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The genetic stability of potato spindle tuber viroid (PSTVd) molecular variants

ANNA GÓRA-SOCHACKA,[1] ANDRZEJ KIERZEK,[1] THIERRY CANDRESSE,[2] and WLODZIMIERZ ZAGÓRSKI[1]

[1]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawinskiego 5A, 02-106 Warsaw, Poland
[2]Station de Pathologie Végétale, INRA, BP81, 33883 Villenave d'Ornon Cédex, France

ABSTRACT

RNA viruses propagate as a population of genetically related entities composing a quasi-species. Specific representatives are the result of both a high mutation rate during replication and competition between the continuously arising sequence variants. Similar to other RNA pathogens, potato spindle tuber viroid (PSTVd) propagates as a population of similar but nonidentical sequences. The sequence of progeny molecules derived from cloned molecular variants of PSTVd were studied after one and six consecutive plant passages. Although the severe parental sequence S23 was found to be genetically stable, all five other parental sequences analyzed, irrespective of their pathogenicity, led to the appearance of complex populations. Divergence of the progeny was observed at the sequence level, but also, more surprisingly, at the level of the pathogenicity of individual progeny molecules. In two cases, the parental sequence was retained in the progeny population. In the other cases, it was completely out-competed and eliminated, sometimes in as little as one plant passage. Although it has been observed previously that artificially mutated PSTVd molecules may revert rapidly to the wild-type sequence, this study presents direct evidence for the rapid evolution of naturally occurring PSTVd sequence variants.

Keywords: infectious clones; pathogenicity; quasi-species; sequence diversity; sequence space

INTRODUCTION

Potato spindle tuber viroid (PSTVd) is a nonencapsidated, single-stranded, circular pathogenic RNA of approximately 360 nt. Because PSTVd does not code for proteins, it is completely dependent on the host plant enzymatic system for its replication and for expression of its pathogenicity (for reviews see Riesner & Gross, 1985; Diener, 1987; Semancik, 1987). Based on sequence similarities, Keese and Symons (1985) have proposed that viroids contain five structural domains: a central conserved region (CCR), the pathogenicity (P) and variable (V) domains, and the right (TR) and left (TL) terminal hairpins. The P domain has been associated with viroid pathogenicity (Visvader & Symons, 1986), and Schnölzer et al. (1985) have reported a correlation between the structure of the VM (virulence-modulating) region of the P domain and symptom severity. Comparison of the calculated thermodynamic stability of the VM regions of different PSTVd isolates suggested that the severity of symptoms increases with decreasing thermal stability of the VM region. This tendency is generally observed, although some isolates (Góra et al., 1994) and several PSTVd mutants obtained by site-directed mutagenesis (Hammond, 1992; Owens et al., 1996) do not fully conform to this rule, supporting the idea that local conformation, rather than local secondary structure stability alone may be crucial in determining pathogenicity (Owens et al., 1996).

The sequence of the genomes of more than 20 different PSTVd isolates is known (Gross et al., 1978, 1981; Herold et al., 1992; Owens et al., 1992; Lakshman & Tavantzis, 1993; Góra et al., 1994). These sequences differ mostly in two regions, the P and the V domains. In the remaining domains, sequence variation has also been observed, but only with low frequency. Many in vitro-generated PSTVd sequence mutants have been studied (Hammond & Owens 1987; Owens et al., 1991; Hammond, 1992; Wassenegger et al., 1994). Mutagenesis often abolished infectivity or the mutations reverted to the original sequence (Owens et al., 1991). A minor fraction of the mutations were stably conserved or resulted in compensatory mutations at other positions of the molecule (Owens et al., 1991; Qu et al., 1993; Wassenegger et al., 1994).

Reprint requests to: Anna Góra-Sochacka, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawinskiego 5A, 02-106 Warsaw, Poland; e-mail: annag@ibbrain.ibb.waw.pl.

