BIOLOGIA PLANTARUM 36 (2): 285-294, 1994

Instability of potato spindle tuber viroid (PSTVd) cDNA in *Agrobacterium tumefaciens*

J. MATOUŠEK*, L. TRNĚNÁ*, S. RAKOUSKÝ* and D. RIESNER**

*Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic**
*Institut für Physikalische Biologie, Heinrich-Heine-Universität, Universitätstrasse 1, 4000 Düsseldorf, FRG***

Abstract

Potato spindle tuber viroid (PSTVd, severe strain) was isolated from tobacco plants transformed with a dimeric infectious expression construct which was maintained for a long time in *Agrobacterium tumefaciens*. After *in vitro* hybridization of PSTVd to complete minus transcripts of PSTVd (lethal), the resulting heteroduplexes were analyzed by electrophoresis under native conditions. Electrophoretic analysis revealed an appearence of electrophoretically distinguishable heteroduplexes, suggesting an accumulation of mutated sequence variants of PSTVd had occurred in the plant transformants. TGGE analysis of PSTVd cDNA, re-cloned from the original expression vector pCB1413 to plasmid pUC18 confirmed the accumulation of mutations in the cDNA and the instability of this sequence in *A. tumefaciens* maintained at 4 °C for 2.5 years. One of these point mutations was identified by sequencing the PSTVd cDNAs isolated from the individual *E. coli* colonies. This transition (GC→AT) was localized at the position of 81 in the PSTVd genome, causing the change of C to U in PSTVd plus RNA. Transformation of tobacco with the freshly prepared expression vector containing the dimeric sequence of PSTVd lethal KF440-2 lead to the propagation of PSTVd electrophoretically identical to that derived from the original sequence and maintained in the tomato by a conventional infection.

Introduction

Potato spindle tuber viroid (PSTVd) is a member of a special class of small autonomously replicating infectious plant RNAs (for review see Diener 1987).

Received 2 February 1993, *accepted* 13 September 1993.
Acknowledgements: The authors would like to thank R. Grüner, V. Rosenbaum, G. Steger and Qu Feng from the Institute of Physical Biology, Heinrich Heine University of Düsseldorf, FRG, for their methodical support and fruitful discussions. The authors would also like to thank Prof. M.J. Fraser from the Children's Hospital, Randwick, Australia, for his help in preparing of this manuscript.

Infectivity has been described also for complete copies of PSTVd cDNA (Cress *et al.* 1983) or when co-inoculation of all the restriction fragments of PSTVd cDNA was performed (Tabler and Sänger 1984). Despite the fact that viroids are well characterized by molecular genetic methods, a lot of basic problems of the molecular genetics of viroids remain to be solved. Some of these questions, particularly those dealing with ability of viroids to propagate in different plant species or the ability of viroid derived from mutated cDNAs to replicate, can be investigated advantageously by gene manipulation methods using plant transformation or agroinfection (*e.g.* Gardner *et al.* 1986, Salazar *et al.* 1988, Owens and Hammond 1990, Matoušek *et al.* 1993).

For plant transformation, usually an *Agrobacterium tumefaciens*-mediated transfer has been used. In this system, the sequence identity of the transferred DNA depends on the bacterial biochemical machinery. In the present study, we describe evidence for instability of the long-term maintained PSTVd cDNA seguence in *A. tumefaciens*. The more general significance of the observation described here is discussed with respect to the techniques of gene manipulations.

Materials and methods

Expression vectors: The expression vector pCB1413 containing a dimeric sequence of PSTVd (severe strain) (Van Wezenbeek *et al.* 1982) was described earlier in detail (Matoušek *et al.* 1989). This plasmid was constructed from the expression vector pCB1399 which contains T-DNA borders and the origin of replication from pGA472 (An *et al.* 1985) and sequences carrying the gene 7 polyadenylation signal. In addition, it carries the coding region of mannopine dual promoter from the T_R-DNA of the octopine expressing Ti-plasmid from the vector pAP2034 (Velten and Schell 1985). Expression of neomycin phosphotransferase II directed from the 1'part of the dual promoter enabled selection of regenerated plant transformants. The vector pCB1413 was found to be highly infectious for transformed potato (Matoušek *et al.* 1989). This plasmid was transferred from the *E. coli* strain DH5α to *Agrobacterium tumefaciens* strain pAL4404, by three-strain conjugation (Comai *et al.* 1983) and maintained in *Agrobacterium* for 2.5 years. The bacteria were transferred monthly to fresh medium.

