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Analysis of thermal stress-mediated PSTVd variation and biolistic inoculation of progeny of viroid "thermomutants" to tomato and *Brassica* species

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Abstract

Thermal stress of PSTVd-infected *Nicotiana benthamiana* led to appearance of a broad PSTVd sequence distribution, where most of mutations accumulated in the left half of the viroid's secondary structure including the "pathogenicity" domain. A similar effect had been reported for hop latent viroid [Virology 287 (2001) 349]. The pool of viroid "thermomutants" progenies was transcribed into cDNA and used for biolistic inoculation of *Raphanus sativa*, where the PSTVd infection was detectable by reverse transcription and polymerase chain reaction (RT-PCR). Newly generated inoculum from *R. sativa* was used for biolistic transfer to *Arabidopsis thaliana* wild-type and silencing-deficient mutants bearing one of *sde1*, *sde2*, and *sde3* locuses. Irrespective to *A. thaliana* silencing mutants, viroid levels in *Brasicaceae* species infected with mutated PSTVd variants were of approximately 300 times lower than it is expected for tomato. At the same time, no systemic infection of *A. thaliana* was achieved with the wild-type PSTVd. In *Arabidopsis*, a population of PSTVd, consisting of frequent and minor variants, was present and the sequence distribution differed from that of the original viroid "thermomutants"; that is, mutations were not predominantly restricted to the left half of viroid's secondary structure. At least 65% of viroid sequences from *Arabidopsis* library accumulated mutations in the upper conserved central region (UCCR). In addition, mutants having changes in "hairpin II" domain (C→A transition at position 229) and in the conserved internal loop element in the left part of viroid structure (single insertion of G at position 39) were detected. All those mutants were inoculated biolistically to tomato and promoted infection especially after prolonged period of plant cultivation (50–80 days pi) when infection reached 70–90%. However, the sequence variants were unstable and reverted to the wild type and to other sequence variants stable in tomato. Our results demonstrate that heat stress-mediated production of viroid quasi-species could be of significance for viroid adaptations.

Keywords: Potato spindle tuber viroid; *Arabidopsis thaliana*; Biolistic infection; TGGE; Viroid mutagenesis

Introduction

Viroids represent the smallest pathogenic RNA replicons and do not code for any protein. Therefore, their propagation is fully dependent on the metabolism of the host plant (for a recent review, see Hadidi et al., 2003). Up to now, there are as much as 27 different viroid species classified and listed in biological databases (Flores et al., 1998; Pelchat et al., 2003), and most of them form, in addition, populations of molecular variants that conform to the quasi-species concept (Eigen, 1993). These molecular variants may serve as a source of adaptations to new hosts and life-cycle conditions. Experiments on viroid mutagenesis indicate that upon exposure to selective pressures, viroids can evolve extremely rapidly, with another fitter component of the quasi-species often

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becoming dominant within days or weeks. This extreme plasticity of their nucleotide sequences establishes viroids as one of the most rapidly evolving biological system known (Diener, 1996).

Potato spindle tuber viroid (PSTVd), type member of the *Pospiviroidae* family, is one of the best characterized viroids. Its replication follows the so-called asymmetrical rolling circle mechanism (Branch and Robertson, 1984) and is catalyzed by the DNA-dependent RNA polymerase II of the host (e.g., Schindler and Mühlbach, 1992). PSTVd has a central conserved region (CCR) important for processing of linear replication intermediates into covalently closed circles (Baumstark et al., 1997; Schrader et al., 2003). Mature viroid molecules are located predominantly in plant nucleoli (e.g., Harders et al., 1989). Natural strains of PSTVd, exhibiting different symptoms on *Lycopersicum esculentum* cv. Rutgers, are known (e.g., Gruner et al., 1995; Schnölzer et al., 1985), as well as sequence variants or populations, which were observed upon inoculation of certain cDNA clones (e.g., Góra-Sochacka et al., 1997, 2001; Qu et al., 1993). This suggests some degree of sequence variability of PSTVd in natural hosts, although it is believed that PSTVd's propagation machinery ensures significantly higher sequence stability in comparison to other viroids, like PLMVd belonging to the *Avsunviroidae* family (Ambros et al., 1999).

The experimental host range of PSTVd includes about 160 species, mostly from *Solanaceae*, but also a few species scattered among 10 other families (Singh, 1973; for review,

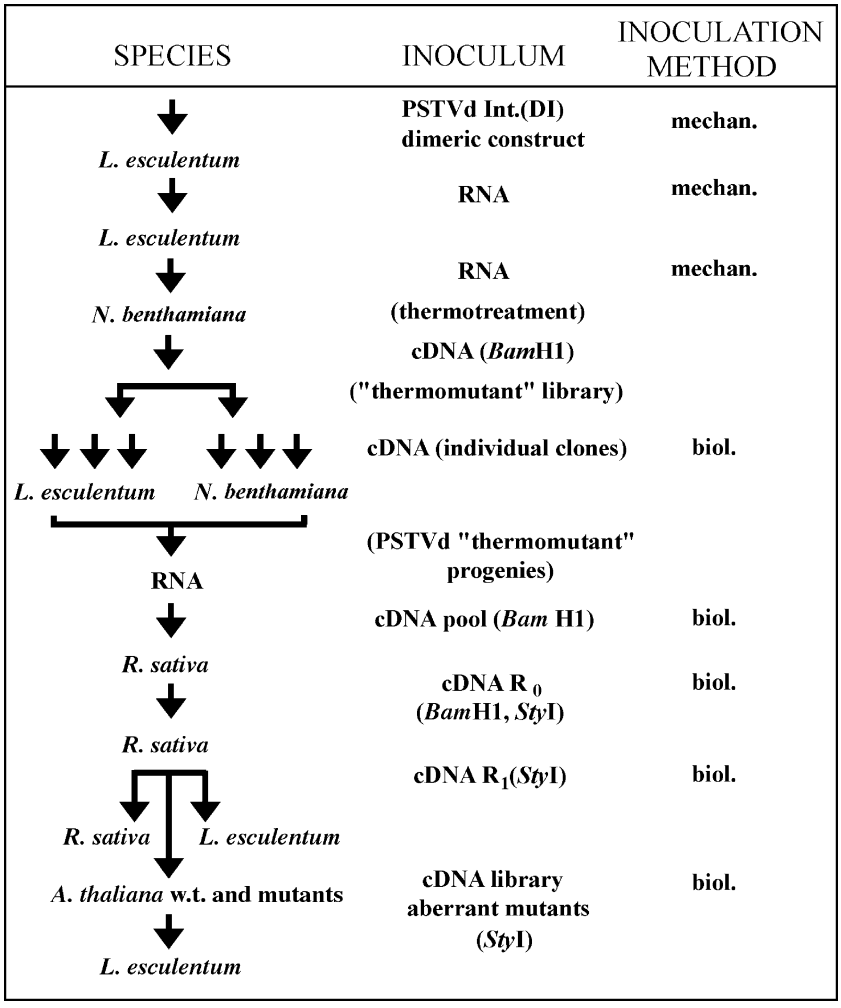

Fig. 1. Preparation of inocula and scheme of inoculations. The sequence of inoculation experiments is shown by the arrows. To prepare an initial, uniform inoculum, the PSTVd strain intermediate (DI) was introduced to tomato by mechanical inoculation (mechan.) with the pBluescript SK(+) plasmid containing dimeric cDNA. For inoculation, DNA was dissolved in 0.04 M sodium phosphate buffer (pH 7.6) and Carborundum was used as an abrasive. The resulting progeny was isolated in GPS buffer and passed once under standard conditions. Then the PSTVd sample was mechanically inoculated to *N. benthamiana* for the thermotreatment and for cDNA library preparation. Individual clones of thermomutant library were then introduced to both *N. benthamiana* and *L. esculentum* as *Bam*H1 restriction fragments (*Bam*H1) using biolistic method (see triple arrows). RNA was purified from infected plants and cDNA was prepared from a mixed RNA sample using PSTVdb primers. This cDNA was *Bam*H1-treated and introduced to *R. sativa* using biolistic inoculation. Resulting R_o generation was biolistically transferred to new plants as mixed *Bam*H1 and *Sty*I fragments. R_1 generation, as well as mutants selected from *A. thaliana*, was inoculated in all further experiments as *Sty*I restriction fragments (*Sty*I) using biolistic method. biol., biolistic inoculation using Gene Gun; mechan., conventional mechanical inoculation using Carborundum; w.t., wild-type *A. thaliana*.

see Diener, 1979). No species from the family of *Brassicaceae* is on the list of suscepts, although a species like *Arabidopsis thaliana* could be advantageously utilized for detailed molecular genetic analysis of viroid–host interaction using advanced genomics developed for this species (for a recent review see, e.g., Mitchell-Olds and Clauss, 2002). Already in 1986 (Gardner et al., 1986), it was attempted to transmit PSTVd to *Brassica rapa* using infection mediated by Ti plasmid of *Agrobacterium tumefaciens.* A more detailed study of possible PSTVd replication in turnip tissue transformed with highly infectious dimeric cDNA constructs was performed by Salazar et al. (1988). Using hybridization methods in combination with reinoculation of tomato indicator plants, these studies showed that no detectable levels of PSTVd-related RNA appear in the foliage of these *Brassicaceae*, thus confirming the high degree of resistance of *B. rapa* and turnip to PSTVd infection.

