Russian Isolates of *Potato spindle tuber viroid* Exhibit Low Sequence Diversity

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ABSTRACT

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Potato spindle tuber viroid (PSTVd) is currently widespread in seed potatoes grown in Russia. Characterization of 39 PSTVd isolates collected over a 15-year period from widely separated areas in Russia revealed the presence of 17 different sequence variants, all but one of which were previously unknown. Most variants were recovered only once, but two were more widely distributed; one of these was a mild variant previously isolated in Germany, the second was a novel variant inducing symptoms similar to those of the type strain in tomato. Despite this apparent lack of population diversity, several informative PSTVd variants were recovered. Sequence changes in the pathogenicity and variable domains were particularly common, but previously unknown changes were also detected within the loop E motif in the central domain, a structural motif known to play a key role in PSTVd replication and host range determination.

The spindle tuber disease of potato was first described by Martin (15), who reported a new disorder affecting the variety Irish Cobbler being grown in southern New Jersey for seed purposes. Evidently, the disease had been imported into New Jersey from Maine, and evidence of its infectious nature was published shortly thereafter (25). Nearly 50 years were to elapse before Diener (5) identified the causal agent as *Potato spindle tuber viroid* (PSTVd), the first example of a new class of subviral RNA pathogens. The first complete nucleotide sequence for PSTVd (that of the Intermediate or type strain) was published in 1978 (9), and databases now contain the sequences of more than 60 naturally occurring variants.

For many years, spindle tuber was common in potato-growing regions of the northern and northeastern United States and Canada (6), but the introduction of a series of improved diagnostic methods has led to its virtual eradication from North America and Western Europe (26). These

methods include bioassay on certain sensitive tomato cultivars (23), polyacrylamide gel electrophoresis (16), nucleic acid dot-blot hybridization (20), and, most recently, real-time reverse transcription-polymerase chain reaction (RT-PCR) (3). In Russia and other regions of the former Soviet Union, however, PSTVd infection still poses significant problems for seed potato production.

Potato “gothic” disease was first reported in European Russia in the early 1930s. Similarities in the symptoms of the two diseases suggested that the causal agents of spindle tuber and gothic disease might be identical (14), but the limited distribution of gothic disease made it of little economic significance. During the 1980s and 1990s, however, the yield and quality of Russian seed potatoes as well as the quality of some cultivars as a whole declined dramatically. Initial suspicions that seed potatoes and in vitro plantlets could be infected with PSTVd later proved to be true; indeed, PSTVd was detected in about 50 to 70% of in vitro potato plants and tuber samples collected from different regions of Russia (12). The main cause of this situation was the widespread adoption of meristem tip culture technology to produce virus-free potato in the former USSR beginning in the early 1970s. In applying this technology, the possibility of PSTVd infection was not considered. Other contributing factors include favorable conditions for viroid replication (e.g., high temperatures) during thermo-therapy and growth in the greenhouse and poor phytosanitation (17,18).

The sale of untested seed potatoes is responsible for the present widespread distribution of PSTVd throughout Russia, and the prevalence of this pathogen poses a serious threat to both seed production and potato breeding. Measures have been taken to control PSTVd (1,2,7,19), but the danger of further spread still remains. There are no regional seed potato certification laboratories in Russia, and farmers lack access to reliable methods of viroid identification. We are currently attempting to fill this gap and, as part of that effort, have characterized the PSTVd isolates in the current VNIIF collection and collected a large number of new isolates from locations throughout the Russian Federation. Here we report the sequences of 17 PSTVd variants recovered from Russian cultivars, only one of which has been previously described. Sequence changes in the pathogenicity and variable domains were particularly common, but previously undescribed changes were also detected within the so-called “loop E motif” in the central domain. A preliminary account of these studies has previously appeared (13).

