UNIVERSIDAD POLITÉCNICA DE VALENCIA
DEPARTAMENTO DE BIOTECNOLOGÍA

ESTUDIOS DE PATOGENICIDAD DE VIROIDES DEL GÉNERO *APSCAVIROID* Y *HOSTUVIROID* EN CÍTRICOS

TESIS DOCTORAL

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Resumen

Estudios realizados en *Atalantia citroides*, un género afín de los cítricos, mostraron que estaba infectada con un viroide no descrito hasta el momento. Este nuevo viroide tiene un genoma de 293–294 nucleótidos con una alta proporción de bases GC, una región central conservada que es característica de los miembros del género *Apscaviroid*, y también la región terminal conservada que tienen este y otros géneros de la familia *Pospiviroidae*. La estructura secundaria de mínima energía libre predicha para este nuevo viroide es una conformación en forma de varilla con un 68.7% de nucleótidos apareados y con una identidad de secuencia respecto a los otros viroides inferior al 90%, que es el límite convenido para separar las diferentes especies de viroides dentro de un mismo género. Los ensayos de infectividad utilizando el cidro Etrog han mostrado que este nuevo viroide produce síntomas característicos suaves. La co-inoculació de este nuevo viroide con *Citrus bent leaf viroid* o con *Citrus dwarfing viroid*, que también son miembros del género *Apscaviroid*, causa interacciones sinérgicas que se manifiestan induciendo síntomas muy pronunciados en las hojas y un enanismo muy marcado. Este aumento en la intensidad de síntomas no está acompañado por variaciones en la acumulación del viroide en la planta ya que su concentración se mantiene inalterada en plantas co-inoculadas. De acuerdo con estas propiedades moleculares y biológicas, así como por su capacidad por replicarse en *A. citroides*, este nuevo viroide que tentativamente hemos nombrado *Citrus viroid V* (CVd-V), ha sido propuesto como una nueva especie del género *Apscaviroid*.

El análisis de 64 muestras provenientes de varias zonas citrícolas ha mostrado que CVd-V está presente en los Estados Unidos, España Nepal, y el Sultanato de Oman. Estos resultados indican que este viroide no ha surgido recientemente y está bastante difundido por diferentes partes del mundo. Los ensayos de transmisión a naranjo, mandarino, híbridos de mandarino, clementito, satsuma, limonero, naranjo amargo, lima Tahití, lima dulce de Palestina, calamondín, bergamoto y kumquat han mostrado que todas estas especies son huéspedes de CVd-V. Se han probado una serie de métodos de detección como el "slot blot", la hibridación "northern" y la RT-PCR utilizando tanto el cidro Etrog como bioamplificador o directamente especies i cultivares comerciales.

Para evaluar el efecto en la expresión de síntomas que produce el intercambio de segmentos discretos de la molécula de CVd-V por los segmentos correspondientes de CDVd, se sintetizaron siete viroides quiméricos que se inocularon mecánicamente a tres plántulas de cidro Etrog. El análisis de estas plantas mediante hibridación y RT-PCR mostró que solo una de las tres plantas inoculadas con la quimera Ch5 (CVd-V con

el dominio Terminal izquierdo de CDVd) estaba realmente infectada. Se ha demostrado que esta quimera es estable salvo por la sustitución 42C→U. Las plantas infectadas con Ch5 no muestran ningún tipo de síntomas y las plantas co-infectadas con Ch5 y CVd-V o CDVd muestran los mismos síntomas que las plantas infectadas solamente con CVd-V o con CDVd, respectivamente. Estos resultados indican que el dominio terminal izquierdo está involucrado en la patogenicidad de CVd-V y que no existen interacciones entre Ch5 y los otros dos viroides. El análisis de las plantas co-infectadas ha mostrado que tanto CVd-V como CDVd desplazan a Ch5 aunque se llega a detectar a concentraciones muy bajas.

La cachexia de los cítricos es una enfermedad producida por *Hop stunt viroid* (HSVd). Las variantes patogénicas y no patogénicas varían en lo que se conoce como "cachexia expression motif" que consta de cinco o seis nucleótidos localizados en el dominio variable de su estructura secundaria. Por medio de mutagénesis dirigida se obtuvieron una serie de mutantes para investigar si todos estos nucleótidos son necesarios para la infectividad y/o expresión de síntomas. Los resultados confirman que el "cachexia expression motif" juega un papel muy importante en la inducción de síntomas y que cambios sutiles dentro de este motivo modulan su expresión, llegando incluso a suprimirla.

Índice

Introducción

1. Los viroides como entidad biológica

En la primera mitad de la década de los 70 se caracterizaron los agentes causales de la enfermedad del tubérculo fusiforme de la patata (PSTV) (Diener, 1971) y de la enfermedad de la exocortis de los cítricos (CEV) (Semancik y Weathers, 1972). Se descubrió así un nuevo tipo de replicón infeccioso con características moleculares propias y de tamaño aproximadamente diez veces inferior a los virus más pequeños conocidos. A esta nueva entidad biológica se la definió de distintas formas como "pathogene" o "metavirus" aunque el término aceptado a día de hoy por la comunidad científica es el de "viroide".

Los viroides son replicones infecciosos que se acumulan de forma sistémica en sus huéspedes. Su ciclo infectivo consta de distintas etapas en las que destacan; la entrada en el huésped, el movimiento para localizar la maquinaria replicativa, la utilización de esta maquinaria para su propia replicación, su traslado hasta el sistema vascular, su difusión sistémica a través de la planta y su transmisión a otras plantas. A pesar de que este ciclo es muy similar al de otro tipo de replicones infecciosos, los viroides tienen propiedades moleculares y biológicas propias que los distinguen de los virus y de los RNAs satélites (Diener, 1991, Flores *et al*. 2005, Ding y Itaya, 2006). La principal característica de los viroides es que su ciclo infectivo está exclusivamente mediado por factores del huésped. Los virus, que molecularmente son muy distintos a los viroides, codifican proteínas que intervienen en distintas etapas de su ciclo como la replicación, el movimiento o la encapsidación para eludir los sistemas de defensa de la planta. Los RNAs satélites son molecularmente muy semejantes a los viroides con los que comparten mecanismos replicativos. Sin embargo, los RNAs satélites no pueden infectar sin la presencia de un virus auxiliar. Funcionalmente por tanto, los viroides son dependientes de la maquinaria transcripcional del huésped mientras que los virus lo son de la maquinaria de traducción. Los RNAs satélites son dependientes de ambas ya que se transcriben con la maquinaria del huésped y requieren la presencia del virus auxiliar al que parasitan.

El estudio de los viroides tiene dos claras vertientes. Por un lado se estudian desde el punto de vista de la patología vegetal ya que se ha demostrado que varias enfermedades de plantas son causadas por viroides. Hasta hoy se conocen una treintena de viroides, la mayoría de ellas asociadas a algún tipo de patología. Desde esta

perspectiva se abordan aspectos prácticos como la búsqueda de variedades y especies tolerantes o resistentes, la puesta a punto de técnicas de diagnóstico o el desarrollo de programas de certificación de plantas libres de viroides que han sido de gran ayuda para la protección de cultivos sensibles. Por otro lado, se estudian aspectos básicos de su biología como su replicación, su movimiento, o las interacciones con sus huéspedes. Los viroides son las entidades biológicas más simples y de menor genoma conocidas hasta el momento. Su estudio no solo nos aporta información acerca de ellos sino que dada su capacidad para interactuar con el huésped son un magnifico modelo para la comprensión de las bases moleculares que rigen los mecanismos celulares.

2. Características moleculares de los viroides

2.1. Estructura primaria

Los viroides son moléculas de RNA circular monocatenario cuyo tamaño oscila entre 246 y 402 nucleótidos. Su secuencia presenta auto-complementariedad de bases generalmente con una alta proporción en citosina y guanina lo que conlleva que la molécula viroidal tenga una robusta estructura secundaria (Flores *et al.* 2005). El genoma de los viroides no codifica peptidos ni proteínas. Sin embargo, el alto grado de conservación de los genomas viroidales, así como la pérdida de infectividad que suele provocar la admisión de cambios en su secuencia, muestran la gran relevancia biológica de su estructura primaria (Zhong *et al*. 2008). Pese a no codificar proteínas, la estructura primaria de los viroides determina la conformación del RNA y la presencia de motivos que deben mediar la interacción del viroide con factores del huésped. Su simplicidad molecular y su genoma tan reducido se contrarrestan con su capacidad para interactuar con la maquinaria celular del huésped y así llevar a cabo todas las funciones necesarias de su ciclo infectivo.

Los viroides han sido clasificados en dos familias atendiendo tanto a características moleculares como biológicas. Los miembros de la familia *Pospiviroidae*, con *el viroide del tubérculo fusiforme de la patata* (PSTVd) como especie tipo, poseen motivos de secuencia y de estructura conservados (Flores *et al*. 1998). Por otro lado los viroides de la familia *Avsunviroidae*, con *el viroide del manchado solar del aguacate* (ASBVd) como especie tipo, no poseen regiones conservadas salvo los motivos

concernientes a la formación de estructuras ribozimáticas que utilizan en su ciclo replicativo (Flores *et al*. 1998).

2.2. Estructura secundaria

Como se ha mencionado anteriormente, los viroides son RNAs monocatenarios aunque su alto grado de estructura secundaria les otorga algunas características moleculares más propias de las moléculas bicatenarias. Un ejemplo en este sentido es la capacidad que tienen muchos viroides para mantenerse disueltos en soluciones de cloruro de litio a diferencia de las moléculas de RNA de cadena simple poco estructuradas (Flores *et al*. 1998). La estructura secundaria de los viroides ha sido estudiada mediante programas informáticos que calculan la conformación termodinámica más estable en unas condiciones estandarizadas (Zuker, 1989). Según estas aproximaciones *in sílico,* las moléculas viroidales en condiciones no desnaturalizantes adoptan conformaciones donde se alternan zonas de bases apareadas en forma de hélice con zonas desapareadas que forman bucles (Gross *et al*.,1978, Riesner *et al*.,1979). Atendiendo a estas estructuras, los viroides de la familia *Pospiviroidae* se caracterizan por adoptar forma de varilla o casi-varilla, mientras que los viroides de la familia *Avsunviroidae*, a excepción de ASBVd, adoptan conformaciones ramificadas (Flores *et al*. 1998).

Las estructuras obtenidas mediante estos programas han sido confirmadas por medio de datos experimentales. Se ha comprobado mediante aproximaciones fisicoquímicas como la microscopía electrónica, el mapeo enzimático o la electroforesis, que *in vitro* los viroides de la familia *Pospiviroidae* adoptan forma de varilla. (Sogo *et al*. 1973; Sänger *et al*. 1976; Riesner y Gross, 1985). Por otro lado, el análisis de variantes de secuencia que presentan mutaciones compensatorias, indican que *in vivo* se mantienen tanto las estructuras predichas de los viroides de la familia *Pospiviroidae* como las de los viroides de la familia *Avsunviroidae* (Haseloff *et al*. 1982; Semancik *et al*. 1994; Navarro y Flores, 1997; de la Peña *et al*. 1999).

2.3. Dominios estructurales, motivos de secuencia y estructuras metaestables

2.3.1. Familia *Pospiviroidae*

Se ha propuesto un modelo que divide la estructura en forma de varilla de los viroides de la familia *Pospiviroidae* en cinco dominios (Keese y Symons, 1985). Según este esquema la molécula se divide en dos dominios terminales, uno en el lado derecho (T_R) y otro en el izquierdo (T_L), en un dominio patogénico (P) y otro variable (V) que flanquean a un dominio central (C) a izquierda y derecha respectivamente. A estos dominios se les han asignado funciones biológicas, aunque en este sentido hay que destacar que este modelo se elaboró a partir de la homología de secuencia de los pocos viroides caracterizados hasta ese momento y estudios posteriores indican una correlación mucho más compleja entre distintas partes de su genoma y las funciones biológicas que desempeña (Ding y Itaya, 2007).

Como se ha mencionado anteriormente, los viroides de la familia *Pospiviroidae* se caracterizan por la existencia de una serie de motivos de secuencia conservados (Flores *et al.* 1998). Estos motivos deben desempeñar un papel importante en el ciclo infectivo ya que una alteración en su secuencia compromete la viabilidad de los mismos. El motivo conservado más estudiado, es la denominada región central conservada (CCR) que consiste en dos series de nucleótidos en ambas hebras del dominio C (Figura 1). La serie de nucleótidos de la hebra superior consta de una zona central flanqueada por dos repeticiones invertidas o palindrómicas. Este motivo parece estar involucrado en el procesamiento del viroide durante su replicación (Diener 1986, Visvader *et al*. 1985). Los viroides de la familia *Pospiviroidae* poseen una segunda región conservada situada en el dominio T_L. Dependiendo del viroide que se trate esta región puede constar de 13 o 16 nucleótidos situados en la hebra superior y que se denomina Región Terminal Conservada (TCR) (Figura 1) (Flores *et al*. 1997), o bien constar de 13 nucleótidos situados en el extremo del dominio y que se denomina Horquilla Terminal Conservada (TCH) (Figura1) (Puchta *et al*.1988, Flores *et al*. 1997). La función de dichas regiones se desconoce hasta el momento.

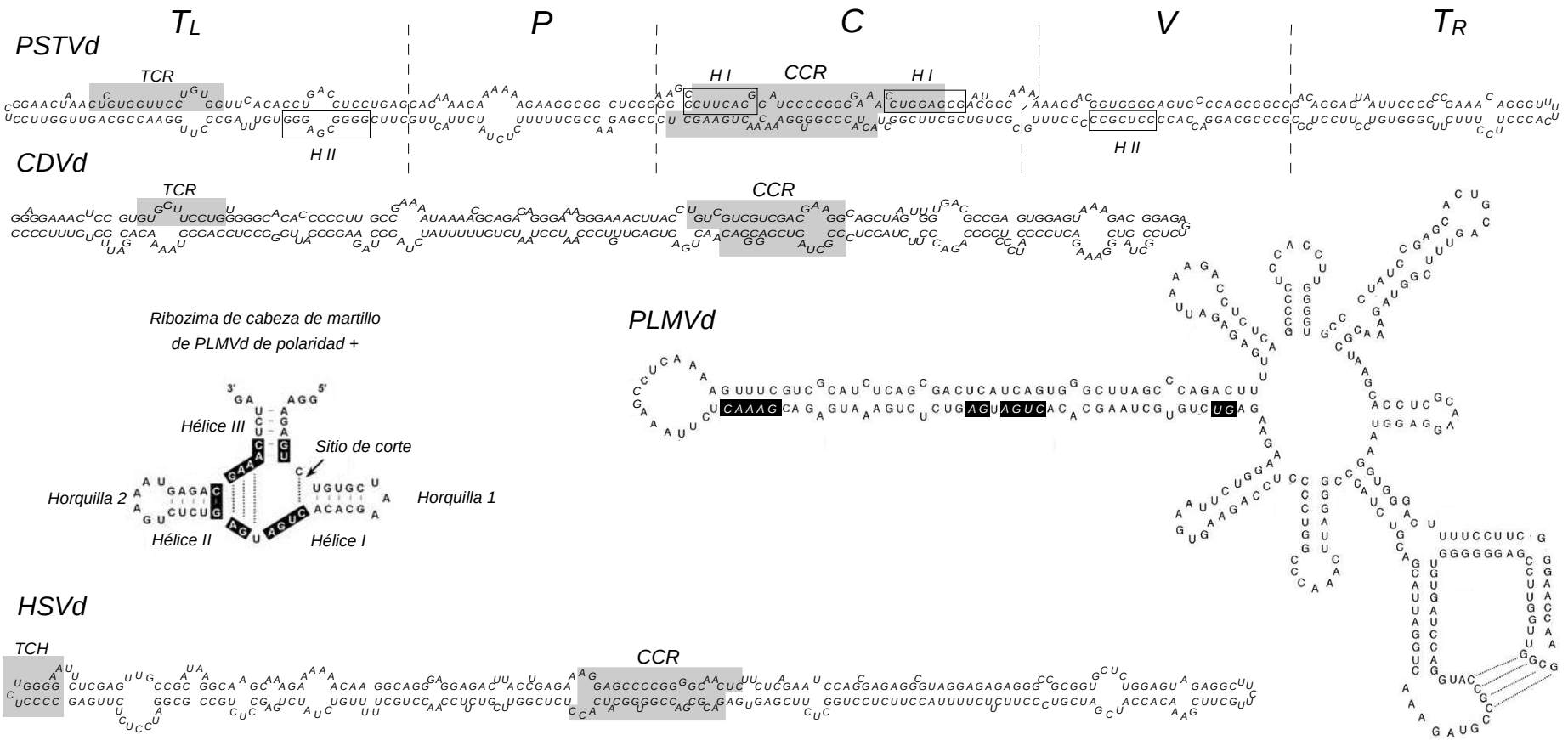


Figura 1. Estructura secundaria predicha para *el viroide del tubérculo fusiforme de la patata* (PSTVd), *el viroide enanizante de los cítricos* (CDVd), *el viroide del mosaico latente del melocotonero* (PLMVd) en el que se detalla la estructura de su ribozima en forma de cabeza de martillo y *el viroide del enanismo del lúpulo* (HSVd). Las zonas sombreadas en gris corresponden a las regiones conservadas CCR y TCR o TCH de PSTVd, CDVd y HSVd. La estructura de PSTVd está dividida mediante líneas discontinuas que separan los dominios de la molécula y se han encuadrado los nucleótidos correspondientes a la formación de la horquilla I (HI) y de la horquilla II (HIII). Las zonas sombreadas en negro corresponden a los motivos conservados de PLMVd implicados en la formación del ribozima de polaridad positiva. Las líneas discontinuas en PLMVd correspondes a interacciones entre bucles de la molécula.

Las estructuras secundarias obtenidas *in sílico* mediante cálculos teóricos se ajustan a los datos experimentales obtenidos mediante curvas de desnaturalización. Sin embargo, se ha comprobado que en la desnaturalización de transcritos monoméricos de PSTVd, la molécula adopta unas conformaciones más estables de lo esperado (Riesner *et al*, 1979). Esto se debe a cambios conformacionales metaestables que la molécula adquiere en su transición hacia la desnaturalización. Especialmente relevante es la reorganización de la CCR mediante el apareamiento del palíndrome de la hebra superior formando la denominada horquilla I (HI) (Figura 1, Figura 2). Esta conformación se ha detectado experimentalmente mediante microscopía electrónica, HPLC y ultracentrifugación analítica (Riesner *et al*, 1979). La HI parece estar involucrada en el procesamiento del viroide como sugiere el hecho que miniviroides artificiales de PSTVd, que solo contienen la CCR y parte de la molécula colindante a este motivo, son capaces de cortarse y circularizarse *in vitro* en presencia de extractos nucleares de patata (Shrader *et al*. 2003).

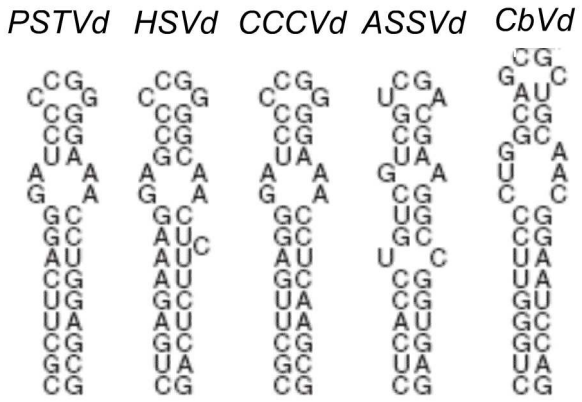


Figura 2. Estructuras metaestables de la horquilla I (HI) del viroide del tubérculo fusiforme de la patata (PSTVd), del viroide del enanismo del lúpulo (HSVd), del viroide del cadang-cadang del cocotero (CCCVd), del viroide de la piel cicatrizada de la manzana (ASSVd) y del viroide 1 del coleus blumei (CbVd).

Una segunda estructura metaestable descrita en PSTVd es la denominada horquilla II (HII). Esta conformación se produce mediante el apareamiento de dos secuencias palindrómicas que se encuentran en la hebra inferior de los dominios T_L y V (Figura 1). La relevancia de esta horquilla se ha puesto de manifiesto en estudios de mutagénesis ya que la introducción de cambios en el palíndrome provoca la pérdida de infectividad del viroide que solo se restaura al revertir dichos cambios (Loss *et al*. 1991, Candresse *et al*. 2001, Owens *et al*. 1991).

Un motivo de secuencia identificado en viroides de género *Pospiviroide* es el denominado bucle E que se localiza en la CCR. Este motivo es un elemento de estructura terciaria ya que al ser irradiado con luz ultravioleta es susceptible de entrecruzar covalentemente la hebra superior e inferior de la molécula (Branch *et al*. 1985). Este tipo de estructura se da *in vivo* como se ha demostrado irradiando directamente material vegetal (Eiras *et al*. 2007, Wang *et al*. 2007). El bucle E parece estar implicado en la replicación del viroide como sugiere el hecho que un único cambio nucleotídico en su secuencia intensifica 10 veces la replicación de PSTVd en cultivos celulares de tabaco (Qi y Ding, 2002). Recientemente se ha comprobado *in vivo* (Gas *et al.* 2007) que el bucle E participa en la etapa de la ligación del procesamiento replicativo de los viroides tal como ya se había previsto mediante datos *in vitro* (Baumstark *et al*. 1997).

2.3.2. Familia *Avsunviroidae*

Los viroides de la familia *Avsunviroidae* no poseen CCR, TCR o TCH. Sin embargo en moléculas de ambas polaridades se localizan motivos conservados que pueden formar estructuras de cabeza de martillo con actividad ribozimática. Estos motivos no aceptan ningún tipo de cambio en los nucleótidos que constituyen el centro catalítico del ribozima (Figura 1). Solo se han observado cambios en las regiones adyacentes que forman los bucles y siempre están asociados a mutaciones compensatorias para no afectar a la estabilidad de las hélices (Navarro y Flores, 1997, Ambrós *et al*. 1998).

Las estructuras secundarias ramificadas predichas *in sílico* para el viroide del mosaico latente del melocotonero (PLMVd), así como para el viroide del moteado clorótico del crisantemo (CChMVd), están apoyadas mediante estudios físicos y biológicos. Entre ellos cabe destacar la insolubilidad que presentan estos viroides en soluciones 2M de cloruro de litio o la aparición *in vivo* de mutaciones compensatorias al introducir cambios que alteran dicha estructura (Navarro y Flores, 1997, De la peña *et al*. 1999).

Análisis *in vitro* de mapeo mediante nucleasas o de hibridación de oligonucleótidos sugieren que existen interacciones entre los bucles de las distintas ramas de la molécula en PLMVd (Bussière *et al*. 2000). Este tipo de interacciones tiene importancia biológica ya que una alteración de la secuencia de estas zonas provoca la

perdida de infectividad como se ha descrito *in vivo* mediante experimentos de mutagénesis dirigida en CChMVd (Gago *et al*. 2005).

Al igual que el denominado bucle E en los viroides de la familia *Pospiviroidae*, se ha identificado en PLMVd un elemento de estructura terciaria susceptible a formar uniones covalentes mediante irradiación con luz ultravioleta. La función biológica de este motivo no está determinada aunque su posición sugiere que puede estar implicado en la iniciación de la trascripción en la replicación del viroide (Hernández *et al*. 2006).

3. Clasificación

Los viroides se clasifican según una normativa establecida por el Comité Internacional de Taxonomía de Virus (ICTV). A pesar de las grandes diferencias entre virus y viroides, en ambas entidades se sigue un criterio de clasificación similar en el que se diferencian familias, géneros y especies.

Las 29 especies de viroides conocidas hasta el momento se engloban en 2 únicas familias según 3 criterios:

- Los miembros de la familia *Pospiviroidae*, cuyo viroide modelo es PSTVd, poseen CCR, no presentan actividad de auto-corte mediada por ribozimas de cabeza de martillo y se replican en el núcleo mediante la variante asimétrica del mecanismo del círculo rodante (Figura 3).
- Los viroides de la familia *Avsunviroidae,* cuyo miembro modelo es ASBVd, no poseen CCR, las moléculas de ambas polaridades presentan actividad de autocorte mediada por ribozimas de cabeza de martillo y se replican en los cloroplastos mediante la variante simétrica del mecanismo del círculo rodante (Figura 3).

En la familia *Pospiviroidae* se distinguen cinco géneros dependiendo de la secuencia de la CCR y de la presencia o ausencia de TCR o TCH. En la familia *Avsunviroidae* se distinguen 3 géneros dependiendo de la proporción de bases G y C, su conformación termodinámicamente más estable y las propiedades de la actividad ribozimática. El nombre del género hace referencia a su especie modelo.

Dentro de géneros, la distinción entre especies y variantes se realiza mediante dos criterios arbitrarios. Dos viroides de un mismo género pertenecen a especies distintas si tienen una identidad de secuencia inferior al 90% y además poseen alguna propiedad

biológica que los diferencie. El nombre que se asigna a la especie suele hacer referencia la manifestación de síntomas que provoca el viroide en huéspedes naturales. Sin embargo no todos los viroides provocan patologías por lo que su nomenclatura sólo hace referencia a su huésped natural. Especies que coinciden en estos criterios incluyen una numeración para diferenciarse. El esquema actual de clasificación de viroides es coherente con los estudios filogenéticos (Elena *et al*. 2001).

Famila	Género	Especie
Pospiviroidae	*Pospiviroide*	**PSTVd (*viroide del tubérculo fusiforme de la patata*)** **TCDVd (*viroide del enanismo clorótico del tomate*)** **MPVd (*viroide de la papita mexicana*)** **TPMVd (*viroide de la planta macho del tomate*)** **CSVd (*viroide del enanismo del crisantemo*)** **CEVd (*viroide de la exocortis de los cítricos*)** **TASVd (*viroide del enanismo apical del tomate*)** **IrVd (*viroide de la iresine*)** **CLVd (*viroide latente de la columnea*)**
	Hostuviroide	**HSVd (*viroide del enanismo del lúpulo*)**
	Cocadviroide	**CCCVd (*viroide del cadang-cadang del cocotero*)** **CTiVd (*viroide del tinangaja del cocotero*)** **HLVd (*viroide latente del lúpulo*)** **CBCVd (*viroide de de la corteza agrietada de los cítricos*)**
	Apscaviroide	**ASSVd (*viroide de la piel cicatrizada de la manzana*)** **CDVd (*viroide del enanismo de los cítricos*)** **ADFVd (*viroide del fruto picado del manzano*)** **GYSVd1 (*viroide 1 del moteado amarillo de la viña*)** **GYSVd2 (*viroide 2 del moteado amarillo de la viña*)** **CBLVd (*viroide de la hoja curvada de los cítricos*)** **PBCVd (*viroide del chancro pustuloso del peral*)** **AGVd (*viroide australiano de la viña*)**
	Coleviroide	**CbVd1 (*viroide 1 de Coleus blumei*)** **CbVd2 (*viroide 2 de Coleus blumei*)** **CbVd3 (*viroide 3 de Coleus blumei*)**
Avsunviroidae	*Avsunviroide*	**ASBVd (*viroide del manchado solar del aguacate*)**
	Pelamoviroide	**PLMVd (*viroide del mosaico latente del melocotonero*)** **CChMVd (*viroide del moteado clorótico del crisantelmo*)**
	Elaviroide	**ELVd (*viroide latente de la berenjena*)**

Tabla 1. Esquema de clasificación de los viroides del Comité Internacional de Taxonomía de Virus (ICTV). Las especies subrayadas corresponden a las especies tipo.

4. Replicación

4.1. Localización y modelo replicativo

La replicación es una etapa fundamental y necesaria para el ciclo infectivo de los viroides. A partir de mínimas cantidades de inóculo, los viroides son capaces de invadir tejidos distales de la planta y acumularse en cantidades detectables. Este hecho conlleva que el viroide se multiplica de forma autónoma dentro del huésped al que infecta.

Experimentos de fraccionamiento de células mediante centrifugación, hibridaciones *in-situ* y microscopía electrónica han mostrado que los viroides de la familia *Pospiviroidae* se acumulan en el núcleo (Diener 1971, Bonfiglioli *et al*. 1996, Harders *et al*. 1989). La molécula viroidal en este orgánulo se encuentra de diferentes formas, siendo la más abundante la unidad monomérica de una determinada polaridad a la que arbitrariamente se le ha denominado polaridad positiva (Branch y Robertson, 1984). También se encuentran, aunque en menor cantidad, oligómeros de polaridad negativa (Grill y Semancik 1978). Estudios recientes apuntan a que ambos tipos de moléculas se localizan en el nucleoplasma pero las de polaridad positiva se acumulan en mayor medida en el nucleolo (Qi y Ding 2003). Los miembros de la familia *Avsunviroidae* se encuentran preferentemente en el cloroplasto y es en este orgánulo donde se localizan monómeros circulares de ambas polaridades así como oligómeros lineales también de ambas polaridades (Mohamed y Thomas 1980, Bonfiglioli *et al.* 1994, Lima *et al*. 1994, Bussière *et al*. 2000).

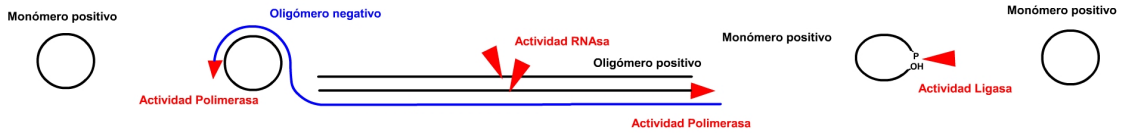
La replicación de los viroides sigue el modelo del círculo rodante. Este modelo ha sido propuesto debido a la naturaleza circular del viroide y a la falta de intermediarios de DNA homólogos o complementarios a su secuencia (Zaitlin *et al*. 1980, Branch y Dickson 1980). En este mecanismo solo intervienen intermediarios de RNA y se sustenta por la presencia de moléculas de ambas polaridades que se acumulan a distintas concentraciones. Atendiendo a la familia de viroide que se trate, el mecanismo de replicación se da de distintas formas.

Los viroides de la familia *Pospiviroidae* siguen la vía asimétrica. En esta variante, la molécula circular de polaridad positiva sirve de molde para la síntesis de oligómeros

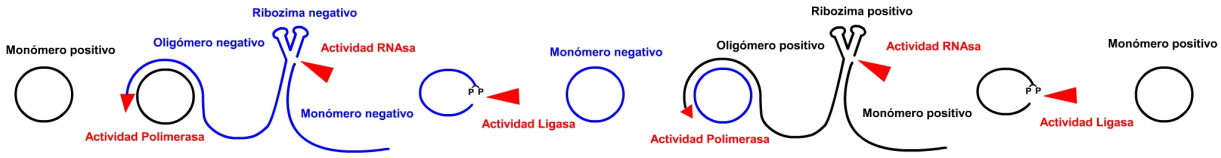
lineales de polaridad negativa que a su vez sirven de molde para la síntesis de oligómeros de polaridad positiva. Estas moléculas son procesadas a unidades

Mecanismo del círculo rodante

Vía asimétrica (familia *Pospiviroidae*)



Vía simétrica (familia *Avsunviroidae*)



monoméricas que por autoligación dan lugar a moléculas circulares de polaridad positiva que son el punto de partida del ciclo replicativo (figura 3).

Los viroides de la familia *Avsunviroidae* siguen la vía simétrica (Branch y Robertson, 1984, Darós *et al*. 1994, Navarro *et al*. 1999). En esta variante la molécula circular de polaridad positiva sirve de molde para la síntesis de oligómeros lineales de polaridad negativa que se autocortan generando monómeros que se circularizan. Así se obtienen moléculas circulares de polaridad negativa que sirven de molde para la síntesis de oligómeros lineales de polaridad positiva que se autocortan generando monómeros que son circularizados y que son el punto de partida del ciclo (figura 3).

Las dos variantes del modelo difieren en el tipo de intermediario del ciclo replicativo, que es una molécula de RNA circular de polaridad negativa detectada en ASBVd (Hutchins *et al*. 1985, Darós *et al*. 1994) y PLMVd (Bussière *et al*. 1999) pero no en PSTVd (Branch and Robertson, 1984, Branch *et al*. 1988 y Feldstein *et al*. 1998).

Otra diferencia entre las dos variantes es el fraccionamiento a unidades

Figura 3. Representación esquemática del ciclo replicativo de los viroides mediante el mecanismo del círculo rodante en su variante asimétrica y en su variante simétrica.

monoméricas de la molécula de polaridad negativa que solo se da en la simétrica.

En ambas variantes del ciclo replicativo se requieren tres actividades catalíticas que son; la síntesis de RNA (actividad RNA polimerasa), el corte del RNA a unidades monoméricas (actividad RNAsa) y la circularización del RNA (actividad RNA ligasa). A diferencia de los virus que codifican proteínas que intervienen en su propia replicación, los viroides requieren que todas estas actividades catalíticas estén mediadas por proteínas del huésped salvo las reacciones autocotalíticas mediadas por ribozimas de los viroides de la familia *Avsunviroide*.

