In vitro recombinants of two nearly identical potyviral isolates express novel virulence and symptom phenotypes in plants

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Six novel chimeric viruses were constructed by sequentially exchanging segments of the viral genomes between the infectious cDNA clone (pPVA-B11) of Potato virus A (isolate PVA-B11) and pUFL, an almost identical infectious cDNA of PVA (isolate U) made in this study. The infectious in vitro transcripts of pUFL and pPVA-B11 caused similar severe mosaic and leaf malformation phenotypes in systemically infected leaves of Nicotiana benthamiana. In contrast, one chimera induced a unique phenotype of yellow vein chlorosis without leaf malformation with viral titres that were equivalent to those of the parental viruses. Furthermore, as opposed to the viral cDNAs from which it was assembled, one chimera showed no detectable infectivity of N. benthamiana plants. Thus, recombination of nearly identical, phenotypically similar virus genomes can give rise to new viral strains with novel virulence and symptom phenotypes, which has not previously been demonstrated with potyviruses. One chimera failed to cause systemic infection in potato plants, but, nevertheless, avirulence could not be attributed to a single genomic region. These data suggest that different parts of the potyviral genome function coordinately. The results provide novel insights into the evolution of the genus Potyvirus.

INTRODUCTION

Potyviruses (family Potyviridae, genus Potyvirus) comprise approximately 30% of the known plant viruses (Van Regenmortel et al., 2000). This group of viruses has successfully adapted to many different hosts and environments. Previous studies have sought explanations for the evolution of single-stranded messenger-sense RNA viruses, such as potyviruses (reviewed by García-Arenal et al., 2001; Roossinck, 2003), utilizing potyvirus isolates and strains obtained from the field, mainly from cultivated plants. Mutation rates are relatively high during RNA virus replication due to the lack of proofreading activity in the viral RNA-dependent RNA polymerase (Reanney, 1987), a characteristic that is also exhibited in potyviruses (García-Arenal et al., 2001). Mutations provide the progeny viral genomes with the potential to adapt to changing environments, such as the infection of new hosts. Diversifying selection, probably owing to host effects, has been found in the 6K2 protein and the coat protein (CP) of Potato virus Y (PVY) (Moury et al., 2002). Since potyviral proteins are multifunctional (reviewed by Kekarainen et al., 2002), they are under different selective constraints that correspond to their various functions. For this reason, as proposed by García-Arenal et al. (2001), viral proteins are never optimized for only one of their functions.

Recombination is another important source of genetic variability in viruses (Bruyere et al., 2000; García-Arenal et al., 2001). Previous studies indicate that recombination between different isolates of potyvirus species may be common. Some examples, predicted based on sequence analyses of virus isolates obtained from different hosts and geographic regions, are PVY (Cervera et al., 1993; Revers et al., 1996; Moravec et al., 2003) and Turnip mosaic virus (TuMV) (Ohshima et al., 2002). However, the parental virus isolates that recombined to produce these strains, as well as their phenotypes, are unknown.

Potato virus A (PVA) is a widespread and harmful potyvirus
infecting potato crops in all major potato production regions (Rajamäki et al., 1998; Spetz et al., 2003). The isolate U of PVA (PVA-U) was originally isolated from a potato breeding line infected in the field in Michigan, USA. It has been maintained in infected tubers of potato cv. Pito and described in previous studies (Valkonen et al., 1995; Rajamäki et al., 1998; Hämäläinen et al., 2000). PVPVA-B11 is an infectious, full-length cDNA (Puurand et al., 1996) constructed from a PVA isolate (PVA-B11) originally isolated from potatoes infected in the field in Germany. The virus derived from pPVA-B11 differs from PVA-U in that it does not infect potato plants systematically (Valkonen et al., 1995; Andrejeva et al., 1999), possibly due to extensive passage in experimental Nicotiana host plants (Rajamäki et al., 1998). However, both PVA isolates infect plants of Nicotiana benthamiana systematically, causing similar severe symptoms of mosaic and leaf malformation (Savenkov & Valkonen, 2002). Comparison of the genomes of PVA-U and pPVBA11 reveals that they are almost identical. The 3’ non-translated region (NTR) is identical in the two isolates, and the different protein-encoding regions and 5’-NTR show >97.5% nucleotide sequence identity, except for the 6K1 protein- and coat protein (CP)-encoding regions, which exhibit 95.5% and 96.8% nucleotide sequence identity, respectively. At the amino acid level, sequence identity is higher than at the nucleotide level, except for the CP (95-99%).

