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BRIEF COMMUNICATION

Hop latent viroid (HLVd) microevolution: an experimental transmission of HLVd "thermomutants" to solanaceous species

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Abstract

The possibility was examined whether the pool of sequence variants of HLVd which accumulated as progeny of "thermomutants" induced upon heat-treatment of hop could initiate infection of non-host solanaceous plants. It was found that HLVd microevolution led to the appearance of HLVd population in tomato. This viroid population was maintained at levels detectable by molecular hybridisation, showing the highest concentration in apical leaves. HLVd was further transferred from tomato to *Nicotiana benthamiana,* where distinct HLVd sequence variants appeared and were stably maintained at low levels. Our results show that replication of HLVd under heat stress resulted in the production of viroid quasispecies, potentially important for viroid evolution in so-called non host plants.

Additional key words: viroid spreading, viroid detection, TGGE, RT PCR, *Humulus lupulus*, *Lycopersicum esculentum*, *Nicotiana benthamiana*.

Viroids are the smallest pathogenic circular single-stranded RNA molecules, which do not code for any protein and therefore they are fully dependent on host plant in their life-cycle (for review see, *e.g.*, Diener *et al.* 1987). Accumulating sequencing data suggest that most viroid species form populations of sequence variants, which according to quasi-species concept (Eigen 1993) may serve as a source of adaptations to new hosts and new life-cycle conditions. Results from site-directed mutagenesis experiments indicate that, upon exposure to selective pressures, viroids can evolve extremely rapidly, with another, fitter, component of the quasi-species often becoming dominant within days or weeks. This extreme plasticity of their nucleotide sequences establishes viroids as the most rapidly evolving biological system known (Diener 1995).

Two viroid species, hop stunt viroid (HSVd) and hop latent viroid (HLVd) which both belong to *Pospiviroidae* family have been described as hop (*Humulus lupulus* L.) pathogens. HSVd has apparently 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). Fifty four sequence variants of HSVd have been published so far (Sano *et al.* 2001). In contrast, despite of a wide experimental screening, no other hosts but hop were found for HLVd (Puchta *et al.* 1988). Only recently, reverse transcription polymerase chain reaction (RT PCR) approach allowed to detect this pathogen in stinging nettle (*Urtica dioica* L.) (Knabel *et al.* 1999). No sequence variants of HLVd have been originally detected (Puchta *et al.* 1988, Hataya *et al.* 1992). However, recently we described HLVd variation which is induced upon heat-treatment of hop (Matoušek *et al.* 2001). While

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Abbreviations: d p.i. - days post inoculation; HLVd - hop latent viroid; RT PCR - reverse transcription polymerase chain reaction; TGGE - temperature-gradient electrophoresis.

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only 1 % of deviating HLVd sequence variants, all having single base deletions, were identified in HLVd population from non-treated hop, heat treatment caused an accumulation of mutations which were localized predominantly in the left half of HLVd molecule. Mutated cDNAs were infectious and initiated an evolution of complex progeny populations (Matoušek *et al.* 2001). Although the concentration of the progeny of "thermomutants" was lower than the wild type HLVd, these populations showed differential levels of accumulation during cultivation and post dormancy periods (Matoušek *et al.* 2001).

In the present work we aimed to study a possible transmission of progeny of HLVd "thermomutants" from hop to so-called non-host plant species *Lycopersicum esculentum* L. and *Nicotiana benthamiana* L. It was found that distinct molecular variants of HLVd formed stable populations on these species.

