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Respuesta de distintos genotipos de cítricos y géneros afines a la infección con viroides

Tesis Doctoral

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RESUMEN

Los especies de cítricos y géneros afines pertenecen a la familia *Rutaceae*. Los cítricos son huéspedes naturales de siete viroides, pero solamente *Citrus exocortis viroid* (CEVd) y variantes específicas de *Hop stunt viroid* (HSVd) causan patologías (exocortis y cachexia, respectivamente) en especies sensibles. La mayoría de los cítricos son tolerantes a las infecciones viroidales salvo algunas especies que son sensibles y manifiestan síntomas. En variedades comerciales infectadas, es frecuente encontrar los viroides como infecciones múltiples. El objetivo de este trabajo es estudiar la respuesta de distintos genotipos de cítricos a la infección con viroides.

Se ha caracterizado un aislado de campo que contenía CEVd (clase A), HSVd (variante no patogénica), CBLVd (variante tipo CVd-Ia) y CDVd (variantes de tipos CVd-IIIa y CVd-IIIb), y se ha estudiado su efecto en el comportamiento de árboles de naranjo dulce 'Navelina' y clementino 'Nules', ambos injertados sobre citrange Carrizo y mantenidos en condiciones de campo durante 10 años. Los árboles de ambos cultivares alcanzaron un tamaño inferior y su cosecha fue menor que la de los controles no inoculados. Las características de los frutos también resultaron afectadas: (i) Los frutos de naranjos infectados presentaban un tamaño, índice de madurez y contenido en zumo superiores a los de los controles no infectados; (ii) los frutos de clementinos infectados presentaban un tamaño y relación diámetro/altura mayores que los de los controles no infectados; (iii) En ambos cultivares, la densidad, contenido en sólidos solubles y acidez del zumo eran inferiores a los de los controles no infectados.

Los árboles infectados tenían un sistema radicular poco desarrollado y mediante histología se demostró que sus raicillas contenían menos amiloplastos que las de los controles no inoculados. El efecto de la infección sobre el desarrollo del sistema radicular se comprobó mediante una aproximación *in vitro* que mostró que explantes de citrange Carrizo infectado poseían una menor capacidad rizogénica que los explantes procedentes de plantas no infectadas.

Estudios preliminares mostraron que *Citrus karna* es susceptible a la infección y que los viroides inoculados alcanzan concentraciones elevadas en las plantas infectadas. Al comparar la distribución de los viroides en *C. karna* y en cidro Etrog, se observó que ambos huéspedes contenían títulos elevados en la corteza de brotes jóvenes, lo que confirma no sólo la infección sino su movimiento a larga distancia a

través del floema. Sin embargo, los análisis de hojas mostraron que los títulos alcanzados en *C. karna* son muy inferiores, y a veces incluso imperceptibles, mientras que en cidro Etrog son mucho más elevados. Todos los viroides resultaron indetectables en preparaciones de protoplastos de mesófilo de hoja de *C. karna* mientras que en cidro Etrog se encontraban a títulos elevados, lo que indica que a diferencia de cidro Etrog, en *C. karna* los viroides se encuentran restrigidos al floema.

En estudios dirigido a identificar genotipos resistentes a la infección con viroides, se seleccionaron *Eremocitrus glauca* y *Microcitrus australis,* dos especies de géneros afines a los cítricos que han evolucionado en un habitat inusual. Se ha estudiado la infección, movimiento y acumulación de CEVd, HSVd, CBLVd, CDVd, CBCVd y CVd-V en plantas auto-enraizadas, en las plantas injertadas sobre el patrón limón rugoso y en plantas sobreinjertadas con cidro Etrog. Las plantas se analizaron por hibridación northern y RT-PCR. Los resultados indican que *M. australis* y, en particular *E. glauca*, no son buenos huéspedes de viroides, pero sin embargo ambos genotipos permiten el movimiento a larga distancia tanto hacia el patrón como hacia el cidro Etrog sobreinjertado.

Se han caracterizado distintos aislados recuperados en cultivos comerciales de tres citriculturas diferentes. En Sudán e Irán, dos ejemplos de citriculturas en las que no se han desarrollado programas de saneamiento y certificación, se ha constatado que la presencia de viroides es muy frecuente como infecciones múltiples. Dado que en ambas citriculturas el patrón mayoritario es el naranjo amargo y que se ha detectado el virus de la tristeza de los cítricos (CTV), la presencia de viroides debe tenerse en cuenta a la hora de diseñar estrategias para controlar los daños potenciales de CTV. La clonación y secuenciación de variantes de HSVd de Sudán permitió identificar secuencias de este viroide en el que la composición del motivo de expresión de cachexia no se ajusta al de las variantes descritas anteriormente. En España, a pesar de la implementación de un Programa de Saneamiento y Certificación de cítricos, todavía existen parcelas establecidas con árboles injertados sobre citrange Carrizo que se hallan infectadas con viroides y que por tanto presentan un comportamiento anómalo que incide en su productividad.

