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Distribution of carnation viruses in the shoot tip: Exclusion from the shoot apical meristem

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Abstract

Cell biological tools were used to localize the genomic RNAs/DNA and/or the coat protein (CP) of *Carnation mottle*, *Carnation vein mottle*, *Carnation latent*, and *Carnation etched ring* viruses in the carnation shoot tip to know whether any of them was capable of infecting the shoot apical meristem. Our results showed that all the viruses studied were excluded not only in single-infected but also in double-infected plants. Despite that, these viruses showed a different infection pattern inside the shoot tip, with different cell types being their main targets. For the mixed infection studied here, our results showed the existence of a spatial separation pattern within the carnation shoot tip which strongly suggests an exclusion phenomenon.
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Keywords: *Carnation mottle virus*; *Carnation vein mottle virus*; *Carnation latent virus*; *Carnation etched ring virus*; *Dianthus caryophyllus*; cv. Dixie; *In situ* hybridization; Immunohistochemistry; Shoot tip; Shoot apical meristem; Single and mixed viral infection

1. Introduction

The systemic infection of a plant depends on the capacity of a given virus to replicate in the initially infected cells, move to adjacent cells through the plasmodesmata and reach distal parts of the plant through the vasculature [1,2]. Although considerable progress has been made during recent few years to elucidate virus invasion patterns of plant organs and tissues, viral infection of the shoot tips have been less studied. The shoot apical meristem (SAM) of higher plants functions as a site of continuous organogenesis within which a small pool of pluripotent stem cells, replenishes the cells incorporated into lateral organs (leaves, stems and flowers) [3]. At present, a large body of evidence suggests that most viruses and viroids are unable to invade the SAM [4–8]. One exception to this general rule is the work of Cohen et al. [9]. These authors used a chimera of *Turnip crinkle virus* (TCV) in which the coat protein (CP) gene was replaced by that of GFP. The consequent GFP expression was detected in the meristematic regions when this chimeric virus was inoculated into transgenic plants expressing TCV CP.

Carnation is susceptible to infection by many viruses, which in some cases may result in serious losses. Those caused by *Carnation mottle* (CarMV), *Carnation etched ring* (CERV), *Carnation vein mottle* (CVMV), *Carnation ringspot* (CRSV), *Carnation Italian ringspot* (CIRSV) and *Carnation latent* (CLV) viruses are the most important. These viruses occur as single and, more frequently, multiple infections. From all the viruses infecting carnation, we have focused on: (i) CarMV, which is the type member of the genus *Carmovirus* within the family *Tombusviridae* of plant viruses. This is a 34 nm icosahedral plant virus consisting of a single-stranded, positive-sense 4.5 kb RNA [10]. CarMV is thought to be involved in synergistic reactions in mixed infections, with a negative impact on cut-flower production [11–13]. (ii) CVMV, which is a *Potyvirus* within the family *Potyviridae* of plant viruses. It has filamentous, usually flexuous virion particles, 790 nm long and 12 nm wide. Its genome consists of a

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single-stranded RNA [14]. The symptoms include chlorotic and darker green spots, flecks and mottling [15]. (iii) CLV, which is the type member of the genus *Carlavirus* with filamentous, usually straight (or slightly curved) virions, 650 nm long and 12 nm wide. Its genome consists of a single-stranded, positive-sense 8.5 kb RNA [14]. The virus causes few or no symptoms; (iv) CERV is a *Caulimovirus*. It is a 45 nm isometric plant virus [16] with a genome of DNA (gDNA); double-stranded; circular and linear (also super coiled) with a size of 7.9 kb [14]. Replication of the vDNA involves the reverse transcription of the largest transcribed RNA. The symptoms vary seasonally and it is often symptomless, although it sometimes causes necrotic flecks and lines.

In the case of many virus–shoot combinations [17], the meristem tip appears as a zone of variable length (usually about 100 μm but up to 1000 μm) near the shoot or root tip that is free or contains very little virus [18,19]. This situation has already been exploited to obtain virus-free clones by growing excised shoot tips in tissue culture. Meristem tip culture has already been used to free carnation cultivars from the different viruses described, although, some of these, such as CERV, are very difficult to eliminate by this method. It is not always clear what the success of such a method depends on: the regular absence of virus from meristem tissue, some meristematic regions in the plant containing viruses and others containing none, or the inactivation of the virus present in the meristem during culture [17].

