Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9397–9401, September 1996
Agricultural Sciences

Mexican papita viroid: Putative ancestor of crop viroids

(plant diseases/potato spindle tuber disease/epidemiology/quarantine/quasispecies)

JUAN PABLO MARTÍNEZ-SORIANO*†, JORGE GALINDO-ALONSO‡§, CLARISSA J. M. MAROON¶‖, IREM YUCEL‖**, DENNIS R. SMITH§††, AND THEODOR O. DIENER¶‖†††‡‡

*Instituto Nacional de Investigaciones Forestales y Agropecuarias-Universidad Autonoma de Nuevo León, Apdo. postal 128-F, San Nicolás de los Garza, N.L., Mexico; ‡Colegio de Postgraduados, Montecillos, Mex., Mexico; ¶Department of Plant Biology, University of Maryland and ‖Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, MD 20742; and ††Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705

Contributed by Theodor O. Diener, June 14, 1996

ABSTRACT **The potato spindle tuber disease was first observed early in the 20th century in the northeastern United States and shown, in 1971, to be incited by a viroid, potato spindle tuber viroid (PSTVd). No wild-plant PSTVd reservoirs have been identified; thus, the initial source of PSTVd infecting potatoes has remained a mystery. Several variants of a novel viroid, designated Mexican papita viroid (MPVd), have now been isolated from *Solanum cardiophyllum* Lindl. (papita güera, cimantli) plants growing wild in the Mexican state of Aguascalientes. MPVd's nucleotide sequence is most closely related to those of the tomato planta macho viroid (TPMVd) and PSTVd. From TPMVd, MPVd may be distinguished on the basis of biological properties, such as replication and symptom formation in certain differential hosts. Phylogenetic and ecological data indicate that MPVd and certain viroids now affecting crop plants, such as TPMVd, PSTVd, and possibly others, have a common ancestor. We hypothesize that commercial potatoes grown in the United States have become viroid-infected by chance transfer of MPVd or a similar viroid from endemically infected wild solanaceous plants imported from Mexico as germplasm, conceivably from plants known to have been introduced from Mexico to the United States late in the 19th century in efforts to identify genetic resistance to the potato late blight fungus, *Phytophthora infestans*.**

Since the discovery of viroids (1), the smallest known agents of infectious disease, many of their properties have been elucidated, such as their molecular structures (2, 3), aspects of their replication mechanisms (4–6), and possible evolutionary relationships among themselves and with viroid-like satellite RNAs (7). One of the most enigmatic problems remaining concerns the evolution of viroids from other RNAs and the source of viroids causing diseases of crop plants (for a review, see ref. 8). In contrast to certain virus diseases of cultivated plants, which are known to have existed centuries ago, the diseases now known to be viroid-incited have come to the attention of farmers and plant pathologists only during the 20th century (9). It appears likely that some changes in agricultural practices, such as large-scale monoculture of genetically identical crop plants, have favored their appearance, which has caused economical problems in the recent past. But where did the causative viroids come from?

Conceivably, viroids could have originated *de novo* in the cultivated plant species themselves, for example, by mutation of normal cellular RNAs, but in view of the fact that genomic DNA of viroid host plants does not contain discernible viroid-related sequences (10–12), a *de novo* origin from host genetic material appears ruled out. More plausibly, viroid reservoirs exist in wild plants, and viroid diseases of crops originate by chance transfer of viroids from wild, possibly symptomless, carrier species to cultivated plant species. Indeed, the experimental host ranges of several viroids include numerous wild species (9), and many of these tolerate viroid replication without the appearance of recognizable symptoms.

Circumstantial evidence indicates that one viroid, the tomato planta macho viroid (TPMVd) (13), has entered tomato plants, possibly by a demonstrated aphid vector (14), from endemically infected, native solanaceous plant species (15).

