VIROIDS

Effect of Sequence Variation on the Biological Properties of *Citrus exocortis viroid* (CEVd)

M. Gandía and N. Duran-Vila

ABSTRACT. Analysis of cloned cDNAs recovered from a *Citrus exocortis viroid* (CEVd)-containing field isolate (E-117) showed that it was composed of a complex population with quasispecies structure containing a predominant sequence (V1) representing 52.8% of the overall population. V1 and eight additional variants (V2 to V9) were selected to construct dimeric clones to perform infectivity assays on citron and *Gynura aurantiaca.* Only citron plants inoculated with V1 and V6 became infected and developed the severe symptoms characteristic of the original isolate. In both cases, the infected citron plants contained populations of CEVd variants with V1 as the predominant sequence. None of the inoculated *Gynura* plants became infected. The results illustrate the existence of host specific requirements for viroid infection.

Citrus exocortis viroid (CEVd) is the casual agent of exocortis disease (15). It is a covalently closed circular RNA with a highly base paired rod-like structure which contains the five structural domains (T_L, P, C, V, T_R) model defined by Keese and Symons (8). CEVd is present in the infected hosts as a complex of sequence variants, which follows the "quasispecies model" proposed by Eigen (3) to describe heterogeneous populations of RNA molecules. The high variability found in viroid populations appears to be the result of their high mutation rate due to the absence of proof-reading by RNA polymerase during their replication.

In a previous study, 44 sequence variants were identified after the molecular characterization of 316 clones recovered from the field isolate of CEVd (E-117) (4). A master sequence (V1) with 98.65% and 98.12% nucleotide identity with CEVd-C (5) and CEVd-A (18) respectively, and accounting for 52.8% of the population, was identified. Another variant (V2) differing from V1 by a single addition (+G) at position 75 located in one of the loops of the P domain of the viroid secondary structure accounted for 25.3% of the population. Seven additional variants (V3 to V9) differing in one or two nucleotides from V1, were found more than once in the overall CEVd population. Most of the nucleotide differences among variants were located in the P domain of the secondary structure without disturbing their base pairing arrangement which is similar to CEVd strains inducing a severe reaction in tomato (19).

Here we report the generation infectious dimeric molecules of these nine variants recovered from CEVd (E117) and their infectivity and biological properties in Etrog citron and *Gynura aurantiaca*.

MATERIALS AND METHODS

Synthesis and cloning of dimeric viroid DNAs. Plasmids containing monomeric DNAs of the nine selected variants (V1 to V9) (4) were used as the viroid-DNA sources to generate infectious dimeric molecules. Monomeric viroid-DNA inserts were recovered as blunt-end products by PCR using the CEVd-specific synthetic oligonucleotides CEVd-1 (complementary to bases 81 to 98 of CEVd-A) and CEVd-2 (homologous to bases 99 to 117 of CEVd-A) (18), in a buffer containing 2 mM $MgSO_4$, 0.13 mM dNTPs, 0.5 µM of each primer and 1 U of Pwo DNA poly-

merase (Boeringer Mannheim). PCR parameters consisted of 35 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, with a final extension of 72ºC for 5 min. The size of the DNA product was determined by electrophoresis in 2% agarose gels.

The DNA products were phosphorylated using 10 mM ATP and 0.3U T4 DNA Kinase (Pharmacia), and were subjected to a ligation reaction with 2U T4 ligase (Gibco) for 16 h at 14°C. The dimeric molecules were purified from 2% agarose gels and were ligated into the positive Eco RV site of the pBS vector and transformed into *Escherichia coli*. Plasmids from transformed cells were subjected to restriction and sequence analysis to verify the correct tandem orientation of the dimeric inserts.

Infectivity assays. Etrog citron 861-S1 was propagated by grafting onto rough lemon rootstock, and *G. aurantiaca* as rooted cuttings. Four Etrog citron and five *Gynura* plants were slash inoculated with each plasmid preparation (4 µg per plant) containing dimeric inserts of each of the nine variants to be assayed. The inoculated plants were kept in the greenhouse at 28-32°C and observed for symptom expression and analyzed every 3 mo.

