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Stability of recombinant plant viruses containing genes of unrelated plant viruses

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The stability of hybrid plant viruses that might arise by recombination in transgenic plants was examined using hybrid viruses derived from the viral expression vectors potato virus X (PVX) and tobacco rattle virus (TRV). The potato virus Y (PVY) NIb and HCPro open reading frames (ORFs) were introduced into PVX to generate PVX-NIb and PVX-HCPro, while the PVY NIb ORF was introduced into a vector derived from TRV RNA2 to generate TRV-NIb. All three viruses were unstable and most of the progeny viruses had lost the inserted sequences between 2 and 4 weeks post-inoculation. There was some variation in the rate of loss of part or all of the inserted sequence and the number of plants containing the deleted viruses, depending on the sequence, the host (*Nicotiana tabacum* vs *Nicotiana benthamiana*) or the vector, although none of these factors was associated consistently with the preferential loss of the inserted sequences. PVX-NIb was unable to accumulate in NIb-transgenic tobacco resistant to infection by PVY and also showed loss of the NIb insert from PVX-NIb in some NIb-transgenic tobacco plants susceptible to infection by PVY. These data indicate that such hybrid viruses, formed in resistant transgenic plants from a transgene and an unrelated virus, would be at a selective disadvantage, first by being targeted by the resistance mechanism and second by not being competitive with the parental virus.

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INTRODUCTION

One of the early successful applications of biotechnology has been the use of transgenic plants expressing plant viral sequences to protect those plants from infection and/or disease caused by the same plant virus (Goldbach *et al.*, 2003; Prins, 2003). However, the use of transgenic plants expressing plant viral sequences has raised a number of environmental concerns regarding various interactions. These include *trans*-encapsidation (when viral capsid protein genes were employed), synergy of disease between the transgene product and a non-target virus (i.e. a virus unrelated to the viral source of the transgene) and recombination between the transgene mRNA and non-target viruses (Palukaitis, 1991; Palukaitis & Kaplan, 1997; Tepfer, 2002). Recombination has been observed between expressed viral transgenes and the same viruses used as the source of the transgene (Adair & Kearney, 2000; Borja *et al.*, 1999; Greene & Allison, 1994, 1996; Schoelz & Wintermantel, 1993; Varrelmann *et al.*, 2000; Wintermantel & Schoelz, 1996), as well as between two related viruses (i.e. in the same genus) co-infecting plants (Aaziz & Tepfer, 1999; Masuta *et al.*, 1998; White & Morris, 1994). Recombination has also been used to explain the presence of plant viral sequences in plant genomes (Bejarano *et al.*, 1996; Harper *et al.*, 1999; Jakowitsch *et al.*, 1999; Tanne & Sela, 2005). Similarly, sequence analyses indicate that recombination had occurred between closely related viruses (Chare & Holmes, 2006), especially potyviruses (Bousalem *et al.*, 2000; Cervera *et al.*, 1993; Desbiez & Lecoq, 2004; Krause-Sakate *et al.*, 2004; Revers *et al.*, 1996; Tan *et al.*, 2004), in some cases altering the virulence of the virus (Glais *et al.*, 2002; Larsen *et al.*, 2005; Zhong *et al.*, 2005). However, to date, no recombination has been demonstrated experimentally between completely unrelated viruses (i.e. in different families), or between a viral-derived transgene and an unrelated virus. Concerns have been raised concerning possible consequences of such recombination events, giving rise to novel viruses with potentially altered host range (reviewed by Tepfer, 2002). Given the precision of recombination required to generate such novel viruses (i.e. integration into a region of the virus genome that would allow expression of the donor gene without affecting either the replication of the acceptor virus or expression of the other genes in the acceptor virus), it may be rather difficult to detect such recombinant viruses. Therefore, as an approach to examine the consequences of recombination between a virus and a transgene derived from an unrelated virus, three hybrid viruses were generated *in vitro*, using viral genes previously used as transgenes and plant viruses

Supplementary figures are available in JGV Online.

previously utilized as gene expression vectors. These hybrid viruses were then assessed for their stability in two host species.

METHODS

Plants and inocula. Tobacco (*Nicotiana tabacum* 'Samsun' NN) and *Nicotiana benthamiana* were inoculated with the following viruses: an empty gene expression vector derived from *Potato virus X* (PVX), PVX containing an insert encoding the HCPro open reading frame (ORF) of *Potato virus Y* (PVY) (PXV-HCPro), PVX containing an insert encoding the PVY NIb ORF (PVX-NIb), *Tobacco rattle virus* (TRV) and a vector derived from TRV expressing the PVY NIb ORF (TRV-NIb). Tobacco plants (*N. tabacum* 'Samsun' NN) transgenic for expression of the *NIb* gene of PVY and either resistant or susceptible to infection by PVY (Audy *et al.*, 1994) were also inoculated with TRV-NIb. The TRV inocula consisted of a mixture of transcript derived from cDNA clones of RNA2 of either wild-type (wt) TRV or TRV-NIb mixed with total RNAs from plants previously infected with TRV RNA1, as described by Mueller *et al.* (1997). The other inocula consisted of either RNA transcripts obtained by *in vitro* transcription of the PVX derivative plasmids, or buffered extracts from plants infected with transcripts 3 days post-inoculation (p.i.), for experiments involving TRV and TRV-NIb (Canto & Palukaitis, 2001, 2002). The various *in vitro* transcripts were prepared using an mMESSAGE mMACHINE T7 transcription kit (Ambion) as previously described (Canto & Palukaitis, 2001; Canto *et al.*, 2004).

