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BRIEF REPORT

Identification and characterization of a new coleviroid (CbVd-5)

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Abstract A viroid-like RNA was detected from coleus (*Coleus blumei*) in China. It consisted of 274 nucleotides and had 66% sequence identity with a member of the closest known viroid species. The predicted secondary structure is rod-shaped with extensive base pairing, and it has the conserved region characteristic of the genus *Coleviroid*. Two terminal sequences that are highly conserved among some members of the genus were also identified. The viroid-like RNA was successfully transmitted to coleus by slash-inoculation. This viroid was identified as a new member of the genus *Coleviroid*, and we tentatively propose the name Coleus blumei viroid 5 (CbVd-5).

Viroids are the smallest known pathogens in plants. They replicate autonomously in susceptible hosts and can cause diseases with significant agricultural implications. These single-stranded, non-protein-encoding, circular, small RNA molecules can fold into a rod-like structure, and viroids appear to rely entirely on host factors for their replication [1, 3, 4]. Previous studies have linked the existence of viroids with pathogenesis, and infection sometimes causes serious disease problems for crops and fruit trees [10, 19]. Several ornamental plants, including coleus (*Coleus blumei*), are known to be infected by viroids. To date, only viroids belonging to the genus *Coleviroid* are known to infect coleus. All of the coleus viroids share a common central conserved region (CCR) and are the members of the family *Pospiviroidae* [5, 6, 12].

Coleus blumei viroid 1 (CbVd-1) was first reported in commercial yellow Coleus in Brazil and was later detected from a variety of Coleus cultivated in many countries including China and Japan [7, 8, 11, 14, 21, 22, 25]. The incidence of CbVd-1 is quite high, ranging from 16 to 68% in the same cultivar (cv.), and sometimes the infection rate is 100%. CbVd-1 is transmissible from coleus to coleus by mechanical and graft inoculation or through the seeds [20]. Its infection can be either asymptomatic or result in symptoms including dwarfing or slight chlorosis, depending on the cultivar [7, 20, 21]. In addition, members of three other distinct viroid species, CbVd-2, CbVd-3 and CbVd-4, have also been detected from coleus in Germany [23, 24]. However, the geographical distribution of CbVd-2, -3, and -4 has not yet been investigated widely, and information is limited, especially on CbVd-4, for which only the nucleotide sequence has been deposited in a DNA database. Here, we report the molecular and biological characterization of a member of a new viroid species in the genus *Coleviroid* isolated from coleus in China.

In April 2007, a total of 20 symptomless coleus plants (cv. Sukang) were collected from Tianjin in China. Low-

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molecular-weight RNAs were extracted for the analysis of viroid infection according to procedures described by Li et al. [13]. RNA extracts were separated using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) under non-denaturing and denaturing conditions and stained with silver [18]. In one of the samples, we detected two RNA bands suggestive of circular, viroid-like RNA (Fig. 1Ia). The smaller one (arrow in Fig. 1Ia) was identified as CbVd-1 based on the migration rate in the 2D-PAGE and also on the result from Northern analysis (data not shown), the larger band (white arrowhead in Fig. 1Ia) is ca. 20–30 nucleotides (nt) larger than CbVd-1 (ca. 250 nt) and is apparently different from CbVd-1.

A major characteristic of viroids is infectivity; i.e., replication in suitable host plants [3, 10, 19]. We therefore carried out slash-inoculation using low-molecular-weight RNAs extracted from the coleus (cv. Sukang) plant into viroid-free coleus seedlings. Cucumber (*Cucumis sativus* cv. Suyo) seedlings at the cotyledon stage were also inoculated with the RNAs by mechanical inoculation. Control plants were inoculated with 100 mM Tris–HCl-10 mM EDTA (pH 7.5). All of the plants were maintained at 28°C in a

