Use of randomly mutagenized genomic cDNA banks of potato spindle tuber viroid to screen for viable versions of the viroid genome

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In an effort to study sequence space allowing the recovery of viable potato spindle tuber viroid (PSTVd) variants we have developed an in vivo selection (Selex) method to produce and bulk-inoculate by agroinfiltration large PSTVd cDNA banks in which a short stretch of the genome is mutagenized to saturation. This technique was applied to two highly conserved 6 nt-long regions of the PSTVd genome, the left terminal loop (TL bank) and part of the polypurine stretch in the upper strand of pre-melting loop 1 (PM1 bank). In each case, PSTVd accumulation was observed in a large fraction of bank-inoculated tomato plants. Characterization of the progeny molecules showed the recovery of the parental PSTVd sequence in 89 % (TL bank) and 18 % (PM1 bank) of the analysed plants. In addition, viable and genetically stable PSTVd variants with mutations outside of the known natural variability of PSTVd were recovered in both cases, although at different rates. In the case of the TL region, mutations were recovered at five of the six mutagenized positions (357, 358, 359, 1 and 3 of the genome) while for the PM1 region mutations were recovered at all six targeted positions (50–55), providing significant new insight on the plasticity of the PSTVd genome.

INTRODUCTION

Viroids are small, single-stranded, circular, non-encapsidated and non-coding RNA molecules causing diseases in various plants (Flores et al., 2005; Hadidi et al., 2003; Tsagris et al., 2008) that have been classified in two families, the family Avsunviroidae and the family Pospiviroidae (Flores et al., 2003). The best-known representative of the family Pospiviroidae is the species Potato spindle tuber viroid (PSTVd), which typifies the genus Pospivirwood. Based on sequence comparison, the rod-like structure of the pospiviroid genome has been divided into five structural domains named central (C), pathogenicity (P), variable (V), terminal right (TR) and terminal left (TL) (Keese & Symons, 1985) (Fig. 1). The C domain together with the TL domain is involved in replication (Baumstark et al., 1997; Kolonko et al., 2006; Schrader et al., 2003; Zhong et al., 2008), whereas the P domain seems to control pathogenicity (Owens et al., 1996; Schnölzer et al., 1985). Structural elements of the TR and V domains are important for viroid trafficking in host plants (Qi et al., 2004; Zhong et al., 2007). Since viroids do not encode proteins, they depend on host enzymes for their biological functions. It is now clear that most viroid functions are collectively regulated by determinants located in different parts of the viroid molecule (Zhong et al., 2008).

Site-directed mutagenesis studies have allowed the unravelling of particular PSTVd structures and their contribution to PSTVd biology (Hu et al., 1996, 1997; Owens et al., 1995, 1996; Qi et al., 2004; Wang et al., 2007; Zhong et al., 2008). However, over 50% of randomly introduced mutations abolish PSTVd infectivity (Owens et al., 1991) and mutations are frequently unstable (Owens et al., 1995, 1996), limiting the usefulness of this strategy to probe in detail the sequence space around a particular viroid sequence.

An alternative strategy is the in vivo selection (in vivo Selex) of viable variants from large collections of mutants (Owens et al., 2003; Owens & Thompson, 2005). Starting from libraries of mutants with genomes partially or fully randomized at key positions, these authors inoculated tomato plants with in vitro-transcribed PSTVd RNAs and analysed the recovered progenies, identifying a number of new, viable variants. Another strategy based on biolistic inoculation was used to screen viroid ‘thermomutant’
propagation in different plant species (Matoušek et al., 2004a, b). In the present work we have developed and used an in vivo Selex approach to study the viable variability in two highly conserved 6 nt-long segments of the PSTVd molecule located, respectively, in the left terminal loop of the TL domain and in the upper strand of pre-melting loop 1 (PM1) of the P domain. The left terminal hairpin is highly conserved between natural PSTVd isolates and in all other pospiviroids characterized to date. A search of over 140 PSTVd sequences present in GenBank revealed only four natural isolates diverging from the C_{357}CUCGG_{3} motif. These isolates, AY532801–804 are also unusual in having been identified from pepper plants (Lebas et al., 2005), a rare natural host for PSTVd.

