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Short Communication

Fluorescent labelling reveals spatial separation of potyvirus populations in mixed infected *Nicotiana benthamiana* plants

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The distribution of potyviruses in mixed infected *Nicotiana benthamiana* plants was investigated by using green and red fluorescent proteins (GFP, DsRed). Full-length cDNA clones of *Plum pox virus* (PPV-NAT-*AgfpS*; PPV-NAT-*red*), *Tobacco vein mottling virus* (TVMV-*gfp*; TVMV-*red*) and *Clover yellow vein virus* (ClYVV-GFP) expressing fluorescent proteins, referred to here as labelled viruses, were used to characterize the distribution of different potyviral populations (e.g. TVMV-*gfp*/PPV-NAT-*red*), as well as populations of identical, but differently labelled potyviruses (e.g. PPV-NAT-*AgfpS*/PPV-NAT-*red*) or in mixed infections of potyviruses with labelled *Potato virus X* (PVX). Plants infected by any of the PVX/potyvirus combinations exhibited synergistic symptoms and large numbers of cells were doubly infected. In contrast, co-infections of differently labelled potyvirus populations appeared non-synergistic and remained predominantly separate in the infected plants, independent of whether different viruses or identical but differently labelled viruses were co-infecting. Contact of differently labelled virus populations that exhibited spatial separation was restricted to a small number of cells at the border of different fluorescent cell clusters.

The occurrence of two or more plant viruses in mixed infections is a common phenomenon of a diverse nature. Early data indicated that interactions between unrelated viruses could result in antagonism or suppression or even in synergistic symptom development, whereas co-infections of virus strains could lead to interference resulting in varying degrees of cross-protection (Bennett, 1951). Hence, cross-protection has been shown to be a strategy that can be used to control several viral diseases (Fulton, 1986), including protection of crops from potyviral diseases (Lecoq *et al.*, 1991; Gonsalves, 1998). Potyviruses belong to the family *Potyviridae* and form the largest genus among plant viruses (reviewed by Riechmann *et al.*, 1992; Shukla *et al.*, 1994; López-Moya & Garcia, 1999). Extensive agronomic losses caused by some members of this genus reflect their economic importance, e.g. the type member *Potato virus Y* (PVY) as well as *Plum pox virus* (PPV) and *Tobacco vein mottling virus* (TVMV). Even when potyviruses display strong host-specialization two or more can occur in mixed infections of common host species (Hollings & Brunt, 1981). The most prominent type of mixed infection is the well studied synergism phenomenon of potyviruses and *Potato virus X* (PVX) (Rochow & Ross, 1955; Goodman & Ross, 1974a, b; Vance *et al.*, 1995; Pruss *et al.*, 1997).

Divéki *et al.* (2002) recently demonstrated that inoculation of differently labelled PVX led to their discrete distribution in *Nicotiana clevelandii* plants. However, there is a general lack of data concerning the distribution at the cellular level of plant viruses in mixed infections. Using differently labelled viruses could probably help to understand the distribution of these pathogens in multiple infections.

Here we describe various types of potyviral mixed infections in *Nicotiana benthamiana* plants at the cellular level using confocal laser-scanning microscopy (CLSM) techniques. To facilitate this analysis genes of an enhanced green fluorescent protein variant (smRS-GFP; Davis & Vierstra, 1998) and DsRed (Living Colours DsRed1-C1; BD Biosciences Clontech) were used to label full-length cDNA clones of the non-aphid transmissible strain of PPV (p35PPV-NAT; Maiss *et al.*, 1992) and of TVMV (pXBS; Domier *et al.*, 1986). p35PPV-NAT was used to generate p35PPV-NAT-*red* and p35PPV-NAT-*AgfpS*, respectively. The T7 promoter of the TVMV full-length cDNA clone pXBS7 was replaced by an enhanced 35S CaMV promoter (Kay *et al.*, 1987) giving pe35TVMV. This full-length cDNA clone was subsequently used to introduce the above mentioned reporter genes into the viral genome, resulting in pe35TVMV-*red* and pe35TVMV-*gfp*. The marker genes were introduced into p35PPV-NAT and pe35TVMV, respectively, upstream of the coat protein-coding sequence as an independent cistron allowing the release of the marker protein from the potyviral polyprotein by the NIa protease (Fig. 1).

