An infectious viroid RNA replicon evolved from an *in vitro*-generated non-infectious viroid deletion mutant via a complementary deletion *in vivo*

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The 359 nucleotides (nt) long potato spindle tuber prototype viroid (PSTVd) is sensitive to experimentally introduced mutations as the substitution or deletion of a single nucleotide usually abolishes its infectivity, although certain sequence alterations are tolerated. This is illustrated by the fact that viroid progeny can evolve *in planta* upon inoculation with substitution mutants generated *in vitro*, and by the existence of genetically stable 356–360 nt long PSTVd field isolates. However, to date, no viable *in vitro*-generated deletion mutant of PSTVd has been reported. We have now found a 341 nt long infectious PSTVd RNA replicon that evolved in agrotransformed plants transformed with the dimeric form of an *in vitro*-deleted, non-infectious 350 bp long PSTVd cDNA unit by an additional complementary deletion of 9 nt *in vivo*. This is the first report that the deletion-abolished infectivity of a viroid is restored by an additional deletion that concurrently restabilized its perturbed secondary structure by abandoning an internal segment of the rod-like molecule. The fact that ~5% of the total PSTVd RNA genome was deleted demonstrates that the maintenance of this viroid-specific rod-like structure is not only essential for nuclease protection but also for the infectivity, i.e. transmissibility, replicability, processibility and pathogenicity of these minimal infectious agents.

Key words: RNA replicon/secondary structure/transgenic tobacco/viroid deletion mutant

Introduction

Viroids, the smallest pathogens presently known, are ~240–380 nt long non-translated circular RNA molecules. Their RNA–RNA replication proceeds in the plant cell nucleus with the aid of the DNA-dependent RNA polymerase II of the host (Sänger, 1987; Schindler and Mühlbach, 1992). Although under native conditions the viroid RNA exists as a highly base-paired rod-like structure, it is assumed to undergo certain structural transitions during its replication process (Henco *et al.*, 1979; Riesner *et al.*, 1979). Thermodynamic analysis of the potato spindle tuber prototype viroid (PSTVd) revealed a highly cooperative main transition leading from the rod-like native structure to the formation of an intermediate form with three stable hairpin (HP) structures (for review see

Riesner, 1990). Sequence comparison of PSTVd field isolates and analysis of *in vitro*-generated PSTVd mutants revealed that HP I and HP II are evidently essential for their infectivity. In all PSTVd field isolates, the sequence of the core regions of both hairpin structures was found to be highly conserved (Schnölzer *et al.*, 1985; Puchta *et al.*, 1990; Herold *et al.*, 1992; Lakshman and Tavantzis, 1993). Nucleotide substitutions and deletions of *in vitro*-generated mutants either abolished their infectivity or resulted in a viroid progeny that had reverted to the sequence of the type strain (Loss *et al.*, 1991; Owens *et al.*, 1991; Lakshman and Tavantzis, 1992; Qu *et al.*, 1993).

Nucleotide substitutions introduced into other structural domains of the PSTVd RNA molecule also destroyed viroid infectivity, in most cases. However, some novel infectious PSTVd variants could be isolated in which the introduced mutation remained stable or in which the substituted nucleotide reverted to a nucleotide that differed from that of the original sequence (Hammond and Owens, 1987; Owens *et al.*, 1991; Hammond, 1992). If one evaluates the results of all these mutagenic studies with PSTVd no general concept emerges by which a clear prediction can be made of whether a given mutation will be tolerated or not.