The second targeted region is part of the so-called oligo- or polypurine stretch (Steger et al., 1984), a hallmark of the P domain of pospiviroids (Keese & Symons, 1985). In PSTVd, the consensus of this domain is AGA(3–5)GA(5–7)GA with only two sequences in GenBank for which a pyrimidine is present in this purine stretch. Such a purine-rich motif is also observed in many other members of the family Pospiviroidae. Despite this conservation, no clear biological function has been associated with this motif, beyond its contribution to the local instability of the molecule (Gast et al., 1996; Steger et al., 1984). Mutations immediately upstream of this region or on the corresponding lower strand of the PSTVd molecule affect the pathogenicity (Hammond & Owens, 1987; Owens et al., 1996; Schnölzer et al., 1985) or the trafficking of PSTVd isolates (Qi et al., 2004; Zhong et al., 2007).

The methods developed allowed the preparation and screening of banks containing all possible mutants of the 6 nt target regions. Sequencing of the PSTVd progenies recovered from the inoculated plants allowed the identification of viable mutants from pools of variants obtained by saturation mutagenesis.

**RESULTS**

**In vivo selection of viable PSTVd variants from the PM1 and TL mutant banks**

Following the PCR-based strategies described in Methods, two Agrobacterium tumefaciens banks containing mutagenized full-length monomeric infectious PSTVd constructs under the control of the cauliflower mosaic virus 35S promoter in the pGreen binary vector (Hellens et al., 2000) were prepared. Both banks correspond to the saturation mutagenesis of a short 6 nt-long region corresponding, respectively, to the left terminal hairpin (TL domain, positions 357–359 and 1–3 of the PSTVd molecule, numbering according to the PSTVd-D1 reference isolate sequence) and to part of the upper portion of PM1 in the virulence modulating (VM; Schnölzer et al., 1985) region (positions 50–55) (Fig. 1). Each bank contained over 50,000 individual colonies and therefore contains with high statistical probability all 4096 (4^6) possible PSTVd variants. Five clones of each bank were chosen randomly and sequenced, confirming the presence of mutations in the target regions. No additional mutations outside the mutagenized regions were observed (data not shown).

Each bank was bulked, amplified and mass-agroinoculated to the leaves of 50 tomato plants cultivar (cv.) ‘Rutgers’. As a control, 10 plants were inoculated with A. tumefaciens carrying the parental PSTVd-S23 cDNA similarly cloned in the pGreen vector. Two weeks after agroinfiltration, PSTVd accumulation was detected in 60 % of the control PSTVd-S23 inoculated plants and this proportion reached 100 % by 4 weeks post-inoculation (p.i.) (Table 1), thus confirming the efficiency of the inoculation method. By contrast, only 58 (29/50) and 24 % (12/50) of plants inoculated with the TL and PM1 isolates, respectively, showed PSTVd accumulation by 4 weeks p.i. (Table 1), but in both cases this proportion reached 92 % by 8 weeks p.i.

**Table 1. Number of plants showing PSTVd accumulation in the upper non-agroinoculated parts of tomato cv. ‘Rutgers’ plants**

<table>
<thead>
<tr>
<th>Agroinoculated construct</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSTVd-S23</td>
<td>6/10</td>
<td>8/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Bank TL</td>
<td>2/50</td>
<td>18/50</td>
<td>29/50</td>
<td>35/50</td>
<td>44/50</td>
<td>44/50</td>
<td>46/50</td>
</tr>
<tr>
<td>Bank PM1</td>
<td>0/50</td>
<td>2/50</td>
<td>12/50</td>
<td>30/50</td>
<td>37/50</td>
<td>43/50</td>
<td>46/50</td>
</tr>
</tbody>
</table>

*The values given represent the number of plants showing PSTVd accumulation over the total number of agroinoculated plants.*
The hybridization signals observed with the bank-inoculated plants were generally lower than those of PSTVd-S23 inoculated controls, but showed a general tendency to increase with time, so that a significant proportion of plants ultimately reached PSTVd accumulation levels similar to those of the control plants (Supplementary Fig. S1, available in JGV Online).

Analysis of the TL bank progeny

The PSTVd progeny present in 18 of the 46 plants inoculated with the TL bank were characterized by direct sequencing of PCR-amplified full-length cDNAs (Góra et al., 1994). In 16 of these 18 plants only the PSTVd-S23 parent was observed and these plants were not analysed further. There was no clear correlation between the onset of detectable PSTVd accumulation and detection of the PSTVd-S23 sequence since the first positive hybridization signal for these 16 plants was observed at between 2 and 6 weeks p.i. The two other plants analysed yielded mutants differing from the PSTVd-S23 sequence at three or four of the six mutagenized positions. No mutations were observed in other parts of the genome of the recovered progenies. Plant number 34 analysed at 3 weeks p.i. accumulated variant TL34/3, which differs from PSTVd-S23 by three mutations in the terminal loop: C358A, U359A and C1A (Table 2 and Fig. 2a). The same variant was observed again in the same plant at 4 weeks p.i. but later analysis, at 7 weeks p.i., revealed the accumulation of another variant, TL34/7, differing from TL34/3 by an A358G mutation. No sequence heterogeneity was observed at any of these three time points. No symptoms were observed at 3 weeks p.i. on plant 34 and the hybridization signal was very weak, but by 4 weeks p.i. the hybridization signal was comparable to that of control S23-inoculated plants and symptoms of intermediate severity were observed. At 7 weeks p.i., the plant displayed typical S23 severe symptoms (data not shown).