The origin of the potato spindle tuber viroid (PSTVd), however, which causes the oldest known viroid disease, potato spindle tuber (16), has been a mystery; the viroid has not been found in wild plants, including plants collected from areas where the disease had first been reported (the northeastern United States) (9). It is conceivable, however, that PSTVd has entered potato cultivars from endemically infected wild plants imported from other geographical locations as germplasm for genetic breeding. Transmission could have occurred by mechanical contact with breeding stock or, because PSTVd is vertically transmissible in some hosts (17–19), through the (true) seed or pollen from infected wild plants. PSTVd variants have indeed been identified in several major germplasm collections, but the source of these viroids cannot be traced back to wild solanaceous plants (20–23). The cultivated potato (*Solanum tuberosum* L.) originated in the Andes of South America (24), but a search for the presence of PSTVd (or of similar viroids) in more than 5000 specimens of Andean solanaceous plants, including some known to have been used in potato breeding, remained fruitless (22).

Solanaceous plants native to Mexico have also been used in potato breeding (24). Although PSTVd is not known to occur in cultivated potato in Mexico (25, 26), one of us (J.G.-A.) considered it possible that the viroid might nevertheless occur in native solanaceous plants (27).

Here we report that several wild, symptomless *Solanum cardiophyllum* Lindl. (papita güera or cimantli) plants from Mexico are infected with a novel viroid, designated Mexican papita viroid (MPVd), that may be an ancestor of TPMVd, PSTVd, and possibly of other PSTVd-group viroids now infecting crop plants.

MATERIALS AND METHODS

Plant Collections and Initial Assays. Leaves and tubers from 13 solanaceous plant species growing wild at Ojuelos in the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PSTVd, potato spindle tuber viroid; MPVd, Mexican papita viroid; TPMVd, tomato planta macho viroid.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L78454 and L78456–L78463).

†Present address: Unidad de Biotecnología e Ingeniería Genética de Plantas, Centro de Investigacion y Estudios Avanzados del IPN, Apdo. postal 629, 36500 Irapuato, Gto., Mexico.
§Retired.
**Present address: Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705.
‡‡To whom reprint requests should be addressed.

Table 1. Replication and symptoms of MPVd, PSTVd, and TPMVd in differential hosts

Differential host	Viroid		
	PSTVd*	MPVd	TMPVd
N. glutinosa	Flower variegation	Flower variegation	No variegation
G. globosa	Slight replication	No replication	Good replication

*PSTVd data from ref. 37.

Mexican state of Aguascalientes were collected and assayed for viroid infection in the greenhouse by mechanical inoculation of small tomato (*Lycopersicon esculentum*, cv. Rutgers) plants. Tomato plants developing symptoms were processed as described below.

Extraction and Isolation of MPVd. MPVd variants were propagated in Rutgers tomato plants mechanically inoculated with leaf or tuber extracts from papita plants. Tomato leaves showing symptoms characteristic of viroid infection were harvested 4–5 weeks after inoculation, RNAs were extracted, and the viroids were purified following a combination of modified, previously described procedures (28, 29). Briefly, leaves frozen in liquid nitrogen were ground to a fine powder with a mortar and pestle and homogenized in TES extraction buffer (0.1 M Tris·HCl/0.01 M EDTA/0.1 M NaCl/1% SDS/5 mM DTT/10 mM diethyldithiocarbamate, pH 8.9) and 10 ml/3.5 g of tissue. The resulting slurry was clarified with an equal volume of phenol/chloroform (1:1, vol/vol), and the aqueous phase was reextracted with an equal volume of phenol/chloroform. Total nucleic acids were recovered by ethanol precipitation at −20°C and resuspension in H_2O. Polysaccharides were removed by ethylene glycol monomethyl ether extraction (29). Nucleic acids recovered by ethanol precipitation were treated with DNAse I. To remove high molecular weight RNAs, the preparations were subjected to CF-11 cellulose chromatography at room temperature. Viroids and other low molecular weight RNAs were adsorbed to CF-11 cellulose (Whatman) in STE buffer (1 M Tris·HCl, pH 7.8/0.1 M EDTA/0.1 M NaCl) plus 35% ethanol and eluted in the same buffer without ethanol. Eluted RNAs were recovered by ethanol precipitation and resuspension in H_2O.

