Molecular sampling of hop stunt viroid (HSVd) from grapevines in hop production areas in the Czech Republic and hop protection

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ABSTRACT

Molecular sampling of HSVd in grapevines in the environs of hop gardens was performed. Specific RT PCR primers were designed to unambiguously distinguish between HLVd and HSVd infections. These primers were used for detection and analysis of HSVd cDNAs from individual samples by thermodynamic methods, TGGE and cDNA heteroduplex analysis. We found that at least 70% of grapevine samples from locations close to hop gardens in Northern Bohemia (Žatec and Úštěk hop production areas) were infected with HSVd forming populations containing quasispecies. Particular sequence variants, dominant in grapevines from wine-growing areas like Znojmo, were also found in minor private vineyards. HSVd was experimentally transmissible (80% success) from these samples to Osvald's clone 72 of Czech hop, where according to the cDNA library screening, one of the dominant HSVdg variants corresponding to AC E01844 was detected in early populations three weeks p.i. HSVd was detected neither in reproduction materials nor in examined hop gardens. However a potential danger for hop cultivation, consisting in the high biological potential of HSVd spread is discussed.

Keywords: viroid spreading; viroid quasispecies; RT PCR analysis; cDNA heteroduplexes; TGGE

Viroids represent the smallest pathogenic RNA replicons, ranging from 246 to 401 nt in size, which are fully dependent on the metabolism of host plant (for review see e.g. Diener et al. 1987, Flores 2001). As many as 27 different viroid species have been classified and listed in biological databases until now (Flores et al. 1998, Pelchat 2000) and most of them form populations of molecular variants that conform to a quasi-species model (Eigen 1993). These molecular variants can adapt themselves to new hosts and life-cycle conditions. Although probably living fossils originating from the RNA world, viroids represent one of the most rapidly evolving biological systems known (Diener 1996), having a high biological potential to spread. Modern agricultural practices, characterized by extensive plantations of genetically uniform species and intensive world-wide movement of plant material, could facilitate the rapid spreading of viroids. Consistently with this view, all viroid diseases were recorded in the 20th century (for review see Flores 2001).

Two viroid species, hop stunt viroid (HSVd) and hop latent viroid HLVd (Puchta et al. 1988a), both belonging to the family *Pospiviroidae*, have been described as hop (*Humulus lupulus* L.) pathogens. HSVd apparently has a wide host range and besides hop it is able to propagate in cucumber, grapevine, citrus, plum, peach, pear (Shikata 1990), apricot and almond plants (Astruc et al. 1996, Canizares et al. 1999). Currently, eighty-four HSVd sequences are present in the subviral RNA database (Pelchat et al. 2000), but as many as 120 HSVd sequence entries are in common biological databases, and about eleven of them are designated as hop-adapted. Very high variability, plasticity of this viroid and wide sequence distribution make it comparable with other extremely variable viroids, such as the peach latent mosaic viroid from the family *Avsunviroidae* (e.g. Ambros et al. 1999). Molecular genetic comparisons, made by Sano et al. (2001), theoretically confirmed a generally accepted opinion that the primary jump of this viroid to hop plants occurred from grapevines. Japan is the only country, where it is known that it happened, probably due to local agricultural and hop cultivation practices, but the viroid itself is pervasive and has spread world-wide. Thus, the devastating potential of HSVd for hops is a potential threat that provokes a question about the need of permanent monitoring of this viroid in connection with hop growing.

In the present paper we initiated a molecular sampling of HSVd from main hop production areas in the Czech Republic. We found approximately 70% incidence of HSVd populations in grapevines planted in the environs of hop gardens. Moreover, there is obviously no obstacle for the particular HSVdg variants to infect hop genotypes of Czech origin.

