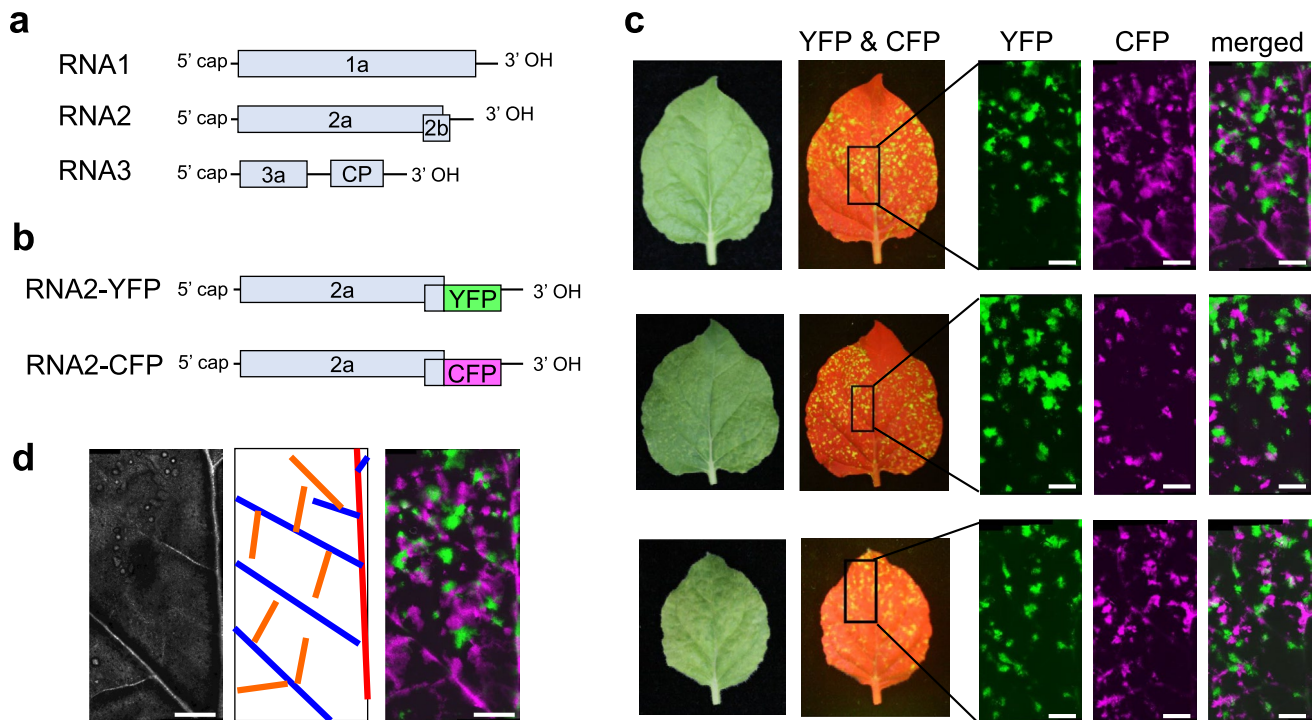


Fig. 1 Spatial separation of cucumber mosaic virus (CMV) RNA2s labeled with yellow (*YFP*) and cyan (*CFP*) fluorescent protein genes after phloem-mediated movement to upper uninoculated leaves of *Nicotiana benthamiana*. **a** Genome structure of CMV. **b** RNA2 variants labeled with *YFP* or *CFP* genes. **c** Observation of three independent uninoculated upper leaves (UL) at 3–5 dpi by macroscopy (two left panels) and microscopy (three right panels). For macro-

scopic fluorescent observation, blue excitation light and a long-pass filter (>515 nm) were used to detect both *YFP* and *CFP* fluorescence. **d** Vein network (left) visualized as a schematic (middle), with primary, secondary, and tertiary veins indicated in red, blue, and orange, respectively, and fluorescence protein observations (right), with *YFP* and *CFP* indicated in green and magenta, respectively. Bars = 1 mm