It is believed that plant resistance to molecular pathogens is at least partly mediated by the branched pathways of posttranscriptional gene silencing (PTGS) (for reviews see, e.g., Vance and Vaucheret, 2001; Voinnet, 2001). Because of the characteristic of RNA cleavage products of about 23–25 bp appearing during the viroid propagation cycle (Itaya et al., 2001; Papaefthimiou et al., 2001), apparently, the gene silencing mechanism operates also during viroid infection. Whether this mechanism could block viroid propagation in resistant host species and whether this pathway(s) can be efficiently suppressed by viroids in their susceptible host species are not known. Infection of silencing-deficient plants with viroids would represent a new approach to solve the species barrier problem. Such *Arabidopsis* mutants, deficient for RNA-dependent RNA polymerase (Dalmay et al., 2000), RNA helicase (Dalmay et al., 2001), or SGS2 and SGS3 genes (Mourrain et al., 2000) were characterized recently.

We have previously shown that heat treatment of infected hop (*Humulus lupulus*) leads to accumulation of sequence variants of hop latent viroid (HLVd), another viroid of the *Pospiviroidae* family (Matoušek et al., 2001). These mutations do not accumulate randomly in HLVd. A "hot spot" region was identified located in the left half of the viroid's secondary structure in a position analogous to the "pathogenicity" domain of PSTVd, whereas no mutations were found in the central part of the upper conserved central region (UCCR). Viroid "thermomutants" promoted formation of complex progeny populations upon cDNA inoculation in the absence of the wild type (Matoušek et al., 2001). Subsequently, the pool of progeny variants was successfully transferred experimentally to so-called nonhost plant species, tomato and *Nicotiana benthamiana*, where distinct HLVd sequence variants were stably propagated (Matoušek, 2003). These results indicate that replication of HLVd under heat stress conditions resulted in the production of viroid quasi-species, having some potential for viroid spread to plants that were considered to be "nonhost" species, because of their resistance to the wild-type HLVd.

In the present work, sequence variation of PSTVd propagated under thermal stress has been analyzed, and biolistic transfer of a pool of PSTVd "thermomutant" progeny to two *Brassica* species *Raphanus sativa* and *A. thaliana* is described. A first attempt to analyze viroid propagation in *Arabidopsis* mutants is presented.

Results

PSTVd sequence variation upon heat treatment of host

Previously, we have shown that cultivation of infected hop mericlones (plants that developed in tissue culture from

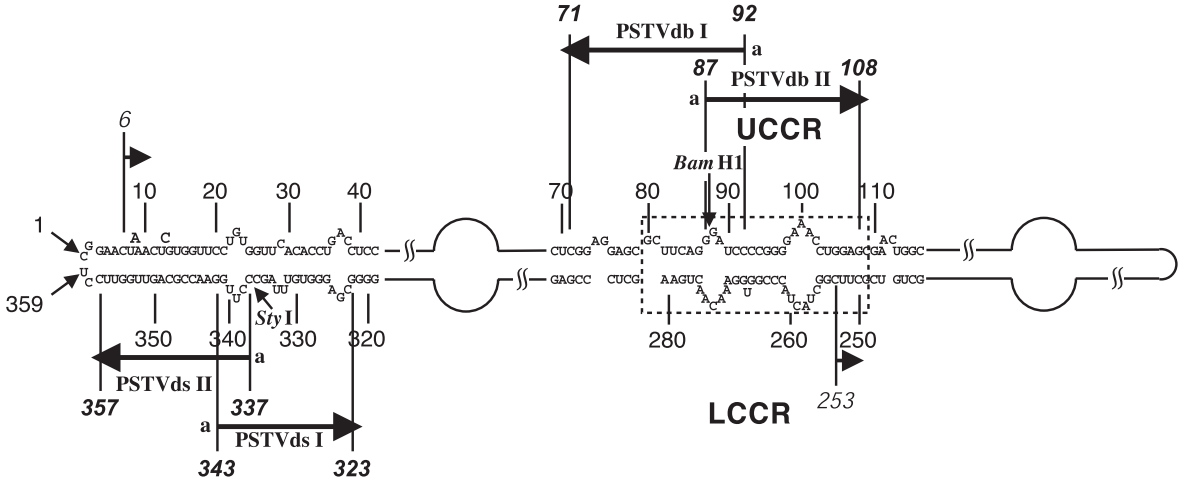

Fig. 2. Schematic drawing of viroid amplification and detection primer systems. The primer pair designated PSTVdb I and II covers the *Bam*H1 restriction site and is localized in the upper central part of viroid secondary structure. Primers PSTVds I and II covers the *Sty*I restriction site and are localized in the lower part of the left half of viroid secondary structure. Primers are represented by arrows, positions are designated by numbers; a, position of nonspecific nucleotide; UCCR and LCCR designate upper and lower part of the central conserved region, respectively. Start and end positions of PSTVd probe from nt 6 to nt 253 are shown. See Material and methods for sequence details.

a single meristem) in repeatedly increasing and decreasing temperature conditions led to an accumulation of HLVd variants. Here, we address at first the question about a more general validity of the effect of thermal stress on viroid sequence stability. The PSTVd strain intermediate (DI) was introduced to tomato using a highly infectious, dimeric cloned cDNA (Fig. 1), and the resulting progeny was passed once under standard conditions to prepare an initial, uniform inoculum. For the thermotreatment, the inoculum was transferred to *N. benthamiana*, because it is a symptomless host that allows PSTVd to replicate to high titers. In Fig. 1, the scheme of this and the consecutive inoculations is depicted. The mean value of PSTVd RNA concentration under optimal growth conditions at temperatures ranging from 22 to 28 °C reached 50 pg/mg fresh weight in young leaves of *N. benthamiana*. A temperature regime (see Material and methods) was applied having a rather devastating effect on the physiological state of this plant; the upper temperature limit of 40 °C was close to heat shock. Under these conditions, leaf blade malformations, local necrotic lesions, and local depigmentations were observed, but plants survived and allowed us to perform RNA extractions. Total RNA was isolated from infected plants after the second temperature cycle, PSTVd cDNA of unit length, having *Bam*H1 restriction sites on each ends, was prepared using high fidelity reverse transcription and amplification by polymerase chain reaction (RT-PCR) with primers PSTVdb I and II (Fig. 2), and analyzed by temperature-gradient gel-electrophoresis (TGGE) (Fig. 3).

TGGE patterns revealed an abundance of heteroduplexes suggesting the presence of significant sequence variation in the thermotreated samples (see Fig. 3A2). It is important to note that the original PSTVd inoculum derived from the cDNA clone of PSTVd intermediate showed high sequence stability in plants cultivated at the temperature ranging from 22 to 28 °C, and no heteroduplexes were observed in TGGE (Fig. 3A1). In order to analyze individual sequence variants, the heteroduplex zone was excised from the gel (Fig. 3A2), cDNA was re-amplified, and a cDNA library was constructed. This library was analyzed by hybridization with wild-type PSTVd cDNA to form DNA heteroduplexes (not shown). From the cDNA library, mutant clones were selected, which we call library of ‘‘thermomutants’’ for simplicity (see Material and methods). Thirteen clones from this library were sequenced and individual base changes localized on the secondary PSTVd structure (Fig. 4A). Except for clones 34, 40, and 98, all clones contained more than one mutation, suggesting accumulation of mutations in subsequent replication cycles. Only one mutation, a U→C change in position 130 in clone 48 was localized in the right part of PSTVd structure and a single G→A base change in clone 26 (position 255) was detected within the LCCR. Most mutations accumulated in the left half of the viroid structure including the ‘‘pathogenicity modulating’’ (PM) domain. Comparison of sequenced clones in this region allowed us

