Nucleotide sequence of the original Brazilian isolate of coleus yellow viroid from *Solenostemon scutellarioides* and infectivity of its complementary DNA

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The complete nucleotide (nt) sequence of the original coleus yellow viroid (CYVd) from *Solenostemon scutellarioides*, 'Golden Bedder', has been determined. The covalently closed single-stranded CYVd RNA molecule consists of 248 nt residues which assumes a rod-like secondary structure when folded in the model of lowest free energy. The sequence was determined by direct sequencing of RNA and from three overlapping cDNA clones. Comparison of the CYVd sequence with that of Coleus blumei viroid 1 (CbVd 1) from Germany demonstrated that they are closely related. The differences observed in the genome organization of CYVd relative to CbVd 1 were at three sites: position 25 (one U deletion), position 26 (a U was replaced by an A) and position 241 (one A insertion). The first two mutations were detected in one A-rich segment of eight nt (between positions 25 and 34). Northern blot hybridization of partially purified nucleic acids from the leaf tissue of *S. scutellarioides* 'Frilled Fantasy', inoculated with double-stranded cDNA, demonstrated that this fragment was infectious. These data enable CYVd to be assigned to the viroid class of plant pathogens, based on its biological properties and molecular structure. This work also gives additional support to the present classification system, in which the viroids isolated from *S. scutellarioides* form a distinct subgroup.

The coleus yellow viroid (CYVd) was first described as a latent infection of *Solenostemon scutellarioides* (L.) Codd (syn. *Coleus blumei* Benth. 'Golden Bedder') in Brazil (Fonseca et al., 1989). Transmission tests using purified CYVd ssRNA were reported later (Fonseca et al., 1990). A similar viroid-like molecule, tentatively named *Coleus blumei* viroid 1 (CbVd 1) from Germany was isolated shortly after from *S. scutellarioides* 'Bienvenue' in Germany and sequenced (Spiker et al., 1990). Although resembling a viroid in size and molecular structure, no conclusive evidence of the viroid nature of CbVd 1 was obtained because Koch's postulates were apparently not completed (Spiker et al., 1990). Even though not fully characterized as a viroid, CbVd 1 was still classified as the prototype of a new class of viroids (Spiker et al., 1990). In this present study, we report the complete nucleotide sequence of the original CYVd from *S. scutellarioides* and its relationship to CbVd 1.

The viroid source for sequence analysis was the original plant of *S. scutellarioides* 'Golden Bedder' (syn. 'Amarelo') from which CYVd was first isolated (Fonseca et al., 1989). Nucleic acids were phenol-chloroform-extracted and partially purified by CF-11 cellulose fractionation as described previously (Fonseca et al., 1989). The viroid RNA was separated from cellular low Mr RNAs by bidirectional PAGE (Schumacher et al., 1983) as modified by Flores et al. (1985). CYVd RNA eluted from the corresponding gel slices was then used for sequence analysis. A partial sequence was established by direct sequencing as described by Hernández et al. (1992). This allowed for the synthesis of an oligonucleotide (S' dCGTTCAGCGTCCAGGA 3'), which was used as a primer for first-strand cDNA synthesis. Second-strand DNA was synthesized by the method of Gubler & Hoffman (1983) and the resulting double-stranded cDNA was cloned in the Smal site of pUC18. The nucleotide (nt) sequences of the cDNA inserts were determined from two full-length clones and from one longer-than-unit length clone by the dideoxynucleotide chain termination method (Sanger et al., 1977) using T,
DNA polymerase. Secondary structure analysis was carried out with the RNA folding program MFOLD version 2 (Zuker, 1989).