Preparation of MPVd cDNAs. cDNA synthesis was carried out using either random hexamer primers or PSTVd-specific primers, namely cPSTVd (5′-CCCTGAAGAAGCGCTC-CTCCGAG-3′) (30) and cd-1 (5′-GGCGCGAGGAAGGA-CACCCGAAGAAA-3′). PCR assays were carried out using the following sets of PSTVd-specific primers: cPSTVd and hPSTVd (5′-ATCCCCGGGGAAACCTGGAGCGAAC-3′) (30); RAO-2 (5′-GCGGATCCGGTGGAAACAACT-GAAGC-3′) and RAO-33 (5′-GCCGGTACCAGTTCGCTC-CAGGTTTCCCC-3′); RAO-14 (5′-AGGGATCCCCGGGG-AAAACC-3′) and RAO-34 (5′-GCCGGTACCAAGGGCT-AAACACCCTCGCC-C-3′) (courtesy of R. A. Owens, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD); and cd-1 and cd-2 (5′-AAAGAAGGCG-GCTCGGAGGAGCGCTT-3′). These primers were chosen because the primer pair cPSTVd/hPSTVd results in full-length DNA copies of PSTVd, whereas primer pairs RAO-2/RAO-33 and RAO-14/RAO- 34 result in the left and right halves of PSTVd, respectively. Primer pair cd-1/cd-2 was used to obtain the regions containing binding sites for the previous primer pairs. Thirty PCR cycles were carried out using a thermocycler (Perkin–Elmer/Cetus) with denaturation for 1 min at 94°C, annealing for 1 min at 55, 62, or 72°C (depending on the primers used), and extension at 72°C for 1 min (8 min at the final stage). Samples were stored at 4°C before analysis by electrophoresis.

Cloning and Sequencing of MPVd PCR Products. PCR products of the expected size were gel-purified by either the "crush soak" method (31) or Prep-A-Gene (Bio-Rad). Gel-purified RAO-14/RAO-33 PCR products digested with *Bam*HI and *Kpn*I were cloned into pUC 129 (32). Gel-purified, full-length PCR products were end-filled and phosphorylated as described (33) and blunt-end ligated with the *Eco*RV- cut pUC 129.

PCR products cloned into pUC 129 were sequenced using Sequenase DNA Sequencing Kit (version 2.0, United States Biochemical) as recommended by the manufacturer. The other PCR products were directly sequenced using the *fmol* DNA Sequencing System (Promega) as recommended by the manufacturer. Both kits employ the standard dideoxy termination methods using primers previously used in PCR.

Sequence Alignment and Phylogenetic Analysis. MPVd sequences were aligned by CLUSTAL, version 5, using DNA-star's (Madison, WI) LASERGENE software. The resulting alignments were visually inspected to logically place gaps and manually adjusted to maximize sequence similarity. Phylogenetic analysis was carried out using the computer program PAUP, version 3.1 (34). Uninformative characters were excluded from the analyses. To search for the tree with minimal branch length, the Branch and Bound Method was used with the hop stunt viroid (35) or MPVd sequence designated as the outgroup. Bootstrap analysis was carried out with 1000 replicates.

Determination of Most Stable Secondary Structures. Thermodynamically most stable secondary structures of MPVd variants were obtained by the algorithm of Zuker and Stiegler (36).

RESULTS

Detection and Propagation of MPVd Isolates. Approximately 1000 leaf and 200 tuber samples from individual plants

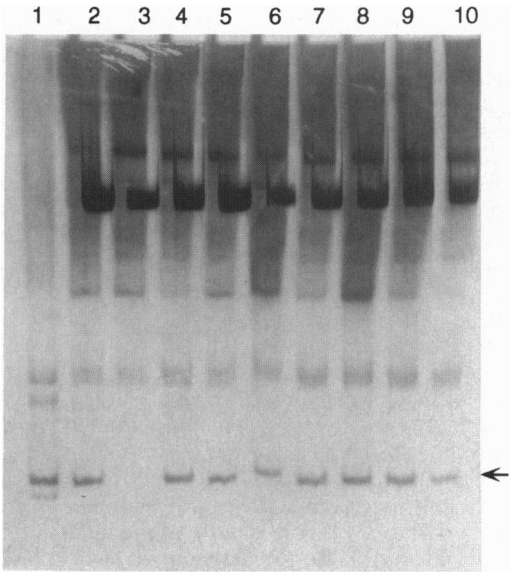

FIG. 1. Return gel electrophoresis of nucleic acid extracts from uninoculated Rutgers tomato, from MPVd-infected *S. cardiophyllum*, and from plants inoculated with previously known viroids. Lanes 1 and 2, low molecular weight RNA and total nucleic acids, respectively, from PSTVd-infected tomato; lane 3, total nucleic acids from uninoculated tomato; lane 4, total nucleic acid from TPMVd-infected tomato; lanes 5–10, total nucleic acid from plants inoculated with sap from six *S. cardiophyllum* plants (OG1 to OG6, respectively).