A third variant, TL4/11, was also detected in a single plant, differing from the S23 parent by four mutations: C357A, U359A, C1A and G3C (Table 2 and Fig. 2a). This mutant was detected at 5 weeks p.i. in a plant showing mild symptoms and a strong hybridization signal but was not further analysed.

Collectively, the three recovered variants differ from the parental sequence at five of the six mutagenized positions, the G at position 2 being the only parental nucleotide retained in all three variants which, in addition, share the same pair of U359A and C1A mutations (Table 2).

The infectivity and genetic stability of these three variants were verified by inoculating tomato seedlings with infectious monomeric cDNAs and resequencing their progeny at 4 weeks p.i. The results (Table 2) confirm the infectivity of all three variants. TL34/3 and TL34/7 induced severe symptoms similar to those of PSTVd-S23, while TL4/11 only caused mild symptoms. All three variants were genetically stable since in each case a sequence identical to that of the inoculated variant was obtained when analysing its progeny at 4 weeks p.i. (data not shown). However, all three variants also showed a somewhat reduced accumulation compared with the S23 parent. Although this effect was rather limited in the case of TL34/3 and 34/7, it was stronger for TL4/11 (Fig. 3).

Analysis of the PM1 bank progeny

The PSTVd progeny was characterized in 33 of the 46 plants infected upon inoculation with the PM1 bank. In six of these plants the parental PSTVd-S23 was detected. As for the TL bank, S23 was observed in plants showing early (4 weeks p.i.) or late (up to 8 weeks p.i.) onset of PSTVd accumulation. The other 28 plants yielded a total of 10 variants differing by between one and five mutations from the parental sequence. Overall, all six of the mutagenized positions were observed to be affected in one or more

| Table 2. Name, sequence at the targeted positions, infectivity and symptomatology of the PSTVd variants recovered from tomato plants agroinoculated with TL bank |
|---------------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| PSTVd variant       | Targeted genome positions* | Infectivity†     | Symptoms‡       |
|                    | 357 | 358 | 359 | 1 | 2 | 3 |                              |
| S23 (parental)     | C   | C   | U   | C | G | G | 5/5  | Severe  |
| TL34/3             | C   | A   | A   | A | G | G | 9/12 | Severe  |
| TL34/7             | C   | G   | A   | A | G | G | 11/12 | Severe  |
| TL4/11             | A   | C   | A   | A | G | C | 25/27 | Mild |

*Numbering according to the PSTVd-DI reference isolate sequence. Nucleotides differing from the parental PSTVd-S23 sequence are indicated in bold.
†The values given represent the number of plants showing PSTVd accumulation over the total number of tomato plants inoculated with plasmids harbouring a full-length infectious cDNA of the relevant variant.
‡Symptoms observed at 5 weeks p.i. on the tomato plants inoculated with plasmids harbouring a full-length infectious cDNA of the relevant variant.

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variants (Table 3). While three of the variants contained additional point mutations outside of the mutagenized region, none of them contained insertions or deletions. Five of the 10 variants were observed in more than one plant and one-third of the plants analysed (11/33) contained more than a single sequence variant (Table 3).

Two of the recovered variants are characterized by single mutations affecting position 50. In plant number 16, variant PSTVd-PM1-16, carrying a single U50C mutation, was observed at 6 weeks p.i. However, 2 weeks later the population was heterogeneous. Sequencing of six cloned PSTVd cDNAs revealed the presence of three variants, PSTVd-PM1-16 (one clone), the parental PSTVd-S23 (four clones) and a new variant, PSTVd-PM1-46 (one clone). The last one differs from PSTVd-PM1-16 and PSTVd-S23 by the presence of an A at position 50. As compared to the parental PSTVd-S23, this U50A substitution corresponds to reversion to the PSTVd-DI sequence (Gross et al., 1978).

Mutant PM1-46 was also detected, alone, in another plant (plant 46, Table 3).

