Integrated pararetroviral sequences define a unique class of dispersed repetitive DNA in plants

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Although integration of viral DNA into host chromosomes occurs regularly in bacteria and animals, there are few reported cases in plants, and these involve insertion at only one or a few sites. Here, we report that pararetrovirus-like sequences have integrated repeatedly into tobacco chromosomes, attaining a copy number of $\sim 10^3$. Insertion apparently occurred by illegitimate recombination. From the sequences of 22 independent insertions recovered from a healthy plant, an 8-kilobase genome encoding a previously uncharacterized pararetrovirus that does not contain an integrase function could be assembled. Preferred boundaries of the viral inserts may correspond to recombinogenic gaps in open circular viral DNA. An unusual feature of the integrated viral sequences is a variable tandem repeat cluster, which might reflect defective genomes that preferentially recombine into plant DNA. The recurrent invasion of pararetroviral DNA into tobacco chromosomes demonstrates that viral sequences can contribute significantly to plant genome evolution.

Materials and Methods

A cloning and Nucleotide Sequence Analysis. Two genomic DNA λ libraries were prepared from Nicotiana tabacum cv. petite havana SR1 DNA digested partially with Sau3AI or completely with EcoRI, respectively, using the λ FIX II (V-clones) or λ ZAP II (E-clones) from Stratagene as described (14). Using a “partial fill-in” strategy, the λ FIX II system is specifically designed to prevent the formation of cloning artifacts. For screening, a cloned 1.0-kilobase (kb) PCR fragment derived from the reverse transcriptase (RT) region of clone V1 was used. The resulting λ clones were subcloned, and nucleotide sequence analysis was performed with a Li-Cor DNA Sequencer Long Read IR 4200 system (Li-Cor, Omaha, NE) using a ThermoSequenase cycle sequencing kit (Amersham Pharmacia) and infrared-labeled oligonucleotides (MWG Biotech, Ebersberg, Germany). Database searches were performed by using the BLAST algorithm (15).

DNA Blot Analysis. Plant genomic DNA used for blot analysis was isolated from leaves of symptomless adult tobacco plants using a Plant DNA Isolation Kit (Roche, Vienna, Austria). DNA gel electrophoresis and transfer to nitrocellulose were done following standard procedures. For DNA slot blots, the MilliBlot-D system (Millipore) was used according to the manufacturer’s instructions. A hybridization probe labeled with 32P was synthesized from an isolated 0.8-kb EcoRI-XhoI fragment of clone V6 (Fig. 1) by using a Multiprime DNA labeling system (Amersham Pharmacia). Hybridization and washing were performed according to Thomashow et al. (16) under moderately stringent conditions [3× standard saline citrate (SSC) at 64°C; 1× SSC = 0.15 M NaCl/0.015 M sodium citrate].

Abbreviations: BSV, banana streak virus; TPV: tobacco pararetrovirus; TPVL: tobacco pararetrovirus-like; kb, kilobase; RT, reverse transcriptase; CsVMV, cassava vein mosaic virus.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AJ238747).

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Northern Blot Analysis. Preparation of poly(A)^+ RNA from tobacco plants and callus tissue and Northern blot analysis were done as described (17). The 32P-labeled DNA probe was synthesized from an isolated DNA fragment extending 5.5 kb from the T7 end of clone V6 (Fig. 1).

Virus Detection. For the attempted preparation of virion DNA from tobacco, the procedure described by Covey et al. (18) was performed following exactly the authors’ recommendations. Two-dimensional gel electrophoresis (18) was used to search for circular forms of virus DNA in tobacco DNA prepared by using the CTAB (N-cetyl-N,N,N-trimethyl-ammonium bromide) procedure (19).

