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e-Xtra*

Diverse Amino Acid Changes at Specific Positions in the N-Terminal Region of the Coat Protein Allow *Plum pox virus* to Adapt to New Hosts

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***Plum pox virus* (PPV)-D and PPV-R are two isolates from strain D of PPV that differ in host specificity. Previous analyses of chimeras originating from PPV-R and PPV-D suggested that the N terminus of the coat protein (CP) includes host-specific pathogenicity determinants. Here, these determinants were mapped precisely by analyzing the infectivity in herbaceous and woody species of chimeras containing a fragment of the 3′ region of PPV-D (including the region coding for the CP) in a PPV-R backbone. These chimeras were not infectious in *Prunus persica*, but systemically infected *Nicotiana clevelandii* and *N. benthamiana* when specific amino acids were modified or deleted in a short 30-amino-acid region of the N terminus of the CP. Most of these mutations did not reduce PPV fitness in *Prunus* spp. although others impaired systemic infection in this host. We propose a model in which the N terminus of the CP, highly relevant for virus systemic movement, is targeted by a host defense mechanism in *Nicotiana* spp. Mutations in this short region allow PPV to overcome the defense response in this host but can compromise the efficiency of PPV systemic movement in other hosts such as *Prunus* spp.**

Plum pox virus (PPV) is considered as one of the most important plant viruses (Scholthof et al. 2011). It causes sharka, a devastating disease that affects nearly all species of the genus *Prunus* (García and Cambra 2007; Sochor et al. 2012). PPV is a member of the genus *Potyvirus* of the *Potyviridae* family (López-Moya et al. 2009), and has a single-stranded RNA genome of 9,786 nucleotides. Its genomic RNA is translated into a single polyprotein and a truncated frameshift product that are proteolytically processed by three self-encoded proteases (Chung et al. 2008; Salvador et al. 2006). RNA-dependent RNA polymerases are responsible for the replication of the genomic RNA, and cause high mutation rates due to lack of proofreading activity (Malpica et al. 2002). As a consequence, heterogeneous virus populations of closely related genomes or "quasispecies" coexist in the host plant during infection (Biebricher and Eigen 2006; Domingo and Holland 1997; Eigen 1996). However, virus populations retain a consensus sequence that can rapidly evolve depending on the environmental conditions and ultimately lead to new consensus sequences. Importantly, the modification of the consensus sequence of a virus population may also modify its pathogenicity (Domingo and Holland 1997). This feature makes these pathogens cause the occurrence of new emerging diseases, which is a major problem in agriculture (Elena et al. 2011; Jones 2009). In recent years, agricultural activity has undergone devastating epidemics caused by new viruses that have switched host species. In fact, the expansion in natural host range is considered one of the major factors driving emergence of new diseases (Jones 2009). However, the molecular mechanisms that determine how plant viruses establish successful infections in a certain host are still largely unknown.

Pathogenicity determinants in *Potyvirus* spp., including PPV, are spread throughout the viral genome. Several viral products such as P1 (Maliogka et al. 2012; Nagyova et al. 2012; Nakahara et al. 2010; Valli et al. 2007), HCPro (Carbonell et al. 2012; Faurez et al. 2012; Moury et al. 2011; Sáenz et al. 2001), P3+6K1 (Dallot et al. 2001; Hjulsager et al. 2006; Jenner et al. 2003; Sáenz et al. 2000; Suehiro et al. 2004; Wen et al. 2011), CI (Abdul-Razzak et al. 2009; Seo et al. 2009; Zhang et al. 2009), 6K2 (Spetz and Valkonen 2004), NIa-VPg (Keller et al. 1998; Masuta et al. 1999; Moury et al. 2004), NIa-Pro (Chen et al. 2008), NIb (Fellers et al. 2002; Gallois et al. 2010; Wallis et al. 2007), and the coat protein (CP) (Andersen and Johansen 1998; Decroocq et al. 2009; Salvador 2008), as well as the 5′ and 3′ noncoding regions (Rodríguez-Cerezo et al. 1991; Simón-Buela et al. 1997), have been reported to contain pathogenicity determinants, which play different roles in all steps of the viral infection (Revers et al. 1999). Analyses of the infectivity of chimeras generated from viruses with different biological properties have been a useful tool for the identification of virus-specific pathogenicity and host-range determinants. Thus, in the case of PPV, complex pathogenicity determinants were identified in herbaceous and woody hosts by using chim-

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eric cDNA clones generated first from the PPV-R and PPV-PS isolates, belonging to two different PPV strains (Sáenz et al. 2000), and then more precisely from the PPV-R and PPV-D isolates, both belonging to the PPV-D strain (Salvador et al. 2008). PPV-R infects herbaceous hosts but has lost the ability to infect *Prunus* spp. after extended propagation in herbaceous plants (Dallot et al. 2001), whereas PPV-D systemically infects *Prunus* spp. but not herbaceous hosts (Salvador et al. 2008). The analysis of chimeras originating from PPV-R and PPV-D demonstrated that, although pathogenicity determinants are extensively spread throughout the PPV genome, relevant host-specific determinants are located at the N terminus of the CP (Salvador et al. 2008). Moreover, the determinant of potyvirus ability to overcome the restricted *Tobacco etch virus* movement (RTM) resistance of *Arabidopsis thaliana* also mapped to the N-terminal region of the CP (Decroocq et al. 2009).

Here, we have further explored the pathogenicity determinants contained in the 3′ end of PPV genome that are related to host adaptation. Our results show that specific amino acid substitutions or a 30-amino-acid (aa) deletion at the N terminus of the CP allow PPV to systemically infect herbaceous hosts with or without reducing its fitness in peach seedlings. The possible mechanisms by which these mutations could affect CP interactions with host factors to promote efficient long-distance movement of the virus or to induce host defenses are discussed.

RESULTS

Infectivity of PPV-D and -R chimeric viruses in woody and herbaceous hosts.

Previous studies with chimeric viruses between PPV-R and PPV-D, two isolates with high sequence similarity but different host specificity, showed that host-specific pathogenicity determinants are largely spread throughout the viral genome (Salvador et al. 2008). To further dissect such determinants, cDNA clones of two new chimeras, PPV-BSD and PPV-BND, were constructed. In these chimeras, a fragment of the 3′ region of the genome of PPV-R including the partial or total CP coding sequence was replaced by the corresponding fragment of PPV-D, in a PPV-R backbone (Fig. 1). Clones expressing PPV-R, PPV-Dc (a variant of PPV-D), and the *Nicotiana* spp.–infecting R/D chimera PPV-SBD were previously described (Salvador et al. 2008) and used here as controls (Fig. 1). The infectivity of the different clones was tested in woody and herbaceous hosts. In all cases, virus-induced symptoms were monitored, and virus accumulation levels were measured by enzyme-linked immunosorbent assay (ELISA) or Western blot analyses.

First, infectivity experiments in *Prunus persica* GF305 seedlings showed that only seedlings inoculated with PPV-Dc but not with PPV-SBD, PPV-BND, or PPV-BSD showed symptoms at 21 days postinoculation (dpi) (Table 1). ELISA analysis of upper leaves at 30 dpi confirmed that PPV was only detected in those seedlings inoculated with PPV-Dc (Table 1).

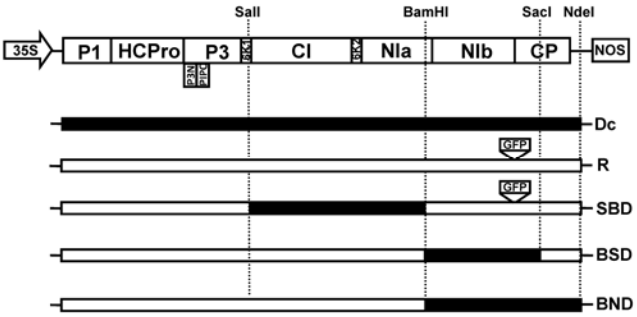

Fig. 1. Schematic representation of *Plum pox virus* (PPV) cDNA clones Dc (in black), R (in white), and PPV-R/D hybrids constructed from them. Positions of the restriction sites used for the cloning of the hybrids are highlighted with vertical dotted lines. The green fluorescent protein (GFP) sequence is represented with a box between NIb and coat protein (CP).

