

# Biolistic inoculation of plants with viroid nucleic acids

J. Matoušek<sup>a,\*</sup>, L. Orctová<sup>a</sup>, G. Steger<sup>b</sup>, D. Riesner<sup>b</sup>

<sup>a</sup> Department of Molecular Genetics, Institute of Plant Molecular Biology, Czech Academy of Sciences, Branišovsk 31, 37005 Česká Budějovice, Czech Republic

<sup>b</sup> Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, D-40225 Düsseldorf, Germany

Received 30 March 2004; received in revised form 13 August 2004; accepted 16 August 2004

Available online 13 October 2004

## Abstract

Parameters for biolistic transfer of viroid nucleic acids using a Helios Gene Gun device were assayed. The main achievement of this method is high efficiency of inoculation with linear monomeric viroid cDNAs and RNAs. This greatly facilitates the study of mutated sequence variants, viroid libraries and mixed populations. The lower limits for efficient inoculation of monomeric cDNA fragments with the sequence of potato spindle tuber viroid (PSTVd) and native PSTVd RNA as detected 21 days p.i. are in the range of 50 ng and 200 pg per tomato plant, respectively. At a higher dose, i.e. 2 ng of native RNA per plant, biolistic transfer causes drastic stunting compared to conventional mechanical inoculation, which points to higher PSTVd titers after the biolistic transfer. Infection is readily achieved with exact length monomeric RNA transcripts having 5'-triphosphate and 3'-OH termini in amounts ranging from 2 to 20 ng per plant, suggesting no need for any supplementary modifications of ends or RNA circularization. The biolistic transfer is efficient for viroid "thermomutants", which exhibit low or no infectivity with conventional mechanical inoculation with Carborundum. The biolistic inoculation is also efficient for two other members of the *Pospiviroidae* family, hop stunt and hop latent viroid.

© 2004 Elsevier B.V. All rights reserved.

**Keywords:** Gene gun; Viroid infection; Viroid pathogenesis; Thermomutants; *Lycopersicon esculentum*; *Humulus lupulus*

## 1. Introduction

Biolistic transfer of nucleic acids was originally developed in the late 1980s using large onion (*Allium cepa* L.) cells as a target for delivery of a chloramphenicol acetyl phosphotransferase (CAT) expression construct, as well as RNA of tobacco mosaic virus (TMV) coated onto tungsten microprojectiles (Klein et al., 1987). Nowadays, biolistic particle bombardment of plant cells (for review, see Mäenpää et al., 1999) is becoming a powerful alternative to *Agrobacterium*-mediated plant transformation (for a recent review, see Gelvin, 2003) and agroinfection described originally by Grimsley et al. (1986).

In plant virology, the biolistic method has been used for inoculation of various viral genomes either in the form of

RNA or cDNA (Klein et al., 1987; Gilbertson et al., 1991; Galon et al., 1995; Fakhfakh et al., 1996; Hämäläinen et al., 2000; Kekarainen et al., 2002; Merits et al., 2002). The Helios Gene Gun (Bio-Rad), a hand-held particle bombardment device, is used quite commonly in various animal and human systems for vaccination, gene therapy and transfection, but is especially useful to inoculate intact plants (Hämäläinen et al., 2000). Therefore, it has a wide application in plant virology. No information, however, is available about the application of this technique for inoculation with viroid genomes.

Viroids are the smallest known plant pathogens (for review, see Hadidi et al., 2003), consisting solely of a circular, non-coding RNA ranging from 246 to 463 nt. Their replication follows a rolling-circle replication mechanism. Viroids are transmissible by mechanical injury of host cells. To date, as many as 27 different viroid species have been classified and listed in biological databases (Pelchat et al., 2003). Most viroid RNAs form rod-like sec-

\* Corresponding author. Tel.: +42 38 777 5529; fax: +42 38 41475.

E-mail address: [jmat@genom.umbr.cas.cz](mailto:jmat@genom.umbr.cas.cz) (J. Matoušek).

ondary structures, which denature in a highly co-operative mode at high temperatures (Riesner et al., 1979), and are highly resistant to certain plant nucleases, as found for potato spindle tuber viroid (PSTVd) (Matoušek et al., 1988, 1995).

Viroid cDNA has also been described to be infectious. For instance, Tabler and Sanger (1984) showed that cloned single- and double-stranded DNA copies of PSTVd as well as co-inoculated subgenomic DNA fragments are infectious. The infectivity of cDNA of other viroid species like hop stunt viroid (HSVd) (Meshi et al., 1984) or citrus exocortis viroid (CEVd) (Visvader et al., 1985) has been studied. Linear viroid molecules prepared by in vitro transcription were also shown to be infectious (Tabler and Sanger, 1985; Rigden and Rezaian, 1992; Rakowski and Symons, 1994). Not all cDNA or RNA forms, however, are equally efficient for inoculation. In general, dimeric and oligomeric molecules are highly infectious, whereas monomeric linear molecules are much less infectious. This difference has been explained by the requirement for duplication of sequences necessary for processing to circles. These processing sites involve either a sequence duplication of the central conserved region (CCR) (Hammond et al., 1989; Candresse et al., 1990) where a well-defined processing structure is formed (Baumstark et al., 1997; Schrader et al., 2003), or of alternative sites (Hammond et al., 1989), or of specific sequence stretches from the cloning vectors (Rakowski and Symons, 1994). Due to low or no infectivity of monomeric viroid cDNAs or monomeric RNA transcripts, usually dimeric or partly duplicated constructs are prepared to study infectivity of specific clones or to analyze viroid infections in so-called non-host plant species (Gardner et al., 1986; Salazar et al., 1988). This approach is particularly laborious in infectivity studies of viroid populations, of quasispecies, or of the numerous sequence variants known for various viroids (e.g. Visvader and Symons, 1985; Rigden and Rezaian, 1993; Gora-Sochacka et al., 2001; Kofalvi et al., 1997; Ambros et al., 1999; Matoušek et al., 2001).

The small size of the viroid genome, and infectivity of its cDNA and transcript RNA are favorable properties to apply the biolistic method for effective inoculation. In the present work, we assayed parameters for biolistic transfer of viroid nucleic acids using a Helios Gene Gun device and we showed that the biolistic method allows for efficient inoculation with monomeric linear viroid constructs derived from the wild type, with individual heat-induced sequence variants, which we characterized previously (Matoušek et al., 2004), as well as with a mixture of clones forming a secondary population of viroid “thermomutants”. In addition, biolistic transfer of native RNA greatly increased the pathogenic effect of PSTVd on tomato. The procedure described here was applied also to two other members of the *Pospiviroidae* group, hop latent and hop stunt viroid. Thus, the use of biolistic transfer of monomeric cDNA as well as of RNA greatly simplifies or even facilitates the molecular genetic analysis of viroid species.

## 2. Materials and methods

### 2.1. Plant and tissue culture cultivation conditions

*Lycopersicon esculentum* cv. Rutgers plants were maintained in clima boxes at a temperature of  $25 \pm 3^\circ\text{C}$ . Plants were grown under natural light with supplementary illumination ( $90 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR) to keep a 16-h day period. Healthy hop mericlones of Oswald’s clone 72 were transferred to soil and maintained under clima box conditions for approximately 3 weeks before inoculation. In vitro-grown potato plants cv. Kamyk were supplied with light (16 h) of intensity  $35 \text{ mmol m}^{-2} \text{s}^{-1}$  PAR. Day/night temperatures were  $25/18^\circ\text{C}$ . Hairy root lines of potato were established after injection of a suspension of *A. tumefaciens* A4–24 strain bearing Ri plasmid (Cardarelli et al., 1987). Hairy roots, which developed usually within 2 weeks, were excised and grown further on MS medium (Murashige and Skoog, 1962) with 200 mg/l Timentin.

