Dahlia latent viroid: a recombinant new species of the family *Pospiviroidae* posing intriguing questions about its origin and classification

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A viroid-like RNA has been detected in two asymptomatic dahlia accessions by return and double PAGE. It appeared smaller than *Chrysanthemum stunt viroid* and *Potato spindle tuber viroid*, the two members of the genus *Pospiviroid*, family *Pospiviroidae*, reported in this ornamental previously. RT-PCR with primers designed for amplifying all pospiviroids produced no amplicons, but RT-PCR with random primers revealed a 342 nt RNA. The sequence of this RNA was confirmed with specific primers, which additionally revealed its presence in many dahlia cultivars. The RNA was named *Dahlia latent viroid* (DLVd) because it replicates autonomously, but symptomlessly, in dahlia and shares maximum sequence identity with other viroids of less than 56 %. Furthermore, DLVd displays characteristic features of the family *Pospiviroidae*: a predicted rod-like secondary structure of minimum free energy with a central conserved region (CCR), and the ability to form the metastable structures hairpins I and II. Its CCR is identical to that of *Hop stunt viroid* (HSVd, genus *Hostuviroid*). However, DLVd: (i) has the terminal conserved region present in members of the genus *Pospiviroid*, but absent in HSVd, and (ii) lacks the terminal conserved hairpin present in HSVd. Phylogenetic reconstructions indicate that HSVd and *Pepper chat fruit viroid* (genus *Pospiviroid*) are the closest relatives of DLVd, but DLVd differs from these viroids in its host range, restricted to dahlia so far. Therefore, while DLVd fulfils the criteria to be a novel species of the family *Pospiviroidae*, its recombinant origin makes assignment to the genera *Pospiviroid* or *Hostuviroid* problematic.

INTRODUCTION

Viroids are small, circular, non-coding, ssRNAs that can infect specific host plants, replicate autonomously and cause disease (Diener, 2003; Ding, 2009; Flores et al., 2005; Tsagris et al., 2008; Verhoeven et al., 2012). Although certain satellite RNAs display similar structural features, they depend for their replication and transmission on helper viruses (Hu et al., 2009). Due to their circular structure, viroid-like RNAs (either genuine viroids or satellites) display a mobility in denaturing polyacrylamide gels significantly slower than linear host RNAs of similar size and, based on this property, several detection procedures have been developed (Flores et al., 1985; Rivera-Bustamante et al., 1986; Schumacher et al., 1986). These procedures are also instrumental for obtaining purified preparations of viroid-like RNAs for subsequent cloning purposes.

To be considered a novel viroid species, an isolated small circular RNA must contain certain sequence or structural regions/motifs typically conserved in viroids and exhibit autonomous replication when mechanically inoculated free of other accompanying nucleic acids. Moreover, these conserved regions/motifs serve as key criteria for classification in one of the two viroid families (Owens et al., 2011). Members of the family *Pospiviroidae*, type species *Potato spindle tuber viroid* (PSTVd), replicate in the nucleus and present a central conserved region (CCR) (McInnes & Symons, 1991) as well as a terminal conserved region (TCR) or a terminal conserved hairpin (TCH) (Flores et al., 1997; Kolunow & Rezaian, 1988), whereas members of the family *Avsunviroidae*, type species *Avocado sunblotch viroid* (ASBVd), can form hammerhead ribozymes that
mediate their replication in plastids (Flores et al., 2000; Hutchins et al., 1986). Within the family Pospiviroidae, PSTVd and Hop stunt viroid (HSVd) are the type species of genera Pospiviroid and Hostuviroid, respectively.

In 2010, three foreign pot-grown accessions of dahlia (Dahlia sp.) without discernible symptoms were examined by return PAGE (r-PAGE) (Roehorst et al., 2000; Schumacher et al., 1986) during a routine inspection of ornamental plants in The Netherlands. As a result, a viroid-like RNA was detected in two of the accessions with a mobility slightly different from that of PSTVd and Chrysanthemum stunt viroid (CSVd), the two viroid species found in dahlia so far (Nakashima et al., 2007; Tsushima et al., 2011). This finding raised a suspicion for the presence of a third (either known or novel) viroid and stimulated its characterization. Here, we report our results showing that a viroid different from PSTVd, CSVd and other known viroids, infects dahlia. We propose to name it dahlia latent viroid (DLVd) because dahlia is the first and only host identified so far, and symptoms have not been recorded. Furthermore, we propose classification of DLVd as a novel member of the family Pospiviroidae, based on its molecular and biological properties. However, the origin inferred for DLVd – a recombinant between members of genera Pospiviroid and Hostuviroid – poses particular problems for its allocation to one or the current genera.