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MATERIAL AND METHODS

Plant material, collection of samples, RNA isolation and inoculation

For an analysis of the rate of HSVd variability in the stock grapevine samples were collected from a vineyard of Ampelos, Breeding and Selection Station of Viticulture, Znojmo and included in three groups, each containing samples from ten plants as follows (sample identification in the vineyard by field, row and plant numbers across the slashes): Group I: Sauvignon, 5/1/5; Neuburger, 18/3/6; Müller Thurgau, 49/6/3; André, 106/3/2; Saint Laurent, 120/4/2; Welschriesling, 140/1/6; Riesling weiss, 160/2/3; Traminer rot, 11/3/4; Aurelius, 13/2/3; Veritas, 17/3/1; Group II: Veltliner gruen, 49/2/2; Silvaner gruen, 92/1/3; Chasselas blanc, 105/2/3; Irsai Oliver, 126/1/4; Welschriesling, 139/3/2; Traminer rot, 2/3/2; Veltliner gruen, 11/2/3; Veltliner gruen, 11/2/3; Welschriesling ryzlink, 28/3/1; Welschriesling ryzlink, 28/3/1; Group III: Sauvignon, 70/1/2; Sauvignon, 70/1/2; Chardonnay, 88/2/3; Chardonnay, 88/2/3; Müller Thurgau, 90/1/4; Veltliner gruen, 102/2/3; Pinot gris, 113/1/2; Pinot gris, 123/1/2; stock SO4, 166/2/3; stock K5BB, 170/2/3. Other identified or unidentified samples from private vineyards were collected at several localities in Northern Bohemia as indicated in the figures, but mainly near the cities of Žatec and Úštěk. Young leaves were collected and stored in cold until RNA was extracted. To assay possible HSVd infections, samples of Czech hop were collected at the same localities as above and in addition from important materials, such as virus-free plants of Osvald's clones 31, 114 and 72, Bor, Sládek, Premiant and Agnus varieties, from virus-free technical and spatial isolates of Hop Research Institute in Žatec and from reproduction material of virus-free rootstocks in TU-FLOR Co. Ltd. in Tušimice. Virus-free plants of Osvald's clones 72 from production hop gardens in Žatec hop production region (Stekník, Oploty, Liběšice, Vědomice, Soběsuky, Brozany, Tuchořice, Obora) were also sampled. Hop leaves were collected and stored as above.

Total RNA was isolated from 100 mg of leaves using RNeasy Plant Total RNA kit (Qiagen). The extraction buffer contained an addition of 1% PEG 6000 to remove polysaccharides and secondary metabolites. For the cDNA inoculation experiment, healthy hop mericlones of Osvald's clone 72 were used. These plants were transferred into soil and maintained under climate chamber conditions for approximately three weeks before inoculation. Temperature conditions in the climate chamber were maintained at 25 ± 3°C. Plants were grown under natural light with additional illumination (90 µmol/m/s PAR) to keep a 16h day period. These plants were inoculated by conventional carborundum method. Aliquots of Qiagen-purified RNA from individual grapevine samples were pooled and the final mixed sample was adjusted to contain 0.04M sodium phosphate buffer (pH 7.6) and used for inoculation. Each plant was inoculated in two young leaves with 20 µl of RNA inoculum per leaf.

RT PCR amplifications, cloning and sequencing

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. To detect HSVd, RT primer designated HSVd I (5'GCGTCTCATCGGAAGAGCC3') and PCR primer HSVd II (5'GACCGGTGGCATCACCTCT3') were used. In parallel, HLVd was monitored in hops using RT primer ($5'_{200}$CCACCGGGTAGTTTCCAACT$_{181}$3') and PCR primer ($5'_{201}$ATACA ACTCTTGAGCGCCGA$_{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 re-amplification of viroid cDNAs. In a typical experiment we used the following amplification conditions: 120 s at 94°C, 35 × (30 s at 94°C, 30 s at 58°C, 60 s at 72°C), 72°C, 10 min. PCR products were purified using 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-labelled standard primers (Thermosequenase Dye Termination Kit, Amersham-Pharmacia).