MATERIALS AND METHODS

Maintenance of PSTVd isolates. Since the early 1990s, most PSTVd isolates in the VNIIF collection have been maintained under field conditions using tubers collected from infected plants during the previous growing cycle. A small number of isolates were stored as low-molecular-weight RNA preparations at -20°C . As a rule, these RNA preparations were isolated from tomato (*Solanum lycopersicum* L. cv. Rutgers) previously inoculated with crude sap from PSTVd-infected potato. To recover these isolates for sequence analysis, greenhouse-grown tomato plants at the four- to five-leaf stage were dusted with Carborundum, aliquots (30 μl) of low-molecular-weight RNA solution corresponding to 100 to 120 μg total RNA were placed on each of two upper leaves, and the leaves were then rubbed with a glass spatula. A month later, the upper leaves of the infected tomatoes were collected for isolation of total RNA.

RNA isolation and sequence analysis. Total RNA was isolated from frozen leaf tissue using Trizol RNA Prep 100 reagent as recommended by the manufacturer (Lab Isogene, Moscow). Full-length double-

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stranded PSTVd cDNAs were synthesized by RT-PCR using primers PSTVd 179 (5'-AAACCCTGTTTCGGCGGGAATTAC-3', complementary to positions 179-156) and PSTVd 180 (5'-TCACCCTCCTTTCTTCGGGTGTC-3', homologous to nucleotides 180-203). *Taq* DNA polymerase (Fermentas, Lithuania or Dialat Ltd., Moscow) was used for all PCR amplifications, and overlapping cDNAs were synthesized using primers PSTVd 112 (5'-ACTGGCAAAAAGGACGGTGGGGA-3', homologous to positions 112-135) and PSTVd 359 (5'-AGGAACCAACTGCGGTTCCAAGGG-3', complementary to positions 359-336) to confirm the sequence under the first pair of primers. Nucleotide sequences of uncloned PSTVd cDNAs were determined by automated sequence analysis using an ABI Prism Genetic Analyzer 3100; those not previously reported were then deposited in GenBank (accession numbers EU879913 to EU879926). Analysis of uncloned PCR products is insensitive to mutations introduced by the comparatively low-fidelity *Taq* DNA polymerase used for amplification and provides the sequence of the predominant variant(s) in each isolate. In contrast, to identify less-abundant variants and assess the sequence diversity of individual isolates would require analysis of cloned cDNAs generated using a proof-reading polymerase.

Phylogenetic analysis. Sequences of 66 naturally occurring isolates of PSTVd were obtained from the Subviral RNA Database (<http://subviral.med.uottawa.ca>). Relevant information about these isolates including their GenBank accession numbers and country of origin is summarized in Supplementary Table 1. Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 4.1 (28) and SplitsTree4 software packages

(11). PSTVd sequences were aligned using Clustal W (10) and then manually adjusted before phylogenetic analysis.

RESULTS

When our project began in 2006, the PSTVd collection at the VNIIF contained approximately 20 isolates that had been collected between 1993 and 1998 and maintained by yearly propagation from tubers in the field. Several isolates had also been stored at -20°C as frozen solutions of total RNA. During the last 2 years, we have made five additional collecting trips to different areas in Russia, resulting in an additional 20 possible new isolates of PSTVd. Many of these new isolates were obtained from plant material maintained by N.I. Vavilov Russian Research Institute of Plant Industry (VIR) and the Russian Research Institute for Plant Protection (VIZR) in Saint Petersburg and the All-Russian Potato Research Institute

(VNIKH) in Moscow; others were collected as symptomatic tubers from local markets or individual farmers. Figure 1 shows the locations of the geographic areas sampled.

Identification of PSTVd sequence variants. In 2007, total RNA was extracted from 275 potato plants in the VNIIF collection and tested for the presence of PSTVd using a combination of dot-blot hybridization and RT-PCR. A large majority of these plants (228) were expected to be PSTVd-infected, but only 107 (about 47%) tested positive by dot-blot hybridization. Among 31 dot-blot positive samples that were also tested by RT-PCR, 26 yielded an amplification product of the predicted size. Possible reasons for the failure of the remaining five samples to amplify include the presence of variable amounts of contaminants/inhibitors in RNA extracted from field-grown tissue.