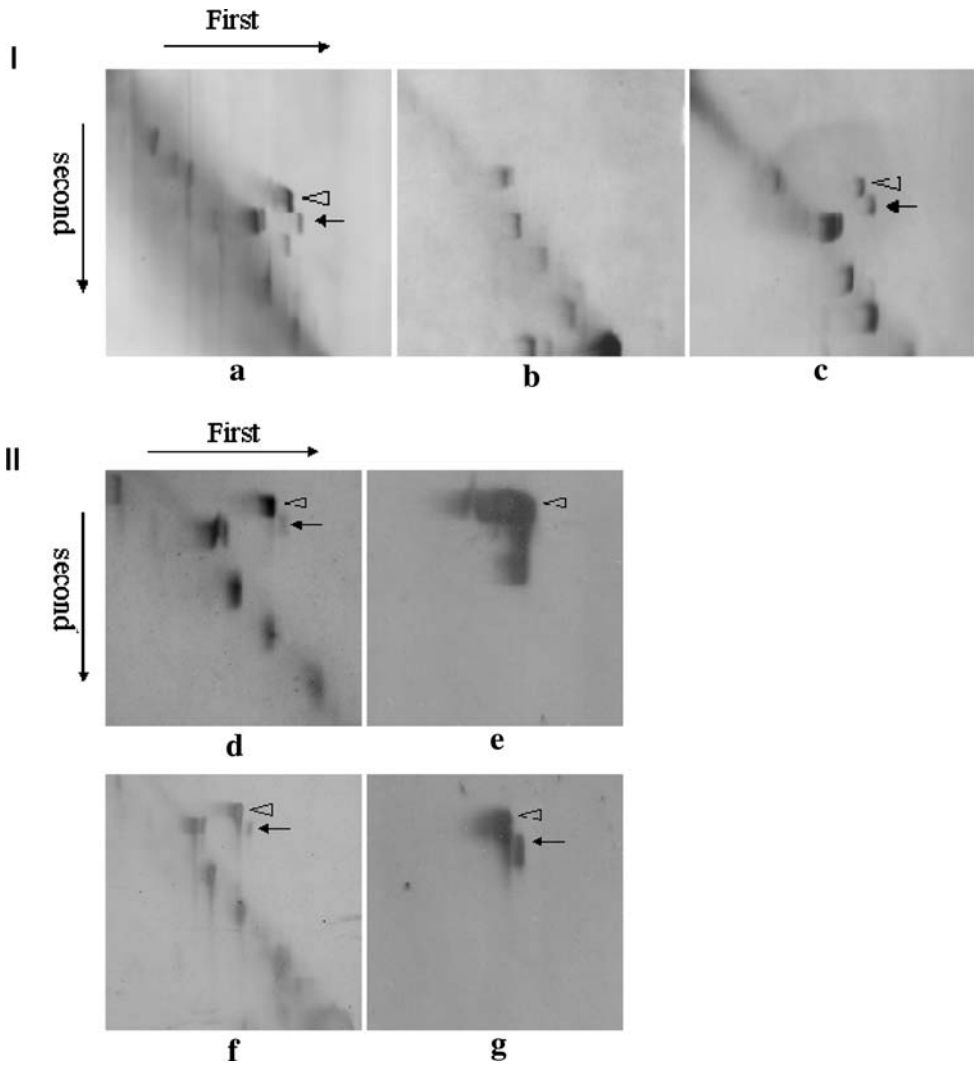


Fig. 1 *I* 2D-PAGE analysis of RNA extracts from coleus plants. **a** coleus (cv. Sukang) collected in Tianjin, China. **b** Healthy coleus seedling. **c** coleus seedling 3 months after artificial inoculation with the RNA extract from the original Coleus (cv. Sukang). Two viroid-like RNA bands are indicated by an *arrow* and a *white arrowhead*, respectively. *Horizontal* and *vertical arrows* with first or second indicate the direction of electrophoresis for the "first" and the "second" dimension, respectively. *II* 2D-PAGE and northern hybridization analysis of RNA isolated from a coleus seedling artificially infected with an RNA extract from Coleus (cv. Sukang). **d** and **f** 2D-PAGE analysis of RNA extract from Coleus seedling 1 year after inoculation of the RNA extract from Coleus (cv. Sukang). Gels were stained with silver. **e** Northern hybridization of (**d**) using a DIG-labeled cRNA probe for CbVd-5. **g** Northern hybridization of (**f**) using DIG-labeled cRNA probes for CbVd-5 plus CbVd-1. The *arrow* indicates CbVd-1, and the *white arrowhead* indicates CbVd-5. *Horizontal* and *vertical arrows* with "first" or "second" indicate the direction of electrophoresis for the first and the second dimension, respectively

greenhouse. Three months later, leaf samples were collected and analyzed for infection with viroid-like RNAs using 2D-PAGE. Two similar RNA bands with the properties of single-stranded circular RNA were again detected in the coleus seedlings inoculated with the low-molecular-weight RNA extract (Fig. 1Ic). The corresponding bands were not detectable in the viroid-free Coleus seedlings (Fig. 1Ib) or the inoculated cucumber seedlings (data not shown), indicating that the larger viroid-like RNA can be mechanically transmitted to, and replicate in, coleus.