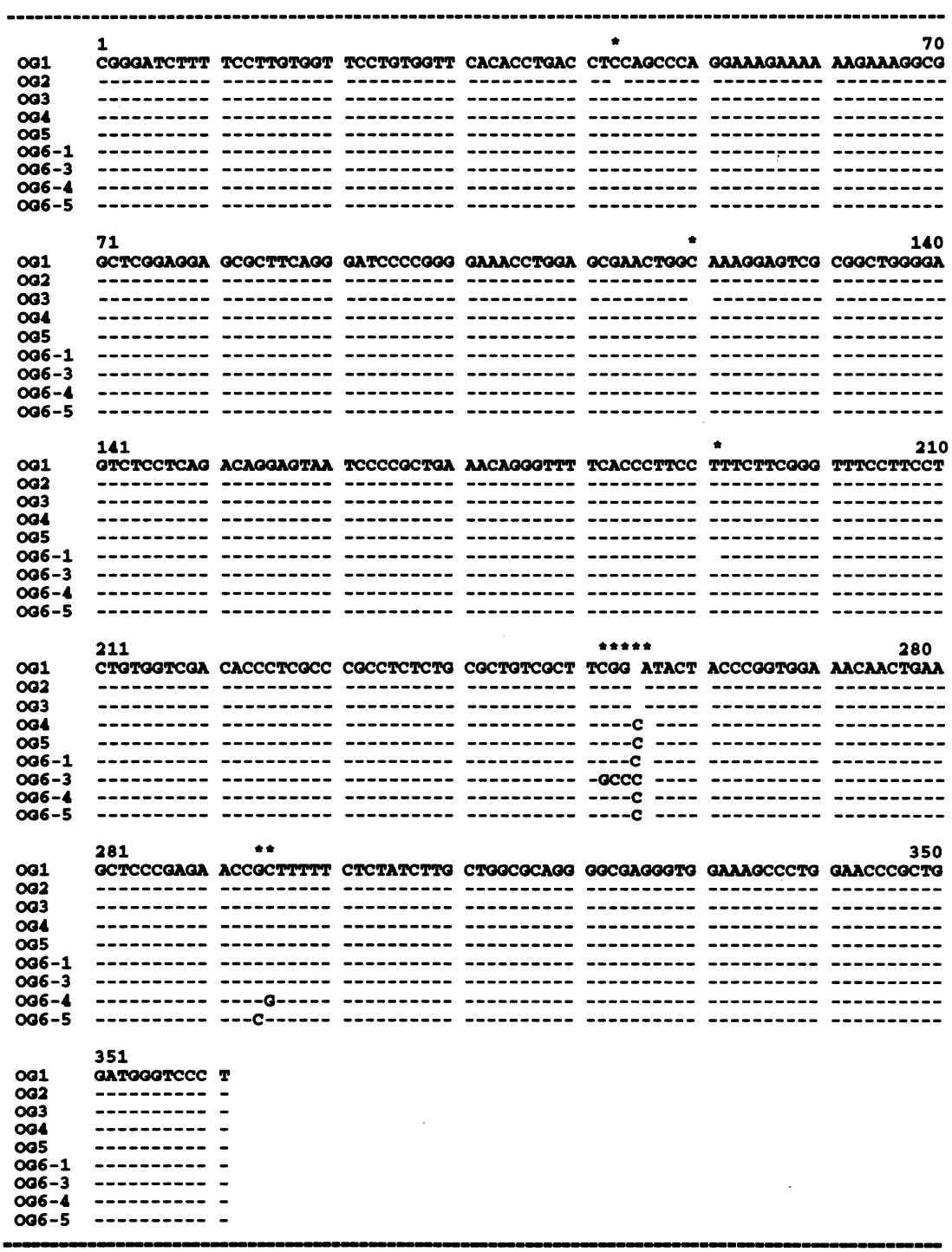

FIG. 2. Nucleotide sequences of the different MPVd isolates (OG1 to OG6).

