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Host-specific loss of sequences of an alfalfa mosaic virus isolate during systemic infection

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ABSTRACT

Infectivity of an alfalfa mosaic virus (AMV) isolate from *Leonotis nepetaefolia* in different tomato cultivars was analyzed. Symptoms typical of AMV infection were observed in indicator plants, but not in Flora Dade and Rio Grande tomato cultivars; however, mild symptoms were observed in cv. Rutgers. Furthermore, at least 1 kb of the 3´ segment of RNA 2 and the coat protein gene were missing in systemic leaves of inoculated Rio Grande and Flora Dade plants, while in cv. Rutgers infected with this AMV strain all genomic components were detected. Northern blot analysis of plants infected with the aforementioned AMV isolate confirmed the absence of the CP gene, but suggested rearrangements in both RNA 2 and 3. Factors that may affect differential movement or systemic accumulation of genomic components in multipartite viruses in plants are discussed.

Weeds, invasive plant species that outcompete native or cultivated plants for resources due to their resilience (for example, the ability to tolerate drought stress and in perturbed areas as well as to produce ingent amount of seeds) may constitute reservoirs for pests and pathogens in general (Piedra-Ibarra et al., 2005). Epidemiological data is shedding light on the global role of weeds in the dispersion of plant pathogens (Jones, 2014). The presence of novel viruses in weeds as well as in crops, identified by large scale sequencing, supports this notion and may suggest a role of viruses in plant adaptability (Roossinck et al., 2010). On the other hand, it is not clear whether certain wild plants (or weeds) display higher levels of tolerance to viruses, or these evolve into less virulent strains within these hosts (Malmstrom and Alexander, 2016).

Among the most successful plant viruses in terms of host range are members of the *Virgaviridae* family [which includes tobacco mosaic virus (TMV)], and members of the *Bromoviridae* family, such as cucumber mosaic virus (CMV) and alfalfa mosaic virus (AMV). The latter is an RNA plus-sense strand tripartite virus with a wide host range that includes 232 species from 58 families. The replicase complex is encoded by RNA components 1 and 2, namely, the methyl transferase and helicase (P1 protein), and the RNA-dependent RNA polymerase (RdRp; P2 protein), respectively. The movement protein (MP, or P3), as well as the coat protein (CP), are encoded by RNA 3; this is transcribed to generate subgenomic RNA 4, which is in turn translated to yield the CP. All genomic components are packaged independently (https://viralzone.expasy.org/133?outline = all_by_species).

We have previously reported the presence of these (as well as a satellite RNA of CMV) in *Leonotis nepetaefolia*, a non-native plant found in Central Mexico that is a virus reservoir, during a survey for viruses in weeds (Piedra-Ibarra et al., 2005). We found that CMV and AMV did not induce all the typical symptoms expected in some indicator host plants tested. Indeed, this CMV isolate did not infect tomato, while AMV Leonotis did not induce necrosis in *C. amaranticolor*. Thus, infection by AMV in these hosts was analyzed with more detail.

Systemic infection and accumulation of different viral genomic components by two distict AMV isolates was analyzed. The AMV Leonotis strain (AMV Leo) was isolated from naturally infected *L. nepetaefolia* growing next to tomato fields in Atlixco, Puebla, as part of a complex that also harbored CMV and TMV, and was separated and identified through several passages by mechanical transmission onto indicator and differential plants, and detected by double Antibody Sandwich Enzyme-linked immunosorbent assay (DAS-ELISA) as previously described (Piedra-Ibarra et al., 2005). Although AMV can induce necrotic lesions in common bean from which it could be isolated, this was not carried out in order to avoid contamination with *Bean common mosaic virus*. The AMV Capsicum strain (AMV Cap) was isolated

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from *C. annuum* (chili pepper cv. Ancho) with yellow mosaic symptoms consistent with AMV infection in the field in Cuautla, State of Morelos, Mexico, and likewise identified, detected and propagated in indicator plants. Independently, symptomatic leaves of infected indicator plants with AMV were liophilized and stored at 4 °C prior to use as inoculum. For the biological characterization of both AMV isolates, some indicator and differential plant species were selected based on those suggested by Kurstak (1981) and May (1985): *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Gomphrena globosa* and *Solanum lycopersicum* (tomato) cultivars Rio Grande, Flora Dade and Rutgers. At least 6 plants per species/cultivar were mechanically inoculated in the cotyledonary stage using the infective tissue from indicator plants that were previously inoculated with AMV Leo or AMV Cap as described before (Piedra-Ibarra et al., 2005). Plants were grown under greenhouse conditions for 21 days post inoculation, with a 16 h light and 8 h dark photoperiod, and a maximum temperature of 30 °C and a minimum of 10 °C. Per each plant lot, negative control plants were mock-inoculated with leaf suspension from healthy control plants.