Next, the infectivity of the PPV-D or -R hybrids was assessed in two *Nicotiana* spp., *Nicotiana clevelandii* and *N. benthamiana*. These species are common experimental hosts used for PPV studies, although neither of them is systemically infected by PPV-Dc. In a first bioassay, four *N. clevelandii* plants were inoculated with PPV-BND, PPV-BSD, or the PPV-SBD control chimera. At 21 dpi, all four plants inoculated with PPV-SBD showed leaf chlorosis as a consequence of viral infection (Table 1). At this time, all the plants inoculated with PPV-BSD or PPV-BND showed neither disease symptoms nor viral accumulation by ELISA analysis. However, at 27 dpi, one plant inoculated with PPV-BND and one plant inoculated with PPV-BSD showed leaf chlorosis symptoms in upper leaves. These symptoms were similar to those of plants infected with PPV-SBD (Fig. 2A; Table 1). Virus accumulation in the symptomatic plants and in two asymptomatic plants inoculated with PPV-BSD was detected by Western blot analysis from extracts of upper leaves at 27 dpi (Fig. 2B). However, CP levels in PPV-BND- or PPV-BSD-infected plants were variable, and lower than those of plants infected with PPV-SBD. Thus, these results show that both PPV-BND and PPV-BSD chimeras have low infectivity rates, and virus accumulation is delayed. Next, we analyzed the sequence of the PPV-D-derived genomic region of virus progenies accumulating in upper noninoculated leaves of those plants that became infected with PPV-BND and PPV-BSD. This analysis indicated the presence of the following amino acid substitutions in the N terminus of the CP: i) Ile at position 16 was mutated to Val in a PPV-BSD-infected plant and in the only PPV-BND-infected plant, ii) Asp in position 11 was mutated to a Gly in another PPV-BSD-infected plant, and iii) amino acids from positions 7 to 36 were deleted in the third PPV-BSD-infected plant (Fig. 2B; Table 1). To further confirm and extend these results, in a second bioassay, 30 additional plants were inoculated with PPV-BSD. PPV-BSD and not PPV-BND was selected for further studies because it was the most infectious chimera (Fig. 2B; Table 1). Almost half of the plants inoculated with PPV-BSD were systemically infected and showed a diverse range of symptom intensity and virus accumulation (Table 1). Sequencing of virus progenies accumulating in the upper leaves of the infected plants showed, in all cases, the presence of mutations in a 30-aa region at the N terminus of the viral CP (Table 1). In summary, five amino acid changes (D11G, I16S, I16V, I16T, and V18A) and one deletion (Δ7-36) were found in the N terminus of the CP of viral progenies in *N. clevelandii*. The most frequent mutation was V18A (Table 1).

The infectivity of PPV-BSD chimera was next analyzed in *N. benthamiana*. Symptom development, virus accumulation, and sequence of the N terminus of the CP were analyzed to verify whether or not mutations could also be selected in the inoculated tissue. In a first bioassay, both PPV-R and PPV-Dc were able to replicate in the inoculated leaves but PPV-R accumulated at higher levels (Fig. 3B). PPV-BSD accumulated at intermediate levels, higher than PPV-Dc, although virus accumulation was variable (Fig. 3B). In all cases, virus progenies accumulating in the inoculated leaves maintained the parental sequence of the N terminus of the CP (Fig. 3B). Neither symptoms nor viral CP could be detected in the upper leaves of plants inoculated with PPV-Dc (Fig. 3A and B). However, at 21 dpi, two of the three plants inoculated with PPV-BSD displayed characteristic viral symptoms such as stunting and leaf chlorosis, although milder than those observed in plants infected with PPV-R (Fig. 3A). Indeed, all those plants inoculated with

PPV-BSD showing symptoms accumulated virus CP as observed by Western blot. Interestingly, virus accumulation levels in upper leaves did not correlate with those detected in the inoculated leaves (Fig. 3B). As in *N. clevelandii*, sequencing of the virus progenies accumulating in the upper leaves of plants infected with PPV-BSD showed mutations in the N terminus of the viral CP (Fig. 3B). In a second bioassay, 27 additional plants were inoculated with PPV-BSD. Thirteen of these new plants were systemically infected (Table 1). Three of the amino acid changes detected in BSD-infected *N. clevelandii* plants (I16V, I16T, and V18A) were also identified in the progeny of some PPV-BSD-infected *N. benthamiana* plants (Table 1). In addition, a Lys to Arg mutation at position 14 was also detected in the virus that systemically spread in one *N. benthamiana* plant. In contrast to *N. clevelandii,* in *N. benthamiana*, some of the virus progenies were able to systemically move without introducing any mutations at the N terminus of their CP (Table 1). The symptomatology of plants infected with a chimeric virus lacking mutations was in general mild and the virus accumulation rather low compared with that of chimeras with amino acid changes (Table 1).

The infectivity of PPV-BSD chimera was also evaluated in two ecotypes of *A. thaliana*, Col-0 and L*er*, that are known to have different susceptibility to diverse PPV isolates (Decroocq et al. 2006) (Supplementary Fig. S1; Table 1). At 40 dpi, none of the Col-0 and L*er* plants inoculated with either PPV-Dc or PPV-BSD displayed symptoms of infection. In contrast, all Col-0 and L*er* plants inoculated with PPV-R displayed strong leaf chlorosis. Western blot analyses of upper noninoculated leaves revealed that PPV-R accumulated to high levels in both Col-0 and L*er* plants. In contrast, no viral CP was detected in any of the eight plants inoculated with PPV-Dc. Viral CP was not detected in any of the Col-0 and L*er* plants inoculated with PPV-BSD except for one L*er* plant. In this plant, no mutations were found in the CP coding sequence of the virus progeny.

In summary, these results indicate that i) the 3′ end of PPV-D genome is not sufficient to confer PPV the ability to infect *P. persica*; ii) PPV chimeras containing sequences from the 3′ end of the PPV-D genome can systemically infect *N. clevelandii*, provided that the N terminus of the CP sequence is modified; and iii) the PPV-BSD chimera can occasionally systemically infect *N. benthamiana* without modifying the N terminus of the CP sequence, although mutations at this region are also usually selected in this host.

Stability of the mutations detected in the progeny of PPV-BSD.

Next, a second round of PPV-BSD infections was performed. The aim was to assess the stability of the mutations introduced during the adaptation process of PPV-BSD in herbaceous plants and their effect on the overall fitness of the virus. Healthy *N. clevelandii* or *N. benthamiana* plants were inoculated with extracts of the plants primarily infected with PPV-BSD (Tables 2 and 3).

In the two *Nicotiana* spp., most of the mutated progenies of PPV-BSD (from either *N. clevelandii* or *N. benthamiana* plants) infected all the inoculated plants and accumulated large amounts of virus. Moreover, they conserved the original muta-

Table 1. Phenotypic and molecular analyses of *Plum pox virus* (PPV)-D/R hybrids in different plant hosts

Host, PPV variant	Infectivity[a]	Amino acid changes[b]	Symptoms[c]	Virus accumulation[d]
Prunus persica				
BND	0/4	–	–	–
BSD	0/4	–	–	–
Dc	4/4	–	+++	+++
SBD	0/4	–	–	–
Nicotiana clevelandii				
BND	1/4	I16V (1)	+++	+
BSD[e]	16/34	Δ7-36 (1)	–	+
		D11G (1)	–	+
		I16S (3)	+ (1), – (2)	+++ (1), ++ (1), ± (1)
		I16T (3)	+ (1), – (2)	+++ (1), + (2)
		I16V (2)	+++ (1), – (1)	++
		V18A (6)	+++ (1), ++ (1), ± (1), –(3)	+++ (2), ++ (2), ± (2)
SBD	4/4	–	+++	+++
N. benthamiana				
BSD[e]	15/30	(–10)	+ (1), ± (2), –(7)	+++ (1), ++ (3), + (6)
		K14R (1)	±	+++
		I16T (1)	+++	+
		I16V (2)	+++	++
		V18A (1)	+	+++
Dc	0/4	–	–	–
R	4/4	–	+++	+++
Arabidopsis thaliana				
Col-0				
BSD	0/8	–	–	–
Dc	0/8	–	–	–
R	8/8	–	+++	+++
L*er*				
BSD	1/8	–	–	+
Dc	0/8	–	–	–
R	8/8	–	+++	+++

[a] Infectivity is defined as the number of systemically infected plants/number of inoculated plants.
[b] Amino acid changes in the N-terminal region of the coat protein are indicated. The minus sign refers to absence of changes and the number in parentheses indicates the number of independent plants in which this specific mutation was observed.
[c] Symptoms of upper leaves are classified according to their intensity as severe (+++), medium (++), mild (+), very mild (±), and absent (-). The number of plants displaying a particular symptom is in parentheses.
[d] Viral accumulation levels, assessed by Western blot or enzyme-linked immunosorbent assay, are classified as high (+++), medium (++), low (+), very low (±), and null (–). The number of plants displaying a particular viral accumulation level is in parentheses.
[e] Number of plants displaying a particular viral accumulation level is in parentheses.

tions and did not undergo further mutations (Tables 2 and 3). There were some exceptions in which the infections were delayed or attenuated. This is the case of the infection of *N. clevelandii* plants inoculated with extracts containing virus progenies with the Δ7-36 or I16T modifications (Table 2). Attenuated infections were also observed in both *Nicotiana* spp. infected with virus progenies with D11G and K14R mutations (Table 2 and 3). A second mutation, V18G, was detected in the virus produced in *N. clevelandii* plants infected with a D11G-containing virus progeny. In addition, a T19P mutation was observed in one *N. clevelandii* and one *N. benthamiana* plant infected with a K14R-containing virus progeny (Tables 2 and 3). The K14R mutation was not stable in the virus accumulating in one *N. clevelandii* plant. R14 was further mutated to Gly, which appeared to cause a notable enhancement of symptoms and viral accumulation in this host (Table 2).