RESULTS

Preliminary indications for the presence of a viroid-like RNA in dahlia

Analysis by r-PAGE of nucleic acid preparations from dahlia revealed a band, present only in the samples from two pot-grown accessions (with yellow and dark red flowers but not in another accession with red flowers), showing the low mobility typical of a circular viroid-like RNA (Fig. S1, available in JGV Online). This band was accompanied by another one, moving faster, presumably generated by the linear forms of the same viroid-like RNA. Because these two bands displayed a mobility significantly higher than the corresponding bands generated by CSVd and PSTVd (297 nt) (lanes 1, 2, 3 and 4, respectively) and of the viroid-like RNAs from two accessions of pot-grown dahlia with yellow and dark red flowers (lanes 5 and 6) after double PAGE in 5% gels. Only the second denaturing gel, stained with ethidium bromide, is presented.

various ornamentals (Owens et al., 2011; Verhoeven et al., 2012) – or a novel viroid species.

To get a preliminary insight into these alternative possibilities, nucleic acid preparations from dahlia containing the viroid-like RNA were examined by RT-PCR with the primer pairs Pospi1-FW/RE and Vid-FW/RE (Verhoeven et al., 2004), designed for detecting all known pospiviroids including PCFVd (Verhoeven et al., 2009). No amplification product was obtained, suggesting that the viroid-like RNA from dahlia would not be a member of the genus Pospiviroid. RT-PCR analysis with primers HS3 and HS4 specific for HSVd (Osaki et al., 1998), a viroid with a broad host range of the genus Hostuviroid, also failed to generate any amplification product.

Molecular characterization: sequence and proposed secondary structure

To clone and determine the sequence of the viroid-like RNA from dahlia, its circular forms were purified by two consecutive PAGEs in denaturing gels (to ensure removal of contaminating host RNAs), eluted and used for cDNA production with a specific RT-PCR approach. Both cDNA strands were synthesized with a 26-mer primer – having its six 3’-terminal positions degenerated – in two consecutive reactions catalysed by reverse transcriptase and DNA polymerase, respectively, with the cDNAs then being PCR-amplified with a 20-mer primer having the non-degenerate sequence of the first primer (Navarro et al., 1996). Analysis by non-denaturing PAGE and ethidium bromide staining revealed amplicons with sizes ranging from 250 to 700 bp, which were subsequently cloned. The resulting recombinant plasmids were restricted with appropriate enzymes, and ten inserts with sizes similar to those of the initial amplicons were sequenced (Fig. S2). Examination of the sequence of the two longest inserts showed that it comprised two repeats in tandem of a 342 nt unit containing regions typically conserved in some
viroids. Four other inserts examined corresponded to slightly longer-than-unit portions of the same sequence. These results were further validated by RT-PCR amplification of the purified circular forms with two adjacent primers of opposite polarity (DLVd-P1 and DLVd-P2) derived from the 342 nt sequence. PAGE analysis revealed a prominent band of the expected size (Fig. S3) that was eluted and cloned. Sequencing of ten clones from each of the two dahlia accessions confirmed the previous sequence with minimal variations (A55→C, G147→A and UUUUUU277→GAUUUU) observed in three independent clones. These mutations could reflect natural variability or amplification artefacts. The master sequence of the viroid-like RNA from dahlia, deposited in the NCBI GenBank (accession no. JX263426), thus consists of 342 nt: 72 A (21.05 %), 69 U (20.17 %), 105 G (30.70 %) and 96 C (28.07 %), making a G+C content of 58.77 %. Analysis of nucleic acids preparations by denaturing PAGE and Northern-blot hybridization, with a DLVd-specific riboprobe for detecting (+) strands, revealed the characteristic circular and linear forms in the two dahlia pot-grown accessions with yellow and dark red flowers, but not in the accession with red flowers (Fig. S3).