Both AMV strains induced mosaic and yellowing symptoms in *G. globosa*, *C. quinoa*, *C. amaranticolor*, and *S. lycopersicum* cv. Rutgers, but these were more marked in plants inoculated with AMV Cap compared with AMV Leo (Fig. 1A–D). Interestingly, while typical symptoms of AMV infection were observed in *S. lycopersicum* cv. Rio Grande and Flora Dade after inoculation with AMV Cap, no detectable symptoms were observed in these plants inoculated with AMV Leo (Fig. 1E–H).

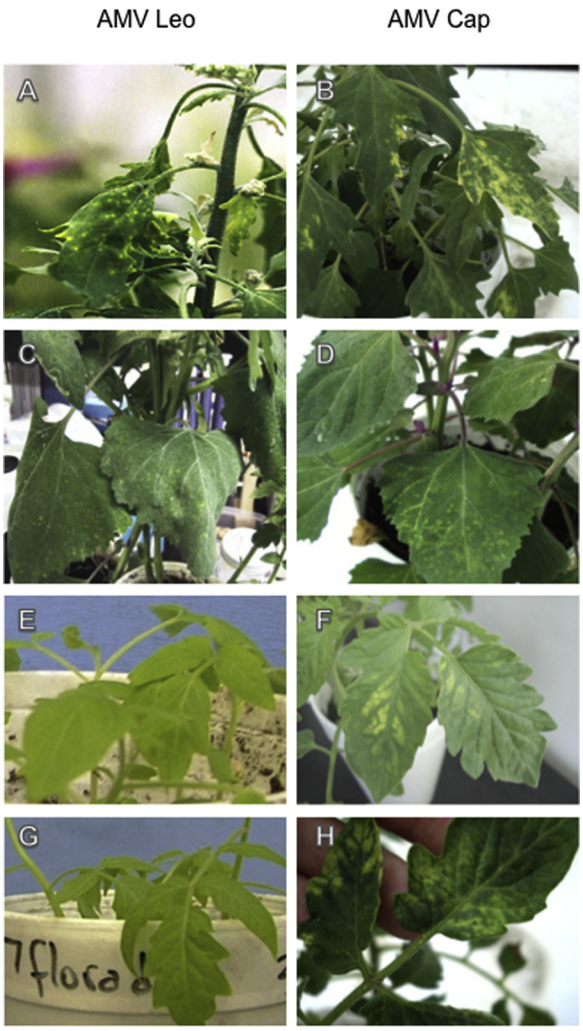

Fig. 1. Symptoms induced by different AMV strains. AMV isolated from *L. nepetaefolia* (AMV Leo) and AMV strain isolated from *C. annuum* (AMV Cap) in different hosts. A, C, E, G: plants inoculated with AMV Leo; B, D, F, H: plants inoculated with AMV Cap. A, B: *C. quinoa*; C, D: *C. amaranticolor*; E, F: *S. lycopersicum* cv. Rio Grande; G, H: *S. lycopersicum* cv. Flora Dade.

To determine whether the absence of symptoms in AMV Leo-inoculated Rio Grande and Flora Dade plants was due to its inability to infect locally and systemically these cultivars, detection of viral fragments by reverse transcription – polymerase chain reaction (RT-PCR) was carried out using RNA from local inoculated and upper non-inoculated leaves. Total RNA from AMV-infected and mock-inoculated control plants was isolated 21 days post inoculation essentially as described (Logemann et al., 1987). Specific primers were used for RT-PCR of RNA 1, RNA 2, and the MP and the CP genes (Fig. S1 and Table S1). For amplification of specific regions of AMV genomic components, cDNA was synthesized from total RNA from mock and infected plants with a SuperScript III/RNAseOUT enzyme mix following the supplier´s recommendations (Thermo Fisher, Waltham MA). The products were amplified with Taq polymerase (New England Biolabs, Beverly MA). In the case of RNA 1 and 2 the target was a region corresponding to a 3′ segment of each component, while for RNA 3 and 4 the open reading frame (ORF) of MP and CP genes were targeted (Fig. S1). PCR products were sequenced to confirm their identity. All genomic components from both AMV isolates were detected by RT-PCR in inoculated and upper non-inoculated leaves of *G. globosa*, *C. quinoa*, *C. amaranticolor*, and *S. lycopersicum* cv. Rutgers. However, in Rio Grande and Flora Dade cultivars when infected with AMV Leo, RNA 2 was not detected in local nor in upper non-inoculated leaves. Furthermore, while the MP gene was detected in local and upper leaves, the CP gene was absent in both cases (Fig. 2A, B). In contrast, such differential accumulation of viral sequences was not observed for AMV Cap- inoculated plants, in which all of the genomic components accumulated locally and systemically even in Rio Grande and Flora Dade (Fig. 2C, D).