Response of several genotypes of citrus and citrus relatives to viroid infection

SUMMARY

Species of citrus and related genera belong to the family *Rutaceae*. Citrus are natural hosts of seven viroids, but only *Citrus exocortis viroid* (CEVd) and specific variants of *Hop stunt viroid* (HSVd) cause diseases (exocortis and cachexia, respectively) in sensitive species. Most citrus are tolerant to viroid infection except some species that are sensitive and develop symptoms. In infected commercial cultivars, viroids are usually found as multiple infections. The aim of this work is to study the response of several citrus genotypes to viroid infection.

A field isolate was characterized and shown to contain CEVd (class A), HSVd (non pathogenic variant), CBLVd (CVd-Ia variant) and CDVd (CVd-IIIa and CVd-IIIb variants). The effect of this isolate on the performance of 'Nules' clementine and 'Navelina' sweet orange trees grafted on Carrizo citrange and maintained during a 10 years in the field was studied. Infection resulted in small trees yielding a crop smaller than that of the non-infected controls. Fruit characteristics were also affected: (i) Sweet orange fruits from infected trees had size, maturity indexes and juice content higher than those from non-infected trees; (ii) clementine fruits from infected trees were larger and presented a diameter/height ratio larger than those from non-infected controls; (iii) in both species, juice density, amount of soluble solids and acidity of fruits from infected trees were lower than those of fruits from non-infected controls. Infected trees also had a poorly developed root system with fibrous roots containing fewer amyloplasts than non-infected trees. The effect of viroid infection on the root system was confirmed through an *in vitro* assay that showed that explants from infected Carrizo citrange had lower rizhogenesis capacity that those of non-infected ones.

Preliminary studies showed that *Citrus karna* was susceptible to viroid infection and that the inoculated viroids reached high concentrations in the infected plants. By comparing the distribution of viroids in *C. karna* and Etrog citron it was found that both hosts contained high viroid concentrations in the bark of young shoots which confirmed not only the infection but their long-distance movement through the phloem. However, analysis of leaves showed that the viroid titters in *C. karna* were very low, and sometimes even imperceptible, whereas in Etrog citron they were much higher. Viroids were undetectable in preparations of mesophyll protoplasts from *C. karna* leaves while

in Etrog citron they were found at high concentrations, indicating that unlike Etrog citron, viroids are phloem restricted in *C. karna*.

In studies addressed to identify genotypes resistant to infection with citrus viroids, *Eremocitrus glauca* and *Microcitrus australis*, two species of citrus related genera were selected because their evolution in an unusual habitat. Infection, movement and accumulation of CEVd, HSVd, CBLVd, CDVd, CBCVd and CVD-V were studied in self-rooted plants, plants grafted on rough lemon and plants top grafted with Etrog citron. The plants were analyzed by northern hybridization and RT-PCR. The results indicated that *M. australis*, and in particular *E. glauca*, are poor viroid hosts, but that both genotypes allowed the long-distance movement of viroids to the rootstock as well as to the top-worked Etrog citron.

Different isolates recovered from commercial cultivars of three different citrus industries have been characterized. In Sudan and Iran, two countries in which sanitation and certification programs have not been developed, viroids were found to be widespread as multiple infections. Since in both citricultures the main rootstock is sour orange and *Citrus tristeza virus* (CTV) has been detected, the presence of viroids should be taken into account when designing strategies to control the damage produced by CTV. Cloning and sequencing of HSVd variants from Sudan led to the identification of sequence variants in which the composition of the cachexia expresion motif differed from the variants described earlier. In Spain, despite the implementation of citrus Sanitation and Certification Program, orchards established with viroid-infected trees grafted on Carrizo citrange can still be found in certain areas in which trees present a poor performance that affects tree size and fruit production.