Here we show that the maintenance of the viroid-specific native secondary structure is evidently an essential prerequisite for the replicability and survival of these small RNA replicons. Our data further demonstrate that the intimate relationship between viroid structure and function can be studied when transgenic plants are used as a bioassay system. In this case, the continuous primary transcription of the genome-integrated mutated viroid cDNA to mutated viroid RNA offers an experimental advantage over mechanical leaf inoculation. The ample supply of these primary transcripts guarantees further secondary viroid RNA–RNA transcription and concurrent additional mutations so that natural selection can act continuously *in vivo* until viable new mutants have evolved. Thus the stable integration of a non-infectious 350 bp long PSTVd cDNA into the tobacco genome led to a viroid-infected transgenic plant. However, the subsequently replicating viroid RNA proved to be a 341 nt long truncated PSTVd that had evolved from the primary transcript of the introduced cDNA by an additional deletion of 9 nt *in planta*. In the native rod-like secondary structure this second *in vivo* deletion is located at exactly the opposite position to the first *in vitro* deletion. It can be assumed that 14 of the altogether 18 internally deleted nucleotides were originally base-paired in the native rod-like molecule, thus forming a distinct secondary structure domain. The deletion of this entire internal domain restored the viroid-specific overall rod-like structure which had been perturbed in the 350 nt long PSTVd because of the *in vitro*-deleted 9 nt. This finding demonstrates that the

Fig. 1. Cloning strategy of the *Bal31*-directed PSTVd cDNA mutagenesis. A *BamHI* monomer of the 359 bp long PSTVd KF440-2 (PSTVd 359) cDNA was cloned into pT3T7-lac, giving pTPSTV-Bm(+). This plasmid was either linearized with *XbaI* or *StyI*. After *Bal31* treatment the DNAs were recut with *EcoRI* and ligated as indicated. After transformation, a series of individual *BamHI* clones that contained PSTVd cDNAs between 300 and 360 bp in size were isolated.

rod-like secondary structure must be important for some step(s) during viroid replication, processing and systemic spreading in the host plant.

Results

Construction of PSTVd cDNA deletion mutants and plant transformation

The cloning strategy of PSTVd cDNA deletion mutants is shown in Figure 1 and described in detail in Materials and methods. Several individual clones that contained a mutated PSTVd cDNA were isolated and one clone with a 9 bp deletion of nucleotides 70–78, and therefore called PSTVd 350, was chosen for further experiments. A dimer of this cDNA was inserted in both orientations into the binary plant transformation vector pPCV702SM between the cauliflower mosaic virus (CaMV) 35S promoter (*P*_{35S}) and the *pAnos* signal sequence. The T-DNAs of the pPCV702SM derivatives were introduced into the genome of SRI tobacco via the *Agrobacterium*-mediated leaf disc transformation and 10 independent transformants of each construct were regenerated. Due to the 350 bp long PSTVd-specific cDNA units that had been introduced, the transgenic plants were named SRI 350(+) and SRI 350(–), respectively.

Analysis of the transgenic tobacco plants

The correct genomic integration of the T-DNA was ascertained by Southern blot hybridization after root formation of the SRI 350(+) and SRI 350(–) plants on kanamycin-containing medium. It revealed that all constructs had been introduced without any rearrangement (data not shown). To monitor the occurrence of autonomous viroid replication, total leaf RNA of all SRI 350(+) and SRI

Fig. 2. Demonstration by Northern analysis of autonomous viroid RNA–RNA replication in transgenic SRI tobacco and in mechanically inoculated tomato and tobacco plants. The SRI tobacco contained the original non-infectious dimeric cDNA integrate of PSTVd 350. For mechanical inoculation of tobacco and tomato, the infectious PSTVd 341 RNA that had evolved *in vivo* was used. Total RNA was glyoxalated, electrophoretically separated by PAGE, electro-transferred to Qiabrane, and hybridized to an *in vitro*-synthesized RNA probe specific for PSTVd (+)RNA. Lane 1, total RNA of uninfected SRI tobacco; lane 2, total RNA of the transgenic SRI 350 tobacco; lane 3, total RNA of SRI tobacco inoculated with PSTVd 359; lane 4, total RNA of tomato inoculated with PSTVd 341; lane 5, total RNA of tomato inoculated with PSTVd 359. The upper and lower bands in each lane represent the circular and the linear 341 and 359 nt long forms of the viroid RNA, respectively.