Plasmids and constructs

Generation of TRV-NIb. The PVY sequence containing the NIb ORF (nt 7010–8566) was amplified by PCR from a full-length cDNA clone of a PVY^O isolate obtained from the Scottish Crop Research Institute collection (unpublished data). The upstream PCR primer (5′-CATGTTAAC**ATG**GCTAAGCACTCTGCGTGG-3′) contained an *Hpa*I site (underlined) and an added ATG initiation codon (bold). The downstream PCR primer (5′-CATTCTAGATTGATGGTGTACTTCATAAG-3′) contained an *Xba*I site (underlined). The PCR fragment was digested with *Hpa*I and *Xba*I and was ligated into the vector TRV-AtALY2-GFP (Uhrig *et al.*, 2004), previously cut with the same two restriction enzymes. The resulting clone, TRV-NIb/GFP, expressed the *NIb* gene as a fusion to the gene encoding the green fluorescent protein (GFP). To remove the GFP-encoding sequence, the *NIb* gene was amplified again by PCR using the above upstream primer and 5′-CATCTCGAGGTAC**CTA**TTGATGGTGTACTTCATAA-3′ as the downstream primer, containing an added termination codon (bold) and a *Kpn*I site (underlined). This PCR fragment was digested with *Sac*I (internal to the NIb sequence) and *Kpn*I, and ligated into TRV-NIb/GFP previously digested with the same enzymes, generating the construct TRV-NIb.

Generation of PVX-NIb. The *NIb* gene was amplified from TRV-NIb/GFP by PCR using 5′-ATGCGGCCG**ATG**GCTAAGCACTCTGCGTGG-3′ as the upstream primer, containing an *Eag*I site (underlined) and an added ATG codon (bold), and 5′-CATGATA**TCA**TTGATGGTGTACTTCATAAGAG-3′ as the downstream primer, containing an added termination codon (bold) and an *Eco*RV site (underlined). The PCR fragment was digested with *Eag*I and *Eco*RV and was ligated into the PVX vector pTX.P3C2 402 (Baulcombe *et al.*, 1995) previously digested with the same restriction enzymes, generating the construct PVX-NIb.

Generation of PVX-HCPro. The PVY sequence containing the HCPro ORF (nt 965–2407) was amplified by PCR from the above full-length cDNA clone of PVY^O. The upstream PCR primer (5′-ATCCGGCCG-**ATG**TCGAATGCTGACAATTTTTGG-3′) contained an *Eag*I site (underlined) and an added ATG starting codon (bold). The downstream primer (5′-AGCTGCAGCCCGGG**TTA**ACCAACTCTATAATG-3′) contained an added stop codon (bold) and a *Pst*I site (underlined). The PCR fragment was digested with *Eag*I and *Pst*I and was ligated into the PVX vector pTX.P3C2 402 previously digested with *Eag*I and *Nsi*I, to generate the construct PVX-HCPro.

Preparation and analysis of plant nucleic acid samples. Total RNAs from either mock-inoculated or virus-infected plants were isolated using a buffer–phenol/chloroform extraction method (Canto & Palukaitis, 1998). RNAs were fractionated by agarose gel electrophoresis, blotted to nitrocellulose membranes and hybridized with the specified digoxigenin-labelled RNA probes for TRV RNA2 and PVX RNA, all as described previously (Canto & Palukaitis, 2001; Canto *et al.*, 2004). The TRV probe was specific to the sequences encoding the capsid protein (CP) in TRV RNA2, while the PVX probe was specific to the 3′-terminal sequences.

The total plant RNAs extracted were used for RT-PCR with primers TRV-3′ (5′-CGAGAATGTCAATCTCGTAGG-3′) or PVX-3′ (5′-TGTACTAAAGAAATCCCCATCC-3′) for the RT step in a 13 µl reaction done at 37 °C for 60 min. The PCR (30 µl) was done using 5 µl RT reaction products and either the primers TRV-5′ (5′-CTGGGAGATGATACGCTG-3′) plus TRV-3′ or the primers PVX-5′ (5′-ATCACAGTGTTGGCTTGC-3′) plus PVX-3′ in a reaction mixture recommended by the supplier of the *Taq* DNA polymerase (Promega). Amplification was done by an initial incubation at 94 °C for 2 min, followed by five cycles of incubation at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min 10 s, followed by 25 cycles at 93 °C for 20 s, 59 °C for 20 s and 72 °C for 2 min. The RT-PCR products were analysed by agarose gel electrophoresis and staining with ethidium bromide.