Finally, nonmutated virus progenies from *N. benthamiana*-infected plants (Table 1) could not systemically infect *N. clevelandii* plants (Table 2). In contrast, these progenies infected *N. benthamiana* plants, although with a noticeable delay (Table 3). These plants showed mild symptoms and variable levels of virus accumulation at later stages of infection (Table 3). Sequencing analysis of the virus progenies showed amino acid changes in the N terminus of the CP (I16T and I16F) (Table 3).

These results indicate that, generally, the mutated progenies (except for progenies with D11G and K14R mutations) have high infectivity, accumulated high amounts of virus, and induced severe symptoms. Moreover, all the mutations selected in the initial adaptation of PPV-BSD, except K14R, were stable after one passage in *N. benthamiana* and *N. clevelandii* plants.

Natural variability in the N terminus of PPV CP.

An extensive bioinformatic analysis was done in the SharCo database of sequences of natural PPV isolates. The aim of the analysis was to verify whether natural variability already existed at those amino acid positions of the N terminus of the CP that mutated during PPV-BSD adaptation to *Nicotiana* spp.

In all, 583 CP sequences from isolates representing all seven existing PPV strains were retrieved. The natural variability existing in PPV isolates in the amino acid positions of study (positions 11, 14, 16, 18, and 19) is summarized in Table 4. The sequence analysis revealed that the amino acids present in PPV-Dc at these positions (D11, K14, I16, V18, and T19) were highly prevalent in isolates of the D strain. Some of the amino acids selected in PPV-BSD progenies were common in other PPV strains (R14 in PPV-M, PPV-W, and PPV-EA; T16 in PPV-M, PPV-Rec, and PPV-C; and A18/19 in PPV-W). In addition, most of the amino acids selected in the experimental

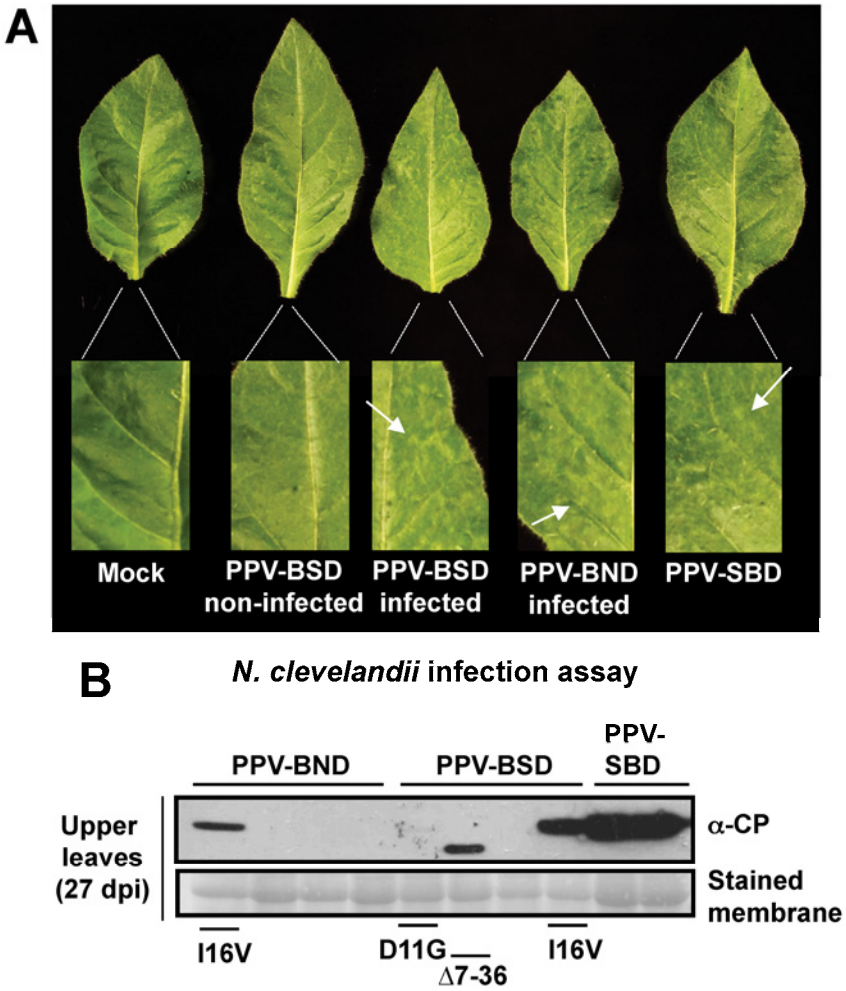

Fig. 2. Infectivity of *Plum pox virus* (PPV)-D/R hybrids in *Nicotiana clevelandii*. **A,** Pictures at 27 days postinoculation (dpi) of upper leaves from plants inoculated with different virus variants. Upper panel, whole leaves. Lower panel, details of leaf sectors in which a few virus-induced chlorotic areas are labeled with white arrows as examples of viral symptoms. Leaf sectors pictures were taken with a 1.7 magnification. **B,** Western-blot detection of virus coat protein (CP) in upper leaves at 27 dpi. The membrane stained with Ponceau red is included as loading control. Mutated residues at the N-terminal region of CP from PPV progenies are shown below the corresponding lane.

adaptation process (G11, R14, T16, V16, and A19) herein already existed in the CP of natural PPV isolates of strain D infecting *Prunus* trees (Table 4). A dN/dS (nonsynonymous/synonymous substitutions) analysis of the codons 11, 14, 16, 18, and 19 of D-type PPV isolates showed a higher incidence of neutral mutations (Supplementary Table S1). This result indicates that these residues are not under positive selection, and that purifying selection pressure is acting to preserve the wild-type sequence. No deletions were observed within the 30-aa region of the N terminus of the CP in the sequences of natural PPV isolates deposited in the SharCo database.

Infectivity in *N. clevelandii* of PPV-BSD clones containing amino acid substitutions in the N terminus of their CP.

Next, we wanted to further clarify the biological relevance of the amino acid mutations at the N terminus of the CP for PPV adaptation to *Nicotiana* and *Prunus* spp. For this purpose, we introduced several mutations in PPV-BSD and PPV-Dc cDNA clones because these two variants replicated efficiently in inoculated leaves of *Nicotiana* and *Prunus* spp., respectively (Figs. 3B and 4B). PPV-BSD-based mutant clones were used in *Nicotiana* spp. and PPV-Dc-based mutant clones were used in *Prunus* spp. The mutant clones contained several of the amino acid changes selected under experimental conditions, which are present (K14R, I16V, and V18A) or absent (Δ7-36, K14G, I16F, and I16S) in natural PPV isolates. Additionally, the I16M change (resulting from a T to G transversion), not selected under experimental conditions and absent in natural PPV isolates, was also introduced. This mutant was selected to further understand how restrictive the host-specific sequence requirements at the N terminus of PPV CP are.

PPV-BSD mutant variants described above were inoculated in *N. clevelandii* (Fig. 4). *N. clevelandii* was selected as the herbaceous host because it was found to be less permissive for PPV-BSD infections than *N. benthamiana* (discussed above). Western blot analysis of extracts from the inoculated leaves showed that plants inoculated with wild-type or mutant PPV-BSD variants accumulated high amounts of virus CP. Plants inoculated with most of the mutants accumulated virus CP similarly to those inoculated with wild-type PPV-BSD or PPV-R (Fig. 4B). The only exceptions were those plants inoculated with PPV-BSD-Δ7-36 mutant, which accumulated lower amounts of virus. However, CP levels in these plants were still noticeably higher than those observed in plants inoculated with PPV-Dc (Fig. 4B). All plants inoculated with mutant PPV-BSD clones, except for the K14R mutant, developed systemic disease symptoms (Fig. 4A; Supplementary Table S2). In most of the cases, such as plants inoculated with PPV-R, the infected plants showed systemic symptoms at 10 dpi. However, symptoms were delayed and milder in plants infected with PPV-BSD-Δ7-36 (Fig. 4A). As in previous experiments, the appearance and the intensity of the symptoms of plants infected with PPV-BSD was variable: only two of four plants developed symptoms, of low to medium intensity, which appeared with a 4- to 10-day delay. Western blot analysis at 21 dpi of extracts from upper leaves showed that plants infected with most PPV-BSD mutants, including the variant with the I16M change, accumulated virus CP to similar levels as plants infected with PPV-R (Fig. 4B). Virus accumulated to lower levels in plants infected with the PPV-BSD-Δ7-36 mutant and wild-type PPV-BSD chimera, in agreement with the milder symptoms of these plants (Fig. 4). The PPV-BSD-K14R mutant, despite accumulating to high levels in the inoculated