Plants were grown in the growth chamber at temperature of 25 ± 3 °C and under natural light with supplementary irradiance of 90 μmol m^{-2} s^{-1} PAR to keep 16-h photoperiod. Viroid inoculum was prepared either from wild type HLVd or as mixed RNA sample from progeny of thermomutants having the following GenBank accession numbers (across the slashes): T50/AJ290406, T61/AJ290408, T75/AJ290409 and T92/AJ290410. The viroid progeny isolates were identical to those described previously (Matoušek *et al.* 2001). RNA inoculation was performed similarly as described earlier for potato spindle tuber viroid using the carborundum method (Matoušek *et al.* 1994). Dot blot hybridisation analysis of viroid was performed according to Matoušek *et al.* (1994) using ^{32}P dCTP ~110 TBq $mmol^{-1}$-labelled cDNA. Viroid level was quantified by means of *STORM PhosphorImag*er device and *ImageQuant* software (*Molecular Dynamics*, Inc., Sunnyvale, CA, USA) using standard reference sample. RT PCR method was employed as described earlier by Matoušek and Patzak (2000) using RT primer ($5'_{200}$CCACCGGGTAGTTTCCAACT$_{181}3'$) and PCR primer ($5'_{201}$ATACAACTCT TGAGCGCCGA$_{220}3'$). cDNA analyses by temperature-gradient-gel electrophoresis and by the method of pre-formed DNA heteroduplexes were accomplished as described previously (Matoušek *et al.* 2001). Automatic sequencing was performed with an *ALF II* system (*Amersham Pharmacia Biotech,* Uppsala, Sweden) using a sequencing kit with Cy5-labelled standard primers (*Thermosequenase Dye Termination Kit, Amersham-Pharmacia Biotech,* Freiburg, Germany). Sequence analyses and alignments were carried out with *DNASIS for Windows, version 2.5* (*Hitachi Software Engineering* Co., Tokyo, Japan).

In order to assay the potential transmissibility of pool of HLVd mutants (Fig. 1*A*) to other species, we inoculated young tomato plants cv. Rutgers. Under the experimental conditions used, no hybridisation signal was detected after inoculation of the wild type HLVd. However, inoculation of thermomutant HLVd population resulted in detectable infection in two out of twenty tomato plants in the first inoculation cycle 30 days post inoculation (d p.i.) and 40 % of infection in the second inoculation cycle, when inoculum was prepared from infected tomatoes. Analysis of cDNA from primary infected tomato by the heteroduplexes method indicated a significant reduction of number of heteroduplexes as compared with the original pool isolated from infected hop. In total, 35 distinct and 14 less discernible bands were seen in the spectra of heteroduplexes of individual

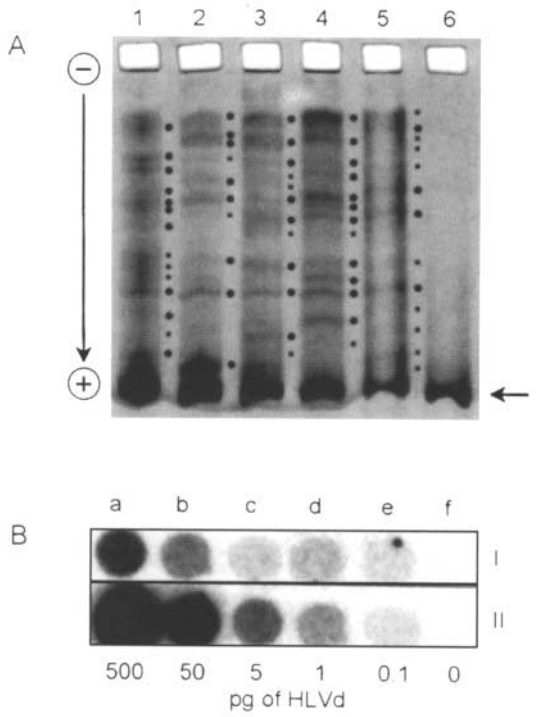

Fig. 1. Analysis of HLVd in hop and tomato. *A*: Hop plants were infected with cDNA from individual viroid thermomutants. HLVd was then extracted 30 d p.i., amplified by RT PCR and analysed for heterogeneity by electrophoresis of DNA heteroduplexes at 42 °C in 6 % acrylamide gel containing 7 M urea. The gel was stained for nucleic acids with $AgNO_3$. HLVd variants as detected in *lanes 1 - 4*, corresponding to samples infected with thermomutants T50, T61, T75 and T92, respectively, were pooled and transferred by RNA inoculation in tomato analysed in *lane 5*. The positions of individual heteroduplexes are marked by the *dots* on the right side of individual lines. *Larger dots* represent more distinct bands and *smaller ones* less discernible heteroduplexes, respectively. The position of DNA homoduplex loaded in *line 6* is indicated by the *arrow*. *B*: Analysis of HLVd levels in different tomato leaf tissue by dot-blot hybridisation (*row I*) and HLVd standard (*row II*). *Spot a* - apex leves; *b* - upper young leaves; *c,d* - leaves from the middle part; *e* - lower old leaves; *f* - mixed leaf sample from healthy plants.