Axiomager M2 microscope (Zeiss, Oberkochen, Germany) and a charge-coupled device (CCD) camera (EXi Aqua; QImaging, Surrey, BC, Canada) at 3–5 days post inoculation (dpi). The resulting images were prepared by merging yellow fluorescence protein (YFP) and cyan fluorescent protein (CFP) images, followed by image tiling using the Stitching plugin in ImageJ Fiji (Preibisch et al. 2009). The inoculated leaves had many foci with YFP and CFP fluorescence (data not shown), suggesting co-infection by RNA2-YFP and RNA2-CFP in these sites, whereas upper uninoculated leaves rarely showed sites with both YFP and CFP fluorescence (Fig. 1c), indicating rare co-infection by RNA2-YFP and RNA2-CFP. Importantly, sites initiated from the same secondary or tertiary veins consisted of both YFP-only and CFP-only sites in upper leaves (Fig. 1d), indicating that a mixture of both RNA2-YFP and RNA2-CFP molecules reached the secondary or tertiary veins, whereas infection after phloem unloading involved a limited number of RNA2 molecules, which led to the formation of separate infection sites. To examine the rate of loss of YFP/CFP genes, we carried out RT-PCR amplification of RNA2-YFP/CFP at 5 dpi, using a primer set for nearly entire RNA2 (a 5'-UTR forward primer Cy677, 5'-GTAAACTCCCTAGACTTGA-3', and a 3'-UTR reverse primer Cy48, 5'-ACCCTGAACTAGCACGT-3') (Fig. 2a, b). The amplified products contained fragments shorter than the expected size, suggesting the accumulation of deletion

mutants, though the amounts and patterns of shorter fragments varied among the replicates. RT-PCR with primers Cy30 (5'-TCAAGTAGATGGTTTCGGA-3', a forward primer within 2a–2b overlapping region) and Cy48 also amplified short fragments, suggesting that the deletion occurred within the YFP/CFP coding region (Fig. 2c). Based on these results, we considered that observation of YFP-only and CFP-only sites in the upper uninoculated leaves can be at least partially explained by the loss of fluorescent genes, in addition to the infection by a limited number of RNA2 molecules, and thus it is difficult to accurately quantify singly or co-infected sites in the systemically infected leaves. Furthermore, asynchronous formation of infection sites in the upper uninoculated leaves brought more uncertainty to site quantification, because it led to the difficulty in distinguishing primary and secondary infections in upper leaves, where infected sites served as the source of infection in the same leaf.

To eliminate the effect of deletion mutations and the timing of infection site formation in quantifying singly or co-infected sites after phloem-mediated CMV movement, we developed a half-leaf inoculation procedure. After inoculating half of a young, expanding *N. benthamiana* leaf with the transcript mixture, the leaf was detached from the plant at 2 dpi and incubated in a humid box for an additional day to enhance virus translocation within the leaf; newly infected sites were detected in the other half at 3 dpi, indicating that