The expression vector designated $PSTVd_{lethal(++)}$ in pKYLX7 contained a dimeric sequence of PSTVd lethal strain KF440-2 (Tabler and Sänger 1985) in plus orientation. The *Eco* RI, *Bgl* II insert containing the dimeric sequence of PSTVd cDNA corresponding to nucleotides 282-359/1-359/1-281 and was re-cloned from plasmid pRH721 (Hecker 1989) into *Sma* I site of the plasmid pUC18 (*Boehringer*, Mannheim, FRG) by blunt end ligation. The dimeric sequence cut out from pUC18 with the restriction enzymes *Kpn* I and *Hind* III was cloned into the expression vector pKYLX7 (Schardl *et al.* 1987). In the resulting vector, the transcription of PSTVd cDNA was directed from the CaMV 35S promoter. A neomycin phosphotransferase II gene controlled by the nos promoter enabled selection of regenerated plant transformants. This vector was transferred to *Agrobacterium* by the triparental

mating described above and used for transformation without prolonged storage at 4 °C.

Plant material and plant transformation: Tobacco (*Nicotiana tabacum* L. cv. White Burley) and potato (*Solanum tuberosum* L. cv. Kamýk) were used for transformation with the expression vectors pCB1413 and $PSTVd_{lethal(++)}$ in pKYLX7. The *Agrobacterium*-mediated transformation was performed using the method of Rogers *et al.* (1986). Transformed regenerants were cloned and maintained on MS medium (Murashige and Skoog 1962) containing 200 mg l^{-1} of kanamycin. All the experiments were performed under aseptic conditions. *In vitro* plants were supplied with light [16 h, 35 μmol(PAR) m^{-2} s^{-1}]. Day/night temperature was 25/18 °C.

Extraction and electrophoretic analysis of PSTVd: LiCl-soluble nucleic acids were extracted from PSTVd positive transformed plant lines as described earlier by Matoušek and Dědič (1988). For further purification, nucleic acids were fractionated with polyethyleneglycol 6000 (PEG). PSTV-specific RNA was recovered by precipitation with 12-20 % PEG (Matoušek and Dědič 1988). The PEG precipitate was dissolved in 0.01 M Tris-HCl (pH 8.0) buffer containing 1 mM EDTA (TE buffer), re-extracted with phenol-chlorophorm and precipitated with 2.5 v/v of ethanol. After precipitation and washing twice with 70 % (v/v) ethanol, the sediment was dried and dissolved in an appropriate volume of TE buffer.

PSTVd was hybridized in solution with [α-P]UTP (18.5 MBq $mmol^{-1}$, *Amersham*, Bucks, U.K.) labelled full-length monomeric minus transcripts of PSTVd lethal KF440-2 from plasmid pRH710 (Hecker 1989) and electrophoresed under non-denaturing conditions. The hybridization and electrophoretic procedure described by Zimmat *et al.* (1990) were used with minor modifications. PSTVd was hybridized to linear monomeric transcripts in 1 mM Na cacodylate buffer (pH 6.8) containing 100 mM NaCl and 1 mM EDTA (sample buffer). The samples were heated to 95 °C in a heat block and cooled in an insultation box to 40 °C in 2 h. The samples were then electrophoresed in 5 % acrylamide, 0.12 % bisacrylamide gel containing 89 mM Tris, 89 mM boric acid, 0.24 mM EDTA, (1 × TBE), pH 8.3, 0.1 % TEMED, 8 M urea, 10 % glycerol and 0.06 % ammonium persulfate. Electrophoreses were carried out at 150 V, and 20 °C for 14 h. Before autoradiography, the gels were incubated in a mixture of 10 % ethanol and 0.5 % acetic acid for 10 min. Autoradiograms were scannned an *ULTROSCAN* (*LKB*, Bromma, Sweden). The pattterns were then compared using the *Gelscan 1D-compare computer program version 2.1* (*LKB*).