4.2. Actividad RNA polimerasa

Estudios *in vitro* e *in vivo* utilizando α-amanitina apuntan a que la enzima DNA dependiende RNA polimerasa II (Pol II) es la encargada de la transcripción de los viroides de la familia *Pospiviroidae* (Mühlbach y Sänger 1979, Flores y Semancik 1982, Yoshikawa y Takahashi 1986). Mediante la utilización de anticuerpos monoclonales para Pol II se ha comprobado *in vivo* la asociación de esta enzima con moléculas de ambas polaridades del viroide (Warrilow y Symons 1999). Sin embargo el descubrimiento de enzimas RNA dependiente RNA polimerasas (RdRs) (Wassenegger y Krczal, 2006) no descarta la posibilidad de que este tipo de enzimas pueda estar también involucrado en la replicación viroidal (Ding y Itaya 2007).

Respecto a los viroides de la familia *Avsunviroidae*, estudios *in vitro* con cloroplastos de aguacate infectados con ASBVd en presencia de tagetitoxina sugieren que la replicación de este viroide está mediada por polimerasas codificadas en el núcleo celular (NEP). La tagetitoxina afecta la actividad de polimerasas codificadas en el cloroplasto (PEP) e inhibe la transcripción de genes en este orgánulo (Navarro *et al*. 2000). Sin embargo no se puede descartar la existencia de PEPs resistentes a este tóxico y por tanto que la replicación viroidal esté mediada por alguna polimerasa de este tipo. Apuntando a esta última hipótesis estudios *in vitro* han mostrado que las RNA polimerasas de *Escherichia coli*, enzimas más próximas a las polimerasas codificadas en el genoma cloroplástico que a las nucleares, son capaces de transcribir PLMVd (Pechalt *et al*. 2001). Por otro lado, se ha comprobado que en sectores albinos de hojas afectadas por "Peach Cálico" moléculas de ambas polaridades de PLMVd se acumulan en proplastidios. Los proplastidios son orgánulos precursores de cloroplastos y tienen muy mermado el tráfico de RNAs mensajeros codificados en el núcleo celular, lo que sugiere que la transcripción de este viroide esté mediada por PEPs (Rodio *et al*. 2008).

4.3. Actividad RNAsa

El procesamiento de oligómeros de polaridad positiva en viroides de la familia *Pospiviroidae* parece estar mediado por enzimas del huésped. La obtención de monómeros a partir de este tipo de moléculas se ha demostrado *in vitro* mediante la incubación de oligómeros de PSTVd con extractos nucleares de patata (Tsagris *et al*. 1987) e *in vivo* mediante plantas transgénicas de arabidopsis que expresan oligómeros de polaridad positiva de viroides representativos de todos los géneros de esta familia (Darós y Flores 2004). Se ha propuesto para PSTVd que esta actividad requiere un plegamiento especial del oligómero viroidal que genera un cambio conformacional donde está involucrado el motivo de estructura terciaria Loop E (Baumstark *et al*. 1997). Sin embargo este modelo no es extrapolable a otros miembros de la familia *Pospiviroidae* en los que no se ha descrito el motivo E.

Estudios recientes utilizando plantas transgénicas de *Arabidopsis thaliana* que expresan dímeros viroidales, proponen que la actividad RNAsa se realiza sobre una conformación entre oligómeros mediada por la interacción de sus horquillas I (HI). Los puntos de corte obtenidos mediante la técnica "primer extensión" sugieren que los oligómeros se cortan en dos puntos próximos de las hebras complementarias de esta configuración (Gas *et al*. 2008a), obteniéndose monómeros con extremos 5'fosfomonoester y 3'con un grupo hidroxilo. Este dato sugiere también que las enzimas del tipo RNAsa III sean las responsables de este procesamiento (Gas *et al*. 2008b). El procesamiento de oligómeros a moléculas monoméricas parece localizarse en el nucleolo de la célula como ocurre con precursores de tRNA o rRNA ya que es en este orgánulo donde se localizan preferentemente los oligómeros de polaridad positiva como previamente se ha descrito.

En los viroides de la familia *Avsunviroidae* la actividad RNAsa no parece estar mediada por factores del huésped ya que puede realizarse mediante ribozimas en forma de cabeza de martillo que se forman en moléculas oligoméricas de ambas polaridades. La reacción de corte es una transesterificación que se produce en el centro activo del ribozima en presencia de Mg^{2+} y cuyo producto es una molécula monomérica con un

grupo hidroxilo en el extremo 5' y un grupo 2', 3'-fosfodiester cíclico en el extremo 3'(Hutchins *et al*. 1986, Prody *et al.* 1986). Estudios recientes han demostrado que la actividad ribozimática no depende únicamente del centro catalítico sino que también influyen las regiones periféricas. Se ha demostrado que al alterar la horquilla 1 y 2 del ribozima se reduce drásticamente la actividad catalítica (De la Peña *et al*. 2003, Khovorova *et al*. 2003). Este hecho pone de manifiesto la importancia de la estructura terciaria de la molécula para llevar a cabo el autocorte. Esta reacción, a pesar de ser autocatalítica, *in vivo* se ve favorecida por la presencia de proteínas cloroplásticas estabilizadoras que actúan a modo de chaperonas (Darós *et al*. 2002).

4.4. Actividad ligasa

En viroides de la familia *Pospiviroidae,* la circularización de los monómeros lineales parece estar mediada por factores del huésped, ya que enzimas del tipo RNA ligasa extraídas de germen de trigo son capaces de circularizar *in vitro* monómeros lineales de PSTVd (Branch *et al*. 1982). De igual modo, se ha comprobado la formación de monómeros circulares a partir de oligómeros lineales de PSTVd incubados en presencia de extractos nucleares de patata (Baumstark *et al*. 1997, Tsagris *et al*. 1987). Este tipo de actividad también se da en plantas transgénicas de *Arabidopsis thaliana* que expresan dímeros de viroides representativos de los distintos géneros de la familia *Pospiviroidae.* A pesar de que esta planta no es huésped de los viroides, la presencia de monómeros circulares viroidales pone de manifiesto que *Arabidopsis thaliana* cuenta con las enzimas necesarias para catalizar la circularización (Darós y Flores, 2004). Los extremos de los monómeros resultantes del procesamiento de *Arabidopsis thaliana* sugieren que la actividad ligasa está mediada por un enzima distinto a las tRNA ligasas (Gas *et al*., 2008b).

En los viroides de la familia *Avsunviroidae,* la actividad ligasa puede ser autocatalítica ya que in vitro monómeros de PLMVd provenientes del autocorte ribozimático son capaces de circularizarse en ausencia de proteínas (Côté y Perreault, 1997). Sin embargo no puede descartarse que esta actividad ligasa esté mediada por algún factor cloroplástico del huésped.

5. Variabilidad de los viroides

Los primeros análisis de secuencia de virus de RNA mostraron que dentro del huésped se encuentra una población viral heterogénea y no una única variante como cabría esperar (Domingo *et al*. 1978). Estudios en plantas infectadas con PSTVd, CEVd (Tabler y Sänger, 1984, Visvader y Symons, 1983) y posteriormente con la mayoría de especies conocidas han mostrado que las infecciones viroidales siguen una dinámica parecida y se encuentran en el huésped mezclas de secuencias más o menos complejas (Rakowsky y Symons, 1989, Hernández y Flores, 1992, Ridgen y Rezaian, 1993, Ambrós *et al*. 1995, Polivka *et al*. 1996, Kofalvi *et al*. 1997, Navarro 1997, Palacio-Bielsa *et al*. 2004, Gandia y Duran-Vila, 2004). El número y la frecuencia de las distintas secuencias definen la variabilidad de una población. Dentro del espectro de secuencias se suele hallar mayoritariamente una que se define como maestra por ser la de mayor eficiencia biológica. La secuencia maestra suele coincidir con la denominada secuencia consenso que se define como la secuencia promedio que en cada posición tiene el nucleótido más frecuente de la nube de secuencias de la población (Eigen 1993).

Las infecciones viroidales siguen el modelo de las cuasiespecies. Este concepto se adoptó para referirse a la distribución de genomas no idénticos pero de secuencias similares que habiéndose generado a partir de un único genoma constituyen poblaciones de replicones cuya complejidad aumenta si la fidelidad del proceso replicativo disminuye. Se ha comprobado que los genomas de RNA evolucionan y varían más rápidamente que los de DNA (Holland *et al.* 1982). La alta tasa de mutación en este tipo de genomas se debe a la ausencia de mecanismos de corrección de errores en las polimerasas implicadas en el proceso de replicación. Estudios *in vitro* han confirmado la elevada tasa de mutación que llega a frecuencias de 10^{-5} (Ward *et al*. 1988, Williams y Loeb, 1992). Las cuasiespecies no están constituídas por un conjunto de mutantes al azar, sino por conjuntos organizados que pueden fluctuar de acuerdo con las presiones que reciben.

En infecciones viroidales, la variabilidad de la población de secuencias depende del genotipo del viroide y del huesped. Se ha comprobado que infecciones naturales de CVd-IIa y CVd-IIb, dos tipos de variantes de HSVd en cítricos, tienen distintas

dinámicas poblacionales, siendo las poblaciones de las variantes tipo CVd-IIa mucho más homogéneas que las del tipo CVd-IIb (Palacio-Bielsa *et al*. 2004).

La presión de selección que el huésped ejerce sobre el espectro de secuencias se evidencia mediante transmisiones de un mismo aislado a distintas especies vegetales. En este sentido, se ha comprobado que aislados de CEVd muestran cambios en la secuencia maestra y en la frecuencia del resto de la población al ser transmitido de haba (*Vicia faba*) a tomate (*Solanum lycopersicum*) (Gandía *et al*. 2007). El dinamismo que tienen las poblaciones viroidales para modificar su espectro de secuencia se evidencia claramente mediante inoculaciones de secuencias únicas que forman rápidamente una nube de secuencias (Owens *et al*. 1986, Gora-sochacka *et al*. 1997, Ambrós *et al*. 1999, Gandía y Duran-Vila, 2004; Gandía *et al.,* 2005). Normalmente la denominada secuencia maestra suele ser la de mayor tasa de infectividad. Inoculaciones con secuencias alternativas del aislado, o alteradas artificialmente, pueden no ser infectivas o acabar revirtiendo a la secuencia maestra para lograr infectar a la planta (Gandia y Duran-Vila, 2004). Aunque en una población viroidal cualquier mutación puntual es posible, aquellas que conllevan la perdida de funciones esenciales como la replicación o el movimiento del viroide, tienden a desaparecer dentro de la población. Por ello existen características estructurales que limitan la divergencia genética como se ha comprobado en PLMVd, miembro de la familia *Avsunviroidae*, cuyas variantes nunca muestran cambios en los motivos implicados en las estructuras de cabeza de martillo (Ambrós *et al*.1998). En viroides de la familia *Pospiviroidae*, no se suele encontrar cambios en los motivos conservados como la hebra superior de la CCR, la TCR, la TCH o la HII. (Lakshman y Tavantzis, 1992, Qu *et al*. 1993, Owens *et al*. 1995).

6. Movimiento

6.1. Aspectos generales

Los viroides tienen capacidad de infectar a las plantas de forma sistémica. En su ciclo infectivo los viroides deben trasladarse al núcleo de la célula (familia *Pospiviroidae*) o al cloroplasto (familia *Avsunviroidae*), replicarse, salir al citoplasma, moverse a través de células, llegar e introducirse en el tejido vascular, moverse a través del floema y salir de este. Los viroides no codifican sus propias proteínas de movimiento ni requieren encapsidarse para moverse a lo largo de la planta. Sin embargo

su genoma posee toda la información necesaria para interactuar directamente con factores del huésped aprovechando rutas y mecanismos endógenos de tráfico molecular para localizarse y completar todas sus funciones biológicas. Dada su simplicidad, los viroides son un buen sistema experimental para el estudio y comprensión de los mecanismos de tráfico intracelular, intercelular y sistémico del RNA. (Ding *et al*., 2005; Flores *et al*., 2005).

6.2. Movimiento célula a célula

Se ha demostrado que PSTVd es capaz de infectar de forma sistémica cuando es inoculado por biobalística en células de la epidermis (Qi *et al*., 2004). Una vez infectadas las primeras células, el viroide debe colonizar las células adyacentes antes de llegar a partes distales de la planta. Tanto virus como RNAs endógenos realizan este tipo de movimiento, denominado movimiento célula a célula, a través de los plasmodesmos, que son los orgánulos de interconexión citoplasmática intercelular. Estudios con transcritos de PSTVd marcados con fluorescencia sugieren que los viroides también utilizan estas vías al transitar entre las células (Ding *et al*., 1997). La mayor velocidad de avance de los viroides respecto a RNAs inmóviles sugiere algún tipo de mediación dirigida. Es probable que los viroides se unan a algún factor del huésped para utilizarlo como medio de transporte.

No todas las uniones entre células son iguales. La planta posee barreras y filtros en puntos estratégicos para regular el tráfico de macromoléculas a los distintos tejidos. Uno de estos puntos se encuentra en el meristemo apical que es una zona restringida a la mayoría de virus y viroides y de gran actividad celular. El sistema vascular en sus proximidades aún no está diferenciado por lo que su acceso solo puede darse por movimientos célula a célula. Mediante hibridación *in situ* se ha comprobado que algunos viroides se aproximan mucho al meristemo apical, llegando casi a invadirlo, como es el caso de PLMVd en su variante cálico (Rodio *et al*., 2007). Un posible mecanismo que impide su invasión parece estar mediado por la maquinaria de silenciamiento génico post-transcripcional, ya que una merma de la función RNA dependiente RNA polimerasa 6 (RdR6) en plantas de patata provoca la invasión de PSTVd al meristemo (Comunicación personal R. Flores). De igual modo ocurre con el virus X de la patata en plantas de *Nicotiana benthamiana* (Schwach *et al*. 2005).

6.3. Movimiento vascular

Macromoléculas como los fotoasimilados se mueven a través del floema desde las fuentes a los sumideros por difusión (Sjölund *et al*., 1997). Tanto virus como viroides son capaces de desplazarse de igual modo. (Palukaitis, 1987, Zhu *et al*., 2001). Sin embargo existen evidencias de que el tráfico floemático de viroides y otros RNAs está mediado y regulado por factores del huésped (Haywood *et al*., 2005). El movimiento de RNAs endógenos, RNAs de interferencia y RNAs virales a lo largo del floema está facilitado mediante la unión a proteínas de la planta (Xoconostle-Cázarés *et al*. 1999, Yoo *et al*. 2004, Scholtof, 2005). En viroides existen varios ejemplos de proteínas que facilitan este transporte. La lectina floemática PP2 de pepino (*Cucumis sativus*) puede unirse a HSVd *in vitro* (Gómez y Pallás, 2001) e interaccionar *in vivo* con el viroide (Gómez y Pallás, 2004). Se han descrito dos proteínas de melón (*Cucumis melo*) que se unen a viroides de ambas familias como CEVd y ASBVd. Se ha comprobado que una de ellas, la proteína CmmLec17, se desplaza a través del floema ya que se puede detectar en plantas de otras especies injertadas sobre melón (Gómez *et al*., 2005).

La entrada de moléculas al sistema vascular se encuentra regulada. Uno de los filtros se localiza en las células de la vaina del haz que separan el mesófilo del floema. El tráfico de RNAs endógenos en este paso está regulado e implica motivos de secuencia y factores del huésped (Hamada *et al*., 2003). Estudios mediante hibridaciones in situ en plantas de tabaco (*Nicotiana tabacum*) indican la existencia de mecanismos similares para los viroides. Se han identificado dos motivos de secuencia en PSTVd que comprometen su entrada y salida del floema. Estos datos sugieren la existencia de interacciones entre el viroide y distintos factores del huésped que regulan únicamente un sentido del paso (Qi *et al*., 2004; Zhong *et al*. 2007).

La descarga a distintos organos de la planta también parece tener barreras específicas. Una evidencia de ello es la existencia de un sistema de regulación que restringe y media el transporte a los órganos florales. En plantas de *Nicotiana benthamiana* y tomate, PSTVd es incapaz de acceder a las flores cuando están en formación y sólo alcanza los sépalos una vez han madurado (Zhu *et a*l., 2002). Esta restricción en algún momento debe inhibirse ya que PSTVd es transmisible por semilla y por tanto ha de acceder hasta los óvulos o el polen.

7. Gama de huéspedes

Hasta el momento la gama de huéspedes de los viroides está restringida al reino vegetal, concretamente a las plantas superiores. Se han descrito infecciones viroidales en plantas herbáceas y leñosas así como en plantas dicotiledóneas y monocotiledóneas.

En general los viroides son capaces de infectar varias especies de una misma familia. Los viroides de la familia *Pospiviroidae* tienen una mayor gama de huéspedes que los viroides de la familia *Avsunviroidae*. En la primera familia tenemos ejemplos extremos. HSVd es capaz de infectar Cucurbitáceas, Rutáceas, Rosáceas, Vitáceas o Cannabeáceas. En contraposición, HLVd tiene al lúpulo (*Humulus lupulus*) como único huésped conocido (Puchta *et al*., 1988).

La transmisión de aislados a distintas especies vegetales es uno de los pilares básicos para el conocimiento de los viroides. Las infecciones pueden manifestarse de manera muy distinta dependiendo de la asociación viroide-huésped. Es por ello una práctica habitual en estudios de patología transmitir artificialmente aislados desde sus huéspedes naturales a nuevas especies denominadas huéspedes experimentales de síntomas más evidentes y que acumulan el viroide a mayor nivel, lo que facilita su estudio.

8. Sintomatología y patogénesis

8.1. Aspectos generales

El reconocimiento de patologías vegetales y la posterior caracterización de sus agentes causales ha sido el punto de partida para el descubrimiento de los viroides, así como para la identificación de la mayoría de sus especies. En este sentido, existe una cierta tendencia a asociar las infecciones viroidales a patologías como es el hecho de que los nombres que se les asignan hacen referencia a la sintomatología que manifiestan las plantas al enfermar. Sin embargo, también se han descrito viroides que reciben el nombre de "latentes" ya que aparentemente no provocan síntomas en sus huéspedes naturales. Por otro lado, viroides cuya infección en determinadas especies provoca síntomas muy agresivos, pueden comportarse como infecciones latentes en huéspedes

distintos. Estos huéspedes reciben el nombre de "huéspedes tolerantes" y ponen de manifiesto que la patogenicidad no es consecuencia únicamente del viroide sino de la asociación viroide-huésped. Los cítricos son un buen ejemplo. El viroide de la exocortis CEVd puede ser letal en cidro Etrog pero es bien sabido que su infección no causa síntomas en la mayoría de especies comerciales de cítricos (Maltifano *et al*. 2005).

Es probable que en la asociación viroide-huésped, la aparición de síntomas sea una excepción. En este sentido, hay que destacar que las patologías de etiología viroidal se han identificado en especies cultivadas de interés comercial y por tanto son plantas que han sufrido algún tipo de selección por parte del hombre. Normalmente, este tipo de selección está dirigida a favorecer caracteres de interés agronómico. En la mayoría de casos esta selección suele comprometer y mermar la eficacia biológica de estas especies "domesticadas" que ya no son competitivas en espacios naturales y su existencia queda relegada a su cultivo en condiciones favorables creadas artificialmente por el ser humano.

No todos los viroides se han descrito en especies cultivadas. Existen algunos que se han identificado en plantas silvestres como es el caso de MPVd y CLVd (Hammond *et al*. 1989, Martinez-Soriano *et al.* 1996). En ambos casos, sus huéspedes naturales se muestran asintomáticos. Sin embargo, CLVd al ser transmitido a tomate o patata provoca síntomas similares a los inducidos por una infección con PSTVd (Owens *et al*. 1978). Recíprocamente, tanto PSTVd como CEVd no provocan síntomas en las especies ornamentales y silvestres en las que se han identificado o inoculado (Verhoeven *et al.* 2004, Bostan *et al*. 2004, Matoušek *et al*. 2007). Este hecho es especialmente relevante en viroides clasificados como organismos patógenos de cuarentena ya que puede malograr programas de erradicación de patógenos al actuar las plantas silvestres como reservorios naturales.

8.2. Expresión de síntomas

Existe una gran similitud en los síntomas causados por virus y viroides. Al igual que ocurre con los virus, las infecciones viroidales pueden provocar una amplia variedad de síntomas en plantas infectadas. A nivel macroscópico, los viroides pueden causar enanismo, reducción del tamaño de hojas, flores y frutos, decoloraciones en hojas y frutos, epinastia, lesiones y necrosis, malformaciones en frutos, perdida de dominancia apical, exudaciones gomosas, descamaciones en corteza y retrasos en la

floración. A nivel ultra-estructural HSVd y CEVd son capaces de producir distorsiones y malformaciones en la pared celular de sus huéspedes que también pueden presentar cloroplastos con tilacoides deformados (Semancik y Conejero-Tomas, 1987). Las plantas infectadas con ASBVd y PLMVd en su variante cálico presentan también cloroplastos desorganizados similares a pro-plastidios (Desjardins, 1987; Rodio *et al*., 2007).

La intensidad de síntomas varia desde muy suave a muy agresiva llegando incluso a provocar la muerte de la planta. El tipo de síntoma y su intensidad dependen de la asociación huésped-viroide así como de las condiciones ambientales en que se dé la infección.

El huésped es un factor determinante en la expresión de síntomas. Un mismo aislado puede actuar como una infección latente en determinadas especies y ser agresivo en otras como es el caso de CEVd al ser transmitido de haba a tomate (Fagoaga *et al*.1995). Se han encontrado aislados de PSTVd que provocan síntomas con distintas intensidades en cultivares de una misma especie (Herold *et al*. 1992) lo que indica que el factor huésped influye en la expresión de síntomas de modo intraespecífico. Es posible que la modulación de síntomas por parte del huésped se dé a nivel genotipo ya que los bioensayos para diagnosticar infecciones viroidales se suelen realizar con genotipos seleccionados por su sensibilidad como es el caso del clon 861-S1 de cidro Etrog (*Citrus medica*) (Roistacher, 1991).

Al igual que el huésped, el papel del viroide es determinante en la manifestación e intensidad de los síntomas que provoca. Existen patologías donde el agente causal de la enfermedad es un tipo de variante. Tanto en el caso de HSVd en mandarinos (*Citrus reticulata*) como en el de PLMVd en melocotoneros (*Prunus persica*) existen variantes que se comportan como latentes y variantes patogénicas que provocan la cachexia o el Peach cálico respectivamente (Reanwarakorn y Semancik, 1998; Malfitano *et al*. 2003). También existen variantes de un mismo viroide que determinan la intensidad de síntomas como es el caso de CEVd cuyas variantes (tipo A y B) inducen en tomate síntomas agresivos y suaves respectivamente (Visvader y Symons, 1985).

La expresión de síntomas está determinada por las condiciones ambientales en las que se desarrolla el huésped infectado. La sintomatología así como la acumulación del viroide en la planta se ven favorecidas por altas temperaturas y altas intensidades lumínicas (Singh 1983, 1989). En general un paso de 20ºC a 35ºC provoca síntomas mas agresivos y una mayor acumulación de PSTVd (Sänger y Ramm, 1974), CEVd

(Semancik *et al*. 1988), ASBVd (da Graça y Van Vuuren, 1981) o CBLVd, HSVd y CDVd (Duran-Vila *et a*l. 1988), aunque existen excepciones como es el caso de ASSVd que se acumula en mayores niveles a temperaturas próximas a los 18ºC (Skrzeczkowski *et al*. 1993). En consecuencia, las patologías viroidales son más comunes y agresivas en zonas cálidas que en zonas templadas (Singh, 1983). Posiblemente el aumento en la expresión de síntomas por causas ambientales sea una combinación de las condiciones óptimas para la replicación del viroide y el estrés provocado al huésped ya que las temperaturas superiores a 28ºC no suelen ser óptimas para su desarrollo.

8.3. Patogénesis

Los viroides son pequeñas moléculas de RNA que no codifican proteínas. Sin embargo su genoma contiene toda la información necesaria para interactuar y utilizar la maquinaria del huésped. Para llevar a cabo la infección, el viroide debe acoplar cada etapa del ciclo infectivo al metabolismo endógeno de las plantas y es probable que en esta interacción existan puntos críticos que comprometan el desarrollo óptimo del huésped.

Los mecanismos por los cuales la infección viroidal provoca la manifestación de síntomas están muy poco elucidados. Estudios de expresión diferencial con macroarrays demuestran que las plantas de tomate alteran su expresión génica al ser infectadas por PSTVd (Itaya *et al.* 2002). Dentro de esta alteración, se ha comprobado que la infección viroidal provoca la expresión de proteínas de defensa PR cuya presencia está asociada al mecanismo de resistencia sistémica adquirida (SAR) (Conejero *et al*.1990). Este tipo de defensa también se da en ataques por hongos, bacterias y virus alterando la concentración de hormonas y metabolitos de la planta (Granell *et al*. 1987). Estos datos sugieren que en la interacción entre el huésped y el patógeno deben existir mecanismos que promuevan una cascada eventos. Tanto la molécula viroidal como sus derivados pueden interaccionar con proteínas y ácidos nucleicos del huésped (Flores *et al*., 2005) por lo que cabe la posibilidad de que cualquiera de ellos actúe como efector directo del inicio de la patogénesis.

Generalmente la intensidad de los síntomas no está correlacionada con un aumento de la acumulación del viroide (Schnölzer *et al.* 1985, Góra *et al*. 1996, Gruner *et al.* 1995, Rodio *et al*. 2006;), aunque también se han obtenido resultados donde si existe una correlación positiva (Sano *et al*. 1992). El hecho de que existan variantes de

un mismo viroide con similar acumulación en el huésped pero que difieren en la sintomatología que manifiestan las plantas infectadas, indica que pocos cambios en la secuencia del viroide conllevan diferencias en sus propiedades biológicas.

Existen muchos ejemplos en los que se han identificado posibles determinantes patogénicos en la molécula viroidal. Visvader y Symons otorgaron la función patogénica al dominio P. Sin embargo muchos de los resultados obtenidos hasta el momento no pueden explicarse según este modelo y parece existir una correlación mucho más compleja entre distintos dominios de la molécula y la patogenicidad. Se ha demostrado que tres o cuatro cambios nucleotídicos en la hebra inferior del dominio P de PSTVd promueven variaciones en los síntomas que muestran las plantas infectadas. Se ha sugerido que la inducción de síntomas se debe a modificaciones en la capacidad de unión del viroide con proteínas del huésped ya que estos cambios nucleotídicos provocan alteraciones en la geometría de la molécula (Owens *et al*. 1996, Schmitz y Riesner, 1998). También se ha propuesto la hipótesis de que la molécula viroidal vea alterada su estabilidad termodinámica provocando nuevas conformaciones estructurales que sean las responsables de la patogénesis (Schnölzer *et al.* 1985). Sin embargo existen datos que evidencian que la conformación de varilla de las variantes más agresivas es termodinámicamente más estable que la de las variantes más suaves (Owens *et al*. 1996).

Fuera del dominio P también se han descrito determinantes de patogenicidad. En PSTVd se ha comprobado que un cambio U→A en la posición 257 del dominio C, provoca un enanismo muy marcado en plantas de tomate. Este enanismo está asociado a una expansión celular deficiente debida a una disminución de la expansina endogena codificada por el gen LeExp2 (Qi y Ding, 2003). Por otro lado, los resultados de estudios con viroides quiméricos sintetizados intercambiando regiones de PSTVd y TASVd indican que los dominios terminales T_L y T_R están tambien involucrados en la expresión de síntomas (Sano *et al*.1992). En HSVd los determinantes de patogenicidad se encuentran en el dominio V (Reanwarakorn y Semancik, 1998).

En los viroides de la familia *Avsunviroidae* también se han descrito motivos involucrados en la patogénesis. En ASBVd se ha asociado la expresión de síntomas con un motivo poli-A en el bucle terminal derecho (Semancik y Szychowski. 1994). En CChMVd un bucle formado por 4 nucleótidos es el responsable de la expresión de síntomas como se ha evidenciado mediante mutagénesis inducida transformando una variante patogénica agresiva en latente (De la Peña *et al*. 1999, De la Peña y Flores

2001). Un resultado muy similar se ha observado en el caso PLMVd. Únicamente variantes que cuentan con una inserción de 12 o 13 nucleótidos inducen la enfermedad del Peach cálico asociada a la alteración de los cloroplastos en las hojas que presentan síntomas (Maltifano *et al*. 2003, Rodio *et al*. 2007).

Todos estos datos evidencian que los determinantes patogénicos se sitúan en posiciones distintas dependiendo de la especie de viroide. Si la patogénesis es atribuible a una interacción directa de la molécula viroidal con alguna proteína del huésped cualquier parte de la molécula puede potencialmente alterar dicha interacción mediante cambios en motivos de unión o cambios conformacionales en la estructura secundaria y terciaria de la molécula.

En los últimos años, ha emergido el mecanismo de silenciamiento génico mediado por pequeñas moléculas de RNA que actúa en plantas y en animales. Hasta este descubrimiento, el conocimiento acerca de la regulación génica se basaba en la expresión de genes mediada por promotores y proteínas que regulan la transcripción. Sin embargo, se ha descubierto que existe un trasfondo regulador regido por RNAs de 21 a 24 nucleótidos (sRNAs de "small RNAs"), que actuando tanto a nivel pre-trascripcional como post-trascripcional, son capaces de redirigir la expresión génica mediada por promotores y factores de trascripción.

Dentro del grupo de los sRNAs se pueden distinguir los microRNAs (miRNA) de 21-24 nucleótidos y que son moléculas endógenas de plantas y animales que se sintetizan a partir de regiones no codificantes del genoma. Los transcritos de estas regiones, denominados pre-microRNAs, presentan autocomplementariedad de bases en distintas partes de la molécula lo que les confiere una conformación en forma de horquilla con fragmentos de doble cadena. Los pre-microRNAs son sustrato de un grupo de enzimas RNAsas tipo III denominadas Dicers que dividen al precursor formando los pequeños miRNAs (Bernstein *et al*. 2001). Estos son captados por el complejo inductor de silenciamiento RISC (Hammond *et al*. 2000) que guía al microRNA hasta un RNA mensajero con el que aparea formando una pequeña región de doble cadena que deja al RNA mensajero inactivo y susceptible a ser degradado (Figura 4). Los microRNAs por tanto tienen la función de reducir la expresión de genes (Carrington y Ambros, 2003) y actúan como un mecanismo endógeno de silenciamiento génico de regulación post-transcripcional. Los resultados de estudios con plantas transgénicas que sobre-expresan o mutan pre-microRNAs muestran que éstos

desempeñan una importante función en el desarrollo de las plantas (Bartel, D.P. 2004). Los tejidos de estas plantas alteran su respuesta a la regulación hormonal y presentan malformaciones de órganos. También se han identificado puntos de regulación en el metabolismo primario así como en el secundario mediados por miRNAs (Bartel, D.P. 2004, Jones-Rhoades y Bartel, 2004), lo que evidencia que este tipo de molécula cumple un importante papel en la fisiología de la planta.