### 2.2. RNA extractions, cDNA preparation, and in vitro transcription

For inoculation, an RNA fraction was prepared by fractionation of 2 M LiCl-soluble nucleic acids with 12–20% PEG 6000 as described earlier (Matoušek and Dedič, 1988). In the PEG precipitated fractions PSTVd amount was usually in the range 0.5–2%. The level of viroid was estimated in these samples using electrophoretic and hybridization methods with HPLC-purified PSTVd as standard. For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was isolated from 100 mg of leaf tissue using the plant RNA purification reagent CONCERT<sup>TM</sup> (Invitrogen) and additionally purified by the RNA cleaning protocol using the RNeasy Plant Total RNA kit (Qiagen).

cDNA fragments from cloned wild-type and thermomutant sequences were amplified using Pwo polymerase (Angewandte Gentechnologie Systeme GmbH, Germany). For PSTVd amplifications, two primer pairs were used (Fig. 1). Primers covering the BamHI restriction site were designated PSTVdb I (5′-aG<sub>92</sub>GATCCCTGAAGCGCTCCTCCG<sub>71</sub>-3′) and II (5′-aG<sub>87</sub>GATCCCCGGGGAAACCTGGAG<sub>108</sub>-3′), and primers covering the StyI restriction site were designated PSTVds I (5′-aC<sub>33</sub>7CAAGGGCTAAACACCCTCGC<sub>35</sub>-3′) and II (5′-aC<sub>34</sub>3CTTGGAAACCGCAGTTGGTTC<sub>32</sub>-3′). For HLVd cDNA amplification, we used primers covering the PstI restriction site (HLVdp I, 5′-aC<sub>233</sub>TGCAGGTAAAGCTCGGC<sub>216</sub>-3′, HLVdp II, 5′-aC<sub>228</sub>TGCAGAAAGTTCACATAAAAAG<sub>249</sub>-3′). For HSVd amplification, we used primers covering the EcoRI restriction site (HSVde I, 5′-aA<sub>12</sub>GAATTC<sup>3</sup>CCCCAGAGGGGCTCA-3′, HSVde II, 5′-aG<sub>5</sub>GAATTC<sup>3</sup>CGAGTTGCCGC-3′) (Fig. 1). The non-specific adenine in each primer (indicated by a small letter “a”) was designed to facilitate cleavage of cDNA fragments; restriction sites encoded in the primers are underlined. For in vitro transcription from double-stranded

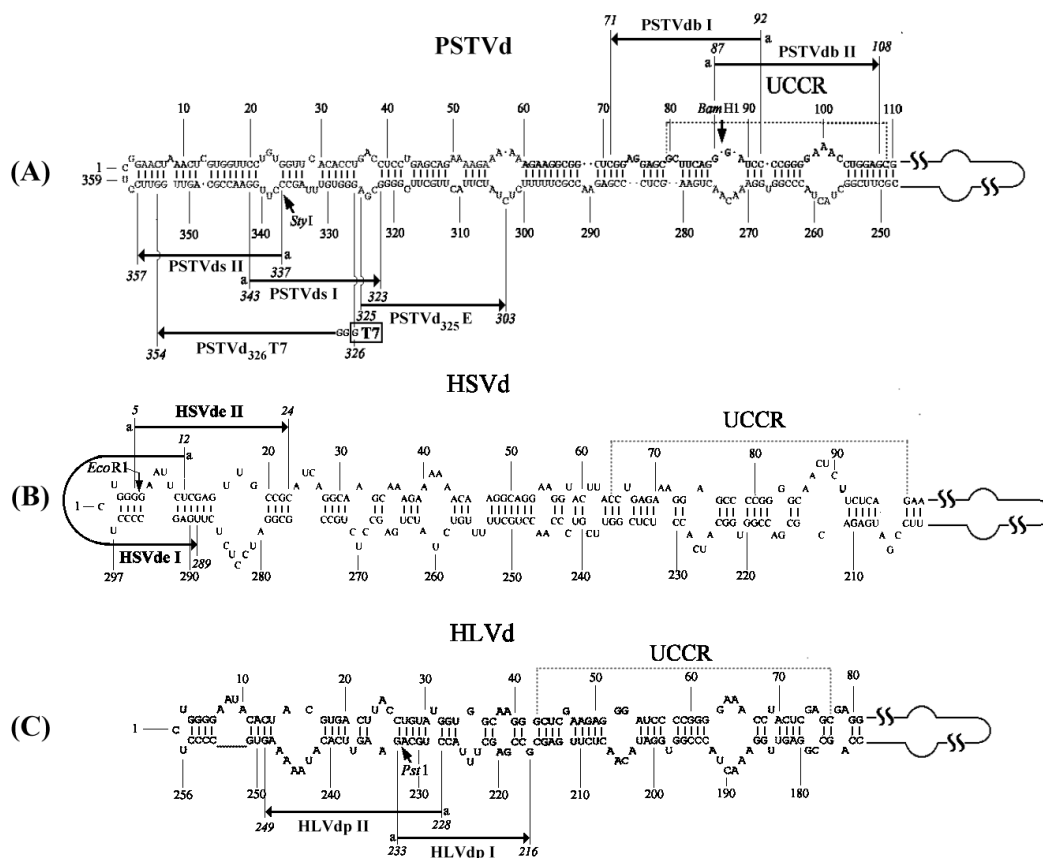


Fig. 1. Schematic drawing of primer systems used for viroid amplification and detection. Primers are localized on the left half part of viroid's rod-like structure. (A) Primer pairs for potato spindle tuber viroid (PSTVd). The primer pair designated PSTVdb I and II covers the *Bam*HI restriction site localized in the upper central part of the secondary structure. Primers PSTVds I and II cover the *Sty*I restriction site localized in the lower part of the left half of the secondary structure. These primers are used for preparation of infectious cDNA fragments. Primers PSTVd<sub>326</sub>T7 with a T7 box and PSTVd<sub>325</sub>E are primers used for transcription of exact PSTVd monomers. (B) Primers designed for preparation of infectious cDNA of hop stunt viroid (HSVd). The primers are localized in the terminal part of rod-like structure and cover the unique *Eco*R1 restriction site. (C) Primers for preparation of infectious cDNA of hop latent viroid (HLVd). Primer binding sites are localized in the lower part of the viroid's structure and cover a unique *Pst*I restriction site. Primers are represented by arrows, positions are designated by numbers; a, position of non-specific nucleotide; UCCR, upper part of the central conserved region.

PSTVd fragments having an attached T7 promoter sequence we used primers (Fig. 1) PSTVd<sub>326</sub>T7 (5'-TAATACGACTC-  
ACTATAGGGTGTTTAGCCCTTGAACCGCAGTTGG-  
3', the attached T7 promoter part is underlined) and PSTVd<sub>325</sub>E (5'-TCGCCCCGAAGCAAGTAAGATAG-3'). The T7 primer was HPLC purified.

We used the following amplification conditions: 94 °C, 120 s; 35 × (94 °C, 30 s; 58 °C, 30 s, 72 °C, 60 s); 72 °C, 10 min. PCR products were phenolized, precipitated with 3 (v/v) ethanol, dissolved in water, and cleaved with the corresponding restriction endonuclease over night. Then the fragments were phenolized and purified by the Qiagen Gel Extraction Kit (Qiagen). Unless stated otherwise, purified fragments were used for microprojectile coating.

RT-PCR reactions were performed using the Titan One Tube RT-PCR system including a high-fidelity *Pwo* polymerase (Roche Molecular Biochemicals) using primers described above. Reactions were carried out in 50 µl reaction volume as recommended by the manufacturer, i.e., reverse transcription was run for 30 min at 52 °C, and after 2 min de-

naturation 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. Unless stated otherwise, RT-PCR was carried out for 38 cycles.

RNA transcription was run from DNA templates purified with Qiagen Gel Extraction kit (Qiagen) using a RiboMax protocol (Promega). After transcription, RNA was extracted once with phenol:chloroform, precipitated with ethanol and dissolved in DNase I buffer. After DNase I cleavage, the sample was phenolized, precipitated with ethanol, dissolved in water and RNA was purified using the RNA clean up protocol from Qiagen. A one-tenth of 10× concentrated sodium-cacodylate hybridization buffer (10 mM sodium-cacodylate buffer (pH 6.8) containing 1 M NaCl and 10 mM EDTA) was then added to the sample. RNA samples were heated to 95 °C in a heat block and cooled slowly in an insulation box to 40 °C in 2 h to pre-form the thermodynamically more stable rod-like conformation, and finally precipitated with ethanol. Before adsorption, the sample was dissolved in 1 mM sodium-cacodylate buffer pH

6.8 without salt. In some cases, RNA samples were adjusted with purified tRNA to make an equal concentration of 0.4 µg RNA per mg of microcarrier for coating.