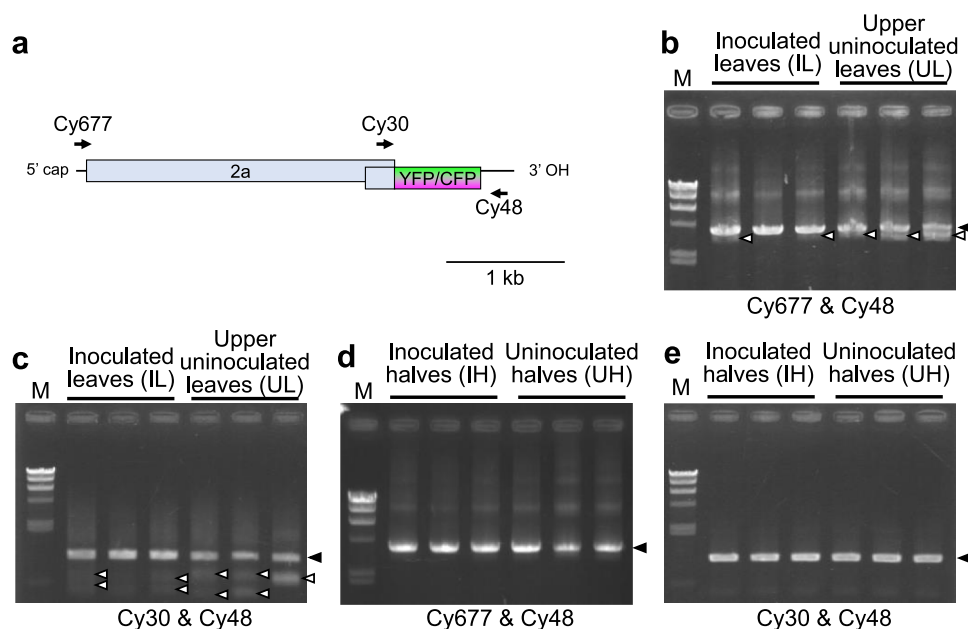


Fig. 2 RT-PCR amplification of RNA2-variants to detect deletion mutants in systemic infection experiments at 5 dpi (**b**, **c**) and in half-leaf inoculation experiments at 3 dpi (**d**, **e**). Bands with expected migration are indicated with black arrowheads, while those with higher migration (i.e., originated in deletion mutants) are indicated with white arrowheads. The left-most lanes show the lambda HindIII DNA size marker (M). **a** Location of the primers used for RT-PCR.

b Amplification of RNA2 variants accumulated in inoculated leaves (IL) and upper uninoculated leaves (UL) using Cy677 and Cy48 at 5 dpi. **c** Amplification of RNA2 variants in IL and UL using primers Cy30 and Cy48. **d** Amplification of RNA2 variants accumulated in inoculated leaf halves (IH) and in uninoculated leaf halves (UH). Primers Cy677 and Cy48 were used. **e** Amplification of RNA2 variants accumulated in IH and UH using primers Cy30 and Cy48

the inoculated half acted as an infection source for the uninoculated half via the phloem, in a similar manner to systemic long-distance movement. In the uninoculated leaf halves, many YFP-only and CFP-only sites from the same veins were observed but co-infected sites were rarely observed (Fig. 3), reproducing our observations in uninoculated upper leaves (Fig. 1). RT-PCR amplification of RNA2-YFP/CFP detected no or only a minor occurrence of deletion mutants both in the inoculated and uninoculated leaf halves (Fig. 2d, e). The numbers of co-infected and singly-infected sites in uninoculated half leaves in five independent experiments are summarized in Table 1. The co-infection rates were

0–1.87%, with an average of 0.88%, demonstrating that co-infection after phloem unloading was very rare.

The frequencies of singly infected and co-infected sites in half-leaf inoculation experiments were used to estimate the size of genetic bottlenecks after CMV phloem unloading (Fig. 4). We assumed that the proportion of RNA2-YFP and RNA2-CFP molecules in the infection source (i.e., phloem sap in the second or tertiary veins of uninoculated leaf halves) vary among leaves, thus providing the following independent parameters to each leaf as the ratio of $r_{(n)}:1 - r_{(n)}$, where n is the leaf sample number (1, 2, 3, 4, or 5). We assumed that the number of viral RNA2 molecules

