Virology 287, 349–358 (2001)
doi:10.1006/viro.2001.1044, available online at http://www.idealibrary.com on IDEAL®

metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connect

The Variability of Hop Latent Viroid as Induced upon Heat Treatment

Jaroslav Matoušek,* Josef Patzak,† Lidmila Orctová,* Jörg Schubert,‡ Lukáš Vrba,*
Gerhard Steger,§ and Detlev Riesner§[1]

*Department of Molecular Genetics, Institute of Plant Molecular Biology Czech Academy of Sciences, Branišovská 31, 37005 České Budějovice, Czech Republic; †Department of Virology, Institute of Hop Research and Breeding, Kadaňská 2525, 438 46 Žatec, Czech Republic; ‡Federal Centre for Breeding Research, Institute for Resistance Research and Pathogen Diagnostics, Theodor-Roemer-Weg 4, 06449 Aschersleben, Germany; and §Institute of Physical Biology, Heinrich-Heine Universität Düsseldorf, Universitätsstraße 1, D-40225 Düsseldorf, Germany

Received March 28, 2001; returned to author for revision March 30, 2001; accepted June 11, 2001; published online August 2, 2001

We have previously shown that heat treatment of hop plants infected by hop latent viroid (HLVd) reduces viroid levels. Here we investigate whether such heat treatment leads to the accumulation of sequence variability in HLVd. We observed a negligible level of mutated variants in HLVd under standard cultivation conditions. In contrast, the heat treatment of hop led to HLVd degradation and, simultaneously, to a significant increase in sequence variations, as judged from temperature gradient–gel electrophoresis analysis and cDNA library screening by DNA heteroduplex analysis. Thirty-one cDNA clones (9.8%) were identified as deviating forms. Sequencing showed mostly the presence of quadruple and triple mutants, suggesting an accumulation of mutations in HLVd during successive replication cycles. Sixty-nine percent of base changes were localised in the left half and 31% in the right half of the secondary structure proposed for this viroid. No mutations were found in the central part of the upper conserved region. A "hot spot" region was identified in a domain known as a "pathogenicity domain" in the group representative, potato spindle tuber viroid. Most mutations are predicted to destabilise HLVd secondary structure. All mutated cDNAs, however, were infectious and evolved into complex progeny populations containing molecular variants maintained at low levels. © 2001 Academic Press

Key Words: HLVd; H. lupulus; thermotreatment; TGGE; DNA heteroduplexes; viroid mutagenesis.

INTRODUCTION

Hop latent viroid (HLVd) is a single-stranded, circular infectious RNA of 256 nt (Puchta *et al.,* 1988). Similar to other viroid species, HLVd completely depends on the metabolism of the host plant: its replication and pathogenesis are mediated by its structural features and its capacity to interact with cellular factors (for a recent review see Diener, 1999). HLVd occurs worldwide in hop (*Humulus lupulus* L.) and is deleterious to production in certain hop cultivars. Quantitative changes of some secondary metabolites in the secretory glands forming lupulin are induced by HLVd infection and in addition the cones of viroid-infected hops are smaller than those from viroid-free plants (Barbara *et al.,* 1990; Adams *et al.,* 1991, 1992). Thus, despite the absence of characteristic morphological symptoms in somatic tissues, the specific biochemical changes of lupulin are in clear contrast to the name of this viroid, which was originally designated "latent." HLVd thus appears of agricultural significance.

The HLVd sequence was first reported by Puchta *et al.* (1988), but no sequence variants of HLVd have so far been characterised. However, many observations suggest that viroids form complex populations of natural sequence variants called "quasispecies." Such variants have been detected, for example, for citrus exocortis viroid (Visvader and Symons, 1985), citrus viroid III (Owens *et al.,* 1999, 2000), avocado sun blotch viroid (Rakowski and Symons, 1989), grapevine viroids (Rigden and Rezaian, 1993; Polívka *et al.,* 1996), peach latent mosaic viroid (Ambros *et al.,* 1998, 1999), potato spindle tuber viroid (Gruner *et al.,* 1995; Góra-Sochacka, 1997), and hop stunt viroid (Kofalvi *et al.,* 1997; Sano *et al.,* 2000).

Previously, we partly characterised the horizontal (Matoušek *et al.,* 1994) and vertical (Matoušek *et al.,* 1995) distribution of HLVd in Czech hop clones Osvald 31 and 72, which we found to be nearly entirely infected with this viroid. We evaluated heat treatment as a possible method to eradicate this pathogen and found that such treatment of hop clones derived from meristematic tissue (mericlones) reduced viroid levels significantly (Matoušek *et al.,* 1995). However, we also found that the infection is usually restored to the original level during a long cultivation period under standard conditions. The rapid decrease of viroid levels following heat treatment is correlated with the induction of a nucleolytic complex, which cleaves viroid RNA (Matoušek *et al.,* 1995), but HLVd infection in heat-treated mericlones has not been investigated further. During the posttreatment period, when infection persists at low viroid level, suboptimal

[1] To whom reprint requests should be addressed. Fax: +(0211) 811 5167. E-mail: riesner@biophys.uni-duesseldorf.de.

0042-6822/01 $35.00
Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

mutated HLVd forms may accumulate having lower fitness and adaptability to the host metabolism. Although the influence of heat-stress conditions on viroid variability has not been described, accidental heat-shock conditions in the greenhouse may be responsible for the unexpected appearance of a highly pathogenic PSTVd strain described by Gruner *et al.* (1995).

Here we report that mutated HLVd accumulates in heat-treated hop plants and find that heat stress leads to destabilisation of regular molecular processes maintaining an optimally adapted HLVd population in hop and to the significant mutability of HLVd.

RESULTS

HLVd instability and sequence variability following the heat treatment of hop

We have previously shown that the cultivation of HLVd-infected hop at temperatures above the standard cultivation temperature of 25 to 36°C for 5 days (the heat-treatment cycle) allows plants to survive without significant morphological damage and, at the same time, the level of viroid is decreased dramatically (Matoušek *et al.,* 1995). After the subsequent application of four such heat-treatment cycles, the content of viroid decreases gradually from approximately 33 to 2 pg/mg of fresh leaf tissue and some plants appear viroid-free. However, as a rule, the viroid level eventually recovers to the original level after 6 months or longer of no further treatment. We proposed that a nucleolytic complex is activated by the thermal stress and leads to viroid degradation (Matoušek *et al.,* 1995). To check whether the long posttreatment recovery of viroid level is due to sequence changes in the surviving molecules, we analysed the sequence variation of HLVd after the heat treatment and during subsequent recovery of HLVd levels.