Resposta de distints genotipus de cítrics i gèneres afins a la infecció amb viroides

RESUM

Les espècies de cítrics i gèneres afins son de la família *Rutaceae*. Els cítrics son hostes naturals de set viroides, però sols *Citrus exocortis viroid* (CEVd) i certes variants de *Hop stunt viroid* (HSVd) causen patologies (exocortis i cachexia, respectivament) en espècies sensibles. La majoria dels cítrics son tolerants a les infeccions viroidals excepte algunes espècies que son sensibles i manifesten símptomes. En les varietats comercials infectades, es freqüent trobar-hi els viroides com a infeccions múltiples. L'objectiu d'aquest treball es estudiar la resposta de distints genotipus de cítrics a la infecció amb viroides.

S'ha caracteritzat un aïllat de camp que contenia CEVd (classe A), HSVd (variant no patogènica), CBLVd (variant tipus CVd-Ia) i CDVd (variants dels tipus CVd-IIIa i CVd-IIIb), i s'ha estudiat el seu efecte en el comportament d'arbres de taronger 'Navelina' i clementí de 'Nules', ambdós empeltats sobre citrange Carrizo i mantinguts en condicions de camp durant 10 anys. Els arbres d'ambdós cultivars assoliren una mida inferior amb una collita que era també inferior a la dels controls no inoculats. Les característiques de la fruita també resultaren afectades: (i) La fruita dels tarongers infectats tenia una mida, índex de maduresa i un contingut en suc superiors als dels controls no infectats; (ii) La fruita dels clementins infectats eren d'una mida i tenien una relació diàmetre/alçada majors que els dels controls no infectats; (iii) En ambdós cultivars, la densitat, el contingut en sòlids solubles i l'acidesa del suc eren inferiors als dels controls no infectats.

Els arbres infectats tenien un sistema radicular poc desenvolupat i per mitjà d'un estudi histològic es va demostrar que les seues arrels tenien molts menys amiloplasts que les dels controls no inoculats. L'efecte de la infecció sobre el desenvolupament del sistema radicular es va comprovar mitjançant una aproximació *in vitro* que va mostrar que les explantes de citrange Carrizo infectat tenien una menor capacitat rizogènica que les explantes procedents de plantes no infectades.

Estudis preliminars mostraren que el *Citrus karna* era susceptible a la infecció i que els viroides inoculats assolien concentracions elevades en les plantes infectades. Al comparar la distribució dels viroides en *C. karna* i en cidre Etrog, es va observar que ambdós hostes contenien títols elevats en l'escorça de brotes joves lo que confirmava no sols la infecció sinó també el moviment a llarga distancia a través del floema. Malgrat tot, els anàlisis de fulles mostraren que els títols assolits en *C. karna* son molt

inferiors, i de vegades imperceptibles, però que en el cidre Etrog son molt mes elevats. Tots els viroides eren indetectables en preparacions de protoplasts de mesòfil de fulla de *C. karna* però que els de cidre Etrog s'hi trovaven a títols elevats, el que indica que a diferencia de cidre Etrog, en el *C. karna* els viroides estan restringits al floema.

En els estudis dirigits a identificar genotipus resistents a la infecció amb viroides, es varen seleccionar l'*Eremocitrus glauca* i el *Microcitrus australis,* dues espècies de gèneres afins als cítrics que han evolucionat en un habitat inusual. S'ha estudiat la infecció, moviment i acumulació de CEVd, HSVd, CBLVd, CDVd, CBCVd i CVd-V en plantes auto-arrelades, en les plantes empeltades sobre en el peu llima rugosa i en plantes sobre-empeltades amb cidre Etrog. Les plantes es van analitzar per hibridació northern i RT-PCR. Els resultats indiquen que *M. australis* i, en particular *E. glauca*, no son bons hostes de viroides, però que ambdós genotipus permeten el moviment a llarga distancia tant cap al peu com cap el cidre Etrog sobre-empeltat.

S'han caracteritzat distints aïllats recuperats de cultius comercials de tres citricultures diferents. Al Sudan i a l'Irán, dos exemples de citricultures en les que no s'han desenvolupat programes de sanejament i certificació, s'ha constatat que els viroides estan molt dispersats com a infeccions múltiples. Ja que en aquestes citricultures el peu majoritari es el taronger bord i que s'hi ha detectat el virus de la tristesa dels cítrics (CTV), la presencia de viroides s'ha de tindre en compte a la hora de dissenyar estratègies per a controlar els danys produïts pel CTV. El clonatje i seqüenciació de variants de HSVd de Sudan ha permès identificar seqüencies d'aquest viroide en les que la composició del motiu d'expressió de cachexia no s'ajusta al de les variants descrites anteriorment. A Espanya, malgrat haver-se implementat un programa de sanejament i certificació de cítrics, encara hi han parcel·les d'arbres empeltats sobre citrange Carrizo que estan infectades amb viroides i que per tant presenten un comportament anòmal que repercuteix en la seua productivitat.