The predicted secondary structure of minimum free energy is a rod-like conformation similar to that proposed for most viroids (Keese & Symons, 1985; Owens et al., 2011; Säng er et al., 1976), except for a short bifurcation in the right terminal domain, wherein 67.84 % of the nucleotides are paired (65.51 % G:C, 26.72 % A:U, and 7.76 % G:U pairs) (Fig. 2).

Conserved regions and structural elements

The rod-like secondary structure predicted for the viroid-like RNA from dahlia contains a CCR, the key structural element (and taxonomic criterion) for assigning viroids to the family Pospiviroidae. Hereafter, we shall refer to this RNA as Dahlia latent viroid (DLVd), because its limited nucleotide identity with other viroids and biological properties (see below) support this notion. Regarding allocation to a specific genus within this family, the class of CCR and the presence of a TCR or a TCH (both seem mutually exclusive) are the main criteria (Flores et al., 1997; Owens et al., 2011). The CCR of DLVd is identical to that of HSVd (Ohno et al., 1983), the type (and single) species of the genus Hostuviroid, and to that of Columnea latent viroid (Hammond et al., 1989). The latter viroid, nevertheless, has been assigned to the genus Pospiviroid because it possesses a TCR, higher overall sequence identity and biological properties resembling those of most members of this genus (Flores et al., 1998). DLVd also has a TCR like pospiviroids, but not the TCH present in HSVd (Fig. 2).

Thermal denaturation studies of PSTVd and four closely related viroids have revealed that they adopt metastable branched conformations containing hairpins. The most prominent is hairpin I (HPI) formed by the upper CCR strand and the flanking inverted repeat (Henco et al., 1979; Riesner et al., 1979). Subsequent phylogenetic analyses have confirmed that this is a general feature in the family Pospiviroidae (Flores et al., 1997; Keese & Symons, 1985), most likely because HPI is involved in promoting cleavage of the oligomeric (+) RNA intermediates generated by replication through a rolling-circle mechanism (Gas et al., 2007), and possibly also in other functions (Abraitiene et al., 2008). DLVd can form a typical HPI, including the capping palindromic tetraloop, the adjacent 3 bp stem, and the stem of 9–10 bp interrupted by two facing nucleotides seemingly unpaired (Fig. 2). When the lower portion of this long stem was compared in PSTVd, HSVd, CLVd and
DLVd, multiple point changes could be observed but, interestingly, without affecting the stability because they are covariations (Fig. 3).

Besides HPI, thermal denaturation analyses unveiled the so-called hairpin II (HPII), typical for all members of the genus Pospiviroid (Henco et al., 1979; Riesner et al. 1979; Riesner, 1991). This structural element with a GC-rich stem of 9 bp results from sequences of the lower strand of the rod-like secondary structure adopting an alternative metastable conformation and is critical for infectivity (Candresse et al., 2001; Qu et al., 1993). Like CLVd, but unlike HSVd, DLVd can also form HPII even if interrupted by two facing nucleotides seemingly unpaired. The sequence differences between DLVd and CLVd do not impinge on the stability of the HPII stem because they are covariations (Fig. 3). DLVd also has the polypurine stretch conserved in the family Pospiviroidae (Keese & Symons, 1985) and the core of the RY motif previously reported in the genus Hostuviroid, whereas all members of the genus Pospiviroid contain at least one complete RY motif (Gozmanova et al., 2003; Maniak et al., 2003).

**DLVd replicates autonomously, is widespread in dahlia, and has an unusually narrow experimental host range**

The molecular properties reported in the preceding sections indicate that DLVd is a new viroid. The biological properties do confirm its viroid nature. Mechanical inoculations of non-infected dahlia plants (cultivar with red flowers) by slashing their stems with a razor blade dipped in gel-eluted DLVd circular forms or in dimeric head-to-tail in vitro transcripts resulted in infection in one out of ten plants (as revealed by Northern-blot hybridization following double PAGE or by RT-PCR). To enhance the transmission efficiency, dahlia leaves were agroinfiltrated with a culture of Agrobacterium tumefaciens containing a plasmid for expressing a dimeric head-to-tail transcript of DLVd. Subsequent analyses showed that this inoculation approach resulted in a high infection rate: viroid circular forms were detected by double PAGE in six out of seven agroinoculated plants (data not shown). Therefore, DLVd is endowed with autonomous replication and fulfils the primary criterion to be considered a viroid. No symptoms were apparent (at least in the aerial part) of the DLVd-infected plants, consistent with the observations in the original dahlia plants.