As a control and for reference reasons, HLVd variability was first assessed in an untreated mericlone of Czech hop Osvald's 31. First, a cDNA library was established and 10 clones were sequenced. We detected no HLVd sequence variation in this control hop and all cDNAs carried the sequence described originally by Puchta *et al.* (1988). We next used temperature gradient gel electrophoresis (TGGE) to look for sequence variants. In primary autoradiograms of TGGE patterns (not shown), we detected about 1.2% of the radioactivity signal from cDNAs having lower thermostability than the wild-type cDNA, suggesting the presence of a minor fraction of mutated HLVd sequence(s). This cDNA of lower thermostability was reextracted from a silver-stained gel, reamplified by high-fidelity PCR, and cloned in a PCR-Script vector. The sequencing of 16 independent HLVd clones revealed two 255-nt variants of HLVd. One variant had a deletion of one G from a run of four G nucleotides (positions 3–6) in the left terminal (T1) domain; the other had a deletion of a G at position 83 or 84. Both deletions

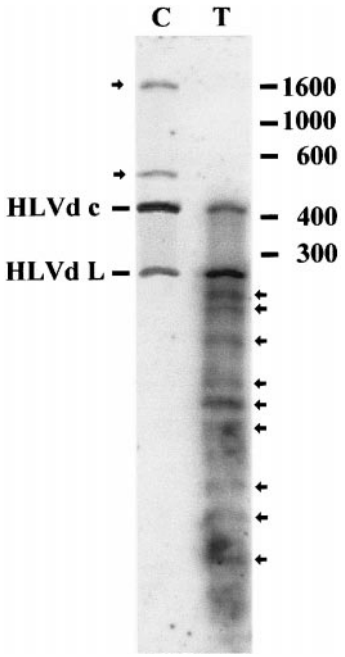

FIG. 1. The electrophoretic analysis of HLVd RNA from control and heat-treated hop. Total RNA was isolated using TriZOL reagent from either control hop mericlones grown *in vitro* (C) or from *in vitro* mericlones collected immediately after the second cycle of heat treatment (T), as described under Materials and Methods. A 2-M LiCl-soluble RNA fraction was analysed in 5% denaturing acrylamide gel. RNA was transblotted onto a nylon membrane and hybridised to a HLVd-specific riboprobe. HLVd c, circular form of HLVd; HLVd L, linear HLVd form. The arrows on the left side indicate (+)-oligomeric HLVd forms. The arrows on the right side designate the shorter than unit length viroid degradation products. The positions of Dig RNA III markers are indicated by numbers.

should indeed destabilise the predicted secondary structure of HLVd. In summary, only 1.2% of the cDNAs were detected in that part of TGGE where mutants were expected, and only two from 16 clones, i.e., 10 to 20%, from this region were identified as variants and the other 14 as wild-type. It follows that less than 0.2% of all HLVd molecules were variants. Thus, HLVd shows a high genetic stability in infected Osvald's hop under standard cultivation conditions.

Viroid destabilisation was initiated by the application of two consecutive heat-treatment cycles interrupted by a 1-day cultivation at 20°C. In heat-treated plants immediately after the heat-treatment cycles, viroid levels dropped to about 20% compared to controls. Electrophoretic analysis of viroid RNAs on a denaturing gel revealed the accumulation of shorter than unit-length RNA species hybridising to a HLVd riboprobe in the heat-treated sample (Fig. 1). Moreover, the (+)-oligomeric RNA species, which are detectable in HLVd-infected control (non heat-treated) are not present after the heat treatment. These results suggest the degradation of viroid RNA upon heat-treatment conditions. The bands with lower electrophoretic mobility correspond to the

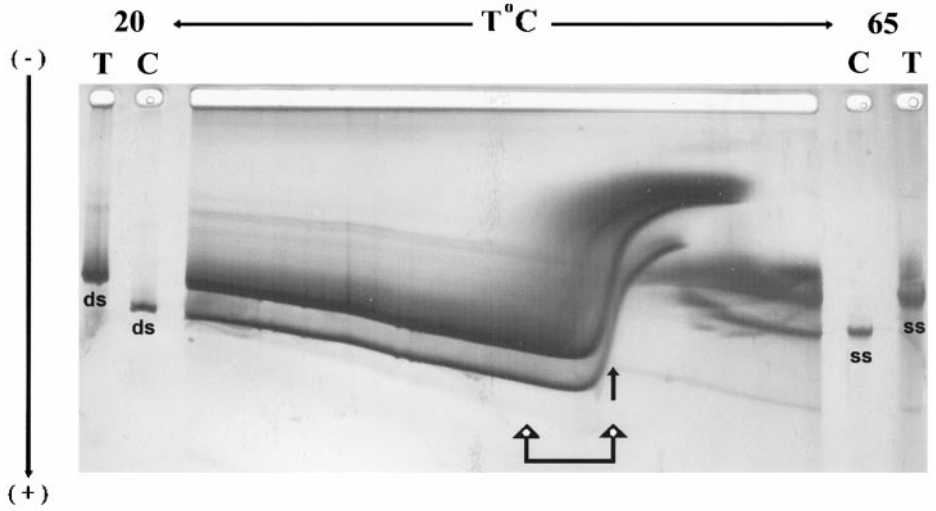

FIG. 2. Analysis of HLVd cDNA on TGGE. cDNA from control plants (C) was loaded as the first sample. After 15 min of electrophoresis at 10°C, cDNA from heat-treated hops (T) was loaded as the second sample. Then the temperature gradient 20–65°C was switched on and the electrophoresis continued until the tracking dye reached the bottom edge of the gel. "ds" and "ss" designate double- and single-stranded parts of DNA patterns, respectively. The melting temperature of the major cDNA form is indicated by a filled arrow. Additional cDNA species having melting temperatures in the range of 42–48°C are indicated by the open white arrows; these cDNA species are present only in HLVd cDNA from heat-treated hop. The gel was stained for nucleic acids with $AgNO_3$.

oligomeric forms of HLVd (Fig. 1C) and not to hybridisation artifacts because they were not detected in RNA extracts from healthy plants.

To examine the effect of temperature on viroid variability, RNA was extracted from heat-treated samples and HLVd cDNAs were synthesised using a high-fidelity RT-PCR system. Analysis of viroid cDNAs from heat-treated and control plants on TGGE gels revealed a significant accumulation of cDNAs exhibiting lower thermostability in the heat-treated material. While a predominant HLVd cDNA form having a single melting temperature of about 48°C was seen in both the heat-treated and the control sample, a significant portion of cDNAs having melting temperatures between 42 and 48°C was observed in the sample from heat-treated plants (Fig. 2), suggesting an accumulation of mutants in this sample. Quantification of the radioactivity signal on autoradiograms of TGGE patterns showed that >54% of the cDNAs from heat-treated plants differed from the control (not shown).

