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In vivo generated *Citrus exocortis viroid* progeny variants display a range of phenotypes with altered levels of replication, systemic accumulation and pathogenicity

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ABSTRACT

Citrus exocortis viroid (CEVd) exists as populations of heterogeneous variants in infected hosts. In vivo generated CEVd progeny variants (CEVd-PVs) populations from citrus protoplasts, seedlings and mature plants, following inoculation with transcripts of a single CEVd cDNA-clone (wild-type, WT), were studied. The CEVd-PVs population in protoplasts was heterogeneous and became progressively more homogeneous in seedlings and mature plants. The infectivity and pathogenicity of selected CEVd-PVs was evaluated in citrus and herbaceous experimental hosts. The CEVd-PVs U30C, G128A and U182C were not infectious; G50A and 108U+ were infectious but reverted back to WT and 62A+, U129A and U278A were infectious, genetically stable and more severe than WT. The 62A+ and U278A and U129A accumulated at higher levels than WT in protoplasts and seedlings respectively. The effect of specific mutations on the predicted secondary structure of the CEVd-PVs' RNA coupled with the infectivity and replication studies suggested complex structure-to-function relationships for CEVd.

Introduction

Viroids are the smallest known plant infectious agents (246–475 nt) and have a single stranded, circular, highly structured, non-protein-coding, non-encapsidated RNA genome. Viroid genomes have approximately equimolar amounts of Watson–Crick base pairs in addition to non-canonical GU base pairing resulting in a self-complementary rod-like or quasi-rod like structure (Flores et al., 2005; Semancik et al., 1975; Tabler and Tsagris, 2004).

Viroids have been identified as the etiologic agents for a variety of maladies in agriculturally significant crops. In citrus, exocortis is an important viroid disease of the commonly used rootstock, *Poncirus trifoliata* and is characterized by bark scaling, leaf epinasty, yellow blotching of twigs, and severe tree stunting (Singh et al., 2003). *Citrus exocortis viroid* (CEVd), the causal agent of the exocortis disease (Fawcett and Klotz, 1948), was the first characterized citrus viroid (Semancik and Weathers, 1972). CEVd is the largest of the citrus viroids with 371–475 nt and belongs to the genus *Pospiviroid* of the family *Pospiviroidae* (Flores et al., 1998; Semancik et al., 1994; Semancik and Duran-Vila, 1999; Szychowski et al., 2005).

All members of *Pospiviroidae* have a quasirod-like secondary structure of minimal free energy with five structural-functional domains; the left-terminal (TL); pathogenicity (P); central domain (C) containing a region conserved in many related viroid species; variable (V); and right terminal (TR) (Keese and Symons, 1985; Sano et al., 1992). In addition to these domains, viroid genomes possess additional structural features such as imperfect palindromes, pre-melting (PM) regions and thermodynamically metastable structures. The functionality of viroid structural features has been demonstrated in several members of *Pospiviroidae* in infectivity (Candresse et al., 2001; Hammond and Owens, 1987; Loss et al., 1991; Wassenegger et al., 1996; Zhu et al., 2002), movement (Hammond, 1994; Zhong et al., 2008), symptom expression (Qi and Ding, 2002, 2003; Wassenegger et al., 1996; Zhu et al., 2002), and replication (Kolonko et al., 2006; Owens et al., 1996; Riesner, 1990; Zhong et al., 2006).

Viroids of the *Pospiviroidae* family replicate in the nucleus by host DNA-dependent RNA polymerase II. They rely on their conserved RNA secondary structures (i.e. hairpin I and loop E) and cellular factors for processing the multimeric replication intermediates into unit length

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molecules that are subsequently ligated to form circular infectious progeny viroids (Flores and Semancik, 1982; Flores et al., 2004; Gas et al., 2007; Mühlbach and Sänger, 1979; Warrilow and Symons, 1999). It is generally accepted that viroids replicate as polymorphic populations which are described as variations around a master sequence (Ambrós et al., 1998; Codoner et al., 2006; Eigen et al., 1989) in accordance with the "quasispecies model" proposed by Eigen (1993). The main factors responsible for the observed genetic diversity during viroid replication are most likely the absence of proof-reading activity of the plant polymerase(s), the large population size, and the rapid rate of RNA replication (Codoner et al., 2006; Domingo and Holland, 1997; Gandía and Duran-Vila, 2004; García-Arenal et al., 2001; Holland et al., 1982).

Genomic diversity of CEVd RNA populations at the whole-plant level has been studied extensively in the past (Gandía et al., 2005, 2007; Semancik et al., 1993; Visvader and Symons, 1985). In an elegant small-scale evolutionary experiment conducted over a period of 10 years, Bernad et al. (2009) demonstrated that the CEVd population profiles changed drastically in susceptible and tolerant citrus hosts. However, little is known about the genomic diversity of the replicating viroid RNA populations at the cellular level. It is conceivable that the selection pressure is much greater and more complex at the plant level as compared to the cellular level in protoplasts. An obvious difference in protoplasts, in comparison with the whole plant, is the absence of both cell-to-cell and long distance movement. Thus, protoplasts transfected with viroid RNAs offer a synchronous system to study the population composition and genomic diversity at the cellular level (Aoki and Takebe, 1969; Faustmann et al., 1986; Flores et al., 2008; Lin and Semancik, 1985; Mühlbach, 1982; Mühlbach and Sänger, 1977; Qi and Ding, 2002; Zhong et al., 2006, 2008). Such a system is very valuable when it is coupled with a rapid, sensitive and quantitative method for monitoring viroid replication. The number of reports on reverse-transcription (RT) quantitative polymerase chain reaction (qPCR) methods for the quantitative detection of citrus viroids is very small and limited to the whole-plant level (Rizza et al., 2009).