viroid populations *i.e.* in progeny of T50, T61, T75 and T92 thermomutants (Fig. 1*A*, *lanes 1 - 4*), while only 4 dominant and 9 minor bands were observed in infected tomato (Fig. 1*A*, *lane 5*). This observation suggests significant elimination of HLVd sequence variants upon the experimental transmission to a new host plant. From pre-selected cDNA clones, six were sequenced; one was double-, three triple-, one quadruple mutant and in one clone (No11) nine mutations were found. Except for the double mutated clone and clone No11, the origin of mutated cDNAs could not be easily established, as no original mutations were found in these clones. The double mutant originated probably from clone T75, since A_{12}->G mutation was found in this clone. The second mutation was deletion of G at position 6. The same deletion was found in one from three triple mutants (Fig. 2). This suggests a possible origin of this mutant from the progeny variant of T75 described above, providing G_{12}->A reversion. However, in the triple mutant an insertion of G at position 116 and G->A base change at position 179 were also found. In clone No11 three mutations A_7->G, U_{229}->C and C_{255}->U corresponded to original clone T61 and were preserved upon transmission (Fig. 2). Five base changes, G_{84}->A, C_{141}->U, U_{145}->C, U_{161}->C, C_{163}->A and one insertion of G at position 139 were new mutations which were not detected in the original pool of progeny of thermomutants from hop analysed previously (Matoušek *et al.* 2001). These results suggest selection, as well as wide molecular changes of HLVd upon transmission from hop to tomato, possibly as the early step of process of HLVd microevolution in this plant species. As judged from molecular hybridization experiments (Fig. 1*B*), the concentrations of mutated HLVd variants in tomato were about 40-times lower than the concentrations of thermomutants in infected hop, probably due to rather low fitness and adaptation of transmitted HLVd variants to this species. Interestingly, the tissue specific concentrations of HLVd population in tomato were very similar to distribution of tomato pathogen-PSTVd (*e.g.* Zhu *et al.* 2001), *i.e.* the highest HLVd concentration was in young sink leaves [0.5 ng g^{-1}(f.m.)], whereas much

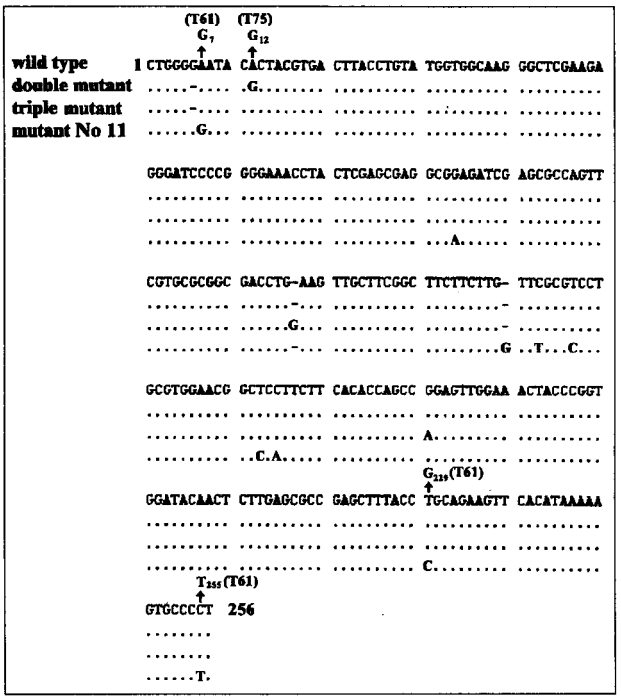

Fig. 2. The multiple alignment of cloned HLVd cDNAs. Three HLVd clones from tomato are aligned together with the wild type HLVd. The positons of mutations in the original thermomutants T61 and T75 from which new viroid variants may originate are indicated by the *arrows*.