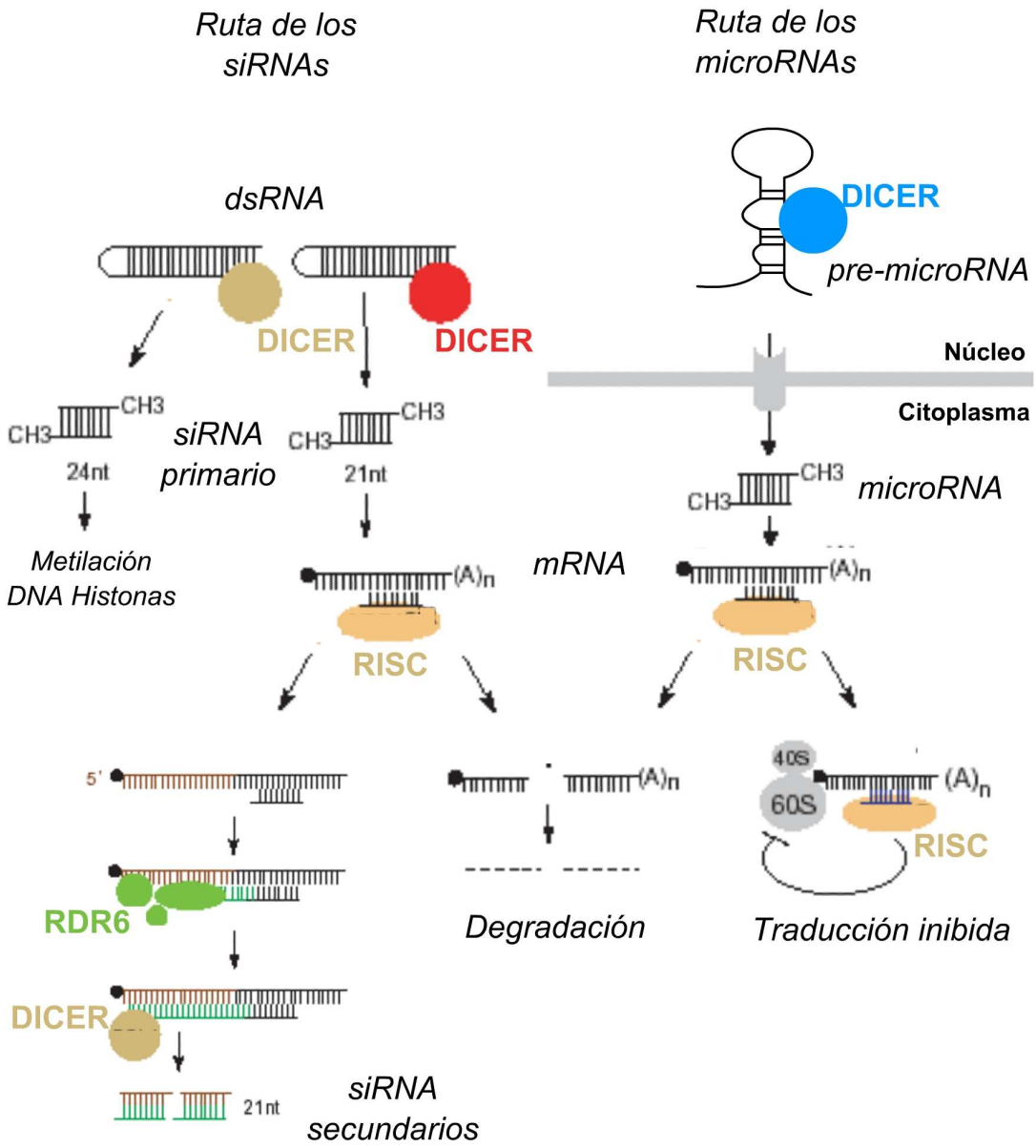


Figura 4. Representación esquemática de la ruta de los siRNAs y de la ruta de los microRNAs

Otro grupo de pequeños RNAs son los denominados RNAs de interferencia (siRNAs, de "small interfering RNAs"). Este tipo de moléculas está involucrado en un mecanismo de silenciamiento y degradación de RNAs exógenos a la planta como transgenes y replicones infecciosos como los virus. Los siRNAs tienen un tamaño de 21 o 24 nucleótidos y son producidos a partir de RNAs de doble cadena (dsRNAs). Este tipo de moléculas se dan en plantas transgénicas donde se han integrado transgenes en sentido y antisentido que se expresan simultáneamente (Stam *et al*. 1997, Metzlaff *et al*.1997). Se ha comprobado que la inserción de transgenes que en su secuencia presentan repeticiones invertidas (IR-RNAs) provoca la aparición de siRNAs (Chuang *et al*. 2000). Tanto dsRNAs como IR-RNAs son fragmentados por enzimas tipo Dicer en siRNAs de 21 nucleótidos que son guiados por RISC a RNAs que presentan homología de secuencia. El RNA queda así inactivo y es digerido en la región apareada con el siRNA produciéndose el silenciamiento post-transcripcional.

Los siRNAs de 24 nucleótidos se forman también mediante el procesamiento de dsRNAs por enzimas tipo Dicer. Este tipo de siRNA está involucrado en la metilación de histonas y regiones de DNA que presentan homología de secuencia. La región metilada del genoma queda inactivada y no puede transcribirse por lo que este mecanismo es capaz de actuar a nivel pre-transcripcional (Hamilton *et al*. 2002, Zilberman *et al*. 2003).

En el caso de los virus, se ha comprobado que sus infecciones provocan la aparición de siRNAs homólogos al genoma viral (Szittya *et al*. 2002). Muchos virus en alguna etapa de su replicación utilizan como intermediarios replicativos moléculas del tipo dsRNAs que son sensibles a ser procesados por Dicers para producir siRNAs. El complejo RISC guía estas moléculas hasta el virus y provoca su degradación (Buchon y Vaury, 2006).

Estudios en *Arabidopsis thaliana* han mostrado la implicación de la enzima RNA polimerasa 6 RNA dependiente (RDR6) en el silenciamiento post-transcripcional. Esta enzima es capaz de crear dsRNAs a partir de RNAs de cadena simple (Dalmay *et al.* 2000, Mourrain *et al*. 2000). Por otro lado, RDR6 es capaz de elongar hebras complementarias de las moléculas hibridadas con siRNAs que actúan como cebadores. A partir de los dsRNAs formados de este modo se obtienen siRNAs secundarios que funcionan como los siRNAs primarios, lo que se traduce en una amplificación del silenciamiento que se difunde a lo largo de la planta (Himber *et al*. 2003). El mecanismo de siRNAs secundarios parece tener gran relevancia en la defensa de la

planta frente a RNAs infecciosos como sugiere el hecho de que plantas con la actividad RDR6 mermada son muy susceptibles a diversos virus (Mourrain *et al*. 2000, Muangsan *et al*. 2004). En *Nicotiana benthamiana* se ha comprobado mediante hibridaciones *in situ* que una deficiencia en esta actividad provoca la invasión de los virus al meristemo apical (Schwach *et al.* 2005).

El silenciamiento post-transcripcional mediado por siRNAs actúa como un mecanismo de defensa sistémico contra virus. Como una estrategia de contraataque, muchos virus codifican proteínas supresoras que merman este mecanismo e interfieren en distintos puntos de su ruta. Un ejemplo es HcPro de potyvirus que evita la acumulación de siRNAs (Kasschau y Carrintong, 2001). Otro ejemplo es la proteína 2b de cucumovirus que es capaz de inhibir la señal sistémica de silenciamiento post-transcripcional (Guo y Ding, 2002). La expresión transitoria de este tipo de proteínas revierte temporalmente genes silenciados post-transcripcionalmente como ocurre con GFP en plantas transgénicas de *Nicotiana benthamiana* (Llave *et al*. 2000, Voinnet *et al*. 2000, Dunoyer *et al.* 2002, Hamilton *et al.* 2002, Pfeffer *et al*. 2002, Takeda *et al.* 2002, Bucher *et al.* 2003). Las proteínas supresoras tienen diversas funciones en el ciclo infectivo de los virus, siendo algunas de ellas proteínas de movimiento, de la cápsida o que intervienen en la replicación.

Se ha comprobado que plantas transgénicas que expresan supresores del silenciamiento muestran una sintomatología similar a la inducida por la infección viral como ocurre en limas mexicanas que expresan la proteína P23 del virus de la tristeza de los cítricos (CTV) (Fagoaga *et al*. 2005, 2006). Está bastante aceptado que la patogénesis de virus se debe a una la alteración del mecanismo de silenciamiento provocada por la expresión de este tipo de proteínas. A pesar de que el contraataque de los virus va dirigido a mermar la ruta de los siRNAs, la expresión de este tipo de proteína también provoca una alteración de los niveles de microRNAs como ocurre en plantas de *Arabidopsis thaliana* que expresan HcPro y muestran malformaciones en su desarrollo (Kaschau *et al*. 2003). La ruta de los microRNAs comparte actividades con la de los siRNAs por lo que ambas son afectadas por los supresores del silenciamiento. Los microRNAs regulan funciones endógenas de las plantas y la alteración de sus niveles parece ser la causa de la patogénesis de los virus (Brodersen y Voinnet, 2006).

Al igual que en los virus, las plantas parecen defenderse de los viroides mediante el mismo mecanismo de silenciamiento post-transcripcional. Las infecciones producidas por viroides de ambas familias provocan la aparición de siRNAs viroidales (Itaya *et al.*

2001, Papaefthimiou *et al*.2001, Martinez de Alba *et al.* 2002). También se ha comprobado que una infección viroidal es capaz de metilar regiones del genoma en las que se ha insertado transgénicamente la secuencia del viroide (Wassenegger *et al*. 1994). La molécula viroidal tiene una estructura secundaria semejante a la de un pre-microRNA y puede ser sustrato de enzimas tipo dicer (Itaya *et al*. 2007) que también pueden actuar sobre intermediarios replicativos del viroide en forma de dsRNAs.

Existen evidencias que indican que los viroides son susceptibles al mecanismo de defensa mediado por siRNAs. Se ha comprobado que su infectividad disminuye al ser coinoculados con dsRNAs viroidales (Carbonell *et al*. 2008). Sin embargo, parece que los viroides son capaces de sortear el mecanismo de defensa ya que esta disminución solo se da en plazos de tiempo relativamente cortos y en condiciones de temperatura no óptimas para la infección. Similares conclusiones se han obtenido en estudios con plantas transgénicas infectadas con PSTVd en las que se ha incluido el gen que codifica la proteína GFP (proteína fluorescente verde) interrumpido con parte de la secuencia de este viroide. En estas plantas no se observa la expresión de GFP debido a que los siRNAs viroidales provocan la degradación del RNA mensajero antes de su traducción. Sin embargo, se ha comprobado que la molécula madura del viroide sortea este mecanismo (Itaya *et al.* 2007, Goméz y Pallás, 2007). Existen hipótesis sobre la resistencia de los viroides a la defensa de la planta. Una posible causa es la robusta estructura secundaria del viroide, que al igual que le confiere resistencia a digestiones con RNAsas, podría impedir la hibridación de siRNAs mediada por RISC. La localización nuclear o cloroplástica de los viroides también puede ejercer un papel en su resistencia ya que el complejo RISC se localiza en el citoplasma celular donde el viroide se encuentra como molécula madura mientras que las formas más sensibles a ser degradadas están localizadas fuera del citoplasma.

Es posible que el mecanismo de defensa de la planta actúe únicamente como regulador de la acumulación del viroide. Así se puede explicar el fenómeno de la protección cruzada en el que la infección de una raza suave protege temporalmente la entrada y acumulación de una raza más agresiva del mismo viroide. Este fenómeno se puede explicar por la acumulación de siRNAs del viroide suave que acciona la defensa de la planta frente al viroide agresivo. Con el tiempo este fenómeno desaparece y aparecen los síntomas de la raza agresiva (Khoury *et al*. 1998, Niblett *et al.*1978).

Como se ha mencionado anteriormente, la sintomatología que provocan virus y viroides son muy parecidas. Este hecho sugiere que la patogénesis de ambas entidades

esté mediada por mecanismos comunes. Los datos acerca de proteínas supresoras de silenciamiento de virus apuntan a que su patogénesis se debe a una interferencia entre mecanismos mediados por sRNAs como son la regulación endógena y la defensa de la planta. En una infección viroidal, sin embargo, no existen proteínas supresoras del silenciamiento ya que los viroides no codifican proteínas. Existen hipótesis sobre los posibles mecanismos de patogénesis:

1. Una patogénesis mediada por la molécula madura del viroide mediante interacciones directas con factores celulares, como sugieren los estudios mencionados anteriormente en los que mínimos cambios en su secuencia provocan la aparición y modulación de los síntomas. Este fenómeno es contradictorio con la hipótesis de que la patogénesis esté mediada por siRNAs. Además estudios en los que se ha secuenciado los siRNAs derivados de PSTVd (Itaya *et al*. 2007) o CEVd (Martín *et al*. 2007) indican una baja proporción de secuencias de los motivos en los que parecen localizarse los determinantes de patogenicidad.

2. Una patogénesis mediada por siRNAs en la que estas moléculas actúen como miRNAs, hibridando con RNAs mensajeros y provocando su degradación. Los resultados de un estudio realizado con plantas transgénicas que contienen repeticiones invertidas no infecciosas de PSTVd mostraron que estas plantas presentaban una sintomatología similar a la producida por la infección del propio viroide (Wang *et al*. 2004). Sin embargo, resulta controvertido que un modelo tan atractivo no se haya repetido o contrastado en estudios posteriores. Tampoco se conoce ningún RNA mensajero de la planta que sea objeto de degradación por siRNAs derivados de virus por lo que la probabilidad de que esto ocurra en un genoma tan pequeño como los viroides es mucho más improbable.

3. Una patogénesis debida a la supresión del silenciamiento mediada por viroides y que conlleve interferencias en la regulación endógena de la planta. No existen evidencias directas que avalen esta hipótesis ya que las infecciones viroidales no son capaces de reestablecer la expresión de transgenes silenciados en *Nicotiana benthamiana* (Itaya *et al*. 2007) ni se han observado alteraciones en

la regulación de miRNAs en plantas de tomate infectadas con CEVd (Martín *et al*. 2007). Sin embargo, existen datos que sugieren este tipo de mecanismo patogénico. Mediante expresión diferencial, se ha observado que plantas de cidro infectadas por CDVd aumentan la expresión de rgsCAM (Tessitori *et al.* 2007), una proteína que puede actuar como supresora endógena de silenciamiento. La sobre-expresión de esta proteína en plantas transgénicas produce una supresión del silenciamiento similar a la producida por el supresor Hc-Pro (Anandalakshmi *et al*. 2000). Por otro lado, CBLVd y CDVd al ser co-inoculados producen un efecto sinérgico. Los efectos sinérgicos que se han descrito en virus parecen estar producidos por la expresión simultanea de sus proteínas supresoras de silenciamiento (Murphy y Bowen, 2006). Los viroides no codifican proteínas y por tanto el efecto sinérgico no puede explicarse de una manera similar. Sin embargo, se ha comprobado que el virus del mosaico necrótico del trebol rojo (RCNMV) es capaz de alterar el mecanismo de silenciamiento post-transcripcional de la planta mediante su replicación y sin la expresión de ninguna proteína que pueda actuar como supresora de silenciamiento (Takeda *et al*. 2005). Es posible que en su replicación, el viroide pueda tener algún efecto en este sentido.

9. Los viroides de los cítricos

Los cítricos son huéspedes naturales de varios viroides pertenecientes a distintos géneros. La mayoria de cítricos son tolerantes a las infecciones viroidales salvo algunas especies que son sensibles y expresan diversas patologías de etiología viroidal. Dado que los cítricos comerciales se propagan vegetativamente, los viroides se encuentran ampliamente difundidos en los países citrícolas, excepto en el caso de cultivares que han sido objeto de programas de saneamiento.

El primer viroide descrito en cítricos fue el CEVd (Semancik y Waters, 1972) como el agente causal de la exocortis. Esta enfermedad había sido descrita mucho antes como una alteración observada en árboles injertados sobre *Poncirus trifoliata* y que presentaban descamaciones en la corteza del patrón asociadas a enanismo y merma de la cosecha (Fawcett y Klotz, 1948). Estudios posteriores demostraron que el agente causal de esta enfermedad era transmisible por injerto por lo que se le atribuyó una etiología viral. La observación de una sintomatología variable, ya que algunas plantas enfermas

mostraban descamación y enanismo mientras que otras presentaban enanismo en ausencia de descamaciones, sugirió la existencia de diversas estirpes del agente causal de la exocortis (Benton *et al*. 1949, 1950). También se observó variabilidad de síntomas al efectuar transmisiones a plantas de cidro Etrog (Calavan *et al*. 1964) por lo que los distintos aislados se clasificaron como agresivos, moderados y suaves (Roistacher *et al*. 1977). La transmisión de los aislados agresivos a *Gynura aurantiaca* provocaba una sintomatología muy evidente en este huésped. El análisis de sus extractos mediante electroforesis evidenció que solo las plantas sintomáticas presentaban la acumulación de una molécula de movilidad característica. Este hecho propició la identificación de CEVd como agente causal de la exocortis (Semancik y Weathers, 1972) y su posterior caracterización (Gross *et al*. 1982, Visvader *et al.* 1982). Sin embargo, a diferencia de los aislados agresivos, las plantas de *Gynura aurantiaca* inoculadas con aislados moderados y suaves se mostraron asintomáticas y no mostraban la banda característica de CEVd. Un resultado similar se observó en extractos provenientes de cidro en los que solo se detectaba CEVd en plantas inoculadas con aislados agresivos siendo indetectable en las plantas inoculadas con aislados suaves.

El desarrollo de método de sPAGE y tinción de plata (Igloi, 1983) ha tenido una gran relevancia en el estudio de los viroides ya que esta técnica ha permitido visualizar las moléculas circulares de los viroides. Así, se observó que los aislados provenientes de plantas afectadas de exocortis contenían moléculas de tipo viroidal de distinta movilidad electroforética. Se observó también que la banda con movilidad característica de CEVd solo se encontraba en plantas inoculadas con aislados agresivos, mientras que los extractos provenientes de plantas inoculadas con aislados suaves o moderados presentaban un patrón de bandas en el que no se encontraba la banda correspondiente a CEVd (Duran-Vila *et al*. 1986, 1988). La evaluación de síntomas en plantas de cidro Etrog inoculadas con las bandas independientes rescatadas de gel, así como los patrones de hibridación obtenidos con sondas creadas a partir de estas bandas, sirvieron para agrupar las moléculas viroidales en cinco grupos (Semancik y Duran-Vila, 1991) que hoy en día corresponden con las cinco especies de viroides descritas en cítricos y aceptadas por el Comité Internacional de Taxonomía de Virus (Flores *et al*. 1998, 2000). Las especies de viroides de cítricos son:

El viroide de la exocortis de los cítricos (CEVd)

Este viroide tiene un tamaño que oscila entre 370 y 375 nucleótidos y pertenece al género *Pospiviroide*. Se ha demostrado que CEVd es el agente causal de la exocortis al verificar los postulados de Koch en *Poncirus trifoliata* (Vernière *et al*. 2004). El cidro Etrog infectado por CEVd manifiesta un fuerte enanismo y epinastia muy marcada acompañada de necrosis en el nervio central de la hoja. CEVd tiene una amplia gama de huéspedes naturales tanto leñosos como herbáceos. Este viroide ha sido descrito en muchas especies de cítricos (Duran-Vila *et al.* 1986), en vid (Flores *et al.* 1985), en tomate (Mishra *et al.* 1991), en haba (Fagoaga *et al.* 1995), en nabo, zanoria, en berenjena (Fagoaga y Duran-Vila, 1996) y en una serie de plantas ornamentales. Se han descrito variantes de este viroide con un tamaño de 463 y 467 nucleótidos que incorporaban duplicaciones de 92 y 96 nucleótidos respectivamente en su dominio T_R (Semancik *et al.* 1994, Fadda *et al.* 2003).

El viroide de la hoja curvada de los cítricos (CBLVd)

Este viroide, antiguamente denominado viroide I de los cítricos (CVd-I), fue descubierto mediante purificación de bandas del patrón electroforético de aislados que se consideraban erróneamente como razas suaves de exocortis (Duran-Vila *et al.* 1986). Hibridaciones moleculares con sondas específicas mostraron una homología de secuencia entre bandas de distinta migración electroforética. Así se evidenció la existencia de dos tipos de variantes denominadas CVd-Ia y CVd-Ib, que fueron confirmadas mediante su caracterización molecular. Estos tipos de variantes presentan un tamaño de 327 y 318 nucleótidos respectivamente (Duran-Vila *et al*.1988, Ashulin *et al.* 1991). CBLVd pertenece al género *Apscaviroide* y tiene una gama de huéspedes restringida a la familia de las Rutáceas. Todas las especies de cítricos testadas se muestran tolerantes frente a este viroide (Vernière *et al.* 2004), salvo la planta indicadora cidro Etrog cuya sintomatología se caracteriza por la aparición de necrosis puntuales en el nervio central de las hojas que provoca una epinastia característica, a veces acompañada de exudaciones gomosas en el tallo y ramificaciones debidas a una pérdida de dominancia apical (Duran-Vila *et al*. 1988). Estos mismos síntomas han sido descritos en una variante descubierta en Japón denominada CVd-I-LSS que presenta una homología de secuencia del 82-85 % con las variantes previamente descritas de

CBLVd (Ito *et al.* 2000).A pesar de no provocar síntomas, la presencia de CBLVd provoca un efecto sinérgico en plantas co-inoculadas con CDVd.

El viroide del enanismo del lúpulo (HSVd)

HSVd se descubrió como el agente causal del enanismo del lúpulo (Sasaki y Shikata, 1977). Estudios posteriores mostraron que este viroide era transmisible a cidro Etrog mostrándose esta planta prácticamente asintomática ante la infección (Sano *et al.* 1986). La primera evidencia de que los cítricos son huéspedes naturales de este viroide se dio mediante la secuenciación y comparación de secuencias de HSVd con dos bandas de distinta movilidad electroforética obtenidas en el estudio de aislados que se consideraban erróneamente como razas suaves de exocortis (Sano *et al.* 1988, Puchta *et al.* 1989, Hsu *et al.* 1994). Estas bandas, habían sido agrupadas mediante patrones comunes de hibridación y se describieron como variantes del viroide II de los cítricos (CVd-II), denominadas CVd-IIa y CVd-IIb (Duran-Vila *et al*. 1986). HSVd es el único miembro del género *Hostuviroide* y sus variantes en cítricos presentan un tamaño que oscila entre 296 y 301 nucleótidos. Todas las variantes de HSVd inducen síntomas de enanismo y rugosidad foliar en pepino (Sano *et al*. 1988) y síntomas muy suaves en cidro (Roistacher *et al.* 1977), donde se encuentran en concentraciones más bajas que los demás viroides de los cítricos (Duran-Vila *et al.* 1993). La caracterización biológica de HSVd mostró que las variantes tipo CVd-IIa no provocan síntomas en la mayoría de especies de cítricos a los que infectan. Sin embargo las variantes tipo CVd-IIb son agentes causales de la enfermedad de la cachexia de los cítricos. Esta enfermedad, también llamada xiloporosis, fue descrita en plantas de lima dulce de Palestina por Reichter y Perlbeger en 1934. Las plantas enfermas presentan hendiduras en la madera que se corresponden con heridas en la cara cambial de la corteza, acompañadas de una decoloración del floema y de exudaciones gomosas. En estadios avanzados de la enfermedad, las plantas sensibles pueden mostrar chancros en tronco y ramas, clorosis foliar y enanismo. En condiciones favorables para la aparición de síntomas esta enfermedad puede acarrear la muerte de la planta. La cachexia afecta tanto a especies que se utilizan como patrón como a especies que se utilizan como variedad. Son especialmente sensibles el *Citrus macrophylla*, los tangelos y los mandarinos. Se ha demostrado que seis posiciones del dominio V de la molécula actúan como determinantes patogénicos de esta enfermedad (Reanwarakorn y Semancik, 1988). Las variantes tipo CVd-IIb presentan cambios de bases y delecciones en estas posiciones

respecto a las variantes tipo CVd-IIa (Palacio y Duran-Vila, 2000). HSVd es el viroide con mayor gama de huéspedes naturales. Se ha descrito en lúpulo, cítricos, pepino (Van Dorst y Peters, 1974), vid (Puchta *et al*. 1988), almendro (Cañizares *et al*, 1999), ciruelo (Sano *et al*. 1989), melocotón (Sano *et al*. 1989), peral (Shikata, 1990) y albaricoque (Amari *et al*. 2001).

El viroide del enanismo de los cítricos (CDVd)

Este viroide, también conocido como viroide III de los cítricos, fue descubierto mediante el análisis molecular de extractos de plantas infectadas con lo que se consideraban erróneamente razas moderadas de exocortis. A partir de patrones electroforéticos y de hibridación se descubrió la existencia de dos tipos de variantes designadas CVd-IIIa y CVd-IIIb (Duran-Vila *et al*. 1988), y su posterior secuenciación constató que tenían tamaños de 294 y 297 nucleótidos respectivamente. Este viroide pertenece al género *Apscaviroide* y su gama de huéspedes está restringida a las rutáceas. CDVd tiene como huésped experimental al cidro Etrog. Su infección en esta planta provoca enanismo acompañado de un decaimiento de las hojas debido a necrosis y anillamiento de los peciolos. Ocasionalmente CDVd puede provocar también epinastia. En especies de interés comercial se ha comprobado que CDVd provoca un enanismo general de la planta (Vernière *et al*. 2004). Este efecto enanizante puede tener interés en algunos países productores ya que una reducción del volumen de la copa puede facilitar las labores del cultivo. La secuenciación de fuentes de CDVd procedentes de diversos países ha mostrado que este viroide mantiene un genoma muy conservado, siendo las variantes tipo CVd-IIIb mucho más frecuentes que las variantes CVd-IIIa (Owens *et al*. 1999).

El viroide de la corteza agrietada de los cítricos (CBCVd)

Este viroide, también denominado viroide IV de los cítricos, se describió inicialmente en California (Duran-Vila *et al.* 1988), en Israel (Hadas *et al*. 1989) y posteriormente en Turquía (Önelge *et al*. 1996). Su caracterización molecular mostró que tenía una secuencia de 284 nucleótidos así como una gran similitud de secuencia con los dominios V y T_R de CEVd (Puchta *et al*.1991). Sin embargo CBCVd pertenece al género *Cocadviroide* debido a la secuencia de su CCR y la de su TCH. La infección en cidro Etrog produce enanismo y epinastia con necrosis en el nervio central. También

se ha asociado a la presencia de grietas en árboles injertados sobre *Poncirus trifoliata* (Vernière *et al.* 2004).

El viroide "OS" de los cítricos (CVd-OS)

Este viroide ha sido descubierto y descrito únicamente en Japón (Ito *et al*. 2001). Tiene un genoma de 330-331 nucleótidos y posee una CCR característica del género *Apscaviroide*. Los cidros infectados muestran síntomas suaves de epinastia y necrosis en el peciolo. Este viroide está en proceso de ser aceptado como una nueva especie por el Comité Internacional de Taxonomía de Virus (ICTV).

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Objetivos

Objetivos

En un trabajo realizado en nuestro laboratorio anterior al desarrollo de la presente tesis, la Dra. Barbosa evaluó la respuesta de un conjunto de especies pertenecientes o afines al género *Citrus*, frente a una infección múltiple con los viroides conocidos que afectan a los cítricos. El análisis biológico y molecular de la especie *Atalantia citroides,* mostró que esta planta no estaba infectada por ninguno de los viroides inoculados. Pero inesperadamente, también mostró la presencia de una molécula con una movilidad característica de los viroides. Los ensayos de transmisión a huéspedes experimentales que se suelen utilizar en el estudio de los viroides de cítricos revelaron que la molécula de característica viroidal es infecciosa únicamente en plantas leñosas.

El presente trabajo comienza bajo estos antecedentes y como primer punto trata de descubrir la naturaleza de la molécula de características viroidales descubierta por la Dra. Barbosa. Así los objetivos del primer capítulo son:

1. Caracterización molecular de una molécula de características viroidales a la que tentativamente denominamos *viroide V de los cítricos* (CVd-V).

2. Caracterización biológica de CVd-V mediante inoculaciones y coinoculaciones con otros viroides de cítricos en plantas de cidro Etrog.

La secuenciación de CVd-V nos otorga información sobre la naturaleza de este nuevo viroide pero también nos abre la posibilidad de utilizar algunos métodos moleculares para su detección. Nos ha parecido interesante como segundo punto del presente trabajo intentar responder si este nuevo viroide ha emergido recientemente como consecuencia de la mezcla de viroides inoculada en *Atalantia citroides,* o por el contrario, es un viroide difundido en la naturaleza y que a pasado desapercibido hasta la fecha. Por otro lado, dada la repercusión que puede tener CVd-V en programas de certificación de plantas libres de patógenos o en programas de cuarentena para la importación y exportación de material vegetal, nos ha parecido necesario estudiar la gama de huéspedes comerciales que tiene CVd-V.

Para ello, y con orden correlativo a los del primer capítulo, los objetivos del segundo capítulo son:

3. Puesta a punto de métodos de detección de CVd-V.

4. Búsqueda y caracterización de variantes de CVd-V en aislados provenientes de distintos lugares del mundo.

5. Elaboración de una gama de huéspedes comerciales para CVd-V.

Los resultados obtenidos en los primeros puntos de la presente tesis nos indican que CVd-V es un nuevo viroide perteneciente al género *Apscaviroide*. Este género se caracteriza por tener una gama de huéspedes restringida a especies leñosas. El difícil manejo que tiene este tipo de plantas, así como los largos periodos de incubación que requieren en comparación con especies herbáceas, ha provocado que existan muy pocos estudios realizados con viroides de este género. En consecuencia, los conocimientos acerca de la biología de los viroides, normalmente obtenidos utilizando especies modelo como PSTVd o HSVd, suelen ser extrapolados a los demás. Dada la poca información que tenemos acerca de los viroides del género *Apscaviroide*, en este punto hemos querido estudiar que partes de la molécula viroidal están involucradas en la expresión de síntomas y por tanto actúan como determinantes de patogenicidad. Para ello, siguiendo con el orden de los anteriores apartados, los objetivos de este tercer capítulo son:

6. Elaboración de una metodología que nos permita intercambiar segmentos discretos previamente definidos de CDVd y CBLVd en CVd-V.

7. Síntesis de moléculas potencialmente infecciosas que contienen viroides quiméricos, así como una evaluación de la infectividad en plantas de cidro Etrog.

8. Evaluación de la expresión de síntomas en plantas inoculadas con los viroides quiméricos así como del efecto que producen en coinoculación con los viroides naturales de los que provienen.

Como cuarto y último punto de esta tesis nos ha parecido oportuno ahondar en el conocimiento de los determinantes de patogenicidad de HSVd como viroide responsable de la enfermedad de la cachexia de los cítricos. Esta enfermedad tiene una especial relevancia en países cálidos, sin embargo, a pesar de que la citricultura española se encuentra muy saneada, el creciente uso del patrón sensible *Citrus macrophylla* nos debe poner en alerta para evitar su difusión. Así, y con orden correlativo a los anteriores puntos de esta tesis, los objetivos de este cuarto y último capitulo son:

9. Síntesis de moléculas infecciosas que incluyen viroides mutantes de HSVd en los que se ha modificado la composición de sus determinantes de patogenicidad.

10. Inoculación y evaluación de la infectividad de viroides mutantes de HSVd en plantas de cidro Etrog.

11. Evaluación de la expresión de síntomas de cachexia provocados por mutantes de HSVd en plantas de mandarino Parson´s Special.

A lo largo de estos años he colaborado en trabajos realizados por mis compañeros de laboratorio. En los anejos de esta tesis se incluyen algunos de estos trabajos.

Capítulo 1

Citrus viroid V: Molecular characterization and synergistic interactions with other members of the genus Apscaviroid

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Abstract

Studies on *Atalantia citroides*, a citrus relative, revealed the existence of a viroid not described previously. The new viroid has a GC-rich genome of 293-294 nucleotides and contains the central conserved region characteristic of members of the genus *Apscaviroid,* and the terminal conserved region present in this and other genera of the family *Pospiviroidae.* The secondary structure of minimum free energy predicted for the new viroid is a rod-like conformation with 68.7% paired nucleotides and showing sequence identities with other viroids always lower than 90%, the conventional limit that separates different species within a given genus. Infectivity assays showed that the new viroid induces mild but characteristic symptoms on the indicator Etrog citron. Co-inoculation of CVd-V with either *Citrus bent leaf viroid* or *Citrus viroid III,* two other members of the genus *Apscaviroid* infecting citrus, disclosed synergistic interactions manifested in enhanced leaf symptoms and very pronounced dwarfing. Viroid titers, however, remained unaltered in co-infected plants. Possible mechanisms underlying the observed synergistic effects are discussed. According to its molecular and biological properties and its unusual ability to replicate in *A. citroides,* the new viroid, tentatively named *Citrus viroid V* (CVd-V), should be considered a new species of the genus *Apscaviroid.*

Introduction

Viroids are small, infectious, circular RNAs, replicating independently in their host plants, in some of which may incite specific disease. They are classified within two families: *Pospiviroidae,* composed of species with a central conserved region (CCR) and without hammerhead ribozymes, and *Avsunviroidae,* encompassing four species lacking CCR but able to self-cleave in both polarity strands through hammerhead ribozymes. Citrus are natural hosts of five viroid species, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Citrus viroid III* (CVd-III), *Hop stunt viroid* (HSVd), and *Citrus viroid IV* (CVd-IV), all belonging to the family *Pospiviroidae* whose type member is *Potato spindle tuber viroid* (PSTVd) (Duran-Vila *et al.,* 1988; Flores *et al.,* 2004). CEVd, CBLVd and CVd-III possess in the left terminal domain of their proposed rod-like secondary structure, a terminal conserved region (TCR) characteristic of species of the genera *Pospiviroid* and *Apscaviroid*, whereas

HSVd and CVd-IV present a terminal conserved hairpin (TCH) characteristic of species of the genera *Hostuviroid* and *Cocadviroid* (Flores *et al.,* 1997).