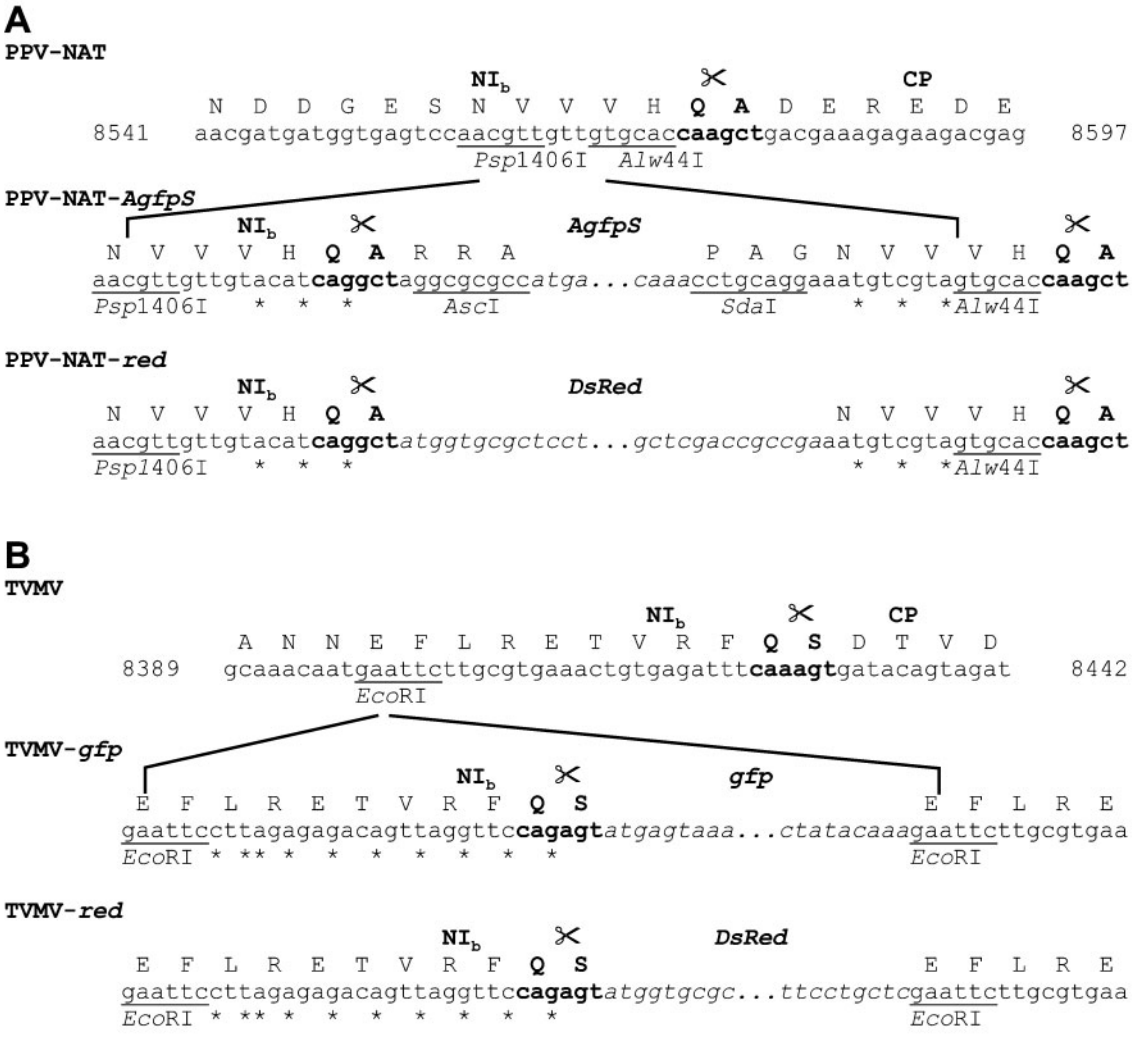

Fig. 1. Construction of DsRed- and GFP-labelled full-length clones of the potyviruses PPV-NAT (A) and TVMV (B). The positions of the first and last given nucleotides is according to sequences published by Maiss *et al.* (1989) and Domier *et al.* (1986). The adjacent sequences of NIb and CP are shown; italic letters indicate the border sequences of the reporter genes. Scissors and bold letters indicate recognition sequences of NIa. NIa, nuclear inclusion body a; NIb, nuclear inclusion body b; CP, coat protein. * Nucleotide substitutions.

Additionally, a PVX full-length cDNA clone (Baulcombe *et al.*, 1995) was labelled with GFP to extend the approach to a taxonomically distinct virus. Also, the recently reported GFP-tagged *Clover yellow vein virus* (Masuta *et al.*, 2000) and the DsRed-expressing PVX vector PVX201-*optRed* (Dietrich & Maiss, 2002) were used in this study. We investigated the distribution of these labelled viruses in doubly infected *N. benthamiana* plants, conducting experiments to mimic either mixed infections of a synergistic nature including PVX with one of the potyviruses, or mixed potyviral infections with different potyviruses. Moreover, differently labelled populations of one virus were examined in *N. benthamiana* and compared to those of different viruses (Table 1).

To infect *N. benthamiana* plants (line 27/1; Timpe *et al.*, 1992) with viral full-length cDNAs, a particle inflow gun (PIG; Gray *et al.*, 1994) was used as described previously (Dietrich & Maiss, 2002). Mechanical inoculation of *N. benthamiana* plants was done by using plant sap from singly infected, PIG-inoculated plants. Infected leaf material (0·5 g) was homogenized in 2 ml 0·03 M HEPES buffer (pH 7) and applied onto three Celite-dusted leaves.

