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In situ hybridization for the localization of two pepino mosaic virus isolates in mixed infections

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ABSTRACT

In situ hybridization (ISH) is an informative and relatively accessible technique for the localization of viral genomes in plant tissue and cells. However, simultaneous visualization of related plant viruses in mixed infections may be limited by the nucleotide similarity in the genomes and the single chromogenic detection over the same sample preparation. To address this issue, we used two *Pepino mosaic virus* isolates and performed ISH over consecutive serial cross-sections of paraffin-embedded leaf samples of single and mixed infected *Nicotiana benthamiana* plants. Moreover, the probe design was optimized to reduce cross-hybridisation, and co-localization was based on the overlapping of consecutive cross-sections from mixed infected leaves; thus, our results showed that both *Pepino mosaic virus* isolates co-localized in the same leaf tissue. In turn, both isolates were localized in the cytoplasm of the same cells. These results provide valuable information for studying mixed infections in plants by using a simple ISH procedure that is accessible to any pathology laboratory.

Mixed infections of plant viruses are common in nature (Juarez et al., 2013; Kassem et al., 2007) and can have important epidemiological and disease outcomes. Mixed viral infections can lead to competitive interactions that may create spatio-temporal distribution patterns throughout plant tissues, and hence, structured sub-populations within the plant (Tromas et al., 2014). A number of experimental approaches have already been used to determine within-plant viral distribution (Bergua et al., 2016; Dietrich and Maiss, 2003; Hall et al., 2001; Takahashi et al., 2007; Takeshita et al., 2004). Many of these studies have employed protein markers from among the broad repertoire of fluorescent proteins, such as the green fluorescent protein (GFP), expressed from engineered viral genomes in order to visualize viral distribution during plant infections (Dietrich and Maiss, 2003; Oparka et al., 1997). Other studies have used *in situ* hybridization (ISH), where the viral genome could be localized at the cellular level (Amari et al., 2009, 2007; Gómez-Aix et al., 2015; Gosalvez-Bernal et al., 2008, 2006), with ISH having the advantage of localizing wild type, unmodified viral genomes. Non-radioactive ISH methods have also been used for chromosome mapping (Gordon et al., 1995), gene expression localization (Zeller et al., 2001), and pathogen detection in animal (Montone and Guarner, 2013) and plant tissues (Alves-Júnior et al., 2009; Bergua et al., 2016; Rentería-Canett et al., 2011; Tanaka, 2009; Wang et al., 2010). Most of the works addressed the localization of a single mRNA type, rarely attempting to co-localize mRNAs that share a significant degree of similarity, as might be the case of RNAs from viral isolates belonging to different strains. We have addressed this issue by using two different isolates of *Pepino mosaic virus* (genus *Potexvirus*, family *Alphaflexiviridae*) in mixed infected *Nicotiana benthamiana* plants.

Pepino mosaic virus (PepMV) is a widespread plant virus that has caused several outbreaks worldwide in tomato crops, resulting in important negative economic consequences (Gómez et al., 2012; Hanssen and Thomma, 2010). The virions are filamentous and the genome is a single-stranded, positive-sense, RNA molecule of approximately 6400 nucleotides with five open reading frames (ORFs) (Aguilar et al., 2002). ORF1 encodes the putative viral replicase containing the methyl transferase, helicase and RNA-dependent RNA polymerase (RdRp) domains; ORFs 2, 3 and 4 form the triple gene block (TGB), essential for cell-to-cell movement and for RNA silencing suppression, and ORF5 encodes the coat protein (CP) (Verchot-Lubicz et al., 2007). Our previous molecular epidemiology studies showed that the PepMV populations sampled in southeastern Spain (Murcia region) between 2005 and 2008 consisted of isolates belonging to two co-circulating genotypes, Chilean (CH2) and European (EU); although the CH2 isolates predominated, the EU isolates persisted in mixed infections (Gómez et al., 2009).