350(–) plants was analysed by Northern hybridization. Only one of the SRI 350(+) tobacco plants was found to be viroid infected, as indicated by strong PSTVd-specific hybridization signals (data not shown). Figure 2 shows a comparison of the PSTVd-specific RNA of SRI 350(+)/1 and of a tobacco plant that had been mechanically inoculated with the RNA of the PSTVd 359 type strain. The electrophoretic mobility of the linear and the circular viroid RNA isolated from SRI 350(+)/1 (upper and lower band, respectively) was clearly faster than the RNA of

Fig. 3. Chain length, nucleotide sequence and secondary structure of PSTVd 341 as compared with the original PSTVd 359 field isolate. The nucleotides that were *in vitro* deleted by *Bal31*-directed mutagenesis in PSTVd 350 are boxed. A thin line in the lower strand of the PSTVd 359 RNA shows the region that was *in vivo* deleted in PSTVd 341. Nucleotides that were substituted, deleted or added are indicated in the revertant. Accession number of PSTVd 341 is 234272

PSTVd 359 (compare Figure 2, lanes 2 and 3). Because the RNAs were completely denatured prior to electrophoresis by glyoxalation, their faster migration was assumed to be based on truncation of the RNA chain rather than on differences in their secondary structures.

Characterization of the SRI 350(+) PSTVd-specific RNA

To determine the chain length and the sequence of the viroid-specific RNA isolated from SRI 350(+)/1, cDNAs were produced using two different PSTVd-specific primer pairs. The same primers were again used for PCR amplification of the synthesized cDNAs, and the resulting PCR products were cloned and sequenced. The corresponding viroid cDNA clones consisted of 341 bp, and not of 350 bp as expected from the genome-integrated PSTVd 350 cDNA deletion mutant. In addition to the *in vitro*-deleted nucleotides 70–78, nucleotides 282–290 had become deleted *in planta*. This *in vivo*-deleted region of the new PSTVd 341 replicon proved to be located exactly opposite and virtually complementary to the primary *in vitro* deletion, as illustrated in the RNA secondary structure model of the original PSTVd 359 (Figure 3).

Considering the resulting secondary structure of the newly evolved and truncated PSTVd 341, it becomes evident that the *in vivo* deletion restored the largely helical rod-like viroid RNA structure which had been grossly perturbed by the preceding *in vitro* deletion of 9 nt that formed a large bulge-loop in PSTVd 350 (Figure 4). In addition to the truncation of the 18 nt, PSTVd 341 exhibited five additional sequence changes. They comprised, according to the numbering of the PSTVd prototype (Gross *et al.*, 1978), (i) a deletion of one of the six A nucleotides at position 55–60, (ii) an addition of a U behind A₆₃ in the pathogenic (P) domain, (iii) a transition of G₁₁₅ to A in the variable (V) domain, (iv) a transition of G₂₅₄ to A, and (v) a transition of C₂₅₉ to U within the central conserved (C) domain (Figure 3).

To ensure that the deletions and changes of PSTVd 341

were not due to mutations of the corresponding cDNAs, PCR amplification with plant genomic DNA was carried out. For this purpose, primers were used that bound to the 3'-end of the 35S CaMV promoter and to the 5'-end of the pAnos sequence, respectively. This strategy allowed the specific amplification of the genome-integrated cDNAs. Sequencing of the cloned PCR products demonstrated that the genome-integrated PSTVd cDNA in the corresponding transgenic tobacco plants had not been altered (data not shown).

Infectivity and genetic stability of PSTVd 341

The infectivity of the PSTVd 341 replicon from the transgenic tobacco was bioassayed in tomato plants by mechanical leaf inoculation. Total RNA extracted from SRI 350(+)/1 and *in vitro* transcripts of a plasmid containing a dimeric cDNA of PSTVd 341 were rubbed onto the leaves of tomato seedlings in the presence of carborundum. After 7 weeks the newly developing young leaves were analysed for viroid replication. In all test plants the truncated circular PSTVd 341 had accumulated to copy numbers similar to those present in control plants inoculated with the PSTVd 359 type strain (compare Figure 2, lanes 4 and 5). In contrast to the severe symptoms caused by the PSTVd 359 type strain the new PSTVd 341 induced only symptoms of intermediate severity in the tomato cultivar 'Rentita' (data not shown).