PSTVd was subjected to temperature-gradient-gel electrophoresis (TGGE) as described by Riesner *et al.* (1989), transblotted to *Biodyne A* (*Pall Bio-Support membranes*, Dreieich, FRG) in 1xTBE buffer. The blots were hybridized as described above using [α-P]UTP (111 MBq $mmol^{-1}$, *Amersham*, U.K.) labelled transcripts from pRH710 as the strand-specific probes for detection of the PSTVd.

Re-cloning and analysis of PSTVd cDNA: For analysis of cDNA, the vector pCB1413 was isolated from *Agrobacterium tumefaciens* and transformed back to *E. coli* strain DH5α. For further analysis, a polycolonial preparation of the vector served as a

source of PSTVd cDNA insert. The insert was cleaved from the vector using *Bam* HI, extracted from agarose gel using *Qiaex* extraction protocol (*QIAGEN Inc.*, Studio City, USA), dephosphorylated and 5'labelled with P[τ-ATP] using polynucleotide kinase (*Boehringer*, Mannheim, FRG). PSTVd cDNA was then subjected to TGGE (Riesner *et al.*1989), for details see the "Results and discussion". For sequencing, the cDNA insert was cloned into the *Bam* HI site of pUC18 and monocolonial preparations were made. The cDNAs from the individual colonies were first analyzed by TGGE as described above in order to verify their homogeneity. Sequencing was performed according to the standard protocol using *Sequenase Version 2.0*, T7 DNA polymerase (*United States Biochemical Corp.*, Cleveland, USA) and the -40 primer.

Results and discussion

Polyacrylamide gel electrophoretic analysis of PSTVd progeny from tobacco transformed with vector pCB1413 revealed appearance of electrophoretically distinguishable heteroduplexes formed between circular forms of PSTVd ($PSTVd_c$) and complete minus transcripts of PSTVd (lethal) (Fig. 1A). This result suggests a

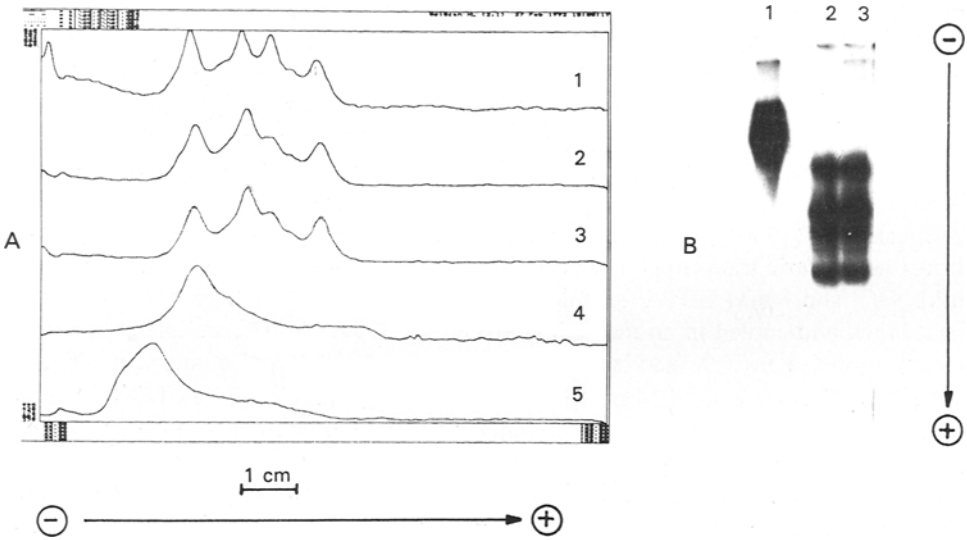

Fig. 1. Analysis of PSTVd by non-denaturing PAGE of circular heteroduplexes: (*A*) The analysis of PSTVd from transformed tobacco leaves (*1*), from transformed tobacco stock (*2*), from untransformed tomato graft (*3*), from transformed potato leaves (*4*). Position of homoduplexes formed with PSTVd lethal strain KF440-2 (*5*). (*B*) Analysis of PSTVd from newly transformed tobacco (*2*) and potato (*3*) with vector pCB1413. Homoduplexes between monomeric transcripts and PSTVd lethal strain KF-440-2 (*1*).