The aim of this study was to investigate whether recombination of nearly identical and phenotypically similar potyvirus isolates could result in new virus strains. Properties that allow viral strains to be distinguished from other known strains are mostly biological, such as the manifestation of different symptoms in a particular host (Matthews, 1999). However, both PVA isolates infect plants of Nicotiana benthamiana systemically, causing similar severe symptoms of mosaic and leaf malformation (Savenkov & Valkonen, 2002). Comparison of the genomes of PVA-U and pPVBA11 reveals that they are almost identical. The 3’ non-translated region (NTR) is identical in the two isolates, and the different protein-encoding regions and 5’-NTR show >97.5% nucleotide sequence identity, except for the 6K1 protein- and coat protein (CP)-encoding regions, which exhibit 95.5% and 96.8% nucleotide sequence identity, respectively. At the amino acid level, sequence identity is higher than at the nucleotide level, except for the CP (95-99%).

The amplified partial cDNAs of the PVA-U genome were used to replace sequentially the corresponding part of pPVBA11 in the cloning vector pUC18 containing the infectious, full-length cDNA copy of PVA isolate B11 placed under the control of the T7 promoter (Puurand et al., 1996). Each PCR product and the plasmid were digested with the same pair of endonucleases: Sphl and Nra1 for replacement of the 5’-proximal region (chimera pBUI), Nra1 and Apal for the central region (chimera pBUII) and Apal and Agel for the 3’-proximal region (chimera pBUIII; Fig. 1). The constructs were cloned in Escherichia coli (strain DHS5; Stratagene) and the composition of all chimeric clones was confirmed by restriction endonuclease analysis based on differences in the restriction maps of pPVBA11 and PVA-U and by sequencing the junctions. Sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an automated DNA sequencer (ABI prism 310 Genetic Analyser; Perkin Elmer).

Transcription from viral cDNA constructs. Transcripts were generated using T7 RNA polymerase (Promega) according to the supplier’s instructions, with minor modifications. Plasmids containing the chimeric viral cDNAs were linearized with Agel and transcribed with T7 RNA polymerase (Promega) in the presence of a m5G(5’ppp5’G) cap analogue (New England Biolabs). The reaction mixture (50 μl) contained Transcription Optimized 5’-proximal region (chimera pBUI), Nra1 and Apal for the central region (chimera pBUII) and Apal and Agel for the 3’-proximal region (chimera pBUIII; Fig. 1). The constructs were cloned in Escherichia coli (strain DHS5; Stratagene) and the composition of all chimeric clones was confirmed by restriction endonuclease analysis based on differences in the restriction maps of pPVBA11 and PVA-U and by sequencing the junctions. Sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an automated DNA sequencer (ABI prism 310 Genetic Analyser; Perkin Elmer).

Infection of plants. Several independently generated chimeric clones were tested for infectivity of N. benthamiana using mechanical inoculation with capped in vitro transcripts. The first three fully expanded leaves on 4-week-old plants were mechanically inoculated with the transcription reaction mixture (3 μl) diluted with 25 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Leaves were dusted with Carborundum prior to inoculation and rinsed with distilled water 5 min after inoculation. Plants were grown in an insect-proof greenhouse at 20/22 °C using a 16 h photoperiod. The PVA CP was detected in plants by double-antibody sandwich (DAS)-ELISA (described below) and by Western blot analysis (data not shown). The clones of pBUI, pBUII and pBUIII that were found to be infectious were used to assemble the full-length PVA-U cDNA (pUFL) by combining the PVA-U-derived parts of the three chimeras into a single plasmid (Fig. 1).