The genetic stability of the PSTVd 341 was examined by cDNA sequencing of the RNA progeny isolated from tomato plants that were mechanically inoculated either with total RNA of SRI 350(+)/1 or with *in vitro* transcripts of dimeric PSTVd 341 cDNA. Several clones containing PCR products that were recovered after amplification with the two primer pairs mentioned above were analysed to guarantee (i) that mutations were not due to PCR errors, (ii) that no additional mutations are located within the sequences covered by the primers, and (iii) that there is no sequence variability among the RNA progeny. This showed that all sequences were identical to the new RNA

Fig. 4. Evolution of PSTVd 341. Schematic representation of the evolution of PSTVd 341 *in planta*. Structural domains: T1, left terminal domain; P, pathogenic domain; C, central conserved domain; V, variable domain; and T2, right terminal domain. The secondary structure model is according to Riesner *et al.* (1979).

replicon present in the transgenic tobacco (data not shown) which demonstrates that PSTVd 341 is not only infectious but also genetically stable.

Discussion

The advantage of transgenic plants as a bioassay system

In the past, practically all experiments with *in vitro* mutagenized PSTVd were performed by mechanically inoculating tomato plants either with the corresponding viroid cDNA or with their respective RNA transcripts (Tabler and Sanger, 1984, 1985). This approach has the advantage of obtaining results within a few weeks. As an alternative assay system the inoculation of plant stems has been performed with *Agrobacterium tumefaciens* whose Ti plasmid contained various viroid cDNA units. This 'agroinoculation', where the primary transcription of the

viroid cDNA is limited to the crown gall tissue, was used for studying the replication (Gardener *et al.*, 1986) and the host range (Salazar *et al.*, 1988) of PSTVd, and the effects of mutagenesis on PSTVd functions (Owens *et al.*, 1986; Hammond, 1994). The much more cumbersome and time-consuming technique of genuine plant transformation with the aid of *Agrobacterium* has the advantage that the primary transcription of the viroid cDNA takes place in virtually all tissues and during all developmental stages of the transgenic plants. So far, this technique has been used only for analysing the host range (Yamaya *et al.*, 1989) and the cross-protection (Matousek *et al.*, 1989) of viroids. We have now used 'agrotransformation' of leaf discs for studying the replicability of *in vitro*-constructed PSTVd deletions mutants in the transformed plant progeny.

Our results show that with this assay system, two potential problems can be overcome that could occur when viroid mutants are assayed after mechanical inoculation of plant leaves. (i) Where *in vitro*-synthesized viroid RNA is used as inoculum, it is conceivable that some of these mutants could carry nucleotide changes or deletions in a domain that is essential for the transport of the viroid RNA to the cell nucleus where viroid RNA–RNA replication is initiated and proceeding. (ii) Mechanical inoculation of host plants with viroid cDNA will be unsuccessful in all those cases where a deletion will interfere with the initial transcription step in which the viroid cDNA must be transcribed to viroid RNA because normal viroid replication proceeds via the RNA–RNA pathway (Sanger, 1987). These assumptions are supported by the observation that we were not able to infect tomato seedlings by mechanical inoculation with either the dimeric PSTVd 350 cDNA or the corresponding *in vitro*-synthesized RNA transcripts (data not shown).

In our transgenic plant system the chances for the development of minimal infectious RNA replicons are relatively high, even if error rates of polymerase II are low, because the 35S CaMV promoter is located 5' to the plant genome-integrated and functionally defective viroid cDNA. Due to this promoter, the corresponding defective viroid RNA is continuously produced in every cell nucleus and is available as template for RNA–RNA transcription by polymerase II. Thus the resulting RNA can be continuously mutated and subjected to the natural selection that acts at the various steps of viroid replication.