Table 1. Geographical distribution of Russian *Potato spindle tuber viroid* (PSTVd) isolates

Variant	Location				
	Northwest	Central	Volga	Caucasus	Far East
Onega Premier_94	1	-	-	-	-
Niva_95	1	-	-	-	-
VNIIF_07_D16	-	1	-	-	-
VIR_06_5L07	1	-	-	-	-
VNIIF_93 [GB M14814]	-	5	-	1	5
Samara_97/2	1	-	1	-	-
Kh_06_29B clone 1	-	-	-	-	1
VIZR_06_6L-07	3	-	-	-	-
Bugry_97	4	3	1	-	1
VNIIF_07_K	-	1	-	-	-
Samara_97_3	-	1	1	-	-
VNIKH_07_42M	-	1	-	-	-
VIZR_06_6L2-08	1	-	-	-	-
Tver'_96	-	1	-	-	-
VIZR_06_4L	1	-	-	-	-
VIR_06_7L	1	-	-	-	-
VIR_06_10L	1	-	-	-	-
Total	15	13	3	1	7

Fig. 1. *Potato spindle tuber viroid* (PSTVd) sampling sites in the Russian Federation. Approximate locations of collection sites in Northwest, Central, Volga River, Caucasus, and Far Eastern Russia (two sites) are indicated by dashed circles.

The VNIIF collection originally contained approximately 30 PSTVd isolates collected during earlier (1993 to 1998) spindle tuber outbreaks in European Russia. These isolates were maintained by repeated tuber passage under field conditions, and only 18 isolates were still available when our study started in 2006. Molecular testing of 16 isolates revealed that only 11 still contained detectable levels of PSTVd. Most of these losses are probably the result of “natural elimination”. After several passages, tubers often became too small to be replanted; in other cases, small tubers showing good symptoms rotted

during storage, and only larger (possibly uninfected) tubers were available for replanting.

As shown in Table 1, the isolates examined were derived primarily from the northwest and central regions of Russia, with smaller number from other regions including the Far East. Collection dates spanned a total of 15 years; i.e., from 1993 (isolate VNIIF_93) to 2007 (isolates VNIIF_07/D16, VNIIF-07/K, and VNIKH_07/42M). In all, complete nucleotide sequences of 39 different PSTVd isolates were determined from their uncloned PCR products. Five of these isolates were derived from tomato plants

that had been inoculated with total potato or tomato RNA preparations extracted from plant material collected during previous spindle tuber outbreaks in European Russia and stored frozen at -20°C.

Geographic distribution of individual variants. Examination of the data summarized in Table 1 revealed that two PSTVd sequence variants are particularly widespread in Russian seed potatoes. One (VNIIF_93/GenBank M14814) is a mild variant that was previously detected in Germany; the second variant (Bugry_97) was first detected in our preliminary characterization of Russian PSTVd isolates and

Table 2. Sequence differences between Russian variants and *Potato spindle tuber viroid* (PSTVd)-Intermediate strain (GenBank M18626)

Isolate name ^a	Potato variety	Region of origin	Size ^b	Sequence changes ^c	Identity
Onega-Premier_94	Nevskii	Northwest	358	A120U -A121	GenBank EF044304 (Russia)
Niva_95	Lugovskoi	Northwest	359	A120U -A121 +U141a	GenBank EF044302 (Russia)
VNIIF_07/D16	Hybrid 801002 (CIP)	Central	358	A120U -A121 C193U	
VNIIF-93 (RNA)		Central	359	A120U	GenBank M14814 (Germany)
VNIIF_06/K	Nevskii	Central		-A121	
Kamchatka_06	Adretta	Far East		A310U	
Osetia_06/17B3	Volzhanin	N-Caucasus		+U313a	
VNIIF_07/D13	Hybrid N 800997	Central			
VNIKH_07/68M	Hybrid 2292-19	Central			
VNIKH_07/49M	Hybrid 2520-154	Central			
Kh_06/28B	Android	Far East			
Kh_06/29B clone 8	Udacha	Far East			
Kh_06/30B	Veteran	Far East			
Kh_06/31B clone 13	Nevskii	Far East			
Samara_97/2	Resurs	Volga River	359	A120U -A121	
Onega_94/2	Nevskii	Northwest		C256U A310U +U313a	
Kh_06/29Bclone1	Udacha	Far East	359	A120U -A121 U306A A310U +U313a	
VIZR_06/6L-07	Nevskii	Northwest	356	A120U -A121	
VIR-06/9L	Resurs	Northwest		-A126	
VIR-06/5L1-08	Nevskii	Northwest		A142U G143U A219G -C237 U309A C311U	
Bugry_97	Lugovskoi	Northwest	358	A120C -A121	GenBank EF044303 (Russia)
VNIKH_94 (RNA)		Central			
Onega_94/1	Lugovskoi	Northwest			
DV_94 (RNA)		Far East			
Penza_96 (RNA)		Volga River			
Tver_97 (RNA)		Central			
VNIKH_06/1	Udacha	Central			
VIZR_06/2L	Agronomicheskii	Northwest			
VIZR_06/8L	Petersburgskii	Northwest			
VIR_06/5L07	Nevskii	Northwest	359	+U62a A120C -A121	