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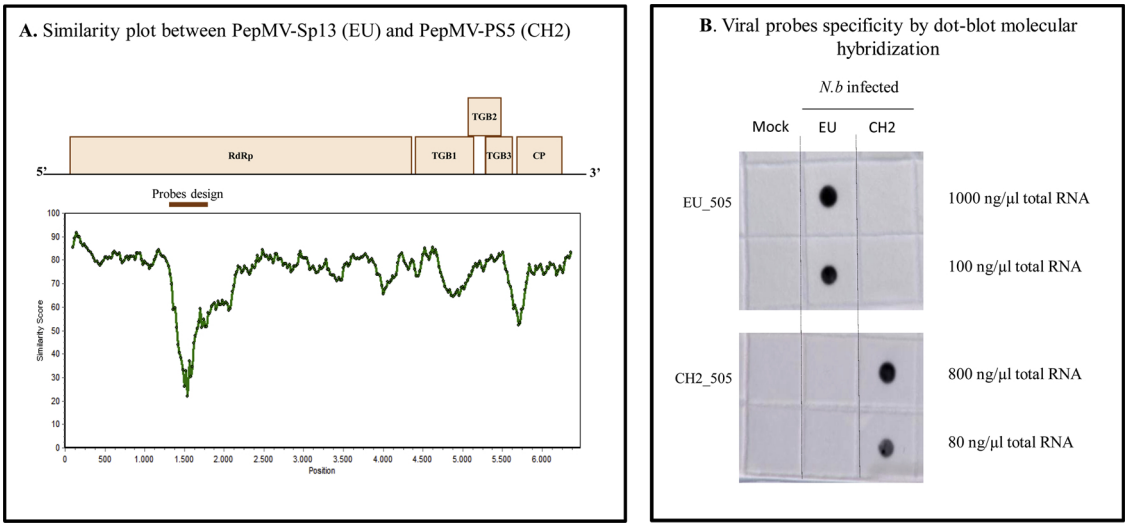

Fig. 1. PepMV probes. A. Similarity plot between PepMV-Sp13 (EU) and PepMV-PS5 (CH2). The similarity plot analysis helped us to identify regions with the lowest nucleotide similarity by showing the variations in the sequence similarity degree between both PepMV isolates along the PepMV genome. It also shows the genomic region that was finally chosen for the probes used corresponding to the RdRp ORF (EU_505 and CH2_505, respectively). B. Viral probes specificity. Dot-blot molecular hybridization on total RNA extracts from mock and single infected *N. benthamiana* plants with the RdRp ORF1 riboprobes. Both riboprobes: PepMV-Sp13 isolate (PepMV-EU type; EU_505) and PepMV-PS5 (PepMV − CH2 type; CH2_505), were specific and no cross-reaction was observed.

The aim of this study was to design digoxigenin-labelled RNA probes that targeted PepMV genomic regions with a high specificity and sensitivity for PepMV isolates of the EU or CH2 genotypes, as well as to obtain a simple and specific ISH procedure to localize both PepMV isolates in tissue sections of mixed infected *N. benthamiana* plants. For this, ISH assays were performed over serial cross sections of paraffin-embedded samples of single and mixed infected plant leaf tissue, to examine the viral RNA distribution in single and mixed infections of *N. benthamiana* plants.

We first compared PepMV-EU and −CH2 reference genomes, which have a sequence similarity ranging from 78% to 86% depending on the genomic region, in order to identify a genomic region that could be used to distinguish both PepMV isolates. A plot similarity analysis identified the regions with the lowest nucleotide similarity (Fig. 1A). A genomic region covering a portion of the RdRp ORF1 was selected for the synthesis of both riboprobes, which were named EU_505 and CH2_505; nucleotide sequence identity within this region among the two reference genomes was on average 60%, with a minimum of 22% and a maximum of 78% when looking at a 525 nt window (Fig. 1A). cDNA fragments that were 349 bp and 481 bp in length from ORF1 of PepMV-EU and PepMV−CH2 isolates (Table 1) were respectively cloned in the pGEM-T easy vector (Promega corporation) and used for transcription of the two probes. The in vitro transcription was performed with the SP6 RNA polymerase (New England Biolabs) and DIG RNA Labeling Mix (Roche) following the manufacturer´s recommendations.