RESULTS

Description of the system

Expression of PVY ORFs encoding either HCPro, a suppressor of RNA silencing, or NIb, the polymerase component of the viral replicase, has been shown to enhance the pathogenicity of PVX in *N. benthamiana* (Brigneti *et al.*, 1998). Therefore, it was considered likely that expression of these sequences might convey some advantage to the virus vector. As HCPro and NIb of potyviruses are translated as a polyprotein that is cleaved into 10 proteins (Urcuqui-Inchima *et al.*, 2001), expression of these sequences from other viruses requires prior terminal modification of the coding sequences. Therefore, ORFs with initiator and terminator codons were constructed from the HCPro and NIb coding sequences of PVY and were inserted into the multiple cloning site of a PVX vector, to generate PVX-HCPro and PVX-NIb (Fig. 1). In addition, to examine the effect of expression of one of these genes from a different virus vector system, the PVY NIb ORF was inserted into RNA2 of a TRV vector, to generate TRV-NIb (Fig. 1). These hybrid viruses were first examined for their stability during infection over a 3 week (*N. benthamiana*) or 4 week (tobacco) period; the different time periods for sampling the two hosts reflect the different rates of systemic infection by the recombinant viruses. Subsequently, the TRV-NIb

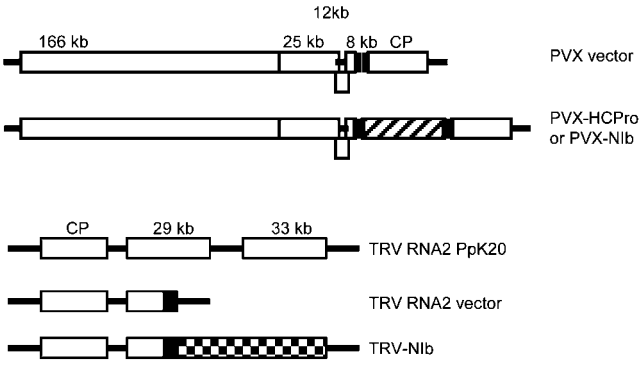

Fig. 1. Viral vector constructs derived from PVX or TRV RNA2. The inserted sequences (HCPro or NIb) are shown as fill patterns. Subgenomic promoters required for expression of inserted genes are indicated by thick, black vertical bars. TRV RNA2 PpK20, wt TRV RNA2.

hybrid virus was examined for its ability to infect PVY NIb-transgenic tobacco plants resistant to infection by PVY. The wt TRV RNA2 of strain PpK20 (Hernández *et al.*, 1995) was chosen rather than the empty TRV RNA2 vector, since wt TRV RNA2 is similar in size to TRV-NIb (Fig. 1). This is because the TRV RNA2 vector has a deletion of the complete *2c* and most of the *2b* genes, which are not required for replication or movement, as well as an insertion of a duplicated CP subgenomic promoter from the related tobravirus *Pea early browning virus* (MacFarlane & Popovich, 2000).

Accumulation and stability of PVX-HCPro and PVX-NIb

Both PVX-HCPro and PVX-NIb could systemically infect and accumulate in tobacco (Fig. 2a) and in *N. benthamiana* (Fig. 2b). However, at all three time points p.i., the levels of accumulation of the hybrid viruses were lower than for the parental virus (the empty PVX vector), regardless of the host species. Furthermore, differences in the size of the genomic RNA were apparent in some samples, and the number of samples with such shorter RNAs increased with time p.i. (Fig. 2). This was true in both species. Although the appearance of smaller-sized RNAs in tobacco occurred at 2 weeks p.i. in one out of four plants infected by either PVX-NIb or PVX-HCPro, in subsequent time samples, the smaller-sized, full-length RNA appeared in more of the samples infected with PVX expressing the *HCPro* gene than in those infected with PVX expressing the *NIb* gene (Figs 2a). That is, in tobacco at 3 weeks p.i., none of the four PVX-NIb samples contained the shorter, wt-sized (6.5 kb) RNA, while at 4 weeks p.i., two of the four samples contained the shorter genomic RNA. By contrast, at 3 weeks p.i., two of the four PVX-HCPro samples contained a mixture of both RNAs and one contained only the shorter RNA, while at 4 weeks p.i., all four samples contained only the shorter RNA. These data suggest that the hybrid viruses