To characterise the mutants, a HLVd-cDNA library was established and screened using the DNA heteroduplex method (Fig. 3). From this cDNA library, 31 clones (9.8%) were identified as forming heteroduplexes with the wild-type cDNA. Nine clones were sequenced and individual mutations identified (Fig. 4). The sequence analysis revealed 23 base changes and one deletion. One single mutated clone, two double mutants, and three clones having three and four point mutations each, were observed. The quadruple mutant T15 and the triple mutant T61 shared two identical base changes A → G and U → C in positions 7 and 229, respectively, and the double mutant T92 shared a C → U transition in position 157

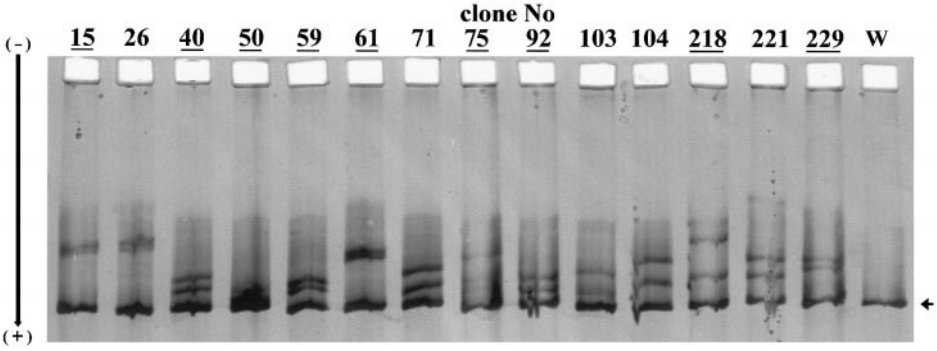

FIG. 3. Analysis of HLVd cDNA library by the method of preformed DNA heteroduplexes. cDNA fragments were prepared from individual clones (numbered) using PCR, hybridised to the wild-type HLVd cDNA, and electrophoresed at a constant temperature of 42°C in 6% polyacrylamide gel containing 7 M urea. The gel was stained for nucleic acids with $AgNO_3$. The position of the wild-type homoduplex (sample W) is indicated by the arrow. Underlined clones were selected for further experiments.

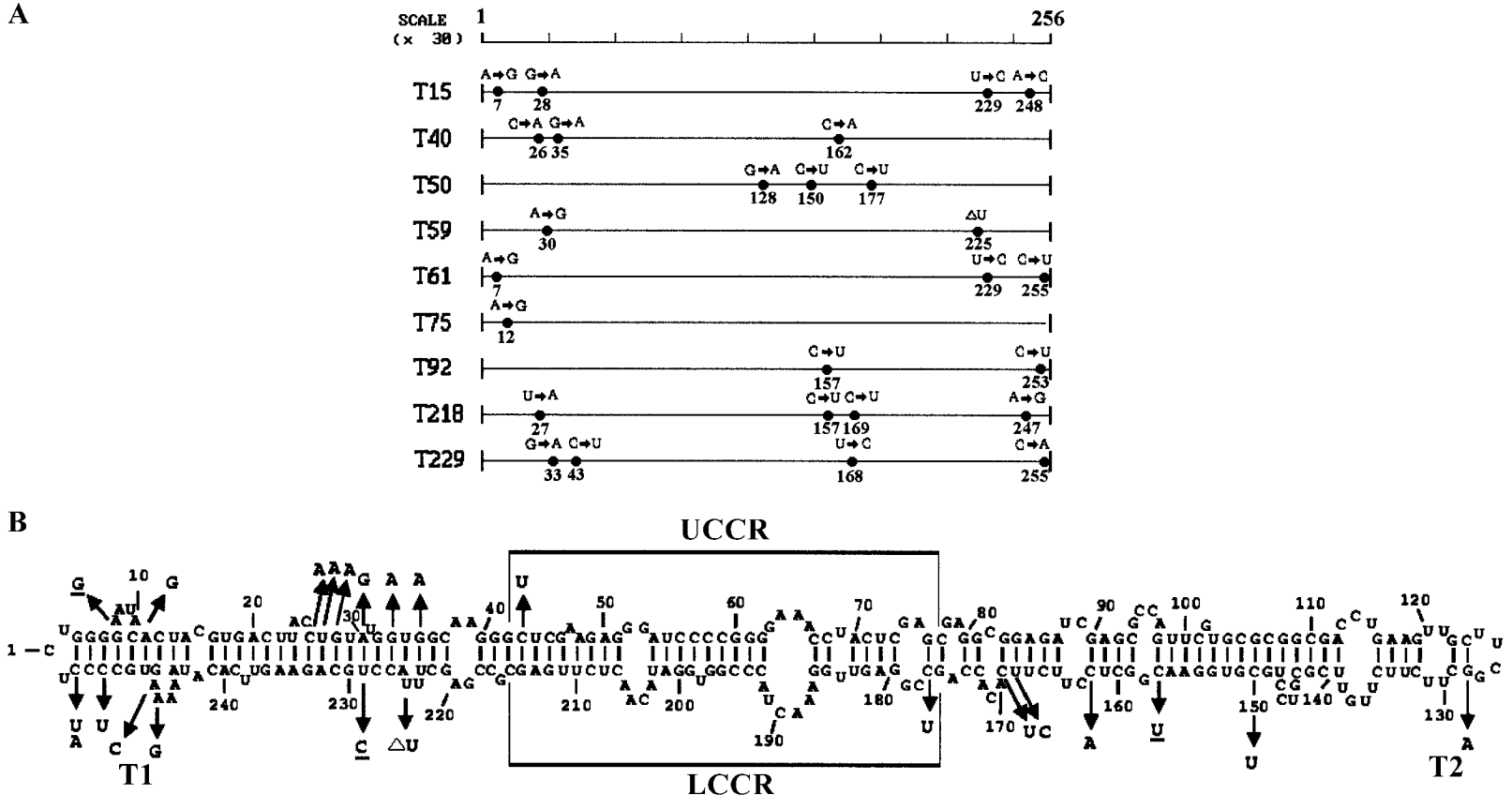

FIG. 4. Nucleotide changes in HLVd variants isolated from heat-treated hop (A) and localisation of individual mutations in the secondary structure of HLVd (B). The secondary structure of HLVd originally described by Puchta *et al.* (1988) was recalculated by the algorithm described by Lück *et al.* (1999) and is approximated to physiological conditions. Individual positions of base changes found within HLVd are indicated by the arrows. Base changes which occurred twice are underlined. UCCR, upper part of the central conserved region; LCCR, lower part of the central conserved region; T1 and T2, left and right terminal domains. Viroid domains were designated as suggested by Keese and Symons (1985).

with mutant T218. This finding, together with the observation of multiple point mutations in individual cDNAs, strongly suggests that the mutagenesis occurred during successive cycles of viroid replication.