### 2.3. Helios Gene Gun conditions for particle bombardment, preparation of viroid inocula, and inoculation methods

In this study, the Helios Gene Gun System from Bio-Rad (USA) was used. The following parameters, recommended by the manufacturer and established for plant tissue by Hämäläinen et al. (2000), were used: the concentration of polyvinyl pyrrolidone (PVP) was 0.05 mg/ml 99.8% ethanol (Merck); gold microcarrier per shot, that is the so-called microcarrier loading quantity (MLQ), was kept at 0.5 mg per shot, i.e. 25 mg of gold per standard length of gold tubing; a 0-cm distance between the leaf and the gene gun spacer was used for intact plants and approximately 2 cm distance for tissue cultures. Other essential parameters varied; f.e., the amount of DNA (DNA loading ratio, DLR) or RNA per mg of microcarrier varied from 0.05 to 2.0 µg/mg. This ratio may not correspond to the amount of loaded viroid-specific DNA or RNA, depending on the types of inocula: in case of plasmid inoculation, amounts of viroid-specific cDNA or RNA per shot were re-calculated to correspond to the viroid cDNA content in the whole construct or to the content of native viroid in the RNA extract. In initial experiments, three sizes of microprojectiles (0.6, 1.0, and 1.6 µm) were applied; in all subsequent experiments 1.0 µm particles were used. Helium pressure varied from 50 to 150 psi, depending on the experiment.

For biolistic inoculations, either viroid cDNA or RNA was prepared as described above. All cDNA constructs—cloned dimeric or monomeric cDNAs, or DNA fragments prepared by high-fidelity PCR and treated with restriction enzymes to form sticky ends—were coated onto gold particles following the manufacturer's protocol. Briefly, gold microcarrier was re-suspended in 0.025 M spermidine and shortly sonicated. Fifty microliters of DNA sample was added to 100 µl of gold suspension, vortexed, and the DNA co-precipitated by adding dropwise 100 µl of 1 M CaCl<sub>2</sub> while shaking. Then the DNA-coated gold was shortly centrifuged and washed three times with 1 ml of absolute ethanol before re-suspension in ethanol/PVP. This suspension was immediately used for coating of the tubing following the procedure recommended by the manufacturer.

For coating with viroid RNA samples, we modified the procedure described by Qiu et al. (1996) developed for gene gun delivery of mRNA, based on the original procedure used for coating tungsten microprojectiles (Klein et al., 1987). Briefly, 25 mg pre-sonicated microcarrier (1 µm) and 12.5 µg RNA were mixed in DEPC-treated 1 mM sodium-cacodylate buffer (pH 6.8) to make a total volume of 100 µl. To this suspension was slowly added 37.5 µl of 0.25 M CaCl<sub>2</sub>, the sample incubated on ice for 5 min and centrifuged for 30 s to prevent aggregation of particles. The sediment washed three

times with 1 ml of absolute ethanol (Merck), followed by transfer to 3 ml of absolute ethanol/PVP solution. This suspension was used immediately for coating. The tubing was rotated in the tubing prep station support cylinder while drying to ensure uniform coating over the inner surface of the Goldcoat tubing.

Attached plant leaves were inoculated while supported with thick cardboard paper. Tissue cultures were bombarded under aseptical conditions after removal from medium and placing in sterile Petri dishes. After inoculation, plants were transferred immediately into polyethylene bags to prevent drying of the shot-wound leaf area. Treated plants were conditioned further by shading them for 24 h, and afterwards by cultivation in holed bags for the next 2 days in the climate boxes under standard conditions as described above. Because the DNA and RNA coated onto microprojectiles appear to be rather stable, laboratory surfaces and equipment used were treated with diluted bleach and the laboratory was irradiated overnight by 260 nm UV light to prevent possible PCR cross-contaminations.

For preparation of conventional DNA or RNA inocula, nucleic acids were dissolved in 0.04 M sodium phosphate buffer (pH 7.6), containing 2 mg/ml RNA extract from healthy plants (Matoušek et al., 1994). For mechanical inoculation, we used 20 µl inoculum per leaf and Carborundum as abrasive. Unless stated otherwise, 8 or 10 plants were used for infectivity tests.

### 2.4. Viroid detection and electrophoretic analyses

RT-PCR detection of hop viroids was performed using the Titan One Tube RT-PCR (Roche) under conditions described above, using primers HLVdp I and II or HSVde I and II. Dot-blot hybridizations were performed as described by Matoušek et al. (1994) using full-length PSTVd, HSVd or HLVd <sup>32</sup>P[dCTP]-labeled probes. The lower detection limit of this method is about 0.03 pg/mg of fresh mass (Matoušek et al., 1994).

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. Hybridization procedure as well as analysis of DNA in gels was described previously (Matoušek et al., 2001). Gels were stained for nucleic acids with AgNO<sub>3</sub> as described by Schumacher et al. (1986). For analysis of RT-PCR products, 2% agarose gel was used and cDNA stained with ethidium bromide. For RNA analysis, nucleic acids were separated under native conditions in 1.5% metaphor agarose using DEPC-treated buffers and afterwards stained with ethidium bromide.

### 2.5. Other methods

PSTVd strain intermediate (DI) (Owens et al., 1986) was used as wild type. Its cDNA was re-cloned as a BamHI fragment from plasmid pRH701 (Hecker et al., 1988) to pBlue-

Table 1  
Effect of helium pressure and microcarrier size on the rate of infection with a dimeric PSTVd construct

Amount of total DNA per shot <sup>a</sup> (μg)	Amount of viroid cDNA per shot <sup>b</sup> (ng)	Microcarrier size (μm)	Number of plants infected/inoculated <sup>c</sup>				
			Helium pressure (psi)				
			50	80	100	130	150
Tomato leaves							
0.25	50	0.6	0/7	0/8	1/8	3/8	3/8
0.25	50	1.0	0/8	2/8	4/8	8/8	7/8
0.25	50	1.6	1/8	2/8	6/8	8/8	7/8
Hairy roots							
1.0	200	1.0	–	–	–	0/8	1/8
1.0	200	1.6	–	–	–	9/12	6/8

<sup>a</sup> DNA loading ratio (DLR) representing the amount of DNA per mg of microcarrier was 0.5 and 2.0 μg/mg for leaf and hairy roots tissues, respectively.

<sup>b</sup> Plants were shot at the stage of two true leaves; one shot per apical leaf or per hairy roots clump was applied.

<sup>c</sup> Detection was performed in upper non-inoculated leaves or in passaged “hairy roots” tissue 21 days p.i. using dot-blot hybridization; –, not performed; p.i., post-inoculation.

script vector to form the infectious *Bam*HI dimer. The “lethal” PSTVd KF440-1 (Schnölzer et al., 1985) was used for inoculation and analysis of symptoms. Clones from our thermomutant library were described previously in detail (Matoušek et al., 2004). The HLVD sample used for inoculation corresponded to GenBank sequence ACX07397 (Puchta et al., 1988) and HSVd variant to ACE01844.

Levels of hybridization signals were assayed by the STORM device and ImageQuaNT software (Molecular Dynamics).

### 3. Results

#### 3.1. Optimized procedure for inoculation of attached intact leaves and hairy roots with viroid using the Helios Gene Gun system

For viroid inoculation mediated by the Helios Gene Gun System, the manufacturer’s recommendations and data obtained during optimization of potato virus A (PVA) inoculation of potato (Hämäläinen et al., 2000) were followed. Based on these data, two basic parameters were selected as constant: the amount of gold microcarrier was kept at 0.5 mg per shot and the concentration of polyvinyl pyrrolidone (PVP) adhesive used for coating the Goldcoat tubing (see Section 2). The gene gun spacer was in direct contact when a leaf was inoculated or kept at a distance of 2 cm for shooting of tissue culture.

In first experiments, an infectious dimeric PSTVd construct cloned into the *Bam*HI site of pBluescript SK(+) vector was coated onto 0.6, 1.0, and 1.6 μm microprojectiles and introduced to tomato leaves at different pressures (Table 1). From these well reproducible results, we roughly estimated the minimum amount of this construct required for infectivity to be in the range of tenths of ng per shot. At pressures higher than 150 psi, tomato leaves were significantly damaged and therefore, this pressure was considered as the upper limit. Infection with small-sized particles (0.6 μm) was unsuccessful

even at higher pressure. The most efficient inoculations were achieved at particle sizes of 1.0 and 1.6 μm and pressures in the range 130–150 psi. Bombardment with 1.6 μm particles tends to rip leaves to a higher extent than with 1 μm particles. Covering plants with polyethylene bags to prevent drying up the shot-wounded area significantly reduced consequences of this damage. To avoid tissue damage, we selected 1.0 μm particles as optimal for inoculation of intact plants in all further experiments. For potato “hairy roots” in vitro culture inoculation was unsuccessful with 1 μm particles, even at maximal DNA loading ratio (DLR), which is recommended for DNA coating using the Helios Gene Gun System. This inoculation was, however, efficient at a higher pressure with particles of 1.6 μm reaching about 75% infected material (Table 1).