representing the following 13 wild solanaceous plant species were collected: *Datura metel*, *Datura stramonium*, *Gomphrena* sp., *Nicotiana glauca*, *Physalis foetens*, *Physalis* sp., *Physalis viscosa*, *Solanum cardiophyllum*, *Solanum cervantesii*, *Solanum ehrenbergii*, *Solanum eleagnifolium*, *Solanum heterodoxum*, and *Solanum rostratum*. Symptoms developed only in plants inoculated with leaf or tuber extracts derived from six *S. cardiophyllum* (papita) plants. Symptoms consisted of epinasty, stunting, and some veinal necrosis typical of infection by PSTVd-group viroids, such as PSTVd or TPMVd. The six isolates were individually subcultured in Rutgers tomato.

Differentiation Between TPMVd and the MPVd Isolates. To investigate whether, biologically, the viroid from papita plants is identical with TPMVd (existing in Mexico), differential host species were inoculated with extracts from MPVd-infected tomatoes and others with TPMVd. No symptoms appeared on *Gomphrena globosa* plants inoculated with either viroid, but MPVd-infected *Nicotiana glutinosa* plants consistently developed pronounced flower variegation, whereas the flowers of TPMVd-infected *N. glutinosa* plants remained normal. Back transfers from inoculated *G. globosa* plants to tomato showed that, in contrast to TPMVd, MPVd is not able to replicate in *G. globosa*. Biologically, MPVd properties are thus similar with those previously determined for PSTVd (37) but dissimilar with those of TPMVd (Table 1).

Characterization of MPVd Isolates. Denaturing return gel electrophoresis of total nucleic acid extracts from infected

tomato plants (Fig. 1) revealed the presence, in all six extracts (OG1 to OG6, lanes 5–10, respectively), of a band with the mobility of PSTVd (lanes 1 and 2) or TPMVd (lane 4), whereas extracts from uninoculated plants lacked a band in this position (lane 3).

Viroids purified by polyacrylamide gel electrophoresis were subjected to reverse transcription PCR and the products sequenced directly or after cloning. Eight variant MPVd sequences differ by from one to five deletions or exchanges (Fig. 2). Comparison of MPVd sequences with those of TPMVd and PSTVd (intermediate strain) revealed identities ranging from 89.4% to 92.5% and from 78.3% to 80.5%, respectively.

When folded to obtain the most thermodynamically stable secondary structure (36), MPVd assumes a rod-like conformation typical of other viroids of the PSTVd group, in which short base paired regions alternate with mismatched internal and bulge loops.

A phylogenetic analysis of MPVd confirmed the close evolutionary relationship of MPVd with TPMVd and PSTVd and the more distant relationships to other viroids (Fig. 3).

DISCUSSION

Both sequence and phylogenetic analyses indicate that MPVd is a member of the PSTVd viroid group and that it is most closely related to TPMVd and PSTVd. According to the criteria suggested by the International Committee for the Taxonomy of Viruses, MPVd is a viroid species distinct from PSTVd, but the extent of sequence difference from TPMVd straddles the borderline (90% identity) between viroid variants and distinct viroid species. But, MPVd can readily be distinguished from TPMVd on the basis of biological properties (Table 1); hence, we consider it as a distinct species.