A second group of five variants is characterized by a pair of G54A-A55G mutations. These variants are by far the most frequently recovered, since they were collectively observed in 82 % of the plants containing non-parental sequences (23 of 28, Table 3). Mutant PSTVd-PM1-40 carries only these two mutations and was detected at 6 weeks p.i. in three plants (plants 11, 21 and 40). In the case of plant 40, heterogeneity at position 50 was observed and sequencing of cloned PSTVd cDNAs revealed four clones with an A50 and one clone with a U50. Interestingly, the variant with the additional U50A mutation, named PSTVd-PM1-9 was the most frequently observed molecule, since it was also detected as the only variant present in nine plants (plants 1, 2, 4, 8, 9, 15, 25, 44 and 50) and, in mixed infection with other variants, in nine other plants (plants 11, 17, 20, 21, 24, 30, 37, 41 and 47) (Table 3). The variant PSTVd-PM1-31, which differs from PM1-40 and PM1-9 by a single U50C mutation, was detected in a single plant (31).

In three of the plants (plants 20, 24 and 30), PSTVd-PM1-9 was observed in co-infection with a variant PSTVd-PM1-30, carrying an additional A51C mutation. In all three cases, this variant showed a tendency to be displaced by PM1-9.

Fig. 2. Predicted secondary structures of the TL domain of recovered PSTVd-TL variants. The mutagenized positions and positions diverging from the parental PSTVd-S23 isolate are shown in bold and underlined, respectively. (a) Classical folding. (b) Alternative branched folding of the same region, shown for PSTVd-S23 and for variant TL4/11.

Fig. 3. Accumulation of PSTVd-TL variants and PSTVd-S23 in cv. ‘Rutgers’ tomato plants. PSTVd accumulation was detected at 4 and 5 weeks p.i. by molecular hybridization with an in vitro-transcribed PSTVd RNA probe. C−, Negative control, plant inoculated with Agrobacterium carrying the empty pGreen vector; C+, positive control, PSTVd-infected plant; Buf., only extraction buffer spotted on the membrane.
Table 3. Name, sequence at the targeted positions, additional mutations observed, infectivity and symptomatology of the PSTVd variants recovered from tomato plants agroinoculated with PM1 bank

Newly described variants deposited in GenBank (accession nos GQ853460–GQ853469)

<table>
<thead>
<tr>
<th>PSTVd variant</th>
<th>Targeted genome positions*</th>
<th>Additional mutations</th>
<th>Observed in plant no.†</th>
<th>Infectivity‡</th>
<th>Symptoms§</th>
</tr>
</thead>
<tbody>
<tr>
<td>S23 (parental)</td>
<td>U A A A G A</td>
<td>–</td>
<td>16, 18, 22, 26, 33, 42</td>
<td>5/5</td>
<td>Severe</td>
</tr>
<tr>
<td>PM1-7</td>
<td>U A A G A A</td>
<td>U311A</td>
<td>7</td>
<td>9/10</td>
<td>Intermediate/severe</td>
</tr>
<tr>
<td>PM1-9</td>
<td>A A A A A G</td>
<td>–</td>
<td>1, 2, 4, 8, 9, 11, 15, 17, 20, 21, 24, 25, 30, 37, 40, 41, 44, 47, 50</td>
<td>9/10</td>
<td>Intermediate</td>
</tr>
<tr>
<td>PM1-14</td>
<td>C A U G U G</td>
<td>G44A</td>
<td>14</td>
<td>7/10</td>
<td>Severe (4); no symptoms (3)</td>
</tr>
<tr>
<td>PM1-16</td>
<td>C A A A G A</td>
<td>–</td>
<td>16</td>
<td>8/10</td>
<td>Severe</td>
</tr>
<tr>
<td>PM1-30</td>
<td>A C A A A A G</td>
<td>–</td>
<td>6, 20, 24, 27, 30, 48</td>
<td>10/10</td>
<td>Intermediate (8); no symptoms (2)</td>
</tr>
<tr>
<td>PM1-31</td>
<td>C A A A A A G</td>
<td>–</td>
<td>31</td>
<td>9/10</td>
<td>Severe</td>
</tr>
<tr>
<td>PM1-37</td>
<td>A A C A A A G</td>
<td>–</td>
<td>17, 37, 41, 47</td>
<td>20/22</td>
<td></td>
</tr>
<tr>
<td>PM1-40</td>
<td>U A A A G A</td>
<td>–</td>
<td>11, 21, 40</td>
<td>10/10</td>
<td>Severe</td>
</tr>
<tr>
<td>PM1-43</td>
<td>C A U A A A A</td>
<td>U306A</td>
<td>43</td>
<td>3/10</td>
<td>Severe (1); mild (2)</td>
</tr>
<tr>
<td>PM1-46</td>
<td>A A A A G A</td>
<td>–</td>
<td>16, 46</td>
<td>9/10</td>
<td>Severe</td>
</tr>
</tbody>
</table>