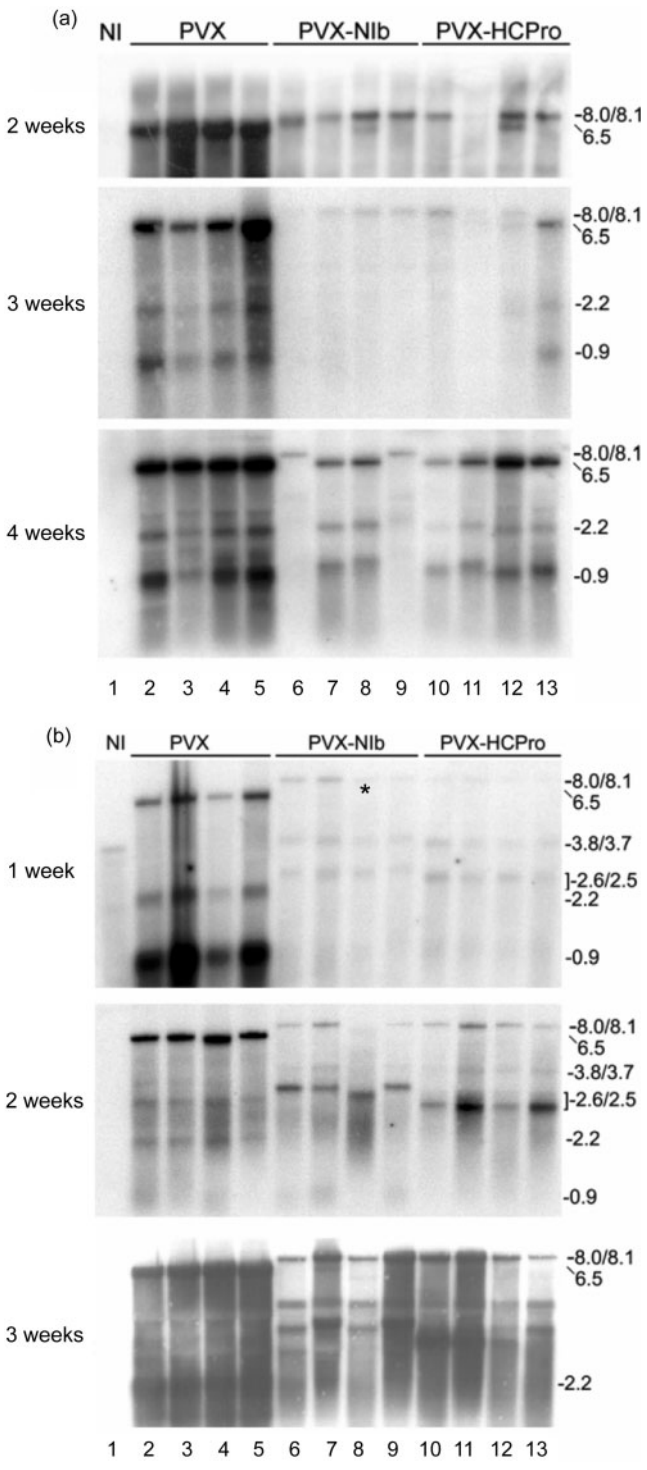

Fig. 2. Northern blot analysis of viral RNA accumulation in (a) tobacco and (b) *N. benthamiana*. Four plants of each species were inoculated with PVX (lanes 2–5), PVX-NIb (lanes 6–9) or PVX-HCPro (lanes 10–13) and systemically infected leaves were extracted at the times indicated p.i. NI, Non-inoculated plant. Size markers: the positions of the PVX genomic (6.5 kb) and subgenomic (2.2 and 0.9 kb) RNAs, as well as both the PVX-NIb genomic (8.1 kb) and subgenomic (3.8 and 2.6 kb) RNAs and the PVX-HCPro genomic (8.0 kb) and subgenomic (3.7 and 2.5 kb) RNAs are indicated. The loading controls are shown in Supplementary Fig. S2 (available in JGV Online).

are not very stable during propagation in tobacco and viruses with smaller or no inserts are preferentially selected. However, the situation in *N. benthamiana* was less clear. In this species, although smaller-sized RNAs appeared, which were different in size from the expected subgenomic RNAs of the hybrid viruses (3.8, 3.7, 2.6 and 2.5 kb), an RNA larger in size (8.0 or 8.1 kb) than the empty PVX vector (6.5 kb) was still present in all but one sample as seen in Fig. 2(b), suggesting that selection of smaller-sized RNA species may not be as strong or rapid in this host. However, this conclusion was not supported by subsequent analysis (see below).

The above data do not really allow a determination of the extent to which the original insert remained in the hybrid viruses, or whether there were multiple co-existing viral species present with different-sized inserts. To determine the size of the inserts and whether single or multiple species existed in the various samples, the same RNA samples extracted at the same time points used for the Northern blots described above were subjected to analysis by RT-PCR. This technique provided a clearer picture of what was happening to the insert within the hybrid virus.

In tobacco, it was apparent that at 2 weeks p.i., very little of the RNA population in the plants infected with PVX-NIb had undergone deletion of the 1581 bp insert (Fig. 3a, lanes 9–12). At 3 weeks p.i, while there was some increase in the number and abundance of minor PCR products, all of the samples from plants infected by PVX-NIb yielded a PCR product corresponding to the full-length insert plus flanking sequences (1882 bp). However, by 4 weeks p.i., only two of the four samples still retained the band reflecting the full-length insert, while the other two samples contained inserts of smaller size, although still larger than the 301 bp product generated from the empty vector (Fig. 3a, lanes 9–12). By contrast, as in the Northern blot (Fig. 2a), only one of the four samples taken at 2 weeks p.i. from plants infected with PVX-HCPro consisted mostly of virus with the 1455 bp HCPro insert deleted, although this sample also still contained virus with little or no deletion present in a fraction of the RNA population (Fig. 3a, lane 15). By 3 weeks p.i., only one of the four samples contained predominantly virus with the 1455 bp insert plus 295 bp flanking sequences expected of the PVX-HCPro inoculated (Fig. 3a, lane 13), while at 4 weeks p.i., none of the samples contained only PVX-HCPro with the full-length insert plus flanking sequences (Fig. 3a).