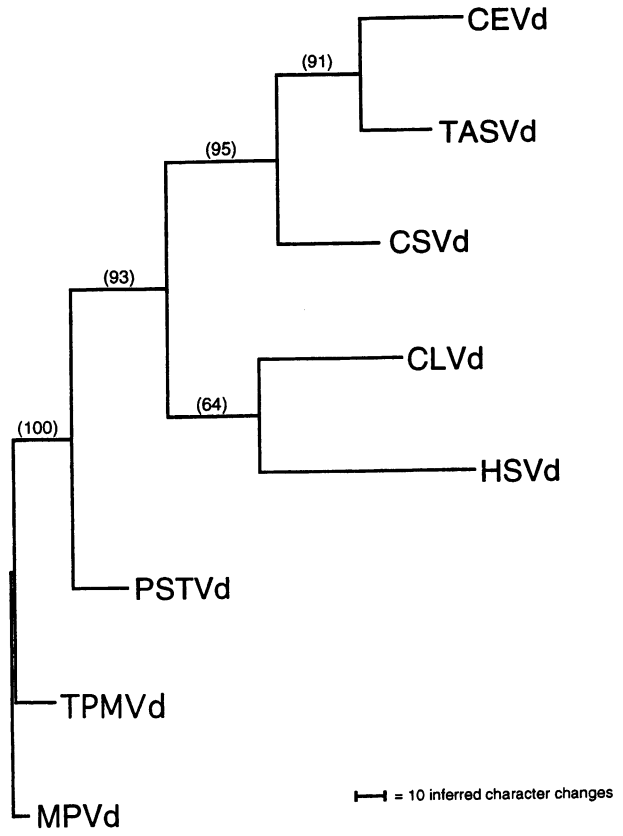

FIG. 3. Consensus phylogenetic tree of MPVd and some previously known viroids. As outgroup, the MPVd sequence was used. Horizontal branch lengths are proportional to the number of character changes. Numbers in parentheses are the percentages of groups being present in 1000 bootstrap replicates. CEVd, citrus exocortis viroid; TASVd, tomato apical stunt viroid (Indonesian strain); CSVd, chrysanthemum stunt viroid; CLVd, *Columnea* latent viroid.

Our discovery of wild, viroid-infected (but symptomless) *S. cardiophyllum* plants growing in rural Mexico, our identification of MPVd as a novel viroid belonging to the PSTVd viroid group, and its close phylogenetic relationship with TPMVd and PSTVd suggest that MPVd represents an ancestral form of extant PSTVd-group viroids that are inciting crop diseases, such as potato spindle tuber, tomato planta macho, and possibly others.

The opposite alternative, that the papita plants might have become viroid-infected by chance transfer of either PSTVd from potato or TPMVd from cultivated tomato plants, can be ruled out for three reasons. (*i*) In Mexico, most commercial potatoes are grown under irrigation, not in rural or undeveloped areas (38). Ojuelos, particularly, is unsuitable for the cultivation of potatoes because of its heavy soil (38). Also, as stated, PSTVd has not been identified in commercial potatoes in Mexico (25, 26). (*ii*) Because of cold winters and lack of rain during summer, Ojuelos is unsuitable for tomato production (38). (*iii*) If papita plants would have acquired their viroids from potato or tomato, one would expect a virulent host–pathogen interaction. Lack of pathology in papita plants suggests coevolution of *S. cardiophyllum* and MPVd. We conclude that MPVd and/or possibly other viroids in wild solanaceous plants are ancestors of extant, PSTVd-group viroids.

How did commercial cultivars become viroid-infected? PSTVd-group viroids have been identified (but not yet sequenced) in wild solanaceous plants growing in the Mexican state of Morelos, close to tomato fields with TPMVd-infected plants (15, 39). Although field observations suggest that tomato plants became infected by spread from wild solanaceous plants (39), the opposite movement cannot be ruled out unequivocally.

How and when PSTVd invaded commercial potatoes can only be speculated upon, but, plausibly, PSTVd or a PSTVd progenitor, such as MPVd, may have entered a germplasm collection by chance transfer from imported, viroid-infected solanaceous plants. Conceivably, this has occurred as a by-product of extensive collections made in Mexico late in the 19th century in efforts to find resistance genes against the late blight fungus (*Phytophthora infestans*) (24), the biological cause of the disastrous Irish potato famine.

Once the viroid had invaded a potato germplasm collection, its eventual worldwide spread is easily understandable in view of the extensive international exchange of potato breeding stocks and centralization of seed potato production that are typical facets of modern agriculture.

TPMVd, in contrast to PSTVd, is not vertically transmitted in tomato (40). This difference, the sexual propagation mode of tomato production, and the geographical separation of commercial potato and tomato production may explain why, in contrast to potato, viroids have not become a problem so far in U.S. tomato production. Even in Mexico, where viroid inoculum was close at hand in wild solanaceous plants, large-scale infection of tomatoes was not observed until the early 1970s (40).