Infectivity of the PVA chimeras was tested in two potato lines, v2-134 and v2-51, by mechanical inoculation. Both potato lines are diploid (2n = 2x = 24) interspecific hybrids obtained from the same cross (Valkonen et al., 1994). The potato plants were clonally propagated in vitro and plantlets were transferred to soil in the greenhouse and inoculated when 6 weeks old. The five lowest leaves were dusted with Carborundum prior to inoculation. Systemically infected leaves of the N. benthamiana plants inoculated with in vitro transcripts were used

METHODS

Construction of the infectious PVA-U cDNA. The full-length cDNA clone of PVA-U, designated pUFL, was constructed as outlined in Fig. 1 and described below. The oligonucleotide primers used in RT-PCR reactions were designed based on the published sequence of PVA-U (Ketakainen et al., 1999) (primer sequences are available from the authors on request). Recombinant DNA techniques were used as described in Sambrook et al. (1989). Total RNA was extracted from leaves of N. benthamiana infected with PVA-U using the RNasy Plant Mini Kit (Qiagen). cDNA synthesis was carried out using Moloney murine leukaemia virus reverse transcriptase (Promega) in a buffer supplied by the manufacturer. Three reverse transcription reactions were carried out independently using primers 3012R, 6823R and 3’polyT-PVA (20 pmol each per reaction) to synthesize cDNAs corresponding to the 5’-proximal, central and 3’-proximal regions of the PVA-U RNA, respectively. The cDNAs were amplified by PCR using the high-fidelity polymerase DyNAzyme EXT (Finzymes) according to the manufacturer’s instructions. The following primer pairs were used: 5’-T7-PVA/3012R for amplification of the 5’ portion of the genome, 2905F/6823R for the central portion and 6055F/3’-polyT-PVA for the 3’ portion of the genome. The PCR mix contained 2 μl of reverse transcriptase reaction mixture, 5 μl 10× Optimized DyNAzyme EXT buffer (Finzymes), 0.2 mM each dNTP, 0.4 μM each primer and 1 U DyNAzyme EXT in a final volume of 50 μl. Amplification conditions for the three cDNAs included an initial denaturation step of 1 min at 94 °C followed by 25 cycles of 15 s at 94 °C, 45 s at 57 °C and 3 min at 72 °C, followed by the final extension for 10 min at 72 °C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) prior to digestion by restriction endonucleases. Restriction products were resolved on agarose gels and the DNA fragments purified from excised gel slices.
Fig. 1. The strategy for construction of a full-length cDNA clone of PVA-U (pUFL) via intermediary chimeric clones. (A) Partially overlapping clones representing the 5'-proximal, central and 3'-proximal regions (designated as UI, UII, UIII, respectively) of PVA-U were obtained by RT-PCR. The numbers of the first and last nucleotide of each clone are indicated. (B) The RT-PCR products were cloned into pPVA-B11, an infectious, previously described cDNA clone of PVA of isolate B11 using the indicated, unique restriction sites to obtain the intermediate infectious clones pBUI, pBUII and pBUIII (the segments derived from PVA-U are indicated with a filled box). They were subsequently used to assemble the full-length cDNA clone pUFL downstream from the T7 RNA polymerase promoter (indicated by an arrowhead). Additional chimeric clones between pUFL and pPVA-B11 were obtained by substituting segments of pPVA-B11 with additional segments of pUFL. The protein-encoding regions are schematically presented: P1, proteinase; HC-Pro, helper component proteinase; P3, third protein; 6K1, 6 kDa protein; CI, cylindrical inclusion protein; 6K2, 6 kDa protein, VPG, viral RNA-linked protein; Nla-Pro, proteinase; Nib, replicase; CP, coat protein. The 5' and 3' non-translated regions are indicated by small boxes at the ends of the polyprotein.
for preparation of inoculum. Virus titres in leaves were determined by DAS-ELISA, the leaves were frozen at −70 °C and the same batches used for inoculation of potato plants in two experiments. The sap was diluted accordingly with distilled water so that similar concentrations of inoculum were used with each virus. pbUII + III was not included in potato experiments due to its poor systemic infection in *N. benthamiana*, making it difficult to obtain inoculum titres comparable with other viruses.

**Virus detection by DAS-ELISA.** PVA CP was detected in plants by DAS-ELISA using a monoclonal antibody (mAb) and the alkaline phosphatase conjugate of the same mAb (mAb 58/0; Adgen, UK) to PVA CP (Rajamäki et al., 1998). Leaves were sampled, weighed, ground in ELISA sample buffer at 1 g per 9 ml and two 100 μl aliquots were transferred to two wells of a microtitre plate coated with mAb 58/0. Known amounts of purified virions of PVA-B11 (0.32, 1.6, 8, 40 and 200 ng) were included to establish a standard curve to estimate the amount of virus in leaves. Colour reactions were developed using *p*-nitrophenyl phosphate as a substrate. Absorbances were measured at 405 nm with a Benchmark microtitre plate reader using Microplate Manager software (Bio-Rad) at the time absorbances were measured at 405 nm with a Benchmark microtitre plate reader using Microplate Manager software (Bio-Rad) at the time point when the absorbance for 200 ng purified virions reached the *A*<sub>00</sub> value of 2.50. Thus, results of different ELISAs could be compared.

