Arch Virol (1996) 141: 2153–2161

Archives of Virology

A new sequence variant of *Coleus blumei* viroid 1 from the *Coleus blumei* cultivar 'Rainbow Gold'*

R. L. Spieker

Max-Planck-Institut für Biochemie, Abteilung Viroidforschung, Martinsried, Federal Republic of Germany

Accepted June 3, 1996

Summary. A viroid was isolated from symptomless *Coleus blumei* cultivar (cv.) 'Rainbow Gold' plants using the bidirectional PAGE method for analysis of small circular RNA molecules. The viriod was transmitted to viroid-free plants of *Coleus blumei* cv. 'Scarlet Dragonfly' by mechanical inoculation. Cloning and sequencing revealed that the viriod from the *Coleus* cv. 'Rainbow Gold' is closely related to the *Coleus blumei* viriod 1-BvA (CbVd 1-BvA) isolated from the *Coleus* cv. 'Bienvenue'. Therefore, new viroid sequence variant has been named *Coleus blumei* viriod 1-RG (CbVd 1-RG). *Coleus blumei* viriod 1-RG consists of 251 nucleotides, 140 G + C, 111 A + U with a GC content of 55.4%. The most stable rod-like secondary structure of this viroid has 53 G:C, 29 A:U and 5 G:U base pairs with a minimum free energy (at 25 °C) of − 81.2 kcal/mol (− 339.4 kJ/mol). The right terminal domain shows a high sequence similarity to the corresponding domain of hop latent viroid (HLVd).

Introduction

Viroids are single-stranded, circular RNA molecules which have so far only been found in higher plants where they may cause disease symptoms or symptomless infections. At present the sequences of about 24 viriod 'species' and of altogether more than 150 sequence variants thereof are known. They show that viroids are characterized by a species-specific distinct chain length in the size range between 240 and 375 residues that can vary by ± 4 nucleotides [8, 10, 26]. Due to their high degree of intramolecular self-complementarity, viroids are presumed to exist in their native state as highly base-paired rod-like structures [1, 2, 16, 17].

* The sequence data reported here have been submitted to the EMBL database and have been assigned the accession numbers X95291 (for CbVd 1-RG) and X57284 (for CbVd 1-BvA).

These properties, particularly the latter, are the basis of a sensitive and selective electrophoretic approach for viroid detection [19].

Viroids infecting ornamental plants such as *Chrysanthemum morifolium* [6], *Columnea erythrophae* [7], *Nematanthus wettsteinii* [22] and *Coleus blumei* [3–5, 21, 23, 24] have been sequenced. The viroids from *Columnea* [7] and *Nematanthus* [22] can infect solanaceous plants including potatoes. The ornamental plant *Coleus blumei* was brought from Indonesia to Europe in 1851 and in 1866 the first *Coleus* hybirds were raised in England. The detection of a viroid in *Coleus blumei* species in Brazil [3, 4] has prompted us to search for viroids in *Coleus blumei* cultivars (cvs) commercially propagated by cuttings for more than 80 years in Germany. Here, we report on some biological and molecular properties of the viroid from the *Coleus blumei* cv. *'Rainbow Gold'*. The viroid consists of 251 nucleotides (nt) and is 93.1% identical to the previously described, 250 nt long *Coleus blumei* viroid 1-BvA (CbVd 1-BvA) [23, 24]. Because of its close genetic relatedness to CbVd 1-BvA, the viroid is designated CbVd 1-RG, to indicate that it is a distinct strain of CbVd 1 occurring naturally in plants of the *Coleus blumei* cv. 'Rainbow Gold'.

Materials and methods

Plant culture and viroid transmission

The *Coleus blumei* cv. 'Rainbow Gold' plants surveyed in our study were grown from their seeds in the greenhouse. All infectivity assays were performed in the greenhouse according to [13] using viroid-free plants of the *Coleus blumei* cv. 'Scarlet Dragonfly'. Test plants were inoculated with 10 µg of total RNA isolated from inspected *Coleus* plants. CbVd 1-RG replication was ascertained at weekly intervals after inoculation by Northern analysis (see below). The sequence stability of CbVd 1-RG in the inoculated plants was ascertained by RT-PCR as described below.