possible accumulation of different sequence variants of PSTVd in tobacco transformants. Analysis of the viroid prepared originaly from potato (natural host species) line transformed with the pCB1413 (Matoušek *et al.* 1989) and stored at

-20 °C as ethanol precipitate showed only one heteroduplex species having low electrophoretic mobility (Fig.1A, lane 4). This difference between PSTVd offspring isolated from transformed tobacco and potato could be caused by PSTVd mutagenesis either at the cDNA level or during PSTVd replication pathway in tobacco. A possible accumulation of PSTVd mutated forms better adapted to the tobacco biochemical machinery cannot be excluded, because tobacco is known to be a host species in which PSTVd infection is much slower than in the potato, probably due to some block(s) of PSTVd transport (Matoušek *et al.* 1993).

According to our experiments (Fig. 1A), performed using grafting of transformed tobacco with untransformed tomato (natural host species), the same spectrum of heteroduplexes was detected in transformed tobacco stocks and tomato grafts (Fig. 1A). Essentially the same results were obtained using a conventional method of infection of tomato with the PSTVd inoculum prepared from transformed tobacco. Also in this case the spectrum of heteroduplexes was the same for viroid isolated from transformed tobacco and infected tomato (data not shown). This suggests that there was not some strong selection system in the tomato, which could effectively abolish replication of mutated molecules originating from transformed tobacco and

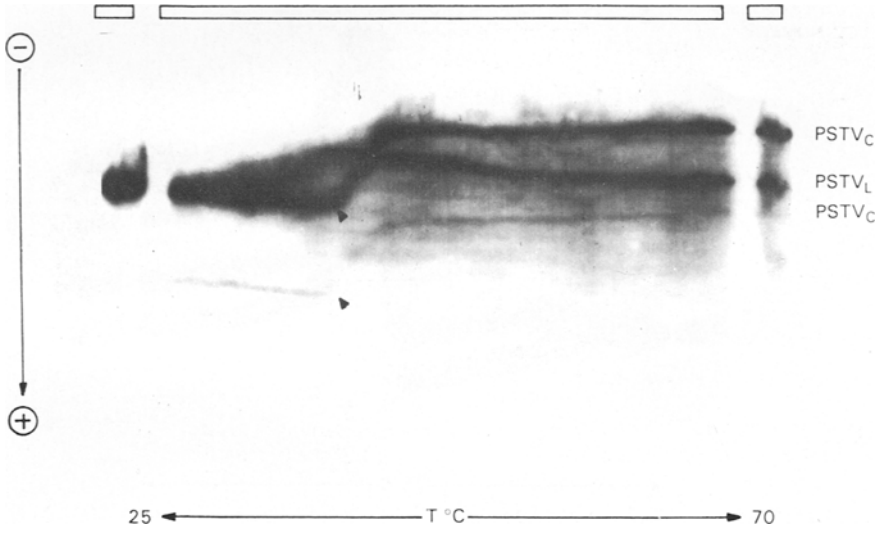

Fig. 2. TGGE analysis of native PSTVd lethal strain KF440-2 from transformed tobacco. Comparison with PSTVd lethal maintained in tomato: The sample containing the PSTVd lethal strain KF440-2 was loaded first and electrophoresed without a temperature gradient for 40 min. at 20 °C. After the loading of the second PSTVd sample from transgenic tobacco, a linear temperature gradient 70→25 °C was established as indicated and electrophoresis continued at 300 V for 75 min. PSTV$_L$ and PSTV$_C$, linear and circular forms of PSTVd. The melting points are indicated by the arrows.

favour replication of a particular "wild-type" sequence variant. A cross-protection process similar to that described for different PSTVd strains (Singh *et al.* 1989) or reversion to the "wild-type" of PSTVd cannot be ruled out and could occur during long term propagation of PSTVd from transformed tobacco in tomato plants.