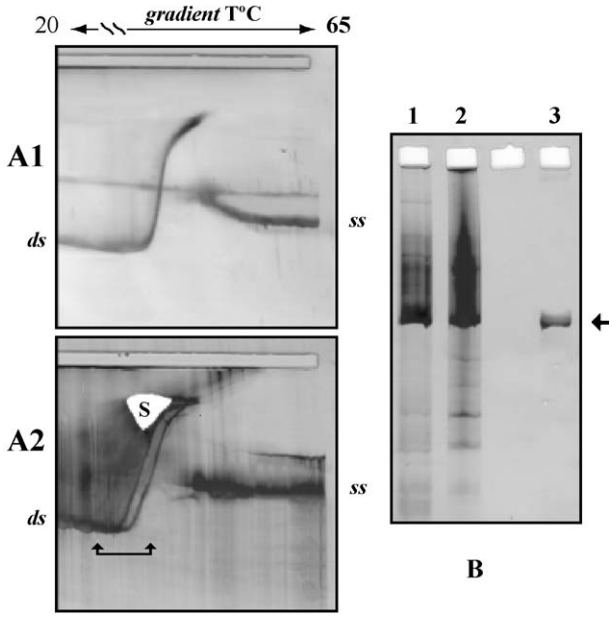

Fig. 3. Selection of PSTVd ‘‘thermomutants’’ using TGGE (A) and analysis of the cDNA pool used for inoculation of *R. sativa* (B). (A1) A sample from control *N. benthamiana* was separated on 6% acrylamide gel containing 7 M urea by electrophoresis using a temperature gradient from 20 to 65 °C and stained afterwards by silver; (A2) TGGE profile of cDNA from thermotreated plants. Homoduplexes are separated from heteroduplexes that contain mismatches in the sequence region responsible for the first melting transition. The wide zone, designated by S, contains heteroduplexes, which appeared in the spectrum of deviating PSTVd cDNAs from heat-treated plants. Two distinct transitions seen on the gel are formed by frequent sequence variants present in the sample. These variants were not included in sample S, which should represent more the wide spectrum of sequences. The arrows indicate positions of melting points for the whole spectrum. From zone S, DNA was extracted, amplified using *Pwo* polymerase, and cloned in pCR-Script SK(+) vector to prepare the library of ‘‘thermomutants’’. cDNA restriction fragments derived from this library were immobilized on the surface of gold microcarrier as used for biolistic inoculation of *N. benthamiana* and *L. esculentum* plants. From these plants, a pool of cDNA was prepared using primers PSTVdb I and II and analyzed for DNA heteroduplexes at 44 °C in a 6% gel containing 7 M urea. The samples shown on panel B correspond to 1, phenolized RT-PCR product; 2, cDNA pool after additional denaturation and re-hybridization; 3, PCR product from cloned control cDNA of PSTVd intermediate (DI) strain. The arrow indicates position of homoduplexes.

to predict structures and relationships of various potential ‘‘thermopathotypes’’.

It is seen in Fig. 4B that there is a wide divergence of ‘‘thermopathotypes’’ ranging from similar to the original intermediate strain to having similarity in this region to highly pathogenic strains like KF440-2 or RG1. There are also types combining different sequence motifs in the upper and lower part of the structure; for example, clones 26, 45, and 67 combine the sequence of intermediate strain on the upper part and an RG1-like sequence in the lower part. Some mutations, like the C→U change in position 286, led to prediction of an unusual loop in this region, and the A insertion in position 60 (clone 37) led to formation of an unusual PM loop-1 containing a stretch of seven A nucleotides. Sequence stability of these clones has not been investigated further. It is

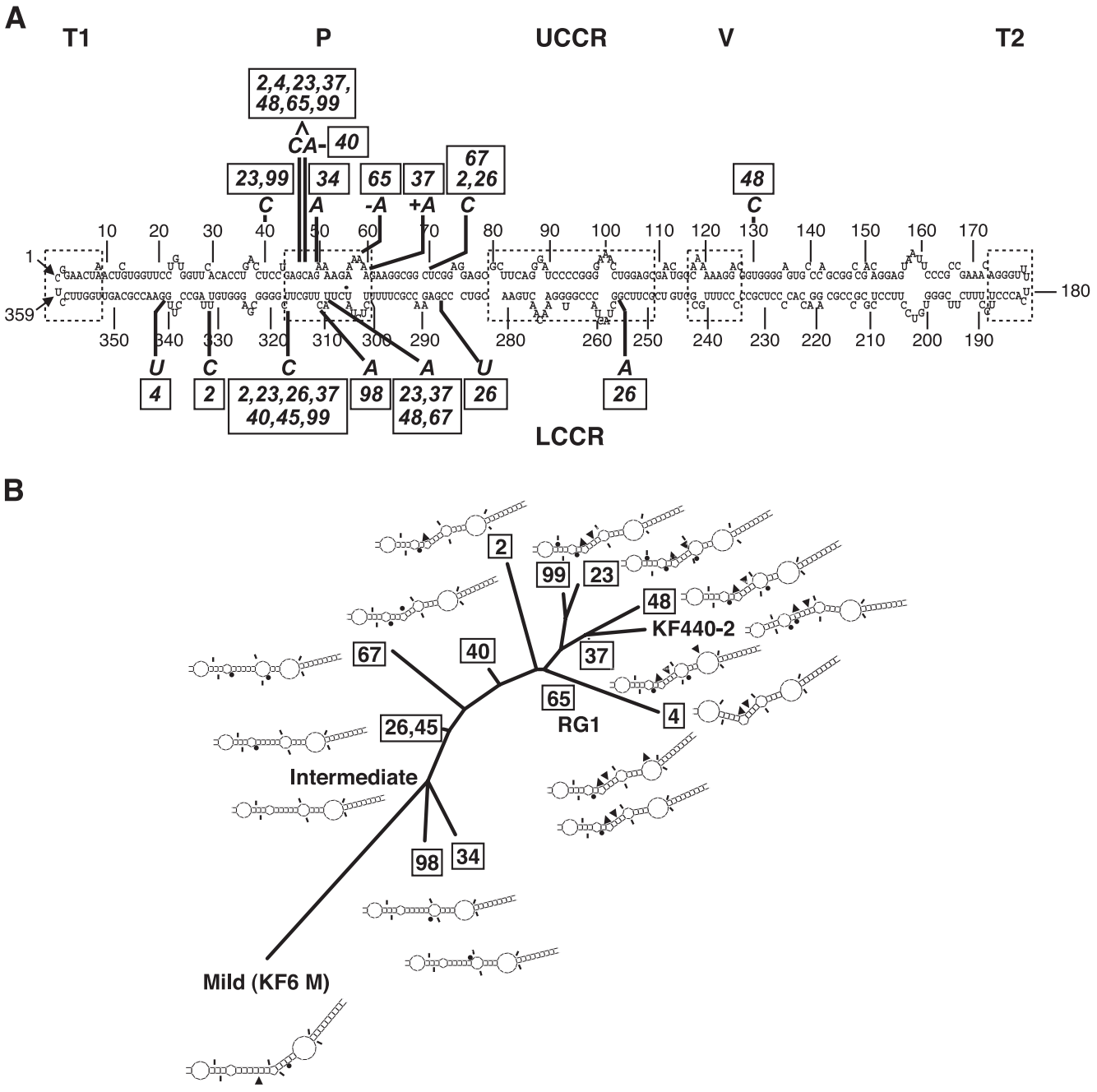

Fig. 4. Localization of mutations of PSTVd from heat-treated *N. benthamiana* on the viroid's secondary structure (A) and comparisons of predicted PM regions of PSTVd "thermopathotypes" (B). Individual base changes found within PSTVd are indicated by the nucleotide symbols and corresponding cDNA clones by the boxed numbers. UCCR, upper part of the central conserved region; LCCR, lower part of the central conserved region; P, "pathogenicity" domain; T1 and T2, left and right terminal domains; V, variable domain. Viroid domains are designated as suggested by Keese and Symons (1985). The phylogenetic tree is based on a structural alignment of full-length sequences. In the structures of PM regions (nucleotides 35–68/292–326 in PSTVd intermediate are shown), base substitutions are marked by dots, base insertions by triangles pointing to the insertion, and base deletions by triangles pointing outwards. The individual branchings in the tree are statistically insignificant, but most thermomutants show closer relationships to pathogenic PSTVd variants (KF440, RG1) than to mild variants (KF6).

important to note, however, that after inoculation of individual clones to tomato (Fig. 1), the plants showed rather mild symptoms. It can be concluded from these experiments that the effect of thermal stress on viroid variability is probably a more general phenomenon, causing significant PSTVd sequence variation and a distribution of mutations similar in character to that described previously for HLVd.

Biolistic transmission of progeny of PSTVd "thermomutants" to Brassica species

In the second part of this work, we aimed to use the heat stress-induced variation of PSTVd to construct "in natura" a pool of sequence variants containing progenies of various thermomutants for possible transfer to *Brassica* species

(Fig. 1). These experiments were stimulated by two earlier studies from our groups. Firstly, the attempts to infect *A. thaliana* and its close relative *Arabis brassica* with the dimeric, highly infectious clone of PSTVd intermediate had been unsuccessful, and secondly, the progenies of HLVd "thermomutants", however, had propagated clearly in host plants resistant to the wild-type HLVd (Matoušek, 2003). On the basis of these experiments and data from the literature (Góra-Sochacka et al., 1997, 2001; Qu et al., 1993), we supposed that the variation of PSTVd can be further enhanced upon inoculation of individual clones from the "thermomutant" library to *N. benthamiana* and *L. esculentum* host plants. This variation was not investigated in detail; subpropagation, however, yielded a pool of cDNA sequences forming a smear after self-hybridization on the denaturing gel, suggesting a plethora of heteroduplexes (Fig. 3B). This RT-PCR product, designated as cDNA pool, was re-amplified, treated with *Bam*H1, and used for the biolistic inoculation of radish (*R. sativa)* (see Fig. 1).