In the frame of a study aimed at defining the response to viroid infection of several species in the genus *Citrus* and in citrus-related genera, *Atalantia citroides* was identified as an unusual viroid host (Barbosa *et al.,* 2002). *A. citroides* plants, propagated on rough lemon (*Citrus jambhiri* Lush) rootstock and graft-inoculated with an artificial mixture of viroids, appeared to be immune to infection with CEVd, CBLVd, CVd-III, HSVd, and CVd-IV. Unexpectedly, sequential PAGE (sPAGE) analysis of RNAs extracted from the inoculated *A. citroides* scion, revealed the presence of a viroid-like RNA with an electrophoretic mobility between those of HSVd and CVd-III that was absent from the non-inoculated controls. The viroid nature of this RNA was inferred from two lines of evidence: (i) denaturing PAGE of purified preparations showed two bands with the mobilities expected for the circular and linear forms characteristic of viroid RNAs, and (ii) the same purified preparations were infectious in Etrog citron (*C. medica* L.), the classical indicator of citrus viroids. Attempts to transmit this RNA to tomato, chrysanthemum, cucumber, pepper, tobacco, *Gynura aurantiaca* and *Tagetes patula,* all of which support the replication of different viroids, failed, suggesting a restricted host range. Riboprobes specific for CVd-III gave a weak hybridization signal whereas riboprobes specific for CEVd, CBLVd, HSVd, and CVd-IV did not hybridize. These results, together with the restricted host range, suggested that the new viroid RNA was probably a novel member of the genus *Apscaviroid* (Barbosa *et al.,* 2005). Here we report its molecular and biological characterization.

Results

Molecular characterization

To clone and sequence the new citrus viroid, nucleic acid preparations from infected citron plants were subjected to sPAGE, and the gel-eluted circular forms were used: (i) to produce a viroid-specific probe by 5'-end ^{32}P-labeling the RNA fragments obtained by partial hydrolysis, and (ii) as template for cDNA synthesis.

The cDNAs obtained using an RT-PCR approach that does not require prior sequence knowledge (Navarro *et al*., 1998) were in the range of 100-300 bp (Fig. 1A), and included cDNAs of the target viroid as confirmed by hybridization with the viroid-specific probe (Fig. 1B). The PCR-amplified products were cloned in a plasmid vector and the resulting inserts

analyzed by PAGE in 5% gels (Fig. 1C). Three plasmids containing viroid-cDNA inserts were identified by hybridization with the viroid-specific probe (Fig. 1D), the sequences of which were used to determine a consensus sequence of 205 nucleotides (Fig. 2, segment in red) that by comparisons with sequences in databases showed similarities with members of the genus *Apscaviroid*. In particular, the upper and lower CCR strands characteristic of this genus could be identified. However, while the sequence of the upper CCR strand was identical to that of the other members of the genus, the sequence of the lower CCR contained a C197→U transition (pointed by an arrow in Fig. 2).

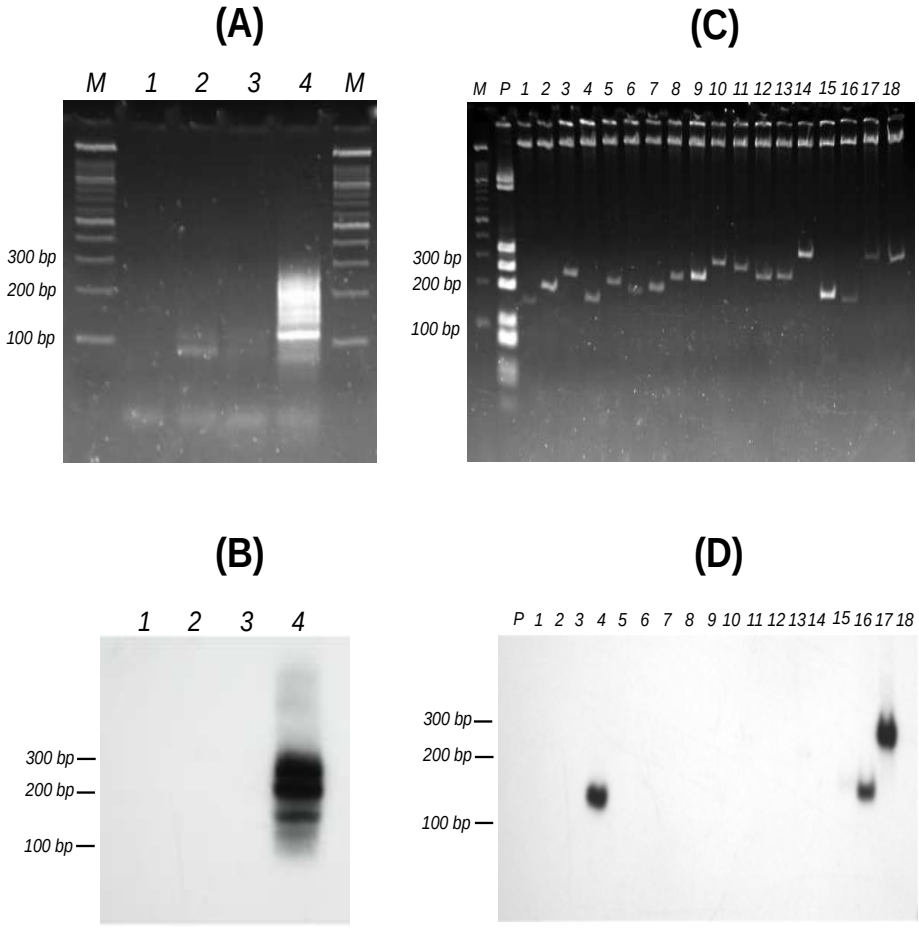

Fig. 1. (A) Analysis by PAGE and ethidium bromide staining of viroid cDNAs in the range of 100-300 nt obtained by RT-PCR following an approach that does not require prior knowledge of its sequence (Navarro *et al*., 1998): (1) PCR control without cDNA, (2) RT-PCR control devoid of AMV-RT, (3) RT-PCR control without RNA template, (4) RT-PCR in which all the ingredients were included, (M) 100-bp ladder. **(B)** Southern analysis of the viroid cDNAs electroblotted to membranes and hybridized with a viroid-specific radioactive probe synthesized by 5'-end labeling viroid RNA fragments obtained by partial hydrolysis. **(C)** Restriction analysis by PAGE and ethidium bromide staining of recombinant plasmids: (M) 100-bp ladder, (P) Bluescript KS+ plasmid digested with *Sau* 3A, (1 to 18) Recombinant plasmids digested with *Eco*RI and *Hind*III. **(D)** Southern analysis of the plasmid inserts electroblotted from to a membrane and hybridized with the viroid-specific radioactive probe.

To obtain the complete sequence of the viroid, two adjacent primers of opposite polarity PI and PII were designed from the 205-nucleotide sequence, and used in an RT-PCR in which purified circular forms of the viroid were included as templates. The amplified cDNA product, which exhibited in non-denaturing PAGE the mobility expected for the size of the new viroid (data not shown), was cloned in a plasmid vector and thirty clones were analyzed by single-strand conformational polymorphism (SSCP). From the electrophoretic mobility of the ssDNAs, six different SSCP profiles or haplotypes were identified, one of which was clearly predominant (data not shown). Sequencing of twelve recombinant plasmids demonstrated that the population indeed contained a predominant variant (V1) of 294 nucleotides (nt) representing 80% of the overall population and five additional variants of 294 nt (V2, V3, V4, V5) and 293 nt (V6) differing from V1 by one to two changes (Fig. 2). Examination of their primary structure revealed the presence of the TCR characteristic of the genus *Apscaviroid* (Fig. 2, shaded nucleotides 18-28). To confirm the sequence of the region covered by primers PI and PII, a new RT-PCR was performed using a second pair of adjacent primers of opposite polarity (PIII and PIV) designed around the TCR. Sequencing of the inserts of four recombinant plasmids corroborated the existence of a dominant variant V1, with 88 G (29.9%), 90 C (30.6%), 53 A (18.0%) and 63 U (21.4%), having therefore a G+C content of 60.5%. All variants presented the C197→U change in the lower CCR strand (Fig. 2).

V1, selected as the reference variant of the new viroid, had a predicted rod-like secondary structure of minimal free energy (Fig. 2) with 68.7% of the nucleotides paired (71.3% G-C, 22.8% A-U and 5.9% G-U pairs). The transition C197→U in the lower CCR strand resulted in the change of a canonic base pair (G-C) between the upper and lower strands into a wobble base pair (G-U) (Fig. 2). The conserved nucleotides of the CCR upper strand and the flanking inverted repeats can form a thermodynamically stable hairpin (hairpin I in Fig. 2), which like in all members of the family *Pospiviroidae* includes a terminal tetraloop, an adjacent 3-bp stem and a long stem at the base. Its structure and nucleotide composition is identical to that proposed for the *Apple scar skin viroid* (ASSVd), the type species of the genus *Apscaviroid* (Koltunow and Rezaian, 1989; Flores *et al.,* 1997). Moreover, the lower strand of the rod-like structure can alternatively form a stable hairpin (Fig. 2) with a GC-rich stem of 7 bp resembling the hairpin II detected, together with hairpin I, in PSTVd during thermal denaturation (Riesner *et al.,* 1979; Loss *et al.,* 1991).

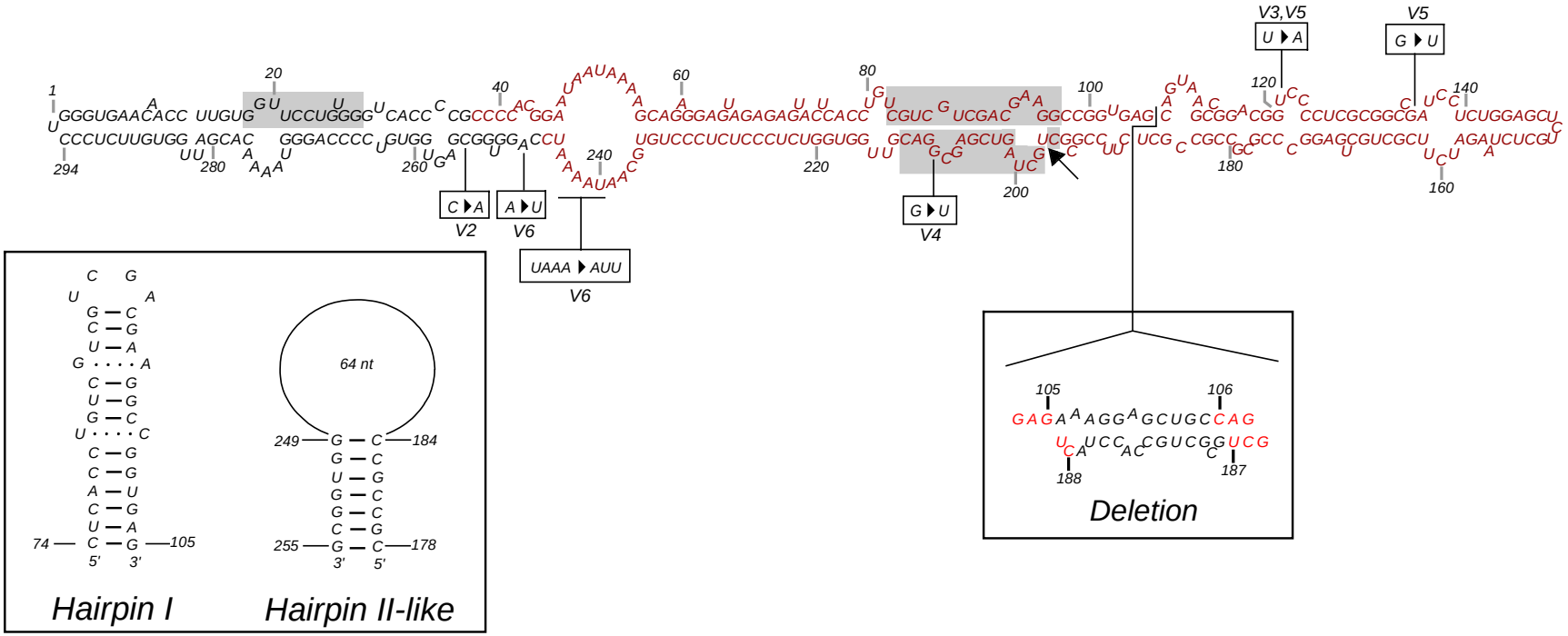

Fig. 2. Primary and proposed secondary structure of minimum free energy of variant V1 (the fragment of 205 nucleotides retrieved from the first cloning experiment is shown in red). The changes observed in five additional viroid variants (V2 to V6) are shown in boxes (V2: C254→A; V3: U121→A; V4: G209→U; V5: U121→A, G123→U; V6: UAAA[240-243]→AUU, A248→U). Variants were obtained with primers PI and PII (identical and complementary to positions 88-107 and 64-87, respectively). The sequence of the region covered by PI and PII was confirmed with additional variants obtained with primers PIII and PIV (identical and complementary to positions 24-41 and 4-23, respectively). Conserved regions (CCR and TCR) in members of the genus *Apscaviroid* are shaded. The arrow points at position 197, which is U instead of C as in all other members of the genus *Apscaviroid.* Left inset, hairpin I and hairpin II-like motifs are metastable structures that can be alternatively formed by sequences from the upper and lower strands of the rod-like structure. Right inset, fragment of ASSVd deleted in CVd-V that accounts for the difference in size between the two viroids.

Phylogenetic relationships

Sequence alignment with the other members of the genus *Apscaviroid* revealed identities (in %) of 73.5 (ASSVd), 64.6 (*Apple dimple fruit viroid* and *Grapevine yellow speckle viroid-2*), 62.6 (CVd-III), 61.6 (*Grapevine yellow speckle viroid-1*), 59.9 (CBLVd), 49.3 (*Pear blister canker viroid*) and 39.1 (*Australian grapevine viroid*) (data not shown). These identities were always lower than 90%, the value adopted by convention to discriminate different species within a given genus. Following the nomenclature used to name citrus viroids (Duran-Vila *et al.,* 1988), the new viroid has been tentatively designated as *Citrus viroid-V* (CVd-V) until more is known about its effects in different citrus hosts.

A consensus phylogenetic tree based on the multiple sequence alignment illustrates the relationship between CVd-V and the other members of the genus *Apscaviroid* (Fig. 3). Comparison between the primary and predicted secondary structures of CVd-V and its closest relative (ASSVd) revealed that nucleotide differences were scattered in distinct regions of the whole molecule including the lower CCR strand; whether this transversion is natural or a cloning artifact remains to be elucidated. Whereas all apscaviroids contain a U-rich segment in the lower strand of the pathogenicity domain (Koltunow and Rezaian, 1989), CVd-V has in the same region several U→A changes that result in a large loop of unpaired nucleotides (delimited by positions 46-56 and 235-245 in Fig. 2). In addition, CVd-V presents two compensatory deletions of 11 and 13 nt in the upper and lower strands of the viroid secondary structure respectively, which account for the difference in size between ASSVd and CVd-V (Fig. 2).

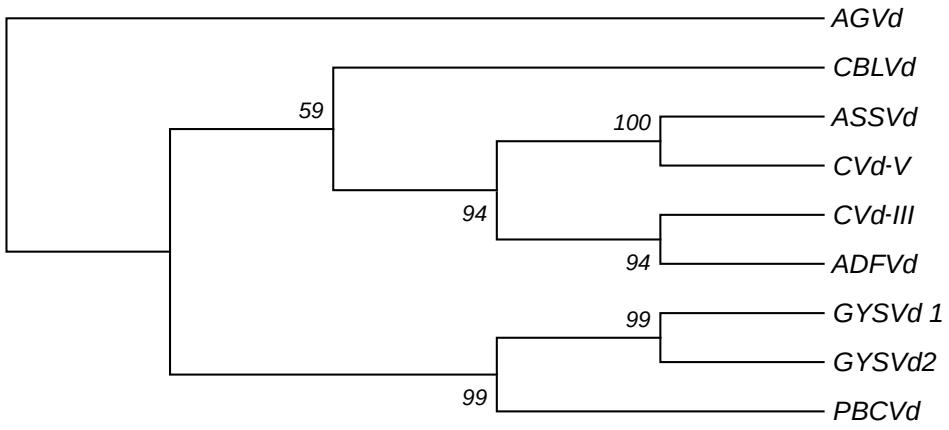

Fig. 3. Consensus phylogenetic tree (based on 10.000 bootstrap replicates) obtained for all members of the genus *Apscaviroid.* Bootstrap values (in %) are indicated in the nodes

Infectivity and symptom expression

To obtain infectious preparations of CVd-V, a head-to-tail dimeric cDNA of the predominant variant V1 was synthesized and used as template to produce the corresponding *in vitro* transcripts that were inoculated mechanically to four citron plants. Analysis by sPAGE and Northern-blot hybridization confirmed infection of the four plants six months after inoculation, when no symptoms had yet developed (data not shown). However, ten months after inoculation, the stems of the infected plants showed very small, necrotic, gum-filled lesions. The sequences of three cDNA clones, obtained by RT-PCR with primers PI and PII and viroid preparations from each of the four infected plants, were identical to that of variant V1.

To better compare the symptoms induced by CVd-V with those characteristic of the other two citrus apscaviroids (CBLVd and CVd-III), three buds from a citron plant infected with CVd-V and three buds from each of two citron plants infected with CBLVd or CVd-III, were graft-propagated on rough lemon seedlings. Three buds from a viroid-free citron plant were similarly propagated as controls. The new growing material of the grafted plants was observed over a 4-month period and showed the symptoms summarized in Table 1. CBLVd-infected citron plants presented the typical "variable syndrome" characterized by flushes of tissue showing mild leaf epinasty alternating with flushes of symptomless leaves (Fig. 4A). The mild epinasty or bending of the leaves (Fig. 4B) was the result of local midvein necrosis on the underside of the leaf (Fig. 4C). The stems presented severe necrotic lesions and cracks releasing gum exudates (Fig. 4D and E). Eventually, the main shoot lost apical dominance, stopped growing, and the plants underwent an unusual branching pattern (Fig. 4A and E). CVd-III-infected citron plants presented the "dropping leaf" pattern (Fig. 4A) due to a moderate epinasty resulting from petiole and mid-vein necrosis (Fig. 4F) and no stem symptoms. CVd-V-infected citron plants showed the mildest symptoms, with only very small necrotic lesions and cracks, sometimes filled with gum, being observed in the stems (Fig. 4G).

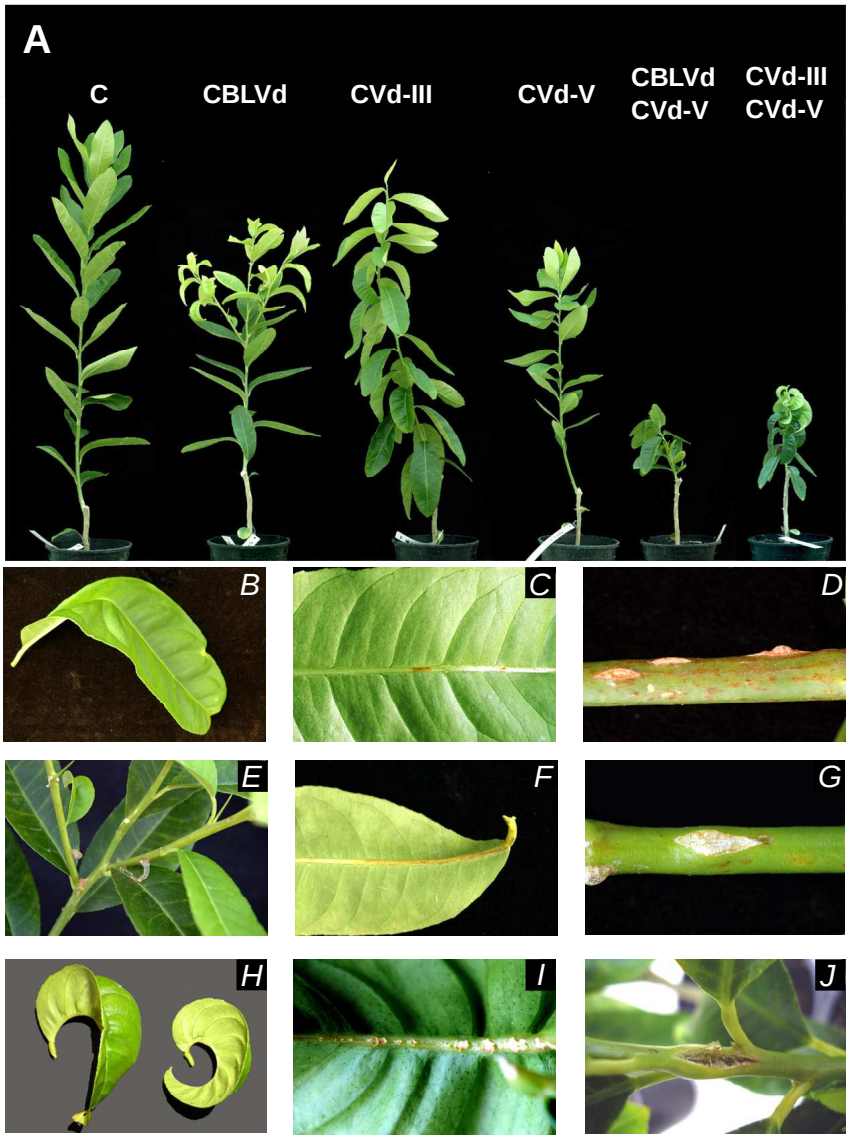

Fig. 4. Symptoms of viroid infection in Etrog citron plants, 4 months post inoculation. **(A)** General aspect of plants non-inoculated (c), infected with a single viroid (CBLVd, CVd-III, or CVd-V), or co-infected with two viroids (CBLVd and CVd-V; CVd-III and CVd-V). **(B)** Bending of the leaves of CBLVd-infected plants. **(C)** Local midvein necrosis on the underside of the leaf blade of CBLVd-infected plants. **(D)** Cracks releasing gum exudates in CBLVd-infected plants. **(E)** Branching pattern and gum exudates in CBLVd-infected plants. **(F)** Petiole and midvein necrosis of CVd-III-infected plants. **(G)** Cracks in the stem of CVd-V-infected plants. **(H)** Leaf curling of plants co-infected with CVd-V and CBLVd (left), or with CVd-V and CVd-III (right). **(I)** Lesions in the midvein of plants co-infected with CVd-V and CBLVd, or with CVd-V and CVd-III. **(J)** Severe cracks devoid of gum in plants co-infected with CVd-V and CBLVd.

Synergistic effects with other Apscaviroids

To see the effect of CBLVd or CVd-III co-infecting CVd-V-infected plants, two additional treatments were carried out. Six buds from a CVd-V-infected citron were each graft-propagated on rough lemon seedlings and, concurrently, three of these plants were graft-inoculated with bark from a CBLVd-infected citron, and the other three with bark from a

CVd-III-infected citron. Co-infection was verified by dot-blot hybridization (data not shown). Periodical examination of plant growth showed that, in contrast to the mild stunting observed in plants singly-infected with CBLVd, CVd-III or CVd-V, both sets of doubly-infected plants (CBLVd and CVd-V, and CVd-III and CVd-V) were very stunted (Fig. 4A), thus revealing a synergistic effect between CVd-V and either CBLVd or CVd-III. Fig. 5 displays data on plant growth over a four-week period for the three sets of treatments (non-inoculated controls, infection with a single viroid and co-infection with two viroids). Comparisons of the average height values, the linear components and the quadratic components using the orthogonal contrast ANOVA confirmed that: i) plant growth was affected as a result of viroid infection, ii) growth of plants co-infected with two viroids were more affected than growth of plants infected with a single viroid, and iii) plants infected with a single viroid presented similar growth patterns, and plants co-infected with two viroids also presented similar growth patterns but distinct from that of plants infected with a single viroid.

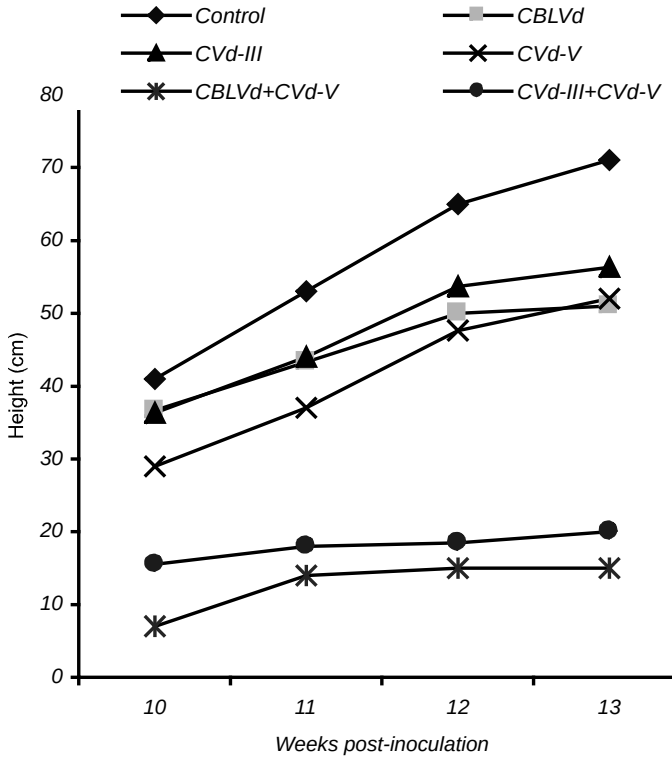

Fig. 5. Growth curves of citron plants infected with a single viroid (CBLVd, CVd-III, or CVd-V), co-infected with two viroids (CBLVd and CVd-V, CVd-III and CVd-V) and non-inoculated controls. Data are the means of the height values determined over a four-week interval. Orthogonal contrast ANOVA revealed: i) significant differences among treatments in the average height (P-value=0.0001<0.05) and the linear component (P-value=0.0008<0.05), but not in the quadratic component (P-value=0.7333>0.05). ii) significant differences between infected and non-inoculated plants in average height (P-value=0.0001<0.05) and linear component (P-value=0.0007<0.05); iii) significant differences between plants infected with a single viroid and plants co-infected with two viroids in average height (P-value=0.0001<0.05) and linear component (P-value=0.0005<0.05); and iv) no significant differences in average height (P-value=0.3135>0.05) and linear component (P-value=0.2812>0.05) in the remaining variability.

As shown in Table 1, all doubly-infected plants also expressed severe epinasty (Fig. 4H) associated with multiple lesions in the midvein (Fig. 4I), a symptom not observed in plants singly-infected with CBLVd, CVd-III or CVd-V. Regarding stem symptoms, plants co-infected with CBLVd and CVd-V presented the severe cracking characteristic of CBLVd but cracks were devoid of gum exudates (Fig. 4J), whereas plants co-infected with CVd-III and CVd-V presented necrotic lesions and the variable syndrome normally associated with CBLVd infection.

Table 1. Symptoms induced by citrus apscaviroids and their synergistic interactions

	Plant growth			Leaf symptoms			Stem symptoms		
Viroid	Variable syndrome[1]	Branching	Stunting	Midvein necrosis	Petiole necrosis	Leaf epinasty	Necrotic lesions	Cracking	Gum exudates
Uninfected	-	-	-	-	-	-	-	-	-
CBLVd	+	+	mild	local	-	mild	severe	severe	severe
CVd-III	-	-	mild	general	+	moderate	-	-	-
CVd-V	-	-	mild	-	-	-	mild	mild	mild
CVd-V+CBLVd	-	+	severe	general	-	severe	-	severe	-
CVd-V+CVd-III	+	-	severe	general	+	severe	moderate	-	-

[1] Variable syndrome refers to the presence in the same shoot of flushes with leaves showing epinasty alternating with flushes of symptomless leaves, as first described by Schlemmer et al. (1985).

Viroid titer remains unaffected in co-infected plants

To find out whether there was any correlation between the dramatic effect of double infection on symptom expression and the titer of the co-infecting viroids, plants were analyzed by sPAGE and Northern-blot hybridization six months post inoculation. Total RNA levels in the preparations were comparable, as revealed by ethidium bromide staining of the first non-denaturing gel (Fig. 6A and 6A'). In plants singly-infected with CBLVd or CVd-V, or co-infected with both, silver staining of the second denaturing gel showed that the titer of CBLVd was always higher than that of CVd-V (Fig. 6B), and Northern-blot hybridization with specific probes failed to detect difference in the titter of these two viroids in singly- or doubly-infected plants (Fig. 6C and 6D).

In plants infected with CVd-III or CVd-V, or co-infected with both, silver staining of the second denaturing gel showed that the titer of CVd-III was lower than that of CVd-V in

singly-infected plants, but the resolution of sPAGE did not allow to discriminate between the two viroids (which moved as a single band) in the co-infected plants (Fig. 5B'). However, Northern-blot hybridization showed that the titer of each of these two viroids was the same irrespective of whether the plants where singly-infected with any of these two viroids or doubly-infected with both (Fig. 5C' and 5D').

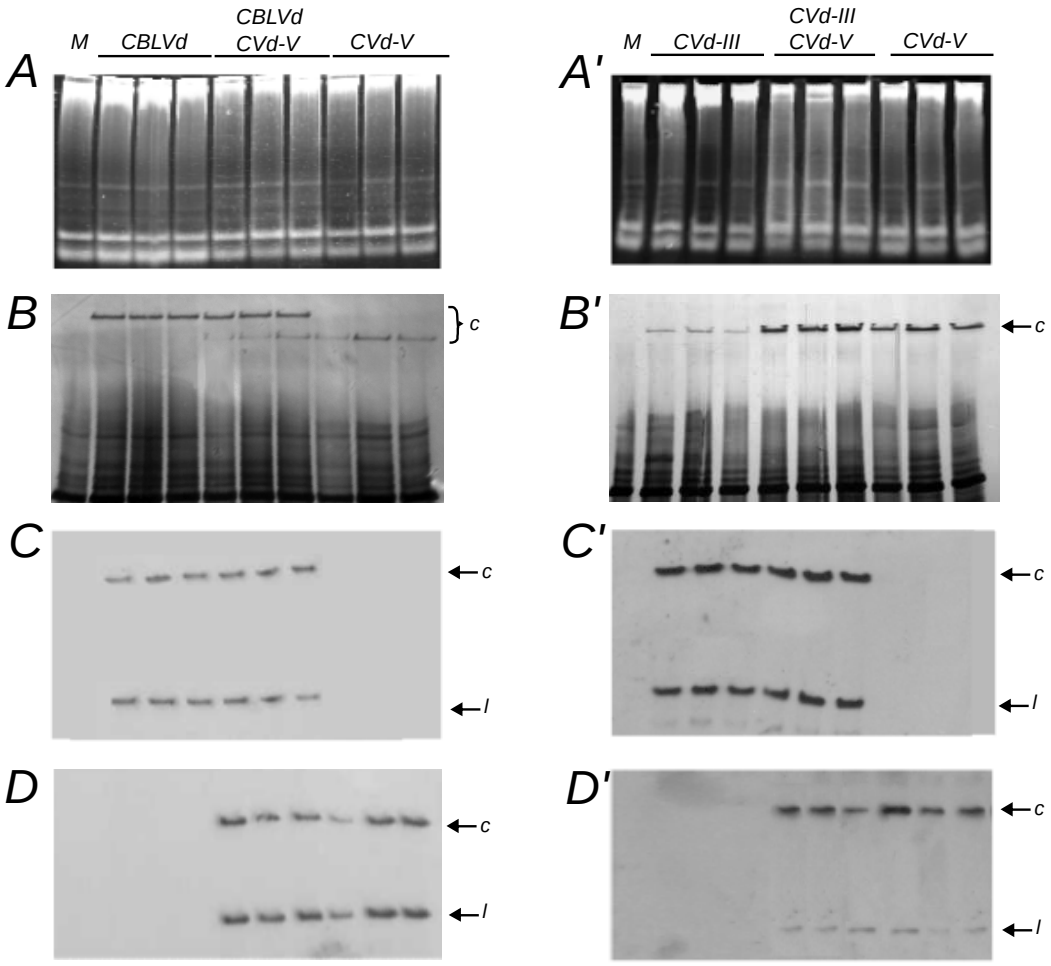

Fig. 6. Analysis by sPAGE and Northern-blot hybridization of citron plants infected with CBLVd, CVd-V or co-infected with both **(A)** to **(D)**, and citron plants infected with CVd-III, CVd-V or co-infected with both **(A')** to **(D')**. M, mock-inoculated control. **(A)** and **(A')** Ethidium bromide staining of the first non-denaturing gel of sPAGE showing that RNA levels in all preparations were comparable. **(B)** and **(B')** Silver staining of the second denaturing gel of sPAGE. **(C)** and **(C'),** and **(D)** and **(D')** Northern-blot hybridizations with DIG-labeled probes specific for CBLVd **(C)**, CVd-III **(C')** and CVd-V **(D)** and **(D')**. Positions of the viroid circular (c) and linear (l) forma are indicated at the right.