واکنش ژنوتیپ های مرکبات و جنس های نزدیک به آن به آلودگی ویروئیدی

خلاصه

مرکبات و جنس های نزدیک به آن به خانواده *Rutaceae* تعلق دارند. بر اساس مطالعات موجود مرکبات به عنوان میزبان طبیعی هفت ویروئید عمده شناخته شده است که در بین آنها ویروئید اگزوکورتیس مرکبات (CEVd) *Citrus exocortis viroid* و سویه های خاصی از ویروئید کوتولگی رازک *Hop stunt viroid* (HSVd) عامل بیماری های اگزوکورتیس و کاککسیا معرفی شده اند. به جز گونه های حساس، اغلب مرکبات نسبت به آلودگی ویروئیدی متحمل اند و علائمی بروز نمی دهند. در ارقام تجاری آلوده معمولاً ترکیبی از ویروئیدهای مختلف وجود دارد. هدف از این تحقیق، بررسی واکنش ژنوتیپ های مختلف مرکبات و جنس های نزدیک به آن به آلودگی ویروئیدی می باشد.

وجود ویروئیدهای مختلف در جدایه ای مزرعه ای مورد بررسی قرار گرفت و مشخص شد که این جدایه واجد ویروئیدهای CEVd (سویه شدید)، HSVd (سویه غیر بیماریزا)، CBLVd (سویه CVd-Ia) و CDVd (سویه های CVd-IIIa و CVd-IIIb) می باشد. تأثیر این جدایه روی درختان پرتقال Navelina و نارنگی کلمانتین Nules با پایه سیترنج Carrizo از نظر مورفولوژی و خصوصیات کمی و کیفی میوه در شرایط مزرعه پس از طی یک دوره ده ساله مطالعه گردید. در مقایسه با درختان سالم، درختان آلوده در هر دو رقم کوتاهتر و محصول آنها کمتر بوده و خصوصیات میوه نیز در آنها تحت تأثیر قرار گرفت: (1) اندازه، فاکتور رسیدگی و میزان آب میوه در درختان آلوده شده پرتقال بیش از درختان آلوده نشده بود. (2) اندازه و رابطه قطر/ارتفاع در میوه درختان آلوده شده نارنگی بیش از میوه درختان شاهد بود. (3) در هر دو رقم غلظت، میزان جامدات محلول و اسیدیته آب میوه کمتر از میوه درختان آلوده نشده بود.

سیستم ریشه در درختان آلوده توسعه کمتری داشته و بررسی های بافت شناسی نشان داد که ریشه های موئین در درختان آلوده تعداد کمتری آمیلوپلاست در مقایسه با درختان سالم دارند. اثر آلودگی ویروئید روی توسعه سیستم ریشه در شرایط *in vitro* نیز مورد بررسی قرار گرفت و مشخص گردید که توانایی ریشه زایی در گیاهچه های حاصله از کشت بافت سیترنج Carrizo آلوده به ویروئیدهای مرکبات کمتر از درختان سالم بوده است.

مشاهدات اولیه نشان داد که *C.karna* گونه ای حساس به آلودگی ویروئیدی بوده و غلظت ویروئیدها پس از تلقیح در این میزبان می تواند به حد بالایی برسد. در مقایسه پراکنش ویروئیدی بین گیاهان آلوده *C.karna* و اتراگ سیترون، غلظت بالایی از ویروئیدها در پوست شاخه های جوان هر دو میزبان مشاهده شد که این موضوع علاوه بر اثبات آلودگی، حرکت آوندی ویروئیدها را در گیاه تأیید می کند. در عین حال، غلظت ویروئیدها در برگ *C.karna* در مقایسه با برگ اتراگ بسیار کم بوده و در مواردی غیر قابل ردیابی بوده است. به همین صورت تمامی ویروئیدهای تلقیح شده در پروتوپلاست استخراج شده از برگ اتراگ قابل تشخیص بودند در حالیکه آنالیز پروتوپلاست برگ گیاهان آلوده شده *C.karna* منفی بوده است که بیانگر این موضوع است که بر خلاف اتراگ که ویروئیدها تمام گیاه آلوده را در بر می گیرند، آلودگی ویروئیدی در *C.karna* فقط محدود به بافت آبکش می باشد.