In *N. benthamiana*, virus containing the full-length inserts in both PVX-HCPro and PVX-NIb predominated in samples taken at 1 and 2 weeks p.i., with only low levels of smaller sized inserts, indicating the presence of deleted forms of the virus, visible at these times (Fig. 3b, lanes 9–16). However, by 3 weeks p.i., plants infected with either hybrid virus showed the presence of multiple deleted forms (Fig. 3b, lanes 9–16). In an independent experiment (Supplementary Fig. S1, available in JGV Online), both PVX-NIb and PVX-HCPro also showed the presence of some deleted forms at 1 and 2 weeks p.i, with no full-length form detectable at 3 weeks p.i. Here, in contrast to the situation in tobacco, PVX-HCPro did not appear to be either more or less stable than PVX-NIb (Figs 2a vs 2b and 3a vs 3b). The absence of any PCR product in one of the PVX-NIb samples taken at 2 weeks p.i. (Fig. 3b, lane 11) is consistent with the smaller-sized genomic RNA seen in this sample on a Northern blot (Fig. 2b, lane 8, asterisk), and may represent a sample in which one of the PCR priming sites was lost. However, it also appears that a population of molecules with some inserts remaining still existed in this plant, as the sample taken 1 week later from a different leaf of the same plant contained PVX-NIb with several intermediate-sized inserts (Fig. 3b, lane 11) as well as the larger-sized genomic RNA species (Fig. 2b, lane 8).

Thus, the data showed that while the levels of virus accumulation in plants infected by PVX-HCPro and PVX-NIb were lower than for the empty PVX vector, the inserts were lost to varying extents depending on the host and, in the case of tobacco, also depending on the nature of the insert sequence. Moreover, the extent to which sequences were lost and the degree of co-existence of viruses with larger inserts and no insert varied in different plants, as well as in different leaves of the same plant and, to some degree, in different experiments.

Accumulation and stability of TRV-NIb

Infection of tobacco with TRV-NIb vs TRV and assessment of the accumulation of TRV RNA2 showed that all of the plants were infected and that the level of accumulation of TRV-NIb at 2 weeks p.i. was not substantially different from that of wt TRV RNA2 (Fig. 4a). There was considerable variation in the level of accumulation of both RNA2s in different plants and in samples taken at different times (Fig. 4a). However, the presence of smaller, abundant PCR products in the samples taken from plants infected by TRV-NIb suggested that the full-length insert was not maintained stably in tobacco. This was verified by an RT-PCR analysis of the same samples (Fig. 4b). Here again, some samples did not yield detectable PCR products, suggesting that one of the primer sites may have been lost due to the deletion. While a mixed population of viruses with full-length (2046 bp) and smaller inserts were present at 2 weeks p.i., the majority of the virus population appeared to have lost most or all of their inserts at 3 and 4 weeks p.i. (Figs 4a, b).

In *N. benthamiana*, TRV-NIb also showed a similar range of accumulation to wt TRV RNA2, but, in contrast to the situation in tobacco, TRV-NIb appeared to retain the insert in more of the population and in more of the samples than was observed in tobacco (Fig. 5a). This was confirmed by the RT-PCR analysis (Fig. 5b). It does not appear from the RT-PCR analyses that, in general, inserts of larger size were retained longer in *N. benthamiana* than in tobacco (Fig. 5b vs Fig. 4b).

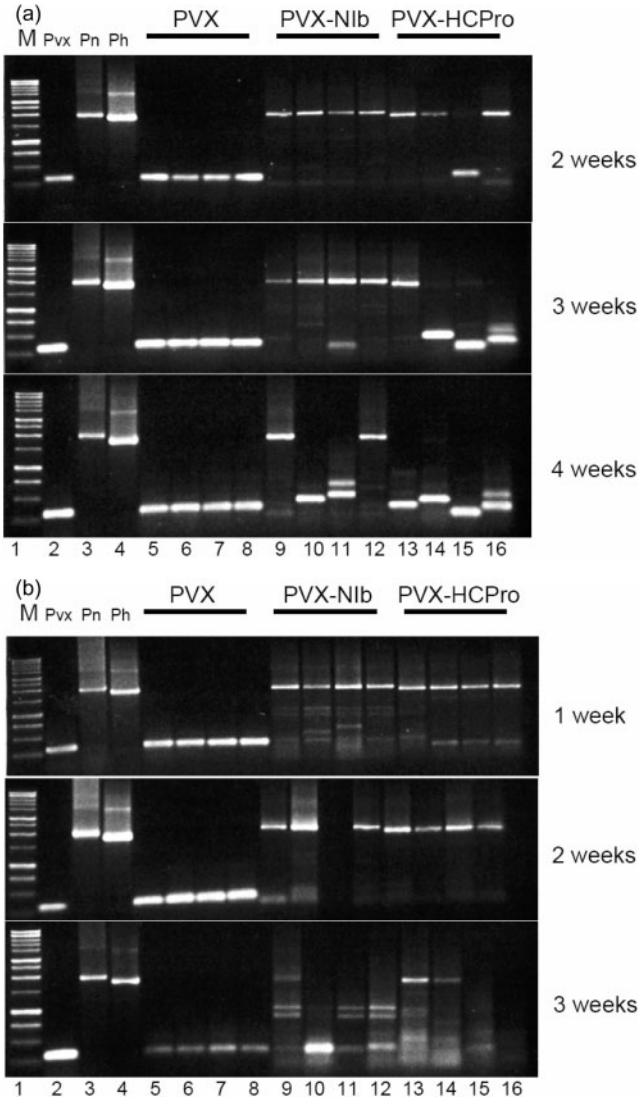

Fig. 3. RT-PCR analysis of insert stability in (a) tobacco and (b) *N. benthamiana*. Four plants of each species were inoculated with PVX, PVX-NIb or PVX-HCPro and systemically infected leaves were extracted at the times indicated p.i. RT-PCR was done using primers flanking the site of insertion. PCR on the plasmids was done with the empty PVX vector (Pvx), PVX-NIb (Pn) and PVX-HCPro (Ph). M, Molecular mass markers (the first six DNA bands in ascending order were 250, 500, 750, 1000, 1500 and 2000 bp).