Gel electrophoretic analysis of cDNA and other methods

Temperature gradient gel electrophoresis (TGGE) was performed in 6% polyacrylamide gels (Riesner et al. 1989) (140 × 140 × 1.8 mm) or in minigels using mini TGGE system (Biometra, Germany). The composition of 6% denaturing acrylamide gels containing 7M urea was the same for both, TGGE and heteroduplex analysis as described previously (Matoušek et al. 2001). Heteroduplexes were prepared by hybridization of cDNA samples to standard (cloned) cDNA or by hybridization without adding this standard cDNA. Hybridization procedure as well as analysis of DNA in gels was the same as described previously (Matoušek et al. 2001) except for the temperature of gels: 45.5°C was used for analysis of HSVd cDNAs. Gels were stained with $AgNO_3$ for nucleic acids as described by Schumacher et al. (1986). For analysis of RT PCR products, 2% agarose gels were used and cDNA was stained with ethidium bromide.

GeneBank database sequences and computing

We employed database described by Pelchat et al. (2000) for HSVdg (grapevine) sequence identification and for the analysis of HSVdh (hop) sequences. For sequence alignments, we used HSVd sequences having the following GenBank (see e.g. Benson et al. 1993) accession numbers: AB039265, AB039266, AB03967, AB039268, AB039269,

Figure 1. Collection of samples for HSVd analysis in the Czech Republic. Solid spots – the main areas of hop growing and their approximate size in hectares. Open square – a place of sample collection in the wine-growing area (Znojmo). See Material and Methods for further details about places of sample collection in two hop production areas Žatec and Úštěk. For orientation, the capitals of Bohemia and Moravia are shown by open spots.

AB039270, AB039271, AB03972, E01844, E01842 and X00009.

Sequence analyses and evolutionary relationships were carried out with DNASIS for Windows, version 2.5 (Hitachi). Structural calculations were performed using mfold v. 3.1 (Mathews et al. 1999, Zuker et al. 1999).

RESULTS AND DISCUSSION

Dominant HSVd sequences in analyzed grapevines from Znojmo

In this study we tried to get the first information about HSVd infections in the Czech Republic, especially in the important hop production areas, including localities close to Žatec and Úštěk hop gardens (Figure 1). Although up to date, there was not any indication of HSVd-like symptoms on any hop in the Czech Republic. This study was stimulated by the fact that HSVd is obviously a very aggressive viroid, in principle representing a potential danger for hop growing. Firstly, we wanted to initiate the sampling of this viroid in the Central European territory and secondly, we wanted to develop a reliable system of HSVd detection.

We started to monitor HLVd several years ago, when we partly characterized the horizontal (Matoušek et al. 1994) and vertical (Matoušek et al. 1995) distribution of HLVd in Czech hop Osvald's clones 31 and 72. The incidence of HLVd infection was nearly 100%. Moreover, it indicated a very high HLVd re-infection rate (Matoušek and Patzak 2000). For this reason we designed new primers for HSVd detection with respect to HLVd/HSVd homology. The primers suggested in Figure 2A did not show any PCR reaction with HLVd due to the extensive gaps and low homology to HLVd (not shown). Recent analyses confirmed the grapevine origin of hop stunt disease (Sano et al. 2001) and, therefore, for the first mapping of HSVd we selected traditional as well as foreign grapevine materials of interest, cultivated in this territory. There are at least ten sequences of HSVd described in the subviral RNA database (Pelchat et al. 2000), forming so-called grapevine-specific (HSVdg) group. However, unlike the strong HSVd symptoms on hop, HSVd infection on grapevines has latent characteristics. Due to this fact, we assumed that the infected grapevines if any, were not recognized and removed from grapevine collections and stock materials in Znojmo, one of the centers of viticulture (Figure 1) and instead, the HSVd populations could be maintained in these selection materials for a long time and possibly accidentally spread to commercial vineyards. For the first screening we solved a problem of the