Next we tested if monomeric cDNA constructs, known for their low infectivity (Tabler and Sängler, 1984), could be efficiently inoculated by particle bombardment. Full-length *Bam*HI and *Sty*I fragments, prepared using primers positioned as shown in Fig. 1A, were compared with respective monomeric clones and an infectious *Bam*HI dimeric construct (Table 2). Inoculations were performed at 80, 100, and 150 psi and various DNA loading ratios. A low pressure of 80 psi was not efficient for inducing disease neither from plasmid nor from fragment DNA constructs. Interestingly, at higher pressure similar infectivity results were obtained for the monomeric fragments having sticky ends as for the dimeric construct at the same amount of delivered DNA. Even a 100% infection was obtained for both monomeric fragments at 200 ng DNA per plant (Table 2), suggesting a high infectivity of cDNA monomers. Delivery of both monomeric constructs at maximal DLR and 220 ng per plant seemed to be unsuccessful, but surprisingly a 40–80% infection was detected at higher psi by the dot-blot hybridization after a more prolonged 45-day period of cultivation. However, most of the resulting dot-blot signals were rather weak (results not shown). This suggests that inoculation with monomeric cloned constructs can be achieved by the biolistic method, but there is a significant delay in comparison to monomeric fragments or the dimeric variant.

Table 2  
Infectivity of various PSTVd constructs after biolistic inoculation

PSTVd cDNA construct	Total DNA per plant ( $\mu\text{g}$ )	Amount of viroid cDNA per plant <sup>a</sup> (ng)	Number of plants infected <sup>b</sup> from eight plants inoculated					
			21 days p.i. at helium pressure (psi)			45 days p.i. at helium pressure (psi)		
			80	100	150	80	100	150
<i>Bam</i> HI dimer in pBluescript SK(+) <sup>c</sup> linearized with <i>Eco</i> RV	0.25	50	2	7	8	–	–	–
<i>Bam</i> HI monomeric cDNA fragment, <i>Bam</i> HI-treated	0.05	50	0	4	6	–	–	–
	0.2	200	1	8	8	–	–	–
<i>Spy</i> I monomeric cDNA fragment, <i>Spy</i> I-treated	0.05	50	0	7	6	–	–	–
	0.2	200	2	8	8	–	–	–
<i>Bam</i> HI monomer in pCR-Script SK(+) linearized with <i>Eco</i> RV	2.0	220	0	1	0	0	3	7
<i>Spy</i> I monomer in pCR-Script SK(+) linearized with <i>Eco</i> RV	2.0	220	0	2	0	0	6	5

<sup>a</sup> Various DNA loading ratios and 1  $\mu\text{m}$  microcarrier particles were used.

<sup>b</sup> Eight plants were shot on the stage of two true leaves twice, once in the cotyledon leaf and once in the apical true leaf; detection was performed in upper non-inoculated leaves by dot-blot hybridization; –, not performed; p.i., post-inoculation.

<sup>c</sup> A mechanical inoculation of dimeric construct yielded 21 days p.i. no infected plant at 250 ng/plant and two infected out of eight inoculated plants at 1  $\mu\text{g}$ /plant, these amounts represent 50 and 200 ng of specific PSTVd cDNA per plant.

### 3.2. Application of the optimized procedure to monomeric DNA of PSTVd variants and thermomutants

Based on the high inoculation efficiency of monomeric fragments, we tested the infectivity of various mutant clones which form part of a mutant PSTVd library obtained from infected thermotreated plants (Matoušek et al., 2004). Most of these “thermomutants” possess more than one mutation (Table 3), which in most cases cause a destabilization of the viroid’s secondary structure (Fig. 2). According to our preliminary experiments with *N. benthamiana* plants, inoculation of the mutants as monomeric *Bam*HI fragments using Carborundum resulted in low or no infections (data not shown). Thus, we analyzed the possibility to induce infection by inoculation of these mutants into tomato by the biolistic Helios Gene Gun method. Two hundred nanograms of viroid cDNA was inoculated at 130 psi to tomato leaves and the infection was assessed by dot-blot hybridization 21 and 45 days p.i. For comparison, 4  $\mu\text{g}$  of *Bam*HI-treated cDNA fragments was inoculated into plants using the conventional Carborundum method. As shown in Table 3, at this concentration the mechanical inoculation of the wild type was quite efficient, reaching 75% of infection 21 days p.i., while none of the “thermomutant” clones produced any detectable hybridization signal. After a more prolonged period, however, infection was detectable for clones T2, T23, T34, T37, and T98, suggesting a very low infectivity of thermomutant clones. In contrast, except for clone T65, biolistic delivery of 200 ng of individual thermomutant cDNA led to a significant number of infected plants already at 21 days p.i. At 45 days p.i. infection ranged from 50 to 100% (Table 3), clearly illustrating that the biolistic transfer is more efficient than mechanical inoculation with these mutated variants.

In parallel, to analyze the possibility of simultaneous transfer of the whole spectrum of mutants, we infected tomato plants by inoculation with a “complex” inoculum prepared by co-precipitation of a mixture of mutated cDNAs (listed in Table 3). In Fig. 3A is shown an example of a temperature gradient gel (TGGE) analysis of cDNA prepared from a viroid sample isolated from a single plant infected with the “complex” inoculum. The cDNA profile (Fig. 3A) shows multiple transition curves, suggesting the appearance of heteroduplexes. This clearly indicates the presence of a population of viroid progenies in the analyzed sample. In contrast, a unique transition curve was observed in TGGE (Fig. 3B) with a mixed sample from 10 plants 45 days after inoculation with the unique wild-type *Bam*HI monomer cloned in PCR-Script vector (compare Table 2). This result suggests that the wild-type PSTVd intermediate (DI) strain was quite stable after the biolistic inoculation, despite the infection proceeded very slowly (Table 2).

### 3.3. Application of the biolistic procedure to native viroid and linear monomeric RNA transcripts of PSTVd, and enhanced pathogenicity reaction due to biolistic delivery

It has been reported by Rigden and Rezaian (1992) that exact length monomeric RNAs of CEVd prepared by in vitro transcription are infectious irrespective of the presence of 5'-triphosphate and 3'-OH termini of untreated transcripts; these molecules, however, were much less infectious than the circular form. Thus, exact length monomeric PSTVd transcripts without any further treatment were tested for infectivity after inoculation of tomato leaves by the biolistic method. Transcription was performed from double-stranded PCR frag-

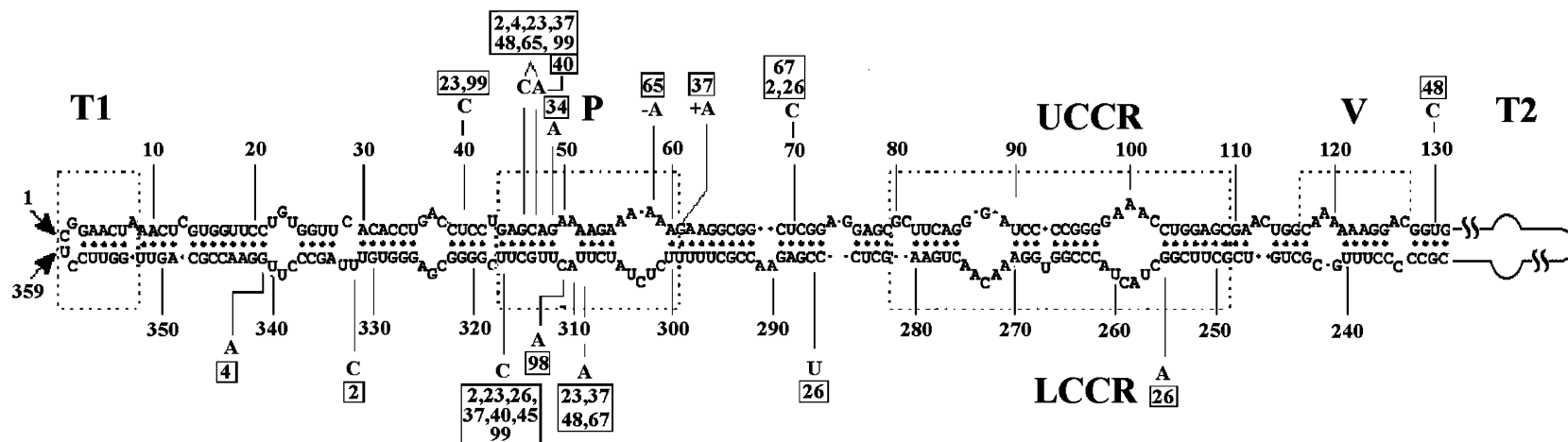


Fig. 2. Schematic drawing of PSTVd's secondary structure including positions of mutations in PSTVd "thermomutants" used for inoculation. Individual base changes are indicated by nucleotide symbols and corresponding cDNA clones by the boxed numbers. UCCR, upper part of the central conserved region; LCCR, lower part of the central conserved region; P, "pathogenicity" domain; T1 and T2, left and right terminal domains. Viroid domains are designated as suggested by Keese and Symons (1985).