Sequence heterogeneity has been observed in natural viroid isolates (Visvader & Symons, 1985; Herold et al., 1992; Lakshman & Tavantzis, 1993; Góra et al., 1994; Polivka et al., 1996). In several cases, variants of different pathogenicity have been simultaneously detected in field isolates (Visvader & Symons, 1985; Góra et al., 1994). Such observations indicate that PSTVd, like many other RNA pathogens, probably propagates in the host as a population of similar but nonidentical sequences, fitting the quasi-species concept defined by Eigen (1993). Replication error rate and natural selection are then decisive factors for the establishment of the "fitness landscape" with condensation of variants in peaks of high fitness. In the present study, the genetic stability of PSTVd upon serial passaging was investigated using infectious cDNA copies of natural PSTVd isolates obtained previously (Góra et al., 1994). This approach led to ab initio creation of a "fitness landscape" with the progressive accumulation of new molecular variants. It should be stressed that viroids offer a unique opportunity for this type of study because their small size allows their genomic information to be analyzed completely by sequencing of full-length cDNA clones.

RESULTS

Six previously characterized (Góra et al., 1994) parental sequences were used: one mild sequence (PSTVd-M), three intermediate sequences (PSTVd-I2 [PSTVd-type], PSTVd-I3, and PSTVd-I4), and two severe sequences (PSTVd-S23 and PSTVd-S27). Following inoculation of tomato plants with plasmids containing monomeric cDNAs, the progenies were isolated after one and six consecutive passages, cloned, and sequenced as described in the Materials and methods.

Progeny of parental sequence PSTVd-M

The parental sequence M was the only variant detected originally in the mild field isolate PSTVd-M (Góra et al., 1994). It is related closely to other mild isolates because it differs from isolate KF 6 (Gross et al., 1981) by a single transition (U310C) in the P domain.

The progeny derived from the M parental sequence was found to be heterogeneous, but the parental sequence was retained up to the sixth plant passage. After the first passage, only one of six sequenced cDNAs was identical to the parental sequence. The five other clones carried point mutations in the left half of the molecule, defining a total of four variants (Fig. 1; Table 1). One of the variants, observed twice, differs from the parent by a UC deletion at positions 300–301 in premelting loop 1 (PM-loop 1) of the VM region. As shown in Table 1 for clone M-I-17, the −UC300–301 deletion almost completely abolished infectivity and only very mild disease symptom were observed. Loop size mutations in this region have been observed in mild KF 5 isolate (Lakshman & Tavantzis, 1993) and in mild–intermediate QFA and QFB isolates (Gruner et al., 1995). Another progeny cDNA clone, clone M-I-40, differs from the parent by a single C-U substitution at position 311 in the VM region (Fig. 1; Table 1), a position known to be variable in PSTVd field isolates. This variant was found infectious, and induced symptoms similar to those of the parent. A similar situation was observed with clone M-I-10, which differs from the parental sequence by an A50G substitution in the upper strand of the VM region (Fig. 1; Table 1).

The last mutation observed in the first-passage progeny was the deletion of an unpaired U (−U333) in the TL domain of clone M-I-42 (Fig. 1; Table 1). This mutation reduced the infectivity strongly (1 plant infected of 15 plants inoculated, Table 1). This observation is not surprising because variation is relatively rare in the TL and TR domains of PSTVd, probably indicating a negative effect of such mutations on the fitness of the molecule.

None of the variants observed after the first passage were recovered at the sixth passage, probably an indication of their reduced fitness. Half of the sixth-passage clones were identical to the parental sequence. The other four clones were identical and differed from the parent by a single deletion (−U312) in the VM region (Fig. 1). This deletion did not affect infectivity of clone M-VI-15 (Table 1), but resulted in a more severe symptomatology. This result is not surprising because the −U312 deletion is observed frequently in intermediate severity isolates.

Progeny of intermediate sequence variants

Three parental sequences I2, I3, and I4, which induce intermediate severity symptoms and differ from each other by single point mutations, were used in separate assays. The I2 sequence is identical to the previously described PSTVd-type sequence PSTVd-DI (Gross et al., 1978).

The progeny of I2 was heterogeneous, but the parental sequence was maintained during all passages. After the first passage, three of seven sequenced clones retained the I2 sequence, whereas the other four had an A310U transversion in the P domain (Fig. 1). This variant is infectious, but the symptoms were significantly milder than those of the parent (Table 1), which may correlate with a predicted increased thermal stability of the VM region.