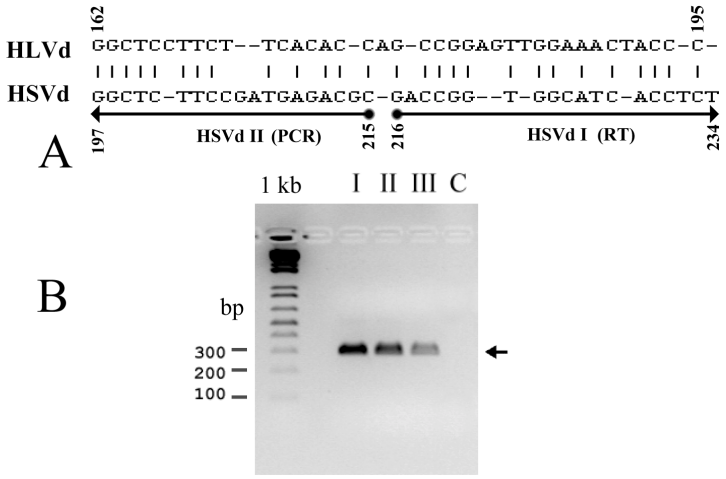

Figure 2. Schematic drawing of HSVd amplification and detection of primer system. Position of primer pair designated HSVd I and II and homology of HSVd to HLVd at this position (A). Detection of HSVd using primers HSVd I and II in grapevine samples collected at Ampelos Breeding and Selection Station of Viticulture, Znojmo (B). HSVd is detected in mixed samples of group I, II and III, C – non-infected sample. Short arrow indicates a specific band of HSVd cDNA. Positions of primers in HSVd genome are indicated by long arrows, and numbers. See Material and Methods for further details.

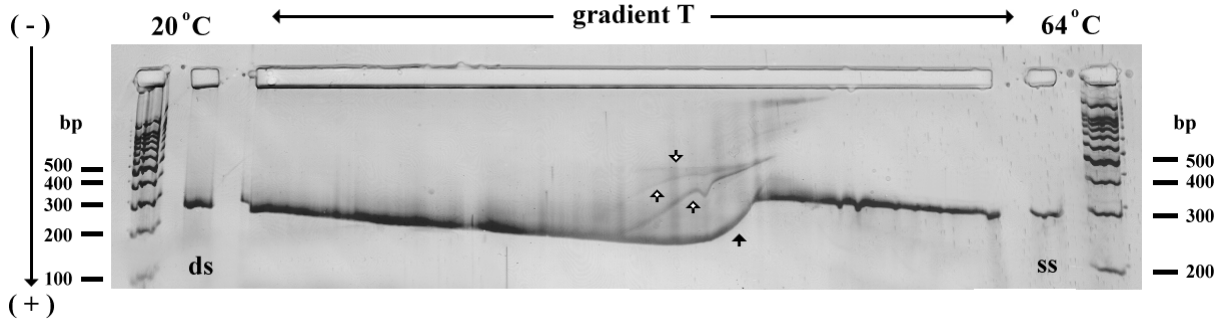

Figure 3. Analysis of HSVd cDNA from grapevines collected in Znojmo on TGGE. Mixed cDNA sample was prepared using primers HSVd I, II, phenolized, heated and allowed to form heteroduplexes as described in Material and Methods. Then the sample was analyzed in 6% acrylamide gel containing 7M urea using a temperature gradient from 20 to 64°C. *ds* and *ss* designate double- and single-stranded parts of DNA patterns, respectively. Dominant HSVd heteroduplexes are indicated by open arrows, homoduplex by solid arrow. The gel was stained with $AgNO_3$ for nucleic acids.