This comparatively fast developing plant has been selected for practical reasons, as it has sufficiently large leaf blades enabling tissue selection surrounding the shot wound area. Both systems of RT-PCR primers (Fig. 2) were used for PSTVd detection in radish plants to follow the stability of the UCCR, covered by PSTVdb but not by PSTVds primers. Already 2 days post inoculation and before local tissue degeneration, a signal specific to PSTVd RNA was detected in the shot wound area (Fig. 5). Most important, PSTVd replication was detectable by RT-PCR in the area surrounding the shot wound, as well as in new non-inoculated rosette leaves. This detection was performed 21 days pi and it clearly indicated PSTVd replication and translocation in the radish tissue. PSTVd from *R. sativa* leaves, which we designated R_0 generation, was amplified, and biolistic reinoculation of radish for longer passage was performed using both *Bam*H1 and *Sty*I restriction fragments (Figs. 1 and 5). RT-PCR analysis of R_1 generation, performed 45 days pi, revealed that only the PSTVds primers yielded a detectable signal (Fig. 5). This result, which was rather surprising at first glance, could mean that some sequence change occurred in the UCCR, where the binding sites for PSTVd primers are. Alternatively, some stabilization of the viroid structure could lead to non-optimal RT-PCR conditions and nonefficient amplification. Due to this result, we applied only the PSTVds primer system for further cloning and analysis of PSTVd from *Brassica* species.

The R_1 generation was amplified as a pool of *Sty*I fragments and was biolistically transferred to *A. thaliana* (Fig. 1). In order to further increase specificity of viroid detection, RT-PCR products were blotted to nylon membrane and probed with PSTVd-specific probe. In *A. thaliana*, PSTVd was detected as a specific band sensitive to RNase A treatment in the shot wound surrounding area as well as in the new non-inoculated leaves within the basal rosette (Fig. 6). The effect of RNase A treatment excludes a misinterpretation of the signal arising from inoculum. R_1

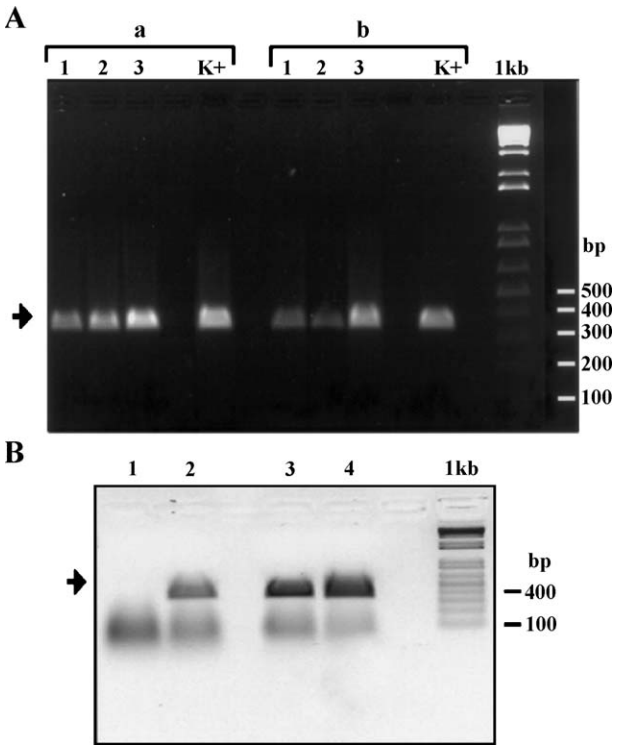

Fig. 5. RT-PCR analyses of PSTVd infection in *Brassica* species. The arrows designate the PSTVd-specific cDNA band. This band does not appear in non-infected controls. (A) Detection of R_0 viroid population in *R. sativa* after biolistic inoculation. RNA samples were purified using the RNeasy extraction protocol (Qiagen), treated with DNAse I, phenolized, and precipitated with ethanol. RT-PCR was performed using primer systems covering either *Sty*I (a) or *Bam*H1 (b) restriction sites as shown in Fig. 2. (1) New non-inoculated rosette leaves, 21 days pi; (2) tissue surrounding the shot wound, 21 days pi; (3) shot wound area, 2 days pi; (K+) 10× diluted PSTVd from tomato. (B) Detection of R_1 viroid population in *R. sativa* after repeated biolistic inoculation. Inoculum was prepared as a mixture of *Bam*H1 and *Sty*I restriction fragments from R_0 viroid population (Fig. 1), and the new R_1 PSTVd population was detected in new non-inoculated rosette leaves 45 days pi. (1) Detection in *R. sativa* using PSTVdb primers; (2) *R. sativa*, PSTVds primers; (3 and 4) detection of R_1 in *L. esculentum* using PSTVdb and PSTVds primers, respectively. 1 kb designates positions of 1 kb ladder.

generation was successfully transferred back to tomato, where it induced severe symptoms, comparable with the lethal strain RG1 in the same conditions (not shown), whereas specific symptoms were observed neither on *R. sativa* nor on *A. thaliana*. It is important to note that the level of PSTVd R_1 generation in both *Brassica* species was roughly about 300 times lower than the level of "thermomutants" in tomato, as was estimated by RT-PCR of diluted samples (not shown). It can be concluded that a "low-level" PSTVd population developed in brassicaceous plants after biolistic inoculation of the original cDNA pool. It has to be noted, however, that no specific signals of systemic PSTVd infection were obtained after biolistic treatment of *A. thaliana* with cloned wild-type PSTVd strains DI, KF440, and RG1, suggesting some specific potential of mutated PSTVd variants to spread.

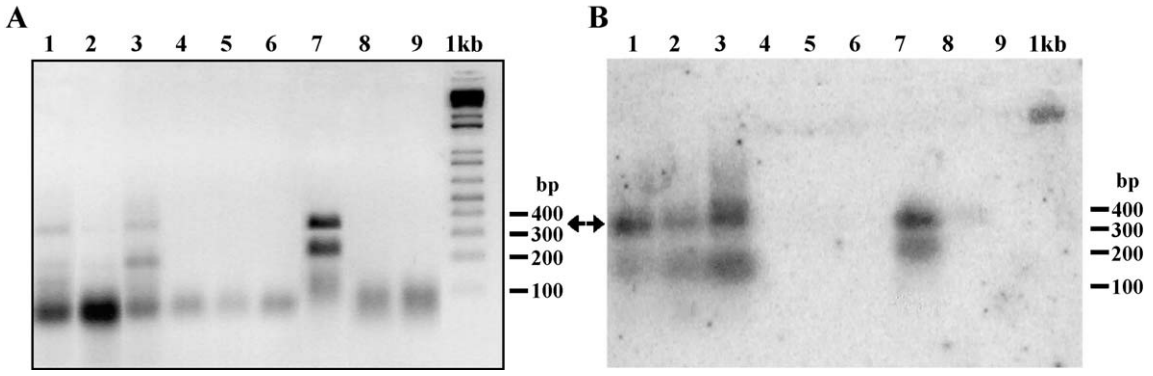

Fig. 6. Detection of viroid in *A. thaliana* and *R. sativa* after biolistic inoculation. Inoculum was prepared as *Sty*I restriction fragments. Detection was performed in new non-inoculated rosette leaves using RT-PCR system covering *Sty*I. Samples were separated by electrophoresis in a 2% agarose gel and stained for DNA with ethidium bromide (A), then the DNA was transblotted onto nylon membrane and probed with the PSTVd-specific probe based on cDNA fragment amplified outside the *Sty*I primer region as described in Fig. 2 (B). (1,2,3) Samples treated with DNAse; (4,5,6) samples treated with DNAse and RNAse. (1 and 4) *A. thaliana* tissue surrounding shot wound, 15 days pi; (2 and 5) *A. thaliana* tissue from new non-inoculated leaves, 28 days pi, (3 and 4) *R. sativa* tissue surrounding shot wound, 15 days pi; (7 and 8) reference samples, PSTVd from *P. hybrida* petals infected with the pool of viroid thermomutants, 28 days pi, DNAse and DNAse plus RNAse treated, respectively; (9) control sample without adding any template. Positions of specific bands corresponding to full-length PSTVd are designated by the arrows. 1 kb designates positions of 1 kb ladder.

The low level of PSTVd propagation in *A. thaliana* could be caused by some posttranscriptional silencing process, as well. To test this hypothesis, R1 generation of PSTVd was biolistically transferred to the wild-type *A. thaliana* and for comparison to three mutants impaired in PTGS (Fig. 1). Mutants are deficient in RNA-dependent RNA polymerase (SDE1), RNA helicase (SDE3), and in a protein with yet non-identified function in PTGS pathway (SDE2), respectively. The level of PSTVd was roughly estimated after RT-PCR using PSTVds primer system and molecular hybridization (not shown). Although the infection was detectable in new non-inoculated leaves of all inoculated plants as a specifically hybridizing band, which was not seen after treatment of the samples with RNase A, no obvious differences were seen in viroid levels in infected wild-type and silencing mutants. Also, no specific signal was detectable on conventional Northern blots (not shown). These results suggest that blocking PTGS is by itself insufficient to allow PSTVd population to reach a higher concentration in *A. thaliana* tissue.