Results
Deduced DNA Sequence of a New Tobacco Pararetrovirus. In a study of plant DNA sequences that flank transgene inserts in tobacco (N. tabacum cv. petit havana SR1), we cloned a 1.8-kb DNA fragment that showed sequence homology to the reverse transcriptase gene of several pararetroviruses. In this clone, the homology to viral DNA terminated abruptly at a junction with nonviral sequences that were presumably derived from tobacco, suggesting covalent linkage of plant and viral DNA. Because pararetroviral sequences are not generally believed to integrate into plant nuclear DNA, we searched for additional pararetrovirus–plant DNA junctions using part of the 1.8-kb fragment to probe two different genomic libraries prepared from healthy tobacco plants. From hundreds of positive clones, 22 were chosen for further analysis. All 22 clones contained fragments of tobacco pararetroviral-like DNA (Fig. 1). From these fragments, an 8-kb virus genome could be assembled based on the order of open-reading frames in cassava vein mosaic virus (CsVMV) (20), the pararetrovirus exhibiting the highest sequence similarity to the putative tobacco pararetrovirus (TPV). Fourteen junctions with plant DNA were isolated. Sequence analysis of PCR fragments synthesized across a number of these junctions con-
confirmed that they were indeed present in tobacco nuclear DNA (Fig. 1).

Attempts to isolate free TPV from tobacco plants using standard procedures for caulimovirus isolation and DNA extraction (18) failed to recover observable amounts of virions or the 8-kb viral genomic DNA. Because the viral DNA-containing clones were isolated from symptomless tobacco plants that did not contain detectable quantities of the corresponding virus, they are referred to as tobacco pararetrovirus-like (TPVL) sequences.

**ORFs and Sequence Comparisons.** A consensus DNA sequence of the putative TPV genome was deduced from overlapping portions of the integrated TPVL fragments. The first nucleotide of the tRNA binding site, which serves as a primer for reverse transcriptase (RT), was designated as nucleotide 1, in accordance with other pararetroviral genomes (20). The nucleotide sequence similarity of the 22 integrated TPVL sequences ranged from 91 to 98%, suggesting that they resulted from relatively recent insertion events. These viral sequences were translationally defective, as indicated by the presence of numerous frameshifts and stop codons, as well as several rearrangements of viral sequences (see below). Some of the observed sequence variation among the integrated TPVL sequences resulted from in-frame deletions that comprised 3, 6, 12, or 15 bp, suggesting that multiple strains of the putative TPV were involved.

The level of amino acid identity between TPV and CsVMV ranges from 43% for the RT gene encoded by ORF3 to 21% for ORF1, which encodes a putative coat protein (Fig. 2). The arrangement of ORFs in the tobacco virus is similar to CsVMV, particularly with respect to the order of coat protein/movement protein/polyprotein genes, which is different in all other plant pararetroviruses (20). Relative to CsVMV, the movement protein of TPV is in a frameshift, and the short ORF2 of CsVMV is missing entirely in TPV (Fig. 2). Both CsVMV and TPV have a recognizable ORF5. Three short TPV ORFs of unknown significance in the putative leader after ORF4 are unlabelled. Such short ORFs are present in the leader regions of other pararetroviruses (21). The percent identity on the amino acid level between TPV and CsVMV is shown below the TPV ORFs. Abbreviations: CP, coat protein; RB, RNA binding site; MD, movement domain; MP, movement protein; POL, polyprotein; PR, protease; RH, RNase H; IBP, inclusion body protein; TAV, transactivation protein.

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**Fig. 2.** Comparison of the genomic organization of cassava vein mosaic virus (CsVMV) (20) and the putative tobacco pararetrovirus (TPV). The putative TPV genome, which was assembled from cloned TPLV sequences, did not contain a recognizable ORF5. Three short TPV ORFs of unknown significance in the putative leader after ORF4 are unlabelled. Such short ORFs are present in the leader regions of other pararetroviruses (21). The percent identity on the amino acid level between TPV and CsVMV is shown below the TPV ORFs. Abbreviations: CP, coat protein; RB, RNA binding site; MD, movement domain; MP, movement protein; POL, polyprotein; PR, protease; RH, RNase H; IBP, inclusion body protein; TAV, transactivation protein.