بر اساس مطالعات گذشته، *Eremocitrus glauca* و *Microcitrus australis* به عنوان ژنوتیپ های مقاوم به آلودگی ویروئیدی شناسایی شدند. حرکت و تجمع ویروئیدهای مرکبات در گیاهان ریشه دارشده، گیاهان پیوند شده روی پایه راف لمون و گیاهان تاپ ورک شده با اتراگ سیترون، پس از آلوده سازی آنها مورد مطالعه قرار گرفت. این گیاهان از طریق هیبریداسیون و RT-PCR مورد آنالیز قرار گرفتند. نتایج بررسی ها نشان داد اگر چه *M. australis* و خصوصاً *E. glauca* میزبان های مناسبی برای ویروئیدها نیستند ولی در هر دو ژنوتیپ ویروئیدها قابلیت حرکت آوندی دارند بطوریکه می توان آنها را در پایه گیاهان پیوندی و در اتراگ تاپ ورک شده ردیابی نمود.

در این تحقیق همچنین مطالعاتی بر روی چندین جدایه از ارقام تجاری سه منطقه مرکبات کاری مختلف انجام گردید. در سودان و ایران، به عنوان کشورهای فاقد برنامه سالم سازی و گواهی، موارد متعددی از آلودگی ویروئیدی به صورت ترکیبی از ویروئیدهای مختلف مشاهده شد. از آنجا که پایه غالب تکثیرمرکبات در هر دو منطقه نارنج بوده و از طرف دیگربیماری تریستزای مرکبات در هر دو کشور در حال گسترش می باشد، آلودگی ارقام تجاری به ویروئیدهای مرکبات در مدیریت کنترل بیماری تریستزا باید مد نظر قرار گیرد. در اسپانیا نیز با وجود برنامه سالم سازی و گواهی، همچنان می توان در باغات مرکبات با پایه سیترنج درختان آلوده به ویروئیدها را بر اساس علائم ظاهری تشخیص داد.

ÍNDICE

INTRODUCCIÓN

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1. LOS CÍTRICOS

Los cítricos y géneros afines pertenecen al orden *Geraniales*, suborden *Geraninas* y familia *Rutaceas*. Las especies pertenecientes a esta familia tienen cuatro características importantes: (i) presencia de glándulas de aceites esenciales, (ii) ovario apoyado sobre un disco nectarífero situado entre los carpelos y los estambres, (iii) presencia de punteaduras transparentes en las hojas y (iv) frutos con placentación axial (Swingle y Reece, 1967). La familia de las *Rutaceas* comprende seis subfamilias, siendo la de las aurantioideas la que contienen a los cítricos y géneros afines.

La clasificación botánica propuesta por Swingle y Reece (1967) divide la subfamilia *Aurantioidea* en dos tribus, *Clauseneae* y *Citreae*. La tribu *Citreae*, a su vez, se subdivide en tres subtribus, *Triphasiinae*, *Balsamocitrinae* y *Citrinae*. La subtribu *Citrinae* se vuelve a dividir en tres grupos subtribales: cítricos primitivos, árboles cercanos a los cítricos y cítricos verdaderos. Este último grupo subtribal incluye seis géneros, *Fortunella*, *Poncirus*, *Eremocitrus*, *Microcitrus*, *Clymenia* y *Citrus*.

El género *Fortunella* está formado por árboles y arbustos de pequeño tamaño que florecen más tarde que las especies de *Citrus,* lo que les hace moderadamente resistentes a las bajas temperaturas debido a su dormancia invernal. En China, los kumquats (nombre genérico con el cual se hace referencia a todas las especies del género *Fortunella*) se han cultivado para el consumo de sus frutos desde tiempos ancestrales. Las especies del género *Fortunella* hibridan fácilmente con las de *Citrus* y las de algunos géneros afines y han sido utilizados en programas de mejora destinados a la obtención de cítricos resistentes a bajas temperaturas. *Fortunella* spp. son resistentes a cancrosis y *Phytophthora* spp. y las plantas son muy atractivas por lo que pueden ser cultivadas como ornamentales en patios y jardines (Krueger y Navarro, 2007).