Analysis of PSTVd variants from A. thaliana

In further experiments, we concentrated on a more detailed analysis of PSTVd variants, which were propagated after biolistic inoculation of the R_1 complex to *A. thaliana* (Fig. 1). TGGE analysis of RT-PCR products from infected *Arabidopsis* (Fig. 7) revealed the presence of a heterogeneous

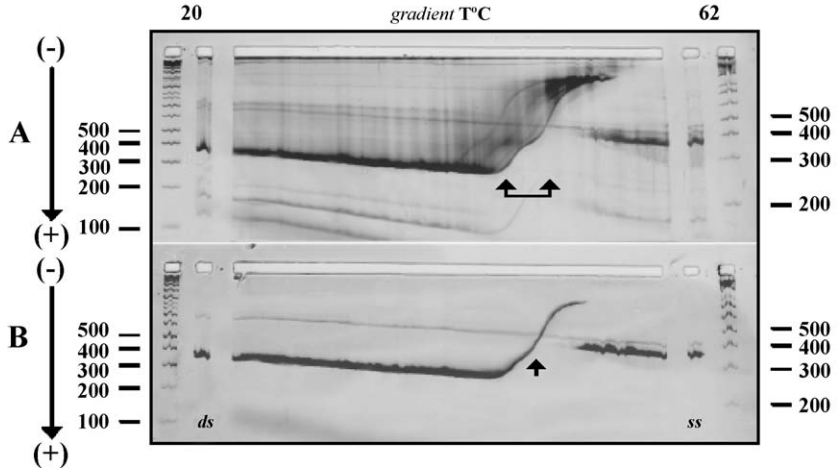

Fig. 7. Analysis of PSTVd cDNA from *A. thaliana* on TGGE. The PSTVd sample in panel A represents the first viroid generation maintained in *A. thaliana* after biolistic inoculation of the R_1 generation from *R. sativa*. The sample in panel B represents cloned viroid cDNA from *A. thaliana* clone 170. cDNA samples were prepared using primers covering *Sty*I site, phenolized, heated, and allowed to form heteroduplexes in 1 mM Na cacodylate hybridization buffer (pH 6.8) containing 100 mM NaCl and 1 mM EDTA. Then the samples were analyzed in 6% acrylamide gel containing 7 M urea using a temperature gradient from 20 to 62 °C. Double- and single-stranded parts of DNA patterns are designated by *ds* and *ss*, respectively. The range of melting points of PSTVd heteroduplexes is indicated by the arrows. The gel was stained for nucleic acids with $AgNO_3$.

PSTVd population, as can be judged from the complex spectrum of frequent and minor heteroduplexes having melting points in the range from 44 to 48 °C. In order to analyze this spectrum, an *Arabidopsis*-passaged PSTVd cDNA library was constructed and screened according to the preformed heteroduplex method for the extent of diversity and content of individual cDNAs (Fig. 8A). Ten clones were finally sequenced and individual base changes were identified (Fig. 8B). It follows from sequencing that there is a quite different character of distribution of individual mutations in the spectrum from *A. thaliana* in comparison to the original "thermomutants" (compare Figs. 4 and 8). The population from *A. thaliana* shows a more disperse distribution of mutations, the "pathogenicity" domain does not form any "hot spot", and there are mutations located in the upper central conserved region. For instance, in clones 6 and 8, which were found by the heteroduplex method to be frequent, mutations were localized directly in the UCCR (Fig. 8). Clones with aberrant CCR were estimated to represent at least 65% of the library, suggesting a high mutation and selection rate for such deviations. The rest of sequenced variants are represented by clones with intact CCR, like clone 19, 46, and 170. In most of these clones, however, mutations accumulated in some sequence and structural motifs, which are considered to be important for conventional propagation of PSTVd in host plants. For instance, the C→A base change at position 229 should disrupt the stem of so-called hairpin II, the structure that is considered to be important for transcription of (−)-strands. Insertion of G in position 38 (clone 170)

Table 1
Infectivity analysis of PSTVd mutants in tomato

Mutant no.	Conserved positions changed in	Plants inoculated	Days pi		
			25	50	80
			Plants showing positive hybridization signal[a]/RT-PCR signal from mix of negative plants[b]		
6	UCCR	10	0/−	2/+	9/−
8	UCCR	10	0/−	2/+	10/none
46	HL II	10	0/−	3/−	7/−
170	position 39	10	3/+	7/+	9/−
DI	none	10	10/none	10/none	10/none
Control	–	10[c]	0/−	0/−	0/−

[a] Detection in upper non-inoculated leaves.
[b] RT-PCR products were hybridized to PSTVd probe.
[c] The control plants were not inoculated.

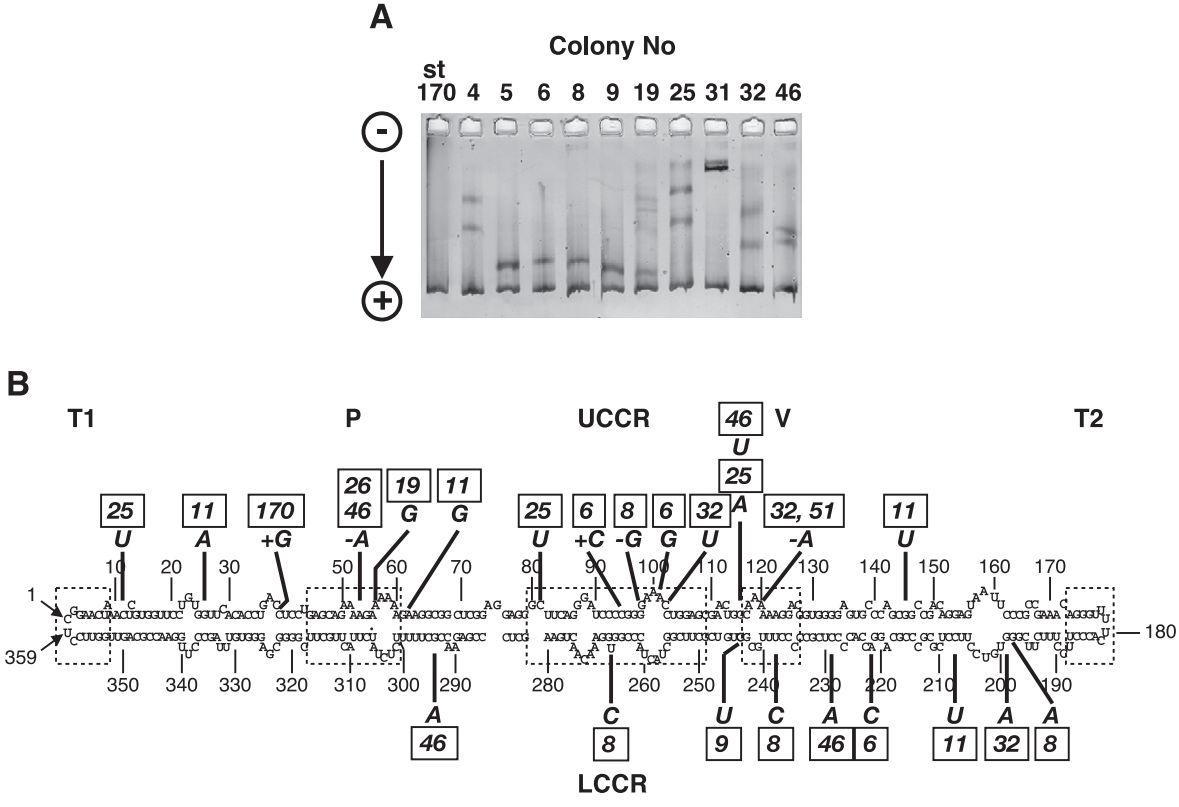

Fig. 8. An example of analysis of a cDNA library derived from a PSTVd population from *A. thaliana* by the method of preformed DNA heteroduplexes (A) and localization of mutations on the viroid's secondary structure (B). cDNA fragments were prepared from individual clones (numbered) using PSTVds primers using PCR, hybridized to the tester cDNA (clone 170), and subjected to electrophoresis at constant temperature of 44 °C in a 6% polyacrylamide gel containing 7 M urea. The gel was stained for nucleic acids with $AgNO_3$. The position of the homoduplex (clone 170 cDNA hybridized to itself) is indicated by the arrow. Individual base changes detected in comparison to the wild-type PSTVd intermediate strain (DI), which appeared in the analyzed population, are indicated by nucleotide symbols and the corresponding cDNA clones by the boxed numbers. The description of viroid domains is as in Fig. 4.

is an example that a thermotreatment stabilized the viroid structure by expanding a helical region and shortening a conserved internal loop in the left half of the viroid structure.