(continued on next page)

^a Isolates listed in Table 1 are shown in bold.

^b Genome sizes are shown in nucleotides.

^c Positions of nucleotide insertions and deletions are indicated by + and -, respectively. Isolates recovered by inoculation of tomato seedlings with RNA stored at -20°C are designated by (RNA). The intermediate strain of PSTVd contains 359 nucleotides (GenBank V01465; 9).

induces intermediate symptoms when inoculated onto sensitive tomato cultivars (13). A third variant (VIZR_06/6L) was recovered three times (and at two different locations) in Northwest Russia. None of the other 14 variants detected have been reported to occur outside Russia.

Table 2 contains a list of all 39 isolates that were sequenced together with (i) their dates of collection and (ii) a description of the sequence changes present. Focusing on the two most widely distributed variants (VNIIF_93 and Bugry_97), their recovery from RNA preparations stored frozen since 1993–1994 as well as living plant material

collected in 2006–2007 demonstrates that both have been present in Russia since at least the early 1990s. Note also that the intermediate variant Bugry_97 was collected from several different locations: Tver' in central Russia, Penza in the Volga River region, and the Far East as well as the northwestern region near Saint Petersburg.

Mutation locations. As shown on the right side of Table 2, comparison of these Russian variants with the type strain (PSTVd-Intermediate; GenBank V01465) revealed several clusters of sequence changes. For example, all 17 variants (i)

contain either an A/U or A/C substitution at position 120 and (ii) lack one of the three A residues at positions 121–123 of the Intermediate strain. The first seven variants listed in Table 2 contain the A/U substitution, and the rest contain an A/C substitution. This A/C change is found only in variants from Russia. A large number of Russian variants also contain two additional changes in the pathogenicity domain: an A/U substitution at position 310 and an extra U residue at position 313a. One final change (either a C/U or C/A substitution at position 256) is located in or near the loop E motif in the central

Table 2. (continued from previous page)

Isolate name ^a	Potato variety	Region of origin	Size ^b	Sequence changes ^c	Identity
VNIIF_07/K	Lugovskoi	Central	359	A120C -A121 A310U +U313a	GenBank EF044305 (Russia)
Samara_97/3 VNIIF_93/Lorkh	Kinel'skaya rosa Lorkh	Volga River Central	359	A120C -A121 C256U A310U +U313a	
VNIKH_07/42M	Hybrid 2567-5	Central	359	A120C -A121 C256A A310U +U313a	
VIZR_06/6L2-08	Nevskii	Northwest	358	G49N A120C -A121 C256A	
Tver'_96	Lugovskoi	Central		A120C -A121 C256U U306A A310C C311U	
VIZR_06/4L	Desnitsa	Northwest	359	+U62a A120C -A121 C214G C216G -A219 A225C +G294a	
VIR_06/7L	Osen'	Northwest	359	A120C -A121 -C212 -G213 -C214 -A219 C223N -A225 +G232a +G249a +G255a +G266a +G278a +G294a	
VIR_06/10L	Temp	Northwest	361	A120C -A121 -A219 C223N A225C +G266a +G281a +G294a +G313a	

portion of the molecule. The potential structural effects of these sequence changes are shown in Figure 2.