*Mutagenized genome positions are numbered according to the PSTVd-DI reference isolate sequence. Nucleotides differing from the parental PSTVd-S23 sequence are indicated in bold.
†For each variant the code numbers of the plants in which the variant was observed are indicated.
‡The values given represent the number of plants showing PSTVd accumulation over the total number of tomato plants inoculated with plasmids harbouring a full-length infectious cDNA of the relevant variant.
§Symptoms observed at 5 weeks p.i. on the tomato plants inoculated with plasmids harbouring a full-length infectious cDNA of the relevant variant. When plants showing symptoms of different severity were observed, the number of plants with each type of symptoms is indicated in parentheses.
||Sequence analysis of the progeny in four plants provided indications of C/A sequence heterogeneity at position 52 (which differentiates PM1-37 from PM1-9) for three plants and the presence only of PM1-9 for the fourth one.

plant 30, PM1-30 was observed alone 3 weeks p.i., but 2 weeks later was observed in mixed infection with PSTVd-PM1-9. The same situation was observed for plant 24 at 6 and 8 weeks p.i. In the case of plant 20, sequence analysis of the uncloned RT-PCR products at 5 weeks p.i. revealed the presence of the two variants, but reanalysis 3 weeks later yielded only PSTVd-PM1-9. The presence of an A at position 51 seems to be favoured over a C. However, PSTVd-PM1-30 was also observed alone in three additional plants (plants 6, 27 and 48), even as late as 6 weeks p.i. in the case of plant 48.

Mixed infection with PSTVd-PM1-9 was similarly observed in four plants (plants 17, 37, 41 and 47) for another variant, PSTVd-PM1-37, which differs from PM1-9 by an additional A52C substitution. Variant PM1-37 was never observed alone.

Lastly, three variants containing additional mutations outside the mutagenized region were observed alone in single plants and late in the experiment (6–7 weeks p.i.). In comparison with the parental S23 sequence, variant PSTVd-PM1-14 has mutations in five of the six targeted positions: U50C, A52U, A53G, G54U and A55G and an additional G44A mutation.

Variant PSTVd-PM1-43 has three mutations in the target region (U50C, A52U and G54A) plus an additional U306A mutation in the lower strand of the VM region, exactly opposite the mutagenized region. Similarly, variant PSTVd-PM1-7 has two mutations in the target region (A53G and G54A) and an additional U311A mutation in the lower portion of the VM.

The infectivity and genetic stability of all PM1 variants were verified as for the TL mutants. The results obtained (Table 3) confirmed the infectivity of all 10 variants, even if the infectivity of variant PM1-43 seems limited, as it infected only three of the 10 inoculated plants. Most variants induced severe symptoms similar to those of the parental S23 isolate, but symptoms of intermediate severity were observed for variants PM1-9, PM1-30 and PM1-7, while heterogeneity in symptom severity was observed in the case of variants PM1-30, PM1-14 and PM1-43. However, this heterogeneity does not appear to correlate with genetic instability since parallel
analysis of progeny molecules from plants with mild or severe infection phenotypes yielded in each case only the sequence of the inoculated parental variant. With the exception of PSTV-PM1-37, all variants were therefore genetically stable over the 5 week study and no evidence for sequence heterogeneity was detected in the progeny sequencing chromatograms.

Mutant PSTVd-PM1-37 displayed high infectivity (20 of 22 inoculated plants) but in three of the four plants in which its progeny was analysed the sequencing chromatograms provided indications of C/A sequence heterogeneity at position 52, which differentiates PM1-37 from PM1-9. The last plant analysed yielded progeny only composed of PSTVd-PM1-9 (Table 3).

Most variants accumulated to levels comparable to that of the PSTVd-S23 parent but four of them (PM1-14, PM1-30, PM1-31 and PM1-43) showed a reduced accumulation (Supplementary Fig. S2, available in JGV Online). For PM1-30 and PM1-31, later resampling at 6 weeks p.i. demonstrated that accumulation levels had caught up with that of S23 (Supplementary Fig. S2).