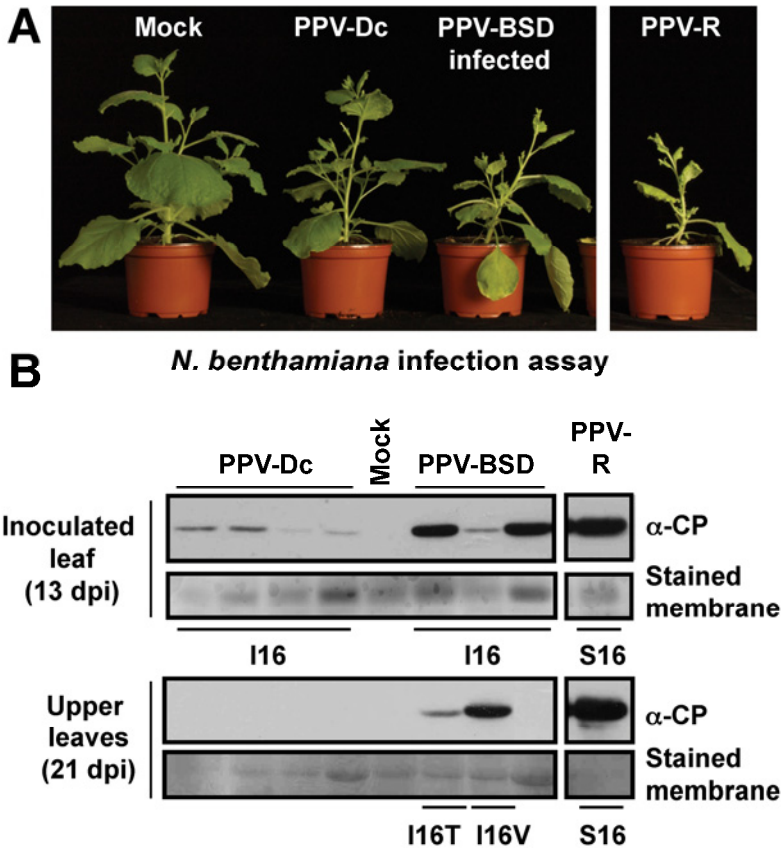

Fig. 3. Infectivity of *Plum pox virus* (PPV)-BSD chimeras in *Nicotiana benthamiana*. **A,** Pictures at 21 days postinoculation (dpi) of plants inoculated with different virus variants. **B,** Western blot detection of virus coat protein (CP) in inoculated (top) and upper leaves (bottom) at 13 and 21 dpi, respectively. Conserved or mutated residues at the N-terminal region of CP from PPV progenies are shown below the corresponding lane. The membrane stained with Ponceau red is included as loading control. Mutated residues at the N-terminal region of CP from PPV progenies are shown below the corresponding lane.

tissue, was not able to move systemically because no symptoms or virus CP were detected in upper noninoculated leaves (Fig. 4A and B). Sequencing analysis of the virus progenies from upper leaves showed that, in all cases, the introduced amino acid changes were stable, and no extra mutations were detected in the N terminus of the CP.

These results confirm that the amino acid changes in the N terminus of the CP are important for the infectivity and fitness of the PPV-BSD chimera in herbaceous plants. Although these changes do not increase the accumulation of PPV-BSD in the inoculated leaves, they do affect the systemic spread of the virus.

Infectivity in *P. persica* of PPV-Dc clones containing amino acid substitutions in the N terminus of their CP.

As mentioned above, the PPV-BSD chimera is unable to infect *P. persica* because the 5′ end of its genome derives from PPV-R (Fig. 1; Table 1). Thus, to analyze the effect of mutations selected during PPV-BSD adaptation to *Nicotiana* plants on PPV fitness in *Prunus* spp., Δ7-36, K14R, K14G, I16S, I16V, I16F, I16M, and V18A mutations were introduced into the PPV-Dc cDNA clone. In a first bioassay, PPV-Dc mutant variants were inoculated into *P. persica* GF305 seedlings (Fig. 5). Western blot analysis of extracts from the inoculated leaves showed that seedlings inoculated with PPV-Dc mutants generally accumulated levels of virus CP similar to those inoculated with wild-type PPV-Dc. However, this was not the case for seedlings inoculated with PPV-Dc-K14G because they accumulated less CP (Fig. 5B). Plants inoculated with mutant clones PPV-Dc-K14R, PPV-Dc-I16S, PPV-Dc-I16V, PPV-Dc-I16F, PPV-Dc-I16M, and PPV-Dc-V18A showed systemic symptoms at 10 dpi, and continued developing symptoms as PPV-Dc. No symptoms were observed in upper leaves of plants inoculated with PPV-Dc-Δ7-36. Very mild symptoms were observed in half of the trees inoculated with PPV-Dc-K14G, and only at a very late time (Fig. 5A; Supplementary Table S3). Western blot analysis at 16 dpi of extracts from upper leaves showed that virus CP accumulated to similar levels in plants infected with PPV-Dc-K14R, PPV-Dc-I16V, PPV-Dc-I16S, PPV-Dc-I16F, PPV-Dc-I16M, or PPV-Dc-V18A compared with plants infected with wild-type PPV-Dc. In contrast, virus CP did not accumulate in plants inoculated with either PPV-Dc-K14G or PPV-Dc-Δ7-36 (Fig. 5B). Whereas CP accumulation was observed in plants inoculated with PPV-Dc-K14G showing mild symptoms at 33 dpi, PPV CP was still not detected in plants inoculated with the PPV-Dc-Δ7-36 mutant at this time (Supplementary Fig. S2). A second bioassay was carried out to verify the defects in systemic infection caused by the Dc-K14G and Dc-Δ7-36 mutations (Supplementary Fig. S3). Virus accumulation was highly variable in the inoculated leaves. In contrast to the result of the previous bioassay, leaves inoculated with PPV-Dc-K14G and PPV-Dc-Δ7-36 clones accumulated higher levels of virus CP than leaves inoculated with the control viruses. In this second bioassay, plants infected with PPV-Dc-K14G showed symptoms with no delay compared with plants infected with PPV-Dc, PPV-Dc-V18A, or PPV-Dc-K14R. However, both the intensity of the symptoms and the virus accumulation levels of plants infected with PPV-Dc-K14G were notably lower than in plants infected with the control viruses. Plants infected with PPV-Dc-Δ7-36 did not show symptoms in this experiment either, and virus CP was not detected by Western blot analysis in upper noninoculated leaves. This result further confirmed the drastic effect of the Δ7-36 deletion in PPV systemic spread in peach. Sequence analysis of virus progenies from upper leaves indicated that all amino acid changes incorporated were stable even at 40 dpi, including the K14G change that reduced the efficiency of the systemic infection. No extra mutations in the N terminus of the CP were selected.

Finally, a competition bioassay was done in GF305 peach seedlings to better understand the effect of the mutations on PPV-Dc fitness in *Prunus* spp. Based on the data collected in

Table 2. Infectivity and sequence analyses of *Plum pox virus* (PPV)-BSD variants after one passage in *Nicotiana clevelandii*

Mutation	Progenies tested[a]	Infectivity[b]	Symptom appearance[c]	Symptoms (21 dpi)[d]	Virus accumulation[e]	Stability of the mutation[f]	New mutations[g]
No	Nb	0/3	…	…	…	…	…
	Nb	0/3	…	…	…	…	…
	Nb	0/3	…	…	…	…	…
	Nb	0/3	…	…	…	…	…
Δ7-36	Nc	3/3	14	++ (2), + (1)	++ (2), + (1)	Yes	No
D11G	Nc	3/3	14	+++ (2), + (1)	+++ (2), + (1)	Yes	V18G[h]
K14R	Nb	2/3	11 (1)	++ (1)	++ (1)	R14G	No
	…	…	…	– (1)[i]	– (1)[i]	Yes	T19P
I16S	Nc	3/3	10	+++	+++	Yes	No
I16T	Nb	2/3	14 (1), 21 (1)	++	+	Yes	No
	Nc	3/3	10	+++	+++	Yes	No
I16V	Nb	3/3	10	+++	+++	Yes	No
	Nc	3/3	10 (1), 14 (2)	+++	+++	Yes	No
V18A	Nb	3/3	10	+++	+++	Yes	No
	Nc	3/3	14	+++	+++	Yes	No
	Nc	3/3	10 (1), 14 (2)	+++	+++	Yes	No

[a] Species of the primarily infected plant used as inoculum. Nb = *N. benthamiana* and Nc = *N. clevelandii.*

[b] Infectivity is defined as the number of systemically infected plants/number of inoculated plants.

[c] Time in days postinoculation (dpi) at which the symptoms were first detected. The number of plants displaying particular behaviors is shown in parentheses.

[d] Symptoms of upper leaves were scored at 21 dpi, and classified according to their intensity as severe (+++), medium (++), mild (+), very mild (±), and absent (–). The number of plants displaying a particular symptom is in parentheses.

[e] Virus accumulation was assessed by Western blot analysis at 21 dpi, unless otherwise indicated, and classified as high (+++), medium (++), low (+), and not detectable (–). The number of plants displaying a particular virus accumulation level is in parentheses.

[f] Conservation or loss of the initial mutation is indicated.

[g] New amino acid changes in residues differing from the original mutated residue are indicated. The number in parentheses indicates the number of independent plants in which this specific mutation was observed.

[h] Three plants were analyzed as a pool.

