A chimeric plum pox virus shows reduced spread and cannot compete with its parental wild-type viruses in a mixed infection

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The effect of a recombination event in the genomic 3' end on the biological properties and competitiveness of plum pox virus (PPV) was investigated. Therefore, a fragment spanning the coat protein (CP) coding region and a part of the 3' non-translated region of a non-aphid-transmissible strain of PPV (PPV-NAT) was replaced by the corresponding region of a PPV sour cherry isolate (PPV-SoC). The resulting chimera (PPV-NAT/SoC) caused severe symptoms in Nicotiana benthamiana, resembling those of PPV-NAT. In mixed infections with either of the parental viruses, the chimera PPV-NAT/SoC was less competitive. Labelling experiments with DsRed showed that PPV-NAT/SoC (PPV-NAT/SoC-red) moved more slowly from cell to cell than PPV-NAT (PPV-NAT-red). In mixed infections of PPV-NAT/SoC-red with a green fluorescent protein-expressing PPV-NAT (PPV-NAT-AgfpS), spatial separation of the viruses was observed. These data suggest that, in PPV infections, symptom severity and competitiveness are independent aspects and that spatial separation may contribute to the displacement of a recombinant virus.

Potyviruses represent the largest genus of plant-infecting RNA viruses and comprise several members causing extensive agronomic losses worldwide (Lopez-Moya et al., 2000; Urcuqui-Inchima et al., 2001). The success of a virus population generally depends on fast adaptation to a changing environment and, therefore, on mutation and recombination as the driving evolutionary forces (Roossinck, 1997). Within potyvirus species, naturally occurring recombinant genomes have been identified, emphasizing their genetic variability (Cervera et al., 1993; Glais et al., 2002; Glasa et al., 2004; Krause-Sakate et al., 2004; Tan et al., 2004). In potyviral populations, strong selection occurs against recombinant genomes with chimeric proteins (Moreno et al., 2004). Thus, natural recombination events often occur in close proximity to sequences encoding protease-recognition sites. In general, recombination in potyviruses has been documented to occur in different parts of the genome, e.g. in the coding regions of protein 1 (P1) and the coat protein (CP) of turnip mosaic virus (Ohshima et al., 2002) or in plum pox virus (PPV) genomes in protein 3 (P3) and at the junction site between nuclear inclusion body b (NIb) and CP (Cervera et al., 1993; Glasa et al., 2004).

Recombinant viruses must compete with the parental viruses immediately after the recombination event. This ensures the emergence of well-adapted, competitive viruses if no fortuitous events, such as aphid transmission, guide an otherwise non-competitive sequence to a new host (Ali et al., 2006). Investigations of competitiveness and population diversity of viruses in mixed infections have been made with isolates of cucumber mosaic virus and other Sindbis-like plant viruses (Schneider & Roossinck, 2000; Takeshita et al., 2004a). A real-time RT-PCR-based approach for quantification of tobacco etch virus (TEV) strains in mixed infections has been established (Carrasco et al., 2006), but data highlighting the course of coexistence in a potyvirus mixed infection with regard to competition between recombinant and wild-type viruses are missing.

To investigate possible effects of a putative recombination event in the genomic 3’ end of the non-aphid-transmissible
strain of PPV (PPV-NAT), a recombinant PPV-NAT full-length clone was constructed. The biological properties and competitiveness of the resulting recombinant were compared with those of the parental viruses.