Each probe was tested for background and cross-detection by dot-blot molecular hybridization on total RNA extracts from mock- and single-infected *N. benthamiana* plants. Plants (mock, single and mixed infected) were grown and inoculated as described by Gómez et al. (2009). The mixed infected plants were inoculated using purified viral particles at a final concentration of 10 ng/µl by mixing equal volumes (1:1) of both viral preparations. No background signal was observed from mock-inoculated plants, and both probes were specific for the corresponding virus isolate, with no cross-reaction with the heterologous viral RNA (Fig. 1B). Probe specificity was then tested in ISH assays on consecutive tissue sections from mock-, single- and mixed-infected *N. benthamiana* plants, basically following the procedures described by Gosalvez-Bernal et al. (2008). Apical leaves from mock, single (PepMV EU or CH2 type) and mixed (PepMV-EU + PepMV−CH2) infected plants were collected, fixed with paraformaldehyde, embedded in paraffin and sectioned (Fig. 2).

For fixation, sections of leaf samples were cut with a sterile blade and immersed in recently prepared 4% paraformaldehyde (PFA, Acros organics, Belgium) in 1x phosphate buffered saline solution (PBS) with 0.1% Triton X-100 (Merck). Immediately after the immersion, samples were vacuum-infiltrated for ≈1 min to help with the penetration of the

Table 1
PepMV nucleotide sequences and genomic region from the PepMV-Sp13 (EU type) and PepMV-PS5 (CH2 type) isolates that were used for the synthesis of the RNA ISH probes.

Probe	PepMV sequence used for probe synthesis	Genomic region	Start and end
EU_505	5' GCAGCTGACTCAATTGGTCAAAAAAGGTACAATATCCTTAGCAGATTTTGAACAGAGAGAACCTGAAATAACTTACACTGAGTTTGAGCCTGAAACT AGGCCCCAAGTGGACTGCGTTACTAATTATAATAATGCAGTAAAAAATTTAGGTCTTTCTGCACTTGATGAACAGCCTCAATGTTCATCTTCTAGCA GTCATATACCCTGCAATGAAATATCCTTAGCAATGACTGATGACGACAATGCTGCGGCCATTCATGAAATTGAATCTCTATTGTCTGAACCGATAAT AGCTCCTCAACTCCCAGCATTGCCACACAAGACATGGGCCAGTTATGCTTCAATCACT 3'	RdRp	1302-1650
CH2_505	5' AAGGCGTAGTAACATTAGCTGATTTCAAAGAAGCGGACCAGCATGTGGAGTACACTCACTTTGATCCTGAGTTAAAATCCACTGTTGACCCCCACCG GAGCTATGAAAATGCCATCAACAATCTTGGCATTGAGATTAATGAGGATGTACCTGAAAGTTCCGGCACCAATGAAACATTGCTTAACAATGAAATA TCTTTAGCAATGTCATCTGCTGAACATGTGCAAGCTGTTCAAGAAATTGAGTCTTTACTCTCTAACCCCGAAGCGGCACCAATATTGCCCCCTGCAC ATGTTAAAACATGGGCTAGCCTTGCATCTGACACTTCAAGCACTAAAAACCGTGAAATCGAAGATATAGTGGCTAAGCTGGAAATACAAAGAAATGA AGCTAGTTGCAGCTACCTTCAACCAAATAAGGAATTGTCAAAACCCAAGGCTGCTGATAACAATCTCCCCTGGAATGCTTGGATCCCATTGCT 3'	RdRp	1325-1805