In order to determine whether all or parts of AMV Leo RNA 2 and RNA 4 segments are lost during infection, Northern analysis was carried out with systemic leaves from Flora Dade plants infected with this virus. These were performed essentially as described (Sambrook and Russell, 2001). Inoculated upper systemic floral leaves were harvested 14 dpi. RNA was extracted as previously described, and 10 µg were run in denaturing conditions, and transferred to a nylon membrane, fixed with UV. Membranes were prehybridized and then hybridized with [α-32 P] dCTP-labeled probes, corresponding to the AMV component fragment cloned in pDrive (Qiagen) (described in supplementary Table 1), and washed, in stringent conditions at 65 °C. Membranes were then exposed to X-ray film and developed. For RNA 1 and RNA 2, the same membrane was used; RNA 1 was used as probe, and after exposure to film, stripped and re-hybridized to the RNA 2 probe.

While RNA 1 was detected, albeit at very low levels, a complex pattern was observed for the other components. Indeed, a band of roughly the size of RNA 2 is detected at similarly low levels along other bands that could correspond to ribosomal RNA or AMV fragments. It is then possible that parts of the 3´ portion of RNA 2 have been lost (including the primer binding sites, which could explain the failure to amplify this component with the primers used in this work) and replaced by other sequences. The band corresponding to AMV RNA 3 (2.1 kb) was also detected, as well as additional bands; this suggests that host host-derived sequences could be replacing most, if not all, the CP coding sequences. This same pattern was observed in infected indicator plants (left lanes), but no signal was detected in mock-inoculated indicator plants (right lanes), suggesting that the observed bands in Flora Dade did not correspond to ribosomal RNA, and point to rearrangement products. Furthermore, and, in agreement with the results obtained with RT-PCR, no signal whatsoever was observed with RNA 4 (CP), confirming the loss of sequences corresponding to AMV CP (Fig. 3). In contrast AMV Cap genomic components were all detected in both indicator and Flora Dade plants (Fig. S3).

In addition, phylogenetic analysis of virtually translated CP and MP gene sequences of AMV Leo was performed using different evolutionary models (Fig. S2). The phylogeny indicated that AMV Leo CP groups