Confocal imaging of GFP- and DsRed-expressing leaf tissues was performed with a True Confocal Scanner (Leica, TCS SP2). Cross-talking of DsRed excited by the argon laser was prevented by a Lambda-Scan procedure to ensure a physically separated excitation of both reporters.

To test the stability of the reporter genes, viruses in plant sap were passaged five times. Another passage was done

Table 1. Combinations of labelled viruses used for mixed infections of *N. benthamiana* plants

Spatial separation of differently labelled viruses is indicated as ◀▷, whereas co-infection of the same cells is indicated as ◀▶. PVX, *Potato virus X*; PPV, *Plum pox virus*; TVMV, *Tobacco vein mottling virus*; ClYVV, *Clover yellow vein virus*.

	PVX201-*gfp*	**PPV-NAT-*AgfpS***	**TVMV-*gfp***	**ClYVV-GFP**
PVX201-*optRed*	◀▷	◀▶	◀▶	◀▶
PPV-NAT-*red*	◀▶	◀▷	◀▷	◀▷
TVMV-*red*	◀▶	◀▷	◀▷	◀▷

after 4 weeks with plant sap from the original PIG-inoculated plants. After all subsequent transmissions the typical appearance of fluorescent cell tissues caused by the GFP- (or DsRed-) labelled viruses was observed under CLSM (see below), indicating the presence and stable expression of the reporter genes. Following the approach of Fernández-Fernández *et al.* (2001), the stability of foreign genes in PPV and TVMV was probably achieved by minimizing the homologous NIa protease recognition sequences at the reporter gene borders and, additionally, by modifying the codons within these sequences (Fig. 1). Moreover, the introduction of two single restriction endonuclease recognition cleavage sites (*Asc*I and *Sda*I) into p35PPV-NAT-*AgfpS* allows the ready exchange of any given cistron between NIb and CP of p35PPV-NAT-*AgfpS* with the NIa protease recognition sequences being retained (results not shown).