After the sixth passage, nine clones were analyzed. Four were identical to the parent, whereas the other five contained single point mutations. Three transitions, A173G (clone I2-VI-50, observed twice), U186C (clone I2-VI-16), and U187C (clone I2-VI-25), were located in the TR domain, whereas the last mutation was

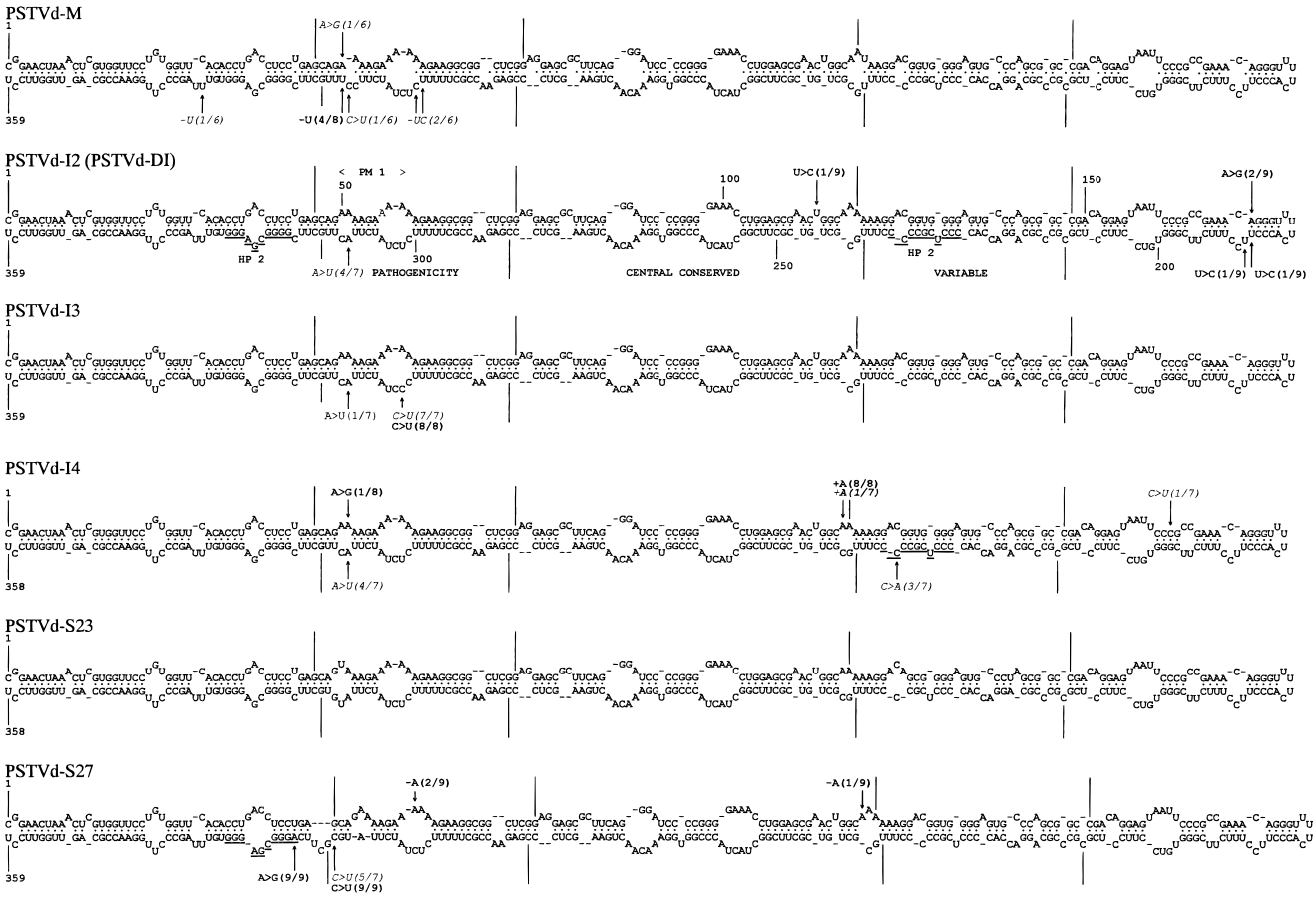

FIGURE 1. Nucleotide sequence and predicted secondary structure of the six parental PSTVd sequences submitted to genetic stability analysis. Nucleotide changes appearing in the progeny of the parental sequences after the first plant passage are indicated italics. Changes detected after the sixth passage are shown in bold type. Number of clones carrying a given mutation per number of sequenced clones is given between brackets. PSTVd structural domains are delimited by vertical bars. Premelting loop I of the virulence modulating region is indicated in PSTVd-DI (PM 1) as well as secondary hairpin two (HP 2, underlined).

the substitution of an unpaired U (U114C, clone I2-VI-27) in the CCR region (Fig. 1). The A173G mutation was found to abolish infectivity in our assay system (Table 1), whereas the U186C mutation decreased infectivity considerably. Interestingly, these two mutations affect the same base pair in the native rod-like structure. Mutations in the terminal domains may affect viroid movement in plants significantly (Owens et al., 1991; Hammond, 1994), a possible explanation for the negative effects of these mutations in our experiments. The two other mutations did not affect infectivity or symptomatology significantly (Table 1).

Parental sequence I3 differs from PSTVd-DI by a single U302C substitution within premelting loop I. This substitution is not stable because complete reversion to the PSTVd-DI sequence was observed after the first passage and confirmed in the sixth-passage progeny. During the first passage, a clone with an additional mutation also detected in the I2 progeny (A310U) was observed. The elimination of the I3 sequence from the progeny is probably a consequence of its very low infectivity compared with PSTVd-DI (Góra et al., 1994).