If MPVd is, indeed, ancestral to viroids now infecting cultivated plants, significant sequence changes must have occurred within an exceedingly short evolutionary time frame. Circumstantial evidence suggests that viroids are replicated by normally double-stranded DNA-dependent host RNA polymerase II (5). The mutation (error) rates of RNA polymerases are much higher than those of DNA polymerases (10^{-3} to 10^{-4} vs. 10^{-8} to 10^{-11} per incorporated nucleotide, respectively), mostly because the former lack proofreading and repair mechanisms (41, 42). It is likely that, with an "unnatural" viroid

template (imperfectly base paired single-stranded RNA instead of double-stranded DNA), RNA polymerase II's error rate is larger still.

As a consequence, progeny in each infected plant represents, in Eigen's terminology, a "quasispecies" (43) consisting of a large number of sequence variants, the distribution of which is centered around one or several master sequences (the "wild type" sequence or sequences) (44), representing the fittest variant(s).

Undoubtedly, the sequence variants obtained from viroid-infected papita plants have been selected as the fittest ones within the particular genetic milieu of each infected, sexually propagated plant, which, once synthesized, will be able to "outgrow" all others. This hypothesis implies that viroid sequences are extremely adaptable, i.e., capable of responding rapidly to selective pressures by sequence changes. Recent evidence, obtained in site-directed mutation experiments, indicates that this is indeed the case; some such mutants revert to wild type as rapidly as 2 weeks after inoculation (45–47).

Our detection of endemically viroid-infected, but symptomless, wild plants and their sequence similarities to TPMVd and PSTVd represent the first plausible clues (to our knowledge) as to the initial source of viroids now causing crop diseases in countries other than Mexico. Our results demonstrate the potentially devastating agricultural consequences associated with the international movement of plants. Our hypothesis implies that in efforts to overcome one serious problem, potato late blight, another serious problem, potato spindle tuber, was created by the unwitting importation of an RNA which, although harmlessly replicating in wild solanaceous plants, has become a serious pathogen in commercial potatoes.

We thank Drs. Dawn Gundersen-Rindal and Tim Friedlander for valuable suggestions and assistance in the phylogenetic analyses.