To construct the recombinant, a fragment of 1136 bp spanning the CP coding region and about 60% of the 3’ non-translated region (3’-NTR) (designated CP/A3’ region) of the sour cherry isolate of PPV (PPV-SoC; Fanigliulo et al., 2003) was amplified by RT-PCR using avian myeloblastosis virus reverse transcriptase (AMV-RT; Promega) and proofreading Taq polymerase (Promega), with primers SoCcp+ (5’-AGGATCCGTTGTCACCAGGCCAAGGAGGAATGATGACG-3’) and SoCcp− (5’-AGGGATCCAGGACATATGGAGGTAAAGCCTCAC-3’). After digestion with Alw 44I/NdeI, the corresponding region in the full-length clone of PPV-NAT (p35PPV-NAT; Maiss et al., 1992) was replaced by the PPV-SoC sequence in frame, giving p35PPV-NAT/SoC. To test infectivity of the recombinant clone, plasmid DNA of p35PPV-NAT/SoC was prepared and used for biolistic bombardment (Dietrich & Maiss, 2002) of five Nicotiana benthamiana plants; p35PPV-NAT served as a control. Plants inoculated with either the recombinant or the parental clone showed symptoms 10–12 days post-infection (p.i.). The presence of the recombinant virus was verified by RT-PCR. For reliable detection and differentiation of PPV-NAT and PPV-NAT/SoC in single and mixed infections, a set of two discriminating pairs of primers was designed: N1, 5’-AGCTGACGAAAGAGAAGACGAGGAGG-3’, and N2, 5’-AAGGATCCACTGAATGACTGCTATTAAAGCGGT-3’, to detect PPV-NAT, and S1, 5’-ACGCCTGCAGTAACAAGCTACAAT-3’, and S2, 5’-AGGGATCCAGGACATATGGAGGTAAAGCCTCAC-3’, for detection of PPV-NAT/SoC. RT-PCR was performed in a Biometra T3 cycler with AMV-RT and HotStar Taq polymerase (Qiagen) using RNA extracts from infected plants (Menzel et al., 2002). After reverse transcription for 45 min at 42 °C, PCR conditions were an initial denaturation of 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 66 °C for 30 s and 72 °C for 90 s, and a final extension step at 72 °C for 7 min. The developed RT-PCR procedure discriminated reliably between PPV-NAT and PPV-NAT/SoC, because N1/N2 only amplified fragments from PPV-NAT and S1/ S2 only detected PPV-NAT/SoC (data not shown).

Symptom development and severity were monitored on N. benthamiana plants inoculated with PPV-NAT, PPV-NAT/SoC or PPV-SoC. Plant sap of infected plants was rub-inoculated onto Celite-dusted leaves in three independent trials using 21 N. benthamiana plants per virus in each trial. As a negative control, 21 plants were mock-inoculated with 0.03 M HEPES (pH 7) only. All plants were placed in climate chambers with a photoperiod of 14 h and a constant temperature of either 22 or 28 °C. Symptoms of PPV-NAT- and PPV-NAT/SoC-infected plants appeared 5–6 days after mechanical inoculation. The symptom development of PPV-SoC-infected plants occurred with a delay of 2–3 days. PPV-NAT and PPV-NAT/SoC caused severe symptoms, typical of PPV in N. benthamiana (Maiss et al., 1992). The plants showed considerable growth reduction, vein clearing, leaf deformation and, in later stages of infection, mosaic symptoms. In the case of PPV-SoC, only mild vein clearing and yellowing, but no growth reduction, occurred (Fig. 1).

The effect of virus infection on plant growth was measured 14 days p.i. by determination of fresh weight. Statistical analyses were performed with SigmaStat 3.1, using an all-pair multiple comparison procedure (Tukey test). Fig. 1 shows a significant reduction of the fresh weight of PPV-NAT- and PPV-NAT/SoC-infected N. benthamiana plants at 22 °C compared with the control. No difference was