Parental sequence I4 differs from PSTVd-DI by a single deletion of an A within a string of six at positions 118–123 in the V domain. A slightly lower infectivity of I4 compared with PSTVd-DI was noted originally (Góra et al., 1994). As in the case of I3, I4 was not stably maintained, although reversion to PSTVd-DI appeared to be slower. Reversion was observed in one of the seven clones sequenced after the first passage, but was complete after the sixth passage (Fig. 1). In addition to this reversion, several other point mutations in domains P, V, and TR were detected.

After the first passage, three mutations were observed (Fig. 1), either alone or in combination, yielding a total of four variants. Two double mutants were detected sharing the A310U mutation, also observed in single mutant I4-I-42: clone I4-I-10 (C235A and A310U) and clone I4-I-37 (C164U and A310U). No infectivity could be observed for clone I4-I-37 (Table 1). Because mutation A310U is shared by infectious molecules, it is likely that the debilitating effects observed are caused by the C164U mutation, which alters the TR region. As noted above, the A310U mutation appears to be asso-

TABLE 1. Symptoms induced and infectivity of plasmids containing a monomeric cDNA copy of the various PSTVd molecules analyzed in this study.[a]

Clone	Mutation frequency	Mutation	Domain	Infectivity	Symptoms
M (parent)	n.a.	n.a.	n.a.	10/10	Mild
M-I-10	1/6	A50G	P	5/15	Mild
M-I-17	2/6	−UC300-301	P	2/21	Very mild
M-I-40	1/6	C311U	P	11/15	Mild
M-I-42	1/6	−U333	TL	1/15	Mild
M-VI-15	4/8	−U312	P	15/19	Mild–intermediate
i2 (parent)	n.a.	n.a.	n.a.	10/10	Intermediate
i2-I-14	4/7	A310U	P	7/15	Mild
i2-VI-16	1/9	U186C	TR	3/19	Intermediate
i2-VI-25	1/9	U187C	TR	10/19	Intermediate
i2-VI-27	1/9	U114C	CCR	19/19	Intermediate
i2-VI-50	2/9	A173G	TR	0/31	n.a.
i4 (parent)	n.a.	n.a.	n.a.	6/10	Intermediate
i4-I-10	2/7	C235A, A310U	P and V	12/18	Mild
i4-I-37	1/7	C164U, A310U	P and TR	0/15	n.a.
i4-I-42	1/7	A310U	P	2/10	Mild
i4-VI-17	1/8	+A118, A51G	P and V	0/17	n.a.
s27 (parent)	n.a.	n.a.	n.a.	2/10	Severe
s27-I-8	5/7	C313U	P	11/22	Mild
s27-VI-19	2/9	−A57, C313U, A319G	P and TL	3/25	Mild
s27-VI-106	6/9	C313U, A319G	P and TL	17/19	Severe