Viroid purification

For the detection of possible viroid infection in *Coleus blumei* and for viroid purification total nucleic acid was isolated from *Coleus* leaves according to [13]. Detection and isolation of viroids by bidirectional polycrylamide gel electrophoresis were performed as described [19].

Northern blotting

Glyoxalation of extracted nucleic acids prior to electrophoresis, Northern blotting to Qiabrane (Qiagen), and molecular hybridization were performed as previously described [25]. For filter hybridization, [γ-^{32}P] ATP-labeled primer pCb1 (see below) was used [14, 24].

RNA sequence determination

End-labeling of viroid-specific DNA olionucleotides and reverse transcription of CbVd 1-RG RNA with M-MuLV reverse transcriptase (BRL) were done as described [14]. For this purpose the following three synthetic oligonucleotides were used as primers: first the 'universal primer' for CbVd-like viroids [24], pCb1 (5'dAGCGCTGCCAGGGAACCCA-

GGTAAG3′, complementary to nt 47-71), and later the two CbVd 1-RG-specific primers pCb 1RGA (5′dTCAGCGAACCAGGACCTTTG3′, complementary to nt 191–210), and pCb1RGB (5′dCGAAGCAACTTCAGGTCGCC 3′, complementary to nt 110–129). The corresponding primer-extended CbVd 1-RG cDNAs were sequenced by a combination of the five G-, G/A-, T/C-, C- and A/C-specific chemical cleavage reactions according to Maxam and Gilbert [11].

cDNA cloning and sequencing

First strand cDNA was synthesized from CbVd 1-RG RNA by reverse trancriptase as described [18] and the primer pCb1 or pCb1RGA. CbVd 1-RG cDNA was PCR amplified using the CbVd 1-RG-specific primer pairs pCb1/pCb1RGR and pCb1RGA/pCb1RGAR. The backward primers have the following sequences: PCb1RGR: 5′dGCAACGGAATC-CATGCCC3′ (corresponding to nt 72–89) and pCb1RGAR: 5′dCCCGGTCTCTTTT-CTAAACCC3′ (corresponding to nt 211–231). The reaction was performed under the following conditions using the Euro TAQ DNA polymerase and reaction buffer (Eurogentec S.A.) and applying 30 cycles: denaturation at 95 °C for 30 sec, primer annealing at 60 °C for 30 sec and DNA polymerization 72 °C for 60 sec. PCR products were purified with the QIA quick-spin PCR Purification Kit (Qiagen). The PCR products were ligated into the *Sma*I site of plasmid pT3T7-lac (Boehringer Mannheim) and cloned in the *Escherichia coli* strain XL1- Blue (Stratagene) following standard protocols [14]. Plasmid DNA was isolated using QIAprep-spin Plasmid Kits (Qiagen). Plasmids containing CbVd 1-RG cDNA were sequenced using T7 DNA polymerase (Sequenase version 2.0, US Biochemicals) following the methods of Sanger et al. [15]. A 19-mer complementary to the T3 promoter (Boehringer) and a 20-mer complementary to the T7 promoter (US Biochemicals) were used to prime the sequencing reactions.

Infectivity of viroid cDNA

Dimeric inserts of the cDNA clones were constructed in a head-to-tail orientation [27] in pT3T7-lac following standard protocols [14]. Dimeric (+) RNA transcripts of Cb Vd 1-RG cDNA clones were synthesized in vitro in standard T3/T7 RNA polymerase reactions [14]. Test plants were inoculated with 1 μg RNA-transcript/plant as described [13].

Computer calculations

The sequence similarity between CbVd 1-RG and the other sequenced prototype viroids was determined with the UWGCG sequence analysis program GAP, version 5.0 June 1987, using a gap-weight of 5 and a gap-length-weight of 0. Secondary structure analysis of the CbVd 1-RG sequence was carried out using the RNA folding program RNAFOLD version 2 [28] for a temperature of 25 °C in a VAX computer (Digital Equipment Corporation).