In the present study, three different citrus experimental systems: protoplasts, seedlings and mature plants were utilized. Following inoculation with transcripts of a single CEVd cDNA-clone (wild-type, WT), a large number of in vivo generated CEVd progeny variants (CEVd-PVs) was analyzed for the estimation of the population composition and genomic diversity of the CEVd progeny RNAs. The infectivity and pathogenicity of selected CEVd-PVs from the recovered progeny populations were evaluated in citrus and herbaceous experimental hosts. Their replication and systemic accumulation were evaluated using a newly developed RT-qPCR assay. The possible effects of specific mutations on the predicted secondary structure of the CEVd-PVs' RNA and their concomitant effects on the observed biological properties are also discussed.

Results

CEVd-WT

The parental CEVd RNA inoculum, which is referred as CEVd-WT in this study, was obtained from the viroid collection of the Citrus Clonal Protection Program (CCPP) at the University of California, Riverside (UCR). CCPP's sweet orange (*Citrus sinensis*) source tree of CEVd isolate E-811 has been repeatedly tested positive for a single disease agent for over 25 years. Viroid RNA from the source tree was isolated, reverse-transcribed and the amplified full length cDNA was cloned and sequenced. The most abundant RNA (83%) identified by the sequencing of 30 CEVd E-811 cDNA clones was used as WT inoculum in the present study. The nucleotide sequence of the CEVd-WT was deposited in the genetic sequence database at the National Center for Biotechnical Information (NCBI) (GenBank ID: GU295988).

CEVd-WT replication in citrus protoplasts at early infection

Using the methods of Grosser and Gmitter (1990) and Grosser et al. (2000), we were able to reliably obtain up to 40 million protoplasts from a 40 ml suspension cell culture of *C. amblycarpa* (viability over 90%). In vitro transcripts of a single CEVd-WT cDNA-clone were transfected into protoplasts and the relative levels of viroid progeny RNAs were monitored with a newly developed RT-qPCR assay.

The observed increase of the viroid RNA titer (2.5 fold) between days 1 and 2 post-transfection (dpt) was not statistically significant ($p = 0.720$). Subsequently, the CEVd-WT progeny RNA (positive sense strands) increased significantly by 80, 100 and 130 folds at 3, 4, and 5 dpt respectively ($F = 134.116$, d.f. = 14, $p < 0.001$) (Fig. 1). Similarly, the increase of replicative intermediates (negative sense strands) was significant after 2 dpt ($F = 134.116$, d.f. = 14, $p < 0.05$ and 2 dpt vs. 1 dpt, $p = 0.159$). The ratio of positive to negative sense viroid strands varied with incubation time and peaked at 3 dpt with a ratio of 15:1 (Fig. 1). The lowest detectable amount of progeny viroid RNA by RT-qPCR was 20 attograms (20×10^{-12} μg) per μg total RNA. The results of the population composition and genomic diversity analysis for the CEVd-WT progeny RNA at the cellular level are presented in Table 1.

CEVd-WT systemic accumulation in citrus seedlings at early infection

The stem of four-week old tube-grown 'Etrog' citron seedlings were slash inoculated with in vitro transcripts of a single CEVd-WT cDNA-clone and the systemic accumulation of the viroid progeny RNA was monitored with RT-qPCR assay.

CEVd-WT progeny RNA was detectable in the stem, at all time points tested, and the titer increased significantly with time from day 1 to day 5 ($F = 730.334$, d.f. = 14, $p < 0.001$) (Fig. 2). At the end of 5 days post inoculation (dpi), a 35 fold increase in viroid titer was recorded at the stem (Fig. 2B). CEVd-WT progeny RNA was first detected in the roots by the end of 3 dpi and interestingly, the viroid RNA accumulation did not increase significantly with time in the root tissue ($p > 0.05$) (Fig. 2B). CEVd-WT progenies were detected in the leaves of the seedlings only at 5 dpi ($H = 13.796$, d.f. = 4, $p = 0.008$) (Fig. 2B). At five dpi, the CEVd-WT progeny RNA accumulation in stems was significantly higher than roots and leaves ($F = 3776.693$, d.f. = 8, $p < 0.001$) while leaves accumulated slightly higher viroid titer in comparison to the roots ($F = 3776.693$, d.f. = 8, $p = 0.046$) (Fig. 2B).

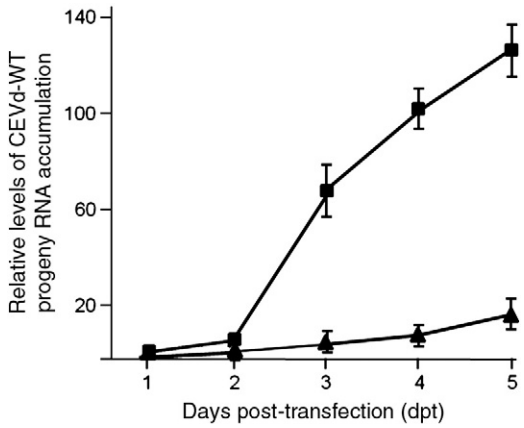

Fig. 1. Replication of *Citrus exocortis viroid* wild-type (CEVd-WT) in citrus protoplasts (*Citrus amblycarpa* Ochse.) was monitored by reverse transcription quantitative polymerase chain reaction. Relative levels of replicating CEVd progeny RNA (+ sense, represented by squares) and their replicative intermediates (− sense, represented by triangles) were monitored over a period of five days post-transfection (dpt). The accumulation levels of CEVd progeny RNA after 1 dpt was arbitrarily set to the value of one. The levels of CEVd progeny RNA from subsequent days were presented as relative levels to this reference value. Data points present the means and standard deviation of triplicate experiments.