[a] Infection was monitored by nucleic acid hybridization of tomato plant extracts 5–6 weeks after inoculation with plasmid DNA (2 μg/plant). Symptoms were assessed 3–6 weeks postinoculation. Frequency of a given mutation in the progeny is expressed as the number of cDNA clones with the mutation per number of sequenced clones. Infectivity is expressed as the number of infected plants per total inoculated plants. n.a., not applicable.

ciated with milder symptoms. This was confirmed for the two viable I4 progeny molecules harboring it (Table 1). The single C235U substitution in the V domain observed three times disrupts a C:G base pair in the core of HP II. This mutation was viable, as demonstrated by the infectivity of variant I4-I-10 (Table 1), but was eliminated from sixth-passage progeny, probably an indication of reduced fitness (Loss et al., 1991; Qu et al., 1993). As noted above, the sixth-passage population showed complete reversion to the PSTVd-type sequence with six A's at positions 118–123. One of the clones additionally contained an A51G mutation in the P domain (Fig. 1). Although located within a loop, this mutation abolished the infectivity under our assay conditions (Table 1).

Progeny of severe sequence variants

Two variants, S23 and S27 (Góra et al., 1994), were submitted to analysis. The S23 parent was found to be stable genetically, all clones sequenced after the first and the sixth passages being similar to the parent. Variant S27 contains a G319A exchange in the terminal part of secondary hairpin II (Góra et al., 1994). Although not detected after the first passage, reversion to the wild-type hairpin II sequence was complete after the sixth passage (Fig. 1).

After the first passage, only one variation was observed: five clones of seven had a C313U transition in the lower strand of the VM region (Fig. 1). This mutation was stably maintained upon further passaging. It increased cDNA infectivity, but reduced the severity of the symptoms (compare parental S27 sequence with progeny S27-I-8 in Table 1). This mutation is predicted to result in an important local rearrangement of the lowest free energy structure (not shown), which may explain its effects on symptom severity.

After the sixth passage, both the C313U substitution and the reversion to the wild-type HP II sequence (A319G), were detected in each of the nine sequenced clones (Fig. 1). A representative clone, S27-VI-106, was highly infectious and induced severe symptoms (Table 1). Two other mutations were observed in some of the sixth-passage clones. The deletion of one A within a string of six at positions 118–123 was observed once. Another deletion of one A within a string of six (positions 55–60) was observed twice (Fig. 1). The plants inoculated with a representative clone (S27-VI-19) showed only mild symptoms, whereas secondary structure calculations predicted a significantly higher thermal stability for the VM region (not shown).

PSTVd variants as a complex quasi-species

The generation of new PSTVd variants and the phenotype conversions observed suggest that PSTVd propagates as a complex population best described by the Eigen theory (Eigen, 1993). A diagram based on the

sequence space concept and showing the statistical geometry of the relationships between the sequences was therefore constructed (Eigen & Winkler-Oswatitsch, 1990), using the sequence of the clones described in this study. Because the sequences used were detected in experiments conducted under stable greenhouse conditions, they can be considered to reflect the diversification of PSTVd under similar selective pressures. The diagram obtained is illustrated in Figure 2. It is clear that in terms of the Eigen theory, these sequences compose a typical quasi-species with a rather high mutation rate, with an average box length of 0.46 and an average protrusion length of 1.13.

DISCUSSION

Field and greenhouse viroid isolates often have been found to be, in fact, a population of molecular variants. This was documented for citrus exocortis viroid (Visvader & Symons, 1985), avocado sunblotch viroid (Rakowski & Symons, 1989), grapevine yellow speckle viroid (Rigden & Rezaian, 1993; Polivka et al., 1996), as well as for PSTVd (Góra et al., 1994; Gruner et al., 1995). The isolate heterogeneity may result from coinfection and co-propagation of different versions of PSTVd or from the accumulation of mutants appearing de novo during the replication of the parental genome. This latter possibility is suggested by the error-prone nature of RNA replication and by isolated cases of phenotype conversion upon propagation of PSTVd-DI (Gruner et al., 1995). In addition, numerous reports indicate that in vitro constructed PSTVd mutants may revert rapidly to wild-type or may accumulate new, compensatory mutations (Loss et al., 1991; Owens et al., 1991, 1995; Qu et al., 1993; Hu et al., 1996).