When PVX201-*optRed* was co-inoculated with one of the GFP-labelled potyviruses, veins on newly developed leaves became necrotic and the plants showed severe stunting. Similar symptom development was observed when PVX201-*gfp* was co-inoculated with PPV-NAT-*red* or TVMV-*red*, respectively. After separate inoculation of PVX and any of the potyviruses onto different leaves of one plant, examination of primary inoculated leaves (2–5 days p.i.) under CLSM showed typical single fluorescent foci and a more or less radial distribution of the invading viruses. Similar patterns were observed in primary infected leaves after inoculation with a mixture of differently labelled viruses. Additionally, in these leaves a mixed fluorescent signal from both reporter genes was detected, indicating that genome expression of a poty- and a potexvirus occurs in the same cells. The areas of mixed fluorescence varied from marginal overlapping of two different fluorescent spots to clusters of epidermal cells that expressed both reporters. Leaves in the early stages of systemic infection revealed patterns of fluorescence similar to those detected in primary infected leaves. No detectable differences were observed in the distribution of fluorescent foci in systemic leaves whether mixed or separate inoculations were used. Cells, now mainly along the major and minor veins, showed the fluorescence of either one or both reporter genes (Fig. 2A–C). Higher resolution examination of these cells revealed a uniform yellow colour, indicating that both reporter genes were expressed (Fig. 2J). Also, in mesophyll cells, both reporter genes were expressed in the same cells (Fig. 2D). At later stages of systemic infection (3–4 days after systemic infection became visible), the specimens were difficult to examine under CLSM, because expanding necrosis caused heavy interfering fluorescent signals, making reliable detection of definite fluorescence patterns impossible. The synergistic effect of mixed infections of PVX with members of the potyvirus group had been demonstrated earlier in the well-studied PVX/PVY interactions (Rochow & Ross, 1955; Goodman & Ross, 1974a, b; Vance *et al.*, 1995; Pruss *et al.*, 1997). Moreover, it was shown for PVX with TVMV or *Tobacco etch virus* (Vance *et al.*, 1995) and PVX with PPV (Sáenz *et al.*, 2001; Yang & Ravelonandro, 2002) that HC-Pro is involved in this synergistic effect. It is notable that all tested combinations of viruses which cause a synergistic increase of symptoms have a second common feature, beside the PVX/potyvirus interactions; i.e. both invading viruses were able to replicate within the same cellular tissues during the entire infection of the host plant, starting in the directly inoculated leaves and continuing to systemically infected leaves. The simultaneous presence of two different viruses was shown earlier for PVX/PVY and PVX/TMV by Goodman & Ross (1974a). Therefore, the results suggest that beside the role of HC-Pro a second important factor for symptom enhancement could be the presence of both viruses in the same cells throughout the infection or that the pathogenicity enhancement function of HC-Pro becomes relevant if a heterologous virus is co-localized in the same cells.

Double infections of identical but differently labelled viruses (e.g. PPV-NAT-*AgfpS*/PPV-NAT-*red*) also revealed a radial spread of the viruses from the initial infection foci. In contrast to fluorescence patterns of PVX/potyvirus combinations, only discrete clusters of red and green cells but no large numbers of cells expressing both marker genes were observed. This separation effect was also detected in systemically infected leaves: both fluorescence signals were visible only in epidermal cells at the border of two neighbouring, different coloured cell clusters (Fig. 2E–G). Images of mesophyll cells confirmed the separation of the different fluorescent signals for this tissue (Fig. 2H). Higher resolution viewing of the specimens (Fig. 2I) revealed the occurrence of only a few epidermal cells that exhibited red and green fluorescence signals (appearing yellow in Fig. 2I), which was restricted to the border region of adjacent, red