In further experiments, we constructed a new vector $PSTV_{lethal}$ in pKYLX7 and made new transformed tobacco and potato lines. No difference was found between PSTVd from transformed potato and tobacco. Only single bands (not shown) corresponding to circular homoduplexes formed between the PSTVd circular form and minus transcripts of PSTVd lethal KF440-2 (Fig. 1A) were detected.

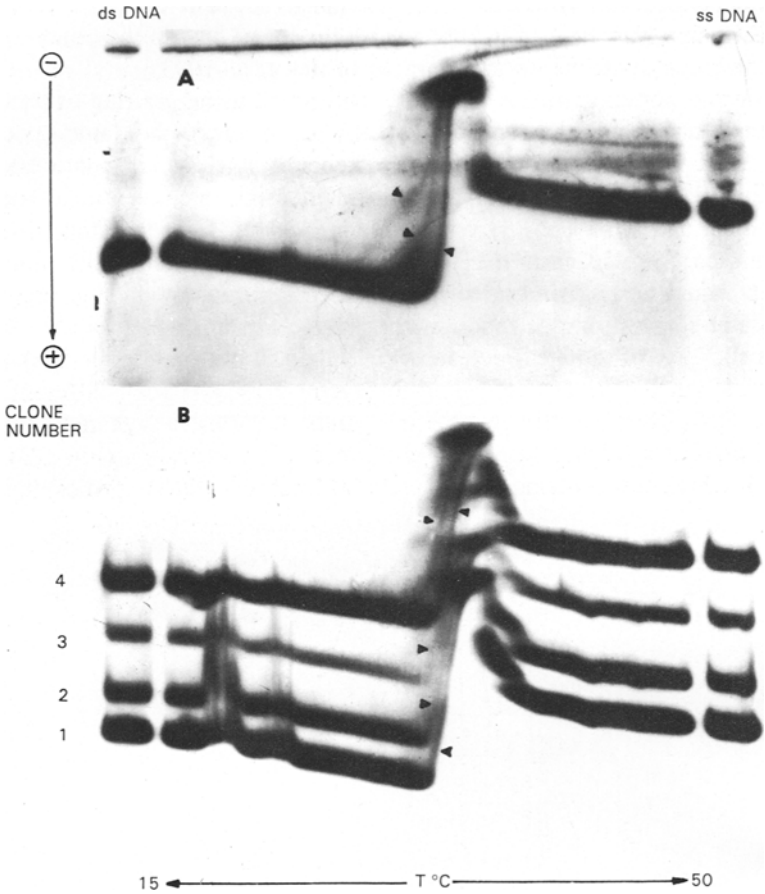

Fig. 3. TGGE analysis of cDNA insert of PSTVd: (*A*) Analysis of PSTVd cDNA cleaved from the vector pCB1413 with *Bam* HI. (*B*) Analysis of PSTVd cDNA re-cloned from pCB1413 to plasmid pUC18. In this case the gel was pre-loaded successively with cDNAs from individual colonies (as indicated by the colony numbers) at 20 min. intervals. Then the linear temperature gradient was established and electrophoresis continued at 220 V for 1 h. The melting transitions are indicated by the arrows.

Temperature-gradient-gel electrophoresis was used in order to compare PSTVd progeny from transformed tobacco (vector $PSTV_{lethal}$ in pKYLX7) with the PSTVd lethal strain KF440-2 maintained in tomato using conventional infections. Only a single band corresponding to PSTVd circular form ($PSTVd_c$) and showing the characteristic melting transition (Riesner *et al.* 1989) was detected (Fig. 2). The

electrophoretic pattern obtained for $PSTVd_c$ from transformed tobacco did not differ from $PSTVd_c$ lethal strain KF440-2, suggesting that there was no accumulation of mutated PSTVd in transformed tobacco.