Viroid analysis. Samples (5 g) of young leaves were homogenized in 5 ml of extraction buffer containing water saturated phenol and the total nucleic acids were partitioned in 2M LiCl (16). The soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1mM $MgCl_2$ pH 7.4). Aliquots of nucleic acid preparations from inoculated citrons and *Gynura* were analyzed by slot-blot hybridization to confirm CEVd infection (14). The CEVd positive samples were used for cDNA synthesis and progeny analysis.

Viroid progeny analysis of CEVd infected plants. First strand synthesis was performed using the CEVd complementary specific oligonucleotide CEVd-1 and the RT-Superscript (Gibco). Second strand synthesis and amplification of dsDNA was performed with CEVd-1 and CEVd-2 primers in buffer containing 1 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 µM of each primer and 1 U of Taq DNA polymerase. PCR parameters were the same as described above. The size of the DNA product was determined by electrophoresis in 2% agarose gels. The PCR products were ligated to the pGEM vector and the plasmids were subject to restriction analysis to verify the presence of the expected inserts.

Heterogeneity (H) of the progeny was estimated following Nei's formula $H = n/(n-1)(1-\sum x_i 2)$ (9) where values range from 0 (all clones have the same nucleotide sequence) to 1 (all clones have different nucleotide sequences).

Identification of variants and sequence analysis. Cloned viroids were recovered from the plasmids by PCR amplification using the same conditions described above. The partially denatured PCR products were subjected to SSCP analysis (13) in 14% PAGE (14 × 11.5 × 0.075 cm gels) run at 200V constant voltage for 16 h. The DNA bands were visualized by silver staining (7).

Inserts from clones containing different variants identified by SSCP analysis were sequenced with an ABI PRISM DNA sequencer 377 (Perkin-Elmer). Alignments of multiple sequences were performed with the program Culstal V (6). Secondary structure analyses were performed with the program MFOLD (circular version) from the GCG package (21).

RESULTS

Infectivity and symptom expression of CEVd variants. Dimeric cDNAs from nine CEVd variants (V1-V9) were synthesized and ligated into the pBS vector. The correct tandem orientation of the

dimeric viroid inserts was verified by restriction and sequence analysis. Three out of the four citron plants inoculated with clone V1 (master sequence) developed the severe symptoms of stunting and leaf epinasty characteristic of the original CEVd (E-117) isolate, 3 mo after inoculation. One out of the four citron plants inoculated with cloned V6 also developed similar symptoms, 9 mo after inoculation. None of the plants inoculated with the other variants developed symptoms and CEVd was undetectable by molecular hybridization analysis. No infection was detected in the inoculated *Gynura* plants over a 12-mo period.

Viroid progeny on citron inoculated with V1. Nucleic acid extracts from citrons inoculated with the infectious clone V1 were subjected to retrotranscription, PCR amplification and cloning, 3, 6 and 9 mo after inoculation. To determine the composition and temporal evolution of the CEVd population in the infected plants, 27 clones were subjected to SSCP and nucleotide sequence analysis. The results (Table 1) indicate that the CEVd population recovered was very similar to the original isolate CEVd (E-117) (4) with V1 and V2 as the major components. As expected, V1 was the predominant variant in the first analysis performed 3 mo after inoculation, but its frequency decreased with the development of new variants whose frequency increased from 7.3% to more than 30% after 3-6 additional months. As a result of these changes, the heterogeneity (H) of the population increased from 0.6 to 0.8. According to the calculated H values, the population appeared to have reached a steady-state equilibrium, 6 mo after inoculation.

The infectivity of variant V1, the viroid progeny recovered upon infection and the expression of symptoms 3 mo after inoculation was similar to that of the original isolate.