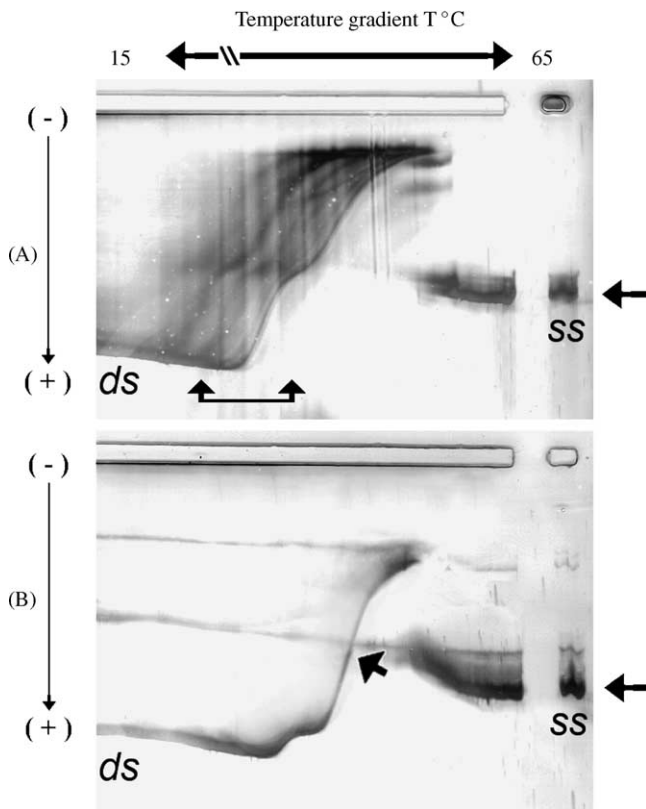


Fig. 3. Temperature gradient gel-electrophoretic analysis of PSTVd cDNA from biolistically infected tomato plants. cDNA was prepared using primers PSTVds I and II, separated on a 6% acrylamide gel containing 7 M urea with a temperature gradient from 15 to 65 °C and stained afterwards by silver. (A) Electrophoretic profile of cDNA from a single plant infected with a mixture of *Bam*HI-treated cDNA from clones listed in Table 3. The cDNA sample was prepared by mixing equal aliquots of thermomutant cDNAs. This mixture was then co-precipitated at a concentration 0.4 µg/mg of gold microcarrier and used for inoculation at 130 psi. Three shots with in total 400 ng of DNA were performed on cotyledon and true leaves. RNA was isolated 21 days p.i. and cDNA was prepared using PSTVds I and II primers. The range of bands (see arrow) represents homo- and heteroduplexes that are formed due to mismatches and are separated according to their thermostability. Distinct transitions seen on the gel are formed by frequent sequence variants within the evolved population. (B) Mixed sample from 10 *L. esculentum* plants infected with a monomeric *Bam*HI cDNA fragment cloned in pCR-Script SK(+) vector. A mixed leaf sample was prepared 45 days p.i. and used for RNA isolation and RT-PCR. Note the single band (arrow) in contrast to the range of bands in (A).

ments fused with the T7 promoter as depicted in Fig. 1A. After removal of the DNA template by DNase I treatment, RNA was purified using the Qiagen protocol and self-annealed to form the thermodynamically stable rod-like structure. This self-annealing greatly stabilizes the RNA during successive handling and immobilization on gold microcarrier (data not shown). Analysis of this RNA in native METAPHOR agarose gels showed a major band corresponding to exact unit length, and minor contaminations with RNA species of about 200 nt length that probably resulted from premature termination of the transcription reaction (Fig. 4). Pre-hybridized samples were immobilized onto 1 µm gold microcarrier using

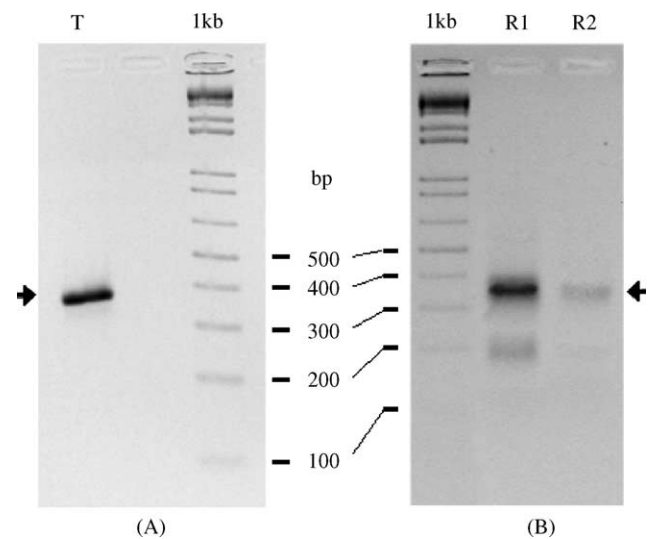


Fig. 4. Synthesis and adsorption of monomeric linear PSTVd. Viroid cDNA containing the T7 promoter was prepared using the primer pair PSTVd<sub>326</sub>T7 and PSTVd<sub>325</sub>E as outlined in Fig. 1, phenolized and gel-purified using the gel extraction protocol from Qiagen. This cDNA served as template for RNA transcription with T7 polymerase using the RiboMax protocol. After transcription the DNA template was cleaved with DNase I, and the RNA self-annealed to pre-form the rod-like conformation. For adsorption, 0.4 µg RNA per mg microcarrier was applied. Nucleic acid aliquots were separated under native conditions in 1.5% metaphor agarose using DEPC-treated buffers and afterwards stained with ethidium bromide. (A) T, T7 cDNA template; (B) R1, amount of RNA transcripts before adsorption; R2, corresponding supernatant after adsorption; 1kb; 1 kb plus ladder (Gibco BLR). Arrows on the sides indicate specific monomeric bands. The shorter transcripts are probably results of premature termination.

the modified calcium co-precipitation procedure (see Section 2). As seen in Fig. 4 this immobilization of RNA was efficient reaching about 90% of RNA co-precipitation. Analysis of infectivity (Table 4) showed that such monomeric RNA is highly infectious, as delivery of 2 ng per plant yielded 40% infection 21 days p.i. (Table 4), demonstrating that monomeric RNA prepared without any subsequent treatment can be successfully used for biolistic transfer.

We used the co-precipitation procedure to coat microprojectiles with various concentrations of native viroid RNAs extracted by LiCl extraction and PEG 6000 precipitation. In these samples, equal concentrations of native linear and circular forms of PSTVd were detected (not shown). Infectivity of such inocula is rather high because RNA amounts in the range 50–200 pg per plant are sufficient for infections detectable 20 days p.i. As judged from plant stunting, biolistic delivery of 200 pg RNA per plant caused a similar pathogenic effect as conventional inoculation with 2 ng PSTVd. Plant heights reached  $32 \pm 8.6$  and  $29 \pm 5.9$  cm after biolistic and conventional inoculation, respectively, while healthy plants reached  $48 \pm 4.2$  cm. In addition, biolistic inoculation with 2 ng of viroid RNA showed drastic stunting of the plants to  $15 \pm 5.2$  cm, i.e., the elongation growth of tomato stems was practically abolished after inoculation (Table 4). These results suggest that biolistic inoculation can lead to a more

Table 3  
Infectivity of thermomutant cDNAs after biolistic and mechanical inoculations

Thermomutant <sup>a</sup> cDNA inoculated	Number of mutations compared to wild type	Number of plants infected from eight plants inoculated <sup>b</sup>			
		Biolistic inoculation <sup>c</sup>		Mechanical inoculation <sup>d</sup>	
		21 days p.i.	45 days p.i.	21 days p.i.	45 days p.i.
T2	5	2	8	0	2
T23	5	5	8	0	3
T26	4	1	4	0	0
T34	1	6	8	0	1
T37	5	4	8	0	2
T40	2	5	8	0	0
T45	1	6	6	0	0
T48	4	2	8	0	0
T65	2	0	7	0	0
T67	2	6	8	0	0
T98	1	5	8	0	1
T99	4	1	5	0	0
Wild type <sup>e</sup>	0	8	–	6	8

<sup>a</sup> See Fig. 2 for positions of individual mutations.