To test the influence of "defects" in characteristic conserved motifs, biolistic transfer of the individual clones 170, 46, 8, and 6 was performed to tomato and their infectivity was compared to the original wild-type PSTVd intermediate (DI) (Table 1). Although PSTVd infection was easily detectable 25 days pi by the use of dot-blot technique in the upper non-inoculated leaves of all plants inoculated with the wild-type PSTVds fragments, only 3 out of 10 plants were dot-blot positive for clone 170 and no infection was found for clones 46, 8, and 6, neither by dot-blot nor by RT-PCR. By RT-PCR, however, a signal was detectable also in dot-blot-negative group of tomato inoculated with clone 170, suggesting low level of infection in this group. Consequently, the concentration was below the detection limit characteristic for the standard dot-blot procedure used, that is, below 0.03 pg/mg of fresh mass. This is consisted with the finding that 70% of plants became dot-blot positive after inoculation with clone 170 at a longer incubation time of 50 days pi. At this time, 2 out of 10 plants became weekly infected for both UCCR defective clones 6 and 8, and 30% of plans inoculated with clone 46 became weakly dot-blot positive, as well. Moreover, except for clone 46, a RT-PCR signal was detectable in the group of dot-blot-negative plants for all clones, suggesting the presence of additional weakly infected individuals in group 6, 8, and 170. Surprisingly, also for

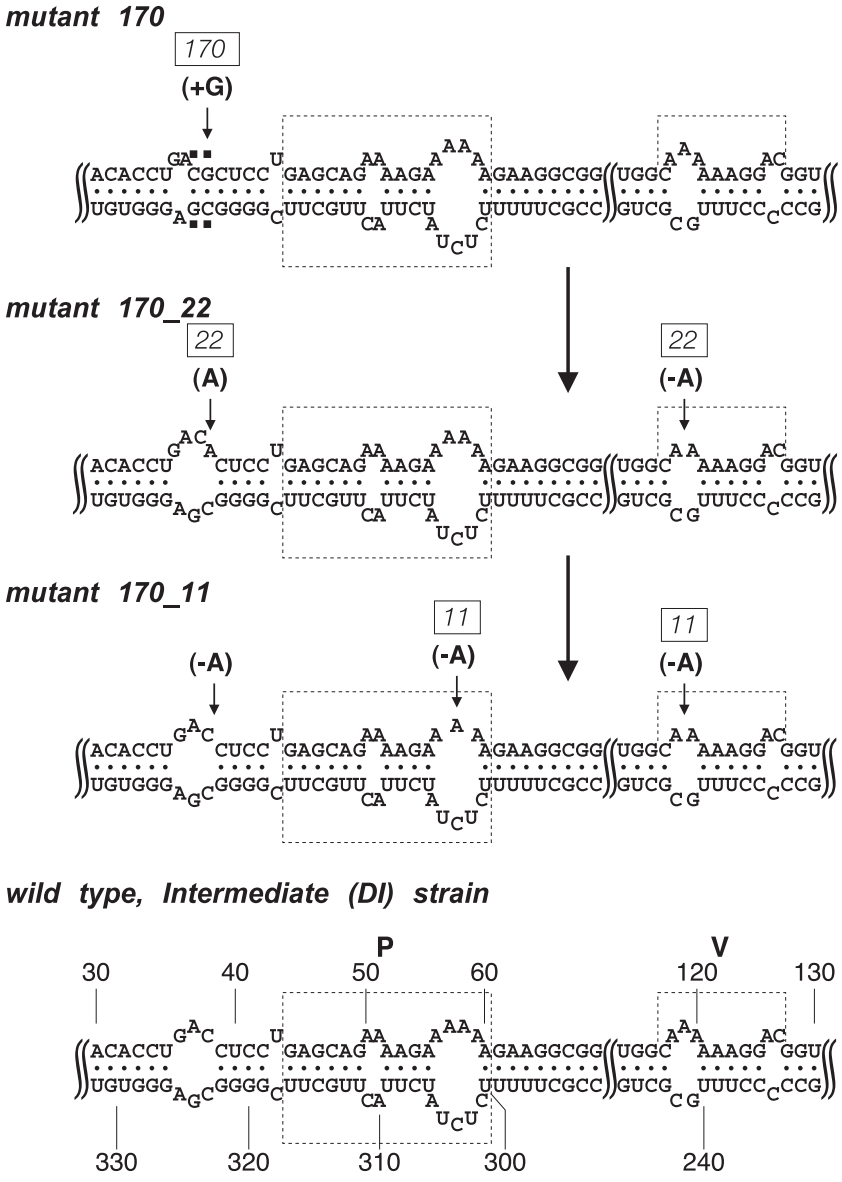

Fig. 9. Some sequence variants of mutant 170 in *L. esculentum*. Parts of viroid's secondary structure are shown, where mutations were localized. P corresponds to "pathogenicity" domain, V corresponds to "variable" domain as suggested by Keese and Symons (1985). Positions of individual base changes that occurred within PSTVd are indicated by the arrows and by nucleotide symbols in brackets. Corresponding cDNA clones are indicated by the boxed numbers. The helix extension in the structure of clone 170 is marked by filled squares. Long arrows indicate possible direction of evolutionary changes.

clone 46, where no RT-PCR signal was found 50 days pi, 70% infection was observed after the prolonged period, 80 days pi. Taking together, these results suggest viability, but at the same time extremely low fitness of most of specific aberrant PSTVd mutants in tomato. Analysis of PSTVd populations of individual clones 50 days pi revealed reversions of original mutations within important domains. For instance, for clone 6, the population contained variants with the wild-type LCCR sequence, but also mutants having a U→C change at position 300 and a C insertion at the same position, and a mutant having a U→C change at position 317. For clone 46, a single mutant was identified in tomato, having retained the original deletion of A at position 54 (see Fig. 8) whereas others reverted. Several new mutations were also identified in the population originating from mutant 170 (Fig. 9). As tested 50 days after infection, the inserted G had mutated to A thereby abolishing the two additional base pairs in the thermomutant, and later in the infection, the A was deleted, that is, establishing the original PSTVd structure of this domain. This highly interesting stepwise reversion of a thermomutant was analyzed independently by using a unit-length RNA of the mutant and inoculating it with the conventional Carborundum method. Exactly the same stepwise reversion to the PSTVd wild-type sequence in that domain was found. At the same time, new mutations appeared: deletion of A at position 119 as detected in clone 170_22 and in addition, new deletion of A at position 57 as identified in clone 170_11 possibly originating from clone 170_22 (Fig. 9). Reversions in important domains demonstrate that re-adaptation changes of viroid variants isolated from *A. thaliana* occurred in tomato during a longer cultivation period.

Discussion

Thermal stress-induced viroid variation is a general phenomenon

In the present experiments and in a recent publication (Matoušek et al., 2001), we described the increasing variability of two viroids of the *Pospiviroidae* family, PSTVd and HLVd, upon thermal stress of their hosts. As for HLVd, enhanced PSTVd sequence variability was detected by TGGE and by sequencing of cloned individual variants. We can conclude that the PSTVd wild-type sequence, which is optimally adapted for high replication and propagation rate and quite stable under normal temperature conditions (i.e., no deviating variants were visible on TGGE pattern), is significantly destabilized in conditions of thermal stress causing a suboptimal physiological state of the host plant. It is obvious that such a strong negative influence of host stress on viroid sequence stability is conditioned by a total biochemical dependence of viroid on the host-encoded metabolical machinery and in particular, on fidelity of replication by the polymerase II complex (Schindler and Mühlbach, 1992) at the elevated temperature. It is not known whether this obviously lower fidelity under thermal stress could be connected to unusual structural properties of viroid representing by highly cooperative RNA structure as transcriptional template. Most of ‘‘thermomutations’’ were localized on the left half of viroid structure and most of them showed a tendency to destabilize the secondary structure of the wild type. Except for one mutation, G→A in position 255, no mutation was detected in the lower part of central conserved region (LCCR). Also in this respect, there is strong similarity to HLVd, that is, the overall distribution of mutations is similar to that observed for HLVd ‘‘thermomutants’’ (Matoušek et al., 2001). We did not follow possible accumulation of mutations in the UCCR covered up by primers PSTVdb. But it has been shown for HLVd that the UCCR is highly preserved in the population of ‘‘thermomutants’’ and such mutations, if any, are probably eliminated upon post-treatment selection in the natural host at standard temperatures (Matoušek et al., 2001).

PSTVd pool of “thermomutant” progenies was transmissible to Brassica *species as a “low-level” population*

As a main goal of this work, we aimed to use the phenomenon of thermal stress-mediated enhanced mutability of viroid to prepare a wide pool of ‘‘in natura’’ occurring sequence variants for possible transfer to plant species that were considered as so-called nonhost plants. This concept is in accordance with the model described by Eigen (1993) that defines a quasi-species as an evolutionarily important collection of sequence variants having some potential to new adaptations. This idea is in agreement with the successful transfer of a pool of ‘‘thermomutant’’ progenies of HLVd to solanaceous species (Matoušek, 2003) that were considered to be so-called nonhost plants, because HLVd infection has not been observed in solanaceous plants during previous inoculation experiments. For this reason, solanaceous species are not listed in the host list for HLVd (e.g., Hadidi et al., 2003).