To estimate the incidence of DLVd and other viroids in dahlia, a survey was carried out in the Netherlands using RT-PCR with primers DLVd-P1 and DLVd-P2, and realtime RT-PCR according to Botermans et al. (2013) for detecting pospiviroids. DLVd was present in five out of ten samples from pot-grown dahlia and in 92 (belonging to 71 cultivars) out of 100 samples from field-grown dahlia. Although part of the sampled plants showed virus-like symptoms, they could not be associated with the presence of DLVd. Sequencing of nine random PCR products generated from different dahlia cultivars showed that all sequences were identical to the DLVd master sequence obtained from the initial plants. In contrast, no known pospiviroids were detected in these samples.

To get a first insight into the experimental host range of DLVd, we inoculated blocks of ten plants of the following species: chrysanthemum and tomato by rubbing carpordon-dusted leaves with DLVd circular forms, Nicotiana benthamiana by agroinoculation, and cucumber and potato using both procedures. In addition, blocks of ten tomato seedlings were inoculated with sap, fingertips or razor blades using DLVd-infected dahlia leaves as source of inoculum. Analysis by Northern-blot hybridization and/or RT-PCR showed that none of these plant species, known to be hosts of different pospiviroids or HSVd, became infected, thus indicating that DLVd appears to have an unusually narrow host range.

To examine whether crop handling could contribute to the spread of DLVd, blocks of ten dahlia cuttings were...
mechanically inoculated with: (i) sap from an infected plant, (ii) fingertips and, (iii) razor blades contaminated by rubbing and cutting infected plants, respectively. RT-PCR using primers DLVd-P1 and DLVd-P2 revealed that while sap and fingertips did not transmit DLVd or only at a low rate (1/10), respectively, razor blade inoculation resulted in a significant fraction of infected plants (4/10). None of the infected plants showed symptoms in the aerial parts (tubers were not produced during the experiment). As a control, blocks of five tomato seedlings were PSTVd-inoculated with sap, fingertips or razor blades. In this case, nearly all plants became infected as they showed symptoms and reacted positively in real-time RT-PCR according to Boonham et al. (2004); none of the mock-inoculated dahlia and tomato plants became infected.

**Phylogenetic reconstruction, putative origin and tentative classification**

Multiple alignment of DLVd with all current species of the genus *Pospiviroid* and construction of a phylogenetic tree based on the neighbour-joining method (Saitou & Nei, 1987) revealed PCFVd as the closest relative to DLVd (data not shown); despite sharing the same CCR, CLVd was more distantly related, whereas the other members of the genus presented the same grouping reported previously (Verhoeven et al., 2009). Yet, the branch that separates DLVd from PCFVd is notably long, indicating lack of close proximity between both viroids: actually, their pairwise alignment revealed a sequence identity of only 56%. These data provide support for considering DLVd as a new species of the genus *Pospiviroid*. However, another multiple alignment including HSVd (Fig. S4), with which DLVd shares the same CCR, and construction of the correspondingly phylogenetic tree showed grouping of these two viroids. This result supports that, alternatively, DLVd could be regarded as a novel species of the genus *Hostuviroid* (Fig. 4), although the branch length and sequence identity (53.3%) (Table 1) inferred from a pairwise alignment indicated a distant relationship between DLVd and HSVd.

Careful inspection of the alignments revealed the unique character of DLVd, which is a mosaic RNA comprising fragments present in: (i) HSVd (the same 19 nt from the upper CCR and 17 out of 19 nt from the lower CCR, the latter preceded by essentially identical stretches amounting to 19 nt) and, (ii) PCFVd (56 nt essentially identical to the upper strand of the terminal left domain, including the TCR and part of the polypurine stretch (Fig. 5).