The mutations observed were not randomly distributed within the genome of HLVd (Fig. 4). More than twice as many point mutations accumulated in the left half of the viroid secondary structure than in the right half. With one single exception, a C → U base change in the left boundary of UCCR in position 43 of clone 229, no mutation was observed in the upper central conserved region (UCCR). A "hot spot" was identified between positions 26 and 35, where base changes destabilising the secondary structure and adenine residues accumulated predominantly. Interestingly, an oligo(A) stretch, forming part of the "pathogenicity domain," is localised in a similar region in PSTVd, the representative member of the family to which HLVd belongs.

Infectivity of mutated HLVd variants and viroid titers

From the analyses performed it can be concluded that the heat treatment led not only to viroid elimination by RNA degradation but also to significant genetic instability of HLVd. Most of the observed mutations are in principle destabilising to the secondary viroid structure predicted for the optimal hop cultivation temperature (25°C) (Fig. 4) and could be deleterious to viroid fitness. Yet, HLVd "thermomutants" should be viable, as most mutations had to accumulate during the successive replication cycles. To assay the viability of individual sequence variants, infection tests were performed with restriction cDNA fragments. For this purpose noninfectious cDNAs, which were originally amplified using primers covering the lower central conserved region (LCCR), were converted to infectious ones using primers specific for the upper central conserved region (UCCR), as described under Materials and Methods, and used to inoculate healthy plants of Osvald's hop growing in the clima box conditions. Viroid titers were assayed using dot-blot hybridisation at different intervals postinoculation and at various cultivation conditions (Fig. 5). In addition, sequence variation was monitored in some of these clones by analysis of DNA heteroduplexes (Fig. 6). Except for clone T15, HLVd infection was easily detectable in all plants and all clones by molecular hybridisation 1 month after inoculation. The detection of HLVd in plants infected with T15 cDNA, less than 0.1 pg/mg of fresh mass, was close to the detection limit. In general, viroid titers were much lower in plants infected with thermomutants and reached a maximum of 34% of the control level in clone T229, 30 days after inoculation. The highest viroid titer, 57% of the control level, was observed for clone T75 120 days after inoculation. Because lower viroid titers were observed for thermomutants than for the HLVd control, a lower fitness of the former can be assumed.

The level of viroid also remained low after the postdormancy period (240 days after inoculation), when the stocks of the hop mericlones were stored cold for 1 month, and then the growth was reinitiated in the greenhouse. There was a differential response of individual clones to different physiological conditions (Fig. 5). The most surprising was the significant increase of viroid levels in clone T15 (240 days after inoculation), for which very low levels were detected before the dormancy, while the postdormancy accumulation titers for clones T61 and T75 were significantly lower than before the dormancy. The major effect was observed after the transfer of hop mericlones to *in vitro* conditions (315 days after inoculation) (Fig. 5). Viroid titers derived from all mutated cDNAs remained very low, about 2 pg/mg of fresh mass, while the wild-type HLVd accumulated up to 33 pg/mg of tissue as expected from our previous experiments.

Sequence distributions in the progeny of mutated HLVd clones

Individual infected plants showing the highest hybridisation signal were selected from clones T50, T61, T75, and T92, 30 days after inoculation and analysis of heteroduplexes were performed to check for viroid stability. Surprisingly, a significant heterogeneity of viroid cDNA was observed for all mutated clones, while only minor fractions of heteroduplexes were detected in plants infected with the wild-type viroid (Fig. 6). Screening of libraries containing the sequence variants resulting from the cDNA inoculations revealed some hot spots in positions 7, 26, 35, 150, 162, 229, and 255; here different mutations appeared repeatedly and independently in the offspring of different clones (results not shown). These results suggest that the progeny from mutated cDNAs form variable populations containing molecular variants maintained at low levels.

Because some specific changes in the complex viroid populations initiated by the individual cDNA clones could be expected during different experimental conditions, we investigated this possibility in detail for clone T75. Because this cDNA contained a single base change A → G in position 12 (Fig. 4), there should be a high probability for reversion to the wild-type. Although this mutation can be considered in thermodynamic terms as neutral or almost neutral, because it changes a A:U base pair into a G:U in the T1 domain of HLVd without disrupting the secondary structure (Fig. 4), inoculation of such a cDNA into hop led to the accumulation of additional sequence variants (Fig. 6). The single mutation reverted to the original but additional mutations accumulated and no complete wild-type sequence was identified in the T75 progeny from the sample collected 30 days after inoculation. To identify specific changes occurring throughout the cultivation period, a second T75 cDNA library was established from *in vitro* grown mericlones (315 days