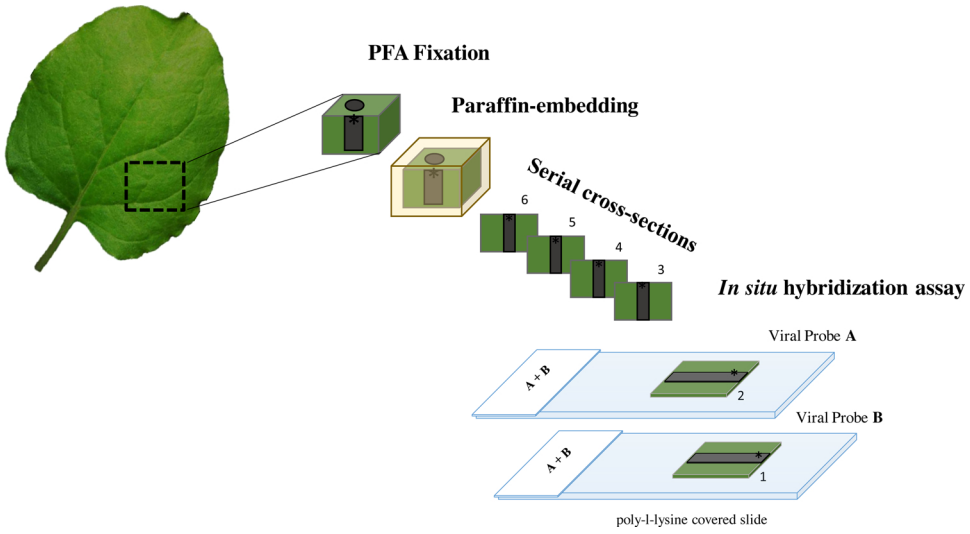

Fig. 2. Schematic representation of the ISH assay. Mock, mixed and single infected *N. benthamiana* leaves were sectioned in rectangular blocks, paraformaldehyde (PFA) fixed, paraffin embedded and sectioned. The ISH was performed on consecutive leaf cross-sections of the same sample by using each probe (A or B) in each slide (1 or 2), respectively.