<sup>b</sup> Plants were inoculated and analyzed by dot-blot hybridization for PSTVd in upper non-inoculated leaves.

<sup>c</sup> 200 ng *Bam*HI cDNA fragments per plant were used for inoculation by shooting plants on the stage of two true leaves twice, once in the cotyledon leaf and once in the apical true leaf with 1.0  $\mu$ m microcarrier particles at 0.2  $\mu$ g/mg DLR (see Table 1) and 130 psi.

<sup>d</sup> 4  $\mu$ g of *Bam*HI cDNA fragments were used per plant by mechanical inoculation using Carborundum as abrasive.

<sup>e</sup> PSTVd intermediate (DI), *Bam*HI fragments; –, not performed.

Table 4  
Biolistic inoculation of tomato with native PSTVd and with monomeric linear RNA transcripts

Inoculum <sup>a</sup>	Inoculation method <sup>b</sup>	Amount of viroid RNA per plant (pg)	Number of plants infected/inoculated <sup>c</sup>	Plant height <sup>d</sup> (cm)
Monomeric RNA transcripts intermediate (DI) strain	Biolistic	$2 \times 10^3$	4/10	–
Native viroid KF 440 strain	Biolistic	$2 \times 10^4$	9/9	–
		$5 \times 10^1$	3/10	–
		$2 \times 10^2$	9/10	$32 \pm 8.6$
Native viroid KF 440 strain	Mechanical	$2 \times 10^3$	10/10	$15 \pm 5.2$
		$2 \times 10^2$	2/10	–
		$2 \times 10^3$	10/10	$29 \pm 5.9$
Control plants	Mechanical (inoculation buffer)	0	0/10	$48 \pm 4.2$

<sup>a</sup> As a source of native viroid inoculum, a 12–20% PEG fraction was used; amount of viroid RNA was estimated from gel comparisons and molecular hybridization using a HPLC-purified sample as standard; the viroid RNA consisted of about equal concentrations of linear and circular forms; self-annealed exact length monomeric PSTVd transcripts were prepared using T7 primers as described in Fig. 1.

<sup>b</sup> Plants were inoculated at the stage of two true leaves twice, once in the cotyledon leaf and once in the apical true leaf. Biolistic inoculation was performed using 1  $\mu$ m microcarrier particles at a ratio 0.4  $\mu$ g RNA/mg of gold microcarrier and 130 psi (see Section 2 for further details); for mechanical inoculation Carborundum was used as an abrasive.

<sup>c</sup> Plants were analyzed by dot-blot hybridization for PSTVd 20 days p.i. in upper non-inoculated leaves.

<sup>d</sup> Confidence intervals are given at  $\alpha = 0.05$ ; –, not measured.

pronounced pathogenic reaction than inoculation using Carborundum.

### 3.4. DNA and RNA inoculation systems for hop viroids

To generalize the validity of parameters assayed for biolistic inoculation of PSTVd cDNA and RNA, we tested the inoculation system for hop viroids HSVd and HLVd, both members of the *Pospiviroidae* family. Primers designed to produce infectious cDNA monomers covered the unique *Eco*R1 restriction site in HSVd and the *Pst*I restriction site in HLVd (Fig. 1). Corresponding cDNAs, treated with the

restriction enzymes to form sticky ends, were coated onto 1  $\mu$ m microprojectiles and inoculated at 0.2  $\mu$ g/mg DLR and 130 psi in hop leaves along the stem. Each plant was inoculated in total with 150 ng of DNA. Both viroids were detected in all inoculated plants (100% infection) 21 days p.i. by RT-PCR (Fig. 5). Leaves were collected from these plants 90 days p.i. and a 12–20% PEG extract was prepared. This extract was coated to microcarriers at 0.4  $\mu$ g RNA per mg gold particles and inoculated into healthy hop mericlones at 130 psi. Each plant was shot with about 100 pg of viroid sample. Dot-blot hybridization was performed 60 days p.i. and, as shown in Fig. 5, a 100% infection was detected for

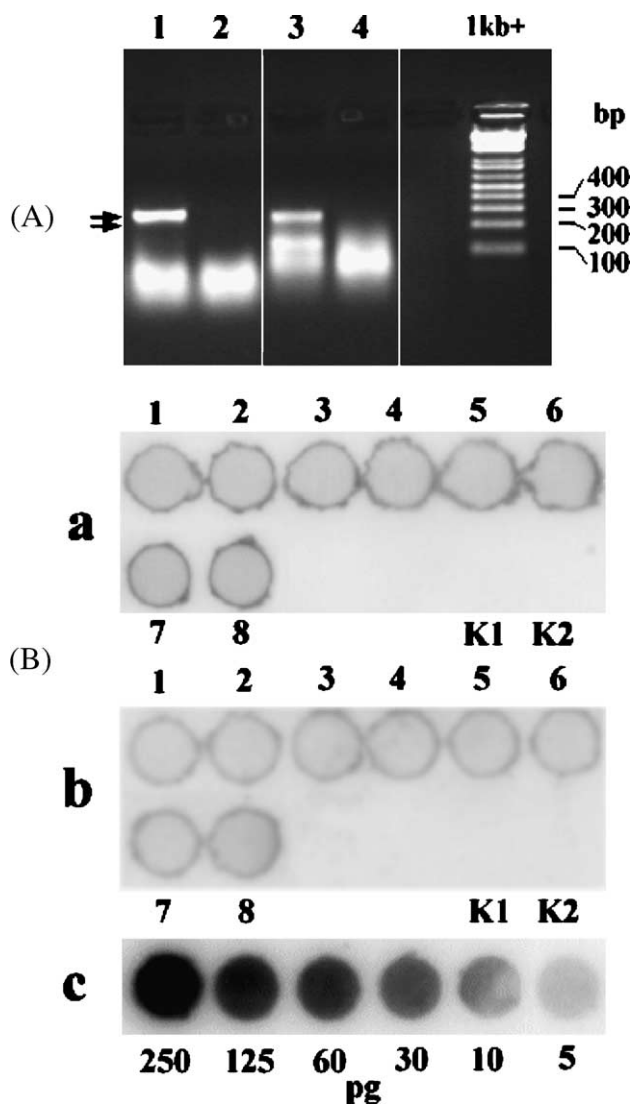


Fig. 5. Detection of hop viroids in *Humulus lupulus* plants infected by particle bombardment. (A) RT-PCR analyses of hop plants infected with HLVd and HSVd cDNA fragments using primers as shown in Fig. 1 and cleaved with *Pst*I or *Eco*R1 in the case of HLVd and HSVd cDNA, respectively. Six plants were shot with 130 psi each three times to individual leaves along the stem in total with 150 ng of viroid cDNA immobilized on 1  $\mu$ m microcarrier at 0.2  $\mu$ g/mg DLR. Samples for RNA preparation and RT-PCR were collected 25 days p.i. and analyzed using HLVdp and HSVde primers. 1, sample from HSVd-infected plants; 2, control from non-inoculated plants, HSVde primers; 3, HLVd-infected plants; 4, non-inoculated control, HLVdp primers. The arrows designate the HSVd and HLVd-specific cDNA bands in samples 1 and 3, respectively. (B) Detection of HLVd (a) and HSVd (b) in hop plants biolistically inoculated with native viroid RNA using dot-blot hybridization. RNA was extracted from plants infected by cDNA inoculation (see panel A) 90 days p.i. A 12–20% PEG RNA fraction prepared from leaves was immobilized at a concentration of 0.4  $\mu$ g RNA per mg microcarrier and used for inoculation of healthy mericlones as described in panel (A). Six plants were shot, in total with approximately 100 pg of native viroid RNA. Analysis was performed 60 days p.i. Amount of extracts per sample blotted onto a Nylon membrane corresponds to 0.4 mg fresh leaf tissue. In part (c), the hybridization signal corresponding to various amounts of purified viroid RNA is given for comparison; the specific activity of all viroid probes was  $4.4 \times 10^7$  cpm/ $\mu$ g cDNA/20 ml hybridization solution. Samples are numbered from 1 to 6; K1 and K2, non-inoculated controls.

both viroids. Plants inoculated with the HSVd variant showed strong symptoms on leaves (changes in coloration, malformations) of Czech hop Oswald's 72, while HLVd variants remained without morphological symptoms (not shown).