**DISCUSSION**

In the present study, highly conserved continuous sequence stretches were targeted and no specific measure was taken to preclude the presence of the parental sequence from the inoculated pool of variants. As a consequence, the complexity of the banks used (4^6=4096 variants) was significantly higher than the 48–3072 variant pools previously used (Owens et al., 2003; Owens & Thompson, 2005). It should be noted that while excluding the parental PSTVd sequence from the mutant pool reduces the sampled sequence space, it does not guarantee that the wild-type sequence will not be recovered, sometimes as the sole variant, during the in vivo Selex step (Owens & Thompson, 2005). In the present study, the wild-type PSTVd-S23 sequence was present in the initial TL and PM1 banks and was recovered in very different proportions following the in vivo selection (89% and 18%, respectively). Although the two target regions are highly conserved between PSTVd isolates, this difference in the rate of recovery of the wild-type parent probably reflects differences in the paucity of viable and/or competitive variants in the banks analysed. The only variants recovered from the TL bank diverged by three to four mutations from the S23 parent, whereas the variant most frequently selected from the PM1 bank, PM1-9, had only two mutations and some other variants differed from S23 by a single mutation.

Of the over 140 PSTVd sequences in GenBank only four natural isolates have left terminal hairpins diverging from the C357UUCGGG motif. There are, in addition, three reports of viable and stable mutants affected in this region. One involves a variant with a U359G mutation recovered during an in vivo Selex experiment (Owens et al., 2003) and the others involve C1U or C1G point mutants (Kolonko et al., 2006; Owens et al., 1991). On the other hand, a U359G mutant was unstable and reverted to the wild-type sequence (Kolonko et al., 2006). Enlargement of the terminal loop from 4 to 6 nt as a result of three mutations (G2U, A4C and C6G), reduced PSTVd replication in *Nicotiana benthamiana* to below 16% of the wild-type sequence (Zhong et al., 2008). The same set of mutations completely abolished PSTVd infectivity in tomato (Hammond, 1994; Hammond & Owens, 1987; Hu et al., 1997). However, restoration of replication and systemic trafficking in tomato only required a C4G mutation (Hu et al., 1997).

Two possible structures for the TL domain were proposed, the well-known rod-like structure and a branched structure involving two terminal hairpins (Gast et al., 1996; Gast, 2003). Nuclear magnetic resonance and thermodynamic analysis of mutations predicted to affect these conformations indicated that the elongated-rod form was the preferred structure in solution (Dingley et al., 2003). Similarly, analysis of *in vivo* Selex variants led Owens & Thompson (2005) to conclude that the branched conformation was unlikely to be important for PSTVd fitness. The analysis of the potential effects on both conformations of the mutations observed in the three TL variants recovered in the present study similarly do not support a significant role for the branched conformation. Indeed, three of these mutations, at positions 357, 358 and 3, are predicted to affect base-pairing in the bifurcated structure. In particular, the C357A and G3C mutations observed in variant PSTVd-TL4/11 are predicted to affect a total of 4 bp in the branched structure, but a single one in the elongated conformation (Fig. 2b).

Some tetraloops may adopt particular tertiary structures involving non-canonical base pairings that significantly contribute to the stabilization of the molecule (Moore, 1999). The PSTVd cCUCGg terminal loop may belong to the CUNG or YNMG tetraloop families (Kolonko et al., 2006; Proctor et al., 2002). In model experiments, a CUCG loop with a closing C:G nucleotide pair had a high thermodynamic stability (Proctor et al., 2002). However, the mutations observed in the present study are all expected to significantly reduce loop stability (ΔT_m of between −3.6 and −6.3 °C, ΔΔG of +1.45 to +4.74 kcal mol⁻¹; Proctor et al., 2002). Although stabilization of the PSTVd molecule through such a tetraloop effect might explain the extreme conservation of the terminal loop sequence in pospiviroids, it appears that this stabilization is not a prerequisite for PSTVd infectivity or stability in tomato plants.

The biological function(s) of the TL domain is (are) not entirely understood, but a contribution to the PSTVd molecule stability (Hammond, 1994; Hammond & Owens, 1987; Hu et al., 1997) and replication process has been demonstrated. Kolonko et al. (2006) showed that the synthesis of the (−) strand is initiated at either position U359 or C1. Site-directed mutagenesis indicated that a C1G mutation was viable and stable but a U359G mutation rapidly reverted to the wild-type sequence (Kolonko et al., 2006).
2006), suggesting U359 as the most probable transcription start site (TSS).