The emergence of PSTVd 341 demonstrates that the analysis of viroid deletion mutants in transgenic plants is a powerful tool to unravel the correlation between the primary and secondary viroid RNA structure and the various biological functions of these minimal infectious RNA replicons. Thus, our data allows the re-evaluation of the effects of some previously observed mutations. The single C→T transition at position 284 and the single A→G substitution at position 77 of PSTVd 359 cDNA have been classified as leading to non-infectious or lethal mutants when assayed by mechanical inoculation onto tomato plants (Owens *et al.*, 1986, 1991). The loss of infectivity of the latter mutant was believed to be due to the close vicinity of A₇₇ to the C-domain. As the original A₇₇ and C₂₈₄ are deleted in PSTVd 341, this substitution might only be significant for the PSTVd 359 type strain sequence. This indeed is the case since, relative to the C-

domain of PSTVd 341, a G is now tolerated at exactly the same position as nucleotide A₇₇ in PSTVd 359.

Mechanisms of RNA replicon evolution

Only one of those plants in which the dimeric PSTVd 350 cDNA was inserted in (+) orientation was, so far, capable of initiating a RNA–RNA replication process. In this plant, the activity of the P_{35S} delivered a viroid-specific (+)RNA which led to the new PSTVd 341 RNA replicon. This experimental precondition is in contrast to our previous transformation experiments with the cDNA of the naturally occurring PSTVd 359 type strain. In this case viroid replication is initiated in tobacco plants irrespective of whether the intact PSTVd 359 cDNA constructs were primarily transcribed to viroid-specific (+)- or (–)RNA (Wassenegger *et al.*, 1994). So far, we are not yet able to analyse a statistically relevant number of SRI 350 transformants because we have obtained only one viroid-infected SRI 350(+) plant. Nevertheless, the most recent results of bioassaying *in vitro*-generated PSTVd mutants in our transgenic tobacco system argue for a (+)RNA-dependent mechanism in the evolution of the PSTVd 341 replicon. In fact, tobacco plants containing a dimeric PSTVd cDNA lacking 4 nt (position 72–75) were all found to be viroid-infected when the cDNA was inserted in (+)-orientation leading to viroid (+)RNA transcripts. However, in 20 independent transformants containing the (–)-oriented viroid cDNA construct delivering viroid (–)RNA no viroid replication could be detected. We therefore assume that the primary cDNA-derived PSTVd 350 (+)RNA transcripts can be processed to monomeric circular viroid (+)RNA molecules which then serve as template for a continuous viroid RNA–RNA replication that gives rise finally to the 341 nt long new RNA replicon. The processibility of the 350 nt long PSTVd RNA might be possible because the C-domain proposed to be involved in PSTVd RNA processing (Riesner, 1990; Steger *et al.*, 1992) has not been mutated in the dimeric PSTVd 350 cDNA. It should be noted, in this context, that the Northern blot experiments carried out to prove PSTVd replication do not allow the detection of the primary transcripts driven by the P_{35S}, because the average copy number of the primary transcripts per cell is much lower than that of the replicating PSTVd progeny.

Regarding the molecular mechanism responsible for the evolution of PSTVd 341, it is conceivable that the viroid-replicating DNA-dependent RNA polymerase II is forced, in some of the replicative rounds, to skip over the bulged-out 9 nt on the strand opposite to the *in vitro*-deleted 9 nt of the PSTVd 350 RNA template. This template switching might be favoured by the base-pairing tendency of the regions on both sides of the secondary structure domain that is finally deleted. Alternatively, the bulged-out loop in the lower strand might be cut out by an RNase and religated.