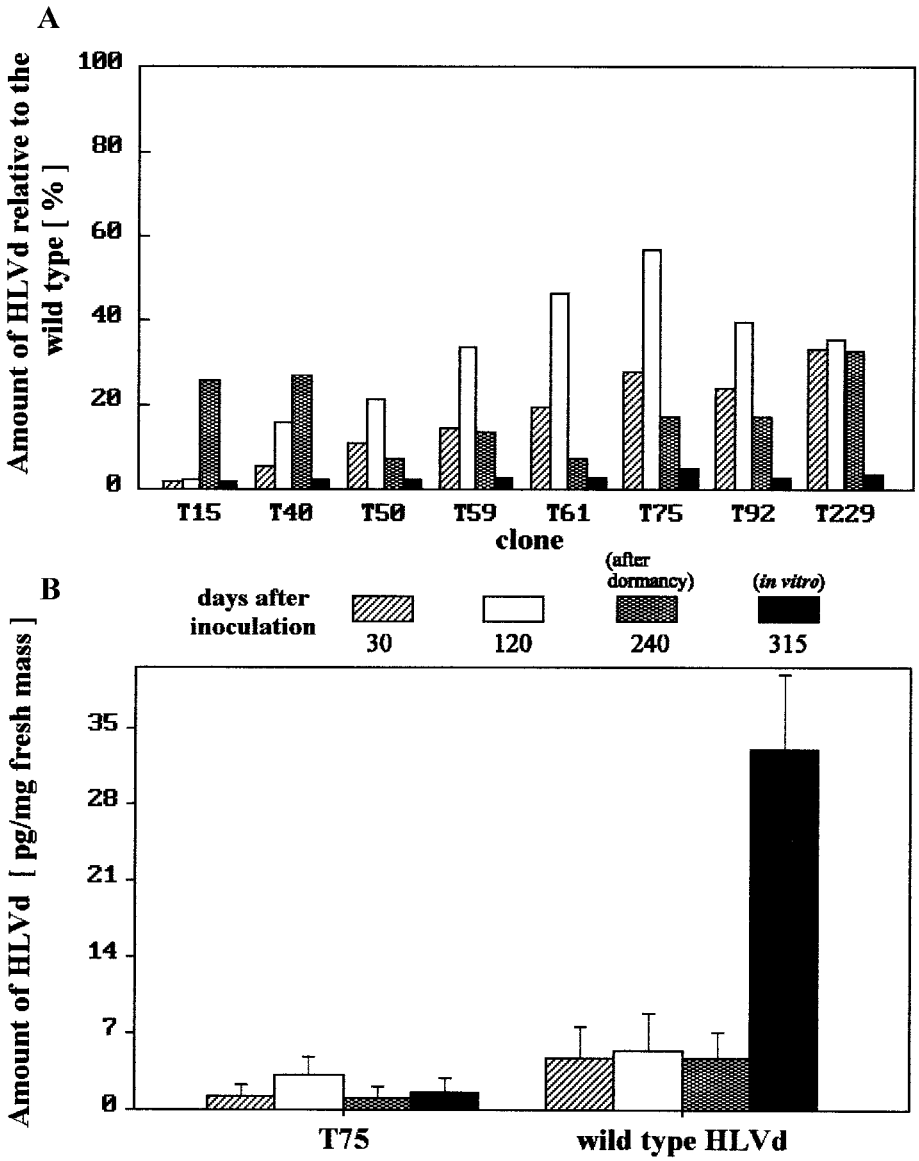

FIG. 5. Levels of HLVd in cDNA inoculated plants. Hop plants were inoculated either with individual mutated cDNAs or with the wild-type HLVd cDNA and analysed for HLVd content at different time intervals as described under Materials and Methods. In A, the mean levels of HLVd are given in percentage relative to the wild-type HLVd (100%). In B, the amount of viroid in plants inoculated with either T75 or wild-type HLVd are shown. Confidence intervals, at 5% level, are designated.

after inoculation). Comparison of cDNAs prepared 30 days after inoculation with those from the *in vitro* sample (315 days after inoculation) revealed a much narrower spectrum of heteroduplexes in the second case. This spectrum was essentially formed by one dominant and several minor bands retarded in the gel (Fig. 7). Sequencing identified the dominant variant within as having a single base change G → A in position 179, i.e., in the right boundary of LCCR. These results clearly show that this dominant HLVd variant bearing a single mutation evolved from the complex population during the cultivation period. Although the mutation is localised in a minor loop (Fig. 4) and should be neutral to the HLVd secondary structure, this viroid variant did not reach the titer of the wild-type, and it is stably maintained at a low level in *in vitro* cultivated hop. Some changes in the spectra of heteroduplexes during cultivation were also detected for other cDNA clones (not shown), although these variants have not been sequenced. The presence of sequence variants maintained at low levels in infected hop is in accordance with the long recovery period of HLVd after the heat treatment (Matoušek *et al.,* 1995).

DISCUSSION

In our experiments described here and in a recent publication (Matoušek *et al.,* 1995), we analysed the sequence distributions of HLVd and the replicability of

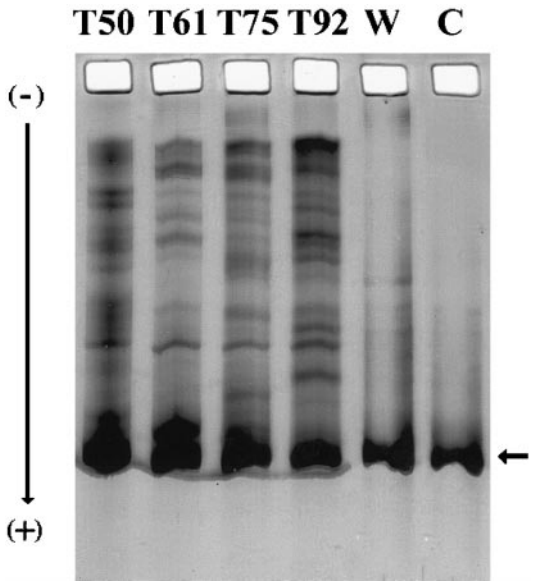

FIG. 6. Analysis of HLVd molecular variants by DNA heteroduplexes. Infectious *Bam*HI DNA fragments from individual mutated HLVd cDNA clones designated as in Fig. 4 and from the wild-type HLVd (W) were inoculated into hop Oswald's 72 as described under Materials and Methods. RT-PCR was performed from samples collected 30 days after inoculation. HLVd cDNAs were reamplified using Pwo polymerase and hybridised without adding the wild-type standard cDNA and resulting heteroduplexes were analysed at 42°C in 6% acrylamide gel containing 7 M urea. In contrast to the experiment of Fig. 3 the wild-type cDNA was not added to avoid a major influence of the wild-type sequence on the heteroduplex pattern. The gel was stained for nucleic acids with $AgNO_3$. C-position of cloned wild-type cDNA homoduplex is indicated by the arrow.

different variants under various *in vivo* conditions. The sequence distributions were shown by TGGE, and individual variants were sequenced after RT-PCR. During the PCR amplification steps, special care was taken to minimise the introduction of artificial variability.

In Osvald's hop, under optimal cultivation conditions, HLVd represents a genetically stable RNA sequence. According to TGGE analysis and sequencing, more than 98% of the HLVd molecules have a sequence identical to that originally described by Puchta *et al.* (1988). We conclude that the HLVd wild-type sequence is optimally adapted to its host and has a high fitness for replication. The few mutants have a relatively low fitness and are overgrown by the wild-type. HLVd sequence stability is also evident in other hop varieties (Hayata *et al.,* 1992). Note, however, that the titer of the HLVd wild-type is relatively low in comparison to PSTVd.

Thermal stress led to destabilisation of regular molecular processes maintaining the optimally adapted HLVd population in hop under normal conditions: after heat treatment the viroid titer is lowered by enhanced degradation, and new sequence variants emerge. The latter is most probably a consequence of a lowered fidelity of replication by the RNA polymerase II complex of the host (Schindler and Mühlbach, 1992) at the elevated temperature. After the heat treatment, a fraction of wild-type HLVd remains in hop mericlones that finally out-competed the mutated variants restoring the original viroid titer.

Although we did not follow the possible accumulation of mutations in the LCCR, which was covered by RT-PCR primers, it is clear from our experiments that mutations accumulate nonrandomly in HLVd with a tendency to destabilise the secondary structure of the wild-type. Most mutations were observed in the left part of the viroid secondary structure and T1 domain, and essentially no mutations were found in the central part of the UCCR and in the upper strand of the right part of HLVd structure. This nonrandom distribution is presumably due to biological constraints similar to those limiting the heterogeneity of peach latent mosaic viroid (PLMVd) populations (Ambros *et al.,* 1998).