The lowest-free-energy structure of PSTVd consists of a series of helical regions separated by interior or bulge loops (27). In a recent study that examined the role of these loops in PSTVd replication and long-distance movement, Zhong et al. (33) assigned a number (1 to 26) to each loop. Following this convention and moving from left to right in Figure 2, we see that insertion of a U residue at position 313a plus an A/U substitution at position 310 is predicted to partially close loop 8. Loop 15 in the central domain is actually not an internal loop at all but rather a highly conserved series of non-Watson-Crick base pairs that is found in many different RNAs and known as a "loop E motif" (4,31). Here, both C/A and C/U changes were found at position 256. The cytosine residue at position 256 is part of an unusual *cis* Watson-Crick/Watson-Crick bifurcated pair involving cytosine 102 that lies just outside the conserved core structure of loop E (31). An earlier study (21) had shown that a C/A substitution at this position does not abolish infectivity, but the effect of a C/U substitution was not tested. Finally, note that the sequence changes at positions 120 and 121 present in all Russian variants likely have only minor effects on the size/structure of loop 17. As shown in Figure 2 (right panel), these two mutations result in a slight enlargement of the loop which separates the central and variable domains of PSTVd.

Phylogenetic analysis of naturally occurring PSTVd variants. These six changes represent only a small part of the sequence diversity uncovered by our survey. As shown in Table 2, three isolates contained a particularly large number of changes: VIR_06/4L, VIR_06/7L, and

VIR_06/10L. The structural effects of certain of these changes (e.g., deletion of an A residue at position 219) appear rather modest, but those associated other changes (insertion of a G residue at position 266a located in the central conserved region, for example) are more difficult to predict. At least two changes were present in all three isolates: the deletion of an A residue at position 219 and the insertion of a G residue at position 294a.

As described in Materials and Methods, the Subviral RNA Database (<http://subviral.med.uottawa.ca>) currently contains the sequences of the type strain (PSTVd-Intermediate = V01465), a mild variant first described in Germany but also found in Russia (VNIIF_93 = M14814), five variants thus far reported only from Russia (EF044302–EFF044305 and EU257478), and more than 60 other naturally occurring variants of PSTVd from around the globe. Including the variants described in Tables 1 and 2, a total of 81 sequences were available for phylogenetic analysis. Several different types of phylogenetic analysis were performed to identify possible evolutionary relationships among the 17 variants known to be present in Russia, and Figure 3 presents the phylogenetic network produced by the SplitsTree4 software package (11). Essentially identical results were obtained by neighbor-joining, maximum parsimony, and minimum evolution analysis using the default settings for the Mega 4.1 software package (results not shown).

Phylogenetic analysis revealed the existence of two large and six smaller clusters of variants with bootstrap values of >70%. One large cluster (PSTVd_010, 28, 45, 97, 100, 101, and 120) contains several PSTVd variants isolated from tomato with possible origins in Oceania (29). Inspection of the underlying sequence alignment revealed that these variants contain ap-

proximately 20 sequence changes concentrated in the pathogenicity and variable domains. A second large cluster contained several less divergent variants isolated in Germany. One of the smaller clusters contained the three most divergent Russian variants (VIR_06/4L, VIR_06/7L, and VIR_06/10L), but as shown in Figure 3, the remaining variants with smaller numbers of sequence changes formed a rather undifferentiated "starburst" pattern with no obvious sign of geographical clustering. Because it does not assume a priori the existence of a bifurcating tree-like pattern of relationships, the SplitsTree algorithm is well-suited for an analysis of this sort.

When standard phylogenetic methods failed to identify possible relationships among our Russian variants, we decided to re-examine this question by manually mapping as many sequences as possible onto a two-dimensional network. As shown in Figure 4, a comparatively simple network involving only three clusters of sequence changes (at positions 120+121, 310+313a, and 256) and two major lineages was able to accommodate almost two-thirds of the variants detected. Several variants from VIR and VIZR lacked the changes at positions 310 and 313a but contained a large number of changes at other positions (Table 2). How more divergent variants like VIR_06/4L, VIR_06/7L, or VIR_06/10L are related to the network shown in Figure 4 is not clear.