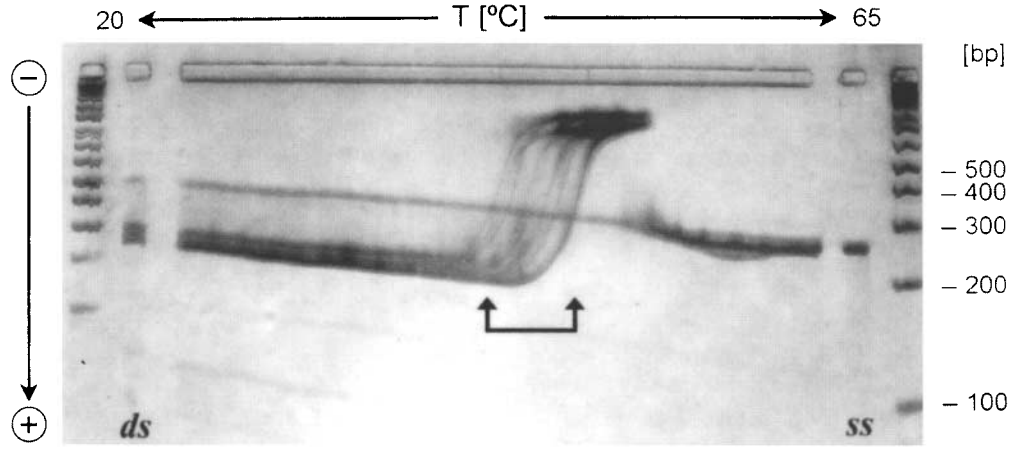

Fig. 3. Analysis of HLVd cDNA from *N. benthamiana* on TGGE. HLVd represents the third viroid generation maintained on *N. benthamiana*. cDNA sample was prepared by RT PCR, heated and allowed to form heteroduplexes. Then the sample was analysed in 6 % acrylamide gel containing 7 M urea using the temperature gradient 20 - 65 °C (*ds* and *ss* designate double- and single-stranded parts of DNA patterns, respectively). The range of melting points of HLVd heteroduplexes is indicated by the *arrows*. The gel was stained for nucleic acids with $AgNO_3$.

lower concentration ranging from 0.1 to 0.01 ng g^{-1}(f.m.) was found in older, fully expanded leaves (Fig. 1*B*).

It was found that the HLVd inoculum from tomato can be easily transmitted to other solanaceous plant: *N. benthamiana.* Fifty percent of HLVd infection was observed after the first transmission from tomato to *N. benthamiana* and more than 70 % infections were achieved in subsequent generations. However, unlike to hops, the concentrations of HLVd in *N. benthamiana* were much lower, about 0.002 ng g^{-1}(f.m.). Using RT PCR, cDNA was prepared from infected plant selected from the third *N. benthamiana* generation and analyzed using temperature gradient-gel electrophoresis (TGGE) system (Fig. 3). At least 9 distinct transition curves were discernible on the TGGE profile, suggesting the presence of quite distinct molecular variants of HLVd. Although these molecular variants have not been sequenced, it is obvious that a discrete viroid population evolved during continuous propagation in this species. This "low level" viroid population persisted in *N. benthamiana* for more than one year of plant maintenance in clima box.

Our results showed that pool of HLVd mutants from hop underwent some microevolutionary changes involving mutagenesis and selection upon transmission to other species. That viroids represent submolecular entities which undergo quick evolutionary changes could be judged from many examples indicating a wide mutagenesis and chimeric origin of some viroids like Columnea latent viroid (CLV) isolated from *Columnea erythrophae* (Hammond *et al.* 1989) or CLV-related variants from *Nematanthus wettsteinii* (Singh *et al.* 1992) or *Bunfelsia undulata* (Spieker 1996). The wide evolutionary changes and adaptation to different hosts can be predicted for the second hop viroid-HSVd that infects many different species in which it forms populations of closely related molecular variants (Sano *et al.* 2001). According to Sano *et al.* (2001) the sequence variants currently being observed in HSVd as it replicates in hops may represent a transition stage in which a viroid that originated in grapevine is in the process of adapting to a new host. It can be assumed in general that as many molecular variants, as higher probability to be transmitted to new species. The wild-type HLVd is well adapted to hop and forms very narrow population. Maybe, this is the reason of limited spreading of this viroid to other species. However, our data clearly show some potential of mutated HLVd variants which appear as progeny of thermomutants to be a source of evolutionary changes and biological contaminations.

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