Accumulation of TRV-NIb in NIb-transgenic tobacco plants

If hybrid viruses are generated by recombination involving non-target viruses infecting transgenic plants resistant to unrelated viruses, one could then expect the recombinant hybrid viruses to become sensitive to the resistance against the (transgene source) target viruses. This situation should create a strong selection pressure for hybrid viruses which have lost most or all of the incorporated transgene sequences. To determine whether this was the case, wt TRV and TRV-NIb were inoculated to tobacco plants of the same cultivar as those used above, but transgenic for expression of the PVY *NIb* gene (Audy *et al.*, 1994) and either susceptible or resistant to infection by PVY. No systemic infection was observed in the NIb-transgenic tobacco resistant to PVY (not shown). Therefore, inoculated leaves were sampled for accumulation of TRV at 4 days p.i. The wt TRV accumulated in non-transgenic tobacco and NIb-transgenic tobacco susceptible or resistant to PVY (Fig. 6a). There was some variation in the levels of accumulation between different plants of the same genotype treated with the same inoculum, but the intact genomic RNA2 was the major form of the TRV RNA2 present (Fig. 6a). Similarly, TRV-NIb inoculated to non-transgenic tobacco showed equivalent levels of accumulation of the genomic TRV RNA2, also with some variation in different plants of the same genotype (Fig. 6a). However, TRV-NIb inoculated to NIb-transgenic tobacco susceptible or resistant to PVY showed a different pattern of accumulation. In the PVY-susceptible NIb-transgenic plants, TRV-NIb accumulated to either similar or much lower levels than in the non-transgenic plants, with only the former plants retaining the full-length TRV-NIb as the major species (Fig. 6a). By contrast, in the PVY-resistant NIb-transgenic plants, TRV RNA2 with the full-length NIb insert did not accumulate and only deletion variants of the TRV-NIb RNA2 accumulated. In several plants, no TRV-NIb RNA2 was detectable (Fig. 6a and data not shown). These conclusions were confirmed by RT-PCR analysis of the same samples (Fig. 6b and data not shown). Therefore, as expected, the loss of the NIb sequence was necessary for accumulation of TRV RNA2 in the PVY-resistant NIb-transgenic plants, and the resistance mechanism increased the rate of selection of the deleted variants, which were then detectable in a few days rather than weeks.

DISCUSSION

There are several studies showing that hybrid viruses formed between unrelated viruses were more pathogenic than their parental viruses (Burgyán *et al.*, 2000; Canto *et al.*, 2004; Li *et al.*, 1999; Pruss *et al.*, 1997; Scholthof *et al.*, 1995; Thomas *et al.*, 2003). In many cases, the hybrid viruses induced necrosis in some hosts and, in one case, the hybrid virus induced an avirulent response limiting infection by that virus (Li *et al.*, 1999). However, these viruses were not assessed for their competitiveness with their parental viruses. In one case where the hybrid virus [*Tobacco mosaic virus* (TMV) with the capsid protein of *Alfalfa mosaic virus*] was not more pathogenic than either parental virus, infection of a new host species (for TMV) was observed by the hybrid virus, although it was also shown not to be as fit as the parental virus (TMV) during infection of a common host (Spitsin *et al.*, 1999).

Although expression of the PVY HCPro and NIb has been reported to enhance the pathogenicity of PVX (Brigneti *et al.*, 1998), we did not observe such effects consistently in