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**Fig. 3.** Variations in the tandem repeat in the putative TPV leader region beyond ORF4. The TPLV sequence clones indicated to the right contained this tandem repeat block (Fig. 1). The 63-bp monomer comprises internal inverted and direct repeats (arrows, top). Length heterogeneity of the 63-bp monomer involves specific sequences (bold) that could form RNA stem-loop structures. Extensions of the 63-bp monomeric unit creating a 76-bp unit involve sequences present in the large loop of a possible RNA hairpin (bold, dotted underline). Partial copies in V4 and V9 consist of sequences in the stem and loop of a possible large hairpin (bold; dotted and heavy underline). Internal deletions (boxed regions; V9, V14) involve sequences in a second putative small stem-loop region (bold and boxed). In V11, the third monomer copy is partial because of the end of clone.

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**TPVL Sequence Junctions with Tobacco DNA.** One complicated and thirteen simple junctions between TPVL sequences and plant DNA were recovered and sequenced. The simple fusions consisted of TPVL sequences leading directly into plant DNA. The complicated junction comprised a 466-bp plant DNA fragment inserted into TPVL sequences that were contiguous apart from a 14-bp duplication at the insertion site (V10). The TPVL sequences at the junctions with tobacco DNA were not randomly distributed across the putative TPV genome (Fig. 1, top arrows). Of the 14 junctions, 7 involved sequences close to the beginning (V11, V14) or end (V3, V7, V9, V10, V14, E22) (Fig. 1).
found adjacent to viral sequences in clone V1. Plant DNA homologous to the 3′ noncoding region of an *N. sylvestris* *Lhcb* gene (GenBank accession no. AB012638) was integrated as a 466-bp filler comprising the complex junction in clone V10. Plant DNA to the left of TPV DNA in clone V8 showed 76% amino acid similarity to the reverse transcriptase region of a gypsy-like retrotransposon of pineapple (23).

**Virus–Virus Junctions and Rearrangements.** Junctions and rearrangements comprising TPVL sequences were present in several clones. These included an inverted repeat (V4); an internal duplication (V7); a short triplication (V14); and a duplication/inversion (E21 vs. V3). Most of these rearrangements of TPVL virus–virus junctions were located in the same two regions as the junctions with plant DNA, namely at the beginning and end of the putative TPV genome and in ORF1.

**TLV Transcription and Fluorescence in Situ Hybridization Analysis.** Faint TPVL sequence transcripts were detected in poly(A)+ RNA isolated from tobacco leaves. No increased levels of RNA were observed in regenerating callus tissue derived from stems and roots (data not shown). We were unable to detect TPVL sequences on tobacco metaphase chromosomes by fluorescence in situ hybridization analysis, possibly because of the relatively small size and dispersed nature of the inserts. Integrated geminivirus (8, 10) and BSV sequences (12) that have been detected by fluorescence in situ hybridization have consisted of large, complex inserts 50–150 kb in size.

**Species Distribution of TPVL-Related Sequences.** DNA blot analysis of uncut tobacco DNA demonstrated that TPVL-specific probes hybridized to high molecular weight DNA (Fig. 4A), as would be expected for integrated viral sequences. When DNA was cleaved with *Xba*I, several stronger bands overlaid on a number of weaker bands were observed (Fig. 4B). The predominant strong band at ~3.5 kb presumably reflects an internal *Xba*I fragment cut from full-length TPV inserts (Fig. 1). The background smear of weaker bands probably represents less than full-length inserts or sequence polymorphism at the *Xba*I site. The diploid progenitors of allotetraploid tobacco (*N. sylvestris* and either *Nicotiana tomentosiformis* or *Nicotiana otophora*) also were tested (Fig. 4A and B). Strong signals were observed with DNA isolated from *N. sylvestris*. Considerably weaker signals were obtained with *N. tomentosiformis* and *N. otophora*. Two other solanaceous plants, Datura and tomato, produced positive signals; a third, petunia, was negative. No hybridization of TPVL probes was seen with DNA isolated from a selection of nonsolanaceous plants, including Arabidopsis and pea (data not shown). The copy number in tobacco was estimated by slot blot analysis to be \(10^3\) copies/diplod genome (data not shown).