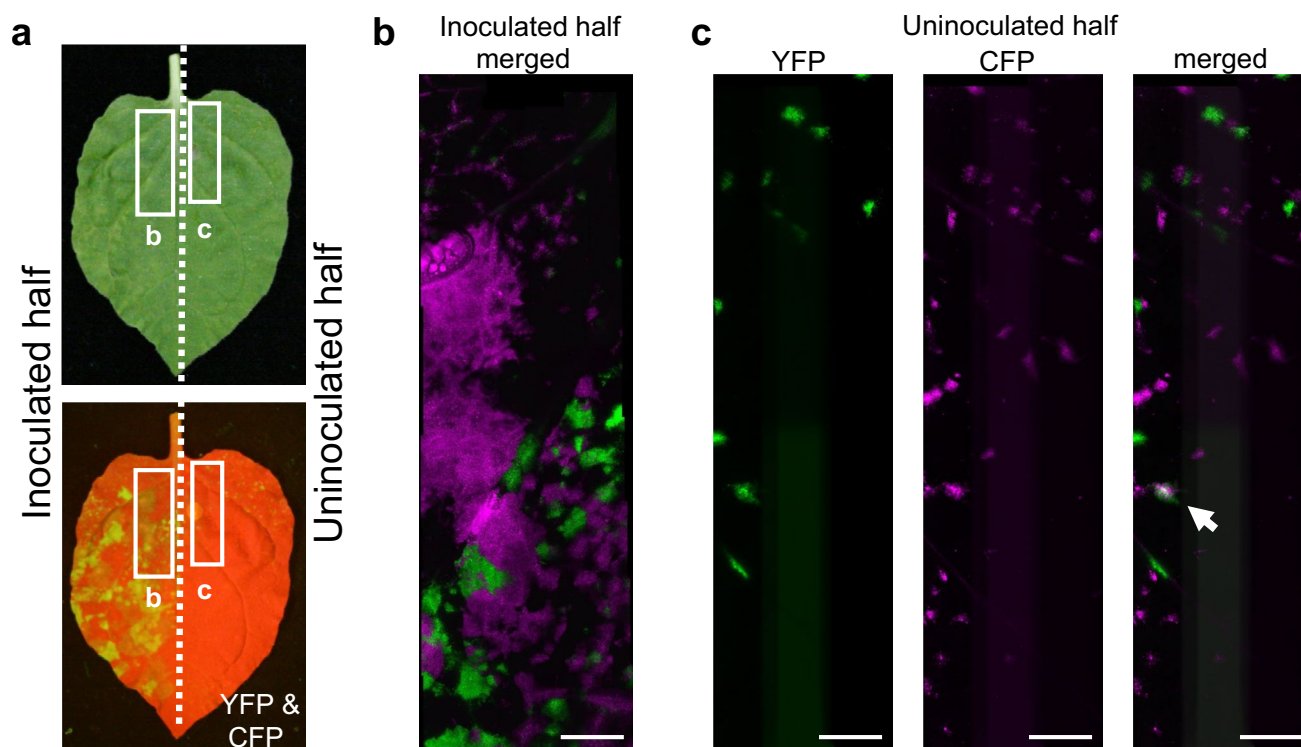


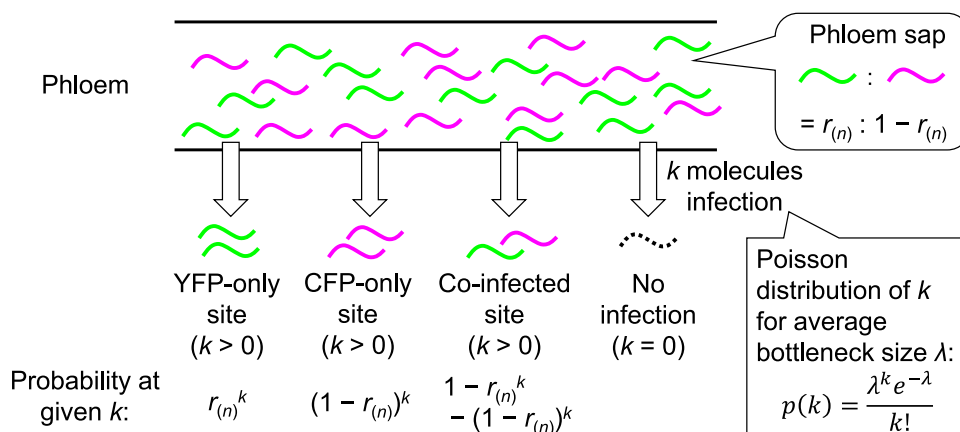
Fig. 3 Half-leaf inoculation of *Nicotiana benthamiana* with a mixture of YFP- and CFP-labeled CMV to observe phloem-mediated movement to the other uninoculated leaf half. **a** Macroscopic observation of the leaf at 3 dpi. **b** Microscopic YFP and CFP fluorescence obser-

vations in the inoculated leaf half. **c** Microscopic YFP and CFP fluorescence observation in the uninoculated leaf half. White arrow indicates the only co-infected site found in this area

Table 1 Numbers of singly and co-infected sites in uninoculated leaf halves. CFP, cyan fluorescent protein; YFP, yellow fluorescence protein

Sample leaf no.	Number of sites formed in uninoculated halves			Co-infection rate (%)	Estimated $r_{(n)}$ (ratio of RNA2-YFP in each infection source)
	YFP only	CFP only	Co-infected		
1	44	61	2	1.87	0.421 ± 0.047
2	16	41	1	1.72	0.286 ± 0.059
3	100	41	0	0	0.711 ± 0.038
4	36	38	1	1.33	0.487 ± 0.057
5	13	61	0	0	0.174 ± 0.044
Total	209	242	4	0.88	NA