In considering possible precursor-progeny relationships among these different variants, it is important to note that (i) the Intermediate (type) strain of PSTVd has not been found in Russia and is included solely as a reference, (ii) any step(s) in this network could be reversible, and (iii) the density of our sampling was not sufficient, either geographically or temporally, to indicate the order in which individual sequence changes may have

Fig. 2. Potential effect of sequence changes on the structure of three *Potato spindle tuber viroid* (PSTVd) interior loops. From left to right, the lowest-free-energy structure of PSTVd contains 2 terminal hairpin and 25 interior or bulge loops. Loops essential for replication (R) or trafficking (T) are marked (35) as are the boundaries of the five pospiviroid structural domains: the terminal left (TL) and terminal right (TR), pathogenicity (P), central (C), and variable (V) domains. Vertical arrows denote the locations of selected mutations in Russian variants located within/near loops 8, 15, and 17 (dashed circles). In the enlarged panels below, these base changes are shown in bold, and small open circles indicate known non-Watson-Crick base pairs. Y, U or C; W, A or U.

appeared. Inspection of the data presented in Table 2, however, indicates that both of the most commonly encountered variants (shown shaded in Figure 4) were present in samples obtained from the All-Russian Potato Research Institute (VNIKH).

DISCUSSION

During the Soviet era, exchange of potato germplasm between Russia and non-Communist countries was restricted, and a combination of factors (e.g., the widespread use of meristem tip culture to produce virus-free plants and the failure to test these plants for the presence of PSTVd) eventually led to an epidemic of spindle tuber in Russian seed potatoes. Several of the PSTVd variants described above were collected during the later stages of this epidemic in the early to middle 1990s. By comparing these older variants with others collected during the last several years, we hoped to reconstruct at least the broad outlines of this epidemic. We also considered it likely that the nature of certain sequence changes would offer new insights into viroid-host interaction. Many different isolates of PSTVd were collected and sequenced during earlier eradication efforts in North America and Western Europe, but to our knowledge, this is the first study to examine PSTVd population diversity over a wide geographical area.

Two PSTVd variants appear to be widely distributed in Russia. One (VNIIF_93 = GenBank M14814) is a mild variant previously isolated from potatoes growing in Germany; the second (Bugry_97) induces symptoms of intermediate intensity in tomato (13) and has not been reported to occur outside Russia. These two variants differ in the nature of the nucleotide substitution at position 120 (A/U versus A/C); in addition, VNIIF_93 contains two additional changes in the lower portion of the pathogenicity domain. Considering the isolation dates for certain other variants in our collection (e.g., VNIIF_07/K), it seems clear that the two lineages represented by these variants have been circulating in Russian seed potatoes for at least 15 years.

Only three of 17 variants were isolated on more than one occasion, and even for the widely distributed variants Bugry_97 and VNIIF_93, the combination of small sample numbers and uneven sampling over time and space prevents any conclusions about their relative abundance from place-to-place or year-to-year. It is probably not a coincidence, however, that both these variants were also recovered from plant material obtained from VNIKH. VNIKH has long played a central role in providing virus-free plantlets to be used by Russian farmers and companies to produce seed potatoes. Any PSTVd variants contaminating this material would be expected to

spread widely and rapidly throughout the country.

Repeated passage of individual cloned PSTVd variants in tomato is often accompanied by the appearance of sequence changes in the progeny (8). In some cases, the founding sequence virtually disappeared from the resulting progeny. It is possible that the sequence network shown in Figure 4 is the result of a similar bottleneck phenomenon in which small numbers of infected plants are dispersed among widely separated locations, but our data are equivocal on this point. The single nucleotide difference between variants VNIIF_93 and Samara_97/2 is consistent with such a scenario; on the other hand, isolation of two other variants (VNIKH_94/Bugry_97 and VNIKH_07/42M) from a single location suggests that sequence change does not require geographical separation. In this case, the differences observed over the 14-year period between the two collections could be due to either sequence drift or introduction of new PSTVd-infected accessions into the VNIKH collection. Additional studies currently underway should yield a more detailed picture of the current distribution of PSTVd variants throughout Russia. For this information to be useful in tracing the source of a particular outbreak, however, a greater number of divergent variants like VIZR_06/4L or VIR_06/7L will be required.