**DISCUSSION**

We have characterized a novel viroid, *Dahlia latent viroid* (DLVd), that is widespread in dahlia. This viroid differs in its molecular properties from the two members of the family *Pospiviroidae* that have been reported in dahlia – CSVd (Nakashima et al., 2007) and PSTVd (Tsushima et al., 2011) – as well as from other known viroids, with which DLVd only has less than 56% sequence identity. The presence of a CCR and other conserved regions/motifs (TCR and core RY motif, but not TCH) together with elements of secondary structure (HPI and HPII), clearly affiliates DLVd to the family *Pospiviroidae*. Together with these molecular features, the narrow host range of DLVd (restricted so far to its natural host) clearly supports that it must be considered a novel species of this family. The question remains to which genus it should be allocated. Similarly to members of the genus *Pospiviroid* but differently from HSVd, DLVd has a TCR, lacks a TCH and can form a stable HPII (Table 1). Conversely, resembling HSVd but differing from members of the genus *Pospiviroid*, DLVd has the CCR of the genus *Hostuviroid* and only exhibits the core of the RY motif (Fig. S5). Furthermore, the highest overall sequence identity of DLVd is with PCFVd and then with HSVd, from the genera *Pospiviroid* and *Hostuviroid* (Table 1). In a phylogenetic analysis derived from a multiple alignment HSVd appears the closest relative of DLVd (Fig. 4), but there is a considerable distance between them. Finally DLVd, in contrast to HSVd, has a narrow host range and in contrast to nine of the ten members of the genus *Pospiviroid* it does not infect potato and tomato.
Therefore, DLVd might be regarded as differing from current members of the family Pospiviroidae not only at the species but even at the genus level. Consequently, we propose to assign the novel viroid to the family Pospiviroidae but not yet to an existing or novel genus.

The primary structure of DLVd indicates that, like CLVd, it is partially a chimeric RNA formed by sequence fragments present in other viroids. However, there are sharp distinctions between both viroids. CLVd is composed of fragments identical with those of HSVd (19 and 17 nt from the upper and lower CCR strands), PSTVd (79 nt from the left terminal domain) and *Tomato apical stunt viroid* (TASVd) (63 nt from right terminal domain), as well as of small fragments from other viroids and fragments unique for CLVd (Hammond et al., 1989). The mosaic structure of CLVd, with predominance of fragments from HSVd, PSTVd and TASVd, is manifested in its biological behaviour: like HSVd, but unlike PSTVd, CLVd replicates and incites symptoms in cucumber; and like PSTVd and TASVd, but unlike HSVd, CLVd replicates and incites symptoms in tomato (Hammond et al., 1989). Moreover, the finding that the striking sequence identities of CLVd with PSTVd and TASVd map at both terminal domains is consistent with the proposal that these domains may be involved in recombination *in vivo* between viroids co-infecting a common host (Keese & Symons, 1985). In contrast, the chimeric structure of DLVd appears more complex and with the boundaries less well-defined: apart from the fragments corresponding to the upper and lower CCR strands of HSVd (together with an adjacent stretch in the second case), and a fragment from the PCFVd containing the TCR, the origin of the remaining part of the DLVd molecule is blurred by the lack of clear nucleotide identities with other known viroids (Fig. 5). Perhaps very ancient recombination events gave rise to a DLVd precursor that subsequently incorporated new mutations to deal with new selection pressures. The singular nature of DLVd is supported by its host range, which does not include the characteristic hosts of HSVd and PCFVd.

These results also raise the question of the molecular determinants responsible for the host range of viroids and, specifically, for DLVd. Because viroids are obligate parasites that do not code for any protein, the genomic RNA must contain sequence/structural motifs for interacting specifically with host proteins that assist invasion, replication, movement and even avoidance of defensive responses (for a review see Flores et al., 2012). Recent studies using PSTVd-infected *N. benthamiana* have provided a genomic map of specific loops/bulges in the predicted secondary structure critical for replication in single cells and for systemic trafficking in the plant (Zhong et al., 2008; for a review see Takeda & Ding, 2009). Therefore, while HSVd most probably contains RNA structural motifs capable of interacting with proteins from a wide spectrum of plants, DLVd has probably evolved structural motifs for infecting only dahlia and, perhaps, some related plant species. The lack of symptoms in DLVd-infected dahlia suggests a long co-evolution that, at least in part, could explain the specificity of the resulting interaction: by adapting to dahlia, DLVd could have lost the potential to infect other plants.