The additional five mutations found in PSTVd 341 are most likely due to copying errors of the RNA polymerase. However, in the PSTVd 341-replicating SRI plant, it is no longer possible to follow the temporal development of these mutations. To analyse their importance for the evolution of PSTVd 341, it is planned to restore, *in vitro* in its cDNA, all mutations in all possible combinations. *In vitro* transcripts of the 'restored cDNAs' will then

be bioassayed and the resulting RNA progeny will be characterized. The combination of polymerase jumping on the template and copying errors finally generate a truncated RNA replicon which can be efficiently replicated, processed, transported and mechanically transmitted. All these properties guarantee that this internally shortened rod-like viroid molecule becomes highly infectious and thus capable of rapidly over-growing all the other less fit ones. The evolutionary advantage of the deletions leading to PSTVd 341 cannot yet be attributed to a single process of the various steps in viroid infection. However, the fact that they re-established the unique and highly base-paired rod-like viroid secondary structure suggests that this is evidently essential for several of these functions.

The strict conservation of the overall secondary structure of the viroid RNA molecule has also been observed in the opposite case i.e. where sequence duplications have led to an extension of their chain length. Thus sequence variants of the 246 and 247 nt long coconut cadang cadang viroid have been found in nature in which the entire right terminal domain has been extended by 41, 50 and 55 nt, respectively. Also, in this case, the original base-pairing sequence within the corresponding terminal domain had been maintained (Haseloff *et al.*, 1982). A similarly extended naturally occurring variant of the 368–375 nt long citrus exocortis viroid has been reported recently in which the duplication of the right terminal domain comprised 92 nt (Semancik *et al.*, 1993).

In the past it has been generally assumed that, together with its circularity, the largely double-stranded structure guaranteed the survival of the viroids by conferring relatively high nuclease resistance and protection during the resting phase of their life cycle. However, the possible deletion and duplication of entire secondary structure domains strongly suggests that the unique secondary structure characteristic for all viroids must confer a great evolutionary advantage to these minimal infectious RNA replicons, not only in a protective sense but also for some other properties essential for their life cycle.

Perspectives

The experimental finding that a newly evolved PSTVd mutant, with a deletion of 18 nt, can be mechanically transmitted to and autonomously replicate in tobacco and tomato plants suggests that RNA replicons shorter than the smallest known 245–255 nt long viroid RNAs could also be constructed. We have analysed PSTVd but it should be possible to carry out similar experiments with some of the smaller viroids. Moreover, RNA replicons can be envisaged which are not necessarily transmissible from plant to plant or from cell to cell because sequences responsible for these functions might not be required for RNA replication, and it is conceivable that they also can be deleted. Furthermore, it is now possible to design truncated or extended viroid-derived RNA replicons, provided that the deleted or inserted sequences in question comprise entire secondary structure domains such as the one described here. Aside from delineating the relationship between the structure and function of viroids, future studies will show whether it is possible to use such RNA replicons as vectors for introducing distinct RNA sequences in high copy numbers into plants. This could be of special interest for gene silencing by ribozymes,

anti-sense RNA, and RNA-directed DNA methylation (Wassenegger *et al.*, 1994).

Materials and methods

Plasmid constructions

A monomer of the 359 nt long PSTVd KF440-2 (PSTVd 359) cDNA was constructed by digesting plasmid pSP-Av6.2(+) (Tabler and Sanger, 1985) with *Bam*HI, and by ligating the gel-eluted 359 bp long DNA fragment into the unique *Bam*HI site of plasmid pT3T7-lac. The plasmid containing the cDNA unit in (+)-orientation, i.e. pTPSTV-Bm(+), was either linearized with *Xba*I (X-type) or *Sry*I (S-type). The unique *Xba*I site is located in the 3'-polylinker sequence of pT3T7-lac, whereas *Sry*I cuts once within the PSTVd cDNA at position 337. Both *Bal*31-treated DNAs were recut with *Eco*RI and 330 bp to 355 bp long *Eco*RI-*Bal*31 fragments of the X-type were ligated with *Eco*RI-*Bal*31 fragments of the S-type that correspond to sizes between 2731 and 2756 bp.