**Discussion**

Sequences derived from a previously unidentified tobacco pararetrovirus (TPV) have been found by molecular cloning and nucleotide sequence analysis to have integrated repeatedly—apparently by illegitimate recombination—into tobacco nuclear DNA. The integrated tobacco pararetroviral-like (TPVL) sequences are characterized by frame shifts and stop codons as well as rearrangements and complex junctions with plant DNA, indicating that they are not functional copies. Although integrated BSV sequences have recently been detected at a few sites in the banana genome (12, 13), our observations extend these results by showing that viral sequences can accumulate to form a family of moderately repetitive, dispersed repeats in a plant genome. The findings raise a number of questions about how and why these pararetroviral sequences have inserted regularly into tobacco DNA, and the extent to which similar viral sequences have invaded other plant genomes.

The frequency of TPVL sequence integration is extraordinarily high, having resulted in \(10^3\) copies per tobacco diploid genome. Insertion into the host chromosomes is not a normal part of pararetrovirus replication and is not thought to occur regularly on a random basis. Indeed, other integrated pararetroviral sequences, including BSV in banana (12, 13) and hepatitis B (hepadnavirus in human liver (7), are present at only low copy numbers in the respective host chromosomes. Therefore, to attain the copy number observed with TPVL sequences, it is likely that something unusual occurred with the TPV genome to facilitate or promote integration. Moreover, as with geminivirus-related sequences in tobacco (10), integration of TPVL sequences must have taken place in cells that contribute to the germ line. Although we cannot yet account for the frequent insertion of TPV DNA into tobacco chromosomes or describe the mechanism of integration, possible clues can be found in the sequence of plant–virus and virus–virus junctions, as well as unusual and variable features in different TPVL sequence clones. On the basis of this information, we propose that failed gap repair in defective versions of open circular TPV DNA led to enhanced recombination between viral and plant sequences.

Even though integration of TPVL sequences did not take place at a specific nucleotide(s), junctions with tobacco DNA and with other TPVL sequences were clustered at two general sites in the putative TPV genome, suggesting that these regions are particularly recombinogenic. About 43% (6/14) of the TPVL sequence junctions with tobacco DNA were concentrated in 11% of the putative TPV genome in ORF1, and 50% were clustered in 11% of the genome comprising the beginning and end of the putative TPV DNA. Three of four virus–virus junctions or duplications/rearrangements were also concentrated in these regions. These findings are strikingly similar to those made with integrated hepatitis B (hepadnavirus) sequences, in which \(40\%\) of the junctions with host DNA and a number of virus–virus junctions were clustered in a region representing 7% of the viral genome (7). The two preferred
an RNA secondary structure (Fig. 3). Secondary structures the involvement of sequences primarily in stem-loop regions of
Jakowitsch et al. repeats 5). Alterations in monomer length might have been produced by
were present in the terminal repeats of the template RNA (Fig.
template switch by reverse transcriptase if the tandem repeat
monomer could have been generated by misalignment during the
multiplication cycle. Changes in the copy number of the 63-bp
genomes that were unable to complete one or more steps of the
cations in this region would be associated with defective virus
 genomes, it is reasonable to assume that the infectious TPV
cluster. Given the general absence of such repeats in viral
DNA and increase opportunities for recombination between
these gaps could conceivably extend the lifetime of open circular
DNA and increase opportunities for recombination between
free ends of TPV DNA at the gaps and plant sequences. In
particular, the presence of one cluster of TPV sequence
junctions and rearrangements close to the beginning or end of
the putative TPV genome would be consistent with the involve-
ment of gap 1, which is present at the tRNA binding site
beginning at nucleotide 1. To explain the second cluster of
junctions, a second gap would be postulated in ORF1, which is
not incompatible with the position of gap 2 in ORFII of
cauliflower mosaic virus open circular DNA (1).