[i] This plant showed neither symptoms nor viral accumulation in Western blot but was PPV positive by immunocapture reverse-transcription polymerase chain reaction.

the previous bioassays, clones PPV-Dc-I16V and PPV-Dc-V18A were selected for the competition experiment because i) both showed similar infectivity in peach and ii) I16V and V18A were the most common mutations observed in *N. benthamiana* and *N. clevelandii*, respectively. Pairs PPV-Dc:PPV-Dc-V18A and PPV-Dc:PPV-Dc-I16V were inoculated in both 1:1 and 1:2 ratios (Table 5).

Persistence of the competing viruses was analyzed at 30 dpi in extracts from systemically infected leaves. Reverse-transcription polymerase chain reaction preceded by immunocapture (IC-RT-PCR) analysis on these extracts was followed by sequencing of the amplified products. PPV-Dc-V18A and PPV-Dc-I16V showed competitiveness similar to wild-type PPV-Dc in peach because all viruses were maintained in the infected plants at the time of the analysis (Table 5). In a second bioassay where plants infected with PPV-Dc:PPV-Dc-V18A and PPV-Dc:PPV-Dc-I16V pairs were subjected to vernalization, the mutant variants were again detected to levels similar to wild-type PPV-Dc (data not shown).

These results show that most of the amino acid changes at the N terminus of the CP selected to adapt PPV to *Nicotiana* plants do not noticeably reduce the fitness of PPV-Dc in peach. However, the K14G mutation appears to lead to slower systemic movement whereas the lack of 30 aa of the CP (Δ7-36) renders the virus unable to move systemically.

DISCUSSION

The N terminus of the CP shows high variability in length and sequence among potyviruses (Shukla and Ward 1989), thus prompting the hypothesis that this region could be involved in specific interactions with host factors and adaptation to new hosts. In agreement with this concept, a 15-aa non-aphid-transmissible (NAT) deletion from this region was observed after PPV propagation in herbaceous species (López-Moya et al. 1995; Maiss et al. 1989). Furthermore, recent studies using chimeric viruses indicated that the N terminus of PPV CP includes host-specific pathogenicity determinants (Salvador et al. 2008) and also determinants that enable the virus to overcome *A. thaliana* RTM resistance (Decroocq et al. 2009).

Here, to further dissect such determinants, we used chimeric viruses engineered from two PPV isolates showing high se-

Table 3. Infectivity and sequence analyses of *Plum pox virus* (PPV)-BSD variants after one passage in *Nicotiana benthamiana*

Mutation	Progenies tested[a]	Infectivity[b]	Symptom appearance[c]	Symptoms at 21 dpi[d]	Virus accumulation[e]	Stability of the mutation[f]	New mutations[g]
No	Nb	1/3	45	–	++ (56 dpi)	…	I16T
	Nb	1/3	36	–	++ (56 dpi)	…	I16T
	Nb	2/3	36 (1), 45 (1)	–	+++ (56 dpi), + (56 dpi)	…	I16F[h]
	Nb	1/3	36	–	++ (56 dpi)	…	n.t.
Δ7–36	Nc	3/3	10	+++	++	Yes	No
D11G	Nc	3/3	21	+++	++	Yes	No
K14R	Nb	3/3	18 (1)	++ (1)	+++ (1)	Yes	T19P
	…	…	…	– (2)[i]	– (2)[i]	Yes	No
I16S	Nc	3/3	10	+++	+++	Yes	No
I16T	Nb	2/3	10	+++	++	Yes	No
	Nc	3/3	10	+++	++	Yes	No
I16V	Nb	3/3	10	+++	+++	Yes	No
	Nc	3/3	10	+++	+++	Yes	No
V18A	Nb	3/3	10	+++	+++	Yes	No
	Nc	2/3	10	+++	+++	Yes	No
	Nc	3/3	10	+++	+++	Yes	No

[a] Species of the primarily infected plant used as inoculum. Nb= *N. benthamiana* and Nc = *N. clevelandii*.
[b] Infectivity is defined as the number of systemically infected plants/number of inoculated plants.
[c] Time in days postinoculation (dpi) at which the symptoms were first detected. The number of plants displaying particular behaviors is shown in parentheses.
[d] Symptoms of upper leaves were scored at 21 dpi, and classified according to their intensity as severe (+++), medium (++), mild (+), very mild (±), and absent (–). The number of plants displaying a particular symptom is in parentheses.
[e] Virus accumulation was assessed by western blot analysis at 21 dpi, unless otherwise indicated, and classified as high (+++), medium (++), low (+), and not detectable (–). The number of plants displaying a particular symptom is in parentheses.
[f] Conservation or loss of the initial mutation is indicated.
[g] New amino acid changes in residues differing from the original mutated residue are indicated. The number in parentheses indicates the number of independent plants in which this specific mutation was observed; n.t. = not tested.
[h] The two plants were analyzed as a pool.
[i] Two plants showed neither symptoms nor viral accumulation in Western blot but were PPV positive by immunocapture reverse-transcription polymerase chain reaction.

Table 4. Natural variability in the N-terminal region of the coat protein (CP) of different *Plum pox virus* (PPV) strains[a]

	PPV strain, number of sequences						
	D	M	Rec	T	C	W	Ea
CP amino acid	**304**	**159**	**102**	**10**	**2**	**4**	**1**
11	D (303) **G**	D (159)	D (102)	D (9) N	D (2)	D (4)	D (1)
14	K (303) **R**	**R** (155) K (4)	K (102)	K (10)	K (2)	**R** (4)	**R** (1)
16	I (300) **V** (3) **T**	**T** (159)	**T** (99) A (2) I	I (10)	**T** (2)	A (4)	L (1)
18	V (304)	V (159)	V (101) **A**	V (9) **G**	T (2)	**A*** (4)	T (1)
19	T (301) **A** (3)	T (158) N	T (100) I (2)	T (10)	T (2)	T* (4)	T (1)

[a] Sequence information was retrieved from naturally infected-trees from the SharCo database. Number in parentheses indicates the number of isolates in which this specific mutation was observed in the database. Amino acid residues in PPV isolates from the database that were recovered from infectivity assays in the present work are in bold. Asterisk (*) indicates the Winona amino acids 19,20, which align with amino acids 18,19 of other PPV strains in sequence comparisons.

quence similarity but different host range: PPV-R, which infects herbaceous species, and PPV-D, which is restricted to *P. persica*. In particular, a 3′ end fragment from the PPV genome, which includes the CP coding sequence, was exchanged for the corresponding fragment of PPV-D in the PPV-R backbone (Fig. 1). Infectivity bioassays showed that the chimeras did not infect *P. persica* (Table 1), indicating that the 3′ end of the PPV-D genome is not sufficient to confer on PPV the ability to infect this host. On the other hand, the chimeras could systemically infect *N. benthamiana* and *N. clevelandii* by replacing or deleting a variety of specific amino acids from the N terminus of the CP (Figs. 2 and 3). Five substitutions and one deletion in the progeny from *N. clevelandii*-infected plants and four substitutions from *N. benthamiana*-infected plants were identified (Table 1; Supplementary Fig. S4). In particular, three of the selected mutations were common in both hosts and mapped into the region of the NAT deletion. In addition, mutations introduced in either *N. clevelandii* or *N. benthamiana* facilitated the systemic infection in the converse *Nicotiana* spp., indicating that requirements at the N terminus of CP for PPV systemic infection in different *Nicotiana* spp. are similar. In contrast, similar mutations allowing for adaptation to *A. thaliana* were not observed (Table 1), which suggests that PPV infection determinants at the N terminus of CP are not specifically conserved in different plant genera.

Most of the mutations introduced in the PPV-BSD chimera allowed an efficient infection and were stable in a subsequent passage (Tables 2 and 3). In contrast, although the D11G and