It has been shown previously in our experiments with HLVd (Matoušek, 2003; Matoušek et al., 2001) and PSTVd (e.g., Góra-Sochacka et al., 1997, 2001; Qu et al., 1993) that cDNA clones of mutated viroid induce complex populations upon inoculation. Having in mind this possibility, we prepared a complex inoculum from progenies of the library of ‘‘thermomutants’’. Although we did not study individual clonal populations in this work, we obviously achieved a broad sequence distribution, as was detected by the heteroduplex method. As a so-called nonhost recipient of PSTVd variants, we selected *Brassica* species, in particular *A. thaliana*, a well-known molecular genetics model. Our preliminary experiments and analyses performed by others (Gardner et al., 1986; Salazar et al., 1988) clearly suggested that propagation of wild-type PSTVd does not occur in these species at levels detectable by traditional hybridization

methodologies. As a result of experiments presented here, low-level propagation of PSTVd, detectable only by RT-PCR, was found after the biolistic transfer of the initial PSTVd pool in *R. sativa* and then in *A. thaliana* inoculated by the R_1 PSTVd generation; a systemic infection could not be detected by classical dot-blot or Northern blot procedures. Our results are consistent with the recent finding that other viroids of the *Pospiviroidae* group (CEVd, HSVd, CCCVd, ASSVd, and CbVd-1) can replicate in *A. thaliana* transformed with infectious constructs in levels lower than in their original hosts, but probably are unable to move, because no systemic infection was detectable after plant agroinfection with infectious viroid constructs (R. Flores, personal communication). In our work, we did not detect systemic infection of *A. thaliana* with various wild-type PSTVd strains either. Very low, but a specific and RNase-sensitive systemic signal, however, was detectable after biolistic transfer of the R1 population. It is possible that some mutated variants had the ability to propagate as low-level population. It can be expected that there is a whole complex of adaptations necessary to achieve high fitness. From this point of view, it can be expected that the *Pospiviroidae* wild-type sequences are sufficient for replication and processing, although only deviating forms have some capability to move. Another mechanism that could be responsible for low viroid level in "nonhost" species is the posttranslation gene silencing pathway. First analyses of PSTVd population in various PTGS-impaired mutants of *A. thaliana*, however, did not show any differences in viroid concentrations, and viroid levels remained low, detectable by RT-PCR only.

PSTVd sequence variation from A. thaliana and the process of re-adaptation in tomato

The PSTVd population from *A. thaliana* contained several frequent sequence variants aberrant in UCCR, and according to our calculations (not shown), even more thermostable structures. Although these aberrancies occurred newly during PSTVd propagation in *Brassica* species, we are now far from understanding which host factors and which particular structural features of PSTVd are involved in the mutual interaction that led to fixing these mutations. For example, each of clones 6 and 8 has two different mutations in the CCR, which might modify the loop E-structure in the extended rod-shaped structure (see Fig. 10). In addition, the same mutations would have an impact on the structure of the CCR thought to be functionally important during processing of linear intermediates to circular molecules (see Fig. 10; Baumstark et al., 1997; Schrader et al., 2003). For example, the A_{101}→G transition in clone 6 would modify the thermodynamically extra-stable tetraloop hairpin, which might be a recognition site for a host factor, whereas the A deletion near position 100 and the U_{267}→C transition in clone 8 might lead to a structural shift. Such modifications might be a consequence of host selection. On the other hand, the existence of clones having an intact CCR, like clone 170 or minor clone 19, suggests also the presence of a processing pathway identical to that operating in solanaceous host species (Baumstark et al., 1997). The existence of minor clone 19 having only a single base change A→G in position 56, which is neutral to the secondary structure, suggests in

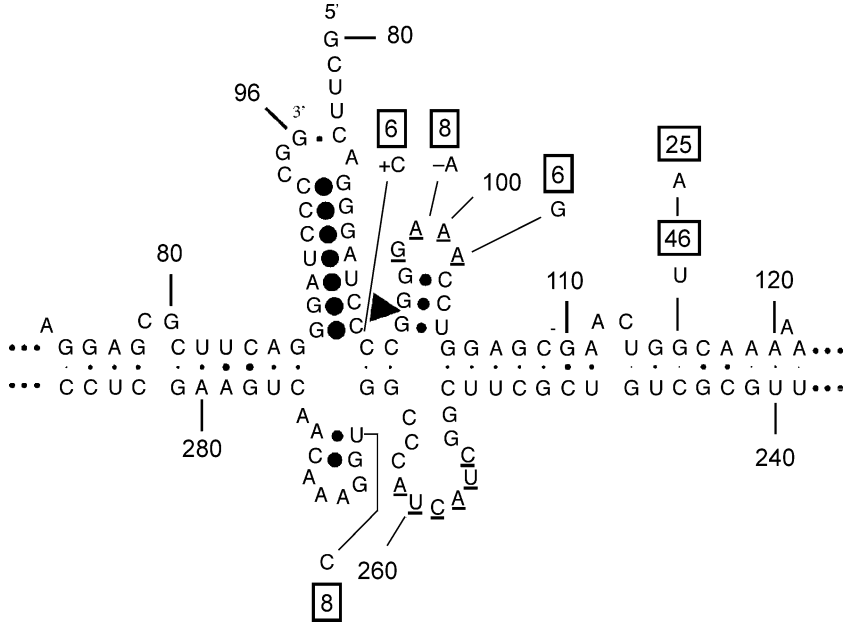

Fig. 10. Consensus structure of the CCR in processing configuration. The sequence and numbering of PSTVd is shown, for which the model was established (Baumstark et al., 1997; Schrader et al., 2003). The structure is based on a consensus model of 11 pospiviroid sequences (PSTVd, MPVd, CEVd, CSVd, TASVd, TPMVd, CCCVd1f, HLVd, CPFVd, HSVd, and AGVd). Size of dots connecting base pairs is proportional to the consensus pairing probability (Lück et al., 1999). The triangle marks the processing site; boxed numbers refer to the respective clone and its mutations located in the CCR. The underlined nucleotides form the loop E in the native rod-like structure (not shown).

addition that the conventional structural viroid motifs can support its propagation in *Arabidopsis*, but these processes are probably too inefficient to maintain such a PSTVd population in *Brassica* species above the detection limit. On the other hand, it has to be noted that some sequence motifs like the RG1-like motifs within the PM region were detected in the original library of "thermomutants", but were not found in the sequence distribution detected in *A. thaliana*. This could mean that there are sequence elements highly unfavorable to PSTVd infection of this species. Similarly, accumulation of mutations in the central conserved region was already observed by Polívka et al. (1996) in the yellow speckle viroid-1, and accumulation of some specific mutations and unusual variants of citrus exocortis viroid following passage through alternative hosts was described by Semancik et al. (1993, 1994). Some of the nucleotide changes were so characteristic that Semancik et al. (1993) proposed the term "host signature" for such host-specific mutations.

The re-adaptation of CCR-defective clones in tomato underlines that the CCR is an essential structural and functional domain for PSTVd replication in tomato, that is, in this case processing step (Baumstark et al., 1997). It shows at the same time that the structural integrity of CCR is not hundred percent required for replication, whereas otherwise the mutants would not have stayed viable in tomato up to the event of reversion. Therefore, one has to assume an unspecific replication/processing mechanism on the basis of a non-adapted CCR structure. Such unspecific mechanism may serve for the infection in *A. thaliana*, and destruction of the tomato-adapted structural domain may even facilitate the unspecific infection mechanism in *A. thaliana*. Adaptation of the viroid structure to an effective propagation in *A. thaliana* would take then many more passages. The contribution of specific and unspecific mechanisms is possibly not restricted to CCR but also valid to domains involved directly in transcription. Other mechanism might affect more general features of viroids like thermostability, flexibility, bending, etc. Mutant 170 with the insertion of G_{39} indeed can be explained by temperature adaptation. Because in one helix (Fig. 9), two base pairs are added, it might have been selected originally because of the thermotreatment of PSTVd in *N. benthamiana* and can replicate only on a very low level at lower temperature. Only if the additional thermostability is reverted by destroying first one GC base pair to an AC mismatch and then deleting the A, the original lower thermostability is reestablished for optimal growth in tomato.

Low propagation rate, which was detectable only by the use of RT-PCR, was observed also for progeny of HLVd "thermomutants" in *N. benthamiana* in our previous work (Matoušek, 2003). If "low-level" viroid populations in so-called nonhost plant species, such as described in the present experiments or previously, would occur in nature, they could be the sources of further evolutionary adaptations and thus might represent a potential danger for cultured plants. Knowing the danger, however, might help to prevent it.