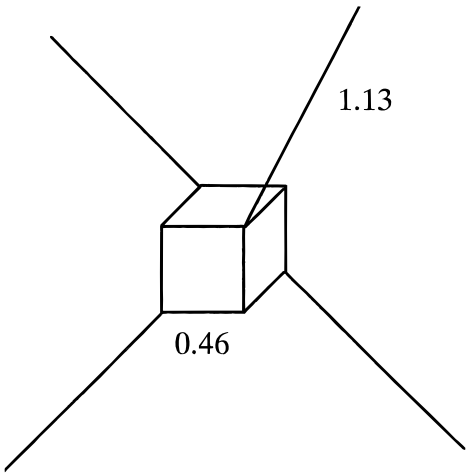

FIGURE 2. Average diagram of $\binom{92}{4}$ = 2,794,155 quartet combinations of 92 PSTVd sequences in RY sequence space.

However, no efforts had been devoted to the study of the build-up and evolution of PSTVd populations from isolated, naturally occurring PSTVd sequences.

To maximize the homogeneity of the starting populations, full-length monomeric cDNA clones were used for plant inoculation during this study. After the first and the sixth plant passage, the fate of the parental genome was analyzed by sequencing several independently obtained, complete cDNA clones. It may be argued that transcription of the cDNA molecules into infectious viroid RNA probably involves different enzymes than the normal PSTVd replication process. This early step may therefore differ significantly from later events in terms of mutation frequency and character. However, this transient expression step probably lasts for only a few days, and thus only represents a minor fraction of the first-passage events. For example, systemic invasion of the plant is usually complete three weeks postinoculation and symptoms begin to be visible at this time (Góra et al., 1996). As a consequence, first-passage progeny, analyzed 5–6 weeks postinoculation, have developed through the natural mutation/selection system for essentially as long as the progenies derived from later passages and should, therefore, be essentially comparable with them.

Only three of the progeny molecules obtained were found to be noninfectious. These may result from errors of the enzymes used during the cloning procedure or may truly represent members of the sampled populations. In the latter case, how can apparently noninfectious molecules be detected among the viroid population? One possibility is that these molecules could be defective only under our assay conditions. The infectivity of plasmids containing monomeric cDNA inserts is known to be two to three orders of magnitude lower than the infectivity of excised monomers or of multimeric constructs (Tabler & Sanger, 1984). The use of a more efficient inoculation technique may therefore have revealed the infectivity of clones found noninfectious under the present assay conditions. Another interesting possibility is that these clones may be defective for a late function, such as movement within the plant. In this respect, it is noteworthy that two of the noninfectious clones contain mutations in the TR region, which has been reported to be associated with movement of the viroid within the plant (Owens et al., 1991; Hammond, 1994).

The present results demonstrate unambiguously that the propagation of unique PSTVd genomes is, in most cases, followed by the accumulation of new sequence variants, creating de novo a population. The quasi-species concept developed by Eigen, defined as "a complex, self-perpetuating population of diverse, related entities acting as a whole" (Eigen, 1993), therefore clearly applies to viroids. Statistical geometry was used to analyze the variability of PSTVd in our data set. The diagram developed for the HIV hypervariable region

indicates much more diversity than for PSTVd, which, in turn, shows higher diversity than the extremely stable poliovirus sequences (Eigen & Winkler-Oswatitsch, 1990).