#### 4. Discussion

In this study, we assayed particle size, helium pressure and amounts of viroid nucleic acid per shot as variables to achieve successful inoculation of viroid cDNAs and RNAs. Particle sizes of 1.0 and 1.6  $\mu$ m at pressures in the range from 130 to 150 psi are suitable for inoculation of tomato plants with viroid nucleic acids. The optimal particle sizes are the same as described by Hämäläinen et al. (2000) for inoculation with PVA, while their optimal helium pressure was higher, about 200 psi for inoculation in potato. This difference could be mainly due to the type and morphology of plant tissue: potato leaves are thicker and rough, while tomato and young hop leaves are rather thin and soft. Correspondingly, the infection of potato "hairy roots", which have hard epidermis cells, was only achieved using 1.6  $\mu$ m microprojectiles. Low pressures, especially 50 and 80 psi, were ineffective. At a helium pressure between 130 and 150 psi we achieved a low level of leaf damage that was further reduced by preventing drying up the shot-wounded area using polyethylene bags.

With the optimized conditions for biolistic inoculations, lower limits of infection as detected 3 weeks post-inoculation were 50 ng and 200 pg per plant for efficient inoculation with monomeric cDNA fragments and native viroid RNA, respectively. Compared to previous reports (Tabler and Sängler, 1984, 1985; Hadidi et al., 2003), these amounts are approximately two orders of magnitude lower for inoculation of PSTVd cDNA fragments and approximately one order of magnitude lower for inoculation of native RNAs. The higher efficiency of the biolistic method can be explained by the delivery of high amounts of nucleic acids immobilized on the microprojectile surface to a small volume within the cell. This "dose effect" can explain the higher efficiency of inoculation of mutant cDNAs, as well as the much stronger pathogenic reaction with native RNA after the biolistic transfer. Furthermore, on the surface of microcarriers the sticky ends of inoculated fragments might be in close proximity. The higher molar concentrations should favor formation of dimers or polymers by base pairing that are ligated in the plant cell and then serve as templates for formation of processive viroid molecules. The importance of such ligation events for infectivity of restriction fragments with viroid sequence was demonstrated by Tabler and Sängler (1984). Ligation of monomeric fragments is also suggested in our system, because in preliminary experiments we were able to detect, by PCR using *Sty*I primers, PSTVd cDNA dimers in RNase-treated DNA from the shot-wounded area after biolistic inoculation with *Bam*HI fragments only a few hours after inoculation. In comparison to mechanical inoculation, a significant increase in efficiency of inoculation by the biolistic method was also observed for

several plant viruses (Gilbertson et al., 1991; Galon et al., 1995; Fakhfakh et al., 1996).

It is conceivable that the biolistic inoculation with the Helios Gene Gun system includes several mechanisms: (1) adsorption of nucleic acids on the cell surface and subsequent cell entry, the mechanism that presumably occurs in more ripped parts of the shot-wound area adjacent to intact cells; (2) RNA delivery into cells and cDNA transcription without integration to plant chromosomes, the mechanism that may occur in shot-surviving cells and finally; (3) cDNA integration into plant chromosomes that should occur after delivery of microparticles into leaf cells (for review, see Mäenpää et al., 1999). For cDNA delivery, the first and the second mechanisms are similar to transient expression, while stable cDNA integration may be similar to viroid agroinfection mechanism used for inoculation of infectious dimeric viroid constructs (Cress et al., 1983; Gardner et al., 1986; Salazar et al., 1988). Most viroids including those of the *Pospiviroidae* group replicate in nucleoli (Harders et al., 1989); it is not clear, however, that this localization may contribute to the efficiency of biolistic delivery of viroid nucleic acids.

Biolistic inoculation with viroid nucleic acids may serve as a good inoculation alternative to conventional inoculation in several respects. Using the biolistic approach, one can infect specific tissues that cannot be inoculated by conventional methods, as we demonstrated in the case of “hairy roots” culture. Because the linear viroid transcripts can be efficiently inoculated, early events of reversions or sequence adaptations can be investigated using defined shot-wound or adjacent leaf areas. As we demonstrated here, monomeric DNA and RNA sequences can be efficiently transferred, which greatly facilitates analysis of spectra of different clones or creation of artificial populations, as shown by the example of thermomutants. Using the biolistic procedure, mutant spectra can be co-precipitated and delivered at once as a mixture to individual cells. This should allow for equal and comparative starting conditions for different mutants. The ligation of different mutant cDNA monomers in the cells could form new combinations of mutations within processive transcripts and then in viroid offspring. This mechanism could even increase the biodiversity of artificial populations. A biolistic transfer of a wide pool of PSTVd sequence variants was used also in our previous study for inoculation in *Brassica* species, where a low-level population of viroid evolved from the diverse pool of mutants (Matoušek et al., 2004).

## Acknowledgements

We thank Prof. H.J. Gross (Institut für Biochemie, Biozentrum, Am Hubland, Würzburg, FRG) for his help and fruitful discussions and Dr. Rudra P. Singh (Potato Research Centre Agriculture and Agri-Food Canada, Fredericton, New Brunswick, Canada) for reading the manuscript. We thank H. Matoušková and M. Matoušková (Institute of Plant Molecular Biology AS CR, Česká Budějovice, Czech Republic) for

excellent technical assistance. Authors would like to thank Bernd Esters (Institute of Physical Biology, Heinrich-Heine-Universität Düsseldorf, Germany) for fruitful discussions and excellent technical support. This work was supported by the bilateral WTZ project between CR and FRG No. ME662 and No. CZE 02/032 entitled: “Analysis of structural and functional properties of viroid thermomutants”, by GA AS CR project AA85051014, NAZV MZe QC1183 and by GACR 521/03/0072.

## References

- Ambros, S., Hernandez, C., Flores, R., 1999. Rapid generation of genetic heterogeneity in progenies from individual cDNA clones of peach latent mosaic viroid in its natural host. *J. Gen. Virol.* 80, 2239–2252.
- Baumstark, T., Schröder, A.R.W., Riesner, D., 1997. Switch from cleavage to ligation is driven by a change from a tetraloop to loop E conformation. *EMBO J.* 16, 599–610.
- Candresse, T., Diener, T.O., Owens, R.A., 1990. The role of the viroid central conserved region in cDNA infectivity. *Virology* 175, 232–237.
- Cardarelli, M., Marriotti, D., Pomponi, M., Spano, L., Capone, I., Constantino, P., 1987. *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. *Mol. Gen. Genet.* 209, 475–480.
- Cress, D.E., Kiefer, M.C., Owens, R.A., 1983. Construction of infectious potato spindle tuber viroid cDNA clones. *Nucl. Acids Res.* 11, 6821–6835.
- Fakhfakh, H., Vilaine, F., Makni, M., Robaglia, C., 1996. Cell-free cloning and biolistic inoculation of an infectious cDNA potato virus Y. *J. Gen. Virol.* 77, 519–523.
- Galon, A., Meiri, E., Huet, H., Hua, W.J., Raccach, B., Gaba, V., 1995. Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus. *J. Gen. Virol.* 76, 3223–3227.
- Gardner, R.C., Kim, R., Owens, R.A., 1986. Potato spindle tuber viroid infections mediated by the Ti plasmid of *Agrobacterium tumefaciens*. *Plant Mol. Biol.* 6, 221–228.
- Gelvin, S.B., 2003. *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiol. Mol. Biol. Rev.* 67, 16–37.
- Gilbertson, R.L., Faria, J.C., Hanson, S.F., Morales, F.J., Ahlquist, P., Maxwell, D.P., Russell, D.R., 1991. Cloning of the complete DNA genomes of four bean-infecting geminiviruses and determining their infectivity by electric discharge particle acceleration. *Phytopathology* 81, 980–985.
- Góra-Sochacka, A., Candresse, T., Zagórski, W., 2001. Genetic variability of potato spindle tuber viroid RNA replicon. *Acta Biochim. Pol.* 48, 467–476.
- Grimsley, N., Hohn, B., Hohn, T., Walden, R.M., 1986. “Agroinfection”, an alternative route for plant virus infection by using the Ti plasmid. *Proc. Natl. Acad. Sci. U.S.A.* 83, 3282–3286.
- Hadidi, A., Flores, R., Randles, J.W., Semancik, J.S. (Eds.), 2003. *Viroids*. CSIRO Publishing, Australia.
- Hämäläinen, J.H., Kekkarainen, T., Gebhardt, C., Watanabe, K.N., Valkonen, J.P.T., 2000. Recessive and dominant genes interfere with the vascular transport of Potato virus A in diploid potatoes. *Mol. Plant Microbe In.* 13, 402–412.
- Hammond, R.W., Diener, T.O., Owens, R.A., 1989. Infectivity of chimeric viroid transcripts reveals the presence of alternative processing sites in potato spindle tuber viroid. *Virology* 170, 486–495.
- Harders, J., Lukács, N., Robert-Nicoud, M., Jovin, J.M., Riesner, D., 1989. Imaging of viroids in nuclei from tomato leaf tissue by in situ hybridization and confocal laser scanning microscopy. *EMBO J.* 8, 3941–3949.