In this work, we studied the CP and/or genome distribution, in the carnation shoot tip, of the main viruses infecting carnation (CarMV, CVMV, CLV, and CERV), when present either in single or mixed (CarMV + CERV) infections, by *in situ* techniques (immunohistochemistry and *in situ* hybridization) at the light microscopy level, demonstrating that they are not able to reach the SAM of the infected plants.

2. Material and methods

2.1. Plants, growth conditions and CarMV inoculation

Carnation plants (*Dianthus caryophyllus* L.) infected with the different viruses studied as well as virus-free carnations produced by meristem tip culture were grown and provided by Barberet & Blanc, S.A. (one of the main carnation breeders in Europe). Plants were grown and maintained, in a greenhouse at temperatures of 35–40 °C day/12–15 °C night, with a day irradiance varying from 300 to 650 μmol photons/m^2 s.

Some of the carnation plants studied were mechanically inoculated with a Dixie isolate of *Carnation mottle virus* (CarMV-Dix) obtained from naturally infected carnation plants (*D. caryophyllus*, cv. Dixie) [20]. The CarMV-Dix was cloned, propagated in *Chenopodium quinoa* plants and purified from leaf tissue as previously described [21]. Thirty five days after the rooting of the carnation cutting, one leaf per plant was mechanically inoculated with 20 μg/ml of purified CarMV in 20 mM potassium phosphate buffer (pH 7.0), using carborundum as abrasive. The inoculation was carried out only on the apical part of the leaves. Mock-inoculated control plants were treated similarly.

2.2. Synthesis of digoxigenin-labelled RNA probes

Digoxigenin-labelled RNA (dig-RNA) probes were synthesized from the corresponding plasmids previously obtained in the lab: pCarM.EC3 [22–24]; and pCVMV2; pCLV-1 and pCERV-1 [25] containing fragments of the genome of the viruses CarMV, CVMV, CLV and CERV, respectively, placed between the T3 and T7 RNA polymerase promoters. Plasmids were linearized with the corresponding restriction enzymes most suited to each one: pCarM.EC3, *Eco* RI; pCVMV2, *Hind* III; pCLV-1, *Eco* RI and pCERV-1, *Bam* HI. Then, the plasmids were digested and purified by phenol-chloroform extraction and ethanol precipitation. The dig-RNA probes were synthesized as previously described [26]. Transcript RNA was recovered by ethanol precipitation and resuspended in sterile water.

2.3. Tissue fixation and embedding

Shoot tip samples were harvested from carnation plants and processed as previously described [23,27]. Briefly, longitudinal sections through their center were obtained with a razor blade and both parts were fixed in a freshly made mixture of 4% *p*-formaldehyde and 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) at 4 °C for 4 h. Samples were vacuum-infiltrated for 2 min immediately after being put into the fixative. They were then washed in the same buffer, dehydrated in a tertiary butyl alcohol series, infiltrated and embedded in paraffin (Paraplast Plus, Sherwood Medical Co., St Louis, MO, USA). Individual blocks of paraffin were sectioned using a Reichert-Jung 2030 (Leica Mycrosistems; Nusloch, Germany) rotary microtome set to 8 or 10 μm. The longitudinal sections were fixed to glass slides precoated with APTES (3-aminopropyl-triethoxy-silane; Sigma-Aldrich Co., St Louis, MO, USA) by heating them at 40 °C on a hot plate. The slides were allowed to stand overnight before further processing to make sure that the sections were tightly adhered to the slide.

2.4. In situ hybridization

In situ hybridization was performed as previously described [23,27,28]. Briefly, tissue sections on slides were dewaxed with xylene (2 × 10 min each) and dehydrated with a series of decreasing ethanol concentrations from absolute ethanol to water. They were then incubated in 0.2 M HCl for 20 min, washed in distilled water, 2 × SSC buffer (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and again in distilled water, 5 min each time. Then, they were treated with proteinase K (1 μg/ml, in 100 mM

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