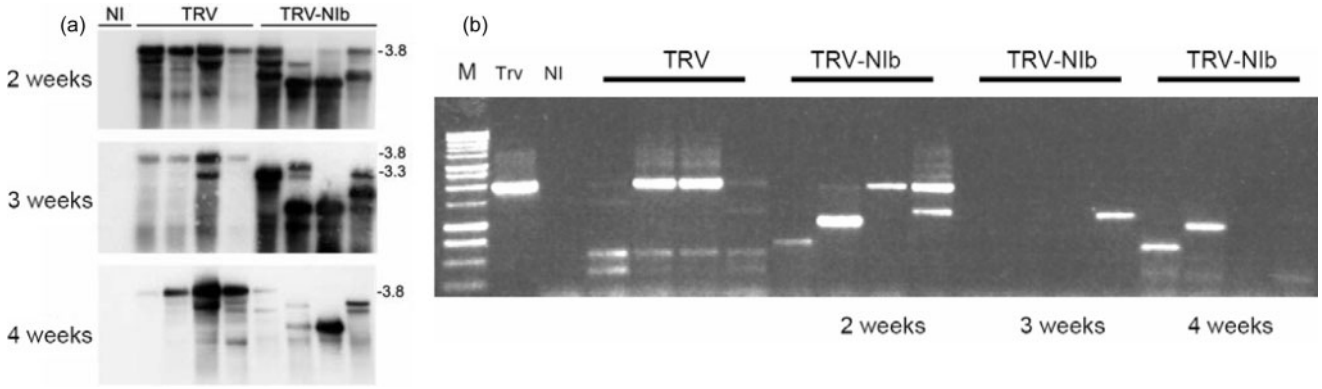

Fig. 4. Replication and stability of TRV-NIb in tobacco. (a) Northern blot analysis of viral RNA accumulation and (b) RT-PCR analysis of insert stability. Four tobacco plants were inoculated with either TRV or TRV-NIb, and nucleic acids were extracted from systemically infected leaves and assayed at the times indicated p.i. NI, Non-inoculated plant; Trv, TRV RNA2 plasmid used for PCR control; M, molecular mass markers. The positions of the 3.8 kb TRV and TRV-NIb genomic RNAs and the 3.3 kb subgenomic RNA are indicated in (a). RNA loading controls for (a) are shown in Supplementary Fig. S3 (available in JGV Online).

this series of experiments. This may have been because our analyses have shown that the HCPro and NIb sequences of PVY were not maintained stably in PVX in either of two hosts, *N. tabacum* or *N. benthamiana*. While some differences were noted in the rate of selection of viruses with most or all of the sequences deleted in one host vs another or depending on the transgene, overall, the effect was the same. That is, the hybrid viruses did not accumulate to as high a level as the empty virus vector and, several weeks p.i., the hybrid viruses had lost most or all of the inserted sequences (Figs 2, 3). A similar result over a similar time frame was observed with PVX expressing a mutant form of the plum pox virus (PPV) HCPro in *N. benthamiana* (Barajas *et al.*, 2006).

In the case of TRV RNA2 expressing the NIb sequence, the hybrid virus accumulated to similar levels to the wt TRV RNA2 and some of the hybrid virus was still present in *N. benthamiana* plants at the last time point tested. Nevertheless, virus with little or no insert accumulated preferentially in both hosts tested (Figs 4, 5). Moreover, in transgenic plants resistant to PVY, mediated by RNA

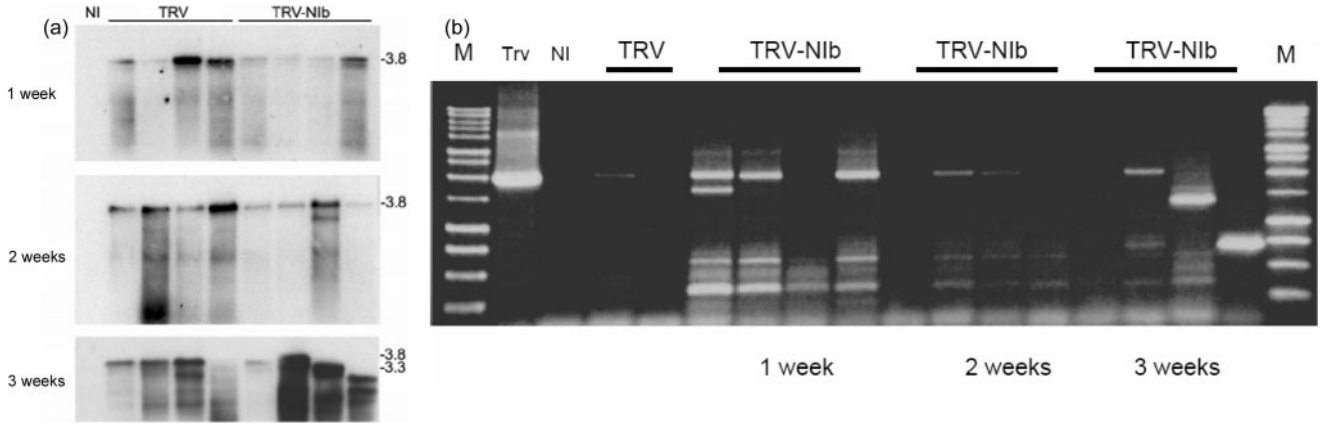

Fig. 5. Replication and stability of TRV-NIb in *N. benthamiana*. (a) Northern blot analysis of viral RNA accumulation and (b) RT-PCR analysis of insert stability. Four *N. benthamiana* plants were inoculated with either TRV or TRV-NIb, and nucleic acids were extracted from systemically infected leaves and assayed at the times indicated p.i. NI, Non-inoculated plant; Trv, TRV RNA2 plasmid used for PCR control; M, molecular mass markers. The positions of the 3.8 kb TRV and TRV-NIb genomic RNAs and the 3.3 kb subgenomic RNA are indicated in (a). RNA loading controls for (a) are shown in Supplementary Fig. S3 (available in JGV Online).