Material and methods

Plant material, cultivation, and heat treatment conditions

L. esculentum and *N. benthamiana* plants were used as conventional host plants. Two so-called nonhost plant species (i.e., *R. sativa* and *A. thaliana* var. Columbia) were included in infection experiments. All these plants were maintained in isolated clima boxes at a temperature of 25 ± 3 °C under natural light conditions. Developing inflorescences were immediately removed from *A. thaliana* wild type grown in a clima box to favor its longer vegetative growth and maintenance at rosette stage. *A. thaliana* mutants SDE1, SDE2, and SDE3 impaired in PTGS silencing were kindly provided by Dr. David C. Baulcombe (Sainsbury Laboratory, John Innes Centre, Norwich, UK).

PSTVd-infected *N. benthamiana* plants were subjected to heat treatment 20 days pi using cycles of gradually increasing temperature (Matoušek et al., 1995) in a special cultivation box to ensure the desired humidity. The temperature increased daily for 1 °C within 1 week starting from 34 °C and reaching maximum 40 °C, followed by 10 days recovery at 25 °C. This heat stress cycle was repeated two times. After the second cycle, PSTVd was extracted and analyzed.

PSTVd extraction, RT-PCR, cDNA cloning, and sequencing

For reverse transcription–polymerase chain reaction (RT-PCR), total RNA was isolated from 100 mg of leaf tissue using CONCERT™ (Plant RNA Purification Reagent, Invitrogen) and supplementary purified by the RNA cleaning protocol using RNeasy Plant Total RNA kit (Qiagen). RT-PCR reactions were performed using the Titan One Tube RT-PCR system including a high fidelity *Pwo* polymerase (Roche Molecular Biochemicals). Two primer systems were used for detection and amplification of PSTVd cDNA (see Fig. 2). Primers covering the *Bam*H1 restriction site were designated PSTVdb I (5′ a$\underline{G_{92}GATCC}$CTGAAGCGCTCCT-CCG$_{71}$ 3′) and II (5′ a$\underline{G_{87}GATCC}$CCGGGGAAACCTGGA-G$_{108}$ 3′), and primers covering the *Sty*I restriction site were designated PSTVds I (5′ a$\underline{C_{337}CAAGG}$GCTAAACACCCT-CGC$_{357}$ 3′) and II (5′ a$\underline{C_{343}CTTGG}$AACCGCAGTTGGTT-C$_{323}$ 3′). The nonspecific adenine in each primer (indicated by a small letter "a") was designed to facilitate cleavage of cDNA fragments at the *Bam*H1 or *Sty*I restriction sites encoded in the primers (underlined). Reactions were carried out in 50 μl reaction volume as recommended by the manufacturer; that is, reverse transcription was run for 30 min at 50 °C, and after 2 min denaturation at 94 °C, the polymerase chain reaction was started with cycles of 30 s at 94 °C, 30 s at 58 °C, and 60 s at 68 °C. If not stated otherwise, RT-PCR was carried out for 38 cycles. In some cases, to study

sensitivity of reaction to RNAse, 10 μg of RNase A from Qiagen was added to reaction mixtures before addition of polymerase complex. Re-amplification of RT-PCR products using *Pwo* polymerase (Angewandte Gentechnologie Systeme, Germany), cloning in pCR-Script SK(+) vector (Stratagene) and automatic sequencing was performed as described previously (Matoušek et al., 2001).

TGGE, cDNA heteroduplex analysis, and electrophoretic analysis in agarose gels

Temperature-gradient gel-electrophoresis (TGGE) was performed in 6% polyacrylamide gels containing 7 M urea (Riesner et al., 1989). Heteroduplexes were prepared by hybridization of cDNA samples to standard (cloned PSTVd intermediate (DI)) cDNA or by hybridization without adding this standard cDNA. Hybridization procedure as well as analysis of DNA in gels was the same as described previously (Matoušek et al., 2001), except for temperature of gels: 44 °C was used for analysis of cDNA prepared using PSTVds primers and 42 °C for analysis of cDNA prepared with PSTVdb primers. miniTGGE system (Biometra, Germany) was used for library screenings for heteroduplexes. Gels were stained for nucleic acids with $AgNO_3$ as described by Schumacher et al. (1986). For analysis of RT-PCR products, 2% agarose gels were used and cDNA was stained with ethidium bromide.

Hybridization probe, Southern blot, and dot-blot hybridization

For Southern blots, we used a hybridization probe amplified from position 6 to position 253 of PSTVd intermediate using primers probe I (5′ G_{253}AAGCGACAGCGCAAAG-G_{236} 3′) and probe II (5′ C_6TAAACTCGTGGTTCCTGTG-G_{26} 3′) to avoid an overlap with the *Sty*I primer system (see above). If not otherwise stated, this α-^{32}P dCTP-labeled probe was prepared by PCR. Southern blot was prepared as a standard alkaline capillary blot from 2% agarose gels onto a Nylon membrane (Charge Modified 0.2 μm; Sigma). Dot-blot hybridization was performed as described by Matoušek et al. (1994) using a full-length PSTVd probe. This analysis exhibited a lower limit of PSTVd concentrations at about 0.03 pg/mg of fresh mass (Matoušek et al., 1994).

Preparation of library of "thermomutants", inocula, and inoculation methods

Tabler and Sänger (1984) found that double-stranded *Bam*H1 fragments of full-length PSTVd are highly infectious. For this reason, we maintained the cDNA clones as *Bam*H1 fragments using primers PSTVdb and later as *Sty*I fragments amplified by PSTVds primers. Preliminary experiments showed that both of these fragments, pretreated with corresponding restriction enzymes to form sticky ends, are infectious. For preparation of the library of "thermomutants", cloned cDNA of 100 positive clones randomly selected from approximately 10^3 original colonies plated after TGGE selection (see Results) was used. cDNA was re-amplified by *Taq* polymerase directly from individual colonies using the same amplification conditions as for *Pwo* polymerase (see above), and screened by the method of preformed DNA heteroduplexes against a PSTVd intermediate (DI) standard clone. Twenty-one clones, showing positions in the gel differing from that of the homoduplex, were selected for sequencing and inocula preparation.

For biolistic inoculation, cloned cDNA was re-amplified using *Pwo* polymerase, phenolized, precipitated with ethanol, treated either with *Bam*H1 or *Sty*I restriction endonuclease, and purified from agarose gels using Qiagen Gel Extraction Kit (Qiagen). Extracted DNA was then immobilized on the surface of 25 mg of gold microcarrier (1 μm) at a concentration of 0.2 μg/mg following the manufacturers protocol for HELIOS Gene Gun System (Biorad, USA). Biolistic inoculation was performed with 150 psi at distance varied from 0 to 2 cm between the leaf and the spacer. A single shot corresponded to approximately 100 ng of cDNA.

The pool for biolistic inoculation of *Brassica* species was prepared as follows: cDNA was amplified by *Pwo* polymerase from individual "thermomutants" using isolated plasmids as templates and immobilized on gold microcarrier. Individual inocula were then applied three times to two *L. esculentum* and two *N. benthamiana* plants over a 1-week period. RNA was then isolated from infected plants for each clone independently, but RT-PCR was performed from a mixed RNA sample using PSTVdb primers. cDNA was then re-amplified with *Pwo* polymerase, treated with *Bam*H1, and immobilized as above at a concentration of 0.4 μg/mg of microcarrier. Inocula of PSTVd wild-type strains, R_1, and PSTVd mutants 170, 46, 8, and 6 were immobilized as cDNA *Sty*I restriction fragments at 0.2 μg/mg of gold.

Conventional RNA inocula were prepared by homogenization of 1 g of leaf tissue in 0.1 M sodium phosphate, 0.2 M glycine (pH 9.6) buffer containing 0.6 M NaCl, and 1% SDS (GPS buffer), followed by phenol/chloroform extraction and ethanol precipitation. The nucleic acid precipitate was dissolved in 500 μl of 0.04 M sodium phosphate buffer (pH 7.6) and used for mechanical inoculation by Carborundum. If not otherwise stated, for infectivity tests, 10 plants were inoculated each into two new leaves, using either the biolistic method or with 20 μl of RNA inoculum per leaf.

Sequence accession numbers and other methods

PSTVd strain intermediate (DI) (Owens et al., 1986) was used as wild type. This cDNA was recloned as a *Bam*H1 fragment from plasmid pRH701 (Hecker et al., 1988) to pBluescript vector to form the infectious *Bam*H1 dimer. This vector was used to prepare fresh RNA inoculum. The wild-type PSTVd sequence corresponded fully to GenBank database accession no. M16826 (Gross et al., 1978). Sequences

of the mild (M) (accession no. M14814), lethal KF440-1 (Schnölzer et al., 1985), and RG1 (accession no. U23058) (Gruner et al., 1995) strains were used for sequence and structure comparisons.

Sequence analyses were carried out with DNASIS for Windows, version 2.5 (Hitachi). The phylogenetic tree was calculated by the Neighbor-Joining method in ClustalW, based on a structural alignment of full-length sequences, and was drawn by the program unrooted (Perrière and Gouy, 1996). Structural calculations were performed using the ConStruct algorithm (Lück et al., 1999). Levels of hybridization signals were assayed by the use of STORM device and ImageQuaNT software (Molecular Dynamics).

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