Results

Electrophoretic mobility of CbVd 1-RG

The search for unknown viroids included bidirectional electrophoretic analysis of small circular RNA molecules from several plants of various *Coleus blumei* cultivars. For comparison the gel-purfied viroids hop stunt viroid (HSVd; 297 nt; [12]), *Coleus blumei* viroid 1-BvA/BvB (CbVd 1-BvA/BvB; 250 nt; [24]) and hop latent viroid (HLVd; 256 nt; [13]) were included in the survey experiments for

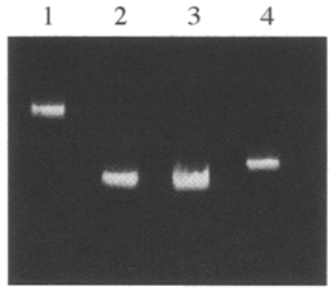

Fig. 1. Comparative mobility of HSVd, CbVd 1-BvA/BvB, CbVd 1-RG and HLVd in the second direction of a bidirectional PAGE (according to [19]). A section from a 5% polyacrylamide gel containing 8 M urea stained with ethidiumbromide is shown. *1* Purified HSVd; *2* purified CbVd 1-BvA/BvB; *3* total RNA of a plant of the *Coleus blumei* cv. 'Rainbow Gold'; *4* purified HLVd

comparison (Fig. 1, lanes 1, 2 and 4). In samples from *Coleus blumei* cv. 'Rainbow Gold' a viroid-like RNA termed CbVd 1-RG, was detected (Fig. 1, lane 3) which migrated faster than HSVd and HLVd and comigrated with CbVd 1-BvA/BvB. Using bidirectional gelelectrophoresis, 40 seed-grown plants of the *Coleus blumei* cv. 'Rainbow Gold' were surveyed in this study. Viroid-like RNA in the CbVd 1 size range was detected in four of these plants (10% of all plants studied).

Transmission studies

The main characteristic of viroids is their infectivity i.e. their ability to initiate replication and the production of viroid progeny when inoculated onto suitable host plants [1, 20]. For infectivity assays five viroid-free plants of the *Coleus blumei* cv. 'Scarlet Dragonfly' were inoculated with 10 µg total RNA isolated from CbVd 1-RG-infected *Coleus blumei* plants cv. 'Rainbow Gold'. Mock-inoculated and untreated plants were used a control, and all plants were maintained in the greenhouse for a period of three months. To monitor the replication of CbVd 1-RG, leaf samples were collected every week and analyzed by Northern blotting. Newly synthesized CbVd 1-RG was first detected two weeks post inoculation (p.i.) in three out of the five inoculated *Coleus* plants. One month p.i. all five plants proved to be CbVd 1-RG-infected. Infected plants were dwarfed significantly as compared with their non-inoculated counterparts. These symptoms were very similar to those incited by the three *Coleus* viroids CbVd 1, CbVd 2 and CbVd 3 [24] in the same host plant.

Complete nucleotide sequence and most stable secondary structure of CbVd 1-RG

The complete sequence of CbVd 1-RG was established at the level of its cDNA which was synthesized by reverse transcription using the purified CbVd 1-RG RNA from one single plant. In pilot experiments a set of DNA primers were