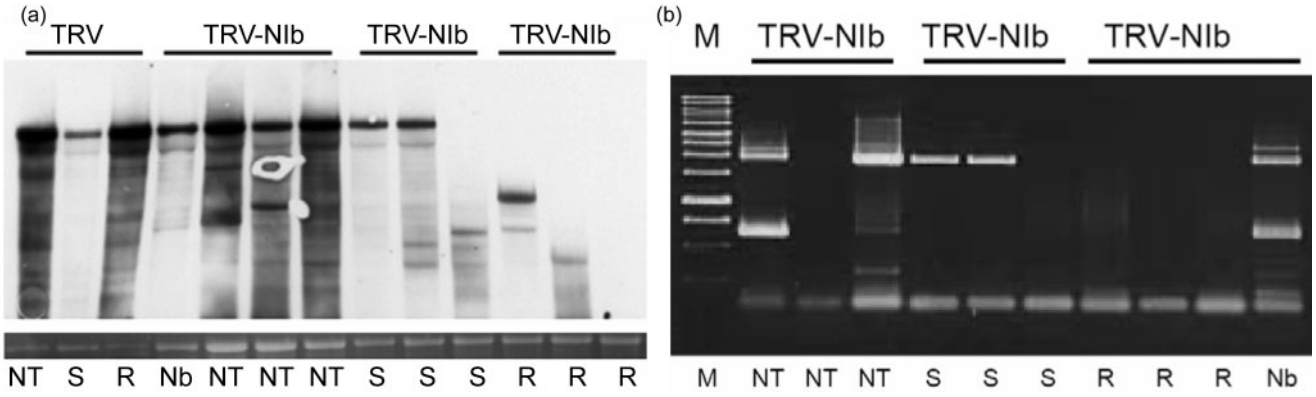

Fig. 6. Accumulation of TRV-NIb in PVY-resistant and -susceptible NIb-transgenic tobacco. TRV and TRV-NIb were inoculated to non-transformed (NT) tobacco plants, as well as to PVY NIb-transgenic tobacco plants resistant (R) or susceptible (S) to infection by PVY. Four days p.i., infected leaves were extracted and analysed by (a) Northern blot hybridization for viral RNA accumulation and (b) RT-PCR for the NIb plus flanking sequences present in the TRV-NIb inoculum. Nb, RNA extracted from TRV-NIb infected *N. benthamiana*; M, molecular mass markers. rRNA loading controls are shown below the blot in (a).

silencing of the *NIb* transgene, TRV-NIb was strongly selected against, with the virus either losing the inserted sequence or not accumulating, as it was now targeted by the resistance against PVY (NIb). Thus, after they are initially formed, it is difficult to see how hybrid viruses could accumulate to detectable levels during competition for further infection with their parental viruses.

The inability of PVX expressing a viral sequence to retain the insert and accumulate in some transgenic plant lines expressing the same viral sequence was also observed in two recent studies (Barajas *et al.*, 2006; Roy *et al.*, 2006). In both of these studies, only PVX with deleted forms of the insert was able to accumulate. In one case, the nature of the partial deletions was examined and found to relate to the regions of the inserts targeted by the small interfering RNAs generated from the transgene (Barajas *et al.*, 2006). Although we did not see such deleted TRV variants arise in the case of the transgenic plants resistant to PVY (Fig. 6), the extent to which different hybrid viruses are able to survive sufficiently to generate such deleted variants seems to depend on the strength of the resistance (Barajas *et al.*, 2006).

There are several conclusions that can be drawn from this and previous studies. (i) Some, if not most, hybrid viruses formed by recombination between a viral transgene and a non-target virus are unlikely to be as fit as the parental, non-target virus, even if they accumulate initially to similar levels. This is supported by several studies showing the instability of some viral vectors expressing non-viral sequences (Chapman *et al.*, 1992; Dolja *et al.*, 1993; Guo *et al.*, 1998; Rabindran & Dawson, 2001). (ii) The size of the insert may be an important factor in the stability of the hybrid virus, since hybrid viruses with shorter remaining inserts persisted longer than the hybrid virus with the full-length insert, although these too were not competitive with the wt virus. This conclusion is similar to those of others using viral vectors to express non-viral sequences (Chapman *et al.*, 1992; Dawson *et al.*, 1989; Dolja *et al.*, 1993; Guo *et al.*, 1998; Rabindran & Dawson, 2001). (iii) While a hybrid virus could in principle be generated by recombination between two co-infecting viruses, this has not been seen to date for unrelated viruses, probably since these novel, hybrid viruses would be present at very low levels and cannot compete efficiently with the parental viruses. (iv) The concern about generation of novel, hybrid viruses by recombination between a viral transgene and a non-target virus can be mitigated to some extent by the observation that the hybrid virus itself is a target of the resistance response. This also was seen in two recent studies in which such hybrid viruses were used to characterize the resistance (Barajas *et al.*, 2006; Roy *et al.*, 2006). Moreover, if the non-target parental virus inhibited RNA silencing-mediated resistance in the infected tissues (which may need to be a pre-requisite for enough transgene mRNA to accumulate for recombination) so that the hybrid virus could accumulate, the latter might then be non-competitive with the non-target parental virus. If somehow the hybrid virus did accumulate and escape to adjacent plants, then it would be sensitive to the RNA silencing-mediated resistance in those plants or, if those plants were pre-infected with the non-target parental virus, the hybrid virus would be subject to cross-protection by that parental virus. Thus, although situations may exist that will favour the hybrid virus, neither of the above circumstances, would facilitate such a hybrid virus becoming established. Data generated using other viral vector/viral inserts will determine how general these conclusions are and whether or not there are exceptions.

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