Fig. 4 Estimation of the average genetic bottleneck size (λ) after phloem unloading, with explanations of parameters and essential equations. Note that we assumed different $r_{(n)}$ for each leaf data



that established infection in a single site follows the Poisson distribution, which have the average molecule number of λ ; thus, the ratio of sites infected with k RNA2 molecules was expressed as $p(k) = \frac{\lambda^k e^{-\lambda}}{k!}$. The probability of observing only YFP fluorescence (not CFP fluorescence) for a given value of k was expressed as $r_{(n)}^k$, such that the overall YFP-only site probability for each leaf was calculated as follows:

$$P_{yfp(n)} = \frac{\sum_{k=1}^{\infty} p(k) r_{(n)}^k}{1 - p(0)}.$$

Note that infection by $k=0$ molecules, which occurs at a probability of $p(0) = e^{-\lambda}$, indicates sites challenged by RNA2 but ended without infection; thus, the YFP-only site probability was divided by $1 - p(0)$ to normalize against total infected sites (but not non-infected sites). Similarly, the overall probability for CFP-only sites was calculated as follows:

$$P_{cfp(n)} = \frac{\sum_{k=1}^{\infty} p(k) (1 - r_{(n)})^k}{1 - p(0)},$$

and that for co-infected sites was calculated as follows:

$$P_{co(n)} = \frac{\sum_{k=1}^{\infty} p(k) [1 - r_{(n)}^k - (1 - r_{(n)})^k]}{1 - p(0)}.$$

The likelihood L of observing the actual frequencies (Table 1) was expressed as follows:

$$L = \prod_{n=1}^5 \frac{(S_{yfp(n)} + S_{cfp(n)} + S_{co(n)})!}{S_{yfp(n)}! S_{cfp(n)}! S_{co(n)}!} P_{yfp(n)}^{S_{yfp(n)}} P_{cfp(n)}^{S_{cfp(n)}} P_{co(n)}^{S_{co(n)}},$$

where $s_{yfp(n)}$, $s_{cfp(n)}$, and $s_{co(n)}$ are the numbers of YFP-only, CFP-only, and co-infected sites, respectively, for the n^{th} leaf. Then, we estimated the most-likely λ and r_n ($n = 1, 2, 3, 4, 5$) that maximized the likelihood L , using a script prepared for this study in R v4.2.1 (R Core Team 2022). The R script is available at GitHub (Miyashita 2024). The average size of the genetic bottleneck at phloem unloading of CMV

RNA2 was estimated as $\lambda = 0.042 \pm 0.021$ (estimate $\pm$ SE), with $r_{(n)}$ ranging between 0.174 ± 0.044 and 0.711 ± 0.038 (Table 1), showing that the bottleneck size for a segment of tripartite virus was similar to that for non-segmented virus [i.e., 0.072 for TuMV (Gutiérrez et al. 2015)]. Note that if we assumed constant $r_{(n)}$ to different leaves, the estimated λ was 0.035 ± 0.018 with a constant $r_{(n)}$ of 0.464 ± 0.023 , indicating that this assumption leads to underestimation of the bottleneck size. Unfortunately, we cannot estimate the bottleneck sizes in phloem unloading of CMV RNA1 and RNA3 due to the lack of techniques to label RNA1 and RNA3 with fluorescent genes without affecting replication or phloem-mediated movement. Breakthroughs in labeling these segments are required to discuss if bottlenecking events occur independently to each segment, or interactions among segments are playing any role.

As the bottleneck size estimate for RNA2 was much lower than one (i.e., $\lambda = 0.042$), we inferred that many sites had been challenged by viral RNA2 molecules in the phloem, but infection was not established. The expected ratio of such abortive sites can be calculated as $p(0) = e^{-0.042} = 0.959 = 95.9\%$ ($k=0$; Fig. 5a). Thus, CMV RNA2 successfully established site infections in only one of 25 challenged sites through phloem unloading. Among the infected sites, 97.9% were estimated to be infected by a single RNA2 molecule, and only 2.1% sites were infected by multiple RNA2 molecules (Fig. 5b). If the other RNA segments independently establish infection and experience similarly narrow genetic bottlenecks, the number of challenged sites will be much more (i.e., $25 \times 25 \times 25 = 15,625$). Thus, it might be reasonable to consider that bottlenecking events do not occur independently to each segment.