The complete sequence and the stable secondary structure of CYVd are shown in Fig. 1. Sequence analysis revealed that the RNA of CYVd consists of 248 nt with a G and C to A and U ratio of 1.23. Seventy-one percent of the nt are base-paired (54 G to C, 29 A to U and five G to U). The secondary structure of CYVd, determined according to Zuker (1989), is helicoidal and rod-like, similar to that of other previously described viroids (Diener, 1991). Comparison of the CYVd nt sequence with the CbVd 1 viroid-like RNA, also from S. scutellarioides, indicated that they are closely related (98.9% of nt matching). Differences between CYVd and CbVd 1 were observed at only three sites: one A insertion at position 241, one U deletion at position 25 and a U to A nt exchange at position 26 (Fig. 1). All mutations were found in the left-hand part of the CYVd molecule. The last two mutations were localized in one A-rich segment composed of eight nt. A similar A-rich segment occurs in the putative pathogenicity (P) domain (Keese & Symons, 1985) of potato spindle tuber viroid (PSTVd) and related viroids (McInnes & Symons, 1991). The P domain has been associated with symptom expression and has a high sequence variability in these viroids (McInnes & Symons, 1991; Schnölzer et al., 1985; Visdaver & Symons, 1983, 1985). However, the functional significance of the A-rich segment in CYVd remains to be determined. The nt differences induced only local structural changes at the sites where they occurred, without causing significant alterations in the overall secondary structure of the CYVd molecule when compared with CbVd 1. Our data support the current classification of viroids and the notion that the viroid-like molecule from S. scutellarioides is the prototype of a new subgroup (Spiker et al., 1990). The present classification system is also supported by a consensus phylogenetic tree obtained for some representative members of viroids and viroid-like satellite RNAs (Elena et al., 1991; Hernandez & Flores, 1992).

A double-stranded cDNA fragment was prepared by the digestion of a clone of CYVd with EcoRI and BamHI. This fragment consisted of a longer-than-unit length copy of the CYVd genome, from position 69 at the 5' end to position 79 at the 3' end, plus short flanking regions derived from the polylinker of pUC18. This cDNA fragment was separated by 5% PAGE, purified by electroelution and inoculated onto 20 plants of S. scutellarioides ‘Frilled Fantasy’. Each plant was mechanically inoculated by slashing with 100 ng of cDNA dissolved in buffer (10 mM-Tris-HCl pH 7.5, 1 mM-EDTA). Ten plants of each cultivar were also slash-inoculated with 100 ng of CYVd RNA purified from infected tissues of the original source, ‘Golden Bedder’. Plants inoculated with each of these and control plants inoculated with buffer alone were kept in a greenhouse at 25 to 30 °C. Infectivity was assayed by Northern blot hybridization of partially purified nucleic acids that had been prepared from leaf tissue of inoculated and control plants 30 days after inoculation. The RNA from 5 g of inoculated leaf material was purified and separated by bidirectional PAGE (Schumacher et al., 1983), blotted onto Hybond-N nylon membranes (Amersham) and hybridized at 68 °C overnight. The digoxigenin-labelled RNA probe used for hybridization was synthesized by in vitro transcription of CYVd cDNA, cloned in the vector pSK+(Stratagene) using T₃ RNA polymerase (Boehringer Mannheim).

Koch's postulates were fulfilled for CYVd using both purified RNA and cDNA. All 10 CYVd RNA-inoculated plants became infected 30 days after inoculation. Infectivity of CYVd cDNA was observed in 17 of 20 inoculated ‘Frilled Fantasy’ plants. Northern blot analysis of the longer-than-unit length cDNA-infected plants demonstrated the presence of RNA molecules with an electrophoretic mobility indistinguishable from that of authentic CYVd RNA (Fig. 2). The production of infectious cDNA clones has become an essential tool for molecular studies of viroid replication and has also been important in determining the effect of in vitro mutagenesis of specific viroid sequences on pathogenesis.
Fig. 2. Northern blot analysis of partially purified nucleic acids from S. scutellarioides 'Frilled Fantasy' isolated 30 days after inoculation with buffer (control plants; lane 1), CYVd RNA (lane 2) or CYVd cDNA (lane 3). The upper and lower bands are the circular and linear forms of CYVd respectively.

(Owens & Hammond, 1991). Construction and analysis of mutant clones will permit the characterization of regions important in CYVd replication.

The majority of S. scutellarioides cultivars have been introduced into South America from European countries (through stem and shoot cuttings) during the last 200 years. The high sequence similarities observed between CYVd and CbVd 1 suggest that either they have a common geographical origin or have emerged from a similar evolutionary process.

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