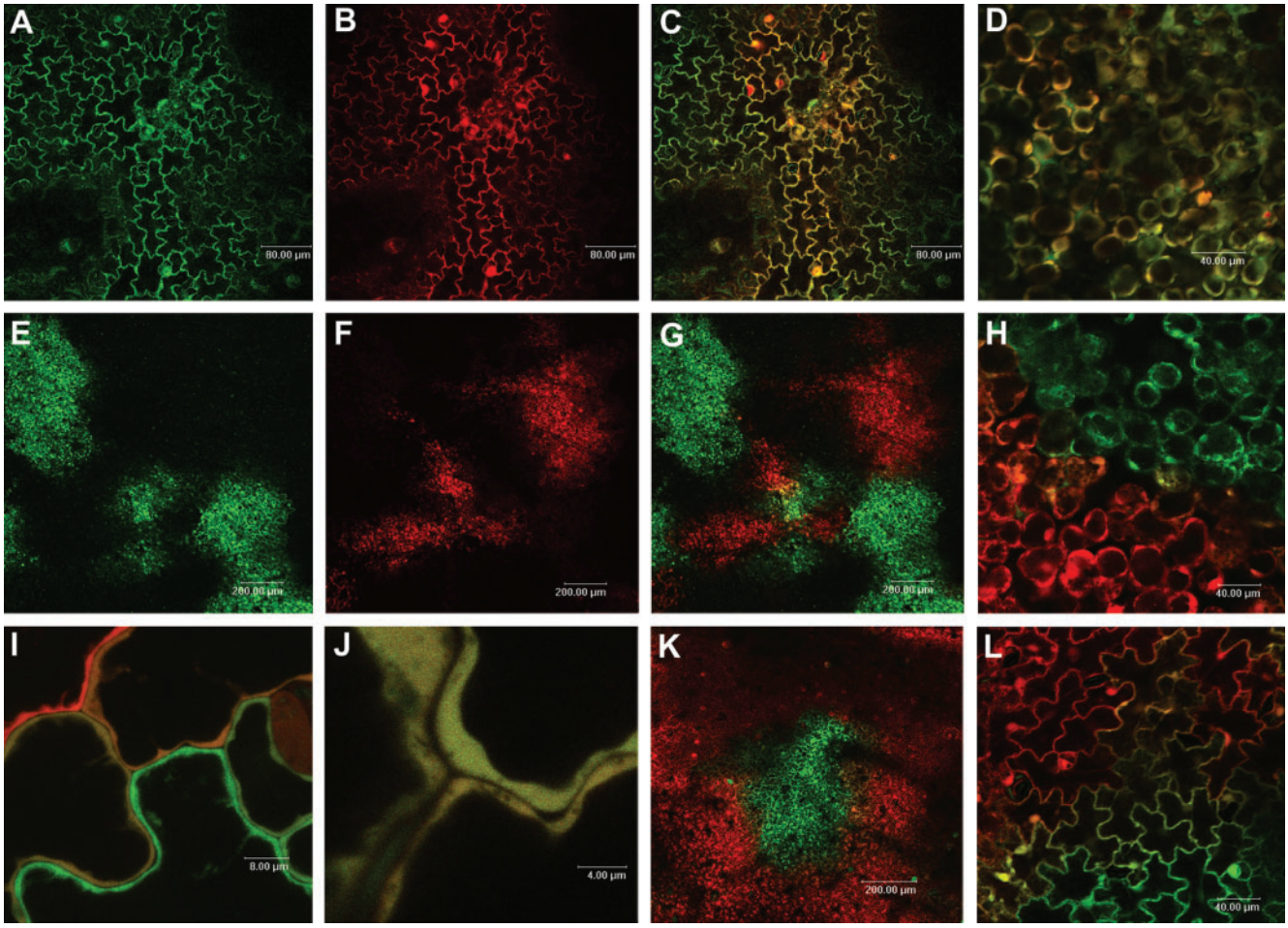

Fig. 2. Virus distribution in mixed infections in systemically infected *N. benthamiana* tissues. Confocal imaging of co-infecting PPV-NAT-*AgfpS* (A) and PVX201-*optRed* (B) reveals extensive double infection of epidermal cells. (C) merged image of (A) and (B). (D) Double infected mesophyll cells. Double infections of PPV-NAT-*AgfpS* (E) and PPV-NAT-*red* (F) result in spatial separation of the two virus populations in epidermal cells as indicated by the different coloured cell clusters in the merged image (G) of (E) and (F). (H) Spatial separation in mesophyll cells. (I) Close-up of (G) at the border region of different fluorescent cell clusters. The co-infected cell appears yellow, whereas the neighbouring cells harbour an excess of PPV-NAT-*AgfpS* (green) or PPV-NAT-*red* (red). (J) Close-up of (C) cells co-infected with PPV-NAT-*AgfpS* and PVX201-*optRed* showing an uniform pale yellow colour. (K) Co-infecting but spatially separate populations of PPV-NAT-*red* and TVMV-*gfp*. (L) Close-up of (K) reveals that contact of PPV-NAT-*red* and TVMV-*gfp* populations is restricted to a few cells at the border of two different fluorescent cell clusters.