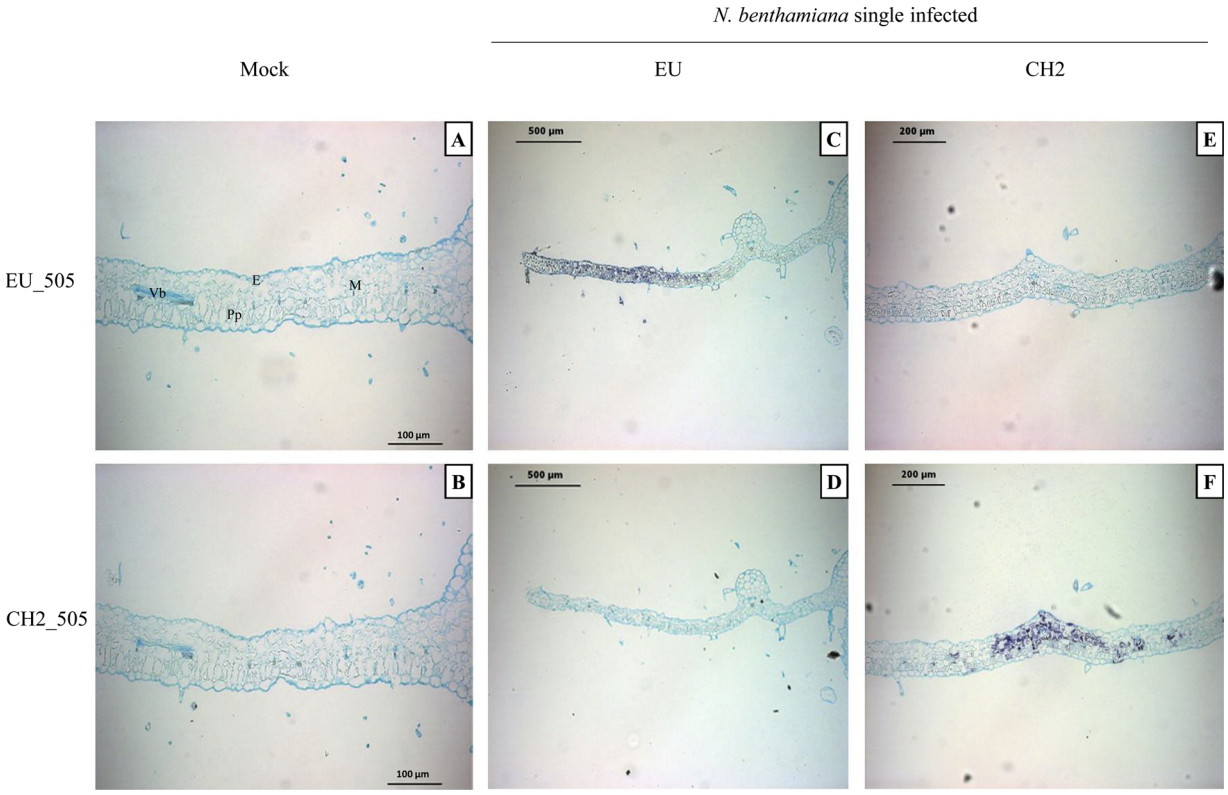

Fig. 3. *In situ* hybridization (ISH) on consecutive leaf serial cross-sections of *N. benthamiana* mock and PepMV single infected samples. A, B: Leaf serial cross-sections from mock inoculated plants used as negative control of the ISH, showing no target reaction with the riboprobes EU_505 (**A)** or CH2_505 (**B)**, respectively. **C, D:** ISH performed on consecutive leaf serial cross-sections from single PepMV-EU inoculated plants with either the riboprobe EU_505 (**C**) or CH2_505 (**D**), the viral RNA of the EU isolate is only located in the **C** panel as a dark-blue coloured area distributed in patches of infected tissue mixed with areas of non-infected tissue. **E, F:** ISH performed on consecutive leaf serial cross-sections from single PepMV – CH2 inoculated plants with either the riboprobe EU_505 (**E**) or CH2_505 (**F**), the viral RNA of the CH2 genotype is only located in the **F** panel as a dark-blue coloured area distributed in patches of infected tissue mixed with areas of non-infected tissue. In all samples, the tissue infected corresponds to spongy mesophyll (M), abaxial and adaxial epidermis (E), palisade parenchyma (Pp) and vascular bundle (Vb) cells. The uniform blue colour is due to the alcian blue post staining. Scale bars are displayed in the images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

fixative into the cells and left overnight at 4 °C. The next day, the PFA was eliminated by performing two washes with 1x PBS (30 min each), then, samples were dehydrated with ethanol (Merck), followed by increasing concentrations of a xylene substitute (Shandon™ Xylene Substitute, Thermo Fisher Scientific), at room temperature. Finally, wax paraffin (Paraplast Plus, Sherwood Medical Co., St Louis, MO) was slowly added and the xylene substitute was replaced slowly for three days.

The 5–7 µm serial cross sectioning of paraffin-embedded leaves was performed with a HM325 Micron microtome (Micron International, Germany). Each individual cross-section was placed in a poly-L-lysine covered slide (VWR International BVBA, Leuven, The Netherlands), and the slides were maintained in order. The order of the slides was very important as the slides were alternatively hybridized with EU_505 and CH2_505