- Hecker, R., Wang, Z., Steger, G., Riesner, D., 1988. Analysis of RNA structures by temperature-gradient gel electrophoresis: viroid replication and processing. *Gene* 72, 59–74.
- Keese, P., Symons, R.H., 1985. Domains in viroids: evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. *Proc. Natl. Acad. Sci. U.S.A.* 82, 4582–4586.
- Kekarainen, T., Savilahti, H., Valkonen, J.P.T., 2002. Functional genomics on *Potato virus A*: virus genome-wide map of sites essential for virus propagation. *Genome Res.* 12, 584–594.
- Klein, T.M., Wolf, E.D., Wu, R., Sanford, J.C., 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327, 70–73.
- Kofalvi, S.A., Marcos, J.F., Canizares, M.C., Pallas, V., Candresse, T., 1997. Hop stunt viroid (HSVd) sequence variants from *Prunus* species: evidence for recombination between HSVd isolates. *J. Gen. Virol.* 78, 3177–3186.
- Mäenpää, P., Gonzalez, E.B., Ahlandsberg, S., Jansson, C.H., 1999. Transformation of nuclear and plastomic plant genomes by biolistic particle bombardment. *Mol. Biotechnol.* 13, 67–72.
- Matoušek, J., Dědič, P., 1988. Acid nucleases in PSTV-infected tomato (*Lycopersicon esculentum* L.). I. Levels of acid nuclease activity in healthy and PSTV-infected tomato leaves and callus tissues. *J. Plant Physiol.* 133, 340–344.
- Matoušek, J., Turková, V., Dědič, P., 1988. Acid nucleases in PSTV-infected tomato (*Lycopersicon esculentum* L.). II. Characterization of sugar non-specific nuclease extracted from healthy and PSTV-infected tomato leaves. *J. Plant Physiol.* 133, 401–408.
- Matoušek, J., Schröder, A.R.W., Trněná, L., Reimers, M., Baumstark, T., Dědič, P., Vlasák, J., Becker, I., Kreuzaler, F., Fladung, M., Riesner, D., 1994. Inhibition of viroid infection by antisense RNA expression in transgenic plants. *Biol. Chem. H-S* 375, 765–777.
- Matoušek, J., Trněná, L., Svoboda, P., Oriniaková, P., Lichtenstein, C.P., 1995. The gradual reduction of viroid levels in hop mericlones following heat therapy: a possible role for a nuclease degrading dsRNA. *Biol. Chem. H-S* 376, 715–721.
- Matoušek, J., Patzak, J., Orctová, L., Schubert, J., Vrba, L., Steger, G., Riesner, D., 2001. The variability of hop latent viroid as induced upon heat treatment. *Virology* 287, 349–358.
- Matoušek, J., Orctová, L., Steger, G., Škopek, J., Moors, M., Dědič, P., Riesner, D., 2004. Analysis of thermal stress-mediated PSTVd variation and biolistic inoculation of progeny of viroid “thermomutants” to tomato and *Brassica* species. *Virology* 323, 9–23.
- Merits, A., Rajamäki, M.-L., Lindholm, P., Runeberg-Roos, P., Kekarainen, T., Puustinen, P., Mäkeläinen, K., Valkonen, J.P.T., Saarma, M., 2002. Proteolytic processing of potyviral proteins and polyprotein processing intermediates in insect and plant cells. *J. Gen. Virol.* 83, 1211–1221.
- Meshi, T., Ishikawa, M., Ohno, T., Okada, Y., Sano, T., Ueda, I., Shikata, E., 1984. Double-stranded cDNAs of hop stunt viroid are infectious. *J. Biochem.* 95, 1521–1524.
- Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497.
- Owens, R.A., Hammond, R.W., Gardner, R.C., Kiefer, M.C., Thompson, S.M., Cress, D.E., 1986. Site-specific mutagenesis of potato spindle tuber viroid cDNA – alterations within premelting region-2 that abolish infectivity. *Plant Mol. Biol.* 6, 179–192.
- Pelchat, M., Rocheleau, L., Perreault, J., Perreault, J.P., 2003. Subviral RNA: a database of the smallest known auto-replicable RNA species. *Nucl. Acids Res.* 31, 444–445.
- Puchta, H., Ramm, K., Sängler, H.L., 1988. The molecular structure of hop latent viroid (HLV), a new viroid occurring world-wide in hops. *Nucl. Acids Res.* 16, 4197–4216.
- Qiu, P., Ziegelhoffer, P., Sun, J., Yang, N.S., 1996. Gene gun delivery of mRNA in situ results in efficient transgene expression and genetic immunization. *Gene Ther.* 3, 262–268.
- Rakowski, A.G., Symons, R.H., 1994. Infectivity of linear monomeric transcripts of citrus exocortis viroid: terminal sequence requirements for processing. *Virology* 203, 328–335.
- Riesner, D., Henco, K., Rokohl, U., Klotz, G., Kleinschmidt, A.K., Domdey, H., Jank, P., Gross, H.J., Sängler, H.L., 1979. Structure and structure formation of viroids. *J. Mol. Biol.* 133, 85–115.
- Riesner, D., Steger, G., Zimmat, R., Owens, A., Wagenhöfer, M., Hillen, W., Vollbach, S., Henco, K., 1989. Temperature-gradient gel electrophoresis of nucleic acids: analysis of conformational transitions, sequence variations, and protein–nucleic acid interactions. *Electrophoresis* 10, 377–389.
- Rigden, J.E., Rezaian, M.A., 1992. In vitro synthesis of an infectious viroid: analysis of the infectivity of monomeric linear CEV. *Virology* 186, 201–206.
- Rigden, J.E., Rezaian, M.A., 1993. Analysis of sequence variation in grapevine yellow speckle viroid I reveals two distinct alternative structures for the pathogenic domain. *Virology* 193, 474–477.
- Salazar, L.F., Hammond, R.W., Diener, T.O., Owens, R.A., 1988. Analysis of viroid replication following *Agrobacterium*-mediated inoculation of non-host species with potato spindle tuber viroid cDNA. *J. Gen. Virol.* 69, 879–889.
- Schnölzer, M., Haas, B., Ramm, K., Hofmann, H., Sängler, H.L., 1985. Correlation between structure and pathogenicity of potato spindle tuber viroid. *EMBO J.* 4, 2181–2190.
- Schrader, O., Baumstark, T., Riesner, D., 2003. A mini-RNA containing the tetraloop, wobble-pair and loop E motifs of the central conserved region of potato spindle tuber viroid is processed into a minicircle. *Nucl. Acids Res.* 31, 988–998.
- Schumacher, J., Meyer, N., Riesner, D., Weidemann, H.L., 1986. Diagnostic procedure for detection of viroids and viruses with circular RNAs by “return”-gel electrophoresis. *J. Phytopathol.* 115, 332–343.
- Tabler, M., Sängler, H.L., 1984. Cloned single- and double-stranded DNA copies of potato spindle tuber viroid (PSTV) RNA and co-inoculated subgenomic DNA fragments are infectious. *EMBO J.* 3, 3055–3062.
- Tabler, M., Sängler, H.L., 1985. Infectivity studies on different potato spindle tuber viroid (PSTV) RNAs synthesized in vitro with the SP6 transcription system. *EMBO J.* 4, 2191–2199.
- Visvader, J.E., Forster, A.C., Symons, R.H., 1985. Infectivity and in vitro mutagenesis of monomeric cDNA clones of citrus exocortis viroid indicates the site of processing of viroid precursors. *Nucl. Acids Res.* 13, 5843–5856.
- Visvader, J.E., Symons, R.H., 1985. Eleven new sequence variants of citrus exocortis viroid and the correlation of sequence with pathogenicity. *Nucl. Acids Res.* 13, 2907–2920.