A set of universal primers specific for all of the known CbVds was designed for cloning and sequencing of the newly detected viroid-like RNA in coleus. CbVd R (5′-CGCTGCCAGGGAACCCAGGT-3′) was used as the primer for reverse transcription (RT), and this primer, together with the forward primer (CbVd F) (5′-GCTGC AACGGAATYCAGKGC-3′), was used for the polymerase chain reaction (PCR). RT-PCR was performed as described previously [26]. Low-molecular-weight RNAs extracted from coleus were used as the template. PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide and visualized under UV light. A PCR product with a size of ca. 270 bp was cloned into the pGEM-T vector (pGEM-T Vector System, Promega) and introduded into *E. coli* DH5α by transformation. Selected clones were sequenced using an automated DNA sequencer (ABI Prism 3730XL DNA Analyzer). The nucleotide sequences obtained were sent to the DNA Data Bank of Japan (DDBJ) (National Institute of Genetics, Shizuoka, Japan) for BLAST homology search.

Some clones obtained using the universal primers matched almost completely to the known CbVd1 sequence, but others showed only limited sequence identity to the known CbVds. Based on the latter sequences, we designed a new set of primers, CbVd5R (5′-AATTGAGGTCAAACCT CTTT-3′) and CbVd5F (5′-GACTAGAACAGTAGTAA AGA-3′). These primer sequences were designed from the newly detected sequences by arranging them to connect with each other in a tail-to-tail orientation. They were used for the RT-PCR amplification of the possible new coleus viroids. RT-PCR, cloning and sequencing of the complete RNA were performed as described above.

Using the primers CbVd5R and CbVd5F, a PCR product with a size of ca. 270 bp was successfully amplified from the original coleus samples (cv. Sukang) and also from the Coleus seedling artificially infected with the RNA extracts. The size of the PCR product is consistent with what we first estimated from 2D-PAGE analysis. A total 20 cDNA clones were sequenced. All of the sequences consisted of 274 nucleotides with minor sequence variations, which will be appear in the GenBank databases with the accession numbers FJ151370-FJ151372. Hereafter, we tentatively named it Coleus blumei viroid 5 (CbVd-5).

The RNA sequence of CbVd-5 (database accession FJ151370) is composed of 52A (18.97%), 66U (24.45%), 80G (29.19%), and 76C (27.37%) and shares 66.0% sequence homology with CbVd-1bv (database accession number X95366), 60.1% with CbVd-4rl (database accession number X97202), and 52.9% with CbVd-3rl [23] (database accession number X95364). Molecular phylogenetic analysis was performed using ClustalW (available on the internet at http://www.ddbj.nig.ac.jp/searches-e.html), and the tree was drawn using TreeView (ver. 3.1). The analysis revealed that CbVd-5 is located between CbVd-1/CbVd4 and CbVd-2/CbVd-3 (Fig. 2).

The sequence was analyzed for putative translation products in both the plus and minus strands, with AUG and GUG as possible initiation codons, using CLC Combined Workbench (http://www.clcbio.com/index.php?id=28). Putative open reading frames were identified, but significant homologies were not detected in BLAST searches (data not shown). Therefore, like other viroids, the genome presumably does not encode any polypeptides [1, 3].

A predicted secondary structure of minimum free energy for CbVd-5 was drawn using the CLC Combined Workbench. The 274-nt sequence has the potential to form a rod-like structure with a high degree of base pairing, like all of the known viroids. We compared the secondary structure with those of all of the known CbVds. The CCR (the red letters in Fig. 3) was highly conserved among the CbVds,