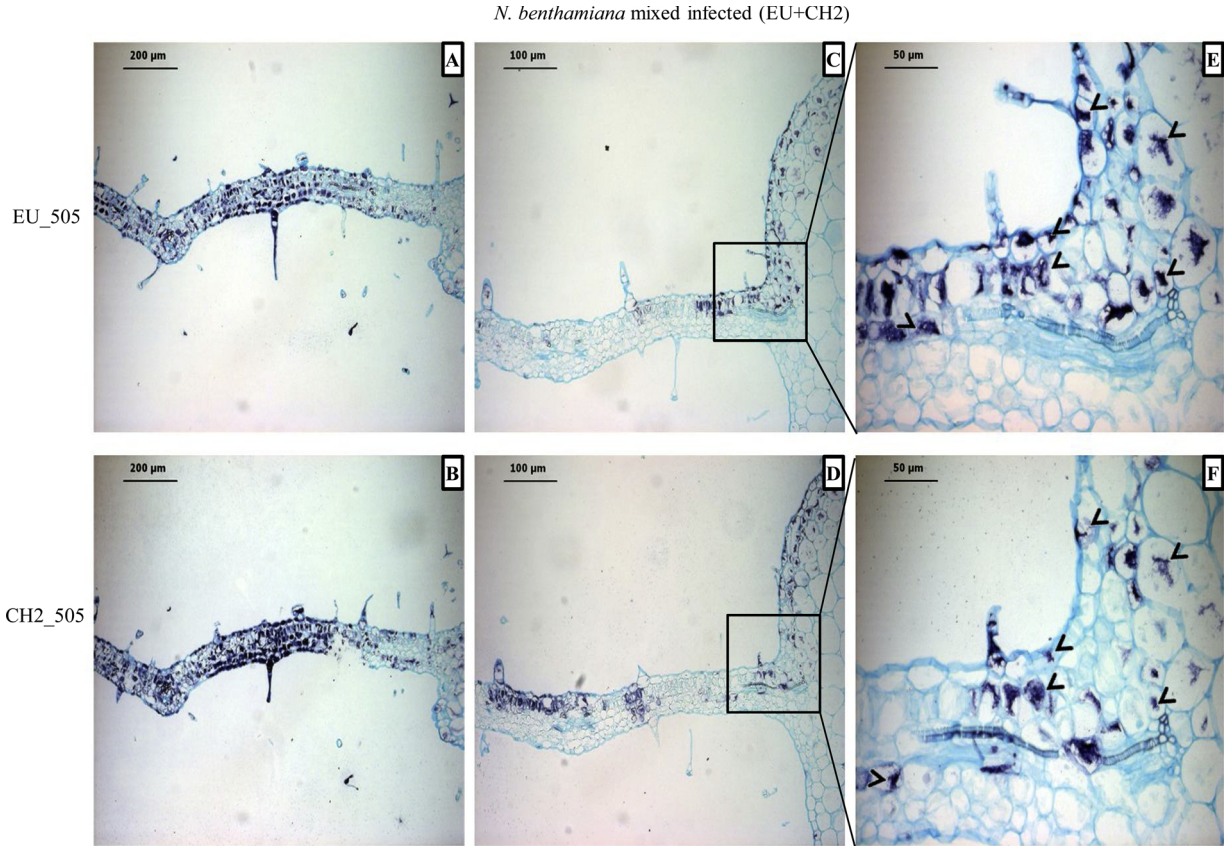

Fig. 4. *In situ* hybridization (ISH) on consecutive serial cross-sections of PepMV mixed infected *N. benthamiana* leaves. **A, B**: Low magnification images of the ISH on a pair of consecutive serial cross-sections of leaves from mixed (PepMV-EU + CH2) inoculated plants with either the riboprobes EU_505 (**A**) or CH2_505 (**B**), the viral RNA of both EU and CH2 genotypes are located in the same extended dark-blue coloured area of the leaf sections distributed in large patches of infected tissue mixed with areas of non-infected tissue. **C, D:** Low magnification images of the ISH done on another pair of consecutive leaf serial cross-sections from mixed (PepMV-EU + CH2) inoculated plants with either the riboprobe EU_505 (**C**) or CH2_505 (**D**); both viruses are located in dark-blue coloured areas of the leaf sections distributed in patches of infected tissue mixed with areas of non-infected tissue. **E, F:** The area boxed in C, D, respectively, at higher magnification, shows that the viral RNA of both EU and CH2 genotypes are located in the same dark-blue coloured cells (arrowheads) of the leaf mixed together with other non-infected tissue cells or only infected by one of the isolates. Scale bars are displayed in the images (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

probes (Fig. 2), resulting in side-by-side comparisons. For ISH, the poly-L-lysine slide mounted tissue sections were first dewaxed with Shandon™ Xylene Substitute (Várallyay and Havelda, 2011). Then, the samples were hydrated in decreasing concentrations of ethanol and the endogenous alkaline phosphatase was inactivated with 0.2 M HCl for 20 min. Afterwards, digestion with proteinase K (1 µg/ml in 100 mM Tris−HCl, 50 mM EDTA, pH 8.0) was performed at 37 °C for 30 min (Lee et al., 2013), followed by a glycine wash (2 mg/ml in 1x PBS for 2 min) to block proteinase K, and PBS washes. The post-fixation was done with 4% PFA in 1x PBS (12 min), washed again twice in 1x PBS (5 min) and dehydrated in an increasing ethanol series. Probes were hybridized at 55 °C overnight in a humid chamber inside an oven. Before hybridization, the probes were denatured at 92 °C for 3 min, and put back on ice. Probes were used at a final concentration of 500 ng/ml, diluted in the hybridization solution (50% formamide, 6x SSC, 1x Denhardt's solution, 10% dextran sulfate and 10 mM DTT) (Lee et al., 2013). After hybridization, a RNase/2x SSC (RNase A, Sigma-Aldrich®) treatment was done in order to eliminate the RNA that did not hybridize (20 µg/ml at 37 °C for 10 min), followed by washes with 2x SSC (0.30 M NaCl plus 0.03 M sodium citrate, pH 7.0) with 0.1% SDS (2 times, 5 min each) and washes with 0.2x SSC plus 0.1% SDS (2 times, 10 min each). A TBS wash (100 mM Tris−HCl, 400 mM NaCl, pH 7.5, 5 min) and incubation with 0.5% BSA/TBS for 1 h (bovine serum albumin fraction V, Acros organics, Fisher Scientific, USA) to block unspecific labelling, were done. Then, the samples were incubated with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Anti-Digoxigenin-AP, Roche Molecular Biochemicals) for 1 h (1:500 in 0.5% BSA/TBS), and additional washes in TBS (3 times, 5 min each) were performed. Samples were washed in detection buffer (100 mM Tris−HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) and incubated for ≈ 1 h in darkness with ready-to-use NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate, Roche Molecular Biochemicals), prepared in detection buffer (1:20). Lastly, slides were counterstained with alcian blue (Várallyay and Havelda, 2011), carefully dried out and mounted on Merckoglass medium before observing them under bright field illumination with a Leica Leitz DMRB light microscope (Leica microsystems). All the steps were performed in Coplin jars except for the hybridization and incubations in proteinase K, RNase, anti-digoxigenin-AP and NBT/BCIP, which were done on the slides with coverslips to help with the spreading of the liquid.