Fig. 1. Fresh weight and symptom development of N. benthamiana plants infected with wild-type and recombinant PPV at 22 °C. Each column represents the mean of fresh weight development of the given virus variant of 21 inoculated plants at 14 days p.i. No significant reduction in fresh weight is caused by PPV-SoC compared with a mock-inoculated plant. No significant differences are observed between PPV-NAT- and PPV-NAT/SoC-infected plants, but the latter show a significant reduction in fresh weight compared with the water control. Only weak vein clearing occurs in PPV-SoC-infected plants, whereas PPV-NAT- and PPA-NAT/SoC-infected plants show heavy vein clearing, leaf curling and growth reduction. Standard errors are indicated as bars. Statistically different variants are indicated by A and B (P<0.05).
observed either between PPV-NAT and PPV-NAT/SoC or between PPV-SoC-infected plants and the uninfected control. A general weak symptom development, but no statistically significant difference in fresh weight, was observed at 28 °C between all three viruses (not shown). Therefore, the CP/A3′ exchange does not influence symptom severity or fresh mass development of PPV-infected *N. benthamiana* plants. The effect of temperature on symptom development and the lack of effect on fresh weight between all viruses at 28 °C are typical of PPV (Glasa et al., 2003) and many other viruses (heat masking). The observed effects can be explained by more effective RNA silencing at higher temperatures (Szittya et al., 2003), which affects PPV-NAT, PPV-SoC and PPV-NAT/SoC in the same manner. Because PPV-NAT and PPV-NAT/SoC only differ in the CP/A3′ region, the silencing-suppressor proteins (P1/HC-Pro; Pruss et al., 1997) are identical. In contrast, the suppressors of PPV-NAT (or NAT/SoC) and PPV-SoC differ by 36 aa (data not shown). Stenger et al. (2006) showed that even minor substitutions in a potyviral HC-Pro can lead to attenuated symptoms. However, other viral genes, such as P3 (Jenner et al., 2003; Saenz et al., 2000), may also be involved in this effect. This would explain the facts that symptom severity at lower temperatures is similar between PPV-NAT and PPV-NAT/SoC, and that PPV-SoC causes mild symptoms.