**RESULTS AND DISCUSSION**

The sequence of the new, infectious cDNA of PVA-U (pUFL) was determined and compared with the previously published sequence of PVA-U (AJ131402; Kekarainen et al., 1999). A total of 25 nucleotide changes were found. Eleven substitutions were silent. Fourteen changes were predicted to result in amino acid substitutions (shown from PVA-U→pUFL) or a short frameshift region (see below). One substitution each was found in the P3 protein (nt 2788, Gly→Ala), the viral genome-linked protein (VPg) (nt 6162, Ser→Pro) and the RNA-dependent RNA polymerase (Nlβ) (nt 8221, Val→Glu). The Nla proteinase domain (Nla-Pro) contained five substitutions (nt 6313, Arg→Gln; nt 6646, Thr→Ile; nt 6658, Gly→Glu; nt 6856, Arg→Lys; nt 6975, His→Tyr). CP contained four substitutions (nt 8550, Gly→Ser; nt 9028, Lys→Ser; nt 9033, Thr→Leu; nt 9036, Arg→Ala). All of the amino acid residues in pUFL were identical to the residues in pPVA-B11 at the corresponding position, making pUFL even more similar to pPVA-B11 than PVA-U. For example, the CP amino acid sequence identity of pUFL and pPVA-B11 was 97.4 %, compared with the 95.9 % sequence identity shared between PVA-U and pPVA-B11. pUFL contained an additional nucleotide at position 7852 and a deletion of nt 7889, compared with PVA-U, resulting in a 13-amino acid frameshift in Nlβ. The position and deduced amino acid sequence of the frameshift region was identical to those previously described in PVA isolates Can and TamMV (Kekarainen et al., 1999).

After their construction, the intermediary chimeras (pBUI, pbUII and pbUII) were pre-selected for infectivity in *N. benthamiana*. We propose that this factor might explain how the aforementioned pPVA-B11-like sequence variants were found from PVA-U. RNA viruses, including potyviruses, exist in infected hosts as quasispecies, i.e. as a population of nearly identical sequences, which are generated by the error-prone viral RNA-dependent RNA polymerase (Eigen, 1996; Domingo et al., 1998). The pPVA-B11-like amino acid residues in the pBUI, pbUII and pbUIII chimeras may have a greater compatibility with the rest of the chimeric virus genome derived from pPVA-B11, which is adapted to *Nicotiana* hosts (see Valkonen et al., 2002). For example, it is noteworthy that pUFL contains an Asp-Ala-Ser (DAS) motif at the CP N terminus (aa 5–7), whereas PVA-U has an Asp-Ala-Gly (DAG) motif at this position. Only DAG is compatible for aphid transmissibility, whereas DAS significantly increases accumulation of PVA in infected leaves (Andrejeva et al., 1999; Valkonen et al., 2002). Indeed, the titres of pUFL were among the highest of all the viruses tested in this study, both in the systemically infected leaves of *N. benthamiana* (Table 1) and the potato line v2-134 (Table 2). Therefore, the spontaneous viral variants with the aforementioned Gly→Ser substitution in CP (aa 7) may benefit

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<th>Virus construct</th>
<th>Amount of virus [μg (g leaf)&lt;sup&gt;−1&lt;/sup&gt;]&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Symptoms</th>
</tr>
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<tbody>
<tr>
<td>pPVAB11</td>
<td>15.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>Severe mosaic and leaf malformation</td>
</tr>
<tr>
<td>pUFL</td>
<td>20.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Severe mosaic and leaf malformation</td>
</tr>
<tr>
<td>pbUI</td>
<td>8.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mild mosaic, no leaf malformation</td>
</tr>
<tr>
<td>pbUII</td>
<td>11.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>Very severe leaf malformation and mosaic</td>
</tr>
<tr>
<td>pbUIII</td>
<td>16.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Very severe leaf malformation and mosaic</td>
</tr>
<tr>
<td>pbUII + II</td>
<td>20.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Vein net chlorosis, no leaf malformation</td>
</tr>
<tr>
<td>pbUII + III</td>
<td>10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Severe mosaic and leaf malformation</td>
</tr>
<tr>
<td>pbUII + III</td>
<td>0</td>
<td>No symptoms at 14 days p.i.&lt;sup&gt;†&lt;/sup&gt;</td>
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</table>