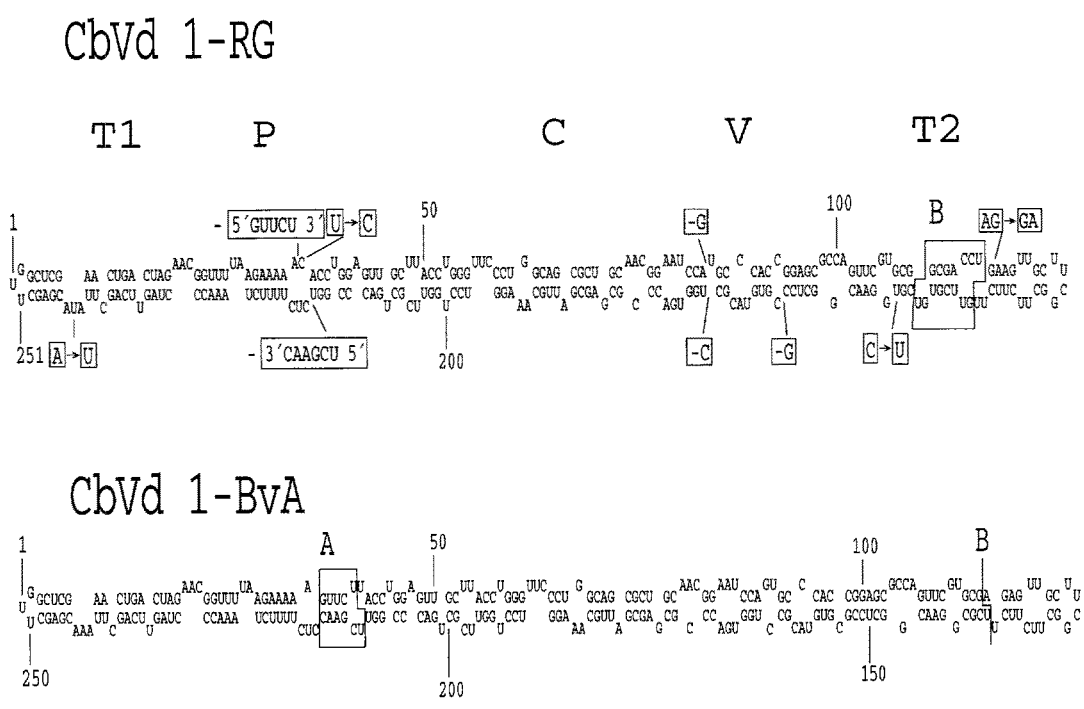

Fig. 2. Nucleotide sequence and secondary structure of CbVd 1-RG as compared with CbVd 1-BvA. The viroid domains are shown according to [9]. The mutations of CbVd 1-RG with respect to CbVd 1-BvA are indicated

tested for their capacity to prime CbVd 1-RG-cDNA synthesis. The 'universal' CbVd-specific primer pCb1 which is complementary to a sequence motif between nt 47–71 in the upper part of the central conserved region of the presently known CbVd-group viroids [5, 23, 24] allowed us to synthesize a CbVd 1-RG-specific cDNA which was sequenced according to the method of Maxam and Gilbert [11]. The sequence information obtained in this way was used to manufacture the CbVd 1-RG-specific primer pCb1RGA. The sequence data obtained with this primer led to the synthesis of the additional primer pCb1RGB. By elongating these three primers it was possible to synthesize and sequence three overlapping CbVd 1-RG cDNAs. Using reverse transcription of CbVd 1-RG RNA and subsequent PCR with two primer-pairs, CbVd 1-RG cDNAs were produced and cloned as described in Materials and methods. Eight cDNA clones were sequenced and the results of the Maxam-Gilbert sequencing procedures could be confirmed. No sequence heterogeneity was found in these cDNA inserts. Sequencing of CbVd 1-RG RNA isolated from the three other *Coleus* plants which were found to be infected by bidirectional gel electrophoresis (see above) confirmed the obtained sequence.

From these data the complete nucleotide sequence of CbVd 1-RG and its secondary structure could be constructed which is presented in Fig. 2. It shows that the circular RNA of CbVd 1-RG consists of 251 nucleotides which can

potentially form a rod-like structure with about 70% of base-pairing like all the other known viroids. The nucleotide composition is 69 G,46 A, 71 C and 65 U, corresponding to 140 G+C, 111 A+U with a GC content of 55.4%. In total 69% of the residues are base-paired and the base-paired residues consist of 53 G:C (58%), 29 A:U (35%) and 5 G:U (7%) base pairs. The most stable secondary structure of this viroid has minimum free energy (at 25 °C) of −81.2 kcal/mol (−339.4 kJ/mol).