These above results were in contrast to the results obtained for vector pCB1413. Therefore, the possibility was examined as to whether or not some mutations had accumulated in the PSTVd cDNA insert of the expression vector pCB1413. This vector was maintained and stored at 4 °C in *Agrobacterium tumefaciens* for a long time, 2.5 years. Approximately 30 passages of the bacterial clone were made since the first transformation of potato was performed by Matoušek *et al.* (1989). The idea about the possible instability of PSTVd cDNA was further supported by the results obtained from additional transformations of potato with the long-term maintained vector pCB1413. We analyzed and compared the spectra of heteroduplexes from newly transformed potato and tobacco lines (Fig. 1B). Several heteroduplexes were detected in both transformed tobacco and potato. No obvious differences were observed between these two electrophoretic patterns, suggesting that accumulation of the same sequence variants from the vector cDNAs has occurred.

Analysis of PSTV cDNA from vector pCB1413 by TGGE (Fig. 3A) revealed several transition curves corresponding to several cDNAs having different thermal stabilities. The difference between melting points was clearly detected. Analysis of individual cDNAs re-cloned to pUC18 confirmed this observation (Fig. 3B). Except for clone 4, which still contained a mixture of at least two cDNA species, the cDNAs

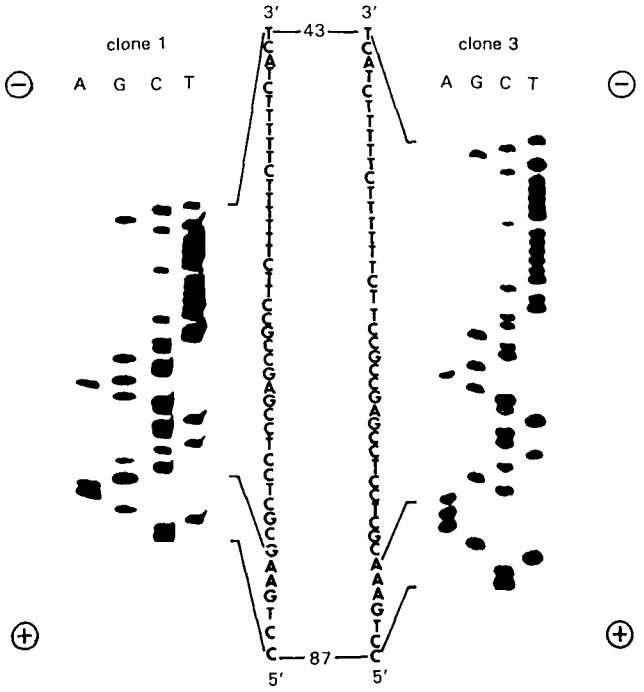

Fig. 4. Sequencing of PSTVd cDNAs corresponding to position 46-87 of PSTVd: The base change G→A is indicated by the arrow. Clone numbers are indicated on the top of each autoradiogram.

were sequenced. The seguence of cDNA clone 1 was completely identical to that found by Schnölzer *et al.* (1985) for the PSTVd severe strain. The cDNA of clone 2 was partially sequenced; that corresponding to the left half of PSTVd was analysed. However, no mutation was identified in this sequence. The predicted mutation(s) therefore must be in the sequence corresponding to the right half of PSTVd. The mutation in the cDNA of clone 3 was identified (Fig. 4) as single point mutation at position 81 in the PSTVd genome and caused GC to AT transition at the cDNA level (Fig. 5). A single base change, C to U, at PSTVd RNA level at this position should not change the secondary rod-like structure of PSTVd. It is not known, however, whether such PSTVd RNA would be viable and replicate, because in the present work we did not identify the mutation at the RNA level. Although it is not known how many mutations accumulated in the PSTVd cDNA and whether all of them would give rise to viable mutated PSTVd species, it is quite clear that instability of the sequence occurred in *Agrobacterium tumefaciens*. Instabitity of plasmid and chromosomal DNAs in the bacteria has also been found by other investigators. For instance, genome rearrangement frequently occurred in wild-type *Agrobacterium* and *Rhizobium* especially under certain stress conditions (Heumann *et al.* 1983). Such mechanisms could give rise to a wide genetic diversity among bacterial strains (Demenzas *et al.* 1991). Certain sequences seem to be unstable in different bacterial strains, for instance, the delta-Endotoxin gene of *Bacillus thuringiensis* (Turner *et al.* 1991) and the chloramphenicol-resistance determinant of *Streptomyces* (Dittrich *et al.* 1991).