or green fluorescing cells. This indicates that both viral populations are replicating predominantly in discrete areas and that co-existence is restricted only to a few cells at the border of these clusters. The observation of spatial separation in primary and systemically infected leaves was also independent of the inoculation method (separate inoculation or with a mixture of viruses; see above for PVX and potyviruses). In addition, when leaves were examined over a period of 10 days the separate patterns of fluorescence remained. Even after a longer period of infection (28 days p.i.) the effect remained unchanged for all combinations of mixed infections of identical but differently labelled viruses.

In mixed infections of different potyviruses (e.g. PPV-NAT-*red*/TVMV-*gfp*) no visible increase in symptom development was observed compared to potyviral single infections. However, the same spatial separation patterns were observed (Fig. 2K–L) as was shown for the mixed infections of identical but differently labelled viruses (Table 1). In all double infections of potyviruses as well as in a double infection of PVX201-*optRed* and PVX201-*gfp* mixed red and green fluorescent signals were obtained only from a few cells which were located between two different fluorescent cell clusters (Fig. 2K–L; and data not shown). The separated fluorescence signals were also observed in the mesophyll (data not shown). This effect was

also unchanged over 10 days in the same leaf position as well as in plants that were examined at 14 and 28 days p.i.

DsRed-expressing vectors have been shown not to silence GFP during co-expression within the same cells (Roberts *et al.*, 2001). Therefore, our data also show that the observed separation effect obtained with GFP and DsRed is not a result of a reporter gene silencing and it can be concluded that the use of these two proteins delivers unambiguous data in mixed infection studies.

The co-existence of different viruses is also documented by the detection of recombinant viruses that necessarily originated from cells co-infected by two virus isolates (Cervera *et al.*, 1993) or different viruses (Aaziz & Tepfer, 1999; Masuta *et al.*, 1998). In addition, complementation experiments (Taliansky & García-Arenal, 1995) suggest the co-existence of two different viruses. However, for many viruses it is unclear whether they generally multiply within the same cells or if co-existence is restricted to a few cells because of spatial separation. This should be considered when data from mixed infections are discussed.

Hence, our results are consistent with the early findings of McKinney (1929) who showed that a TMV mutant that causes a yellow mosaic occurs in separate leaf areas in a mixed infection with wild-type TMV. Infection with two strains of *Alfalfa mosaic virus* (AMV) was shown by electron microscopy to produce a similar effect (Hull & Plaskitt, 1970), as discussed in terms of cross-protection (Hull, 2002). Moreover, it was shown that cross-protection in a potexvirus/tobravirus system is subjected to an RNA-mediated cross-protection phenomenon based on gene silencing (Ratcliff *et al.*, 1999). Thus, the question arises as to whether the spatial separation of closely related viral populations is also a silencing phenomenon.

Spatial separation patterns have now been documented for three different virus genera from at least two families: three different potyviruses (*Potyviridae*, this study), the type member of the potexviruses, PVX (this study and Divéki *et al.*, 2002), and for the alfamovirus AMV in the family *Bromoviridae* (Hull & Plaskitt, 1970). All these viruses exhibit different genome organizations and expression strategies and differ widely in their host range, including herbaceous (PVX, AMV) and woody plants (PPV). Thus, the spatial separation of different viral populations may be a more common phenomenon. Further studies will be needed to clarify whether spatial separation is a silencing phenomenon or if other models, which have been used to characterize cross-protection (Pennazio *et al.*, 2001), can explain this virus distribution.

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