In addition to these potential forensic applications, the pattern of sequence variation uncovered by our studies also throws new light on certain fundamental aspects of viroid biology. For example, RNA folding studies involving PSTVd and all but one of the other currently recognized viroid species have demonstrated an evolutionary trend toward increased structural robustness during viroid radiation (24). As pointed out by the authors, such a result implies that, because of the filtering effect of natural selection, sequence variation in natural viroid populations should have a smaller effect on predicted secondary structure than randomly generated changes.

Until recently, nucleotides in viroid loops and bulges were generally considered to be unpaired/unstructured. It is now clear, however, that most of these loops and bulges are actually highly structured, three-dimensional motifs, formed by non-Watson-Crick base pairing, base stacking, and other base interactions, and serving as

sites for RNA-RNA, RNA-protein, and RNA-small molecule interaction. Examples of loops known to be important for PSTVd function include (i) the loop E motif in the central domain (34) and (ii) a small 2-nt internal loop on the left side of the pathogenicity domain that is necessary for PSTVd movement from the bundle sheath into the phloem (32). Recently, Zhong et al. (33) examined the effect of closing each of its 27 loops/bulges on the ability of PSTVd to replicate in single cells and move systemically. As shown in Figure 2, the motifs most critical for replication are clustered in the distal end of the left terminal domain and central region of PSTVd; trafficking motifs, in contrast, are located in the variable and right terminal domains, the proximal end of the left terminal domain, and at the junction of pathogenicity/central domains. Several of the sequence changes most commonly observed among our Russian variants are located outside these critical regions, sug-

gesting that they may be essentially neutral mutations.

For example, all 17 Russian variants contain changes at positions 120 and 121 in loop 17, a small internal loop located at the boundary between the central and variable domains. Six variants also contain changes at positions 310 and 313a in loop 8. The appearance of a similar pattern of sequence changes was previously reported by Gora-Sochacka et al. (8), who monitored the stability of individual cloned PSTVd variants upon repeated passage in tomato. Unlike the mutations studied by Zhong et al. (33), these naturally occurring changes are not predicted to close either of the loops involved.

Several naturally occurring sequence changes within the loop E motif of PSTVd are known to have dramatic effects on host range and symptom expression (22,30). Located on the right side of the central domain, this motif (loop 15) has recently been the subject of intensive mutational

Fig. 4. Sequence relationships among selected *Potato spindle tuber viroid* (PSTVd) variants. All Russian variants lack one of six A residues found at positions 118-123 in the Intermediate (type) strain. These variants can be divided into two lineages by the nature of the nucleotide found at position 120: U (left) or C (right). Additional changes at positions 310, 313a, and 256 in both lineages account for much of the sequence diversity observed, and the dashed box (right side) indicates a hypothetical intermediate in this diversification process. The most common Russian variants (Bugry_97 and VNIIF_93) are indicated by shading. For clarity, three variants differing from Omega Premier_94 or Bugry_97 by only a single change (Niva_95, VNIIF_07/D16, and VIR-06/5L) have been omitted.

analysis (31). The C/U and C/A substitutions found at position 256 in several Russian variants lie just outside the conserved core of loop E. In PSTVd-Intermediate, C256 forms a *cis* Watson-Crick/Watson-Crick bifurcated pair with the cytosine residue at position 102. Information about such bifurcated base pairs is scarce, but the fact that all three variants are viable suggests that the overall dimensions of C:C, C:U, and C:A pairs are very similar. Further studies will be required to determine the biological effects of these substitutions, but they too may be phenotypically neutral. Another naturally occurring variant from the United States known as "Wisconsin B" (GenBank M88677) contains a G/A substitution at position 254 just outside the loop E motif. As shown in Figure 2, this mutation converts a predicted G:U base pair to an A:U pair and is likely a neutral change.

In conclusion, we have identified a number of what appear to be neutral mutations among 16 novel PSTVd sequence variants recovered from Russian seed potatoes. Russia is the largest country in the world, covering more than one-eighth of the Earth's land area and including a wide range of climates and growing conditions; to our knowledge, ours is the first study of PSTVd population diversity within a single country. Although population diversity appears to be relatively low, the 16 new variants identified represent an approximately 20% increase in the number of naturally occurring PSTVd variants known. More importantly, these new variants provide insight into the selective pressures guiding PSTVd evolution.

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