Discussion

Citrus plants are natural hosts of several viroids, all of which belong to the family *Pospiviroidae* (Duran-Vila *et al*., 1988; Flores *et al*., 2004). Except in countries where sanitation programs have been implemented, viroids are widespread in commercial plantations that have been propagated from symptomless but viroid-infected budwood. All citrus viroids induce specific symptoms on the Etrog citron indicator but only two, CEVd and HSVd, have other sensitive citrus hosts in which they cause exocortis and cachexia diseases, respectively (Semancik and Weathers, 1972; Semancik *et al.,* 1988; Reanwarakorn and Semancik, 1998). Other citrus viroids, particularly CBLVd and CVd-III of the genus *Apscaviroid*, do not induce easily recognizable effects in citrus used commercially as scions or as rootstocks, but infection may cause reduction in tree size and yield (Semancik *et al*., 1997; Vernière *et al*., 2004; Vidalakis *et al*., 2004).

Results of a previous study revealed that *A. citroides* is an unusual viroid host because it is resistant to all previously known citrus viroids, yet capable of replicating a viroid not reported earlier (Barbosa *et al.,* 2005). Even though the origin of this new viroid (CVd-V) is still uncertain, it was probably present, but overlooked, in the inoculum sources of HSVd or CVd-III, two viroids with very similar electrophoretic mobility in sPAGE. Molecular characterization of CVd-V showed that: (i) it contains the characteristic CCR of members of the genus *Apscaviroid*, as well as the TCR present in this and other genera of the family *Pospiviroidae,* (ii) its most stable secondary structure is a rod-like conformation with 68.7% of paired nucleotides, (iii) its sequence identity with other viroids is lower than 90%, the conventional limit used to separate different species within a given genus, and (iv) it induces specific symptoms in citron. From the above properties, we propose that CVd-V should be considered as a new species in the genus *Apscaviroid*. CVd-V propagates *in vivo* as a population of closely related variants with changes distributed throughout the viroid rod-like secondary structure, as also happens with CVd-III and CBLVd (Owens *et al.,* 1999; Foissac and Duran-Vila, 2000). However, a unique feature of CVd-V is that, unlike the other members of the genus *Apscaviroid*, presents in the lower CCR strand the transition C197→U that results in the change of a canonic (C-G) base pair between the upper and lower strands into a non-canonic (G-U) base pair. This change C197→U was retained in all CVd-V variants identified, therefore discarding that it could be an artifact.

Interactions between co-infecting agents, today known to be viroids, were described long ago. The best example is cross-protection, in which a plant first infected with a mild strain of a viroid and then challenge-inoculated with a severe strain of the same viroid, continues expressing only mild symptoms, with the severe strain usually accumulating to low levels. Cross-protection between mild and severe strains of PSTVd, *Peach latent mosaic viroid* (PLMVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd), were exploited for biological indexing even before viroids were discovered (Fernow, 1967; Horst, 1975; Desvignes, 1976). These early observations were extended in further studies to strains of PSTVd, CEVd, HSVd and CChMVd (Khoury *et al.,* 1988; Duran-Vila and Semancik, 1990; Semancik *et al.,* 1992; De la Peña *et al.,* 1999). In addition, cross-protection was shown to also occur between different but closely related viroids (Niblett e*t al.* 1978; Pallás and Flores, 1989). Even though the effect is less dramatic, co-inoculations with two strains of the same viroid or with two closely related viroids also result in modulation of symptom expression and changes in the titer of one of the viroids (Branch *et al.,* 1988; Pallás and Flores, 1989; Semancik *et al.,* 1992; De la Peña and Flores, 2002). Although cross-protection seems to be a general phenomenon in viroids of both families, intriguingly it has never been described between members of the genus *Apscaviroid.*

In addition to cross-protection, several observations indicate the existence of a second class of interactions in plants co-infected with mixtures of distantly related viroids. They can result either in no effect or in symptoms being much more severe than those expected for purely additive effects. More specifically, enhancement of symptom expression in citron was described with viroid mixtures containing CBLVd and CVd-III (Semancik and Duran-Vila, 1991; Duran-Vila *et al.,* 1988), but no further study of this apparently synergistic interaction was conducted. We report here a detailed examination and evaluation along time of the synergistic effects between two pairs of viroids (CBLVd and CVd-V, and CVd-III and CVd-V), manifested in enhanced leaf symptoms and pronounced dwarfing without discernible changes in viroid titers. A similar synergistic effect was observed in a separate assay in which citron plants were co-inoculated with CBLVd and CVd-III (data not shown), thus confirming previous results and showing that effects of this kind are not restricted to mixtures in which one of the components is CVd-V.

Regarding the underlying mechanism, we can only advance some ideas derived from recent studies on interactions between co-infecting viruses. RNA-mediated cross-protection between viruses has been shown to be mechanistically equivalent to post-transcriptional gene silencing (PTGS) (Ratcliff *et al.,* 1999). This mechanism, mediated by the small interfering

RNAs (siRNAs) generated by one or more dicer-like enzymes, could also operate in cross-protection between viroids, with the siRNAs from the first inoculated strain loading the RNA induced silencing complex and targeting the RNA of the challenging strain for degradation (Flores *et al.,* 2005). Because PTGS additionally regulates plant development, and because the defensive and the developmental PTGS pathways share common components, co-infection by two distinct viruses may result in enhanced symptom expression as a result of their silencing suppressors acting at distinct sites of the RNA silencing pathways (Pruss *et al.,* 1997; MacDiarmid, 2005). A parallel interpretation cannot be extrapolated to explain synergism between viroids because, lacking any messenger RNA activity, they do not encode silencing suppressors. However, new data indicate that a plant RNA virus suppresses RNA silencing as a consequence of sequestering for its replication enzymes involved in the biogenesis of the siRNAs and the microRNAs, the final effectors of PTGS (Takeda *et al.,* 2005). It is possible that viroids could also interfere with the RNA silencing machinery of their hosts through a similar mechanism, and that the synergistic effects between distantly related pairs of co-infecting apscaviroids could result from affecting more than one component of this machinery.

Materials and methods

RNA analysis by sPAGE and purification of viroid circular forms

Aliquots of the nucleic acid preparations from viroid-infected citrons were examined by two consecutive polyacrylamide gel electrophoreses (sequential PAGE, sPAGE), the first under non-denaturing and the second under denaturing conditions (Rivera-Bustamante *et al.,* 1986). The denaturing gel was stained with ethidium bromide and the viroid circular forms were eluted overnight with TEP buffer (0.1 M Tris-HCl, pH 9.0, containing 0.1 M, 2-mercaptoethanol, 10 mM EDTA and 1% SDS) in the presence of phenol/chloroform. The RNA was recovered by ethanol precipitation and resuspended in water. For analytical purposes, the denaturing gel was stained with silver (Igloi, 1983) or electrobotted to nylon membranes for Northern-hybridization.

Northern and slot-blot hybridization

For Northern-blot hybridization, the RNAs separated by PAGE or sPAGE were electroblotted (400 mA for 2 h) to positively-charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA). RNAs were immobilized by UV cross-linking and hybridized with ^{32}P- or DIG-labeled probes. Prehybridization (at 50ºC for 2-4 h) and hybridization (at 50ºC overnight) were performed in 50% formamide and 6X SSPE as described by Sambrook *et al.* (1989). After hybridization the membranes were washed twice in 2X SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1X SSC, 0.1% SDS at 60ºC for 60 min, and revealed by autoradiography (radiolabeled probes) or with an anti-DIG alkaline phosphatase conjugate and the chemiluminiscence substrate CSPD (Roche Applied Science) (DIG-labeled probes).

Preparation of viroid-specific probes

Viroid-specific probes were synthesized essentially as described earlier (Negruk *et al.,* 1980) with minor modifications. A preliminary time-course experiment, conducted to optimize the hydrolysis treatment with deionized formamide at 100ºC prior to 5'-end labeling with polynucleotide kinase and [γ-^{32}P]ATP, showed that hydrolysis for 15 min yielded the highest amount of labeled full-length linear RNAs. Therefore, an aliquot of the purified viroid preparation was treated with deionized formamide at 100ºC for 15 min and, after cooling on ice, the partially hydrolyzed RNA was recovered by ethanol precipitation. RNA fragments were 5'-end labeled for 60 min at 37 ºC with 10 U of T4 polynucleotide kinase, 20 U of RNase inhibitor and 10 µCi of [γ-^{32}P]ATP (3000 Ci/mmol) in 20 µl of 50 mM Tris-HCl, pH 8.3, containing 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 7% glycerol and 1 mM spermidine. The non-incorporated [γ-^{32}P]ATP was removed by Sephadex G-50 chromatography. DIG-labeled viroid probes were synthesized by PCR using as a template plasmids containing full-length viroid sequences (Palacio *et al.,* 2000).

RT-PCR amplification, cloning and sequencing

Viroid cDNA clones were obtained by an approach that uses minimal amounts of template and does not require prior knowledge of its sequence (Navarro *et al*., 1998). In brief,

first strand cDNA was synthesized at 42ºC for 30 min with avian myeloblastosis virus reverse transcriptase (AMV-RT) and an oligonucleotide containing a defined sequence at its 5' moiety and six randomized positions at its 3' end (5'-GCCCCATCACTGTCTGCCCGNNNNNN-3'). Synthesis of second strand cDNA was primed by the same oligonucleotide and catalyzed by the Klenow fragment of the *Escherichia coli* DNA polymerase I at 37ºC for 30 min. The resulting DNA was subjected to PCR amplification with *Taq* DNA polymerase (Roche Applied Science) and a primer having the same sequence as that used for cDNA synthesis with the exception of the six degenerated positions at the 3' end. The cycling profile consisted of 30 cycles of 40 s at 94°C, 30 s at 60°C and 2 min at 72°C, with an initial denaturation at 94°C for 2 min and a final extension at 72°C for 10 min. The PCR-amplified product was ligated in the vector pTZ57R/T (Fermentas) with protruding 3'-terminal Ts, and the recombinant plasmids were used to transform DH5α *E. coli* cells. The resulting plasmids were digested with *Eco*RI and *Hin*dIII and analyzed by PAGE in 5% gels and hybridization to identify those containing viroid-cDNA inserts.

From the consensus sequence of three partial-length viroid-cDNA clones, a pair of adjacent viroid-specific primers of opposite polarities PI (5'-TCGACGAAGGCCGGTGAGCA-3') and PII (5'-CGACGACAGGTGAGTACTCTCTAC-3') homologous and complementary to positions 88-107 and 64-87, respectively, of the viroid reference sequence (see Fig. 2), were designed and applied for RT-PCR amplification of the complete viroid sequence with *Pfu* DNA polymerase (Stratagene) using the same conditions described above. The amplification products were cloned in pBluescript II KS (+) (Stratagene) digested with *Eco*RV. From the sequence of the first series of full-length viroid cDNA clones, another pair of adjacent viroid-specific primers of opposite polarities PIII (5'-TGTGGGTCACCCCGCCCC-3') and PIV (5'-GGAACCACAAGGTTGTTCAC-3') homologous and complementary to positions 24-41 and 4-23, respectively, was synthesized and used for generating a second series of full-length viroid cDNA clones with the same protocol. Sequencing was performed automatically with an ABI PRISM 377 apparatus (Perkin Elmer).

Single-strand conformation polymorphism (SSCP) analysis

PCR amplifications of full-length viroid-cDNA clones with primers PI and PII was carried out in a final volume of 50 µl containing 4 µl of the corresponding overnight cultures.

Aliquots (3 µl) of the amplified products were mixed with 2 µl of denaturing solution (90% formamide, 25 mM EDTA [pH 7], 0.05% xylene cyanol and 0.05% bromophenol blue), heated at 95°C for 10 min and cooled immediately on ice. The denatured DNA strands were separated by PAGE in 14% gels and visualized by silver staining (Igloi, 1983). Under these electrophoretic conditions the DNA migrates as two partially-denatured single strands (hDNA and cDNA) homologous and complementary to the viroid sequence, respectively (Palacio and Duran-Vila, 1999).

Sequence analysis and prediction of RNA secondary structure

Alignment of multiple sequences was performed using the program Clustal W (Thompson *et al.,* 1994). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) based on 10.000 replicates. Genetic distances were calculated following the method of Jukes and Cantor (1969) after manual adjustment for keeping aligned the CCR and TCR. All these analysis were conducted using the MEGA 3.1 program (Kumar *et al.,* 2004). The most stable secondary structure analysis was obtained with the MFOLD program (circular version) from the GCG package (Zuker et al.,1989), and with RNAviz program (De Rijk and De Wachter, 1997). The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases (**EF617306**).

Infectivity assays

Monomeric viroid-DNA inserts were recovered as blunt-end PCR products using phosphorylated primers PI and PII and *Pfu* DNA polymerase. The DNA products were subjected to ligation with 2 U of T4 DNA ligase (Gibco) and the dimeric molecules were cloned in pBluescript II KS (+) digested with *Eco*RV. Plasmids from transformed cells were sequenced to verify the desired head-to-tail orientation of the dimeric inserts. Clones with these inserts were linearized with *Hind*III and used as a template in transcription reaction with 1 mM NTPs, 1 mM DTT and 50 U of T7 RNA polymerase to produce dimeric transcripts homologous to the viroid sequence. Four Etrog citron (selection 861-S1) plants graft-propagated on rough lemon rootstocks were slash-inoculated (50 ng of transcript per plant) and kept in the greenhouse at 28º-32ºC.

Viroid infected plants were obtained with two strategies: i) graft-propagation of buds from viroid-infected citron plants, or ii) graft-inoculation of bark from viroid-infected citron plants on rough lemon seedlings.

Statistical analysis

Plant growth data were subjected to orthogonal contrast ANOVA (Bewick *et al.,* 2004) taking into consideration for each plant and treatment: i) the average height values (mean of the measurements made at weakly intervals) as an indicator of plant size, ii) the linear component that refers to the slope of the growth curve, and iii) the quadratic component that refers to the non-linear growth. In addition, also using the orthogonal contrast ANOVA, the average height values and the linear components were compared for: i) infected plants versus non-inoculated controls, ii) plants infected with a single viroid versus plants co-infected with two viroids, and iii) the remaining variability accounting for differences among plants infected with a single viroid and differences between the plants co-infected with two viroids.

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Capítulo 2

Citrus viroid V: Occurrence, Host Range, Diagnosis, and Identification of New Variants

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Abstract

The recently described citrus viroid V (CVd-V) has been proposed as a new species of the genus *Apscaviroid* within the family *Pospiviroidae.* Analysis of 64 samples from different citrus-growing areas has shown that CVd-V is present in the USA, Spain, Nepal and the Sultanate of Oman. CVd-V found in six sweet orange sources from the Sultanate of Oman was identical to the reference CVd-V variant, whereas three new variants with sequence identities of 98.6% (CVd-V^{CA}), 97.3% (CVd-V^{ST}) and 94.9% (CVd-V^{NE}) were identified in sources from California, Spain and Nepal, respectively. These results suggest that this viroid has not emerged recently and that is relatively widespread. Transmission assays to sweet orange, mandarin and mandarin hybrids, clementine, satsuma, lemon, sour orange, Tahiti lime, Palestine sweet lime, calamondin, bergamot and kumquat have shown that all these citrus species and citrus relatives are hosts for CVd-V. Several indexing approaches, including slot-blot, Northern-blot hybridization and RT-PCR, have been evaluated for detecting CVd-V, either using Etrog citron as an amplification host or directly from commercial species and cultivars.

Introduction

Viroids are non-encapsidated, small, circular, single-stranded RNAs that replicate autonomously when inoculated in their host plants. Citrus sp. are natural hosts of several viroid species of the genera *Pospiviroid (Citrus exocortis viroid,* CEVd), *Hostuviroid (Hop stunt viroid,* HSVd), *Cocadviroid (Citrus viroid IV,* CVd-IV, recently renamed *Citrus bark cracking viroid,* CBCVd) and *Apscaviroid* (*Citrus bent leaf viroid,* CBLVd, and *Citrus viroid III,* CVd-III, recently renamed *Citrus dwarfing viroid*, CDVd) (Flores *et al*. 2005). In addition, two other citrus viroids (*Citrus viroid original source,* CVd-OS, *and Citrus viroid V,* CVd-V) have been proposed as tentative species of the genus *Apscaviroid* (Ito *et al*. 2001; Serra *et al*. 2008a). CVd-V has a GC-rich genome of 293-294 nucleotides (nt) and its predicted secondary structure of minimum free energy is a rod-like conformation with 68.7% paired nucleotides. Biological characterization on the indicator Etrog citron (*Citrus medica* L.) showed that CVd-V induces mild but typical symptoms, and that in co-infections interacts synergistically with either CBLVd or CDVd producing enhanced leaf symptoms and very pronounced dwarfing (Serra *et al*. 2008a). Most citrus viroids (CEVd, HSVd, CBLVd, CDVd

and CBCVd) are widespread, usually occurring as complex mixtures co-infecting the same plant. CVd-OS has only been reported in Japan (Ito *et al*. 2001). CVd-V was initially identified after transmission to *Atalantia citroides*, a viroid host that appeared to be immune to CEVd, CBLVd, CDVd, HSVd, and CBCVd but allowed replication of CVd-V (Barbosa *et al*. 2005). Even though its origin is uncertain, CVd-V was probably present, but overlooked, in the inoculum source containing HSVd and CDVd, two viroids with similar electrophoretic mobility as CVd-V in sequential polyacrylamide gel electrophoresis (sPAGE) (Barbosa *et al*. 2005; Duran-Vila *et al*. 1993). The recent identification of a new variant of CVd-V in a tangelo Orlando grown in Spain (Serra *et al*. 2008b) suggests that this viroid may be more widespread than initially anticipated. The information available indicates that all citrus species and most species of citrus-related genera sustain replication of citrus viroids that reach variable titers (Barbosa *et al.* 2002). Even though citrus viroids elicit diseases in sensitive hosts such as exocortis and cachexia (Semancik *et al*. 1973; Semancik *et al*. 1988, Semancik and Weathers, 1972) or affect tree size and crop (Vernière *et al*. 2004), most susceptible species act as symptomless carriers in which viroids may be overlooked unless they are subjected to indexing tests. The present work reports: i) the characterization of a new variant of CVd-V in a source from California, ii) the identification of additional sources of CVd-V, iii) the identification of additional hosts of CVd-V, and iv) the screening of currently available viroid detection tools for CVd-V indexing.

Results

Characterization of a viroid-like RNA identified in California as a new variant of CVd-V.

The results of a series of transmission assays conducted in California revealed the presence of a viroid-like RNA that was transmissible to Etrog citron but not to tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.) and datura (*Datura stramonium* L.). As a preliminary attempt to characterize this viroid-like-RNA, a nucleic acid preparation from infected Etrog citron was examined by Northern blot hybridization with some DIG-labeled viroid-specific probes. A hybridization signal was obtained with the *Apple scar skin viroid* (ASSVd) probe but not with the CEVd, HSVd, or CBCVd probes (J.S. Semancik, personal communication). To clone and sequence this viroid-like RNA, nucleic acid preparations were fractionated by sPAGE and the gel-eluted circular forms were used as template for cDNA synthesis using an RT-PCR approach that does not require prior sequence

knowledge (Navarro *et al.* 1998). The cDNAs obtained were mostly in the range of 150-300 bp (Fig. 1A, lane 3), and some were derived from the viroid-like RNA, as confirmed by Southern-blot hybridization with a probe obtained by partial hydrolysis of the viroid-like RNA and 5'-end radiolabeling of the resulting fragments (Serra *et al*. 2008a) (Fig. 1B, lane 3). The PCR-amplified products were cloned into a plasmid vector and the inserts were analyzed by PAGE after proper restriction (Fig. 1C). Four plasmids containing the largest cDNA inserts of the viroid-like-RNA, confirmed by hybridization with the specific probe (Fig. 1D, see arrows), were selected for sequencing.

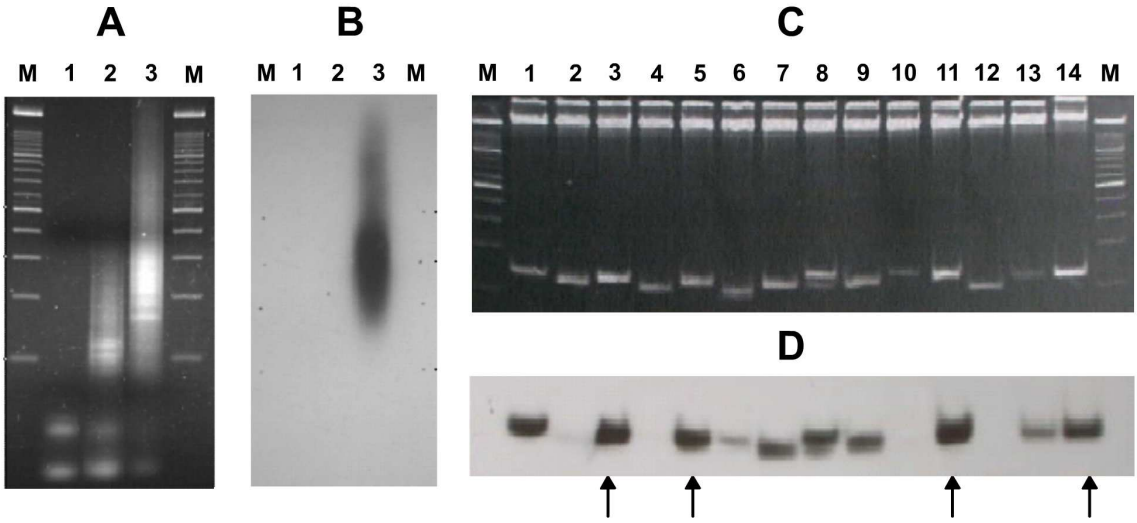

Fig. 1. (A) Analysis by non-denaturing PAGE and ethidium bromide staining of viroid-like cDNAs obtained by RT-PCR with a primer degenerated in its six 3'-terminal positions. (1) PCR control mixture without template; (2) RT-PCR control mixture without template; (3) complete RT-PCR mixture; (M) markers of 100-bp multimers. **(B)** Southern-blot analysis of the viroid-like cDNAs with a specific radioactive probe. **(C)** Restriction analysis by non-denaturing PAGE and ethidium bromide staining of recombinant plasmids digested with *Bam*H I and *Xba* I (1 to 14); (M) markers of 100-bp multimers. **(D)** Southern-blot analysis of the inserts with radioactive probe specific for the viroid-like RNA. Arrows indicate the clones that were sequenced.

The consensus sequence of 88 nt contained a segment of 16 nt identical to the upper CCR (Central Conserved Region) strand of members of the genus *Apscaviroid*. This consensus sequence differed only in two substitutions (65U→A and 69G→A) from the segment of CVd-V delimited by positions 39 to 116 (shaded in Fig. 2, CVd-V^{CA}).

Northern-blot hybridization with a full-length CVd-V-specific probe corroborated the sequence similarity of CVd-V with the viroid-like RNA, but neither with CDVd nor with HSVd included as controls (Fig. 3A, B). To obtain the complete sequence of the viroid-like RNA the 88-nt consensus sequence was used to design two adjacent primers of opposite polarity, (5'-GACGAAGGCCGGTGAGCAGTAAGCC-3') and (5'-GACGACGACAGGTGAGTACTTTC-3'), corresponding to CVd-V positions 90-114 and 69-89, respectively. The cDNA product obtained by RT-PCR amplification of the purified

circular forms of the viroid-like RNA exhibited in non-denaturing PAGE the mobility expected for the CVd-V cDNA (data not shown). This cDNA was cloned in a plasmid vector and sequencing of four independent inserts demonstrated that the viroid-like RNA was indeed a 294-nt sequence variant of CVd-V (CVd-V^{CA}). Two of the inserts had identical sequence, which in addition to the substitutions reported above (65U→A, 69G→A) presented two additional transitions (24C→U, 25C→U) in the lower strand of the rod-like secondary structure predicted for CVd-V (Fig. 2); their overall sequence identity with the reference variant of CVd-V was 98.6%. The two other inserts differed in two changes each showing that CVd-V^{CA}, like CVd-V, contains a population of sequence variants.

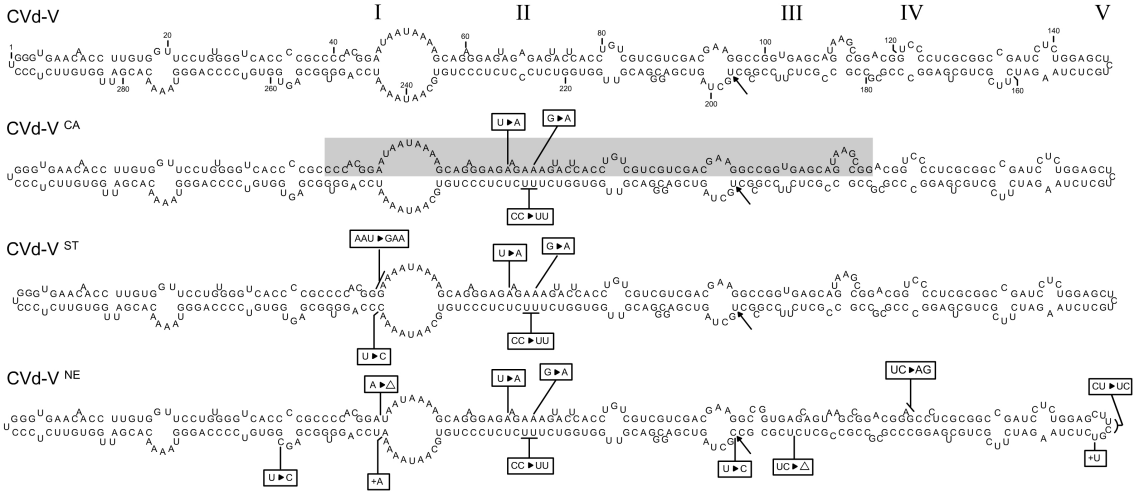

Fig. 2. Primary and predicted secondary structure of minimum free energy of CVd-V showing the changes identified in the variants obtained from an Etrog citron from California (CVd-V^{CA}), a Seminole tangelo (CVd-V^{ST}) from Spain and a sweet orange from Nepal (CVd-V^{NE}). The fragment of 88 nucleotides retrieved from the first cloning experiment of CVd-V^{CA} is shaded. Segments I, II, III, IV and V present deviations of the base pairing between the upper and lower strands as a result of the changes found in CVd-V^{CA}, CVd-V^{S} and CVd-V^{NE}. Arrows point at position 197, which is U in CVd-V, CVd-V^{CA} and CVd-V^{ST}, instead of C as in CVd-V^{NE} and in all other members of the genus *Apscaviroid.*

Identification of additional sources and variants of CVd-V.

Nucleic acid extracts from Etrog citrons graft-inoculated with 62 field isolates collected in different citrus-growing countries and from one single sweet orange sample collected in Nepal were examined by Northern-blot hybridization with a CVd-V-specific DIG-labeled DNA probe. CVd-V was identified in one single source of Seminole tangelo (*C. paradisi x C. tangerina*) from Spain, in the sweet orange from Nepal and in six isolates of sweet orange from the Sultanate of Oman. Identification of CVd-V in the Seminole tangelo was accomplished by sPAGE, Northern-blot hybridization and RT-PCR as reported by Serra et al.

(Serra *et al*. 2008b). Its complete primary structure was determined by sequencing the inserts of three recombinant plasmids containing the amplified cDNAs obtained using two adjacent primers of opposite polarity, with the region covered by the primers being then confirmed by directly sequencing the amplified cDNA obtained with a second set of adjacent primers of opposite polarity (Serra *et al*. 2008b). This Seminole tangelo variant (CVd-V^{ST}), with a 98.6% sequence identity with the reference CVd-V variant, presented the same changes identified in CVd-V^{CA} plus two additional changes in the upper and lower strands of the rod-like secondary structure (Fig. 2). Northern-blot hybridizations showed the presence of CVd-V in the sweet orange collected in Nepal (Fig. 3C) and six Etrog citrons graft-inoculated with six field isolates of sweet orange ('Baladi', 'Washington navel', 'Valencia' and 'Succari') collected in two locations (Tanuf and Sohar) in the Sultanate of Oman (Fig. 3D). These results were confirmed by RT-PCR using two pairs of adjacent primers of opposite polarity, PI and PII, or PIII and PIV (20). The consensus sequences obtained for the amplicons of the six sources from the Sultanate of Oman were identical to the reference CVd-V variant. The amplified product obtained from the isolate from Nepal was cloned into the pGEM-T vector (Promega), and sequencing of the inserts of four recombinant plasmids showed that three had identical 293-nt sequences (CVd-V^{NE}), which differed from that of the reference CVd-V variant in 15 positions (Fig. 2), whereas the remaining insert presented an additional 124C→U substitution.

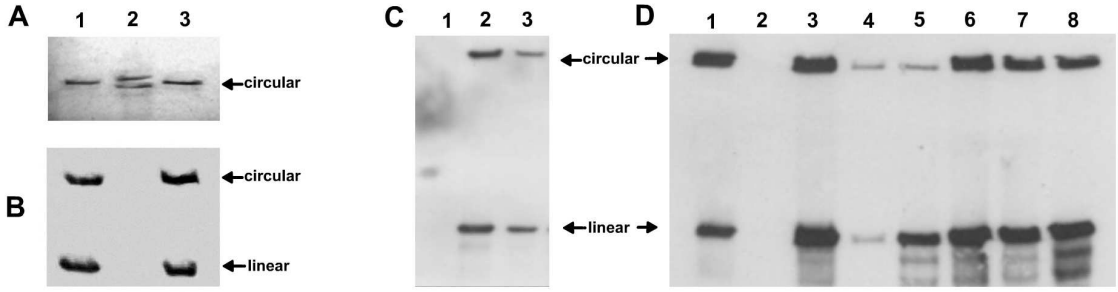

Fig. 3. Detection of CVd-V in samples from California **(A, B)**, Nepal **(C)** and the Sultanate of Oman **(D)**. The sample from California was analyzed by sPAGE and silver staining **(A)** and Northern blot hybridization with a CVd-V specific probe **(B):** (1) Etrog citron infected with the viroid-like RNA from California; (2) Etrog citron co-infected with HSVd and CDVd used as a negative control; (3) Etrog citron infected with CVd-V used as a positive control. Samples from Nepal were analyzed by Northern blot hybridization with a CVd-V specific probe **(C)**: (1) non-inoculated Etrog citron used as a negative control; (2) Etrog citron infected with CVd-V used as a positive control; (3) sweet orange collected in Nepal. Samples from the Sultanate of Oman were analyzed by Northern blot hybridization with a CVd-V specific probe **(D)**: (1) Etrog citron infected with CVd-V used as a positive control; (2) non-inoculated Etrog citron used as a negative control; (3-8) Etrog citrons graft-inoculated with 'Baladi' (3-4), Washington navel (5), Succari (6-7) and Valencia (8) sweet orange sources collected in the Sultanate of Oman.

Molecular characteristics of CVd-V variants.

The three CVd-V variants identified in the present study differed in four (CVd-V^{CA}), eight (CVd-V^{ST}) and 15-16 changes (CVd-V^{NE}) from the reference CVd-V variant (Serra *et al*. 2008a). These three variants have a predicted rod-like secondary structure of minimal free energy with 69.4% (CVd-V^{CA} and CVd-V^{ST}) and 71.7% (CVd-V^{NE}) paired nucleotides (Fig. 2). Among the changes with respect to the reference CVd-V variant there is a cluster of four substitutions in the upper (65U→A, 69G→A) and lower (24C→U, 25C→U) strands of the rod-like secondary structure resulting in an extended base pairing (see region II in Fig. 2). The changes of the left side of CVd-V^{ST} are located in a large loop except for two compensatory mutations that switch an A-U pair adjacent to the large loop into a G-C pair in CVd-V^{ST}, or into two A-U pairs in CVd-V^{NE} (see region I in Fig. 2). No deviations from the predicted secondary structure were found in the right side of CVd-V^{CA} and CVd-V^{ST}. However, the changes in the right side of CVd-V^{NE} result in a theoretical reorganization of the rod-like secondary structure with less conspicuous loops (see regions III and IV in Fig. 2) and a prominent terminal right loop (see region V in Fig. 2).

Unlike the other members of the genus *Apscaviroid*, variants CVd-V^{CA} and CVd-V^{ST}, but not CVd-V^{NE}, present in the lower CCR strand the transition C197→U reported previously in CVd-V that results in the change of a C-G base pair between the upper and lower strands into a wobble G-U base pair (see arrows in Fig. 2).

Indexing methods for detection of CVd-V.