As PPV-NAT/SoC and the parental PPV-NAT did not show differences in their ability to infect *N. benthamiana* or to induce symptoms in single infections, we tested whether both viruses were able to compete in a mixed infection. To inoculate *N. benthamiana* plants with equal amounts of virus, plant sap from PPV-NAT- and PPV-SoC-infected plants was taken and the virus titres were determined by ELISA using PPV-specific polyclonal antibodies. Based on the ELISA results, plant-sap dilutions were adjusted to ratios of PPV-NAT:PPV-NAT/SoC of 1:1, 1:2, 1:5 and 1:10 and used for mechanical inoculation of *N. benthamiana* plants. Six days p.i., systemic symptoms became visible. RNA was extracted at 7, 14 and 21 days p.i. and the presence of viruses was verified by RT-PCR (see above). Surprisingly, PPV-NAT/SoC was nearly outcompeted by the parental virus after 21 days when a starting ratio of 1:1 was used (Fig. 2). Moreover, PPV-NAT was able to compete with PPV-NAT/SoC, even if the initial titre was tenfold lower. This result was also confirmed by co-bombarding equal amounts of plasmid DNA of both full-length clones (data not shown). Serial passages of mixed infections of PPV-NAT + PPV-NAT/SoC, PPV-NAT + PPV-SoC and PPV-NAT/SoC + PPV-SoC were carried out to compare the competitiveness of all three viruses. Three *N. benthamiana* plants were inoculated with each virus combination as described above. The presence of the viruses was verified by RT-PCR using common PPV primers PPVcom1 (5′-GATTGGAGGC-AATTTGTGCATC-3′) and PPVcom2 (5′-ACTCTTTTC-ATACCAAGTTTGGAAAC-3′), located in the Nlb/CP coding region. A subsequent digest with EcoRV resulted in fragments specific for PPV-NAT (496 bp), PPV-SoC (751 bp) and PPV-NAT/SoC (547 bp). After two passages using plant sap from systemically infected leaves, it was observed that PPV-NAT outcompeted PPV-SoC and PPV-NAT/SoC, whereas PPV-NAT/SoC was not fully outcompeted by PPV-SoC (see Supplementary Fig. S1, available in JGV Online). These results were confirmed in a repetition of the experiment. Altogether, these experiments demonstrate that, although PPV-NAT and PPV-NAT/SoC do not show differences in their ability to initiate an infection in *N. benthamiana*, the chimeric virus is less competitive in a mixed infection. Moreover, PPV-NAT/SoC is also not able to compete with either of the parental viruses, even the milder PPV-SoC. Therefore, the exchange of the CP/A3′ region contributes to reduced competitiveness of the chimeric virus. This effect might be explained by impaired movement efficiency of PPV-NAT/SoC, because it is known that the CP of potyviruses is involved in cell-to-cell and long-distance movement (Dolja et al., 1994).
To investigate whether recombination influenced the movement of PPV-NAT/SoC, the reporter gene DsRed was inserted into p35PPV-NAT/SoC (Matz et al., 1999) and used to monitor virus movement. According to our previously published strategy (Dietrich & Maiss, 2003), DsRed1-C1 (Clontech) was cloned into p35PPV-NAT/SoC at the junction between the Nib and CP coding regions. The resulting clone was designated p35PPV-NAT/SoC-red. As demonstrated previously for similar constructs (Dietrich & Maiss, 2003), the fluorescent tag did not influence symptom development and, when compared with PPV-NAT/SoC, no difference in the rate of systemic infection was observed. To determine the movement efficiency of p35PPV-NAT/SoC-red, the clone was co-bombardered with the green fluorescent protein (GFP) expression plasmid CD3-328smRS-GFP (Davis & Vierstra, 1998). For comparison with PPV-NAT, this was also done with the DsRed-labelled variant of p35PPV-NAT (p35PPV-NAT-red; Dietrich & Maiss, 2003). Four days after biolistic bombardment, the radial spread of the viruses was documented by confocal laser-scanning microscopy (CLSM; Leica TCS SP2). Five biolistically inoculated leaves were taken in three repetitions and analysed by CLSM. In each inoculation spot, around ten infection foci became visible. Fig. 3(a, b) shows typical small infection sites of PPV-NAT/SoC-red and larger ones of PPV-NAT-red. Thus, the movement efficiency of the chimeric virus was lower than that of the parental virus. The observed effect on movement of PPV-NAT/SoC-red is probably a result of the CP exchange, because the CP of TEV has been shown to have functions in virus assembly and in cell-to-cell and long-distance movement, but not in genome replication (Dolja et al., 1994, 1995; Rojas et al., 1997). The variable N and C termini of the CP are dispensable for TEV assembly, but are required for efficient long-distance transport. In contrast, the core region is essential for TEV assembly and cell-to-cell movement. In the case of PPV-NAT and PPV-SoC, the core regions [according to van Boxtel et al. (2000)] and the C-terminal parts are highly conserved, with only 10 amino acid substitutions (six conserved; data not shown). Additionally, the conserved amino acid residues S122, R154 and D198 of the TEV CP, which have been shown to be important for cell-to-cell movement (Dolja et al., 1994, 1995), are also present in PPV-NAT and in PPV-NAT/SoC CPs. Therefore, reduced cell-to-cell movement of PPV-NAT/SoC is unlikely to result from alterations in the core region or the C-terminal part of the CP. The N-terminal part of PPV-NAT and PPV-NAT/SoC CP is highly variable. It shows 42 amino acid substitutions from aa 1 to 103 of the PPV-NAT/SoC CP and a deletion of 18 residues in the PPV-NAT CP. As shown in Fig. 3, the deletion does not affect PPV-NAT cell-to-cell movement. With regard to the work of Dolja et al. (1994, 1995), it seems likely that the N-terminal domain of PPV-NAT/SoC CP negatively influences the movement function of the recombinant. Because the 3'-NTR of many viruses is required for genome replication (Dreher, 1999), the possibility that the exchange of 8 nt in the 3'-NTR affected the replication process and, therefore, had an indirect impact on viral movement, cannot be excluded.