presence and sequence variability of HSVd in the collection maintained in Ampelos, Breeding and Selection Station of Viticulture, Znojmo. The mixed samples that were analyzed for group I, II and III (see Material and Methods) all showed positive signals (Figure 2) that allowed us to isolate corresponding cDNAs. These cDNAs were subjected to a thermodynamic analysis on TGGE (Figure 3) using the perpendicular temperature gradient 20–64°C. This technique allowed to recognize different sequences of HSVd forming heteroduplexes exhibiting retardation in the gel and having transition curves that differed from homoduplex cDNA. As expected, besides homoduplex, there were also some minor and dominant heteroduplexes present in the TGGE spectrum (Figure 3), suggesting a mixture of different sequence variants. In order to identify the dominant sequences, cDNA library was prepared and screened by the method of pre-formed heteroduplexes, similarly like described earlier for HLVd (Matoušek et al. 2001). As a result of these experiments we identified two clones designated 1270 and 1271 as major cDNA variants. Both these variants were spread elsewhere and described in the GenBank database. Clone 1270 is identical to sequence having AC E01844, described by Yomo et al. (1989) and clone 1271 is identical to that described by Puchta et al. (1988b) under AC X06873. The cloned cDNAs were further used as molecular standards for HSVd analysis by the method of DNA heteroduplexes and for sequence comparisons.

Molecular sampling of HSVd from grapevines grown in hop production areas by the method of pre-formed heteroduplexes

In further experiments we assayed HSVd incidence in minor private vineyards surrounding hop gardens in northern Bohemia, mainly near Žatec and Úštěk (Figure 1), but also close to the towns of Mělník, Lovosice and Most. Some samples for comparison were collected in Slovenia, close to the town of Žalec, the center of hop

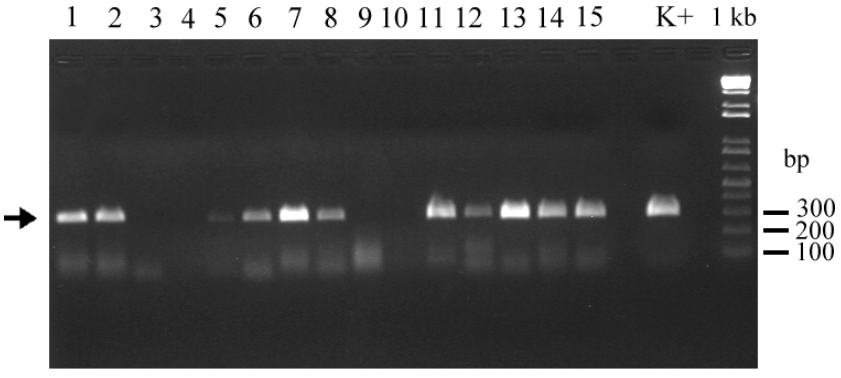

Figure 4. An example of RT PCR analyses of HSVd infections in private vineyards in Žatec. The arrow designates a specific HSVd cDNA band. This band does not appear in non-infected plants. RNA samples were extracted using RNeasy extraction protocol from Qiagen and subjected to one step RT PCR using primers HSVd I and II. Then the samples were analyzed in 2% agarose gels stained with ethidium bromide. 1. Veltliner fruehrot, 2. non-identified I, 3. Saint Laurent I, 4. Saint Laurent II, 5. Saint Laurent III, 6. Müller I, 7. Müller II, 8. Müller III, 9. Otello, 10. non-identified II, 11. non-identified III, 12. MOPR, 13. Portugieser blau, 14. Madeleine, 15. Portugieser grau. K+ = HSVd positive sample from Znojmo, 1 kb = marker DNA (1 kb ladder, BRL).