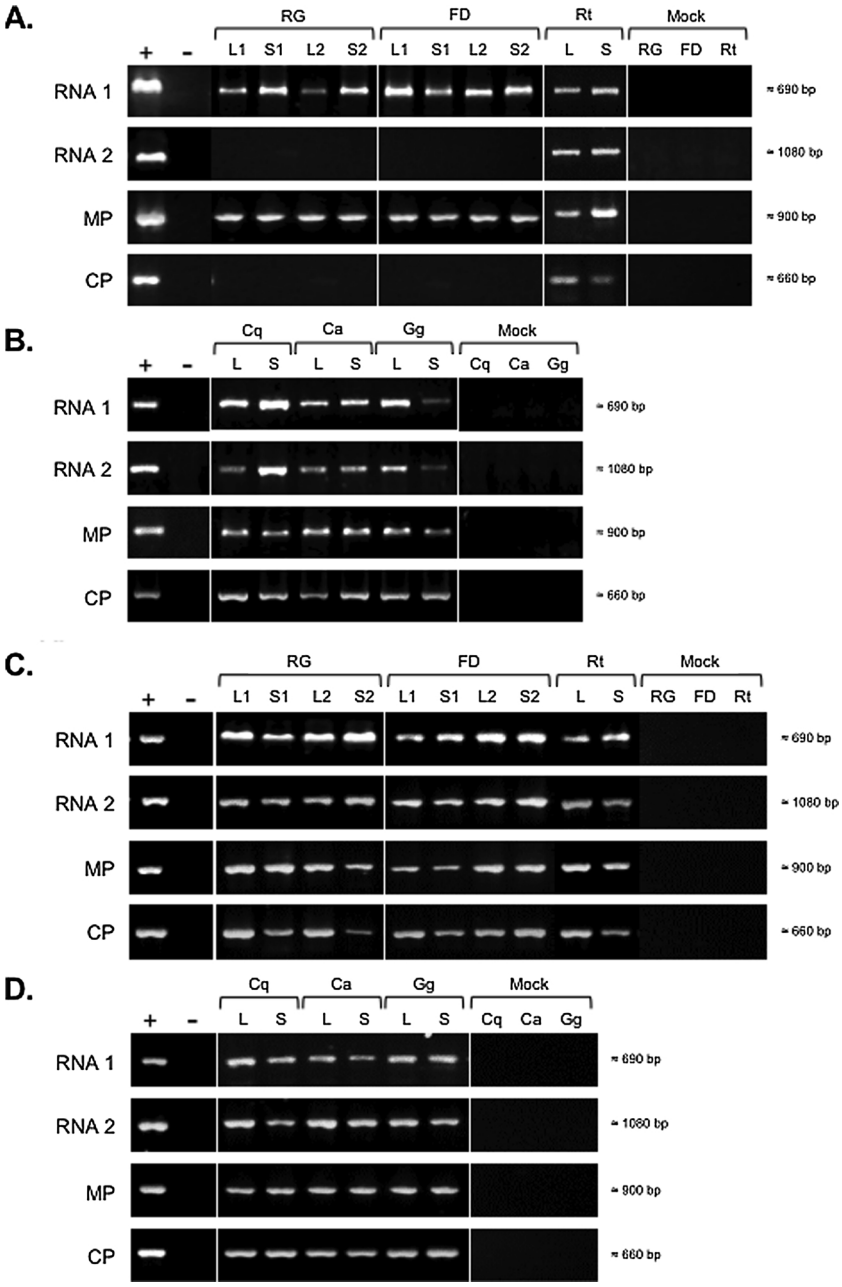

Fig. 2. Differential accumulation of genomic components of AMV Leo and AMV Cap in local and upper non-inoculated leaves. Detection in local (L) and upper non-inoculated (S) leaves of AMV genomic components in different plant hosts. RT-PCR analysis for detection of RNA 1, RNA 2, MP gene and CP gene in: A, B: plants inoculated with AMV Leo; C, D: plants inoculated with AMV Cap. A, C: two independent plants (1, 2) of *S. lycopersicum* cv. Rio Grande (RG) and Flora Dade (FD), and a pool from 4 independent plants of Rutgers (Rt); B, D: *C. quinoa* (Cq), *C. amaranticolor* (Ca) and *G. globosa* (Gg) indicator plants. In each case, amplification from mock-inoculated plants is shown. Positive controls (+): amplification of AMV genomic components cloned in plasmid and diluted to 1 ng/μL. Negative controls (-): corresponded to non-template reactions.

closely with the VRU strain (in tobacco experimental host), which forms unusually long particles (Thole et al., 1998) (Table S2). Indeed, both strains differ only in 2 amino acids (99.1% identity). It has been reported that the Ser 66 and Leu 175 residues are involved in the formation of such structures (Thole et al., 1998). Interestingly, while both AMV Leo and VRU, as most other isolates, share the Ser 66 residue, only these and few more harbor a Leu at position 175, which are part of the same clade (Fig S2). This suggests that AMV Leo may form similar structures. In contrast, the AMV Leo MP amino acid sequence is more closely related to the CP from isolate 175 (accession number AXP79051.1) from *S. tuberosum* (99% identity, not shown). These two isolates share differences with most other AMV isolates, but none reside in residues that appear important for MP structure (Mushegian and Elena, 2015). On the other hand, the MP amino acid sequence from AMV Leo and AMV VRU are more divergent (96% identity), as are the CP sequences from the former and isolate 175 (95% identity). This suggests that, at least regarding their CP, AMV Leo and VRU isolates could behave in a similar manner.

The CP gene is involved in the replication of AMV, as well as enhancing translation of viral RNAs (Bol, 2005; Pallás et al., 2013), and is required for cell-to-cell and long-distance movement (Tenllado and Bol, 2000; Sanchez-Navarro and Bol, 2001). Thus, AMV CP-mutants are defective in local and systemic movement. This is paradoxical in this particular case, in which the CP gene and at least half of RNA 2 (which encodes the RdRp) are missing. However, *Brome mosaic virus* RNA 3 is transported to systemic tissues even in the absence of replication (Gopinath and Kao, 2007). On the other hand, a CP unable to form virions can engage in AMV cell-to-cell movement, although in this case it enables its replication (Sánchez-Navarro et al., 2006). In any case, this suggests that host factors could replace viral proteins involved in replication and transport in some instances. Another possibility is that AMV Leo fragments and/or components are replicated by a hypothetical helper virus co-purified along the serial passages for the isolation of AMV Leo. However, RT-PCR and Northern blot analyses failed to detect TMV and CMV, viruses that were originally found along AMV in *L. nepetaefolia* after AMV Leo isolation in indicator plants (not shown).