Mixed infections were also performed with PPV-NAT/SoC-red and the GFP-labelled PPV-NAT (PPV-NAT-

PPV-NAT/SoC-red + PPV-NAT-red + PPV-NAT-AgfpS

Fig. 3. Spread of labelled PPV-NAT/SoC and PPV-NAT in single and mixed infections. (a, b) Radial cell-to-cell movement in N. benthamiana of PPV-NAT/SoC-red and PPV-NAT-red 4 days after co-bombardment of the respective full-length clones with a GFP-expressing plasmid. Red fluorescence results from DsRed expression of PPV-NAT/SoC-red (a) and PPV-NAT-red (b). The green-fluorescing cell in the centre of red-fluorescent spots indicates the initial infection site. Four days after biolistic inoculation, PPV-NAT-red spreads rapidly from cell to cell, whereas PPV-NAT-red only infected adjacent cells. (a) and (b) suggest a lowered cell-to-cell movement of the chimeric PPV-NAT/SoC-red compared with PPV-NAT-red. (c) An N. benthamiana leaf infected systemically with PPV-NAT-AgfpS (green) and PPV-NAT/SoC-red (red). PPV-NAT/SoC-red is surrounded by PPV-NAT-AgfpS and, due to spatial separation, the radial spread by cell-to-cell movement is blocked. Bars, 220 μm.
AgfpS; Dietrich & Maiss, 2003). First, in mixed infections of PPV-NAT/SoC-red and PPV-NAT-AgfpS, the spatial separation phenomenon described previously (Dietrich & Maiss, 2003) was observed (Fig. 3c). The different labelled viruses remained in discrete clusters and double-infected cells were observed only along the borderline of two adjacent cell clusters. In these mixed infections, the fluorescence of PPV-NAT-AgfpS was detected more frequently than DsRed fluorescence of PPV-NAT/SoC-red. It was evident that PPV-NAT/SoC-red was often surrounded partially or completely by PPV-NAT-AgfpS (Fig. 3c). This was observed regularly in primary inoculated leaves and in systemically infected leaves. From these results, it can be concluded that, due to less effective cell-to-cell movement of the chimeric PPV-NAT/SoC-red, this virus cannot colonize the plant to an extent similar to that of the parental PPV-NAT-AgfpS.

Altogether, the results demonstrate that the exchange of the CP and the adjacent part of the 3′-NTR in the PPV genome does not influence symptom severity or host colonization of *N. benthamiana* considerably in a single infection. Even if the CP/Δ3′ exchange leads to impaired movement function, this does not affect viral fitness severely in a single infection. In contrast, if the recombinant virus occurs in a mixed infection with a parental virus, the recombination becomes disadvantageous and initiates outcompeting of the recombinant virus. This effect may also affect the assertiveness of a recombinant virus because, under natural conditions, a recombinant must compete with both parental viruses. Moreover, our experiments are in accordance with the results of Hall et al. (2001) and French & Stenger (2003) and suggest that outcompeting might be supported by spatial separation, because faster movement of PPV-NAT-AgfpS often results in trapping of PPV-NAT/SoC-red. Thus, the results contribute to general understanding of viral fitness and virus evolution (Roossinck, 2005), because viral genomes with disadvantageous features are less competitive and possibly out-competed, if a selection pressure such as the presence of (parental) viruses with better fitness exists. The observed separation and competition effects may also play a role in diversifying a population that originated from a single inoculation event, as demonstrated and discussed by Iridi et al. (2006). Because spatial separation was also observed in cucumovirus and potexvirus infections (Dietrich & Maiss, 2003; Diveki et al., 2002; Takeshita et al., 2004b), it is likely that spatial separation effects may also influence evolving virus populations in other virus groups directly. Therefore, it should be taken into consideration whether spatial separation could function as a population-imposed bottleneck in RNA virus evolution.

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**References**