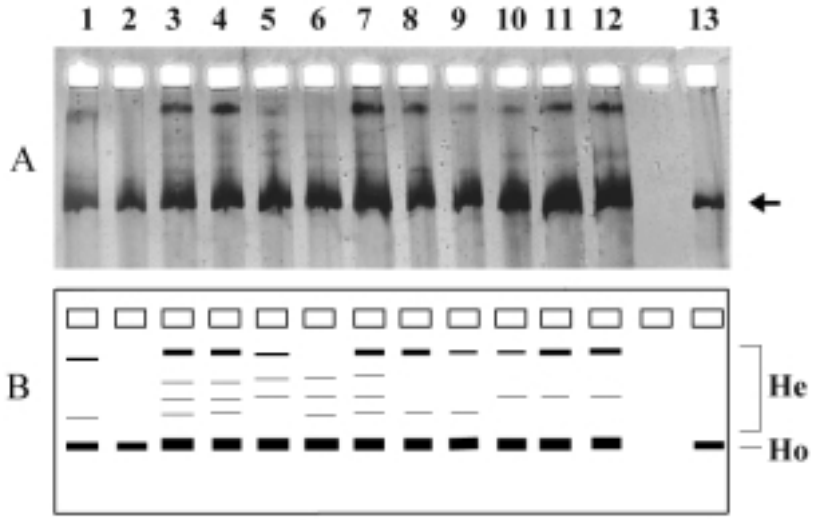

Figure 5. An example of analysis of cDNA samples of HSVd by the method of pre-formed DNA heteroduplexes. Gel stained with $AgNO_3$ (A), schematic drawing of the gel (B). cDNA fragments were prepared using one step RT PCR system from individual RNA samples. Then cDNA was treated as described in Figure 2, selfhybridized and electrophoresed at a constant temperature of 45.5°C in 6% polyacrylamide gel containing 7M urea. Samples 1–6 were collected in Žatec, i.e. 1. Veltliner fruehrot, 2. non-identified I, 3. Müller II, 4. Portugieser blau, 5. Madeleine, 6. Portugieser grau, 7. non-identified sample from Mělník, samples 8–9 are non-identified samples from Úštěk hop production area, i.e. 8. sample I from Úštěk, 9. sample II from Úštěk, 10. sample from Jemnice, samples 11–12 are non-identified samples from Slovenia, i.e. 11. sample from Levec, 12. sample from Žalec. The standard cDNA from 1270 (Znojmo) was loaded in slot 13. The monoduplex position is indicated by the arrow in panel A. Ho designates the position of homoduplex and He the zone of heteroduplexes in panel B.

production. The mean value of HSVd incidence in these samples was about 70% as can be documented by the typical results obtained for Žatec hop production area (Figure 4). As we suggested previously from a comparison of HLVd and HSVd, the level of molecular variants could determine viroid potency for expansion and new adaptations, i.e. with many viroid molecular variants there exists higher probability to be transmitted to new species (Matoušek 2003). This idea is in principle consistent with quasispecies model (Eigen 1993). In this respect we solved the problem of molecular variability of HSVd in individual samples from different grapevine localities. We employed the heteroduplexes method (Figure 5) and found that HSVd formed dominant populations practically in all examined samples. In some of them, codominant heteroduplexes were found, but mainly minor heteroduplexes were observed (Figure 5), suggesting the presence of quasispecies. The fact that HSVd forms plethora of sequence variants follows from the results of many authors (e.g. Amari et al. 2001, Sano et al. 2001). The majority of molecular variants of HSVd was described for a citrus-specific group (see the viroid database, Pelchat et al. 2000).

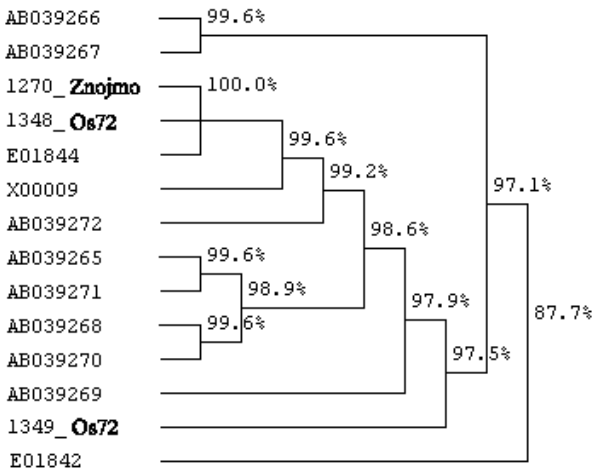

Figure 6. Cluster analysis for a group of hop-specific HSVd cDNAs available in genetic databases and supplemented with early cDNA clones from infected Czech hop. Trees were generated with Clustal W option of DNASIS (Hitachi). Percents of nucleotide identity are included in a phylogenetic tree.