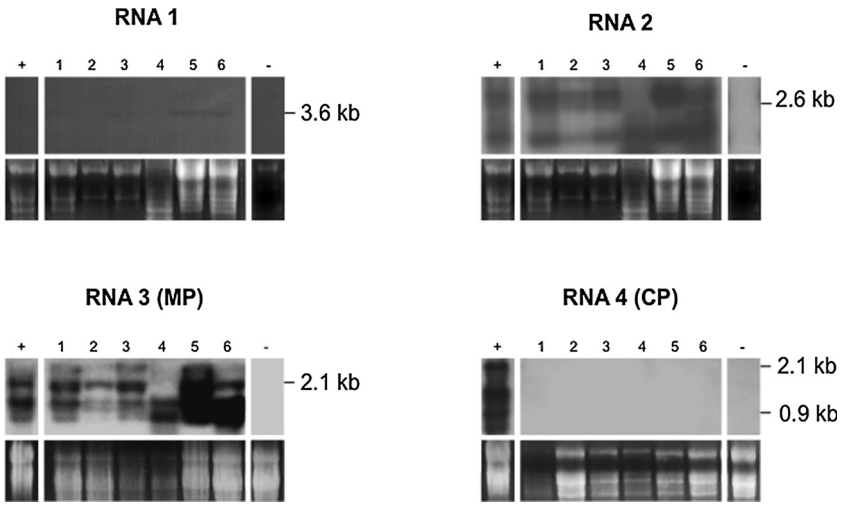

Fig. 3. Northern Blot analysis of systemic tissues from Flora Dade plants inoculated with AMV Leo. Total RNA from systemic leaves was isolated from 6 independent Flora Dade plants (1–6) inoculated with AMV Leo. RNA was hybridized with ^{32}P-labeled probes for AMV RNA1, RNA2 (upper panels), MP and CP (lower panels). RNA1 probe yielded a faint band corresponding to the expected size, while additional bands to RNA2 and RNA3 are observed, while no signal corresponding to CP was detected. Positive control (+) corresponded to total RNA from systemic 1 leaves isolated from *C. quinoa* inoculated with AMV Leo. Negative control (-) corresponded to total RNA from mock-inoculated *C. quinoa* plants.

Conversely, it could not be discarded that RdRp is synthesized in local leaves and is then transported long-distance to systemic tissues where it would replicate the viral RNA sequences. A similar situation could be envisaged for the CP gene. It has been observed that a chimeric construct harboring the AMV MP gene and other MP genes belonging to the 30 K superfamily have the ability to support long-distance movement of such construct (Fajardo et al., 2013). It remains to be determined whether such complementation occurs between AMV CP and replicase and the corresponding proteins encoded by other viruses. It must be emphasized that, while RNA 2 was not detected by RT-PCR, Northern blot analysis revealed the presence of several bands in plants infected with AMV Leo when hybridized to RNA 2 and 3 probes, suggesting that rearrangements may have occurred. Similar bands were observed in *C. quinoa* inoculated with AMV Leo.

No symptoms were observed in cultivars upon infection with AMV Leo, which is likely explained by the fact that the AMV CP interferes with the defense response through interaction with a transcription factor involved in salicylic acid-mediated defense signaling, accounting for the absence of symptoms in Rio Grande and Flora Dade infected with AMV Leo (Aparicio and Pallás, 2017).

Since part of RNA 2 and the CP coding region are missing in both inoculated and upper non-inoculated leaves, it cannot be discarded an inhibition of the corresponding viral RNA movement. It is likely that the phenomenon observed in Rio Grande and Flora Dade upon AMV Leo infection, is due to an impediment in the cell-to-cell movement or to a defense reaction of the plant that prevents the virus from progressing. In fact, the success of a systemic infection is determined by the speed of cell-to-cell movement and the number of primary infection sites (Rodrigo et al., 2014). Further work will help to elucidate the molecular mechanisms underlying this phenomenon, and how general it is in plants.

Authors contributions

RR-M and BX-C designed the experiments, wrote the manuscript and analyzed the data; BC-P carried out most of the experiments; EP-I helped with plant inoculation and along with RT-A characterized both AMV Leo and AMV Capsicum isolates.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197703.

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