1. Diener, T. O. (1971) *Virology* **45,** 411–428.
2. Riesner, D. (1990) *Semin. Virol.* **1,** 83–99.
3. Diener, T. O. (1991) *FASEB J.* **5,** 2808–2813.
4. Branch, A. D. & Robertson, H. D. (1984) *Science* **223,** 450–455.
5. Schindler, I.-M. & Mühlbach, H.-P. (1992) *Plant Sci. (Shannon, Irel.)* **84,** 221–229.
6. Owens, R. A. & Diener, T. O. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 113–117.
7. Elena, S. F., Dopazo, J., Flores, R., Diener, T. O. & Moya, A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5631–5634.
8. Diener, T. O. (1996) *Virus Genes* **11,** 47–59.
9. Diener, T. O. (1979) *Viroids and Viroid Diseases* (Wiley Interscience, New York), pp. 1–252.
10. Zaitlin, M., Niblett, C. L., Dickson, E. & Goldberg, R. B. (1980) *Virology* **101,** 1–9.
11. Branch, A. D. & Dickson, E. (1980) *Virology* **104,** 10–26.
12. Hadidi, A., Cress, D. E. & Diener, T. O. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6932–6935.
13. Galindo, J. A., Smith, D. R. & Diener, T. O. (1982) *Phytopathology* **72,** 49–54.
14. Galindo, J. A., Lopez, C. & Aguilar, T. (1989) *Rev. Mex. Fitopatol.* **7,** 61–65.
15. Orozco Vargas, G. (1983) M.S. thesis (Colegio de postgraduados, Chapingo, Mex., Mexico), pp. 1–58.
16. Diener, T. O. (1987) in *The Viroids*, ed. Diener, T. O. (Plenum, New York), pp. 221–233.
17. Fernow, K. H., Peterson, L. C. & Plaisted, R. L. (1970) *Am. Potato J.* **47,** 75–80.
18. Diener, T. O. & Raymer, W. B. (1967) *Science* **158,** 378–381.
19. Singh, R. P. (1970) *Am. Potato J.* **47,** 225–227.
20. Owens, R. A., Smith, D. R. & Diener, T. O. (1978) *Virology* **89,** 388–394.
21. Puchta, H., Herold, T., Verhoeven, K., Roenhorst, A., Ramm, K., Schmidt-Puchta, W. & Sänger, H. L. (1990) *Plant Mol. Biol.* **15,** 509–511.
22. Owens, R. A., Khurana, S. M. P., Smith, D. R., Singh, M. N. & Garg, I. D. (1992) *Plant Dis.* **76,** 527–529.
23. Harris, P. S., Miller-Jones, D. N. & Howell, P. J. (1979) in *Plant Health: The Scientific Basis for Administrative Control of Plant Parasites*, eds. Ebbels, D. I. & King, J. E. (Blackwell, Oxford), pp. 231–237.
24. Hawkes, J. G. (1990) *The Potato: Evolution, Biodiversity, and Genetic Resources* (Smithsonian Institution Press, Washington, DC), pp. 1–211.
25. Anonymous (1992) in *Secretaría de Agricultura y Recursos Hidraulicos, Direccion General de Sanidad Vegetal* (Centro Nacional de Referencia en Diagnostico Fitosanitario, Mexico City, Mexico), p. 4.
26. Villarreal-García, L. A. (1994) in *Memorias de la II Asamblea Anual del CONAFOFI-SAGAR de 1992* (Secretaría de Agricultura y Desarrollo Rural, Mexico City, Mexico), pp. 67–73.
27. Galindo-Alonso, J. (1987) in *Temas en Virologia II*, eds. Alvizo-Villasana, H. F. & Lozoya-Saldana, H. (Sociedad Mexicana de Fitopatologia, CONACyT., Mexico City, Mexico).
28. Semancik, J. S., Morris, T. J., Weathers, L. G., Rodorf, B. F. & Kearns, D. R. (1975) *Virology* **63,** 160–167.
29. Diener, T. O., Hadidi, A. & Owens, R. A. (1977) *Methods Virol.* **6,** 185–217.
30. Levy, L., Lee, I. M. & Hadidi, A. (1994) *J. Virol. Methods* **49,** 295–304.
31. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, New York), 2nd Ed.
32. Keen, N. T., Tamaki, S., Kobayashi, D. & Trollinger, D. (1988) *Gene* **70,** 191–197.
33. Woodgett, J. R. (1991) *Methods Enzymol.* **200A,** 504–577.
34. Swofford, D. L. (1990) PAUP: Phylogenetic Analysis Using Parsimony (Illinois Natural History Survey, Champaign), version 3.0.
35. Sasaki, M. & Shikata, E. (1978) *Rep. Res. Lab. Kirin Brewery Co., Ltd.* **21,** 41–48.
36. Zuker, M. & Stiegler, P. (1981) *Nucleic Acids Res.* **9,** 133–148.
37. O'Brien, M. J. & Raymer, W. B. (1964) *Phytopathology* **54,** 1045–1047.
38. Anonymous (1981) in *Síntesis Geográfica de Jalisco* (Coordinatión General de los Servicios Nacionales de Estadística, Geografía e Informormática, SPP, Mexico City, Mexico), pp. 1–306.
39. Orozco Vargas, G. & Galindo, J. A. (1986) *Rev. Of. Soc. Mex. Fitopatol.* **4,** 19–28.
40. Belalcazar, S. C. & Galindo, J. A. (1974) *Agrociencia* **18,** 79–88.
41. Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. & Vande Pol, S. (1982) *Science* **215,** 1577–1585.
42. Eigen, M. & Biebricher, C. K. (1988) in *RNA Genetics*, eds. Domingo, E., Holland, J. J. & Ahlquist, P. (CRC, Boca Raton, FL), Vol. 3, pp. 211–245.
43. Eigen, M. (1993) *Sci. Am.* **269,** 42–49.
44. Eigen, M. (1971) *Naturwissenschaften* **58,** 465–523.
45. Keese, P., Visvader, J. E. & Symons, R. H. (1988) in *RNA Genetics*, eds. Domingo, E., Holland, J. & Ahlquist, P. (CRC, Boca Raton, FL), Vol. 3, pp. 71–98.
46. Qu, F., Heinrich, C., Loss, P., Steger, G., Tien, P. & Riesner, D. (1993) *EMBO J.* **12,** 2129–2139.
47. Owens, R. A., Chen, W., Hu, Y. & Hsu, Y. H. (1995) *Virology* **208,** 554–564.