Our results on the sequence distribution of HLVd after heat treatment are in certain respects similar but in others different to those obtained after an accidental heat shock of PSTVd-infected tomato plants (Gruner *et al.,* 1995). There, the high-temperature conditions led also to enhanced mutability of PSTVd, but after several rounds of selection a highly pathogenic, lethal strain RG1 was generated that accumulated to high levels in plants; i.e., PSTVd RG1 was able to out-compete the wild-type PSTVd, whereas the HLVd mutants have a lower fitness than the wild-type HLVd. PSTVd RG1 is a triple mutant having lower thermodynamic stability than the original intermediate DI strain used for plant inoculation. Additional PSTVd sequence variants, QF A and QF B, represented by double and triple mutants, respectively, were identified in the same population. All mutations found in

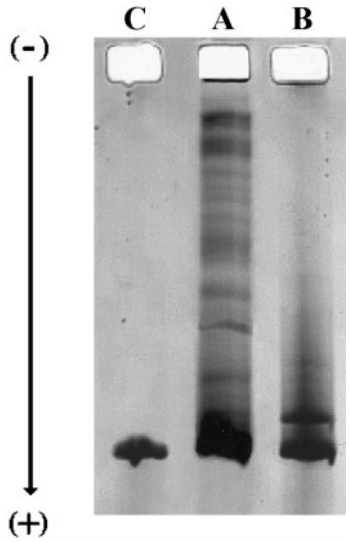

FIG. 7. Analysis of HLVd T75 population by the method of DNA heteroduplexes. RNA was extracted from plants infected with T75 cDNA either 30 days (A) or 315 days (B) after inoculation and cDNA was analysed similarly as described in Fig. 6. In slot C the position of wild-type cDNA homoduplex is shown.

these PSTVd variants mapped to the pathogenicity domain, located in the secondary structure at a position similar to that where the mutational hot spot was found here in HLVd. In both systems, HLVd and PSTVd, the mutants exhibited lower thermal stability than the wild-types. More stable mutants might have appeared at high temperature but would not be viable in cultivation conditions after heat shock.

No wild-type HLVd competitor was present in plants inoculated by particular cDNA clones obtained from mutants after heat treatment, and these mutants have in common a low accumulation titer. They induced, upon transferring to standard conditions, a broad sequence distribution. We interpret this result as a consequence of the low ability to propagate the original as well as the progeny mutants: their replication fitness is so low that selection of a dominant form is not achieved by overgrowing or out-competition but simply by dying out of the most unfavourable variants. This is a clear difference from the evolutionary behaviour of PSTVd after heat treatment. One might speculate that this difference is connected to pathogenicity. A viroid selection process related to pathogenesis has been also observed for grapevine yellow speckle viroid-1 (GYSVd-1) (Szychowski *et al.,* 1998), whereas for progeny variants of PLMVd (Ambros *et al.,* 1998), it has been shown that a variant inducing a reproducible symptomatic infection gave rise to a more uniform progeny; variants inducing variable and latent phenotypes generated more variable populations. The appearance of different viroid variants after inoculation is consistent with previous experiments using different viroid mutants or sequence variants for plant inoculation (e.g., Qu *et al.*, 1993; Góra-Sochacka *et al.,* 1997; Ambros *et al.,* 1998) and shows that viroids are propagated in their hosts as complex populations known as quasispecies (Visvader and Symons, 1985; Rakowski and Symons, 1989; Rigden and Rezaian, 1993; Polívka *et al.,* 1996; Góra-Sochacka, 1997; Kofalvi *et al.,* 1997; Ambros *et al.,* 1998, 1999; Owens *et al.,* 1999, 2000; Sano *et al.,* 2000).

We found in our experiments that different viroid populations responded differentially to physiological conditions during hop cultivation. This is also in good accordance with the expectations for the quasispecies model, although we are far from understanding which host factors and which particular structural features of HLVd are involved in this regulation. For one clone (T75), we identified a dominant sequence, which evolved from its progeny and is stably maintained at a low level in infected hop grown *in vitro*. The transition G → A in position 179 seems to be neutral to the secondary structure characteristic for (+)-viroid progeny. Although this mutation is localised within a small loop positioned on the right boundary of the LCCR, it is not included in the region of HLVd homologous to the PSTVd processing site (Baumstark and Riesner, 1995; Baumstark *et al.,* 1997). However, this base change could be important for some interaction of the tetraloop structure containing the processing site or for other transient viroid structures interacting with cellular components involved in processing and/or replication.

MATERIALS AND METHODS

Hop mericlones, heat treatment, and plant cultivation conditions

Hop mericlones of clone Osvald 31 were used as a source of HLVd. If not otherwise stated, these plants were cultivated *in vitro* and subjected to heat treatment as described earlier (Matoušek *et al.,* 1995). For the cDNA inoculation experiment, healthy hop mericlones of Osvald's clone 72 were used. These plants were transferred in soil and maintained under clima box conditions for approximately 3 weeks before inoculation. Temperature conditions in the clima box were maintained at 25 ± 3°C. Plants were grown under natural light with supplementary illumination [90 μmol m^{-2} s^{-1} PAR] to keep 16-h-day periods from September 1999 to March 2000. Then the stems were cut off and the stocks were transferred to a cold room and kept at 10°C for 1 month (dormancy period) before placing them in greenhouse conditions, where new shoots developed. The plants were kept in the greenhouse for 3 months until the last collection of samples was performed. Then the stems and buds from individual plants were sterilised and maintained further in *in vitro* conditions on MS medium supplemented with vitamins and 40 g/l glucose. *In vitro* plants were supplied with light (16 h) with an intensity of 70 μmol m^{-2} s^{-1} PAR. Day/night temperature was 25/18°C.

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

For the reverse transcription-polymerase chain reaction (RT-PCR), total RNA was isolated from 100 mg of leaf tissue or from *in vitro* plants of individual mericlones by RNeasy Plant Total RNA kit (Qiagen). For the analysis of viroid-specific degradation products, total RNA was isolated using TriZOL reagent (Life Technologies), essentially according to the manufacturer's recommendations. RNA soluble in 2 M LiCl was analysed.