From a plant anatomical perspective, phloem unloading of plant viruses appears to be a multi-step event, involving movement from sieve elements to companion cells, companion cells to phloem parenchyma cells, phloem parenchyma cells to bundle sheath cells, and bundle sheath cells to mesophyll cells (Silva et al. 2002), although there may be minor differences among host plant species. Our results suggest that

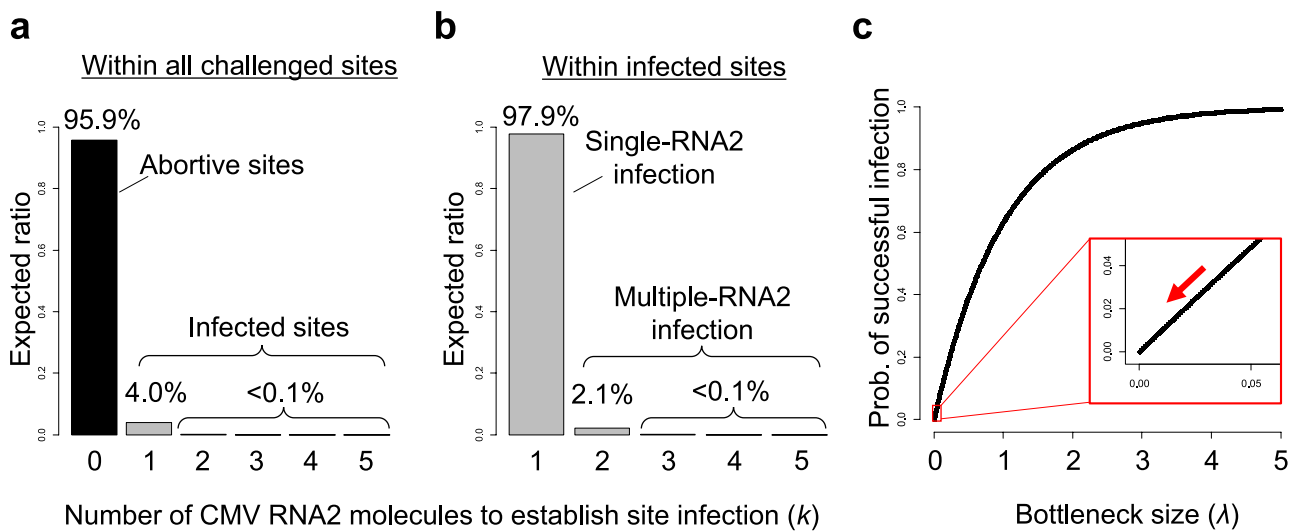


Fig. 5 Consequences of the narrow genetic bottleneck ($\lambda=0.042$). **a** Expected ratio of abortive and infected sites within all challenged sites. **b** Expected ratio of sites infected by single and multiple RNA2 molecules within all the infected sites. **c** Probability of successful

infection for a given value of λ . Inset shows a magnified image of the low- λ region ($\lambda < 0.05$), where a decrease in λ causes a linear decrease in the probability of successful infection

the infection source in secondary and tertiary veins consisted of both RNA2-YFP and RNA2-CFP molecules, such that one or more genetic bottlenecking events occurred after the virus reached the sieve elements. However, we were unable to clarify at which cell infection steps such bottlenecking events occurred, because phloem cells are very small and it is difficult to judge whether they are co-infected or singly infected. Further efforts are required to investigate phloem-unloading events in such detail, for example with higher-resolution microscopic observations of fine slices of newly infected sites.