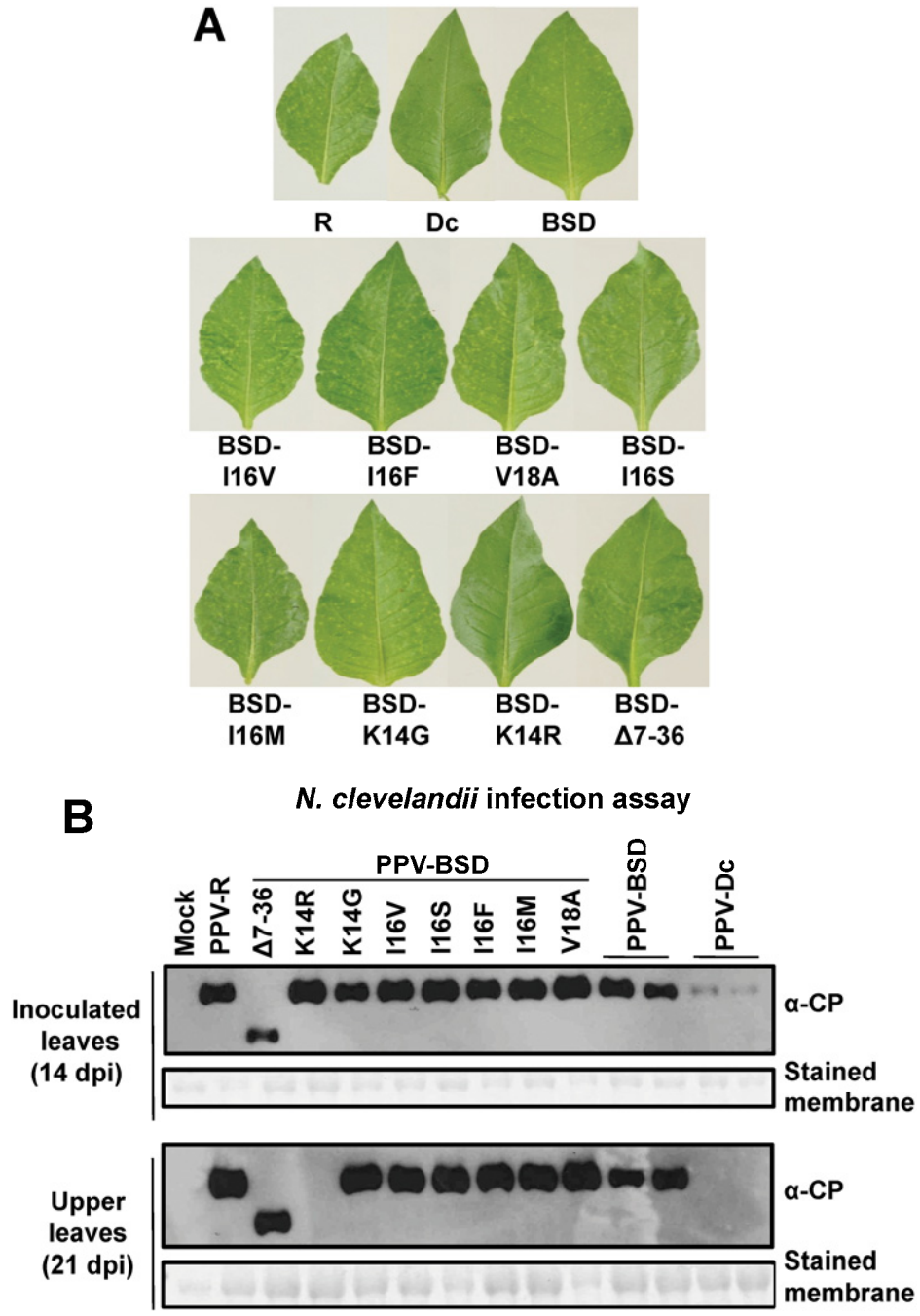

Fig. 4. Infectivity in *Nicotiana clevelandii* of *Plum pox virus* (PPV)-BSD hybrids containing mutations at the N-terminal region of PPV coat protein (CP). **A,** Symptoms observed at 21 days postinoculation (dpi) in upper leaves from plants inoculated with different virus variants. **B,** Western blot detection of virus CP in inoculated (top) and upper (bottom) leaves at 14 and 21 dpi, respectively. The membrane stained with Ponceau red is included as loading control.

K14R mutations facilitated PPV-BSD systemic infection, they conferred low fitness because their virus progeny further evolved in the *Nicotiana* plants (Tables 2 and 3). Thus, in the second round of *N. clevelandii* infections with the virus progeny carrying the K14R mutation, either the residue 14 was further mutated to Gly or a second T19P mutation was selected. It is noteworthy that the T19P mutation, which was not observed in any of the plants infected with the wild-type PPV-BSD chimera,

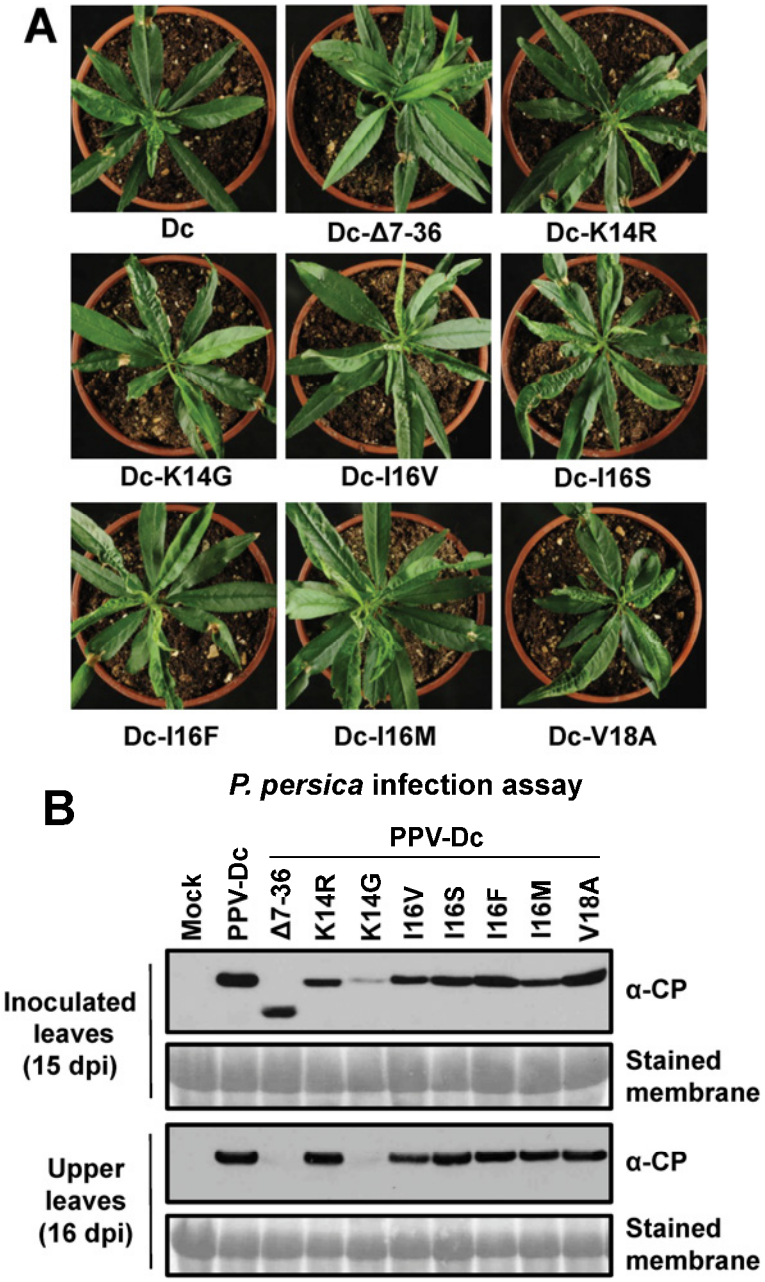

Fig. 5. Infectivity in *Prunus persica* of *Plum pox virus* (PPV)-Dc variants containing mutations at the N-terminal region of PPV coat protein (CP). **A,** Symptoms observed at 30 days postinoculation (dpi) in upper noninoculated leaves from plants inoculated with different virus variants. **B,** Western blot detection of virus CP in inoculated (top) and upper (bottom) leaves at 15 and 16 dpi, respectively. The membrane stained with Ponceau red is included as loading control.

Table 5. Virus detection following co-inoculation experiments with *Plum pox virus* (PPV) Dc mutants in *Prunus persica*[a]

	Inoculum 1:1[b]		Inoculum 1:2[b]	
Virus[c]	Dc: Dc-V18A	Dc: Dc-I16V	Dc: Dc-V18A	Dc: Dc-I16V
Dc	4/4	4/4	4/4	4/4
Dc-V18A	4/4	–	4/4	–
Dc-I16V	–	4/4	–	4/4

[a] Data shown are the number of plants infected with the indicated virus/total number of inoculated plants.
[b] Ratio for DNA mixtures in the inocula used for biolistic inoculation.
[c] Recovered virus at 30 days postinoculation.

was detected together with K14R in progenies from *N. clevelandii* and *N. benthamiana* (Tables 2 and 3). This result suggests a coordinated function of amino acids at positions 14 and 19. A second mutation, V18G, was also introduced together with the D11G mutation after a passage in *N. clevelandii*. Interestingly, although V18A mutations were detected in virus progenies of seven *Nicotiana* plants directly inoculated with PPV-BSD (Table 1), the V18G mutation was never found alone. This further suggests coordinated actions of different residues of the N terminus of the CP in PPV adaptation to *Nicotiana* plants. Collectively, all these results indicate that a variety of mutations at the N terminus of CP allow limited or efficient PPV adaptation to *Nicotiana* hosts.

The selection of a mutation appeared to depend on both the gain of viral fitness and the ease with which it could be generated. The most frequent mutations, I16V, I16T, and V18A, are transitions (ATT→**G**TT, A**C**T, G**T**T→G**C**T). These mutations increased the infection efficiency of PPV in *N. clevelandii* when engineered in the cDNA clone of PPV-BSD (Fig. 4). Other mutations at residue I16 (I16S, I16F, and I16M) supported PPV-BSD infection of *N. clevelandii* with efficiency similar to I16V or I16T mutations (Fig. 4). However, they were selected less frequently (I16F or I16S) or were not selected at all (I16M) (Tables 1–3). This is probably because they are transversions (ATT→**T**TT, AT**G,** A**G**T), which are more difficult to generate during genomic replication. The importance in the adaptive process of the ease with which appropriate mutations may occur is even more clearly illustrated in the case of the mutations of the K14 residue. Although the K14G substitution allows for very efficient infection of PPV-BSD in *N. clevelandii* (Fig. 4), it was never detected in plants infected with wild-type PPV-BSD. This is most likely because this substitution requires two mutation events (AAG→**GG**G). In contrast, a virus variant with a K14R mutation, which derives from a single nucleotide change (AAG→A**G**G), was detected in the first round of infection despite its low fitness in the *Nicotiana* plants (Table 1).