As shown for other citrus viroids (Duran-Vila *et al*. 1993; Palacio *et al.* 2000b), bioamplification in Etrog citron followed by sPAGE (Fig. 4A), slot-blot (Fig. 4B) or imprint hybridization (Fig. 4C) are suitable methods for detecting CVd-V. Conversely, direct sPAGE of RNAs from sweet orange, clementine, Tahiti lime and lemon trees that had been co-inoculated with CEVd, CBLVd, HSVd, CDVd, CBCVd and CVd-V led to inconclusive results because of the low and variable viroid titers in these species (Fig. 4D). However, CVd-V could be detected in these species by slot-blot (Fig. 4E) or Northern-blot (Fig. 4F) hybridization with a CVd-V specific probe. RT-PCR approaches using CVd-V specific primers also gave the expected results (Fig. 4G).

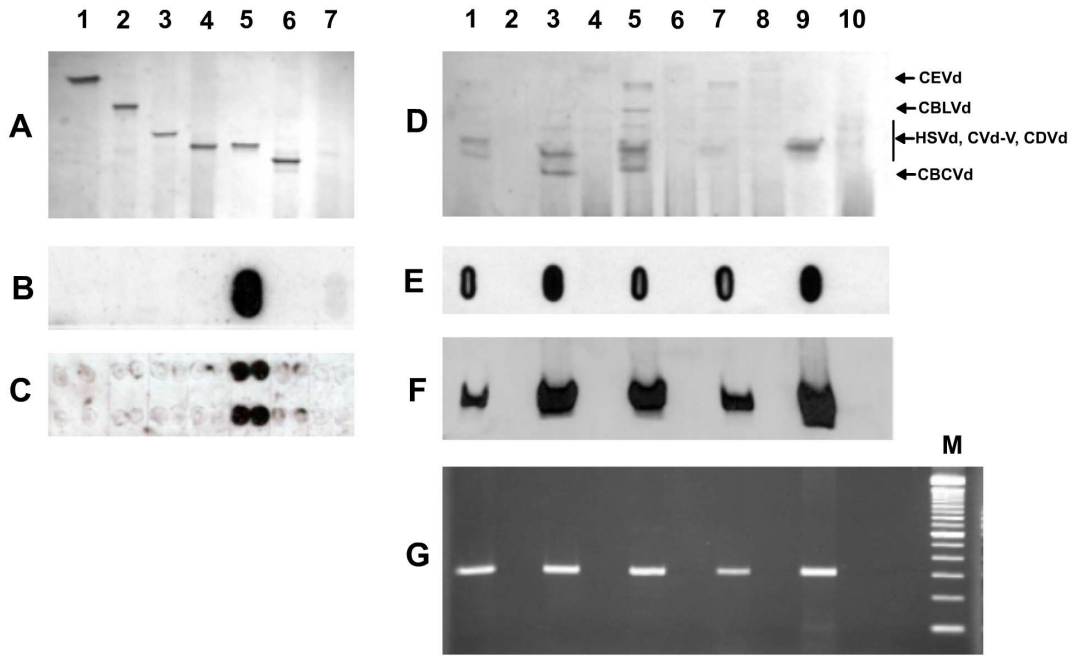

Fig. 4. (A) Detection of CVd-V in Etrog citron by sPAGE **(a)**, dot-blot hybridization **(b)** and imprint hybridization **(c)** using a CVd-V specific probe. Samples were from Etrog citrons infected with (1) CEVd, (2) CBLVd, (3) HSVd, (4) CDVd, (5) CVd-V and (6) CBCVd, and (7) from a non-infected control. **(B)** Detection of CVd-V in sweet orange, clementine, Tahiti lime and lemon by sPAGE **(d)**, dot- and Northern-blot hybridization with a CV-V specific probe **(e and f)**, and RT-PCR with CVd-V specific primers **(g).** Samples were collected from sweet orange, clementine, Tahiti lime and lemon plants co-inoculated with CEVd, CBLVd, HSVd, CDVd, CBCVd and CVd-V (1, 3, 5, 7) and from non-inoculated controls of the same species (2, 4, 6, 8). Samples from Etrog citrons infected with CVd-V (9) and non-inoculated (10) were included as controls. (M) markers of 100-bp multimers.

*Host range of CVd-V***.**

RNA preparations from twenty citrus genotypes that had been graft-inoculated with the original CVd-V source (Serra *et al*. 2008a) were analyzed by Northern-blot hybridization with a CVd-V specific probe. Results confirmed that all were infected (Fig. 5A), whereas no signals were observed in the non-inoculated controls (Fig. 5B).

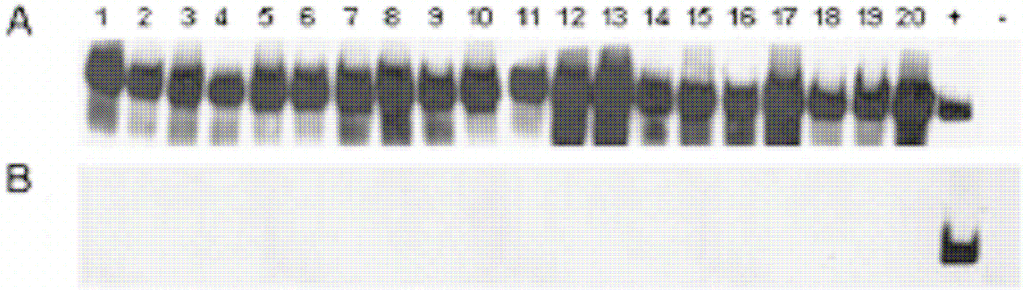

Fig. 5. (A) Detection of CVd-V by Northerm-blot hybridization with a CVd-V specific probe in (1) 'Hernandina' clementine, (2) 'Ricart navelina' sweet orange, (3) 'Nagami' kumquat, (4) 'Cajel' sour orange, (5) 'Oroval' clementine, (6) 'Orlando' tangelo, (7) 'Salustiana' sweet orange, (8) 'Clausellina' satsuma, (9) 'Nova' mandarin, (10) 'Page' mandarin, (11) 'Sanguinelli' sweet orange, (12) 'Fino' lemon, (13) Palestine sweet lime, (14) 'Verna' lemon, (15) calamondin, (16) 'Calabria' bergamot, (17) 'clemenules' clementine, (18) 'Sevillano' sour orange, (19) Temple mandarin and (20) Tahiti lime. Plants were inoculated with a CVd-V source. **(B)** Absence of CVd-V in non-inoculated plants of the same species. Etrog citrons infected with CVd-V (+) and non-inoculated (-) were included as controls.

Discussion

Transmission studies using *Atalantia citroides* revealed the existence of a new member of the genus *Apscaviroid* (Barbosa *et al*. 2005), which induces mild, but characteristic, symptoms on Etrog citron and displayed synergistic effects when co-inoculated with other members of the same genus (Serra *et al*. 2008a). Even though the original source of CVd-V is uncertain, it was most likely present, but overlooked, in field sources containing HSVd or CDVd, which have electrophoretic mobilities in sPAGE similar to CVd-V. Detection of CVd-V in diverse hosts collected in different citrus growing areas (Spain, USA, Nepal and Sultanate of Oman) indicates that even though this viroid does not seem to be as widespread as other citrus viroids, it might have remained unnoticed in indexing programs. The identification of sequence variants which differ from CVd-V in four (CVd-V^{CA}), eight (CVd-V^{ST}) and 15-16 (CVd-V^{NE}) changes, four of which (U65→A, G69→A, C224→U and C→225U) are present in these three variants, suggests an evolutionary link among them. CVd-V occurrence in different geographic locations is probably the result of exchange of plant material followed by the adaptation of mutants under different environmental conditions.

As reported previously for CVd-V (Serra *et al*. 2008a), the sequence of the upper CCR strand of variants CVd-V^{CA}, CVd-V^{ST} and CVd-V^{NE} is identical to that of the other members of the genus *Apscaviroid*, while the sequence of the lower CCR strand contains in CVd-V^{CA} and CVd-V^{ST} but not in CVd-V^{NE}, a C197→U transition that results in the change of a C-G base pair between the upper and lower strands into a wobble G-U base pair. Another feature of the new variants identified in the present study is the presence of two sets of changes in the upper and lower strands (U65→A, G69→A) and (C224→U, C→225U), which result in a rearrangement of the predicted rod-like secondary structure.

The host range study reported here illustrates that, like other citrus viroids, CVd-V can infect all the citrus species and cultivars tested. The phenotypic alterations incited by CVd-V infection on commercial species and cultivars are not known, but the synergistic effects described in Etrog citron co-infected with CVd-V and CBLVd or CDVd (Serra *et al*. 2008a) suggest that similar interactions may result in reduced tree size and yield, as reported for clementine trees grafted on trifoliate orange (*Poncirus trifoliata* L.) co-infected with several viroids (Vernière *et al.* 2006).

In conclusion, the recently described CVd-V (Serra *et al*. 2008a, Serra *et al*. 2008b), is present in a number of citrus growing areas and at least three new variants have been identified and characterized. Even if the effect of CVd-V in commercial rootstock/scion combinations is yet unknown, this viroid must be taken into consideration by agencies involved in phytosanitation, variety improvement, and certification programs.

Materials and methods

Viroid sources.

Unless otherwise stated, viroid sources were graft-inoculated on the sensitive selection 861-S1 of Etrog citron grafted on rough lemon (*C. jambhiri* Lush.) rootstock and maintained at 28 to 32ºC for at least six months before nucleic acid extraction. One of the viroid sources, kindly provided by J.S. Semancik (Department of Plant Pathology, University of California, Riverside) was recovered from Etrog citron after a series of transmission assays. Additional sources were collected in different citrus-growing countries: Spain (18 samples), Brazil (15 samples), Italy (4 samples), Colombia (16 samples), Turkey (3 samples), Sultanate of Oman (6 samples) and Nepal (1 sample). These samples included a range of commercial species: sweet orange (*C. sinensis* (L.) Osb.), mandarin (*C. reticulata* Blanco), tangor (*C. reticulata* × *C. sinensis*), tangelo (*C. paradisi x C. tangerina*), lemon (*C. limon* (L.) Burn.f.), Tahiti lime (*C. latifolia* Tan.), Mexican lime (*C. aurantifolia* (Christm.) Swing.) and citron.

Nucleic acid extraction.

Tissue samples (5 g of young leaves and/or stem bark) were homogenized in a mixture of phenol and extraction buffer [0.4 M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA, pH 7.0; 4% (v/v) 2-mercaptoethanol]. Total nucleic acids were partitioned in 2 M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in 300 µl of TKM buffer (10 mM Tris-HCl, pH 7.4; 10 mM KCl; 0.1 mM $MgCl_2$) (Semancik *et al*. 1973).

Polyacrylamide gel electrophoresis.

Aliquots (20 µl) of the nucleic acid preparations (equivalent to 333 mg of fresh weight tissue) were fractionated by non-denaturing PAGE in a 5% gel that was stained with ethidium bromide (Morris and Wright, 1975). For sequential PAGE (sPAGE), the segment of this gel delimited by CEVd and 7S RNA was cut and applied onto a second denaturing gel containing 8 M urea (Rivera-Bustamante *et al*. 1986; Sänger *et al.* 1979), which following electrophoresis was stained with silver (Igloi, 1983).

Northern-blot, slot-blot and imprint hybridization.

For Northern-blot hybridization, the RNAs separated by PAGE or sPAGE were electroblotted (400 mA for 2 h) to positively-charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA). For slot-blot hybridization, samples (10 µl) were pretreated in 6X SSC containing 8% formaldehyde for 15 min at 60ºC and blotted onto the membrane. For imprint hybridization, freshly-cut stem sections were firmly pressed onto the membrane (Romero-Durban *et al*. 1995). In all instances, RNAs were immobilized by UV cross-linking and hybridized with ^{32}P-RNA or DIG-labeled-DNA probes.

Viroid-specific riboprobes were synthesized by hydrolysis of a purified viroid RNA preparation followed by 5'-end labeling with polynucleotide kinase and [γ-^{32}P]ATP essentially as described earlier (Negruk *et al*. 1980) with minor modifications (Serra *et al*. 2008a). DIG-labelled DNA probes were PCR-synthesized as described by Palacio *et al.* (Palacio *et al*. 2000a) using a plasmid containing the full-length CVd-V sequence and a pair of specific primers.

Prehybridization (at 50 or 60ºC for 2-4 h) and hybridization (at 50 or 60ºC overnight) were performed in 6X SSPE containing 50% formamide as described by Sambrook *et al.* (Sambrook *et al*. 1989). After hybridization the membranes were washed twice in 2X SSC, 0.1% SDS at room temperature for 15 min, once in 0.1X SSC, 0.1% SDS at 60ºC for 60 min, and revealed by autoradiography (radiolabeled probes) or with an anti-DIG alkaline phosphatase conjugate and the chemiluminiscence substrate CSPD (Roche Applied Science) (DIG-labeled probes).

Viroid characterization.

Aliquots of nucleic acid preparations from viroid-infected citrons were examined by sPAGE, and the viroid circular forms were eluted overnight from the ethidium bromide stained gel with TEP buffer (0.1 M Tris-HCl, pH 9.0, containing 0.1 M 2-mercaptoethanol, 10 mM EDTA and 1% SDS) in the presence of phenol/chloroform. The RNAs were recovered by ethanol precipitation and resuspended in water. Viroid cDNA clones were obtained by an approach that uses minimal amounts of template and does not require prior knowledge of its sequence (Navarro *et al*. 1998; Serra *et al*. 2008a). The PCR-amplified products were ligated into vector pTZ57R/T (Fermentas) with protruding 3'-terminal Ts and, following transformation of *Escherichia coli* DH5α cells, the plasmid inserts resulting from digestion with *Bam*H I and *Xba* I were separated by non-denaturing PAGE in 5% gels, blotted and hybridized with a 5'-end labeled viroid-specific probe to identify those containing viroid-cDNAs.

Sequencing and sequence analysis.

RT-PCR amplicons or cloned viroid-cDNAs were sequenced with an ABI PRISM DNA sequencer 377 (Perkin Elmer). Multiple alignments were performed using the program Clustal W (Thompson *et al*. 1994). Secondary structure analyses were obtained with the MFOLD program (circular version) from the GCG package (Zuker *et al*. 1999), and the RNAviz program (De Rijk and De Wachter, 1997).

Host range and detection studies.

A selection of eighteen genotypes of different citrus species and cultivars were graft-propagated on rough lemon and graft-inoculated with a CVd-V isolate. The selected genotypes were: 'Sanguinelli', 'Salustiana' and 'Ricart navelina' sweet oranges, 'Oroval' and 'Hernandina' clementines (*C. clementina* Hort. ex Tan.), 'Fino' and 'Verna' lemons, 'Sevillano' and 'Cajel' sour oranges (*C. aurantium* L.), 'Clausellina' satsuma (*C. unshiu* (Mak.) Marc.), Temple mandarin (*C. temple* Hort. ex Tan.), Tahiti lime, Palestine sweet lime (*C. limettioides* Tan), calamondin (*C. madurensis* Lour), 'Calabria' bergamot (*C. bergamia* Risso and Pot.), 'Orlando' tangelo (*C. paradisi x C. tangerina*), 'Nova' mandarin (*C.*

clementina x (C. paradisi x C. tangerina)), 'Page' mandarin ((*C. paradisi x C. tangerina*) *x C. clementina*), and 'Nagami' kumquat (*Fortunella margarita* (Lour.) Swing.).

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Capítulo 3

An artificial chimeric derivative of *Citrus viroid V* involves the terminal left domain in pathogenicity

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Molecular Plant Pathology (Accepted with minor changes)

Abstract

To evaluate the effect of exchanging discrete segments of the CVd-V molecule for the corresponding segments of CDVd, on symptom expression, seven chimeric viroids were synthesized and were inoculated mechanically to three citron seedling plants, each. Northern-blot hybridization and RT-PCR showed that a single plant from the three that had been inoculated with the chimeric variant Ch5 (CVd-V containing T_L domain of CDVd) was actually infected. Sequencing the DNA amplicon confirmed that the chimeric variant Ch5 was stable, except for a single 42C→U substitution. Plants infected with Ch5 were symptomless, and plants co-infected with Ch5 and CVd-V or CDVd developed the same symptoms as plants singly infected with CVd-V or with CDVd, respectively. These results suggest that the terminal left domain is involved in the pathogenicity of *Citrus viroid V* and that there were no interactions between Ch5 and these two related viroids. Analysis of the co-infected plants showed that CVd-V and CDVd displaced Ch5 that was found to be present in extremely low titers.

Introduction

Viroids are infectious, small, single stranded, circular RNAs (246-401 nt) that fold into compact secondary structures due to the high degree of self-complementarity of their sequences. In spite of their apparent simplicity and their inability to code for proteins, viroids are able to complete their biological cycle relying almost entirely on host factors. The sequence of the viroid genome provides catalytic motifs (only in the case of members of the family *Avsunviroidae*), specific motifs responsible for the interaction with the host, and maintenance of their secondary and probably also tertiary structures. In order to fulfill their infectious cycle, viroids must be transported to the replication site (the nucleous for members of the family *Pospiviroidae*, or the chloroplast for members of the family *Avsunviroidae*), use the transcription machinery of the host cell to generate a progeny that must be released to the cytoplasm, invade neighboring cells (cell-to-cell movement) and/or distant cells through the phloem (long distance movement) and overcome plant defense mechanisms.

Viroids are classified within two families: *Pospiviroidae,* composed of species with a central conserved region (CCR) and without hammerhead ribozymes, and *Avsunviroidae,* encompassing four species lacking CCR but able to self-cleave in both polarity strands through hammerhead ribozymes. Citrus are natural hosts of several viroid species all of them

belonging to the family *Pospiviroidae* whose type member is *Potato spindle tuber viroid* (PSTVd) (Duran-Vila *et al.* 1988; Flores *et al.* 2004) and adopting in vitro a rod-like or quasi-rod-like secondary structures of minimal free energy. Upon denaturation the viroid may acquire metastable branched structures due to the base pairing of inverted repeats leading to the formation of stem loop structures (hairpins I and II) (Riesner *et al*. 1979; Loss *et al*. 1991). Depending on the composition of the CCR, and on the presence or absence of a terminal conserved region (TCR) and terminal conserved hairpin (TCH), the viroids of this family are classified into five genera. Following the model proposed by Keese and Symons (Keese and Symons, 1985), five structural-functional domains: P (pathogenicity), C (central), V (variable), T_L (terminal left) and T_R (terminal right) of the rod-like secondary structure have been taken into consideration. Even though the viroids of the genus *Pospiviroid* appear to be well adjusted to this model, there are evidences showing that the P domain is not the sole determinant of symptom development and suggest a complex correlation between different structural domains and pathogenicity. In the case of PSTVd, a single nucleotide mutation in the lower strand of the CCR causes the flat-top syndrome in tomato (Qi and Ding, 2003b). In citrus, the pathogenicity of the only member of the genus *Hostsuviroid* is not associated with the P domain but with the V domain and that a single nucleotide mutation modulates symptom expression (Reanwakaron and Semancik 1998; Serra *et al*. 2008c).

Studies with heterologous chimeras between variants of the same viroid (Visvader and Symons 1986; Gora *et al*. 1996; Reawarkaron and Semancik 1998), between closely related viroids of the same genus (Sano *et al*. 1992), and to a less extend between viroids of different genera (Sano and Ishiguro, 1998) provided very valuable information regarding the relationship between specific regions of the viroid molecule and symptom expression. Heterologous chimeras between *Citrus exocortis viroid* (CEVd) and *Tomato apical stunt viroid* (TASVd) showed that both T_L and T_R play a role in pathogenesis (Sano *et al*. 1992).

In the case of members of the genus *Apscaviroid*, all of them restricted to woody species, studies addressed to understand the relationship between different segments of the secondary structure of the molecule and their biological properties have only been undertaken by induced mutagenesis (Owens *et al*. 2005). Since citrus species are natural hosts of several viroids of the genus *Apscaviroid*, they offer a suitable system to study the response to infection with artificially constructed chimeras, which is the main objective of the present study.

Citrus viroid III (CVd-III) recently renamed *Citrus dwarfing viroid* (CDVd) with sequence variants of 297, 294 and 291 nt has a rod-like conformation and induces in Etrog

citron (*Citrus medica* L.*)*, stunting and a characteristic "leaf dropping pattern" due to the moderate epinasty resulting from petiole and mid-vein necrosis (Rakowsky *et al*. 1994; Semancik *et al*. 1997). The recently described *Citrus viroid V* (CVd-V) with sequence variants of 293-294 nt and also a rod-like conformation, induces in Etrog citron mild stunting and very small necrotic lesions and cracks, sometimes filled with gum (Serra *et al*. 2008a; 2008b). Moreover, since Etrog citron plants co-infected with CVd-V and CDVd disclose synergistic interactions manifested in enhanced leaf symptoms and very pronounced dwarfing (Serra *et al*. 2008a), these host-viroid combinations provide an ideal model to identify the pathogenicity determinants in viroids of the genus *Apscaviroid.* The aim of the present study was to evaluate the effect of exchanging discrete segments of the CVd-V molecule for the corresponding segments of CDVd, on symptom expression. Construction of the desired chimeras has been pursued using a novel PCR-based approach, that do not have the limitations associated with the availability of restriction sites to exchange viroid segments as used in previous studies conducted with other viroids.

Results

*Design and production of chimeric variants***.**

Figure 1 illustrates the composition of the seven chimeras designed by replacing discrete segments of the secondary structure of CVd-V by the corresponding segments of CDVd. Unlike the strategy followed previously for the construction of chimeric viroids (Visvader and Symons, 1986; Owens *et al.* 1990; Sano *et al.* 1992; Gora *et al,* 1996; Spieker, 1996; Sano *et a.,* 1998; Reawarkaron and Semancik, 1998), the PCR-based novel approach used in the present study did not rely on the availability of shared restriction endonuclease sites for the exchange of segments between the two viroids. With this method, any choice of chimeric viroids can be constructed. Taking into consideration that structural-functional domains have not been clearly defined in members of the genus *Apscaviroid*, in the present work the choice of segments to be replaced was delimited on the basis of preserving the CCR and the flanking inverted repeats responsible for the formation of haripin I and the TCR, both located in upper strand of the viroid secondary structure. Four discrete segments were then defined taking into account the secondary structure of the two viroids (Fig. 1): (**S1**) segment containing the left terminal region of the viroid secondary structure and delimited in the upper strand by the right end of the TCR (positions 28 and 27 of CVd-V and CDVd, respectively), and the

corresponding segment of the lower strand; (**S2**) segment containing the segment delimited by the end of the TCR (positions 29 and 28 of CVd-V and CDVd, respectively) and the left flanking repeat of the CCR (position 73 of CVd-V and CDVd), and the corresponding segment of the lower strand; (**S3**) segment containing the CCR and both flanking repeats, and the corresponding segment of the lower strand; (**S4**) segment containing the right terminal region and delimited in the upper strand by the end of the right flanking repeat of the CCR, and the corresponding segment of the lower strand.

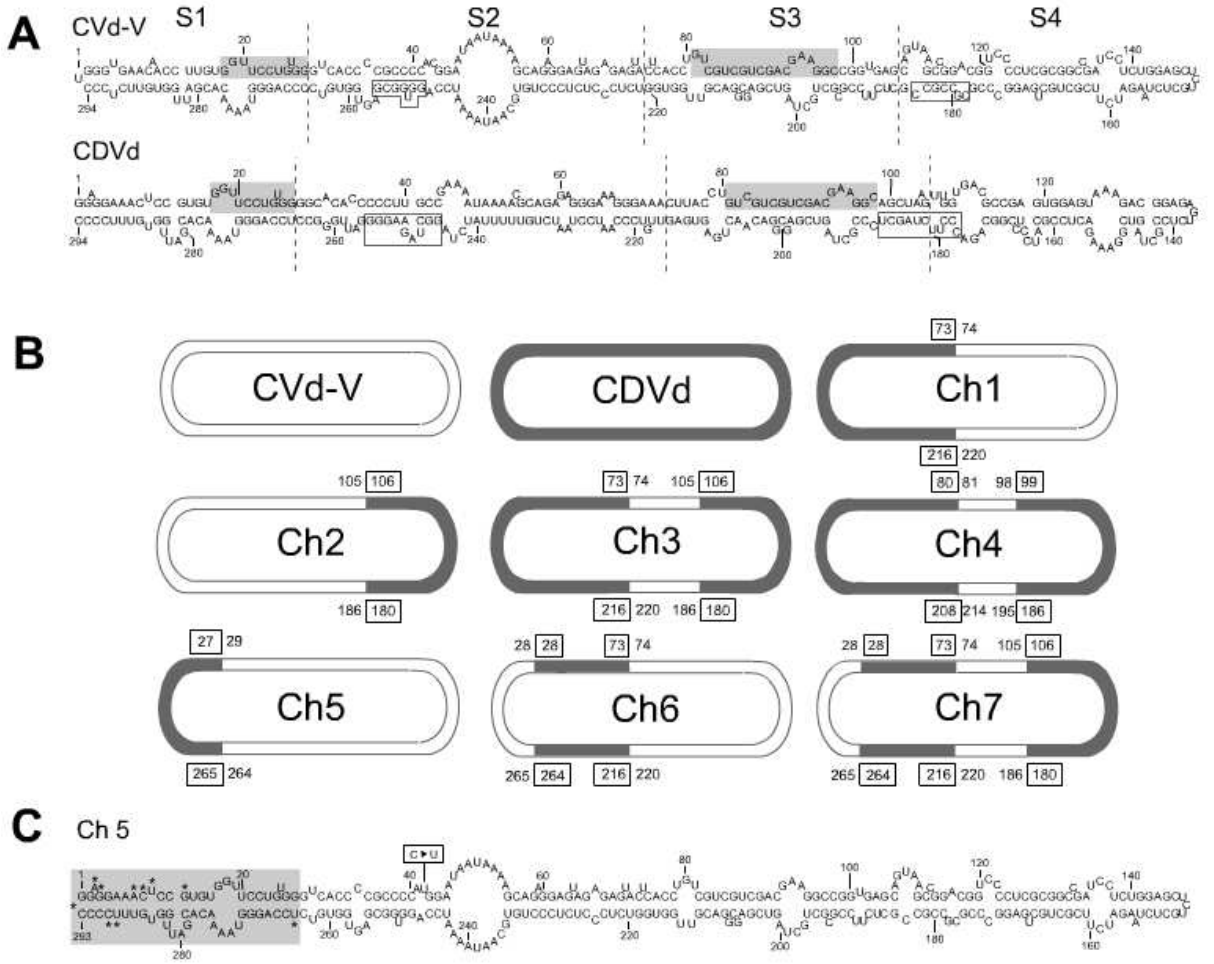

Fig. 1 (A) Primary and proposed secondary structure of minimum free energy of CVd-V and CDVd. Discontinuous lines divide the secondary structure in four segmentes (S1, S2, S3 and S4). Conserved regions (TCR within S1 and CCR within S3) of members of the genus *Apscaviroid* are shaded. Boxed nucleotides show the putative motives involved in the formation of hairpin II-like. **(B)** Schematic representation of the seven chimeric viroids (Ch1 to Ch7) and their parental viroids (CVd-V and CDVd). Shaded and non-shaded regions show the segments from CDVd and CVd-V, respectively. Boxed and non-boxed numbers indicated the first and last nucleotides of the segment from CDVd and CVd-V. **(C)** Primary and proposed secondary structure of minimum free energy of Ch5. Shaded region show the S1 segment from CDVd that replaced the S1 of CVd-V. Nucleotide changes between CVd-V and Ch5 are shown (*). The change C→U found in the progeny of Ch5 is shown boxed.

Even though the hairpin II has only been observed in PSTVd and closely related viroids (Schröder and Riesner, 2002), CVd-V and CDVd contain in the lower strand of their secondary structures, two stretches of 7 bp (CVd-V) or 11 bp (CVd-III) (boxed in Fig. 1) that could form a stable hairpin with a GC-rich stem resembling hairpin II (hairpin II-like) (Serra *et al*., 2008a). With the purpose of preserving this putative structure, an additional segment (**S3'**) resembling S3 but containing the CCR without the flanking repeats, and the corresponding segment of the lower strand, were also defined. The utilization of S3' required the corresponding modifications of the adjacent segments S2 and S4 into **S2'** (S2 plus the left inverted repeat of the CCR and the corresponding segment of the lower strand) and **S4'** (S4 plus the right inverted repeat of the CCR and the corresponding segment of the lower strand), respectively.

The segments of CDVd to replace the corresponding segments of CVd-V were obtained by PCR using p(CDVd) as a template and different primer sets phosporilated at the 5' end (Table 1). The receptor CVd-V sequences were also obtained by PCR using p(CVd-V) as a template and different primer sets (Table 2), that yielded the plasmid vector with the CVd-V insert devoid of the segment to be replaced. Ligation of receptor CVd-V sequences with the corresponding segments of CDVd yielded the designed chimeric variants (Table 2, Fig. 1): **Ch1** (CVd-V containing segments 1 and 2 of CDVd), **Ch2** (CVd-V containing segment 4 of CDVd), **Ch3** (CVd-V containing segments 1, 2 and 4 of CDVd), **Ch4** (CVd-V containing segments 1, 2' and 4' of CDVd), **Ch5** (CVd-V containing segment 1 of CDVd), **Ch6** (CVd-V containing segment 2 of CDVd), and **Ch7** (CVd-V containing segments 2 and 4 of CDVd). The sequences of the chimeric inserts were confirmed by sequencing a selection of clones and those containing segments introduced in the wrong orientation or inserted in tandem were discarded.

Table 1. Primers used to amplify monomeric viroid sequences, specific viroid segments and plasmids containing partial viroid sequences.

Primer	Primer sequence[1]	Orientation[2]	Amplification product	Position
D1	5' TTGTCCCAATCCTAATCTGT 3'	sense	S1+S2	216-235 CDVd
D2	5' TTTCCCTTTCCCTCTCTGCG 3'	antisense	S1+S2	73-54 CDVd
D3	5' TCCAGGGTAAAACACGATTGGTGTTTCCCCGGAGG 3'	sense	S1	265-5 CDVd
D4	5' CCACAGGAACCACACGGAGTTTCCTCCGGGG 3'	antisense	S1	27-291 CDVd
D5	5' GGCACACCCCCTTGCCGAAA 3'	sense	S2+S3+S4+pBluescript	28-47 CDVd
D6	5' GGCCCAATCCCCTTCTAGCC 3'	antisense	S2+S3+S4+pBluescript	264-245 CDVd
D7	5' TTGGTGACGCCGAGTGGAG 3'	sense	S4	106-124 CDVd
D8	5' AAGGGTCTGCCGGAGGATG 3'	antisense	S4	180-162 CDVd
D9	5' AGCTAAGTTGGTGACGCCGA 3'	sense	S4'	99-118 CDVd
D10	5' AGCTAGAAGGGTCTGCCGGA 3'	antisense	S4'	186-167 CDVd
D11	5' TGAGTGAGTTGTCCCAATCC 3'	sense	S1+S2'	208-227 CDVd
D12	5' AGGTAAGTTTCCCTTTCCC 3'	antisense	S1+S2'	80-62 CDVd
V1	5' CTCACCTGTCGTCGATCAAG 3'	sense	S3+S4+pBluescript	74-87 CVd-V
V2	5' CCACCAACGTCCGCTCGACT 3'	antisense	S3+S4+pBluescript	220-193 CVd-V
V3	5' GTCACCCCGCCCCACGGAAT 3'	sense	S2+S3+S4+pBluescript	29-48 CVd-V
V4	5' GACACCACTCGCCACCTGGA 3'	antisense	S2+S3+S4+pBluescript	264-245 CVd-V
V5	5' CCCAGGGTAAAACACGATTGGTGTTCTCCCTGGGTG 3'	sense	S1	265-6 CVd-V
V6	5' CCACAGGAACCACAAGGTTGTTCACCCAGGGAG 3'	antisense	S1	28-290 CVd-V
V7	5' CTCTTCCGCGCTGCTAGTCG 3'	sense	S1+S2+S3+pBluescript	186-205 CVd-V
V8	5' CTCACCGGCCTTCGTCGA 3'	antisense	S1+S2+S3+pBluescript	105-88 CVd-V
V9	5' GCTGCTAGTCGAGCGGACGT 3'	sense	S1+S2+S3'+pBluescript	195-214 CVd-V
V10	5' GCCTTCGTCGAATCGAATTC 3'	antisense	S1+S2+S3'+pBluescript	98-88 CVd-V
V11	5' GTCGTCGATCAAGCTTATCG 3'	sense	S3'+S4+pBluescript	81-87 CVd-V
V12	5' ACGTCCGCTCGACTAGCAGC 3'	antisense	S3'+S4+pBluescript	214-195 CVd-V
P1	5' TCGACGAAGGCCGGTGAGCA 3'	sense	Viroid molecule	88-107 CVd-V
P2	5' CGACGACAGGTGAGTACTCTCTAC 3'	antisense	Viroid molecule	87-64 CVd-V
P3	5' TCGACGAAGGCCGGTGAGTT 3'	sense	Viroid molecule	88-105 CVd-V; 106-107CDVd
P4	5' CGACGACAGGTGAGTTTCCC 3'	antisense	Viroid molecule	87-74 CVd-V; 73-68 CDVd
P5	5' TCGACGAAGGCAGCTAAGTTGG 3'	sense	Viroid molecule	88-98 CVd-V; 99-109 CDVd
P6	5' CGACGACAGGTAAGTTTCCC 3'	antisense	Viroid molecule	87-81 CVd-V; 80-68 CDVd
CVd-III-F1	5' GGCAGCTAAGTTGGTGACGC 3'	sense	Viroid molecule	96-105 CDVd
CVd-III-R1	5' TTCGTCGACGACGACAGGTA 3'	antisense	Viroid molecule	95-76 CDVd
C1	5' AGGTTGTTCACCCAGGGAG 3'	antisense	cDNA CVd-V	14-290 CVd-V
C2	5' CGGAGTTTCCTCCGGGGA 3'	antisense	cDNA CDVd	13-290 CDVd

[1] Underlined nucleotides of primers D3 and D4, or V5 and V6 show overlapping regions of the primers. Bold nucleotides in V1, V10 and V11 correspond to plasmid sequences in the *Eco* RV site.