Our survey on viroids in dahlia has shown that DLVd is widespread in this plant. Because of international trade, DLVd is expected to occur in dahlia crops worldwide. However, the pospiviroids reported in dahlia previously, CSVd and PSTVd (Nakashima et al., 2007; Tsushima et al., 2011), were not found, indicating that their presence in dahlia is more erratic and possibly more recent than that of DLVd. The absence of symptoms in dahlia infected by DLVd explains why it has remained unnoticed in this ornamental crop. The vegetative propagation of dahlia has most likely favoured the persistence of DLVd, while its mechanical transmission – although not very efficient – may have played a role in the spread of this viroid between different dahlia cultivars. Our study has revealed the
molecular and major biological properties of DLVd, and provides testing methods for the control of this viroid in dahlia.

**METHODS**

**Source of infected material, transmission assays and survey.** Samples of two accessions of pot-grown dahlia (*Dahlia* sp.) with yellow and dark red flowers infected by DLVd, but without discernible aerial symptoms, were initially analysed. Samples were found free of viruses by mechanical transmission to a series of indicator plants, electron microscopy and PCR with primers for *Dahlia mosaic virus*.

To test the autonomous replication of DLVd, purified preparations of either gel-eluted circular forms or in vitro-synthesized head-to-tail dimeric transcripts of DLVd resigned in 50 mM K$_2$HPO$_4$, were inoculated by stem incision to blocks of dahlia cuttings (50 ng per plant) of a pot-grown accession with red flowers free of DLVd. Leaves from cuttings of this accession were also inoculated by agroinfiltration with a culture of *A. tumefaciens* (strain C58) harbouring a plasmid expressing the same head-to-tail dimeric transcript of DLVd. The presence of DLVd in the inoculated plants was evaluated (6 weeks later) by Northern-blot hybridization or RT-PCR as indicated in Results.

DLVd was also inoculated using different procedures (see Results) to *tomato* (*Lycopersicon esculentum* L.) cv. Moneymaker and Rutgers, *potato* (*Solanum tuberosum* L.) cv. Nicola, *N. benthamiana*, *chrysanthemum* (*Chrysanthemum morifolium* L.) cv. Bonnie Jean and cucumber (*Cucumis sativus* L.) cv. Suyo. Viroid standards included PSTVd and PCFDV from tomato, CSVd from chrysanthemum and HSVd from cucumber.

To assess the transmission efficiency of different mechanical procedures, blocks of dahlia cuttings of an accession free of DLVd were inoculated by: (i) rubbing carborundum-dusted leaves with sap from an infected plant, (ii) rubbing non-carborundum-dusted leaves with fingertips contaminated by rubbing young leaves of the inoculum source, and (iii) making stem incisions with a razor blade previously contaminated by cutting an infected plant (Verhoeven et al., 2010).

Dahlia plants, collected during a survey in 2012, were examined for DLVd by RT-PCR using primers DLVd-P1 and DLVd-P2, and for known pospiviroids by real-time RT-PCR according to Botermans et al. (2013).

**Nucleic acid extraction, fractionation, analysis and purification by PAGE.** Nucleic acid preparations for r-PAGE and for the initial RT-PCRs with the generic primers for pospiviroids and with specific primers for HSVd, were obtained as reported (Botermans et al., 2013; Roenhorst et al., 2000; Verhoeven et al., 2004). Preparations enriched in RNAs with a high content in secondary structure from DLVd-infected dahlia leaves (20 g) – and from similar amounts of *N. benthamiana*, chrysanthemum, tomato and cucumber leaves infected with PSTVd, CSVd, PCFDV and HSVd, respectively – were obtained by phenol extraction, fractionated with non-ionic cellulose (CF11; Whatman; Pallas et al., 1987) and resuspended in water (500 µl).

Aliquots were subjected to double PAGE (Flores et al., 1985). In brief, following electrophoresis and staining with ethidium bromide, the region of the first non-denaturing gel delimited by the DNA markers of 250 and 400 bp was excised and applied on top of a second denaturing gel, wherein the viroid circular forms migrate with an electrophoretic mobility significantly slower than their linear counterparts of the same size. For preparative purposes, the DLVd circular forms identified by ethidium bromide staining were eluted by crushing the corresponding gel piece and shaking the resulting fragments for 12 h at 4°C with buffer (Tris/HCl 10 mM, pH 7.5, EDTA 1 mM, SDS 0.1%), recovered by ethanol precipitation and subjected to another round of double PAGE.