Analysis of experimental transmission of HSVdg populations to Osvald's hop and initial screening of Czech hop for possible HSVd infections

The fact that there is not any indication of HSVd infection in Czech hop could mean that the used agricultural practices are beneficial for the prevention of this infection or that the particular hop genotypes are immune to HSVd populations present in the ecotope or agrotope in these hop production areas. To clarify these assumptions partly, we used experimental inoculation of HSVdg populations we pooled from all collected grapevine samples from hop production areas, to healthy hop meri-

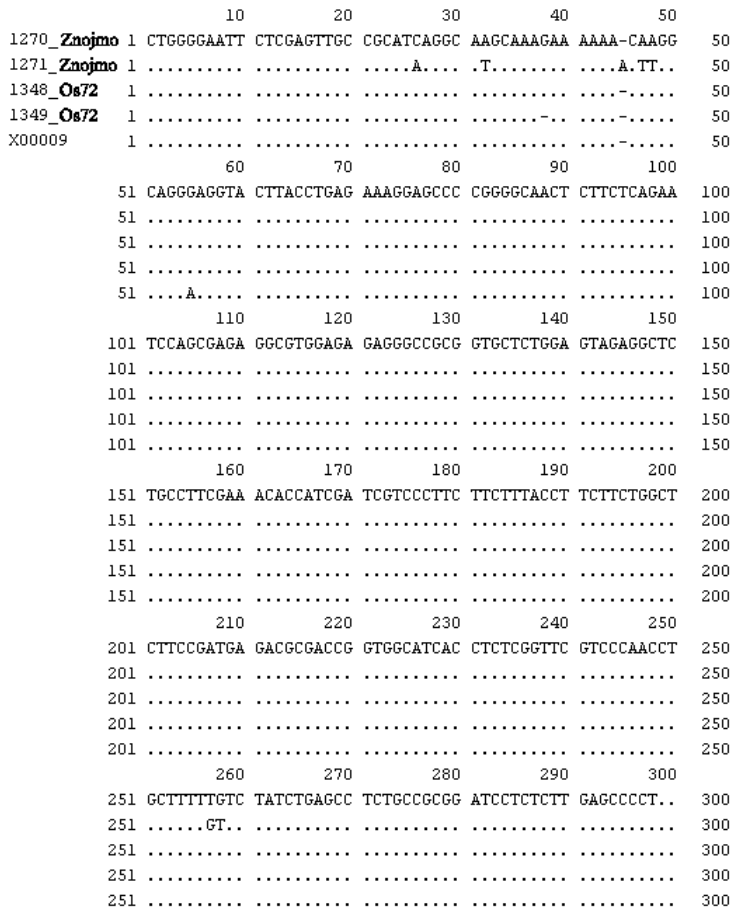

Figure 7. The multiple alignment of HSVd clones. Two dominant clones from Znojmo and two early clones from infected Osvald's hop 72 are aligned together with the sequence AC X00009, representing the first characterized HSVd sequence from hop in Japan.

clones of Osvald's clone 72. The infectivity of this HSVdg sample was significant because 80% of inoculated plants were clearly infected and the RT PCR signal was detectable already 3 weeks post inoculation (p.i.). At that time we collected samples for cDNA analysis of early HSVd variants. Library screening revealed one dominant sequence and some minor variants that were not sequenced (except for one). A dominant variant, clone 1348, was identical to clone 1270 from Znojmo (sequence AC E01844), which was observed in Japanese hop before (Figure 6). The minor variant (clone 1349) had single G deletion at position 38 (Figure 7) and is not present in any database. Whether this variant could represent one of the minor quasispecies that occurred in grapevines or in hop upon inoculation was not analyzed further. It follows from these results that at least one of two major HSVd sequence variants spread in the territory of the Czech Republic is clearly infectious for Osvald's hop. Both sequences isolated from Osvald's hop differ from the first hop-specific HSVd at position 57 (Figure 7), where A instead of G nucleotide was found in the first sequence from hop (AC X00009). It is clear that this G > A base change is not the only one that determines hop specificity of HSVd. About 11 sequences have been isolated from hops so far (Figure 6). Except for sequence AC E01842, all these sequences are related for more than 97%. According to Sano et al. (2001) the sequence variants currently being observed in HSVd as it replicates in hop in Japan may represent a transition stage in which a viroid that originated in grapevine is in the process of adapting itself to a new host.