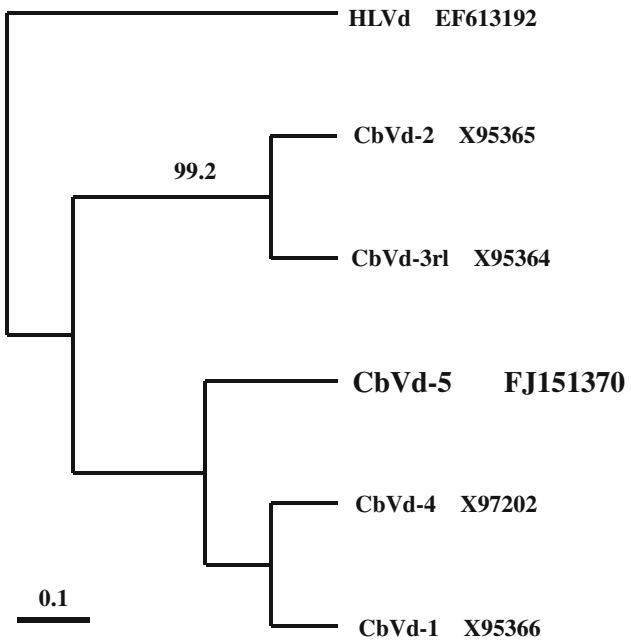
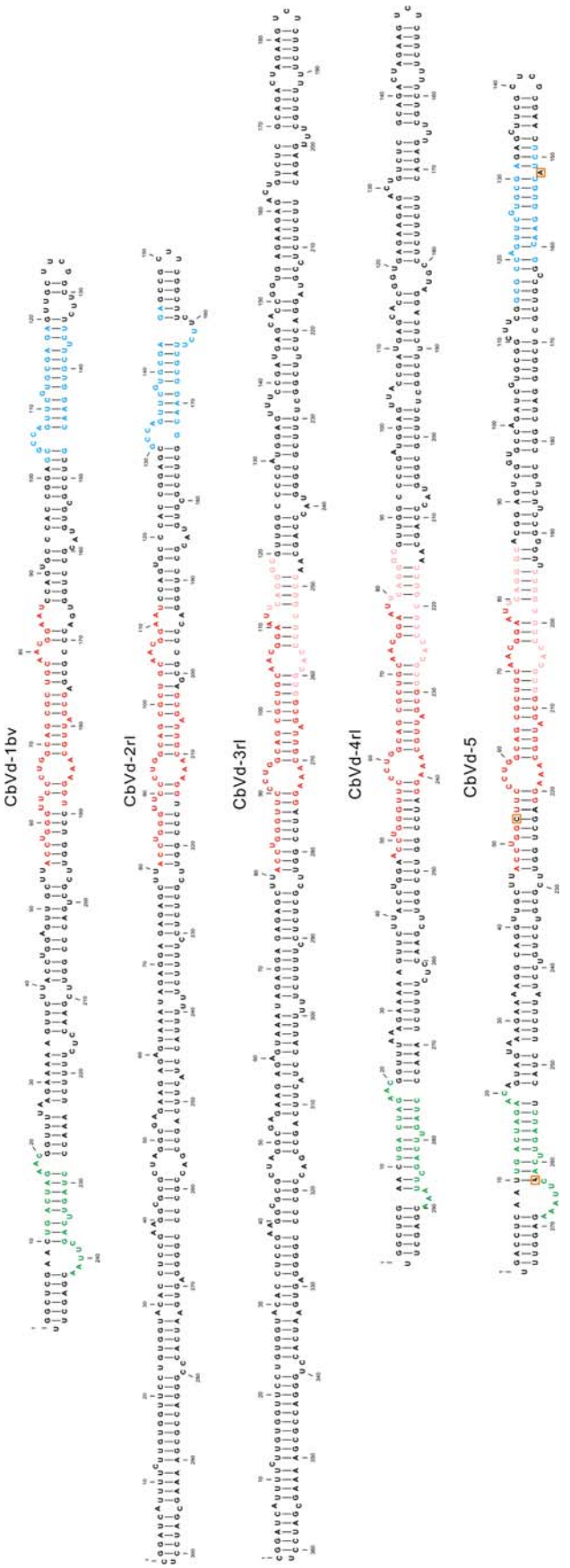


Fig. 2 A phylogenetic tree of Coleus blumei viroids. Branches with bootstrap support less than 70% were collapsed. Numbers in the branches indicate bootstrap support from NJ (1000 replicates). The new Coleus blumei viroid identified in this report is designated in the figure as CbVd-5. Hop latent viroid (HLVd) was used as an out-group



◀ **Fig. 3** Comparison of the predicted rod-like secondary structures of CbVd-5 and members of the known CbVd species in the genus *Coleviroid*. Colored sequences were conserved in all or among some of the *Coleviroid* members. *Red* sequences are the CCR of members of the genus *Coleviroid*. *Pink* sequences are also conserved among CbVd-3, CbVd-4 and CbVd-5, at the right boundary of CCR. *Green* sequences are conserved among CbVd-1, CbVd-4 and CbVd-5, at the terminal left region. *Blue* sequences are conserved among CbVd-1, CbVd-2 and CbVd-5, at the terminal right region. Unique nucleotide substitutions found in CbVd-5 within in the CCR, the semi-conserved sequence in the terminal left and the terminal right regions are boxed. The nucleotide sequences of the known coleviroids were obtained from GenBank; CbVd-1bv (X52960), CbVd-2rl (X95365), CbVd-3rl (X95364), and CbVd-4rl (X97202) (color in online version)

but only CbVd-5 showed a unique nucleotide substitution at position 53, in which G in CbVds -1 to -4 was replaced by C in CbVd-5. The right boundary sequences of the upper (7-nt) and the lower (14-nt) strand of the CCR were also conserved among CbVd-3, -4 and -5 (the pink letters in Fig. 3).