Comparison of the sequences of CbVd 1-RG and CbVd 1-BvA

The overall calculation shows that CbVd-RG shares a similarity of 93.1% with CbVd-BvA whereas the similarity with all the other viroids ranges between 33%–51%. Comparing the sequences of CbVd 1-RG and CbVd 1-BvA, most mutations are due to an insertion and a deletion of two segments of 15 and 11 nt, respectively. The insertion of element B in CbVd 1-RG is located within the right

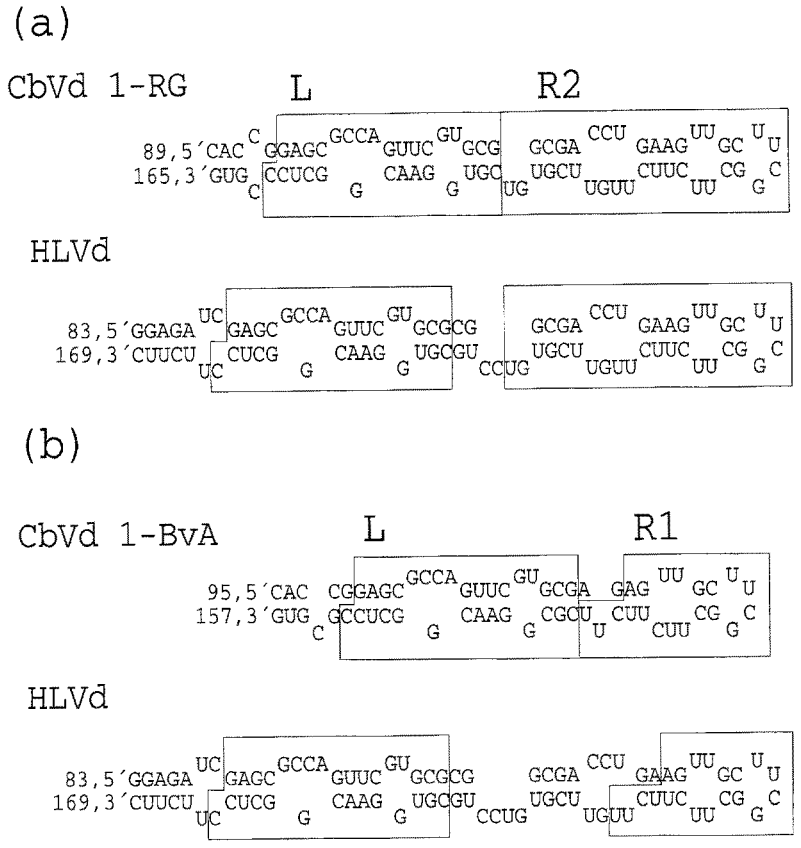

Fig. 3. Nucleotide sequence and secondary structure of the right terminal domains (T2 domains) of CbVd 1-RG, CbVd 1-BvA and HLVd. **a** Comparison of the T2 domains of CbVd 1-RG and HLVd. The identical sequence elements are boxed and named L and R2. **b** Comparison of the T2 domains of CbVd 1-BvA and HLVd. The identical sequence elements are boxed and named L and R1

terminal domains (T2 domain). The comparison of the right terminal domains of CbVd 1-RG and HLVd is presented in Fig. 3a. Two sequence elements named element L and R2 in Fig. 3a show an identical nucleotide sequence in CbVd 1-RG and HLVd. There are 31 nucleotides in element L and 37 nucleotides in element R2. Comparing the T2 domains of CbVd 1-BvA and HLVd, element L can also be found. But, in contrast to CbVd 1-RG, the right element R1 shows only 20 identical nucleotides. Thus, CbVd 1-RG shares 17 additional identical nucleotides with HLVd compared to CbVd 1-BvA (element B in Fig. 2).