RT-PCR reactions were performed using Titan One Tube RT-PCR system including a high-fidelity Pwo polymerase (Roche Molecular Biochemicals) in 50 μl reaction mixtures with RT primer ($5'_{200}$CCACCGGGTAGTTTCCAACT$_{181}3'$) and PCR primer ($5'_{201}$ATACAACTCTTGAGCGCCGA$_{220}3'$), as described by Matoušek and Patzak (2000). Reverse transcription was run for 30 min at 50°C. After 2 min denaturation at 94°C, the polymerase chain reaction started 30 s at 94°C, 30 s at 58°C, and 60 s at 68°C for 35 cycles. Pwo polymerase (Angewandte Gentechnologie Systeme GmbH, Germany) was used for

reamplification of viroid cDNAs. In a typical experiment 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 purified by Qiagen Gel Extraction Kit (Qiagen) and cloned in the vector pCR-Script SK(+) (pCR-Script Cloning Kit, Stratagene). Automatic sequencing was performed with an ALF II system (Amersham-Pharmacia) using a sequencing kit with Cy5-labeled standard primers (Thermosequenase Dye Termination Kit, Amersham-Pharmacia).

cDNA and RNA analysis in polyacrylamide gels

Temperature-gradient gel-electrophoresis was performed in 6% polyacrylamide gels (Riesner *et al.,* 1989) (140 × 140 × 1.8 mm) containing 19:1 acrylamide:bisacrylamide (w/w), 17.8 mM Tris, 17.8 mM boric acid, 0.048 mM EDTA, (0.2× TBE), pH 8.3, 0.1% TEMED, 7 M urea, 2% glycerol, and 0.06% ammonium persulfate. Samples were loaded at 180 V and 10°C, and then the temperature gradient was applied and electrophoresis continued at 220 V until the tracking dye (bromphenol blue) reached the bottom edge of the gel. The same gel system was used for the analysis of cDNA by the method of DNA heteroduplexes. Heteroduplexes were prepared by hybridisation of cDNA samples to standard (wild-type) cDNA or by hybridisation without adding this standard cDNA in 1 mM Na cacodylate buffer (pH 6.8) containing 100 mM NaCl and 1 mM EDTA. The samples were heated to 95°C in a heat block and slowly cooled down in an isolation box to 40°C in 2 h. An amount of 30 μl of hybridisation mixture was phenolysed, mixed with an equal volume of electrophoretic dye containing 0.4× TBE, and electrophoresed at a constant temperature of 42°C. Polyacrylamide gels were either stained for nucleic acids with $AgNO_3$ according to the method described by Schumacher *et al.* (1986) or nucleic acids were transblotted on to a nylon membrane charge-modified 0.2 μm (Sigma) using a semidry blotting procedure in 8 mM Tris–phosphate pH 8.3 containing 0.2 mM EDTA at 150 mA for 60 min. For the analysis of HLVd-specific RNA degradation products, we used denaturing gels containing 8 M urea as described by Schumacher *et al.* (1986). These samples were electroblotted onto a nylon membrane using TB buffer. DNA or RNA samples were hybridised to ^{32}P [UTP]-labeled HLVd riboprobe as described earlier (Matoušek *et al.,* 1995).

cDNA "conversion," infectivity assays, and viroid quantification

Tabler and Sänger (1984) found that double-stranded *Bam*HI fragments of full-length PSTVd are infectious. For this reason we converted individual cloned HLVd-cDNAs to *Bam*HI clones, which enabled us to prepare viroid-specific *Bam*HI fragments. The following procedure was used to convert the clones: cDNA was reamplified with Pwo polymerase from individual clones using phosphorylated RT and PCR primers and blunt-end ligated to prepare head-to-tail dimers or oligomers in the ligation mixture. For this purpose the individual components from the pCR-Script Cloning Kit (Stratagene) were used. After ligation was accomplished, cDNA was purified using QIAquick PCR purification protocol (Qiagen) and PCR was carried out as described above, using primers RTb ($5'A_{57}$ggatccCTCTTCGAGCCCTTGC$_{36}3'$) and PCRb ($5'A_{52}$ggatccCCGGGGAAACCTACTC$_{73}3'$), which were derived from the upstream conserved region containing the unique *Bam*HI site (designated by the lowercase letters). A specific primer RTb2 was used to maintain the C → U transition in clone 229 within the left boundary of UCCR ($5'A_{57}$ggatccCTCTTCGAACCCTTGC$_{36}3'$). Resulting DNA fragments were isolated from the gel, cloned in pCR-Script SK(+) vector, and sequenced to ensure the clone identity. *Bam*HI fragments were cleaved out from the vector and used for plant inoculation.

For infectivity tests 12 hop plants were inoculated each on two new leaves (25 μl of inoculum per leaf) using the Carborundum method. Freshly prepared inoculum of 0.04 M sodium phosphate buffer (pH 7.6) contained 0.2 μg/μl of DNA (*Bam*HI fragments of HLVd) and 3 μg/μl of total nucleic acids isolated from healthy tomato plants to increase stability of the inocula.

Viroid detection and quantification was performed using a dot-blot hybridisation method described earlier (Matoušek *et al.,* 1995) with a ^{32}P [dCTP]-labeled HLVd probe and a HLVd reference sample prepared as described by Matoušek *et al.* (1994). Viroid level was quantified from the autoradiograms by means of STORM PhosphorImager device and ImageQuant software (Molecular Dynamics).

Sequence Accession Nos. and other methods

The wild-type HLVd was identical to GenBank AC X07397 (Puchta *et al.,* 1988). The wild-type HLVd clone, which we used in our experiments, was kindly provided by Professor H. L. Sänger from Max Planck Institute of Biochemistry, Martinsried, Germany. Sequenced clones from our experiments have the following GenBank Accession Nos. (across the slashes): T15/AJ290404, T40/AJ290405, T50/AJ290406, T59/AJ290407, T61/AJ290408, T75/AJ290409, T92/AJ290410, T218/AJ290411, and T229/AJ290412.

Sequence analyses were carried out with DNASIS for Windows, version 2.5 (Hitachi). Structural calculations of HLVd secondary structure having high probability at low (physiological) temperature were performed using the ConStruct algorithm (Lück *et al.,* 1999).

ACKNOWLEDGMENTS

The authors thank Prof. H. J. Gross (Institut für Biochemie, Biozentrum, Am Hubland, Würzburg, Germany) for help and fruitful discus-

sions and Prof. C. P. Lichtenstein (School of Biological Sciences, Queen Mary and Westfield College, University of London) for help in preparation of this manuscript. We thank H. Matoušková (Institute of Plant Molecular Biology AS CR, České Budějovice, Czech Republic) for excellent technical assistance. This work was supported by the bilateral WTZ project between CR and FRG CZE 00/003 entitled "Analysis of Anti-Viroid Antisense Systems Driven by RNA Polymerase III Promoter Elements," by GA AS CR AA85051014, by NAZV Project EP0960996299 entitled "Molecular Diagnosis and Elimination of Hop Viroids" and by the project number FKZ 0311338 of Federal Ministry of Science and Technology of FRG.