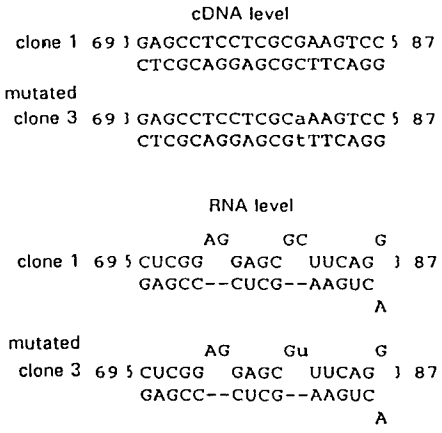

Fig. 5. Schematic drawing of PSTVd sequence and the secondary structure at the DNA and RNA levels. Mutation is indicated by the small letters, the position of the upper strand with regard to PSTVd genome is indicated by nucleotide numbers. The PSTVd RNA structure is predicted on the basis of known secondary structure of PSTVd (Riesner *et al.* 1979).

The exact mechanism which caused an accumulation of point mutation(s) in the PSTV cDNA sequence described here is not known, but it is quite possible that this phenomenon has more broaded implications for other sequences and genes which are manipulated by genetic engineering methods using *Agrobacterium tumefaciens*,

especially sequences, that are not under strong selection pressure in *Agrobacterium*. Mutated cDNAs could give rise to plant chimeras and defective mRNAs and in this way change gene expression.

References

An, G., Watson, B.D., Stachel, S., Gordon, M.P., Nester, E.W.: New cloning vehicles for transformation of higher plants. - EMBO J. **4**: 277-284, 1985.

Comai, L., Schilling-Cordaro, C., Mergia, A., Houck, C.: A new technique for genetic engineering of *Agrobacterium* Ti plasmid. - Plasmid **10**: 21-30, 1983.

Cress, D.E., Kiefer, M.C., Owens, R.A.: Construction of infectious potato spindle tuber viroid cDNA clones. - Nucl. Acids Res. **11**: 6821-6835, 1983.

Demenzas, D.H., Reardon, T.B., Watson, J.M., Glibson, A.H.: Genetic diversity among *Rhizobium-leguminosarum* BV. *trifolii* strains revealed by allozyme and restriction fragment length polymorphism analyses. - Appl. environ. Microbiol. **57**: 3489-3495, 1991.

Diener, T.O.: The Viroids. - Plenum Press, New York - London 1987.

Dittrich, W., Beltzer, M., Schrempf, H.: An amplifiable and detectable chloramphenicol-resistance determinant of *Streptomyces-lividans*-1326 encodes a putative transmembrane protein. - Mol. Microbiol. **5**: 2789-2797, 1991.

Gardner, R.C., Chonoles, K.R., Owens, R.A.: Potato spindle tuber viroid infections mediated by the Ti plasmid of *Agrobacterium tumefaciens*. - Plant mol. Biol. **6**: 221-228, 1986.

Hecker, R.: Die Struktur der Intermediären Ribonukleinsäuren bei der Replikation von Viroiden. - Ph.D. Thesis, Heinrich-Heine-Universität, Düsseldorf 1989.

Heumann, W., Merkt, B., Rösch, A., Springer, R., Rüger, B., Horn, D., Bauer, I., Winkler, K., Wagner, E.: Genome rearrangement of the *Rhizobiaceae*. - In: Pühler, A. (ed.): Molecular Genetics of the Bacteria-Plant Interaction. Pp. 373-379. Springer-Verlag, Berlin - Heidelberg 1983.

Matoušek, J., Dědič, P.: Acid nucleases in PSTV-infected tomato (*Lycopersicum esculentum L.*) I. Levels of acid nuclease activity in healthy and PSTV-infected tomato leaves and callus tissues. - J. Plant Physiol. **133**: 340-344, 1988.