We have previously suggested that the bottleneck size in cell-to-cell movement of ToMV could be determined by $E \times R \times p/d$, where E and R indicate the number of viral genome RNAs to enter the cell and the number of sites for replication complex (RC) formation in a cell, respectively, while p/d indicates the ratio of RC formation per degradation of the RNA (Miyashita et al. 2015). The mechanism of extremely narrow genetic bottlenecks could be attributed to the differences in these parameters between mesophyll cells and phloem cells. For example, the number of sites for RC formation, R , can be smaller in phloem cells due to smaller cell sizes, which would explain less amount of membrane fractions and/or other host factors required for RC formation in those cells. E can be also smaller in phloem cells than in mesophyll cells due to the differences in the number and the structures of plasmodesmata connections. We also need to note that E can be affected by the amount of viral genomes accumulated in the phloem, and thus the parameter value in the half-leaf inoculation method may differ from that in systemic infection; however, frequent single infections by RNA2-YFP or RNA2-CFP in upper uninoculated leaves

(Fig. 1c) imply that the parameter values are not largely different. Importantly, the above situations cannot be applied to phloem-limited viruses, which can infect phloem cells but not mesophyll cells efficiently (i.e., opposite to CMV). Comparison of the sizes of phloem-unloading bottlenecks and the above parameter values between phloem-limited viruses and the others may provide further insights into different surviving strategies between the two groups of viruses.

In principle, narrow genetic bottlenecks will strongly enhance genetic drift to allow survival of the luckiest. However, in the case of phloem unloading bottlenecks, because many infection sites formed in the upper leaves as shown in Fig. 1c, competition among the subpopulations forming these infection sites would select the fittest. In addition, as we have theorized for cell-to-cell infections (Miyashita and Kishino 2010), viral *trans*-acting genes or elements will receive strong purifying selection due to the strong separation effect of the narrow genetic bottlenecks in phloem unloading. Therefore, it is probable that narrow bottlenecks in phloem unloading are an important factor allowing viruses to achieve rapid adaptation.

Regarding virus–host interactions, our previous study suggested that host plant resistance target narrow genetic bottlenecks at cell-to-cell infections, by further decreasing their size to stop viral infections (Abebe et al. 2021). Figure 5c shows the theoretical probability of successful infection for different genetic bottleneck sizes. Comparing the bottleneck sizes for cell-to-cell infections [$\lambda \sim 5$, Abebe et al. (2021)] and for phloem unloading estimated in this study ($\lambda=0.042$), it is evident that the slope is much steeper at the lower λ value, indicating that a decrease in the genetic bottleneck

size results directly in a decrease in infected cells at a lower λ . Thus, plant resistance may work more efficiently at phloem unloading of plant viruses than at cell-to-cell infections; this finding will contribute to the design of more efficient strategies to protect plants from viruses. The half-leaf inoculation method applied in this study may be useful in such studies.

Acknowledgements We thank Dr. Chikara Masuta, Dr. Masashi Suzuki, and Dr. Minoru Takeshita for providing the pCY1, pCY2-A2, and pCY3 constructs used in this study. We also thank the anonymous reviewers for their insightful comments.

Author contributions SM conceived and designed the study. SK and SM performed the experiments. SM, SK, WZ, and HT analyzed the data. SM and SK prepared the manuscript.

Funding This study was partially supported by Japan Society for the Promotion of Science (JSPS) KAKENHI grants (nos. 21K05591, 24K21860, and 24K01756 to SM and nos. 19H02953, 22KK0081, 23K18017, and 24K01755 to HT). WZ was supported by the JYPE program of Tohoku University and a Japan Student Services Organization (JASSO) scholarship for visiting research in our study group. The funders were not involved in the study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