Infectivity of in vitro-synthesized CbVd 1-RG(+) RNA transcripts

The cloned CbVd 1-RG cDNAs were dimerized and cloned in head-to-tail orientation [27] in plasmid pT3T7-lac in order to produce infectious CbVd 1-RG(+) RNA dimers as described in Materials and methods. When in vitro synthesized dimeric CbVd 1-RG (+)RNA was bioassayed, CbVd 1-RG was found to accumulate in all inoculaetd *Coleus blumei* plants of the cv. 'Scarlet Dragonfly' after one month. There was no difference in time of symptom apperance, symptom severity or viroid concentration between plants inoculated with dimeric (+) RNA transcripts and those inoculated with equivalent amounts of viroid RNA. The sequence stability of CbVd 1-RG was proven by cloning and sequencing of CbVd 1-RG RNA isolated from infected *Coleus* plants. No sequence heterogeneity to the original CbVd 1-RG sequence could be detected.

Discussion

The viroid nature of CbVd 1-RG

We have accumulated structural and biological data which clearly demonstrate that the circular RNA we have found to occur in apparently healthy *Coleus blumei* plants of the cv. 'Rainbow Gold' is a viroid, tentatively named CbVd 1-RG to indicate that it is a distinct strain of CbVd 1. Its circular RNA in the viroid size range has the potential to assume by intramolecular base pairing the viroid-specific rod-like structure. Comparison of the nucleotide sequences of CbVd 1-RG and CbVd 1-BvA revealed a sequence similarity of 93.1%. Finally, we have ascertained that the naturally occurring RNA and the RNA transcribed in vitro from the molecularly cloned CbVd 1-RG cDNA are infectious in that they are capable of initating viroid replication and accumulation when mechanically inoculated onto viroid-free plants of the *Coleus blumei* cv. 'Scarlet Dragonfly'. CbVd 1-RG caused stunting symptoms in plants of the *Coleus blumei* cv. 'Scarlet Dragonfly' similar to those of the other *Coleus blumei* viroids CbVd 1-BvA, CbVd 2-Bv and CbVd 3-Bv in the same cultivar [24]. Of 40 seed-grown plants tested, 10% were found to be infected with CbVd 1-RG. Thus, CbVd 1-RG is seed-transmissible like other CbVd 1-variants [21].

We compared the nucleotide sequence of CbVd 1-RG with the sequences of all known other viroids. The T2 domains of CbVd 1-RG and HLVd show a high degree of similarity (92%), whereas the comparison of the corresponding

domains of CbVd 1-BvA and of HLVd leads to more differences (71% sequence similarity). A possible explantion for this finding is a RNA recombination event involving a transfer of parts of the T2 domin of a HLVd-like viroid to a CbVd 1-like viroid. Alternatively, the observed sequence similarity can be explained by convergent evolution of the T2 domains of HLVd, CbVd 1-BvA and CbVd 1-RG.

Acknowledgements

I thank Dr. H. L. Sänger and Dr. M. Wassenegger for helpful discussions, Mr. S. Marinkovic for excellent technical assistance and the German Research Foundation (DFG) for financial support under project Sa 123/6-3. I thank Mrs. Bauer, Mr. Gudinski and Mr. Koller for their taking care of the plants in the greenhouse.