[2] Orientation refers to the positive strand of the viroid sequence.

Table 2. Strategies for construction of chimeric viroids.

Chimeric variant	Composition		Amplification of segments			Amplification of plasmid containing viroid devoid of segment to be replaced			Amplification of full-length chimeric viroid	
	CVd-V	CDVd	Primer	Primer	Template	Primer	Primer	Template	Primer	Primer
Ch1	S3,S4	S1,S2	D1	D2	pCDVd	V1	V2	pCVd-V	P1	P4
Ch2	S1,S2,S3	S4	D7	D8	pCDVd	V7	V8	pCVd-V	P3	P2
Ch3	S3	S1,S2,S4	D7	D8	pCDVd	V7	V8	pCh1	P3	P4
Ch4’	S1,S2,S3’	S4’	D9	D10	pCDVd	V9	V10	pCVd-V	None	None
Ch4	S3’	S1,S2’,S4’	D11	D12	pCDVd	V11	V12	PCh4’	P5	P6
Ch5	S2,S3,S4	S1	D3	D4	None	V3	V4	pCVd-V	P1	P2
Ch6	S1,S3,S4	S2	V5	V6	None	D5	D6	pCh1	P1	P4
Ch7	S1,S3	S2,S4	V5	V6	None	D5	D6	pCh3	P3	P4

Infectivity and symptom expression of chimeric constructs

To obtain infectious preparations of the seven chimeric viroids, head-to-tail dimeric cDNAs were synthesized and used as templates to produce the corresponding *in vitro* transcripts that were inoculated mechanically to three citron seedling plants, each. Dimeric *in vitro* transcripts of CVd-V and CDVd were also synthesized and inoculated as positive controls, and three non-inoculated plants were maintained as negative controls. Three months post-inoculation, plants inoculated with CVd-V or with CDVd developed symptoms, whereas plants inoculated with chimeric variants remained symptomless and indistinguishable from the negative controls over a nine-month period. Analysis by Northern-blot hybridization and RT-PCR showed that a single plant from the three that had been inoculated with the chimeric variant Ch5 was actually infected. Sequencing the DNA amplicon confirmed that the chimeric variant Ch5 was stable, except for a single 42C→U substitution in the S2 segment (Fig. 1). In a parallel study with chimeric variants constructed by replacing discrete segments of the secondary structure of CVd-V by the corresponding segments of *Citrus bent leaf viroid* (CBLVd), none of the inoculated plants resulted infected (data not shown).

To confirm the absence of symptoms in plants infected with Ch5, buds from sensitive selection 861-S1 of Etrog citron were graft propagated on rough lemon seedlings and, concurrently, these plants were graft-inoculated (three plants each) with bark from the Etrog citron seedlings infected with the chimeric variant Ch5, with CVd-V or with CDVd. Three additional non-inoculated plants were also maintained as negative controls. The growing material of the grafted plants was observed over a 4-month period and infection was confirmed by sPAGE (Fig. 2A) and RT-PCR (data not shown). CVd-V-infected citron plants were stunted and presented very small necrotic lesions with gum exudates in the stem and mild leaf epinasty (Fig. 3A,B). CDVd-infected citron plants were also stunted and presented the characteristic “dropping leaf” pattern due to a moderate epinasty resulting from petiole and mid-vein necrosis (Fig. 3A,C). Ch5-infected citron plants were symptomless (Fig. 3A,D). The titers of the three viroids were comparable (Fig. 2A) and RT-PCR analysis and amplicon sequencing confirmed the stability of the inoculated sequences.

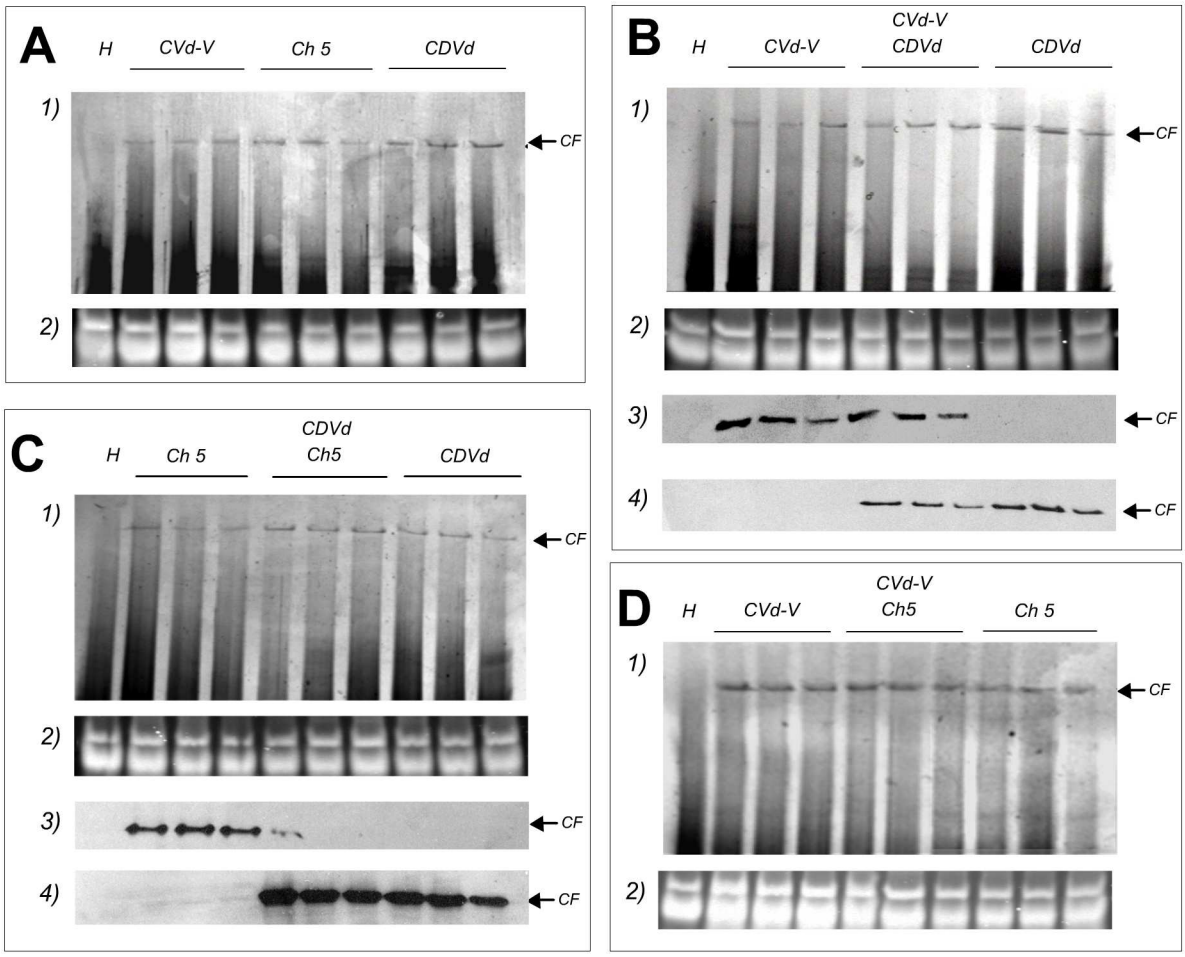

Fig. 2 Analysis by sPAGE of citron plants infected with CVd-V, Ch5 or CDVd **(A1),** citron plants infected with CVd-V, CDVd or co-infected with both **(B1)**, citron plants infected with Ch5, CDVd or co-infected with both **(C1)** and citron plants infected with CVd-V, Ch5, co-infected with both **(D1)**. Silver staining of the second denaturing gel of sPAGE shows the viroid circular forms (CF). H (mock-inoculated control) is shown in all the analysis. Ethidium bromide staining of the first non-denaturing gel of sPAGE shows that RNA levels in all preparations were comparable **(A2 to D2)**. Northern hybridisation analysis of samples electrotransferred from the second denaturing gel of sPAGE of citron plants infected with CVd-V, CDVd or co-infected with both using a DIG-labelled probe specific for CVd-V **(B3)** and a DIG-labelled probe specific for CDVd **(B4)** and citron plants infected with Ch5, CDVd or co-infected with both using a DIG-labelled probe specific for Ch5 **(C3)** and a DIG-labelled probe specific for CDVd **(C4)**.

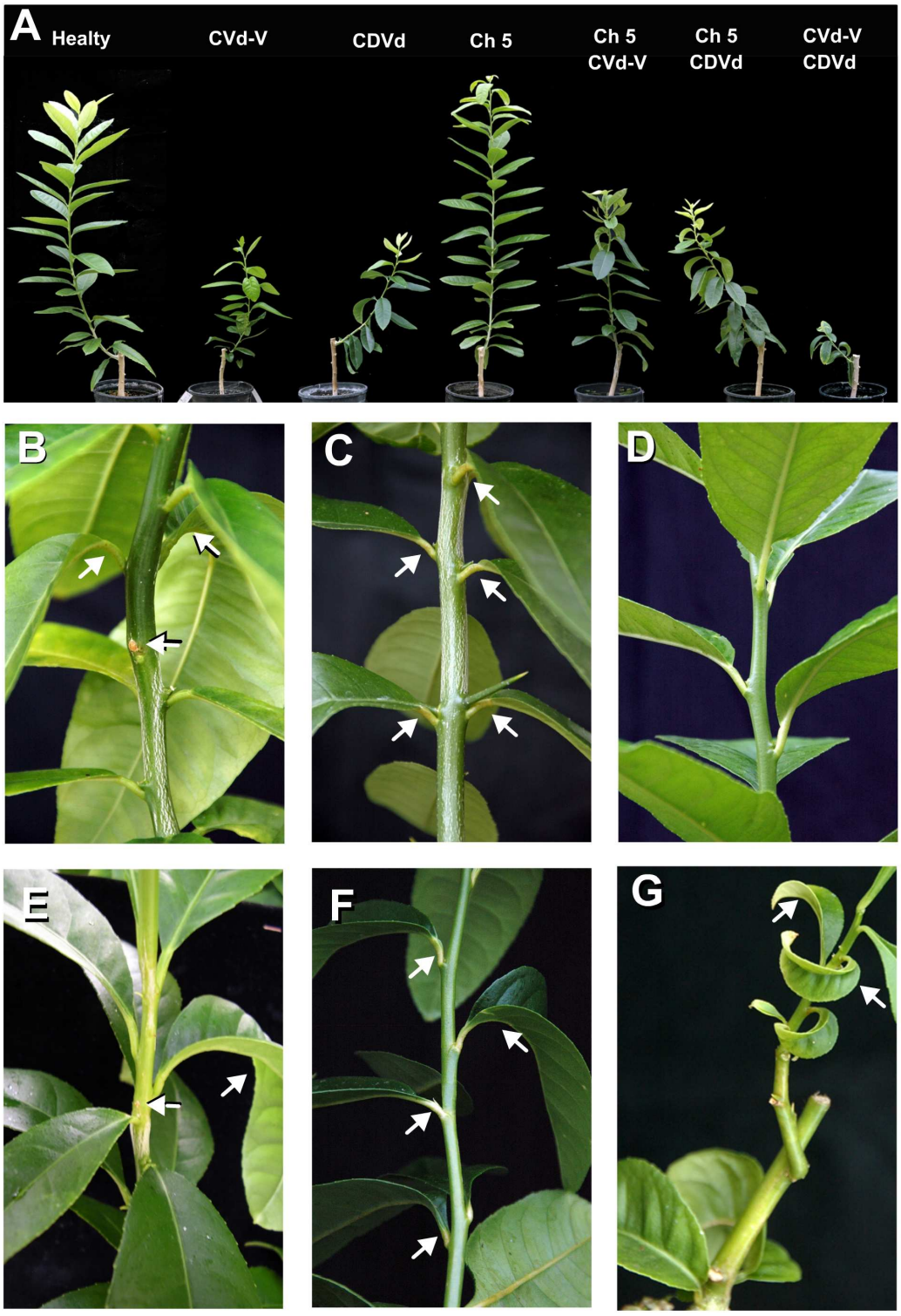

Fig. 3 Symptoms of viroid infection in Etrog citron plants, 4 months post inoculation. **(A)** General aspect of plants non-inoculated (healthy), infected with a single viroid (CVd-V, CDVd or Ch5), or co-infected with two viroids (Ch5 and CVd-V, Ch5 and CDVd or CVd-V and CDVd). **(B)** Leaf epinasty and cracks and gum exudates in the stem of CVd-V-infected plants. **(C)** Petiole necrosis and leaf epinasty of CDVd-infected plants. **(D)** Symptomless plant infected with Ch5. **(E)** Leaf epinaty and cracks and gum exudates in the stem of plants co-infected with Ch5 and CVd-V. **(F)** Petiole necrosis and leaf epinasty of plants co-infected with Ch5 and CDVd. **(G)** Severe leaf epinasty in plants co-infected with CVd-V and CDVd.

Symptom expression in Ch5 infected plants co-inoculated with CDVd or with CVd-V.

Since CDVd and CVd-V co-infecting the same plant discloses synergistic interactions manifested in enhanced leaf epinasty and very pronounced dwarfing (Serra *et al*., 2008a), two additional treatments were carried out to see the effect of Ch5 co-infected with CVd-V or CDVd. Six buds from a Ch5-infected citron were each graft-propagated on rough lemon seedlings and, concurrently, three of these plants were graft-inoculated with bark from a CVd-V-infected citron, and the other three with bark from a CDVd-infected citron. Three additional buds from a CVd-V-infected citron were also graft-propagated on rough lemon seedlings and, concurrently, these plants were graft-inoculated with bark from a CDVd-infected citron as positive controls for synergistic effect. Three buds from CDVd infected plants, three from CVd-V infected plants, three from Ch5 infected plants and three from non-inoculated plants were graft-propagated as controls.

Plant height and number of internodes were measured four months after inoculation and the values were analysed using one-way ANOVA and the Student-Newman-Keuls post-test that compares all pairs of means (Table 3). This showed that: (1) there were significant differences among treatments; (2) no significant differences were found among plants singly infected with CDVd or CVd-V or co-inoculated with Ch5, which however were significantly smaller that the non-infected controls; (3) plants co-inoculated with CVd-V and CDVd were significantly smaller than the other treatments; (4) plants infected with Ch5 were significantly larger than the non-infected controls.

Table 3. Size of Etrog citron plants infected with single viroids or co-infected with two viroids.

Viroid	Plant size	
	Height[1] (cm)	Internodes[1] (number)
Uninfected	63.3±1.8 a	32.3±1.5 ab
CVd-V	37.0±1.0 b	23.5±0.5 bc
CDVd	33.0±0.1 b	20.0±2.6 bc
Ch5	76.0±3.1 c	39.0±1.5 a
Ch5+CVd-V	49.0±1.2 b	27.0±2.9 b
Ch5+CDVd	46.3±7.2 b	30.4±3.8 ab
CVd-V+CDVd	16.5±3.5 d	15.0±1.0 c

[1]Data show the mean and standard error of height and internode numbers. Data followed by the same letter are not significantly different according to analysis by one-way ANOVA and the Student-Newman-Keuls post-test (95%)

In contrast with the synergistic effect observed in plant co-inoculated with CVd-V and CDVd (Fig. 3A, G), plants co-infected with Ch5 and CVd-V (Fig. 3A, E) or with Ch5 and CDVd (Fig. 3A, F) developed the same symptoms as plants singly infected with CVd-V (Fig. 3A, B) or with CDVd (Fig. 3A, C), respectively.

Viroid titers in co-infected plants.

As already reported (Serra *et al.,* 2008a), the synergistic effect observed in plants co-infected with CVd-V and CDVd was not associated with changes in the titers of these two viroids. Northern blot hybridization analysis using CVd-V and CDVd specific probes confirmed that the titers of these two viroids remained unaltered in co-infected plants (Fig. 2B). Unexpectedly, northern hybridization analysis of plants co-infected with Ch5 and CDVd showed that Ch5 was present in very low titers or even undetectable (Fig. 2C). The presence of low titers of Ch5 was confirmed using a more sensitive hybridization test (Murcia et al., unpublished) (Fig. 4A) and by RT-PCR using P1 and P2 primer pair (Fig. 4A).

Analysis by sPAGE and silver staining showed that plants co-infected with Ch5 and CVd-V presented similar viroid titers than plants infected with Ch5 or with CVd-V (Fig. 2E). Given the high sequence identity (96%) between CVd-V and Ch5, these viroids could not be discriminated by northern hybridization but infection was confirmed by RT-PCR using discriminating primer pairs (Table 1) C1 and V7 for CVd-V (data not shown) and C2 and V7 for Ch5 (Fig. 4B). In order to estimate the relative concentrations of CVd-V and Ch5 in the co-infected plants, an RT-PCR approach was designed as follows: (i) The efficiency of reverse transcription to generate cDNAs from CVd-V and Ch5 using primer P2 was evaluated using serially diluted (1:2, 1:20, 1:200) cDNAs generated from equalized preparations of CVd-V or Ch5. Agarose gel electrophoresis of the products amplified using P1 and P2 confirmed that CVd-V and Ch5 were amplified with the same efficiency (Fig. 4C); (ii) The suitability of this approach was confirmed using mixtures (1:1, 1:9 and 9:1) of equalized preparations of CVd-V and Ch5 that were subjected to RT-PCR. The amplicons were ligated into the pGem-T vector and PCR analysis using discriminating primer pairs of eleven clones from each ligation reaction, gave the same ratios of CVd-V and Ch5 as in the mixtures. Using this approach, preparations from each of the three plants co-infected with CVd-V and Ch5 were subjected to RT-PCR and the amplified products were ligated to the pGem-T vector. PCR Analysis of fifteen clones from each preparation showed that all of them contained the

CVd-V sequence. These results show that as in the case of plants co-infected with CDVd and Ch5, plants co-infected with CVd-V and Ch5 contained only very low Ch5 titers.

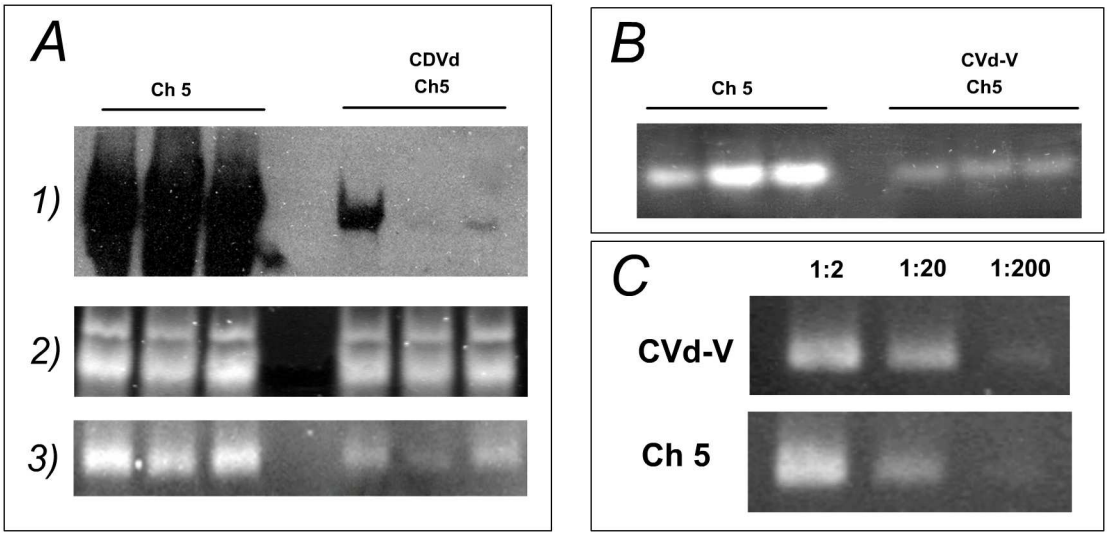

Fig. 4. Northern hybridisation analyses of samples electrotransferred from a non-denaturing gel using a DIG-labelled probe specific for Ch5 of citron plants infected with Ch5 or co-infected with Ch5 and CDVd **(A1)**. Ethidium bromide staining of the first non-denaturing gel of sPAGE shows that RNA levels in all preparations were comparable **(A2)**. RT-PCR analysis of citron plants infected with Ch5 or co-infected with Ch5 and CDVd using primers P1 and P2 **(A3)** and citron infected with Ch5 or co-infected with Ch5 and CVd-V using primers C2 and V7 **(B1)**. Efficiency of reverse transcription to generate cDNAs from CVd-V and Ch5 using primer P2 evaluated using serially diluted (1:2, 1:20, 1:200) cDNAs generated from equalized preparations of CVd-V or Ch5 **(C).**

Discussion

Studies addressed to understand the relationship between different segments of the secondary structure of viroid molecules and their biological properties in members of the genus *Apscaviroid*, all of them restricted to woody species, are very limited. Even though a CCR and a TCR have been clearly identified, the presence and putative role of the P, V, T_L and T_R domains remains unknown. Studies conducted by induced mutagenesis indicated that in spite of apparent variability of the lower strand of CCR and adjacent segments found in naturally occurring variants of CDVd (Owens *et al.* 2000), its genome admits only a very limited range artificially induced mutations (Owens *et al*. 2005).

In the present study this issue was addressed using synthetic chimeras obtained by exchanging discrete segments of the CVd-V molecule for the corresponding segments of CDVd, two closely related viroids naturally infecting citrus in which they accumulate at similar titers and induce distinct and characteristic symptoms. Chimeric viroids have been

produced using a novel PCR-based approach, that do not have the limitations associated with the availability of restriction sites to exchange viroid segments, thus allowing the synthesis of the desired type of constructs. In contrast with the success achieved with heterologous chimeras between CEVd and TASVd two closely related viroids of the same genus (Sano *et al*. 1992), and to a less extend between viroids of different genera CEVd and HSVd (Sano and Ishiguro, 1998), only one out of the seven chimeric constructs between CVd-V and CDVd resulted infectious. Unexpectedly, Ch4, which differs from CDVd in only four nucleotide changes in the lower strand of the CCR, was not infectious. These changes that appear to be lethal for Ch4 infectivity, were also present in all the other chimeric constructs that with the exception of Ch5 were also not infectious. The success of Ch5 is probably due to the fact that it is essentially identical to CVd-V in which its T_L was replaced from that of CDVd. It should be noted that the survival of Ch5 in only one of the three inoculated plants was associated with a 42C→U substitution in the S2 segment, a change that is probably essential for its viability. The present study describes for the first time the synthesis of an artificial chimeric viroid within the genus *Apscaviroid* that replicates, becomes systemic and it is readily transmissible.

Overall, Ch5 differs in 11 changes with respect to CVd-V that impose an organization of the secondary structure of T_L identical to that of CDVd. Unlike the results of previous studies conducted with PSTVd in which mutations in the loops of the T_L domain resulted in low replication efficiency and viroid trafficking being impaired or defective (Zhong *et al*. 2008), the replication efficiency of Ch5 was comparable to that of CVd-V and it was trafficking competent. However in contrast with plants infected with CVd-V or with CDVd, plants infected with Ch5 were slightly taller than the non-infected controls and did not display any of the symptoms induced by either of these two viroids. Plants infected with Ch5 were symptomless lacking the necrotic lesions and cracks, sometimes filled with gum characteristic of CVd-V and the stunting and moderate epinasty resulting from petiole and mid-vein necrosis charactersitic of CDVd. This observation suggests the implication of the T_L domain in blocking the primary elicitor of symptom expression. A different situation was found by Sano et al., (1992) in the case of heterologous chimeras between CEVd and TASVd both belonging, to the genus *Pospiviroid*, in which the T_L did not block but modulated pathogenesis.

Plants co-infected between two viroids or two strains of the same viroid may display two distinct types of interactions termed “cross-protection” and “synergism”. When a plant infected with a latent or mild strain of a viroid becomes protected against subsequent infection

with a severe strain of the same viroid, the typical symptoms of the second strain and its accumulation level is abolished or attenuated, a phenomenon known as "cross-protection" (Pallas and Flores, 1989; Semancik *et al*. 1992). Conversely, co-inoculation with two unrelated viroids may result in a synergistic interaction, with symptoms being more severe that those expected for purely additive effects of the two viroids (Vernière *et al*. 2006, Serra *et al*. 2008a). Given the high sequence identity between Ch5 and CVd-V, Ch5 could be regarded as a latent strain of CVd-V and a "cross-protection" phenomenon should be expected in co-infected plants. Since plants co-infected with CVd-V and CDVd displayed a synergistic interaction (Serra *et al*. 2008a), the same type of interaction was to be expected in the case of plants co-infected with Ch5 and CDVd. Unexpectedly, plants co-infected with Ch5 and CVd-V or CDVd developed the same symptoms as plants singly infected with CVd-V or with CDVd, respectively. These results suggest that there were no interactions between Ch5 and these two related viroids.

Even though co-infected plants were produced by graft-propagation of Ch5 infected buds and further inoculated with either CVd-V or CDVd, analysis of the co-infected plants showed that CVd-V and CDVd displaced Ch5 that was found to be present in extremely low titers. These results explain the lack of interaction regarding symptom expression. In spite of the fact that Ch5 was able to replicate, became systemic and reach the same titers as CVd-V or CDVd in single infection, it loosed these properties in the presence of related viroids. Similar results were found in the case of *Gynura aurantiaca* and tomato co-infected with PSTVd and CEVd where CEVd displaced PSTVd in *Gynura aurantiaca* whereas PSTVd displaced CEVd in tomato (Pallás and Flores, 1989). These observations suggested that most of the host factor that was initially used by one of the viroids, was later monopolized by the other viroid due to a higher affinity. The same explanation could explain the displacement of Ch5 by either CVd-V or CDVd. Ch5 is an artificial viroid with the primary and secondary structures of the T_L different than its CVd-V progenitor that appears to be well fit to the Etrog citron host. However, in contrast to the naturally occurring CDVd and CVd-V that have been subjected for a long time to natural selection under different hosts and environmental pressures, the fitness of Ch5 has not been challenged in other hosts nor in co-infection with other viroids. Therefore the ability of CDVd and CVd-V to displace Ch5 in co-infected plants suggests that Ch5 has a lower affinity for certain host factors than naturally occurring viroids.

The mechanisms involved in viroid-induced symptoms are poorly understood. Since sequence variants with changes affecting specific domains of the viroid secondary structure are responsible for the induction or not of certain diseases (Reanwarakorn and Semancik,

1998; Palacio-Bielsa *et al.* 2000), or with pronounced changes in symptom severity (Serra *et al*. 2008b), the hypothesis that the viroid RNA is the primary effector has been entertained for many years. According to this hypothesis, the interaction of viroid RNA with a cell factor would interfere with its normal function and would lead to symptom expression. Recently, since the discovery that small interfering RNAs (siRNAs) and microRNAs (miRNAs) can mediate host gene expression, the involvement of gene silencing has been proposed as a novel hypothesis to explain viroid pathogenesis (Baulcombe, 2004; Carrington and Ambros, 2003). According with this hypothesis, viroid siRNAs acting like endogenous miRNAs, might target host mRNAs and promote their degradation (Papaefthimiou *et al*. 2001). The results of the present study using chimeric constructs show that the T_L domain is involved in the pathogenesis of CVd-V. In spite of the fact that the presence of siRNAs related to the sequence of the T_L domain has been demonstrated in PSTVd and CEVd infected tomato plants (Itaya *et al*. 2007; Martin *et al*. 2007), the involvement of gene silencing cannot easily explain the T_L-related pathogenesis of CVd-V. The demonstration that the symptoms of CVd-V cannot be induced when its T_L domain is replaced by that of CDVd would implicate the siRNAs generated from this domain as the direct effectors of CVd-V pathogenesis. Since Ch5 should also be able to generate siRNAs from its T_L domain, which is identical as the T_L domain of CDVd, the lack of symptoms expression could only be explained if the pathogenesis of CDVd were mediated by siRNAs generated from another domain of the viroid molecule. However, since the symptoms induced by CDVd and CVd-V share similar features, the above explanation seems rather unlikely.

The results reported here can be best explained taking into consideration the mature viroid RNA as the primary effector of pathogenesis. The replacement of the T_L domain of CVd-V by that of CDVd alters the structure of the viroid RNA and its affinity for a host factor may also be consequently altered. Recently, it has been demonstrated that viroid infection alters the expression of genes that can be down regulated or up regulated (Itaya *et al*. 2002; Tessitori *et al*. 2007). This cascade of events must be mediated by elicitor mechanisms that require specific interactions between the viroid RNA and still unknown host factors. The range of symptoms associated with CVd-V infection that is not induced in Ch5 infected plants suggests that the elicitor mechanisms cannot be adequately activated.

Materials and methods

Viroid sources

Plasmids p(CVd-V) and p(CDVd) consisting of pBluescript KS+ (Promega) containing in the EcoRV site the full-length sequence of CVd-V and CDVd (Genebank accessions NC010165 and EU934004, respectively), and were used as a source of viroid cDNA sequences. The inserts of CVd-V and CDVd with a positive orientation from the T7 promoter, had been generated by RT-PCR with the primer pairs CVd-III-F1 / CVd-III-R1 (Bernad and Duran-Vila, 2006) and P1 / P2, respectively (Table 1).

Construction of chimeric variants

The composition of the chimeric variants is outlined in Table 2 and Figure 1. Unless otherwise stated, PCR amplification of CDVd segments to be inserted into CVd-V devoid of the segments to be replaced, was performed with 2.5 U *Pfu* DNA polymerase (Stratagene), the appropriate template (1 ng) (Table 2), the forward and reverse phosphorylated primer sets (Tables 1 and 2) (0.2 μM each) and dNTPs (0.3 mM each). PCR parameters consisted of a denaturation step at 95^{o}C for 2 min, followed by 30 cycles (94^{o}C for 30 s, 55^{o}C for 30 s and 72^{o}C for 45 s). Segments S1 from CDVd and CVd-V were synthesized with 2.5 U *Pfu* DNA polymerase by elongation of overlapping phosphorylated primers D3 and D4 (CDVd) or V5 and V6 (CVd-V) (0.2 μM each) (Table 1) in the presence of dNTPs (0.3 mM each). The reaction consisted of a denaturation step at 95^{o}C for 1 min, followed by five cycles (95^{o}C for 30 s, 40^{o}C for 30 s and 72^{o}C for 30 s).

Amplification of the plasmid containing the viroid or a previously constructed chimeric variant devoid of the segment to be replaced, was performed with 2.5 U *Pfu* Turbo DNA polymerase (Stratagene), the appropriate template (0.2 ng) (Table 2), the forward and reverse primer sets (Tables 1 and 2) (0.2 μM each) and dNTPs (0.3 mM each). PCR parameters consisted of a denaturation step at 95^{o}C for 2 min, followed by 25 cycles (94^{o}C for 30 s, 55^{o}C for 30 s and 72^{o}C for 210 s).

Electrophoretic analysis in 2% agarose gels confirmed the synthesis of DNA products of the expected sizes. After a second electrophoresis in 1% agarose gels, PCR-amplified products of viroid segments or full-plasmid length (including the partial viroid cDNA insert) were purified with the QIAquick gel Extration kit (QIAGEN). The construction of each

chimeric variant was performed by restoring the amplified segment into the corresponding plasmid devoid of such segment (Table 2) in a ligation reaction performed in a 15 µl volume with 2 U T4 DNA ligase (Gibco) at 15ºC overnight. The ligation product was used to transform DH5α *Escherichia coli* cells. In all instances, sequencing confirmed that the inserts contained only the desired chimeric constructions.