Avoiding and assessing background noise as well as non-specific hybridizations is essential for guaranteeing the specificity of the observed signal. The RNase treatment described above was introduced with this objective, and several specificity controls were used, such as: i) mock inoculated sections (Fig. 3A, B); ii) single infected sections hybridized with the heterologous probe (Fig. 3D, E, respectively); and iii) ISH on single and mixed infected sections without adding any probe to the hybridization solution (data not shown). No ISH signal was detected in mock-inoculated consecutive serial cross-sections of leaves, which showed a homogeneous blue colour of the leaf tissue due to post-staining with alcian blue (Fig. 3A & B). ISH on two consecutive serial cross-sections of leaves from single PepMV-EU inoculated plants

showed that only the EU_505 probe reacted, observed as a dark-blue coloration, as a result of positive RNA target detection, while there was no reaction for the CH2_505 probe on a consecutive leaf cross-section (compare Fig. 3C & D). Similarly, ISH on two consecutive leaf serial cross-sections of PepMV − CH2 inoculated plants showed a dark-blue signal only for the CH2_505 probe (Fig. 3F), and no signal at all was detected with the EU_505 probe (Fig. 3E). These results demonstrated the specificity of both riboprobes for detecting their viral RNA targets. The low magnification images in Fig. 3C to F showed that viral RNA distribution of both PepMV isolates was discrete, creating a mosaic of infection patches of different sizes, including the abaxial and adaxial epidermis, spongy mesophyll, palisade parenchyma and vascular bundle cells (Fig. 3C & F). We then performed ISH over consecutive serial sections of mixed infected samples of *N. benthamiana* leaves (Fig. 4). At low magnification, in two consecutive serial cross-sections of leaves hybridized either with the EU_505 (Fig. 4A) or the CH2_505 (Fig. 4B) probes, both isolates were localized to the same area of the tissue, as shown by the leaf region with a dark-blue colour. A more detailed observation in a new pair of consecutive leaf serial cross-sections showed that viral RNAs from both isolates were localized in patches of consecutive tissue sections (see low magnification Fig. 4C & D). Images, from the same pair of sections above, obtained at higher magnification showed that both isolates were infecting the cytoplasm of the same cells at least in some cases (Fig. 4E & F, arrowheads). Counting the cells (Fig. 4E & F) that were infected by one or both of the viruses, we found that from a total of 62 cells infected, 47% were infected by the EU isolate, 18% were infected by the CH2 isolate and 35% were infected by both isolates. In all samples, the infected tissue corresponded to the abaxial and adaxial epidermis, spongy mesophyll, palisade parenchyma and bundle sheath cells. Note that a good structural preservation is essential for working at this high magnification (Fig. 4E & F) and for clearly distinguishing the infected cells in specific, discrete plant tissue areas.

Thus, the designed probes specifically detected their targets. This is very important for the success of our work, as sequence similarity of the two PepMV isolates was high. These and similarly designed probes and ISH experiments may assist in the finding of the distribution and the characterization of plant viruses under single or mixed infections during the infection processes.

A very important improvement of our ISH method was the use of consecutive serial cross sections of leaves from the same mixed infected samples with a specific thickness that allowed us to have the same cells in consecutive slides and to determine that both isolates can co-infect host cells.

In conclusion, this *in-situ* hybridization technique is remarkably useful for pathologists, as it is an informative and accessible tool for the localization of nucleotide targets in plants, which in turn, could assist in the finding of the distribution and the characterization of plant pathogens under single or mixed infections during infection processes.

Conflict of interest

The authors declare that there is no conflict of interest.

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