The diagram analysis shows that mutations occur rather freely (average box length, 0.46; average protrusion, 1.13). Appropriate values are almost identical for V (0.22 and 0.81) and P domains (0.33 and 0.19) (result not shown). The P domain being decisive for phenotype determination (Góra et al., 1996), a distinct possibility exists for phenotype conversion upon passaging. Indeed, conversion to either more severe or, on the contrary, milder symptomatology was observed in a number of cases in these experiments. Although phenotype conversion has also been reported upon propagation of a natural PSTVd-DI population (Gruner et al., 1995), it appeared to be rather infrequent, maybe due to the relative stability of PSTVd-DI and to the capacity of some PSTVd variants to mask the symptomatology of other isolates (Fernow, 1967; Visvader & Symons, 1985; Branch et al., 1988; Góra et al., 1994). Gruner et al. (1995) reported that PSTVd strains of higher pathogenicity may out-compete lower pathogenicity ones. They correlated this phenomenon with a higher replicability linked to lower thermal stability and, therefore, to the ability to undergo more readily the structural transitions thought to play an important part in the replication process. From our experiments, it is not clear that a selective advantage is linked systematically to increased (or high) pathogenicity. In particular, after one passage, severe parent S27 was almost eliminated (only two of seven clones analyzed) by a mild variant. Similarly, intermediate parent I4 was eliminated from the infective pool by variants of similar severity, but harboring mutations in the V domain, in line with observations of Hu et al. (1996), who showed that mutations in this region may affect PSTVd replicability significantly.

Due to the fact that PSTVd does not have its own replication machinery, one should assume that, in the same host, the replication error rate is equal for all variants. Therefore, the genetic stability of the S23 sequence cannot be explained by a lower replication error rate. We rather interpret this stability in terms of the local shape of fitness landscape, with S23 being "trapped" in a very steep local maximum of fitness. Any point mutation would likely produce mutants of lower fitness, which would be outcompeted by the parental sequence. Thus, the distribution of the progeny sequences of a high fitness parent such as S23 will be biased strongly toward the parental sequence. By opposition, parental sequences that are out-competed, such as I3 and I4, can be seen as occupying valleys of low fitness so that there are easy routes to the accumulation of variants of higher fitness, which can be detected easily by the sparse sampling used.

MATERIALS AND METHODS

Viroid isolates

The sequence variants M, S23, S27, I2, I3, and I4 were detected initially in natural PSTVd isolates and cloned as infectious monomeric cDNAs (Góra et al., 1994).

Plant passages of PSTVd variants and infectivity assays

Tomato seedlings (cv. Rutgers) were inoculated with 2 μg of recombinant plasmid (Góra et al., 1994) containing a complete monomeric cDNA copy of the appropriate variants. Five to six weeks after inoculation, leaves collected from five infected plants (2 g) were ground with phosphate buffer and used to infect the next set of tomato seedlings. Six consecutive passages were performed, spanning a period of 8 months. Plants were kept under greenhouse conditions and monitored weekly for symptom appearance. For infectivity assays, a similar inoculation procedure was used. Plants were assayed for PSTVd infection by molecular hybridization with a PSTVd-specific RNA probe 5–6 weeks after inoculation (Welnicki & Hiruki, 1993).

PSTVd RNA extraction

Total nucleic acids from infected leaves were phenol extracted and PEG fractionated as described (Welnicki et al., 1989). Viroid RNAs were further purified by small-scale batch chromatography on DEAE cellulose (Whatman DE52) (Góra et al., 1994).

cDNA synthesis, cloning, and nucleotide sequence analysis

Full-length, infectious double-stranded cDNAs were synthesized and cloned into pUC9 as described previously (Góra et al., 1994). Sequences were determined on plasmid DNA using a fluorescent primer sequencing kit and an automated A.L.F. sequencer (Pharmacia). The sequences were compiled using the GCG programs (Wisconsin Package version 8, Genetics Computer Group, Madison, Wisconsin, USA). Progeny cDNA clones are named after their parental sequence followed by a roman numeral identifying the passage number and by the appropriate arabic clone number. Thus, for example, M-I-17 denotes a sequence identified in clone no. 17 obtained from the first-passage plants inoculated with the M parental sequence.

Diagram of binary sequence

The 92 PSTVd sequences detected in this study were converted into binary sequences by assigning unity to purines and zero to pyrimidines. Statistical diagrams were then calculated according to the algorithm presented in Eigen and Winkler-Oswatitsch (1990) implemented by a program written by the authors.

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