*Mean of two plants at 14 days post-inoculation, as measured using DAS-ELISA using known amounts of purified pPVAB11 virions for comparison. Means followed by a different letter differ from each other. The least significant difference of means = 12.8 (P=0.05).
†Systemic infection with transiently expressed mild yellow mosaic symptoms and low, positive ELISA absorbances was observed by 28 days p.i.
from higher fitness in their hosts under experimental conditions, but they may be lost in the field where transmission to new hosts depends on aphid transmissibility.

The plants of *N. benthamiana* inoculated with the capped *in vitro* transcripts of pUFL and pPVA-B11 showed similar symptoms of severe mosaic and leaf malformation in systemically infected leaves (Fig. 2). Subsequently, *N. benth-
amiana* plants were inoculated with the capped transcripts derived from chimeras pBUI, pBUII, pBUIII and three additional chimeras (Fig. 1). The additional chimeras (pBUI+II, pBUI+III and pBUII+III) each contained two-thirds of the viral genome from pUFL, as opposed to the original three chimeras (pBUI, pBUII and pBUIII), in which two-thirds of the genome was derived from pPVA-B11. The six different chimeras of PVA varied in symptom phenotypes and viral accumulation in systemically infected leaves of *N. benthamiana*. This result was consistently observed in three experiments (six plants per chimera). Novel yellow vein chlorosis (net chlorosis) symptoms, but no leaf malformation, were observed in leaves infected with pBUI+II (Fig. 2, Table 1). Similarly, chimera pBUI produced titres that were 2-5-fold less than the titres of pBUI+II and pUFL (P<0.05) (Table 1). Infection with pBUI induced mild mosaic symptoms (Fig. 2), indicating that there is no direct correlation between viral titres and symptom severity. These observations support the idea that chimera-specific host interactions are a more important determinant for the phenotypic differences than the viral titre.

The systemic infection and symptoms associated with pUFL, pPVA-B11 and the chimeric viral constructs were fully developed by 14 days post-inoculation (p.i.), with the exception of pBUI+III, which showed a systemic infection delayed by approximately 2 weeks. Titres of pBUI+III were low, as detected by DAS-ELISA, in the systemically infected leaves that showed transient mild yellow chlorosis symptoms (data not shown). The junction regions in progeny viruses of all chimeras were cloned from the systemically infected leaves by RT-PCR and the DNA was sequenced to ensure that the chimeric sequences were maintained during virus multiplication. No mutations or deletions were found.

Two potato lines, v2-134 and v2-51, were then tested for their ability to be infected with the PVA chimeras. Previous studies have shown that line v2-134 is systemically infected with PVA-U but not with PVA-B11, although both viruses can infect the inoculated leaves and accumulate to high titres (Hämäläinen *et al.*, 2000). Indeed, pPVA-B11 infected the inoculated leaves, but not the non-inoculated leaves, as determined by DAS-ELISA, whereas pUFL infected the

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<tr>
<th>Virus construct</th>
<th>Line v2-134</th>
<th>Line v2-51</th>
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<tr>
<td></td>
<td>IL 14 days p.i.</td>
<td>SL 14 days p.i.</td>
</tr>
<tr>
<td>pPVA-B11</td>
<td>2887b</td>
<td>0</td>
</tr>
<tr>
<td>pUFL</td>
<td>3528a</td>
<td>3973</td>
</tr>
<tr>
<td>pBUI</td>
<td>2272b</td>
<td>1153</td>
</tr>
<tr>
<td>pBUIII</td>
<td>2631b</td>
<td>3057</td>
</tr>
<tr>
<td>pBUIII</td>
<td>2973b</td>
<td>3508</td>
</tr>
<tr>
<td>pBUI+II</td>
<td>1768b</td>
<td>0</td>
</tr>
<tr>
<td>pBUI+III</td>
<td>2509b</td>
<td>2643</td>
</tr>
<tr>
<td>LSD0.05*</td>
<td>1421</td>
<td>2774</td>
</tr>
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*Least significant difference of means at *P*=0.05.