Most of the mutations favoring PPV adaptation to *Nicotiana* spp. did not significantly affect virus infectivity in *Prunus* spp. No significant differences in competitiveness were observed in experiments of coinfection of wild-type PPV-Dc with virus variants, including the most frequent mutations I16V and V18A. These results further support the assumption that mutations in the N terminus of CP enabling systemic infection in the herbaceous host genus *Nicotiana* do not necessarily reduce viral fitness in the natural host genus *Prunus*. In agreement with this conclusion, the analysis of PPV sequences deposited in the SharCo database, obtained from naturally infected-trees, confirmed that most of the mutations selected under experimental conditions in *Nicotiana* spp. already existed in natural PPV isolates (Table 4). Moreover, a dN/dS analysis of the codons involved in the adaptation to *Nicotiana* spp. clearly indicated that they are not subjected to positive selection. Thus, these codons may not be contributing significantly to PPV diversification in *Prunus* hosts.

On the other hand, the dN/dS analysis showed that the residues mutated for adaptation to *Nicotiana* spp. were under purifying selection in *Prunus* spp. This suggests that, although several amino acids can competently occupy the positions affected, they are also subjected to important sequence constraints. Thus, the large Δ7-36 deletion that enables systemic infection of PPV-BSD in *Nicotiana* spp. (Fig. 4) completely prevented PPV-Dc systemic infection in seedlings of *P. persica* (Fig. 5). Interestingly, the smaller NAT deletion (Δ13-27), which was found to be associated with PPV propagation in herbaceous plants (discussed above), was neither essential for infection in this host nor prevented systemic infection in *Prunus* spp. (Salvador et al. 2008). However, the wild-type sequence and the NAT deletion were associated with enhanced fitness in *Prunus* and *Nicotiana* spp., respectively (Salvador et al. 2008). A similar situation occurred with PPV variants with different amino acids at position 14. K14 and R14 supported efficient systemic infection of PPV in *P. persica* seedlings but the systemic spread of the virus with G14 was partially compromised (Fig. 5). In contrast, only G14 virus systemically infected *Nicotiana* plants with efficiency (Fig. 4).

The relevance of the N terminus of the CP in potyvirus long-distance movement was previously demonstrated (Andersen and Johansen 1998; Arazi et al. 2001; Dolja et al. 1995; López-Moya and Pirone 1998). Here, the high variability of virus accumulation observed in the inoculated leaves, probably due to the mechanical damage produced in the inoculation procedure, precluded a detailed analysis of local infection. However, our results suggest that those mutations identified in PPV adaptation to *N. clevelandii* affect virus long-distance movement but do not have relevant effects on local infectivity in the inoculated leaves. In this regard, it is important to remark that both the wild-type PPV-BSD chimera and its derivative mutants accumulated to higher levels than PPV-Dc in *N. benthamiana*-inoculated leaves (Fig. 3). This is in agreement with the fact that the first 6,931 nucleotides of the chimera were derived from the *Nicotiana* spp.-adapted isolate PPV-R. This is probably the reason that virus variants able to systemically spread emerge when *Nicotiana* plants are inoculated with PPV-BSD or PPV-BND but not with PPV-Dc.

Two models could explain the mechanisms by which the selected mutations in the N terminus of PPV CP affect systemic infection in *Nicotiana* and *Prunus* plants. In the first scenario, a specific interaction between a host movement factor (MF) and the N terminus of PPV CP is required for the systemic movement of the virus (Fig. 6A). The host factor of *Nicotiana* spp. appears to be quite permissive. It interacts with a large number of variants, including a deletion mutant, but is unable to functionally interact with the CP of PPV-D. Interestingly, the specific host factor of *Prunus* spp. is also permissive to most of these selected variants but will not fit with the truncated CP Δ7-36 (Fig. 6A). Whereas this model involving a large number of gain-of-function mutants cannot be ruled out, a more appealing model is that *Nicotiana* hosts might express a defense factor (DF). This DF could interact with the N terminus of PPV-D CP, preventing virus systemic movement by disturbing a productive interaction of the viral protein with an MF of the plant (Fig. 6B). Specific changes at the N terminus of the CP would disrupt its interaction with the DF without causing a drastic disturbance of the interaction with the MF, resulting in systemic movement of the virus. In the absence of a similar DF in *Prunus* spp., the mutants selected in *Nicotiana* spp. would still be functional in these plants although, in many cases, with some lower efficiency when the mutation affects the interaction with the MF of *Prunus* spp. However, a drastic mutation like the large Δ7-36 deletion could more severely affect the CP-MF interaction in *Prunus* spp. and prevent virus movement (Fig. 6B).

More broadly, the results reported here provide direct evidence supporting the idea that adaptation to new hosts, regardless of the mechanism, results in diversification of the viral population. This diversification could potentiate further adaptive evolution, highlighting the evolutionary power of host shifts.

In summary, the fine dissection of PPV pathogenicity determinants presented here clearly assesses a key role of a few specific amino acids at the N terminus of the CP in PPV adaptation to different host species. However, the subset of host factors, enhancers or inhibitors of the infection, that specifically interact with the viral CP in a potyvirus infection still remain to be elucidated.

MATERIALS AND METHODS

Plant hosts.

Viral infectivity assays were performed in *A. thaliana*, *N. benthamiana, N. clevelandii*, and *P. persica* (peach) 'GF305' plants. All plants were grown in a greenhouse maintained at 16 h of light with supplemental lighting and a temperature range of 19 to 23°C.

Construction of full-length hybrid clones and mutants.

The full-length cDNA clones pICPPV-Dc (Dc) (Salvador et al. 2008), pICPPV-NK-GFP (PPV-R) (Fernández-Fernández et al. 2001), and the PPV-D/R hybrid pICPPV-SBD (SBD) (Salvador et al. 2008) were previously described. The two chimeras pICPPV-BSD (BSD) and pICPPV-BND (BND) were constructed by exchanging fragments using the restriction enzymes indicated in Figure 1.

For the generation of BSD chimeras carrying K14R, K14G, I16S, and I16V mutations at the N-terminal region of the CP, an IC-RT-PCR product amplified with primers number 272 (5′-TTTAACGATGATGGTG-3′) and number 55 (5′-CTATGC ACCAAACC-3′) from the BSD virus progeny carrying the respective mutation and digested with *Bpi*I/*Sac*I was ligated with two fragments generated from the pICPPV-BSD plasmid digested with *Bam*HI/*Bpi*I and *Bam*HI/*Sac*I, respectively. The BSD-I16M mutant was similarly generated but the I16M change in the PCR product was created by site-directed mutagenesis because no virus progeny carrying the specific mutation was naturally selected. First, two different PCR amplifications were done using pIC-PPV-BSD as a template and primers number 80 (5´-TTGGGTTCTTGAACAAGC-3´) and number CP-I16M-Rev (5´-AGT AAC TAC CAT CGG CTT GCC-3´), and number CP-I16M-For (5´-GGC AAG CCG ATG GTA