In addition to the CCR, two conserved sequences were also identified in the terminal left and the terminal right regions (the green and the blue letters in Fig. 3, respectively), in which sequence was conserved among some, but not all, CbVds. That in the terminal left region was conserved among CbVd-1, -4 and -5 (the green letters in Fig. 3); on the other hand, that in the terminal right region was conserved among CbVd-1, -2 and -5 (the blue letters in Fig. 3). It should be noted that only CbVd-1 and -5 shared both sequences. In the genus *Coleviroid*, it was already pointed out that the terminal conserved region (TCR) was found only in the two largest members, CbVd-2 and CbVd-3 [6, 10]. The conserved sequence in the terminal left region of CbVd-1, -4 and -5 (the green letters in Fig. 3), found in this analysis, may be a different type of terminal conserved region for the three smaller members of the genus; i.e., the equivalent element for TCR of the two largest members.

Using a digoxigenin (DIG)-labeled cRNA probe for CbVd-5, we examined the infection of the viroid in the original coleus plant and the artificially infected coleus seedling. The RNA extract was separated in 2D-PAGE and was contact-blotted to a nylon membrane (Hybond-N^+, Amersham Biosciences) overnight at room temperature [15]. After blotting, the gel was stained with silver (Fig. 1IId, f), and the nylon membrane was hybridized with DIG-labeled cRNA probes for CbVd-5 only or CbVd-1 plus CbVd-5. Hybridization was performed using DIG RNA Labeling Kit and Detection Starter Kit 1 (Roche). Northern analysis clearly showed that the small one hybridized with CbVd-1 and the larger one (ca. 270-nt) hybridized with the CbVd-5 probe. Since the result was the same, only the data from the artificially infected coleus seedlings are shown in Fig. 1IIe and g, which confirmed

that CbVd-5 can be mechanically transmitted to, and replicate in, coleus.

Taking into consideration the unique nucleotide sequence, a predicted rod-like secondary structure, the conserved regions in the molecule and the ability to replicate in coleus plants, the 274-nt viroid detected from coleus (cv. Sukang) in China was identified as a member of a new CbVd species in the genus *Coleviroid*. Since four CbVds have been reported so far, although CbVd-4 was recorded only in the DNA database, it should be appropriate to propose the name Coleus blumei viroid 5 (CbVd-5) for the species.

CbVd-5 shared the CCR for members of the genus *Coleviroid* and two additional semi-conserved sequences at the left and the right terminal regions of the predicted secondary structure. Neither terminal sequence was conserved among all members of the genus. It should be noted that the CCR and the conserved sequences were interrupted by blocks of unique sequences that are quite different from each other among the species. The result seems to indicate that CbVd-5 was also created by extensive recombination or by natural shuffling of putative Coleus viroid ancestors [24].

The CCR is known to be an important structural element for pospiviroid replication; i.e., processing and ligation [2, 9]. The nucleotide substitution ($G^{53} \rightarrow C$) found in CCR of CbVd-5 (boxed sequence, Fig. 3) may have some influence on the replication of CbVd-5. In addition, other nucleotide substitutions were found in the semi-conserved terminal left ($G^{262} \rightarrow A$) and the terminal right ($U^{152} \rightarrow A$) sequences. The terminal right and/or the terminal left region of the genus *Pospiviroid* is/are responsible for the regulation of pathogenicity and accumulation/replication of the viroid [16, 17]. The concentration of CbVd-5 was similar to that of CbVd-1 in the early stage of infection but reached a higher level than CbVd-1 in the later stage (unpublished data). Since CbVd-1 and CbVd -5 share the two terminal sequences, the difference in the sequence may be correlated to the different accumulation patterns. The Coleus viroids may provide a suitable system for the molecular dissection of viroid replication and pathogenicity, and also of viroid evolution.

Given the small number of plants tested, we have not yet identified how CbVd-5 infection influences the growth of Coleus, which will require further investigation. Further study will also be essential to understand the distribution of CbVd-5 in the world commercial coleus industry.

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