Matoušek, J., Rakouský, S., Trněná, L., Riesner, D.: PSTV infection in tobacco (*Nicotiana tabacum L.*) transformed with PSTV cDNA.- Biol. Plant. **35**: 131-135, 1993.

Matoušek, J., Schubert, J., Vlasák, J., Dědič, P.: Viroid infection in potato (*Solanum tuberosum L.*) plants transformed with potato spindle tuber viroid cDNA. - Arch. Phytopathol. Pflanzenschutz **25**: 611-613, 1989.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **14**: 473-497, 1962.

Owens, R.A., Hammond, R.: Mutational analysis of viroids. - In: Symons, R.H. (ed.): Seminars in Virology, Vol 1. Pp 101-106. Saunders Scientific Publications/W.B.Saunders Company, Orlando 1990.

Riesner, D., Henco, K., Rokohl, U., Klotz, G., Kleinschmidt, A.K., Domdey, H., Jank, P., Gross, H.J., Sänger, H.L.: Structure and structure formation of viroids. - J. mol. Biol. **133**: 85-115, 1979.

Riesner, D., Steger, G., Zimmat, R., Owens, A., Wagenhofer, M., Hillen, W., Vollbach, S., Henco, K.: Temperature-gradient gel electrophoresis of nucleic acids: Analysis of conformational transitions, sequence variations, and protein-nucleic acid interactions. - Electrophoresis **10**: 377-389, 1989.

Rogers, S.G., Horsch, R.B., Fraley, R.T.: Gene transfer in plants: production of transformed plants using Ti plasmid vectors. - Methods Enzymol. **118**: 627-641, 1986.

Salazar, L.F., Hammond, R.W., Diener, T.O., Owens, R.A.: Analysis of viroid replication following *Agrobacterium*-mediated inoculation of non-host species with potato spindle tuber viroid cDNA. - J. gen. Virol. **69**: 879-889, 1988.

Schardl, C.L., Byrd, A.D., Benzion, G., Altschuler, M.A., Hildebrand, D.F., Hunt, A.G.: Design and construction of a versatile system for expression of foreign genes in plants. - Gene **61**: 1-11, 1987.

Schnölzer, M., Haas, B., Ramm, K., Hofmann, H., Sänger, H.L.: Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTV). - EMBO J. **4**: 2181-2190, 1985.

Singh, R.P., Boucher, A., Somerville, T.H.: Cross-protection with strains of potato spindle tuber viroid in the potato plant and other solanaceous hosts. - Phytopathology **78**: 246-250, 1989.

Tabler, M., Sänger, H.L.: Cloned single- and double-stranded DNA copies of potato spindle tuber viroid (PSTV) RNA and co-inoculated subgenomic DNA fragments are infectious. - EMBO J. **3**: 3055-3062, 1984.

Tabler, M., Sänger, H.L.: Infectivity studies on different potato spindle tuber viroid (PSTV) RNAs synthesized *in vitro* with SP6 transcription system. - EMBO J. **4**: 2191-2199, 1985.

Turner, J.T., Lampel, J.S., Stearman, R.S., Sundich, G.W.: Stability of the delta-Endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* in a recombinant strain of *Clavibacter xyli* subsp. *cynodontis*. - Appl. environ. Microbiol. **57**: 3522-3528, 1991.

Van Wezenbeek, P., Vos, P., Van Boom, J., Van Kammen, A.: Molecular cloning and characterization of a complete DNA copy of potato spindle tuber viroid RNA. - Nucl. Acids Res. **10**: 7947-7957, 1982.

Velten, J., Schell, J.: Selection-expression plasmid vectors for use in genetic transformation of higher plants. - Nucl. Acids Res. **11**: 6981-6998, 1985.

Zimmat, R., Grüner, R., Hecker, R., Steger, G., Riesner, D.: Analysis of mutations in viroid RNA by non-denaturing and temperature-gradient-gel electrophoresis. - In: Sarma, R.H., Sarma, M.H. (ed.): Structure and Methods. Vol.3. DNA and RNA. Pp. 339-357. Adenine Press, New York 1990.

Communicated by T. GICHNER