Viroids: Sequence Variability and Evolution of Pathogenic RNA

J. MATOUŠEK

Institute of Plant Molecular Biology Academy of Sciences of the Czech Republic, 370 05 České Budějovice, Czech Republic

Tel.: +420 387 775 529, Fax: +420 385 300 356, E-mail: jmat@umbr.cas.cz

Abstract

Viroids as the smallest pathogenic circular single-stranded pathogenic RNAs form populations of quasi-species, which has been recently identified by thermodynamic methods like TGGE pre-selection and heteroduplex analysis. It was found that replication under thermal stress led to enormously high level of viroid mutagenesis. Mostly multiple mutants having non-random distribution of base changes were found. A specific "hot spots" were identified in the regions, where a characteristic "pathogenicity domains" are localised in different viroids of the pospiviroidae family. Specific viroid microevolution was observed upon artificial inoculation of non-host plant species. Our results suggest that viroid propagation under physiological stress can be assumed as important factor, which is among others, responsible for an appearance of viroid quasi-species in the nature. Evolution and new viroid patotypes could accumulate due to environmental stress including various pollutants may be a potential danger for cultured plants.

Keywords: viroid mutagenesis; quasi-species; DNA heteroduplexes; stress factors; viroid pathotypes

Viroid populations and evolution

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. 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). New viroid variants have been detected, for example, for citrus exocortis viroid (VISVADER & SYMONS 1985; OWENS *et al*. 1999, 2000), avocado sun blotch viroid (RAKOWSKI & SYMONS 1989), *Coleus blumei* viroid (SPIEKER *et al*. 1990), grapevine viroids (RIGDEN & 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). The last viroid represents the clear example of diverse viroid species having fifty four variants (e.g. SANO 2001). HSVd is now spreaded on on cucumber, grapevine, citrus, plum, peach, pear, apricot and almond plants (ASTRUC *et al*. 1996; CANIZARES *et al*. 1999). Despite the existence of viroid quasi-species, the mechanisms responsible for viroid mutagenesis and adaptations are poorly understood.

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The powerful methods to study viroid diversity

Viroid RNA adopts highly cooperative rod-like structure. This circular RNA can be easily analysed for variability using either RNA (e.g. MATOUŠEK *et al*. 1994) or DNA heteroduplexes methods. Thermodynamic parameters of viroid cDNA can be easily analysed using temperature gradient-gel electrophoresis (TGGE) (RIESNER *et al*. 1989) and based on these parameters DNA heteroduplexes method can be developed (MATOUŠEK *et al*. 2000). Using these methods, low level sequence variants, recombinant RNA species, or some particular quasi-species within the viroid populations can be studied. Quasi-species could exhibit differential adaptability in different plant tissue and genotypes, at different ontogenetic stages, or/and under different growth conditions. TGGE and DNA hetroduplexes methods enable specific *in vitro* pre-selection of mutants by extracting, re-PCR and re-cloning of deviating cDNAs from polyacrylamide gels. Such experiments we performed to analyse low replicating thermomutants of HLVd (MATOUŠEK *et al*. 2001). TGGE pre-selection and cDNA library screening enabled to select specific deviating forms for sequencing, saving time valuable materials for analysis of nucleic acids. The high resolution of mixtures of sequence variants, as well as the possibility to pre-select mutated cDNA forms, make these approaches suitable for easy analysis of complex populations of plant molecular pathogens like viruses and viroids.

Stress factors as possible motor of viroid evolution

It was found that replication of hop latent viroid (HLVd) in thermotreated hop led to enormously high level of viroid mutagenesis i.e. heat stress increased significantly the HLVd variation. While only about 1% of HLVd-specific sequences isolated from untreated control plants formed less thermostable cDNA structures distinguishable from TGGE pattern characteristic for the wild type cDNA homoduplex, more than 54% of cDNAs isolated from thermotreated plants differed from the control. A specific HLVd cDNA library was screened using electrophoresis of pre-formed cDNA heteroduplexes and 31 clones (9.8%) were identified as deviating forms. Nine of these clones were sequenced. The sequencing analysis revealed mostly triple and quadruple mutants, suggesting an accumulation of mutations in HLVd during successive replication cycles. In was found that the distribution of mutations in the HLVd genome is not random. No mutations were identified in the central part of CCR and in the region where general promoter motifs can be predicted by the analogy to potato spindle tuber viroid sequence and structure i.e. start position ($_{73}$CGAGCG<u>A</u>$_{79}$-putative start position underlined) and GC box (18 nucleotides upstream the transcription start). 71% of base changes were localised in the left half and 29% in the right part of the secondary structure of HLVd. A specific "hot spot" was identified in the regions, where a characteristic "pathogenicity domain" is localised in different viroids of the pospiviroidae family. Although all mutations destabilised the secondary structure of HLVd, the most of mutated HLVd cDNA clones were found to be infectious. However, mutated HLVd accumulated with much lower rate than wild type HLVd. While quite uniform band was detected by the cDNA heteroduplexes method after inoculation of wild type cDNA, several co-existing viroid variants were detected at early stages of infection with various mutants, probably due to the processes of reversion and viroid re-adaptation. On the one hand, these results suggest rather low exactness of viroid replication by the host-specific complex of RNA polymerase II under heat stress conditions and on the other hand, the replication under the physiological stress can be assumed as important factor which is among others responsible for the appearance of viroid quasi-species in the nature. It cannot be ruled out that other environmental stress factors could contribute to the appearance of viroid quasi-species. The pool of new mutants could serve as a substrate for viroid evolution in new hosts including economically important crops.