Plasmid pT3T7-lac (Boehringer Mannheim) was used for all primary cloning steps and PCR products were cloned into pTPCR. The pTPCR plasmid was constructed by cloning a synthetic 26 bp long *Kpn*I-*Bam*HI fragment that comprises two *Xcm*I sites into the corresponding sites of pT3T7-lac. The variable nucleotides of the *Xcm*I recognition site were designed in such a way that restriction of the plasmid with *Xcm*I results in overhanging Ts at both ends. By cloning into this plasmid PCR products which were amplified with enzymes that have a terminal transferase activity, two *Hind*III sites are generated. Plasmids were propagated in *Escherichia coli* strain INVαF⁺ (Invitrogen).

Methods used for plasmid construction were as described in Sambrook *et al.* (1989) and plasmid DNA was isolated using QIAprep-spin Plasmid Kits (Qiagen). Restriction enzymes and DNA modifying enzymes were from Boehringer Mannheim and New England Biolabs.

RNA isolation and Northern blotting

Total RNA was extracted from leaf material according to the procedure of Logemann *et al.* (1987). Glyoxalation of RNA prior to PAGR electrophoresis, Northern blotting and molecular hybridization were performed as described (Spiesmacher *et al.*, 1985).

Plant tissue culture and transformation

Nicotiana tabacum plants (cv. Petit Havana SRI) were maintained under sterile conditions. For plant leaf disc transformation (Horsch *et al.*, 1985), selected derivatives of the plant binary vector pPCV702SM (M. Wassenegger, unpublished) were mobilized to the *A. tumefaciens* strain GV3101(pMP90RK) (Koncz and Schell, 1986) and transformed tobacco was grown on MS medium (Murashige and Skoog, 1962) containing 100 mg/l kanamycin sulfate and 500 mg/l carbenicillin.

The pPCV702SM vector is a modified pPCV702 plasmid (Koncz and Schell, 1986) that contains the streptomycin/spectinomycin resistance gene instead of the carbenicillin resistance gene. In addition, a polylinker sequence was inserted between the P_{35S} and the pAnos signal sequence. The restriction sites of the polylinker that were also found in the original vector were destroyed.

Viroid infection

Viroid infection of tomato and tobacco plants was performed by rubbing the primary leaves in the presence of carborundum with 10 µg/ml of total RNA isolated from either transgenic plants containing PSTVd 341 or tomato plants infected with the PSTVd KF440-2 type strain. PSTVd replication was ascertained at weekly intervals post-inoculation by Northern analysis.

PCR

Genome-integrated PSTVd cDNA was PCR amplified out of 3 µg of nuclear plant DNA using the 35S promoter-specific primer P1, the pAnos-specific primer P2 and the PSTVd-specific primers 325-H, 326-R, 175-H and 176-R. The reaction was performed under the following conditions using the EuroTAQ DNA polymerase and reaction buffer (Eurogentec S.A.) and applying 30 cycles: denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s and DNA polymerization at 72°C for 60 s.

Before PSTVd RNA was PCR amplified under the conditions described above using the primer pairs 325-H/326-R and 175-H/176-R, the RNA was reverse transcribed using the cDNA Synthesis Kit (Boehringer Mannheim) and the primer 325-H or 175-H.

PCR products were purified with the QIAquick-spin PCR Purification Kit (Qiagen), cloned and sequenced.

P1: 5'-CCTTCCTCTATATAAGGAAG-3'
P2: 5'-GCCAAATGTTTGAACGATCGGG-3'
325-H: 5'-TCGCCCCGGAACAAGTTA-3'
326-R: 5'-GGGTGTTTAGCCCTTGGAAACCG-3'
175-H: 5'-TCCTGTCGGCCGCTGGGCACTCCC-3'
176-R: 5'-GTAATCCCGCCGAAACAGGG-3'

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