Ethical statement This study did not include any experiments involving human participants or experimental animals.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Abebe DA, van Bentum S, Suzuki M, Ando S, Takahashi H, Miyashita S (2021) Plant death caused by inefficient induction of antiviral R-gene-mediated resistance may function as a suicidal population resistance mechanism. *Commun Biol* 4:947. <https://doi.org/10.1038/s42003-021-02482-7>
- Betancourt M, Fereres A, Fraile A, García-Arenal F (2008) Estimation of the effective number of founders that initiate an infection after aphid transmission of a multipartite plant virus. *J Virol* 82:12416–12421. <https://doi.org/10.1128/jvi.01542-08>
- Gutiérrez S, Pirolles E, Yvon M, Baecker V, Michalakakis Y, Blanc S (2015) The Multiplicity of cellular infection changes depending on the route of cell infection in a plant virus. *J Virol* 89:9665–9675. <https://doi.org/10.1128/jvi.00537-15>
- Hall JS, French R, Hein GL, Morris TJ, Stenger DC (2001) Three distinct mechanisms facilitate genetic isolation of sympatric wheat streak mosaic virus lineages. *Virology* 282:230–236. <https://doi.org/10.1006/viro.2001.0841>
- Karino G, Abebe DA, Saijo Y, Takahashi H, Miyashita S (2023) Artificial coat protein variants of cucumber mosaic virus induce enhanced resistance upon recognition by an R gene. *J Gen Plant Pathol* 89:277–287. <https://doi.org/10.1007/s10327-023-01133-5>
- Li H, Roossinck MJ (2004) Genetic bottlenecks reduce population variation in an experimental RNA virus population. *J Virol* 78:10582–10587. <https://doi.org/10.1128/jvi.78.19.10582-10587.2004>
- Miyashita S, Kishino H (2010) Estimation of the size of genetic bottlenecks in cell-to-cell movement of soil-borne wheat mosaic virus and the possible role of the bottlenecks in speeding up selection of variations in trans-acting genes or elements. *J Virol* 84:1828–1837. <https://doi.org/10.1128/jvi.01890-09>
- Miyashita S, Ishibashi K, Kishino H, Ishikawa M (2015) Viruses roll the dice: the stochastic behavior of viral genome molecules accelerates viral adaptation at the cell and tissue levels. *PLoS Biol* 13:e1002094. <https://doi.org/10.1371/journal.pbio.1002094>
- Miyashita S (2024) An R script to estimate the size of genetic bottleneck at phloem unloading. <https://github.com/ShuheiMiyashita/PhloemUnloading2024>
- Moury B, Fabre F, Senoussi R (2007) Estimation of the number of virus particles transmitted by an insect vector. *Proc Natl Acad Sci USA* 104:17891–17896. <https://doi.org/10.1073/pnas.0702739104>
- Preibisch S, Saalfeld S, Tomancak P (2009) Globally optimal stitching of tiled 3D microscopic image acquisitions. *Bioinformatics* 25:1463. <https://doi.org/10.1093/bioinformatics/btp184>
- Qu F, Zheng L, Zhang S, Sun R, Slot J, Miyashita S (2020) Bottleneck, isolate, amplify, select (BIAS) as a mechanistic framework for intracellular population dynamics of positive-sense RNA viruses. *Virus Evol* 6:veaa086. <https://doi.org/10.1093/ve/veaa086>
- R Core Team (2022) R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. <https://www.R-project.org/>
- Ren R, Zheng L, Han J, Carvalho CP, Miyashita S, Zhang D et al (2023) Intracellular bottlenecks permits no more than three tomato yellow leaf curl virus genomes to initiate replication in a single cell. *PLoS Pathog* 19:e1011365. <https://doi.org/10.1371/journal.ppat.1011365>
- Sacristán S, Malpica JM, Fraile A, García-Arenal F (2003) Estimation of population bottlenecks during systemic movement of tobacco mosaic virus in tobacco plants. *J Virol* 77:9906–9911. <https://doi.org/10.1128/jvi.77.18.9906-9911.2003>
- Silva MS, Wellink J, Goldbach RW, van Lent JWM (2002) Phloem loading and unloading of cowpea mosaic virus in *Vigna unguiculata*. *J Gen Virol* 83:1493–1504. <https://doi.org/10.1099/0022-1317-83-6-1493>
- Suzuki M, Kuwata S, Kataoka J, Masuta C, Nitta N, Takanami Y (1991) Functional analysis of deletion mutants of cucumber mosaic virus RNA3 using an *in vitro* transcription system. *Virology* 183:106–113. [https://doi.org/10.1016/0042-6822\(91\)90123-S](https://doi.org/10.1016/0042-6822(91)90123-S)
- Takahashi T, Sugawara T, Yamatsuta T, Isogai M, Natsuaki T, Yoshikawa N (2007) Analysis of the spatial distribution of identical and two distinct virus populations differently labeled with cyan and yellow fluorescent proteins in coinfecting plants. *Phytopathology* 97:1200–1206. <https://doi.org/10.1094/PHYTO-97-10-1200>
- Takeshita M, Koizumi E, Noguchi M, Sueda K, Shimura H, Ishikawa N et al (2012) Infection dynamics in viral spread and interference under the synergism between cucumber mosaic virus and turnip mosaic virus. *Mol Plant-Microbe Interact* 25:18–27. <https://doi.org/10.1094/MPMI-06-11-0170>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.