**RT-PCR amplification, cloning and sequencing.** First-strand DLVd-cDNA was prepared by incubation with avian myeloblastosis virus reverse transcriptase (AMV-RT) and the primer 5'-GCC-CCTGACCTGTGCGCCGNNNNN-3' with six randomized positions at its 3' end at 42°C for 30 min. This primer was used for synthesis of the second-strand cDNA with the Klenow fragment of the DNA polymerase 1 from *Escherichia coli* at 37°C for 30 min. The resulting dsDNAs were PCR-amplified with Taq DNA polymerase (Roche Applied Science) and a primer identical to that used for cDNA synthesis but without the six degenerated positions at the 3' end. After 30 cycles of 40 s at 94°C, 30 s at 60°C, and 2 min at 72°C, preceded by an initial denaturation at 94°C for 2 min and followed by a final extension at 72°C for 10 min, the PCR-amplified DNAs were ligated in the vector pTZ57R/T (Fermentas) with protruding 3' terminal Ts. The recombinant plasmids were amplified by transforming DH5α cells of *E. coli*, and the inserts released with EcoRI and HindIII were fractionated by PAGE in non-denaturing 5% gels, eluted and sequenced (see below) (Navarro et al., 1996; Serra et al., 2008). From the DLVd sequence, two adjacent primers, DLVd-P1 (5'-GGGGCAACTCCGAGAAGTGCTG-3') and DLVd-P2 (5'-GGGGCCCTCCTCACAGTGCTC-3'), homologous with and complementary to positions 106–126 and 86–105, respectively, were designed and applied for RT-PCR amplification of the complete viroid sequence with *Phu* DNA polymerase (Stratagene) using the buffer recommended by the supplier and the same cycling conditions described above. The amplification products were cloned in plBluescript II KS (+) (Stratagene), digested with EcoRV, and the inserts were sequenced.

Head-to-tail dimeric DLVd-dsDNA was prepared by ligation of unit-length inserts and cloning in plBluescript II KS (+) digested with EcoRV, with the results being examined for proper orientation by restriction analysis using the following primer pairs: 106–126 and 86–105 respectively. The corresponding DNA was generated by transcription in *vitro* catalysed by the T7 RNA polymerase using conventional protocols (Sambrook & Russell, 2001). For agroinoculation, the dimeric viroid-dsDNA insert was recovered as a PCR product with primers 5'-ATATATCCATGTGGATCCTCCCGGTGCTGGAAGG-3' and 5'-ATATATCACGTGGCCCTCCTGAGGTCTCGAGG3'-flanking the EcoRV site of the polylinker and containing *Ncol* and *Pml* sites, respectively (underlined). The dimeric viroid-dsDNA obtained by digestion with these two restriction enzymes was inserted into the pCambia binary vector restricted with the same two enzymes. *A. tumefaciens* C58 cells were transformed with this plasmid and the resulting bacterial culture was used for leaf infiltration (1–2 ml per plant).

**Routine detection by Northern-blot hybridization and RT-PCR amplification.** For Northern-blot hybridization, RNA preparations from dahlia or from other inoculated plants were separated by double PAGE and electrophoretically transferred to positively charged nylon membranes (Roche Applied Science). RNAs were immobilized by UV cross-linking, hybridized with a DIG-labelled riboprobe complementary to the DLVd sequence, and revealed with an anti-DIG alkaline phosphatase conjugate and the chemiluminescence substrate CSPD (Roche Applied Science) (Serra et al., 2008). Serial detection of DLVd by RT-PCR was performed with the standard format (using DLVd-P1 and DLVd-P2 and the conditions described above).

**Comparative sequence analyses, RNA secondary structure and phylogenetic reconstructions.** The nucleotide sequence of cloned RT-PCR products from DLVd was determined with an ABI 3100 Genetic Analyzer (Applied Biosystems). Multiple alignments were performed using CLUSTAL X (Larkin et al., 2007). The secondary structure of minimal free energy at 37°C of DLVd was predicted with
interaction of potato spindle tuber viroid RNA (PSTVd) and the tomato protein Virp1. *Nucleic Acids Res* 31, 5534–5543.