The mutants reported here indicate that both mutations U359A and C1A are viable and stable. Mutations affecting the other mutagenized positions were also recovered, with the exception of position 2, for which the parental G was always observed. Both strands of the PSTVd genome are replicated by the host DNA-dependent RNA polymerase II (Pol II; Schindler & Mühlbach, 1992). Pol II promoters show extensive variability, even if a −1 Y and +1 R (+1 being the TSS) nucleotide dimer sequence seems to be significantly over-represented in plant Pol II promoters (Shahmuradov et al., 2003; Yamamoto et al., 2007). In this context it may be noteworthy that PSTVd seems to stably tolerate any nucleotide at position 1: a C in the wild-type sequence, A in the mutants recovered in the present study and a G or a U in previously observed or constructed mutants (Kolonko et al., 2006; Owens et al., 1991). By contrast, position 359 seems to be somewhat more constrained as a U359G mutation proved unstable (Kolonko et al., 2006) and only an A was recovered at this position in the present work. Taken together these results suggest that U359 could represent the TSS during PSTVd (−) strand transcription.

The second targeted region, in the polypurine stretch of PM1 (Steger et al., 1984) is also almost absolutely conserved in natural PSTVd isolates and, when analysed, mutations introduced in this region have proven to be unstable (Owens, 1990; Zhong et al., 2008).

The rates of recovery of PM1 variants showed an almost 20-fold variation, ranging from 57 (19 of 33 plants) to 3% (one of 33 plants). Since the strategy used should have ensured an unbiased representation of all possible mutants in the inoculum, the variation observed in recovery rate most probably reflects the competitiveness of the individual variants. However, it is not possible to directly equate the recovery rate with genetic stability, as demonstrated by the recovery of the stable parental PSTVd-S23 (Góra-Sochacka et al., 1997) and of the unstable variant PM1-37 at roughly similar rates (18 and 12%, respectively). It seems however noteworthy that with the exception of PM1-37, all the PM1 variants recovered proved stable over a 5 week assay period, whereas previous limited mutagenesis efforts in that region had yielded only unstable molecules (Owens, 1990; Zhong et al., 2008).

It is remarkable that molecules in which one or more of the target bases was replaced by a pyrimidine represented nine of the 11 recovered variants, including the parental PSTVd-S23 sequence. In fact, some of the pyrimidine-containing variants, such as PSTVd-S23 and PM1-30, were among the most frequently recovered molecules. While mutations were recovered at every target position, purine to pyrimidine mutations were observed at positions 50, 51, 52 and 54. Despite the extremely high conservation of the polypurine stretch in pospiviroids, the results reported here demonstrate that PSTVd isolates with pyrimidines in one or more of the target positions can be viable, genetically stable and have significant fitness, as judged from their recovery rates and accumulation levels.

The two recovered variants without pyrimidines are PM1-46 and PM1-9. Their rate of recovery (6 and 57%, respectively) are not correlated with the frequency of natural PSTVd isolates with the same local sequence. Indeed, isolates with the AAAAGA sequence found in PM1-46 represent 60% of the PSTVd sequences in GenBank while those with AAAAGG, as PM1-9, represent a mere 2% (three isolates). We have no explanation for this observation, in particular since PM1-9 and PM1-46 accumulated to comparable levels (Supplementary Fig. S2).

If one considers the range of mutants present in the initial inoculum, the potential local secondary structures vary from a 50–55 region completely base paired to positions 305–310 of the lower strand to a completely unpaired region (Fig. 4). The corresponding folding ΔG values for the complete PSTVd molecule calculated using MFold 3.2 (Zuker, 2003) range from −165.7 to −156.5 kcal mol⁻¹. Similar computations provide a −159.5 kcal mol⁻¹ for the parental S23 sequence and a range of −158.4 (PM1-9 and PM1-37) to −162.3 (PM1-7) kcal mol⁻¹ for the recovered variants, indicating that they only represent a central fraction of the secondary structure stability levels. It is noteworthy that for two of the mutants with additional mutations, PM1-7 and PM1-14, these mutations lower the ΔG value (0.4–0.5 kcal mol⁻¹), suggesting that the most stable structures/mutants could have been counter-selected (Fig. 4). Such an observation would be in keeping with results showing that complete base pairing of the target region though pairs of mutations affecting either positions 50–51 or 310–311 resulted in mutants that were either non-infectious (Owens et al., 1996) or severely affected in their replication and unstable (Zhong et al., 2008). Taken together, the results reported here suggest that viability, genetic stability and fitness of the PSTVd molecule can be achieved even if pyrimidines are introduced in the polypurine stretch, provided that the local secondary structure remains in a limited range of stability, with mutations either stabilizing or further destabilizing the region showing detrimental effects.

Although it is unlikely that all possible viable variants in the target mutagenized regions were recovered in the experiments reported here, the approach used allowed the identification of numerous genetically stable variants that fall outside the natural variability of PSTVd and provide new insights into the plasticity of the PSTVd genome. Further efforts employing the same strategy, in particular with larger target regions or targeting other genomic regions should improve our knowledge of the viable sequence space surrounding the genomes of viroids.