Despite suggestive data indicating the involvement of gaps in
viral DNA integration, it is unclear which features of the TPV
sequences could have contributed to delayed gap repair and
accumulation of open circular DNA. One unusual and conspic-
uous characteristic of many TPV sequence clones is the
putative leader region that contains the variable tandem repeat
cluster. Given the general absence of such repeats in viral
genomes, it is reasonable to assume that the infectious TPV
 genome contained a single copy of the 63-bp sequence; dupli-
cations in this region would be associated with defective virus
genomes that were unable to complete one or more steps of the
multiplication cycle. Changes in the copy number of the 63-bp
monomer could have been generated by misalignment during the
template switch by reverse transcriptase if the tandem repeat
were present in the terminal repeats of the template RNA (Fig.
5). Alterations in monomer length might have been produced by
aberrant reverse transcription through the shorter inverted
repeats/direct repeat within the 63-bp monomer, as indicated by
the involvement of sequences primarily in stem-loop regions of
an RNA secondary structure (Fig. 3). Secondary structures
formed in this region could possibly prevent repair of one or
more gaps, leaving them free to recombine with plant DNA.
Further identification of other integrated pararetrovirus se-
quences as well as characterization of the natural TPV genome
are required to determine the validity of this proposal.

The absence of sequence homology between target plant DNA
and TPVL sequences and frequent rearrangements of TPVL
sequences at the insertion site are consistent with integration by
illegitimate recombination. Insertion into tobacco DNA ap-
ppeared random, as indicated by the different types of plant DNA
that flanked TPVL sequences, including a highly repetitive
tandem element, a moderately repetitive retrotransposon, and a
low copy gene. Interestingly, TPVL sequences are much more
abundant in the N. sylvesteris genome than in N. tomentosiformis
or N. otophora, suggesting that most copies in tobacco are
present in the N. sylvesteris fraction of the genome. Consistent
with this, two of the identifiable flanking plant DNA sequences,
the NTS9 tandem repeat (22) and the Lheb gene (GenBank
accession no. AB012638), are indeed specific to the N. sylvesteris
genome. The species-specific accumulation of TPVL sequences
indicates that integrated viral DNA can contribute to genome
divergence between two closely related plant species.

The putative TPV inferred from integrated TPVL se-
quences would be the first pararetrovirus described for to-
bacco. It is more related to CsVMV than to the other known
pararetrovirus of a solanaceous species, petunia vein-
clearing virus (11). Similarities extend to the unique features
of CsVMV, including a low GC content and modified order of
coat protein/movement protein/polyprotein compared with
all other plant pararetroviruses. Therefore, it can probably be
placed in the same group as CsVMV, which itself has previ-
ously been classified as the sole member of a new pararetro-
virus group separate from caulimoviruses and badnaviruses
(20). Similar sequences in the high molecular weight DNA of
other solanaceous plants suggest that additional integrated
pararetroviruses remain to be discovered.

We obtained no evidence that infectious episcopal genomes
could result from homologous recombination of TPVL se-
quences out of the tobacco genome, as has been reported for
integrated BSV sequences in banana (12, 13). All TPVL clones
that were sequenced contained frameshifts and stop codons,
which would not allow reconstitution of an infectious virus. We
were also unable to obtain evidence for virus replication or a
productive infection in intact plants or regenerating callus, where

In addition to transposable elements, tandem repeats, and
 microsatellites, integrated pararetroviral-like sequences must
now be considered a type of repetitive DNA in higher plants. The
discovery of a new class of repeated DNA justifies sequencing
nongenic regions to identify additional novel components of
plant genomes (28). Not only are such sequences important for
understanding plant genome evolution, they can also potentially
provide information about interactions between host genomes
and parasitic elements and the vagaries of viral genome repli-
cation.

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