Viroid progeny on citron inoculated with V6. Nucleic acid extracts from citrons inoculated with the infectious clone V6, whose frequency was 1.3% in the original population, were subjected to retrotranscription, PCR amplification and cloning, 9 mo after inoculation. After SSCP and sequence analysis of 30 full length clones, 13 different variants were identified, with V1 representing 46% of the population. It must be noted that variant V6 was not recovered, but new variants were identified. The long incubation period required for CEVd detection and symptom expression suggests that citron is a very restrictive host in terms of its ability to be infected with certain CEVd variants. The observed results

TABLE 1
EVOLUTION OF THE POPULATION OF CEVd VARIANTS RECOVERED UPON INFECTION WITH V1

	Frequency of CEVd variants		
Variants[z]	3 mo	6 mo	9 mo
V1	52.0%	26.0%	36.8%
V2	37.0%	37.0%	30.6%
V3	3.7%	3.7%	0%
Others	7.3%	33.3%	32.6%
H[y]	0.611	0.811%	0.756%

[z]CEVd variants V1, V2 and V3 with the same nucleotide sequences found in the original population (4). Others = those variants found only once (low frequency variants). Their frequency is the sum of those variants found only once.

[y]H: Heterogeneity of the population was calculated as $H = n/(n-1)(1-\sum x_i 2)$, where n is the number of clones analyzed, x_i is the frequency of each variant in the population.

suggest that replication and accumulation of CEVd to detectable levels was possible only after a more fit variant (V1) was generated by a single point mutation.

DISCUSSION

CEVd has a very wide experimental and natural host range, inducing symptoms or replicating as latent infections. CEVd has also been shown to be a highly variable viroid with a large number of sequences reported. Pathogenicity studies with viroids have been accomplished with natural variants, induced mutants and synthetic chimeras of CEVd (19, 20), *Potato spindle tuber viroid* (10, 12) and *Tomato apical stunt viroid* (11) using tomato as a host. These studies demonstrated that alterations or changes within conserved regions of the viroid molecule modulate infectivity and replication. However, due to the difficulties inherent in conducting this kind of assays in woody species, information regarding the pathogenic effects of CEVd variants in citrus hosts is still lacking.

The results reported here regarding the infection of citron with nine different clones of CEVd obtained from a single field isolate, suggest that infectivity in citron is restricted to a few specific variants. V1, the predominant variant in the population of the original isolate was readily infectious showing detectable replication/accumulation levels, whereas other variants containing single nucleotide changes affecting the P domain, had a very low infectivity (V6) or were non-infectious. It must be noted that V2, which appears to be non-infectious, was identified in high frequencies, both in the original CEVd source and in the plants infected with V1. This suggests that the specific change in the P domain characteristic of this nucleotide variant impaired its capacity to infect the host plant, but is able to replicate once inside an infected cell.

The results obtained with the inoculation of citron with V6 show that this clone has a very low specific infectivity and CEVd was detectable only 9 mo after inoculation. This observation suggests that V6 is infectious but replicates inefficiently and accumulates below detection levels. This is supported by the results of the progeny analysis in which the inoculated variant was undetected, whereas V1 was found as the most frequent. These results suggest that the infection became apparent only after V1 with high replication/accumulation properties was generated from V6 as result of a single A→U mutation at position 309. V1, once available, probably displaced V6 in the progeny.

Since the nucleotide changes present in the nine variants tested affected the P domain without disrupting its secondary structure or any of the other regions reported as being critical for replication, the lack of infectivity found indicates that the capacity of citron to become infected and replicate CEVd variants is much more restricted than anticipated. The progeny recovered from citrons inoculated with V6 illustrates the major role of V1 and the effect of the host in selecting the best fit variants (2).

In contrast with the high specific infectivity found when *Gynura* was inoculated with field isolates, none of the tested variants were infectious in this host. Comparison of the primary structure of the clones tested with the sequences obtained from infected *Gynura* (1, 17) indicated that U→A at position 6; CU→AG at position 265-266 and U→A at position 281 are common to those sequences recovered from infected *Gynura* which seem confer host specificity. The results of this study are in agreement with previous work demonstrating the effect of host and tissue selection on the recovery of specific CEVd sequences (17).

In conclusion, the results of the present study illustrate how one of the variants present in a viroid iso-

late may play a major role in terms of infectivity and symptom expression. The high frequency of V1 found in the original source as well as in those inoculated citrons that become infected, even when inoculated with clone V6, reveals that it is the fittest variant within the population. Therefore a careful choice of the most biologically active variant within a given isolate must be made when a CEVd isolate is being characterized. In this regard, the information gathered from alternate hosts must be interpreted with caution.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Luis Rubio for his helpful comments. This work was supported by IVIA fellowship to the first author and INIA grants SC97-108 and RTA01-119.