**METHODS**

**Construction of the PSTVd-PM1 bank.** Random saturation mutagenesis of positions 50–55 of a full-length infectious PSTVd-
S23 (Góra et al., 1994) cDNA was achieved by PCR amplification using two primers, one of which was fully degenerate at the six target positions. Primer, PSTVd-P1 (5′-GGGATCCGGGGAAACCT-3′, BamHI site of the central conserved region in bold) corresponds to PSTVd positions 86–104. Primer PSTVd-P4mut (5′-GGGATCCCTGAAGCGCTCCTCCGAGCCGCCTTTTTTNNTNNTTCTGATCA-GGAGTCAAGTCTG-3′) is complementary to PSTVd positions 93–30. The amplification reaction was performed using Pfu DNA polymerase (Fermentas) and the following cycling scheme: 30 s at 94°C, 30 s at 64°C and 1 min at 74°C, for 31 cycles. The PCR product was cloned into pGEM-T-Easy (Promega), generating a bank of mutagenized PSTVd monomers. Recombinant plasmids were purified from the bulked bacterial colonies (Qiagen plasmid purification kit), the full-length PSTVd inserts excised using BamHI and subcloned into the corresponding site of a pGreen vector (Hellens et al., 2000) modified by replacing the original polylinker by that of pUC9. Depending on the orientation of the viroid cDNA, this strategy generates an 8 or 11 bp duplication of the central conserved region of the PSTVd genome that is sufficient for infectivity (Candresse et al., 1990). Following transformation of A. tumefaciens (C58C1 strain carrying the virulence helper plasmid pCH32; Hamilton et al., 1996) with a pool of the recombinant pGreen vectors, a bank of over 50000 bacterial colonies was obtained.

**Construction of the PSTVd-TL bank.** Two terminally overlapping PSTVd-S23 PCR products were first obtained using two different primer pairs. The first PCR product covers positions 86–24 of the circular genome and was obtained using primers TL1 (5′-GGGATCCGGGGAAACCT-3′, PSTVd positions 86–104, BamHI site of the central conserved region in bold) and TL5 (5′-CAGGAAACCAGATTTAGTTNNNNNNNACACATGGTTCCAAGG-3′, PSTVd positions 24–1/359–336, with fully randomized positions 357–359 and 1–3 indicated by Ns). The second PCR product, spanning positions 4–93 was obtained using primers TL3 (5′-AATCTAA-
ACTCGTGTTTCCTGT-3', positions 4–24) and primer TL2 (5'-GGGATCCCTAAGCGCTC-3', complementary to positions 74–93, BamHI site in bold). The two PCR products were purified and joined into full-length PSTVd monomeric cDNAs by a fusion-PCR using primers TL1 and TL2 for this second round of amplification. All PCRs were carried out using Pfu polymerase and all further steps for the preparation of an agroinfiltrable bank were carried out as described above for the PM1 bank.

Mass inoculation of tomato plants and recovery of infectious PSTVd progeny molecules. One-month-old tomato plants cv. ‘Rutgers’ were inoculated by agroinfiltration of the bulked TL or PM1 banks as described by Bendahmane et al. (2000). From 2 to 8 weeks after inoculation plants were regularly observed for symptom development, leaf samples were collected and the presence of PSTVd assessed by dot-blot hybridization. Total RNAs were extracted from individual plants with the RNase Plant mini kit (Qiagen) and subjected to RT-PCR using PSTVd-specific primers complementary to the central conserved region (Góra et al., 1994). Uncloned PCR products were directly sequenced and chromatograms checked for signs of sequence heterogeneity. Individual monomeric infectious cDNAs of progeny molecules cloned in plasmid pUC9 were obtained as described previously (Góra et al., 1994) and their sequences verified.

Verification of the infectivity and genetic stability of progeny molecules. Tomato cv. ‘Rutgers’ seedlings were inoculated as described previously (Candresse et al., 1990) with purified plasmids (2 µg per plant) containing monomeric full-length cDNAs of all PSTVd progeny variants. The inoculated plants were monitored for symptom development, their infection status determined by molecular hybridization and the genetic stability of the PSTVd variants determined by direct sequencing of PCR-amplified progeny PSTVd cDNAs as described above.

Secondary structure and stability predictions for the isolated variants. Secondary structures and folding stability predictions at 37 °C in 1 M NaCl were performed for all recovered variants using the MFOLD 3.2 program (Zuker, 2003).

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REFERENCES