IL, Inoculated leaves; SL, non-inoculated upper leaves.

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\text{Table 2. Amounts of PVA antigen in two diploid potato lines following inoculation with the infectious cDNA clones pPVA-B11 and pUFL and their chimeric derivatives}
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Plants were inoculated with sap from the systemically infected leaves of *Nicotiana benthamiana* plants initially infected with *in vitro* RNA transcripts of the viral cDNAs. Mean (three plants) amounts of viral coat protein antigen [ng (g leaf)$^{-1}$] are given, as determined by DAS-ELISA using known amounts of purified PVA-B11 virions for comparison. Means followed by a different letter differ from each other compared per time point of measurement.
potato plants systemically and accumulated to very high titres in the upper non-inoculated leaves (Table 2). This result suggests that the new, full-length cDNA of PVA-U (pUFL) will be a useful tool for future studies with potato plants. Furthermore, all the chimeric viruses except pBUI+II infected line v2-134 systemically. In inoculated leaves, titres of the parental viruses and chimeras were similar, with the exception of pBUI+II, whose titres were significantly lower than those of pUFL (P<0.05; Table 2). In systemically infected leaves, viral titres were more variable, showing the highest values for pUFL and pBUIII and the lowest values for pBU and pBUII at 28 days p.i. (Table 2). As expected from the results of Hamalainen et al. (2000), plants of potato line v2-51 were not systemically infected with any virus tested. However, inoculated leaves accumulated high titres of all viruses, except for pBUI+II, which exhibited a significantly lower titre than pBU and pBUIII (P<0.05; Table 2). Inoculated and systemically infected leaves showed no visible symptoms with any virus tested in the two potato lines.

These studies of chimeric constructs of pUFL and pPVA-B11 revealed novel information about the functions of the potyviral genome. In addition to pUFL, the chimeras pBU, pBUIII and pBUIII, each of which contain a different segment of pUFL in the pPVA-B11 background, caused systemic infection in potato line v2-134. This was unexpected because a previous study demonstrated that a virus chimera (B11-UCP), in which the CP-encoding region of pPVA-B11 was substituted for that of PVA-U, did not infect potato plants systemically (Andrejeva et al., 1999). The CP of pUFL is not identical to pPVA-B11 or PVA-U, which may explain why pBUIII, but not B11-UCP, can infect v2-134 systemically. Furthermore, in the previous study, four out of six and two out of four amino acid residues of HC-Pro and VPG, respectively, that differed between PVA-U and pPVA-B11 were substituted to make B11-UCP resemble PVA-U. The new, mutated viral chimera moved systemically but at a relatively slow rate, and accumulated only to low levels in the upper, non-inoculated leaves of potato line v2-134 (Hamalainen et al., 2000). Therefore, the systemic infection we observed with pBU and pBUII in potato is consistent with the previously demonstrated importance of the HC-Pro and 6K2-VPG regions in systemic infection in potato (Hamalainen et al., 2000; Rajamaki & Valkonen, 2002). Taken together, the results suggest that complicated coordinated functions between different parts of the potyviral polyprotein and possibly NTRs are required for a successful infection cycle in the host and that pre-selection of the intermediary chimeras for infectivity in N. benthamiana might have been important for finding sequence variants in which the different parts of the chimeric viral genome could carry out the required coordinated functions.