Infectivity assays

The seven chimeric-DNAs inserts were recovered as blunt-end PCR products using phosphorylated primers and *Pfu* DNA polymerase (Table 2). Monomeric CVd-V-DNA, CDVd-DNA were obtained using phosphorylated primers P1 and P2 or CVd-III-F1 and CVd-III-R1, respectively (Table 1). The PCR products were analyzed by electrophoresis in 1% agarose gels to confirm the synthesis of products of the expected size. The DNA bands eluted from the gel with the QIAquick gel Extration Kit were subjected to ligation in a 15 µl volume with 2 U of T4 DNA ligase (Gibco) at room temperature for 40 min. The ligation product was analyzed in 1% agarose gels and the dimeric bands eluted as before were cloned in pBluescript II KS (+) digested with *Eco*RV. Plasmids from transformed cells were sequenced to verify the desired head-to-tail orientation of the dimeric inserts. Clones with these inserts were linearized with *Bam*HI or *Eco*RI depending of the orientation of the insert and used as a template in a transcription reaction with 1 mM NTPs, 1 mM DTT and 50 U of T7 or T3 RNA polymerase (Roche Applied Science) to produce dimeric transcripts homologous to CVd-V, CDVd and the seven quimeric constructions. Three Etrog citron seedlings were slash-inoculated (50 ng of transcript per plant) and kept in the greenhouse at 28º-32ºC for 9 months.

Transmission and symptom evaluation assays.

For viroid transmission and symptom evaluation, viroid infected plants were obtained by two strategies: (i) graft-inoculation on the sensitive selection 861-S1 of Etrog citron graft-propagated on rough lemon (*C. jambhiri* Lush) rootstock seedlings; (ii) graft propagation of buds from viroid-infected 861-S1 of Etrog citron plants. , or ii) graft-inoculation of bark from viroid-infected citron plants on rough lemon seedlings.

RNA analysis by Northern-blot hybridization

Tissue samples of inoculated Etrog citron plants (5 g of young leaves and/or stem bark) were homogenized in a mixture of phenol and extraction buffer [0.4 M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA, pH 7.0; 4% (v/v) 2-mercaptoethanol]. Total nucleic acids were partitioned in 2 M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl, pH 7.4; 10 mM KCl; 0.1 mM $MgCl_2$) (Semancik et al. 1975). Aliquots (20 µl) of the nucleic acid preparations (equivalent to 300 mg of fresh weight tissue) were examined by two consecutive polyacrylamide gel electrophoreses (sequential PAGE, sPAGE), the first under non-denaturing and the second under denaturing conditions (Rivera-Bustamante *et al.* 1986) and silver staining (Igloi, 1983).

For northern hybridization, the RNAs fractionated by non-denaturing PAGE in 5% gels or by sPAGE were electroblotted (400 mA for 2 h) to positively-charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA). RNAs were immobilized by UV cross-linking and hybridized with DIG-labeled probes specific for CDVd, for CVd-V or for chimeric constructs. The probes were synthesized by PCR using as a template plasmids containing full-length sequences of the corresponding templates (Palacio *et al.* 2000). Prehybridization (at 50ºC for 2-4 h) and hybridization (at 50ºC overnight) were performed in 50% formamide and 6X SSPE as described by Sambrook *et al.* (1989). After hybridization the membranes were washed twice in 2X SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1X SSC, 0.1% SDS at 60ºC for 60 min, and revealed with an anti-DIG alkaline phosphatase conjugate and the chemiluminiscence substrate CSPD (Roche Applied Science).

Reverse transcription and PCR amplification

First-strand cDNA was synthesized with 15 U of the reverse transcriptase ThermoScript^{TM} Rnase H^{-} (ThermoScript-RT, Invitrogen®) and the reverse primer P2 for CVd-V and Ch5 or CVd-III-R1 for CDVd (0.75 µM) (Table 1) and dNTPs (1 mM each) for 1 h at 55ºC. Second-strand synthesis and PCR amplification was performed using 4 µl of the first strand cDNA reaction mixture, 1 U of *Taq* DNA and the reverse and forward primers P2 and P1 for CVd-V and Ch5 or CVd-III-R1 and CVd-III-F1 for CDVd (0.5 µM each) and dNTPs (0.12 mM each) (Bernad and Duran-Vila, 2006). The cycling profile consisted of a denaturation step at at 95^{o}C for 5 min, followed by 30 cycles (94^{o} C for 30 s, 55^{o} C for 30 s

and 72^{o} C for 2 min) to finish with and extension step at 72^{o} C for 5 min. Electrophoresis in 2% agarose gels confirmed the synthesis of products of the expected size. Purified RT-PCR amplicons were sequenced with an ABI PRISM DNA sequencer 377 (Perkin Elmer).

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Capítulo 4

A single nucleotide change in Hop stunt viroid modulates citrus cachexia symptoms

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Abstract

Cachexia disease of citrus is caused by Hop stunt viroid (HSVd). In citrus, pathogenic and non-pathogenic strains differ by a "cachexia expression motif" of five to six nucleotides located in the variable domain of the proposed rod-like secondary structure. Here, site-directed mutants were generated to investigate if all these nucleotides were required for infectivity and/or symptom expression. Specifically, an artificial cachexia-inducing mutant M0 was generated by introducing the six-nucleotide changes of the "cachexia expression motif" into a non-pathogenic sequence variant and M0 was used as a template to systematically restore some of the introduced changes. The resulting mutants in which specific changes introduced to generate M0, were restored presented a variety of responses: (i) M1, obtained by introducing two insertions forming a base-pair, was infectious but non-pathogenic; (ii) M2, obtained by introducing an insertion and restoring a substitution, presented low infectivity and the resulting progeny reverted to M0; (iii) M3, obtained by restoring a single substitution in the lower strand of the viroid secondary structure, was infectious but induced only mild cachexia symptoms; and (iv) M4, obtained by restoring a single susbtitution in the upper strand of the viroid secondary structure, was non-infectious. These results confirm that the "cachexia expression motif" plays a major role in inciting cachexia symptoms, and that subtle changes within this motif affect symptom severity and may even suppress symptom expression.

Short communication

Cachexia was first described in 1950 as a disease of Orlando tangelo with the following symptoms: discoloration, gumming and browning of phloem tissue, wood pitting and bark cracking (Childs, 1950). The disease affects mandarins (*Citrus reticulata* Blanco), clementins (*C. clementina* Hort. ex Tan.), satsumas [*C. unshiu* (Macf.) Marc.], some mandarin hybrids such as tangelos (*C. paradisi* Macf. × *C. tangerina* Hosrt. ex Tan.), alemow (*C. macrophylla* Webster), Rangpur lime (*C. limonia* Osb.) and kumquats (*Fortunella* spp). The most common symptoms observed in these sensitive hosts are: (i) discoloration and gum impregnation of the bark, which are easily seen by scrapping or removing the outer bark layers, and (ii) pegs on the cambial side of the bark, with the corresponding depressions or pits in the wood. Affected trees are stunted and chlorotic, and may decline and die.

The causal agent suspected to be a viroid by Roistacher (1983), was indeed identified and characterized as a strain of *Hop stunt viroid* (HSVd) (Semancik et al., 1988; Levy and Hadidi, 1993). HSVd (295-303 nucleotides) is the only member of the genus *Hostuvioid* within the family *Pospiviroidae* (Flores et al., 2005) and its predicted most stable secondary structure is a rod-like conformation with five domains (Fig. 1), a "central conserved region" (CCR) and a "terminal conserved hairpin" (TCH).

Based on the pathogenic properties of HSVd in citrus, at least two distinct strains of the viroid have been reported: "pathogenic strains" that induce the cachexia disease in sensitive hosts, and "non-pathogenic strains" that infect the same sensitive hosts without inducing symptoms. The differences between these two types of strains are very subtle, with only six nucleotides appearing to determine symptom expression (Reanwarakorn and Semancik, 1998). Unlike members of the genus *Pospiviroid*, in which pathogenesis has been found in the "pathogenicity domain" (P) located at the left of the "central domain" (C), the specific nucleotide differences that determine expression of cachexia symptoms ("cachexia expression motif") has been found in the "variable domain" (V) located at the right of the C domain. Molecular characterization of HSVd sources from different geographic origins has confirmed that this motif is highly conserved, although some minor differences have been identified (Velázquez et al., 2002; Palacio-Bielsa et al., 2004).

In an effort to understand pathogenesis more thoroughly, a number of artificial HSVd mutants were produced and their biological properties evaluated. Mutants were generated following a PCR-based protocol (Byrappa et al., 1995) with minor modifications (Gago et al., 2005). Briefly, 5 ng of a derivative of plasmid pBluescript II KS (+) containing the full-length viroid sequence were amplified with 250 ng each of pairs of adjacent and phosphorylated primers, complementary and homologous to different segments of the viroid sequence, in which appropriated changes were introduced to incorporate the desired mutations (Table 1). The PCR cycling profile for amplifying the complete plasmid with *Pfu* Turbo DNA polymerase consisted of 30 cycles of 30 s at 94°C, 30 s at 60°C and 3.5 min at 72°C, with an initial denaturation at 94°C for 2 min and a final extension at 72°C for 10 min. After electrophoresis in 1% agarose gels, PCR-amplified products of full-plasmid length (including the viroid cDNA insert) were purified with the QIAquick kit (QIAGEN), circularized with T4 DNA ligase (Gibco), and used to transform DH5α *Escherichia coli* cells. In all instances, sequencing confirmed that the inserts contained only the desired mutations.

In a first mutagenesis experiment the characteristic nucleotide changes of the "cachexia expression motif" were introduced into a non-pathogenic HSVd variant. A plasmid containing the full-length sequence of the non-pathogenic isolate CVd-IIa-117 (Genebank accession no. AF213503) was used as template to introduce mutations affecting the upper strand of the viroid secondary structure (A107→G, A109→Δ and A115→Δ; Table 1 and Fig. 1). Next, the resulting plasmid was circularized and used as template to introduce a second set of mutations (C197→U, U194→C and U189→Δ) affecting the lower strand of the viroid secondary structure (Table 1 and Fig. 1). To obtain infectious preparations of this mutant (termed M0), a head-to-tail dimeric cDNA was synthesized and used as template to produce the corresponding *in vitro* transcripts (dimeric transcripts are essentially identical to the oligomeric positive strands produced during the replication of viroids that follow the asymmetric pathway of the rolling-circle mechanism), which are highly infectious. Briefly, the M0 insert was recovered as a blunt-end PCR product using the phosphorylated primers HSVd-R1 and HSVd-F1 (Bernad and Duran-Vila, 2006) and *Pfu* DNA polymerase. The amplification products were ligated with T4 DNA ligase and the dimeric molecules were cloned into pBluescript II KS (+) digested with *Eco*RV. The recombinant plasmids from transformed cells were sequenced to verify the head-to-tail organization of the dimeric inserts and, according to their orientation, these plasmids were linearized with *Hind*III or *Xba*I and used as a template in transcription reactions with 1 mM NTPs, 1 mM DTT and 50 U of RNA polymerase T7 (in the case of *Hind*III) or T3 (in the case of *Xba*I), to produce dimeric transcripts of the M0 mutant. Three Etrog citron seedlings were slash-inoculated with 50 ng of transcript per plant and kept in the greenhouse at 28º-32ºC. Dimeric head-to-tail transcripts of the natural cachexia (X-701-1, Genebank accession no. AF213484) and non-cachexia (CVd-IIa-117) variants, used as controls were also inoculated in three Etrog citron seedlings. These two variants were selected because only differ in a single deletion (T154→Δ) in addition to the "cachexia expression motif". As expected, the inoculated Etrog citron seedlings remained symptomless over a six-month period but they were infected as determined by Northern blot hybridization (data not shown). To that purpose, tissue samples were homogenized in extraction buffer containing water-saturated phenol and partitioned in 2M LiCl (Semancik et al., 1975). The RNAs were separated by two consecutive polyacrylamide gel electrophoreses (sequential PAGE, sPAGE) (Rivera-Bustamante *et al.,* 1986), electroblotted to positively-charged nylon membranes (Roche Applied Science) and and hybridized with a DIG-labelled HSVd probe (Palacio et al., 2000) as described by Sambrook et al (1989). Moreover, infection was confirmed by RT-PCR using the HSVd-

specific primers HSVd-R1 and HSVd-F1 (Bernad and Duran-Vila, 2006) that generated amplicons of the expected size and sequence.

One of the three Etrog seedlings of each class (M0, CVd-IIa-117, X-701-1) was biologically indexed on three plants of the Parson's Special mandarin, a cachexia-specific indicator (Roistacher et al., 1973). To this aim, a Parson's Special mandarin bud was grafted on a rough lemon (*C. jambhiri* Lush.) rootstock seedling and immediately thereafter two bark pieces of the infected Etrog citron were grafted immediately below the mandarin bud. Only the mandarin bud was allowed to grow. Three non-inoculated indicator plants were kept as negative controls and plants were maintained in a greenhouse at 28º-32ºC. Eighteen months after inoculation, the bark of the indicator plants was removed for observation of symptom expression. As expected, plants inoculated with either M0 or X-701-1 presented at the bud-union severe stem pitting and gumming that affected the Parson's Special mandarin scion but not the rough lemon rootstock, with pitting being also observed along the stem and, in particular, at the branching points. Plants inoculated with CVd-IIa-117 were symptomless and indistinguishable from the non-inoculated controls. These results confirmed with an artificial variant the requirement of the "cachexia expression motif" for symptom development as described by Reawankaron and Semancik (1998). Even though effects due to changes in the V domain have not been reported in other members of the family *Pospiviroidae*, the "cachexia motif" and "non-cachexia motif" of the V domain of HSVd play a relevant role, not only for its implication in pathogenicity but also by carrying the host-specific changes identified in isolates recovered from different crops (Cañizares et al., 1997; Kofalvi et al., 1997; Amari et al., 2001).

Additional mutagenesis experiments were conducted to elucidate if all these six changes were required for symptom expression. Four mutants (M1, M2, M3, M4) were generated with the same PCR strategy described above, but using as a template the plasmid containing the sequence of the artificial cachexia variant M0 and primer pairs designed to introduce the desired changes (Table 1). The four mutants were obtained as follows: M1 by incorporating two insertions (+A115 and +U189, forming a base-pair) that had been deleted to generate M0; M2 by incorporating an insertion (+A109) and a substitution (C194→U) to revert the changes introduced to generate M0; M3 by restoring a single substitution (U197→C) in the lower strand of the viroid secondary structure; and M4 by restoring a single substitution (G107→A) in the upper strand of the viroid secondary structure. The changes and

the predicted secondary structure (Zuker et al.,1989; De Rijk and De Wachter, 1997) of the region containing the "cachexia expression motif" are shown in Fig. 1

Table 1
Primers for site-directed mutagenesis of HSV

Site-directed mutagenesis		Primers	Oligonucleotide sequence (5′ → 3′)	Positions[b]
Template → mutant	Nucleotide changes[a]			
(CVd-IIa-117) → M0	A107 → G, A109 → Δ, A115 → Δ (upper strand)	Sense	CAGCGGGGGCGTGGAGAGAG	103–124
		Antisense	GATTCTGAGAAGAGTTGCCCCG	102–81
	U189 → Δ, U194 → C, C197 → U (lower strand)	Sense	TTTACCTCCTTCTGGCTCTTCG	187–208
		Antisense	GAAGAAGGGACGATCGATGG	186–167
M0 → M1	+A115 (upper strand)	Sense	CAGCGGGGGCGTAGGAGAGAG	103–124
		Antisense	GATTCTGAGAAGAGTTGCCCCG	102–81
	+U189 (lower strand)	Sense	TTTACCTCCTTCTGGCTCTTCG	187–208
		Antisense	GAAGAAGGGACGATCGATGG	186–167
M0 → M2	+A109 (upper strand)	Sense	CAGCGGAGGCGTGGAGAGAG	103–124
		Antisense	GATTCTGAGAAGAGTTGCCCCG	102–81
	C194 → U (lower strand)	Sense	TTTACCTTCTTCTGGCTCTTCG	187–208
		Antisense	GAAGAAGGGACGATCGATGG	186–167
M0 → M3	U197 → C (lower strand)	Sense	TTTACCTCCTCCTGGCTCTTCG	187–208
		Antisense	GAAGAAGGGACGATCGATGG	186–167

a Nucleotide insertion (+) and nucleotide deletion (Δ)
b Nucleotide positions refer to those shown in the secondary structure of CVd-IIa-117 (Fig. 1).

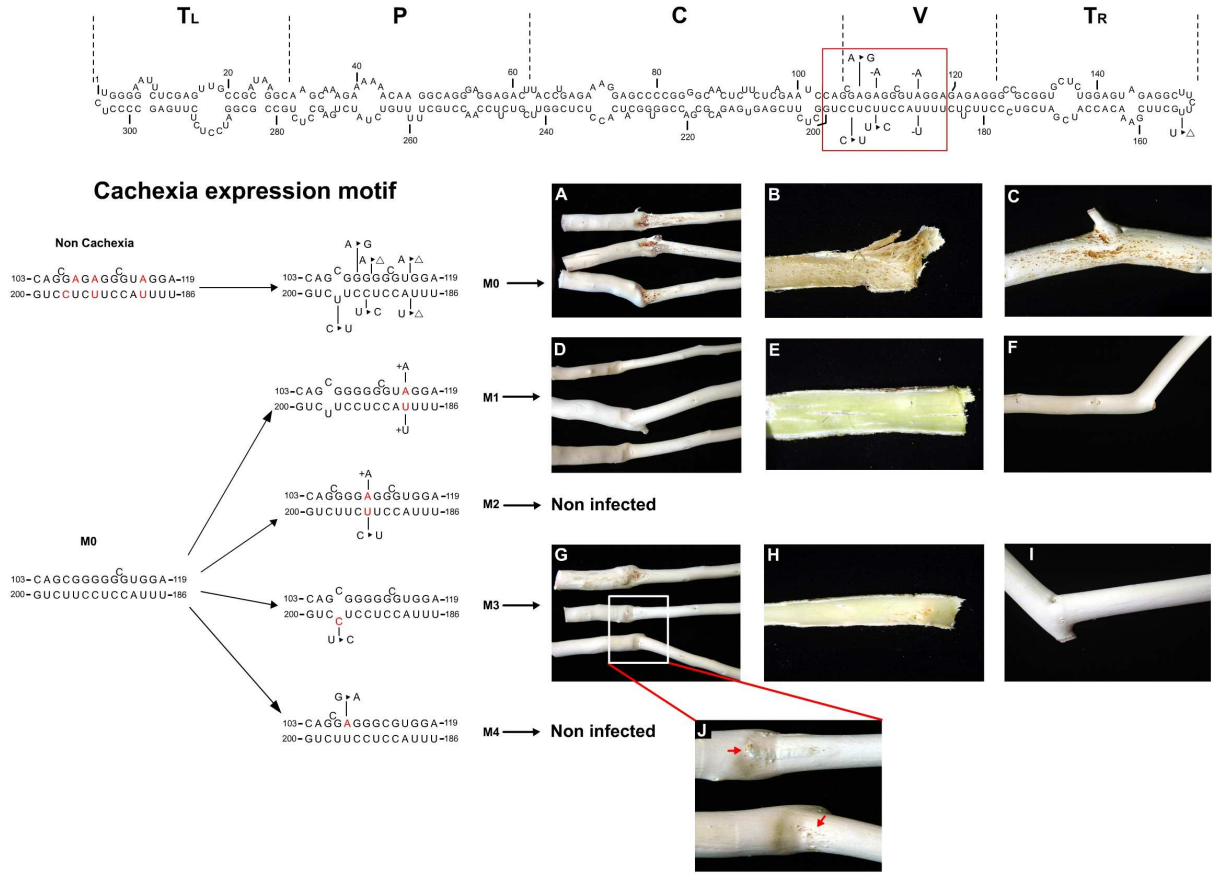

Fig. 1. Primary and predicted secondary structure of HSVd variant CVd-IIa-117 (GeneBank accession AF213484). The 6-nt motif discriminating "cachexia" and "non-cachexia" variants is boxed. M0, M1, M2, M3 and M4 show the composition of the cachexia motif in the six mutants studied (nucleotides in red highlight the mutated positions). Symptoms induced in the Parson's Special mandarin scion: A) severe stem pitting and gumming observed in the wood of the bud-union after removing the bark; B) pegs in the bark; C) conspicuous stem pitting in the wood along the stem of the Parson's Special mandarin scion; D) bud-union devoid of stem pitting and gumming in the wood; E) bark devoid of pegs; F) wood along the stem devoid of stem pitting; G) mild stem pitting and gumming observed in the wood of the bud-union after removing the bark; H) mild pegs in the bark; I) wood along the stem devoid of stem pitting; J) detail of the mild stem pitting.

Infectivity of the four mutants was tested on three Etrog citron seedlings by inoculating the corresponding dimeric transcripts. Northern blot hybridization and RT-PCR amplification showed that mutants M1, M2 and M3, but not M4, were infectious (Table 2). Sequence analysis of the resulting progenies revealed that M1 and M3 were genetically stable, whereas the progeny of the single plant that became infected with M2 reverted to M0.

Table 2
Infectivity and bioassay of mutants constructed by site-directed mutagenesis

Variant/mutant	Etrog citron			Parson's Special mandarin		
	Infectivity[a]	Progeny[b]	Symptoms	Infectivity[a]	Progeny[b]	Symptoms
CVd-IIa-117	3/3	CVd-IIa-117	Symptomless	3/3	CVd-IIa	Symptomless
X-701-1	3/3	X-701-1	Symptomless	3/3	X-701	Severe
M0	2/3	M0	Symptomless	3/3	M0	Severe
M1	2/3	M1	Symptomless	3/3	M1	Symptomless
M2	1/3	M0	Symptomless	NT	-	-
M3	2/3	M3	Symptomless	3/3	M3	Mild
M4	0/3	-	Symptomless	NT	-	-

a Infectivity on Etrog citron and Parson's Special mandarin was determined by Northern Blot hybridization and RT-PCR, 6 months (Etrog citron) and 16 months (Parson's Special mandarin) post-inoculation.
b Progeny was determined by sequencing the RT-PCR amplicons.

One Etrog citron plant infected with M1 and another infected with M3 were each biologically indexed on three cachexia-specific Parson's Special mandarins. Citron plants infected with CVd-IIa-117, X-701-1 or M0 were also bioassayed as controls. Northern blot hybridizations showed that sixteen months after inoculation all the inoculated plants were infected (Fig. 2A and Table 2). RT-PCR analysis and sequencing of the amplicons and individual clones confirmed that the infected plants contained the inoculated variant (Table 2). Eighteen months after inoculation, symptom expression was evaluated by removing the bark of the Parson's Special indicators. Plants infected with M0 developed severe stem pitting at the bud-union and gumming that only affected the Parson's Special scion (Fig. 1A), with pitting extending along the stem and being particularly conspicuous at the branching points (Fig. 1C). The bark presented pegs that corresponded to pits in the wood (Fig. 1B). Two of the three plants infected with M3 only presented extremely mild symptoms at the bud union wood and bark (Fig. 1G, H, J), with the remaining M3-infected plant displaying moderate symptoms that did not extend along the stem as in the case of M0 (Fig. 1I); these enhanced symptoms were associated with a higher viroid titter, as revealed by Northern blot hybridization (Fig. 2B, lane M3-1). Plants infected with M1 were symptomless (Fig. 1D, E, F). Attempts to obtain Parson's Special mandarin plants infected with M2 and M4 were performed by slash inoculating dimeric transcripts but were unsuccessful.

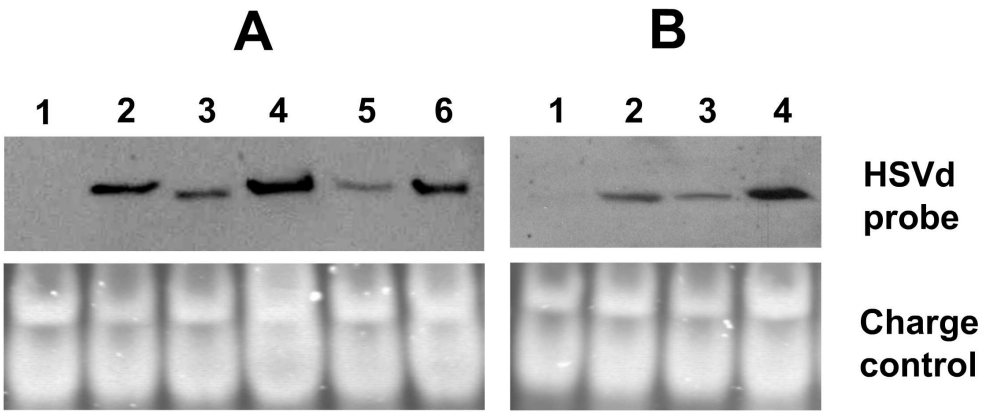

Fig. 2. A) Analysis by sequential PAGE and Northern-blot hybridization with a HSVd-specific probe of representative Parson's Special mandarin plants inoculated with "cachexia" (X-701-1, lane 3) and "non-cachexia" (CVd-IIa-117, lane 2) sequence variants of HSVd, and with the infectious mutants M0 (lane 4), M1 (lane 5) and M3 (lane 6). An RNA preparation from a non-inoculated plant was included as negative control (lane 1). B) Comparison of viroid titers in the three plants (lanes 2, 3 and 4) infected with mutant M3 and analyzed with the same probe (lane 1 corresponds to the non-inoculated control).

Variants of HSVd have been identified in many crops, including vegetable species, pome and stone fruits, grapevine and citrus, and have been phylogenetically classified in five groups (Amari et al., 2001). Variants recovered from citrus appear to fall into a single group (citrus-type). However, based on their biological properties, two types of variants have been identified: those that induce citrus cachexia and those that replicate in cachexia-sensitive hosts but induce no symptoms (Reanwarakorn and Semancik, 1998, Palacio-Bielsa et al., 2004). A "cachexia expression motif" located in the V domain has been shown to be responsible for the induction of cachexia symptoms in sensitive citrus hosts (Reanwarakorn and Semancik, 1998).

In fact, studies addressed to understand the pathogenicity of HSVd as the causal agent of cachexia disease are very scarce due to the limitation of the biological systems available to conduct this type of studies. The study reported here is based on the response of Parson's Special mandarin, the most sensitive citrus host available but a host which requires an incubation period up to 18-month under suitable environmental conditions before reliable results can be achieved. The results here presented confirm the role of the "cachexia expression motif" of HSVd. This motif allows only for limited variation, as revealed by the artificial mutant M3 that has a "cachexia expression motif" with only five nucleotides resulting in only mild symptoms. An identical motif of five nucleotides was identified previously in a natural cachexia isolate (Palacio-Bielsa et al., 2004). Based on field observations and results of routine biological indexing tests, there is a general consensus about the existence of strains of the citrus cachexia agent with different degree of virulence.

The results reported here with mutant M3 provide the first experimental evidence showing that expression of cachexia symptoms can be modulated by a single nucleotide change.

The lack of infectivity of mutant M4, and the poor infectivity of mutant M2 coupled with a reverting progeny, further support the low flexibility of the "cachexia expression motif", in spite of its location in the variable V domain in which many differences have been found in HSVd variants from distinct crops (Amari *et al*., 2001). The insertion into the "cachexia expression motif" of two nucleotides (+A115, +U189) potentially forming a base-pair did not abolish the infectivity, but the resulting variant did not induce symptoms. These two nucleotides, which are present in most non-cachexia inducing variants of HSVd have been found in other variants recovered from citrus (Velázquez et al., 2002), almond (Cañizares et al., 1997), Prunus (Kofalvi et al., 1997) and grapevine (Puchta et al., 1989), thus confirming that their presence or absence does not impair infectivity. Similarly, the U197→C transition responsible for the shift from severe to mild symptoms is also present in different sources of HSVd (Ragozzino et al., 2004; Ito et al., 2006). The changes introduced in the non-infectious mutants M2 and M4, however, have not been reported in natural variants.

Since viroids lack protein-coding capacity, pathogenesis must result from direct interactions between the viroid RNA, or a derivative thereof, and cellular factors affecting the regulation of cellular processes. Most studies on viroid pathogenesis have been undertaken with PSTVd, the type species of the family *Pospiviroidae*, or with *Peach latent mosaic viroid* (Malfitano et al., 2003) and *Chrysanthemum chlorotic viroid* (De la Peña et al., 1999) as representative members of the family *Avsunviroidae*. In PSTVd, sequence variations related to symptom severity have been essentially mapped to the P domain (Schnölzer et al., 1985; Owens et al., 1991, 1995, 1996), but more recent reports have shown that the left and right terminal domains, as well as a single substitution in the loop E within the C domain, also contribute to symptom expression (Sano et al., 1992; Qi and Ding, 2003). The results of the present study confirm that in HSVd, the "cachexia expression motif" located in the V domain, plays a major role in inciting cachexia symptoms, and those subtle changes within this motif affect symptom severity and may even suppress symptom expression.

Although the molecular basis of symptom expression are still unknown, it is generally accepted that viroid induced symptoms are caused by specific interference of host gene expression. In this line, with a macroarray-based approach it has been shown that in tomato, PSTVd alters the regulation of specific genes in a strain dependent manner (Itaya et al., 2002). Therefore, specific interactions between the viroid RNA and cell factors would depend on strain- dependent structural motifs probably affecting the secondary and even tertiary

conformations acquired by the viroid RNA *in vivo*. The effect of structural motifs on putative conformational changes has been suggested in HSVd (Palacio and Duran-Vila, 2000) as well as in the case of the bipartite motif of PSTVd required for trafficking from bundle sheath to mesophyll (Qi et al., 2004). Recently, since the discovery that small interfering RNAs (siRNAs) and microRNAs (miRNAs) can mediate host gene expression, the involvement of gene silencing in viroid pathogenesis has also been proposed (Baulcombe, 2004; Carrington and Ambros, 2003). According with this novel hypothesis that is still controversial, viroid siRNAs acting like endogenous miRNAs, might target host mRNAs and promote their degradation (Papaefthimiou et al., 2001; Martinez de Alba, et al., 2002). This hypothesis is also consistent with the observation that minimal changes, a single nucleotide in the case of HSVd, are sufficient to transform a severe into a mild strain.

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Conclusiones

Conclusiones

Como consecuencia de los trabajos desarrollados en esta tesis doctoral hemos generado un conocimiento que ha sido aportado a la comunidad científica y que puede resumirse en las siguientes conclusiones:

1. Hemos caracterizado molecularmente un nuevo viroide de cítricos que cumple los criterios moleculares establecidos por el Comité de Taxonomía de Virus para ser considerado como una nueva especie del género *Apscaviroide* a la que tentativamente hemos denominado CVd-V.

2. Hemos caracterizado biológicamente a CVd-V, demostrando que este nuevo viroide posee características biológicas propias que lo diferencian de los demás viroides de cítricos conocidos hasta el momento y que es un segundo criterio establecido por el Comité de Taxonomía de Virus para ser considerado como una nueva especie de viroide.

3. Hemos demostrado que CVd-V tiene efectos sinérgicos al ser co-inoculado con viroides de cítricos que pertenecen al género *Apscaviroide.*

4. Hemos demostrado que el efecto sinérgico no se debe a cambios en la acumulación de los viroides ya que no existen diferencias de acumulación en plantas co-inoculadas o inoculadas únicamente con un viroide.

5. Hemos identificado la presencia de CVd-V en España, Estados Unidos, Nepal y el Sultanato de Omán, lo que demuestra que este viroide no ha aparecido recientemente y se encuentra difundido a lo largo del mundo.

6. Hemos caracterizado tres nuevas variantes de CVd-V a partir de las cuales se puede intuir una posible dinámica evolutiva para este viroide.

7. Hemos demostrado que CVd-V puede ser detectado mediante las técnicas de detección de viroides disponibles hasta el momento.

8. Hemos demostrado que CVd-V tiene un amplio rango de huésped en cítricos ya que es capaz de infectar todas las especies que han sido testadas.

9. Hemos sintetizado un viroide quimérico de CVd-V y CDVd al que hemos denominado Ch5 que es capaz de replicarse, traslocarse y ser transmitido en plantas de cidro Etrog.

10. Hemos demostrado que Ch5 no provoca síntomas en plantas de cidro a pesar de acumularse a niveles similares de CVd-V. Este hecho sugiere al dominio terminal izquierdo de CVd-V como un determinante de patogenicidad.

11. Hemos comprobado que la quimera Ch5 queda desplazada por CVd-V o CDVd en plantas de cidro co-infectadas con estos viroides.

12. Hemos demostrado que cinco nucleótidos de HSVd actúan como determinantes de patogenicidad de la enfermedad de la cachexia de los cítricos.

13. Hemos demostrado que un único cambio nucleotídico en HSVd es capaz de modular la expresión de síntomas de cachexia.

14. Hemos demostrado que la modulación de síntomas se debe a cambios en las propiedades biológicas del viroide y no a cambios de su acumulación en la planta.