Although we observed a successful transfer of HSVd to hop, we did not assay any possible pathogenicity reaction of HSVd population in Osvald's hop. It is not known whether some HSVd variants are latent or not and therefore they are not easily observable according to characteristic stunting and other symptoms. Bearing in mind this possibility, we initiated primary screening for HSVd in valuable hop materials and materials from selected hop gardens (see Materials and Methods). As a result of this screening, no HSVd infection was observed by the RT PCR method, suggesting freedom of examined hop materials.

HSVd as persisting molecular danger and hop protection

Viroids are known to spread mainly by a mechanical injury of recipient plant (e.g. Diener 1987). Some molecular variants of HSVd spread in our territory could in principle infect hop and so they obviously represent a persisting molecular danger for hop growing. This potential threat is also of some probability because no specific measures are taken by hop growers to prevent viroid infections although some suggestions to use chemical pre-treatments to prevent viroid spreading were pub-

lished already twenty-four years ago (Singh et al. 1989). According to our own unpublished analyses, pollen extracellular nucleases (Matoušek and Tupý 1985) could be a very cheap source of efficient biochemical agent for possible disinfection of equipment to prevent mechanical viroid spreading. The fast rate of hop re-infection with the second hop viroid – HLVd (Matoušek and Patzak 2000), which was shown to increase its variability due to thermal stress (Matoušek et al. 2001), is another argument for regular checks of both viroids, i.e. HLVd and HSVd. It has been shown by our groups recently that HLVd (Matoušek 2003) could infect so-called non-host plant species in which viroid quasispecies are constantly propagated as low level populations. Because HSVd represents a very aggressive viroid species, one can not rule out its presence in some residual plant populations of weeds within the hop agrotope or surrounding hop gardens.

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ABSTRAKT

Molekulární analýza vzorků viroidu zakrslosti chmelu (HSVd) z révy vinné ve chmelařských oblastech ČR a ochrana chmelu

Byla provedena molekulární analýza vzorků HSVd z révy vinné z bezprostředního okolí chmelnic. Pro jednoznačné odlišení viroidní infekce chmelu, způsobené HSVd od rozšířené infekce HLVd, byly navrženy specifické RT PCR primery. Tyto primery byly použity pro detekci i analýzu HSVd cDNA z individuálních vzorků pomocí termodynamických metod, TGGE a analýzy heteroduplexů cDNA. Bylo zjištěno, že přinejmenším 70 % vzorků révy vinné z lokalit blízkých chmelnicím v severních Čechách (chmelařské oblasti na Žatecku a v okolí Úštěku) je infikováno HSVd, který v nich tvoří populace obsahující minoritní varianty tzv. quasispecies. Zvláštní sekvenční varianty, dominantní ve vzorcích révy vinné z vinařské oblasti u Znojma, byly rovněž nalezeny na těchto minoritních privátních vinicích. HSVd je z těchto vzorků snadno experimentálně přenosný (úspěšnost 80 %) na Osvaldův klon 72 českého chmelu. Jak bylo zjištěno pomocí screeningu knihovny cDNA, v tomto infikovaném chmelu převládala v raných populacích (tři týdny po inokulaci) jedna z dominantních variant HSVdg mající AC E01844. HSVd nebyl detekován ani v základních množitelských materiálech, ani na sledovaných chmelnicích, nicméně je diskutováno potenciální nebezpečí pro pěstování českého chmelu, které představuje vysoký biologický potenciál HSVd k šíření.

Klíčová slova: šíření viroidů; viroidní kvaziformy; analýza RT PCR; heteroduplexy cDNA; TGGE

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