References

1. Diener TO (ed) (1987) The viroids. Plenum Press, New York
2. Diener TO (1991) The frontier of life: the viroid and viroid-like satellites RNAs. In: Maramorosch K (ed) Viroids and satellites: molecular parasites at the frontier of life. CRC Press, Boca Raton, pp 1–20
3. Fonseca MEN, Boiteux LS, Singh RP, Kitajima EW (1989) A small viroid in *Coleus* species from Brazil. Fitopatol Brasil 14: 94–96
4. Fonseca MEN,Boiteux LS, Singh RP,Kitajima EW(1990) A viroid from *Coleus* species in Brazil. Plant Dis 74: 80
5. Fonseca MEN, Marcellino LH, Kitajima EW, Boteux LS (1994) Nucleotide sequence of the original Brazilian isolate of coleus yellow viroid from *Solenostemon scutellaroides* and infectivity of its complementary DNA. J Gen Virol 75: 1447–1449
6. Gross HJ, Krupp G, Domedy H, Raba M, Alberty H, Lossow CH, Ramm K, Sänger HL (1982) Nucleotide sequence and secondary structure of citrus exocortis and chrysanthemum stunt viroid. Eur J Biochem 121: 249–257
7. Hammond R, Smith DR, Diener T (1989) Nucleotide sequence and proposed secondary structure of *Columnea* latent viroid: a natural mosaic of viroid sequences. Nucleic Acids Res 23: 10083–10094
8. Herold T, Haas B, Singh RP, Boucher A, Sänger HL (1992) Sequence analysis of five new field isolates demonstrates that the chain length of potato spindle tuber viroid (PSTVd) is not strictly conserved but as variable as in other viroids. Plant Mol Biol 19: 329–333
9. Keese P, Symons RH (1985) Domains in viroids: evidence of intermolecular rearrangements and their contribution to viroid evolution. Proc Natl Acad Sci USA 82: 4582–4586
10. Koltunow AM, Rezaian MA (1989) A scheme of viroid classification. Intervirology 30: 194–201
11. Maxam AM, Gilbert W (1989) Sequencing end-labeled DNA with base-specfic chemical cleavages. Methods Enzymol 65: 499–560
12. Ohno T, Takamatsu N, Meshi T, Okada Y (1983) Hop stunt viroid: molecular cloning and nucleotide sequence of the complete cDNA copy. Nucleic Acids Res 11: 6185–6197
13. Puchta H, Ramm K, Sänger HL (1988) The molecular structure of the hop latent viroid (HLV), a new viroid occuring worldwide in hops. Nucleic Acids Res 16: 4197–4216
14. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York

15. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467
16. Sänger HL, Klotz G, Riesner D, Gross HJ, Kleinschmidt AK (1976) Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. Proc Natl Acad Sci USA 73: 3852–3856
17. Sänger HL (1987) Viroid replication. In: Diener TO (ed) The viroids. Plenum Press, New York, pp 117–166
18. Schnölzer M, Haas B, Ramm K, Hofmann H, Sänger HL (1985) Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTV). EMBO J 4: 2181–2190
19. Schumacher J, Randles JW, Riesner D (1983) A two-dimensional electrophoresis technique for the detection of circular viroids and virusoids. Anal Biochem 135: 288–295
20. Semancik JS (ed) (1987) Viroids and viroid-like pathogens. CRC Press, Boca Raton
21. Singh RP, Boucher A, Singh A (1991) High incidence of transmission and occurrence of a viroid in commercial seeds of *Coleus* in Canada. Plant Dis 75: 184–187
22. Singh RP, Lakshman DK, Boucher A, Tavantizis SM (1992) A viroid from *Nematanthus wettsteinii* plants closely related to the *Columnnea* latent viroid. J Gen Virol 73: 2769–2774
23. Spieker RL, Haas B, Charng YC, Freimüller K, Sänger HL (1990) Primary and secondary structure of a new viroid 'species' (CbVd 1) present in the *Coleus blumei* cultivar 'Bienevenue'. Nucleic Acids Res 18: 3998
24. Spieker RL (1991) A new class of viroids in *Coleus blumei:* sequencing, cloning, structure/function-analusis by site-directed mutagenesis and expression in *Nicotiana tabacum.* Karlsruhe Contr Plant Phys 22: 1–226
25. Spiesmacher E, Mühlbach H-P, Table M, Sänger HL (1985) Oligomeric forms of potato spindle tuber viroid (PSTV) and its complementary RNA are present in nuclei isolated from viroid-infected potato cells. Biosci Rep 5: 251–265
26. Symons RH (1990) The fascination of low molecular weight pathogenic RNAs. Semin Virol 1: 75–81
27. Tabler M, Sänger HL (1984) Cloned single and double-stranded DNA copies of potato spindle tuber viroid (PSTV) and coinoculated subgenomic DNA fragments are infectious. EMBO J 3: 3055–3062
28. Zuker M (1989) On finding all suboptimal foldings of an RNA molecule. Science 244: 48–52

Authors' address: Dr. R. L. Spieker, Max-Planck-Institut für Biochemie, Abteilung Viroidforschung, Am Klopferspitz 18a,D-82152 Martinsried, Federal Republic of Germany.

Received April 9, 1996