This idea is supported by our recent finding that HLVd microevolution led to the appearance of HLVd population on tomato which was maintained at levels detectable by molecular hybridisation, showing the highest concentration in apical leaves. HLVd was further transferred from tomato on *N. bentamiana,* where distinct HLVd sequence variants appeared and were stably maintained at low levels (MATOUŠEK *et al*. submitted).

Evolution and new viroid patotypes: a potential danger for cultured plants

Our previous work on HLVd revealed not only a highly erroneous replication mode of viroid under heat stress conditions, but also the fact that heat-treatment led to HLVd sequence destabilisation (MATOUŠEK *et al*. 2001). Several sequences, which evolved from original populations of thermomutants were isolated and characterised using specific computer programs (LÜCK *et al*. 1999). They all showed low thermostability

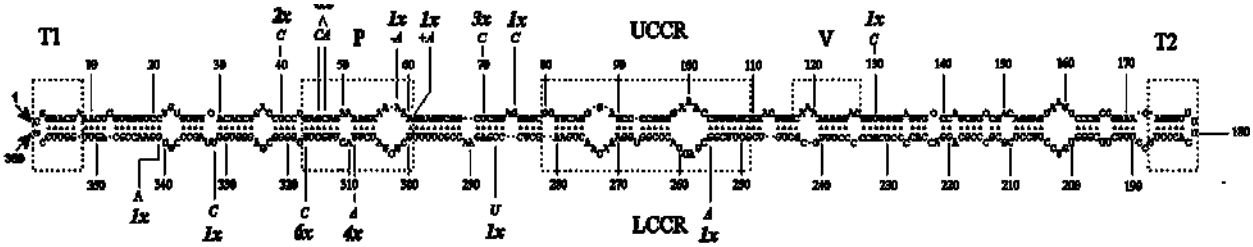

Figure 1. Localisation of PSTVd thermomutations. *N. benthamiana* plants infected with PSTVd intermediate strain were subjected to thermotreatment and PSTVd population was analysed by sequencing. Incidence of individual mutations is shown. UCCR and LCCR, upper and lower part of conserved region, respectively. T1 and T2 = terminal domains; P = pathogenicity domain

although induced by heat. This apparent contradiction was interpreted in the sense, that replication at high temperature generates less stable as well as more stable mutants, but the more stable mutants cannot survive at conditions of normal growth. Consequently, it can be assumed that the wild type viroid is adapted already to optimum stability at normal growth conditions. We showed recently (unpublished) that the phenomenon of induction of viroid variability under heat treatment is phenomenon of more general validity. For both, HLVd and PSTVd we found similar rates of quick accumulation of multiple mutants and formation of specific quasi-species soon after thermal stress (Figure 1). The strength of viroid pathogenicity domain is connected to its thermostability and structure. Viroid mutagenesis under stress conditions could results in the appearance of more pathogenic variants. This probably happened in the past (GRUNER *et al.* 1995), where highly pathogenic RG1 viroid strain evolved in tomato from viroid population maintained in greenhouse conditions under repeated heat shock conditions. Recently we characterised new potential thermopatotypes of PSTVd all having changed premelting loop within pathogenicity domain. This resulted in different bending of PSTVd molecule. Other viroid populations and patotypes are strongly fixed to plant genotype (e.g. SZYCHOWSKI *et al.* 1998; VISVADER & SYMONS 1985), however the mechanism of accumulation of these patotypes is not known.

CONCLUSION

Viroids form populations of quasi-species which can accumulate in host plants due to erroneous replication under various stress conditions. These quasi-species are important for viroid evolution and spreading to new hosts. New patotypes having changed pathogenicity determinants can evolve under environmental stress conditions due to changes of viroid thermostability and structure.

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