REFERENCES

Adams, A. N., Barbara, D. J., and Morton, A. (1991). Effects of hop latent viroid on weight and quality of the cones of the hop cultivar Wye Challenger. *Ann. Appl. Biol.* **118**(Supplement), 126–127.

Adams, A. N., Morton, A., Barbara, D. J., and Ridout, M. S. (1992). The distribution and spread of hop latent viroid within two commercial plantings of hop (*Humulus lupulus*). *Ann. Appl. Biol.* **121,** 585–592.

Ambros, S., Hernandez, C., Desvignes, J. C., and Flores, R. (1998). Genomic structure of three phenotypically different isolates of peach latent mosaic viroid: Implications of the existence of constraints limiting the heterogeneity of viroid quasispecies. *J. Virol.* **72,** 7397–7406.

Ambros, S., Hernandez, C., and 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.

Barbara, D. J., Morton, A., Adams, A. N., and Green, C. P. (1990). Some effects of hop latent viroid on two cultivars of hop (*Humulus lupulus*) in the UK. *Ann. Appl. Biol.* **117,** 359–366.

Baumstark, T., and Riesner, D. (1995). Only one of four possible secondary structures of the central conserved region of potato spindle tuber viroid is a substrate for processing in a potato nuclear extract. *Nucleic Acids Res.* **23,** 4246–4254.

Baumstark, T., Schröder, A. R., and Riesner, D. (1997). Viroid processing: Switch from cleavage to ligation is driven by a change from a tetraloop to a loop E conformation. *EMBO J.* **16,** 599–610.

Diener, T. O. (1999). Viroids and the nature of viroid diseases. *Arch. Virol. Suppl.* **15,** 203–220.

Góra-Sochacka, A., Kierzek, A., Candresse, T., and Zagórski, W. (1997). The genetic stability of potato spindle tuber viroid (PSTVd) molecular variants. *RNA* **3,** 68–74.

Gruner, R., Fels, A., Qu, F., Zimmat, R., Steger, G., and Riesner, D. (1995). Interdependence of pathogenicity and replicability with potato spindle tuber viroid. *Virology* **209,** 60–69.

Hataya, T., Hikage, K., Suda, N., Nagata, T., Li, S., Itoga, Y., Tanikashi, T., and Shikata, E. (1992). Detection of hop latent viroid (HLVd) using reverse transcription and polymerase chain reaction (RT PCR). *Ann. Phytopathol. Soc. Jpn.* **58,** 677–684.

Keese, P., and Symons, R. H. (1985). Domains in viroids: Evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. *Proc. Natl. Acad. Sci. USA* **82,** 4582–4586.

Kofalvi, S. A., Marcos, J. F., Canizares, M. C., Pallas, V., and Candresse, T. (1997). Hop stunt viroid (HSVd) sequence variants from Prunus species: Evidence for recombination between HSVd isolates. *J. Gen. Virol.* **78,** 3177–3186.

Lück, R., Gräf, S., and Steger, G. (1999). ConStruct: A tool for thermodynamic controlled prediction of conserved secondary structure. *Nucleic Acids Res.* **27,** 4208–4217.

Matoušek, J., Trněná, L., Svoboda, P., and Růžková, P. (1994). Analysis of hop latent viroid (HLVd) in commercial hop clones in Czech Republic. *Rostlinná výroba* **40,** 973–983.

Matoušek, J., Trněná, L., Svoboda, P., Oriniaková, P., and 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. Hoppe-Seyler* **376,** 715–721.

Matoušek, J., and Patzak, J. (2000). A low transmissibility of hop latent viroid (HLVd) through a generative phase of hop (*Humulus lupulus* L.). *Biol. Plant* **43,** 145–148.

Owens, R. A., Thompson, S. M., Feldstein, P. A., and Garnsey, S. M. (1999). Effects of natural sequence variation on symptom induction by citrus viroid III. *Ann. Appl. Biol.* **134,** 73–80.

Owens, R. A., Yang, G., Gundersen-Rindal, D., Hammond, R. W., Candresse, T., and Bar-Joseph, M. (2000). Both point mutation and RNA recombination contribute to the sequence diversity of citrus viroid III. *Virus Genes* **20,** 243–252.

Polívka, H., Staub, U., and Gross, H. J. (1996). Variation of viroid profiles in individual grapevine plants: Novel grapevine yellow speckle viroid 1 mutants show alterations of hairpin I. *J. Gen. Virol.* **77,** 155–161.

Puchta, H., Ramm, K., and Sänger, H. L. (1988). The molecular structure of hop latent viroid (HLV), a new viroid occurring world-wide in hops. *Nucleic Acids Res.* **16,** 4197–4216.

Qu, F., Heinrich, C., Loss, P., Steger, G., Tien, P., and Riesner, D. (1993). Multiple pathways of reversion in viroids for conservation of structural elements. *EMBO J.* **12,** 2129–2139.

Rakowski, A. G., and Symons, R. H. (1989). Comparative sequence studies of variants of avocado sunblotch viroid. *Virology* **73,** 352–356.

Riesner, D., Steger, G., Zimmat, R., Owens, A., Wagenhöfer, M., Hillen, W., Vollbach, S., and 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., and Rezaian, M. A. (1993). Analysis of sequence variation in grapevine yellow speckle viroid 1 reveals two distinct alternative structures for the pathogenic domain. *Virology* **193,** 474–477.

Sano, T., Mimura, R., and Ohshima, K. (2000). Phylogenetic analysis of hop and grapevine isolates of hop stunt viroid supports a grapevine origin for hop stunt disease. *Virus Genes* **22,** 53–59.

Schindler, I. M., and Mühlbach, H.-P. (1992). Involvement of nuclear DNA-dependent RNA-polymerases in potato spindle tuber viroid replication: A reevaluation. *Plant Sci.* **84,** 221–229.

Schumacher, J., Meyer, N., Riesner, D., and Weidemann, H. L. (1986). Diagnostic procedure for detection of viroids and viruses with circular RNAs by "return"-gel electrophoresis. *J. Phytopathol.* **115,** 332–343.

Szychowski, J. A., Credi, R., Reanwarakorn, K., and Semancik, J. S. (1998). Population diversity in grapevine yellow speckle viroid-1 and the relationship to disease expression. *Virology* **248,** 432–444.

Tabler, M., and Sänger, H.L. (1984). Cloned single- and double-stranded DNA copies of potato spindle tuber viroid (PSTV) RNA and coinoculated subgenomic DNA fragments are infectious. *EMBO J.* **3,** 3055–3062.

Visvader, J.E., and Symons, R.H. (1985). Eleven new sequence variants of citrus exocortis viroid and the correlation of sequence with pathogenicity. *Nucleic Acids Res.* **13,** 2907–2920.