The virulence of a given chimera could not be predicted from the virulence of the parental chimeric constructs. For example, the chimera pBU+II displayed very poor virulence in N. benthamiana, even though the chimeras containing the individual segments of pUFL (pBU and pBUIII) and pUFL itself exhibited high virulence in N. benthamiana (Table 1, Fig. 2). Similarly, pBU+II showed poor virulence in potato, in contrast to pBU, pBUII and pUFL (Table 2). Moreover, chimera pBU showed low accumulation and mild symptoms in N. benthamiana, whereas pBUIII and pBU caused more severe symptoms in N. benthamiana plants than the parental viruses. These findings once again emphasize that virulence depends on the presence of compatible, coordinated functions between different parts of the PVA genome. Viral component incompatibilities and virulence defects exemplified by the chimeras pBU, pBU+III and pBU+II have not previously been reported in potyvirus studies. Furthermore, pBU+II displayed unique, novel symptoms in N. benthamiana not observed with the parental viruses or any of the other chimeras. In the pBU+II and pBU+III constructs, the 3′-proximal or the 5′-proximal genomic segment, respectively, is derived from pPVA-B11, whereas the remainder of the genome is derived from pUFL. In pBU, the 5′-proximal segment is derived from pUFL and the rest of the genome from pPVA-B11. Thus, the results may be explained by disrupted interactions between different parts of the polyprotein or between mature proteins produced from distant parts of the polyprotein. For example, interactions have been demonstrated between HC-Pro (genomic segment I in this study; Fig. 1) and CP (segment III) (Blanc et al., 1997; Roudet-Tavert et al., 2002), HC-Pro and VPG (segment II) (Yambao et al., 2003) and suggested to occur between CI (segment II) and CP (Rodriguez-Cerezo et al., 1997). These proteins mediate potyviral movement functions (Dolja et al., 1994; Cronin et al., 1995; Schaad et al., 1997; Carrington et al., 1998; Rajamaki & Valkonen, 1999) and are also involved in viral propagation (Kekarainen et al., 2002). Furthermore, interactions between VPG and the 5′ end or the 3′ end of viral RNA (reviewed by Rajamaki et al., 2004) could differ between pUFL and pPVA-B11. Our data suggest that there are complex coordinated functions encoded by different regions of the potyviral genome and that these functions may be affected by subtle mutations in viral RNA and/or proteins. Since different virus chimeras showed defective functions in a host-specific manner, it is also possible that host factors may play a role in the coordinated virus-encoded functions. These findings demonstrate that there are numerous constraints encountered by potyviruses during their evolution.

No studies prior to this have demonstrated creation of novel phenotypes (virus strains) following recombination of phenotypically similar, nearly identical potyviral isolates. In some studies, the symptoms induced by the final
infectious potyviral cDNA, but not the chimeric intermediates required for cloning, have been described (e.g. Puurand et al., 1996; Nicolas et al., 1997; Yang et al., 1998). A few reports describe symptoms of chimeric potyviruses in susceptible hosts. These studies have aimed to reveal which genomic region(s) of the virus determines the observed differences in symptoms/virulence between virus strains isolated from plants infected in the field (Chu et al., 1997; Saeñz et al., 2000; Ullah & Grumer, 2002; Jenner et al., 2003). Additionally, most studies have focused on the discovery of avirulence determinants in specific regions of the potyviral genome by exchanging genomic segments between an avirulent virus strain and a strain that can overcome resistance (e.g. Andersen & Johansen, 1998; Keller et al., 1998; Revers et al., 2003, and references therein). This reverse genetics approach for identifying the determinants of symptoms, virulence or avirulence shows which genetic differences are important to the observed, pre-existing phenotypic differences between virus strains, but does not reveal how the genetic changes have occurred. Our current study differs from these previous studies in that it shows that new virus strains (sensa Matthews, 1991) with novel symptom and virulence phenotypes in a host (N. benthamiana) can be obtained following recombination of two nearly identical PVA genomes that do not differ in their symptoms and virulence in this host. Taken together, these results demonstrate a mechanism by which genetic variation may be converted to phenotypic (strain) diversity in potyviruses.

Recombination between homologous viral genomes is considered to be common during natural co-infection of viruses in plants (Garcia-Arenal et al., 2001). Revers et al. (1996) analysed the CP-encoding region in 29 isolates of PVY and found evidence of frequent recombination. Also, recombination events were detected within the P1- or CP-encoding regions in approximately one in ten isolates of TuMV analysed (Oshshima et al., 2002). Homologous recombination between potyviral sequences also occurs in experimental situations where different viruses containing homologous regions of the genome replicate in the same cells (Varelmann et al., 2000). Our results with the chimera pBUI+II show that recombination between two nearly identical viral genomes can result in a new virus strain exhibiting novel symptoms and high virulence in one host but diminished virulence in another host. These features are typical of virus strains isolated from the field, including those of the previously described PVA strains (Rajamäki et al., 1998). Our results also emphasize the importance of coordinated functions between different parts of the potyviral genome. In conclusion, we propose that these findings provide new insights into the origins of the continuous genotypic and phenotypic variability observed with potyviruses.

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