LITERATURE CITED

1. Chaffai, M.
   2000. Mecanismos de defensa asociados a enfecciones por viroides. Tesis doctoral. Universidad de Valencia.
2. Domingo, E. and J. J. Holland
   1997. RNA virus mutations and fitness for survival. Annu. Rev. Microbiol. 51: 151-178
3. Eigen, M.
   1993. The origin of genetic information: Viruses as models. Gene 135: 37-47.
4. Gandía, M., A. Palacio, and N. Duran-Vila
   2000. Variability of citrus exocortis viroid (CEVd). In: *Proc. 14th Conf. IOCV*, 265-272. IOCV, Riverside, CA.
5. Gross, H. J., G. Krupp, H. Domdey, M. Raba, P. Jank, C. Lossow, H. Alberty, K. Ramm, and H. L. Sänger
   1982. Nucleotide sequence and secondary structure of citrus exocortis and chrysanthemum stunt viroid. Eur. J. Biochem. 121: 249-257.
6. Higgins, P. G. and P. M. Sharp
   1989. Fast and sensitive multiple sequence alignment on a microcomputer. Comp. Appl. Biosci. (CABIOS) 5: 151-153.
7. Igloi, G. L.
   1983. Silver stain for the detection of nanogram amounts of tRNA following two-dimensional electrophoresis. Analyt. Biochem. 134: 184-188.
8. Keese, P. and R. H. Symons
   1985. Domains in viroids: Evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. Proc. Natl. Acad. Sci. USA 82: 4582-4586.
9. Nei, M.
   1987. *Molecular Evolutionary Genetics*, Columbia University Press, New York.
10. Owens, R. A., R. W. Hammond, R. C. Gardner, M. C. Kiefer, S. M. Thompson, and D. E. Cress
    1986. Site-specific mutagenesis of potato spindle tuber viroid cDNA. Plant Mol. Biol. 6: 179-192.
11. Owens, R. A.
    1990. Mutational analysis of viroid pathogenicity: Tomato apical stunt viroid. Mol. Plant-Microbe Interact. 6: 374-380.
12. Owens, R. A., G. Steger, Y. Hu, A. Fels, R. W. Hammond, and D. Riesner
    1996. RNA structural features responsible for potato spindle tuber viroid pathogenicity. Virology 222: 144-158.
13. Palacio, A. and N. Duran-Vila
    1999. Single-strand conformation polymorphism (SSCP) analysis as a tool for viroid characterisation. J. Virol. Meth. 77: 27-36.
14. Palacio, A., X. Foissac, and N. Duran-Vila
    2000. Indexing of citrus viroids by imprint hybridization: Comparison with other detection methods. In: *Proc. 14th Conf. IOCV*, 294-301. IOCV, Riverside, CA.
15. Semancik, J. S. and L. G. Weathers
    1972. Exocortis virus: An infectious free nucleic acid plant virus with unusual properties. Virology 47: 456-466.
16. Semancik, J. S., T. J. Morris, L. G. Weathers, G. F. Rordorf, and D. R. Kearns
    1975. Physical properties of a minimal infectious RNA (viroid) associated with the exocortis disease. Virology 63: 160-167.
17. Semancik, J. S., J. A. Szychowski, A. G. Rakowski, and R. H. Symons
    1993. Isolates of citrus exocortis viroid recovered by host and tissue selection. J. Gen. Virol. 74: 2427-2436.

18. Visvader, J. E., A. R. Gould, G. E. Bruening, and R. H. Symons
1982. Citrus exocortis viroid: Nucleotide sequence and secondary structure of an Australian isolate. FEBS Lett. 137: 288-292.
19. Visvader J. E. and R. H. Symons
1985. Eleven new sequence variants of citrus exocortis viroid and the correlation of sequence with pathogenicity. Nucleic Acids Res. 13: 2907-2920.
20. Visvader, J. E. and R. H. Symons
1986. Replication of *in vitro* constructed viroid mutants: Location of the pathogenicity modulating domain of citrus exocortis viroid. EMBO J. 5: 2051-2055.
21. Zuker, M.
1989. On finding all suboptimal folding of an RNA molecule. Science 244: 48-52.