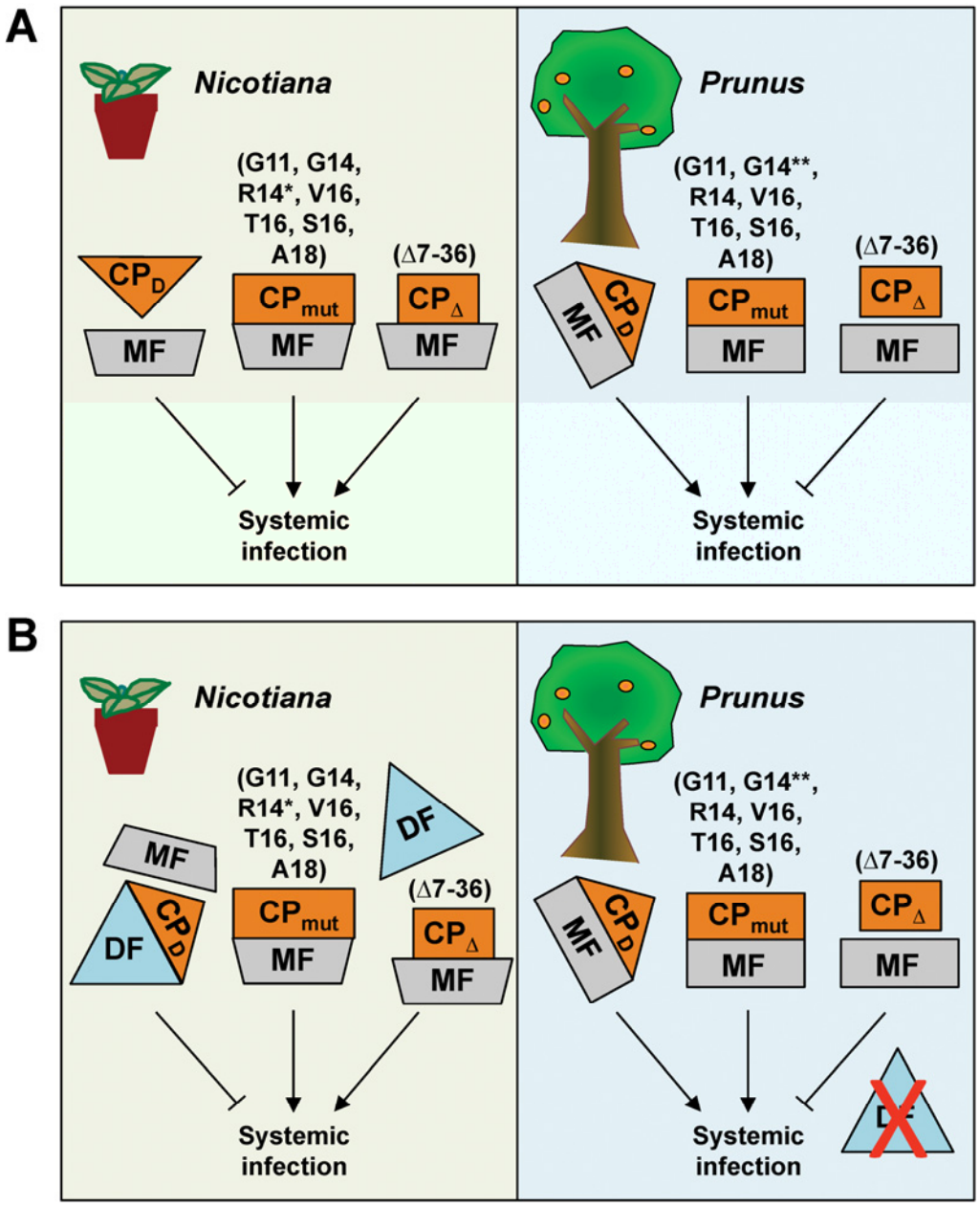

Fig. 6. Models explaining the infectivity of the *Plum pox virus* (PPV)-D/R hybrids containing mutations in the coat protein (CP). **A,** Viral movement requires a compatible interaction between the CP and a movement factor (MF). **B,** Virus movement is preserved when the CP escapes the interaction with a host defense factor (DF). Asterisks: * indicates that PPV with R14 only infects systemically *Nicotiana benthamiana* but not *N. clevelandii*; ** indicates that systemic infection of PPV with G14 is reduced when compared with the rest of the mutants listed in parentheses.

GTT ACT G-3′) and number 55ext (5′-CATTCTCTATGCA CCAAACC-3′) respectively. These were followed by a second PCR with primers number 80 and 55ext, in which the products from the first two PCR assays were included as templates. pIC-PPV-BSD-I16F, pIC-PPV-BSD-V18A, and pIC-PPV-BSD-Δ7-36 constructs were generated by ligating the *Bam*HI/*Sac*I fragment from the respective pIC-PPV-Dc-I16F, pIC-PPV-Dc-V18A, and pIC-PPV-Dc-Δ7-36 constructs with the *Bam*HI/*Sac*I-digested pIC-PPV-BSD plasmid.

Likewise, for the generation of pIC-PPV-Dc-K14R, pIC-PPV-Dc-K14G, pIC-PPV-Dc-I16S, pIC-PPV-Dc-I16F, and pIC-PPV-Dc-V18A constructs, an IC-RT-PCR product amplified with primers number 272 and 55 from the PPV-BSD virus progeny carrying the respective mutation and digested with *Bpi*I/*Sac*I was ligated with two fragments, generated from pIC-PPV-Dc plasmid digested with *Bam*HI/*Bpi*I and *Bam*HI/*Sac*I, respectively. The pIC-PPV-Dc-I16M construct was generated in a similar way but the I16M change in the PCR product was created by site-directed mutagenesis, as described above. The pIC-PPV-Dc-I16V construct was generated by ligating the *Bam*HI/*Sac*I fragment from pIC-PPV-BSD-I16V with *Bam*HI/*Sac*I digested pIC-PPV-Dc plasmid. pIC-PPV-Dc-Δ7-36 was generated by ligating a PCR product amplified with primers number 921 (5′-AGGAGCTCTACGTCACTGATCC-3′) and number 55 from the PPV-BSD virus progeny carrying the respective deletion, and digested with *Bst*XI/*Sac*I, with the *Bst*XI/*Sac*I-digested vector pIC-PPV-Dc.

Biolistic and manual inoculation.

A. thaliana, *N. benthamiana*, and *N. clevelandii* plants and *P. persica* GF305 seedlings were biolistically inoculated using the Helios Gene Gun System (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Microcarrier cartridges were prepared from two different clones per construct, with 1.0 µm gold particles coated with the different plasmids at a DNA loading ratio of 2 µg/mg of gold and a microcarrier loading of 0.5 mg/shooting. Helium pressures of 7.5 and 10 bar were used for inoculating herbaceous hosts (*A. thaliana*, *N. benthamiana*, and *N. clevelandii*) and peach GF305 seedlings, respectively. Each cartridge was shot twice onto two leaves of each plant and, for each plant, one or two cartridges were administered for herbaceous and GF305 hosts, respectively. Usually, eight plants per construct (four per clone) were inoculated in each experiment.

For manual inoculation (second passage) of the virus progeny with or without mutations at the N terminus of the CP, upper leaves from infected *N. clevelandii* or *N. benthamiana* plants were ground with 5 mM sodium phosphate (pH 7.5) with an ice-cold pestle (1 g in 2 ml). Extracts were centrifuged to eliminate tissue fragments. For each extract, two leaves were dusted with Carborundum and inoculated with a total of 15 µl of extract. Usually, each virus progeny was inoculated onto three *N. clevelandii* and *N. benthamiana* plants. Virus infection was monitored by the observation of symptoms.

ELISA and Western blot assays.

Inoculated or systemically infected leaves were harvested, ground to a fine powder under liquid nitrogen, and stored at –80°C until use. Control samples corresponding to noninfected leaves were taken from equivalent areas. The tissue was homogenized in 5 mM sodium phosphate buffer (SPB), pH 7.5, (1 g in 2 ml) and virus accumulation was assessed using double-antibody sandwich indirect ELISA with a REALISA kit (Durviz, Valencia, Spain) as previously described (Salvador et al. 2008). For Western blot analysis, plant extracts were further diluted in a ratio of 1:1 in disruption buffer (150 mM Tris-HCl [pH 7.5], 6 M urea, 2% sodium dodecyl sulfate, and 5% ß-mercaptoethanol). Samples were boiled for 10 min, and cell debris were removed by centrifugation at 18,000 × *g* at 4°C for 10 min. Supernatants were resolved on sodium dodecyl sulfate-polyacrylamide gels (12% acrylamide), electroblotted to a nitrocellulose membrane, and subjected to Western blot analysis. Virus CP was detected using anti-CP rabbit serum as primary antibody, and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Jackson, West Grove, PA, U.S.A.) as secondary reagent. The immunostained proteins were visualized by enhanced chemiluminiscence detection with a Life-ABlot kit (Euroclone, Milano, Italy). Ponceau red staining was used to check the global protein content of the samples.

IC-RT-PCR and DNA sequencing.

Leaves from infected *A. thaliana*, *N. benthamiana, N. clevelandii*, and *P. persica* GF305 plants were homogenized in 5 mM SPB, pH 7.5, (1 g in 2 ml) and incubated in tubes previously coated with anti-PPV IgG overnight at 4°C. The incubation was followed by two washing steps with phosphate-buffered saline (PBS)-Tween buffer (16 mM PBS, 0.1 M NaCl, and Tween 20 at 0.5 g/liter; pH 7.2). RT-PCR was done using the Titan kit (Roche Molecular Biochemical, Branchburg, NJ, U.S.A.) with primers number 80 and 82 (5′-TGGCACTGTAA AAGTTGG-3′), targeting the 5′ end of the CP coding sequence. For further sequence analysis, PCR fragments were purified using a MinElute PCR purification kit (Qiagen, Hilden, Germany) and sequenced by Macrogen (Seoul, Korea). IC-RT-PCR and subsequent sequencing analysis was done once for each individual plant unless otherwise stated.

Sequence analysis.

PPV sequences from different isolates were obtained from the SharCo database. Only those SharCo sequences obtained from naturally infected trees were used in the analyses. A phylogenetic analysis of the PPV sequences followed by a selection analysis was done. Redundant sequences were deleted from the dataset according to the workflow of Moury and Simon (2011). The phylogenetic tree was obtained using MEGA5 software (Tamura et al. 2011), and the best substitution model by Modeltest, maximum likelihood, and neighbor-joining methods were used. The inferred tree was used to find the codon sites under positive selection (dN/dS > 1). For this purpose, HyPhy software (Pond et al. 2005) was used. In particular, single-likelihood ancestor counting, fixed-effects likelihood, and random-effects likelihood methods were applied.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The SharCo *Plum pox virus* database: w3.pierroton.inra.fr:8060