Poncirus es un género constituido por una sola especie, *Poncirus trifoliata.* Esta especie es utilizada como patrón de forma casi exclusiva en Japón, Uruguay, Argentina y otras citriculturas del mundo. Sus híbridos con naranjo dulce, los citranges 'Carrizo' y 'Troyer' (*C. sinensis* x *P. trifoliata*) se emplean también como patrones ya que dan lugar a combinaciones patron/variedad tolerantes al decaimiento causado por CTV (*Citrus tristeza virus*), y son los principales patrones utilizados en España. El citrumelo (*C. paradisi* x *P. trifoliata*) es otro patrón utilizado en algunas zonas de cultivo y se emplea como parental en programas de mejora genética ya que es resistente al

nematodo de los cítricos (*Tylenchulus semipenetrans*), *P. parasitica, P. citrophthora*, CTV y tolera las bajas temperaturas.

Eremocitrus es un género monoespecífico (*Eremocitrus glauca*) compatible sexualmente con *Citrus* y otros géneros afines. *E. glauca* es una especie xerofítica, por lo que es tolerante a la sequía, a altas temperaturas y a elevadas concentraciones salinas. Además, es tolerante al boro y a la pudrición de las raíces causada por *Phytophthora* spp. (Krueger y Navarro, 2007).

El género *Microcitrus* está constituido por seis especies sin valor comercial destacado: *Microcitrus australasica*, *M. australis*, *M. garrowayi*, *M. inodora*, *M. maideniana* y *M. warburgiana*. El prefijo 'micro' alude al reducido tamaño de las hojas, flores y frutos en comparación con las especies de *Citrus*. Las especies de este género se han descrito como resistente a nematodos y *Phytophthora* spp. y tolerantes a la sequía. El género *Microcitrus* hibrida fácilmente con *Citrus*, *Fortunella* y otros géneros afines (Krueger y Navarro, 2007).

Clymenia es el género más primitivo del grupo subtribal de cítricos verdaderos y es el que muestra mayores diferencias morfológicas respecto a *Citrus*, sobre todo en el tipo de hojas y las vesículas de zumo.

El género *Citrus*, al cual pertenecen casi la totalidad de las especies cultivadas de cítricos, se originó probablemente en el sudeste asiático y China hace unos 20 millones de años (Iwamasa, 1988). Las principales especies cultivadas son naranjo dulce (*C. sinensis*), mandarino (*C. reticulata*), limonero (*C. limon*), lima (*C.aurantifolia*), pomelo (*C. paradisi*), pummelo (*C. grandis*) y naranjo amargo (*C. aurantium*) (Swingle y Reece, 1967).

Los cítricos, con una producción mundial aproximada de 117 millones de toneladas al año, constituyen el cultivo frutal de mayor importancia en el mundo. Los principales países productores, de mayor a menor, son: China, Brasil, EEUU, México, India, España e Irán (FAO, 2007). Son cultivados en regiones tropicales y subtropicales con una amplia gama de condiciones climáticas. Los cítricos en general, y en particular las especies y variedades comerciales, están sujetos a importantes estreses abióticos y bióticos. Entre estos últimos destacan los causados por agentes patógenos como hongos, bacterias, micoplasmas, fitoplasmas, virus y viroides (Peña y Navarro, 1999). Los viroides son los agentes causales de dos enfermedades de importancia económica, exocortis y cachexia, causadas por el viroide de la exocortis de los cítricos (*Citrus exocortis viroid,* CEVd) y el viroide del enanismo del lúpulo (*Hop stunt viroid,* HSVd), respectivamente. La mayoría de especies comerciales se comportan como portadores asintomáticos frente a la infección con viroides. Sin embargo, poco se conoce acerca de la susceptibilidad, sensibilidad y tolerancia de

muchas especies frente a los demás viroides descritos en cítricos, algunos de los cuales están asociados a síntomas y/o a distintos grados de enanismo en naranjos y clementinos injertados en el patrón *P. trifoliata* (Vernière *et al.,* 2002, 2004 y 2006; Semancik *et al.,* 1997; Roistacher *et al.,* 1993; Duran-Vila y Semancik, 2003).

2. LOS VIROIDES

Los viroides son moléculas de RNA monocatenario covalentemente cerradas sin capacidad codificante, pero capaces de replicarse de forma autónoma utilizando la maquinaria transcripcional de las células a las que parasitan. Éstos constituyen la forma más extrema de parasitismo en el mundo vegetal. Estos agentes fitopatógenos infecciosos son entidades sencillas con propiedades estructurales, funcionales y evolutivas propias.

El tamaño de los viroides oscila entre 246 y 399 nucleótidos, correspondientes respectivamente al viroide del Cadang-cadang del cocotero (*Coconut cadang-cadang viroid,* CCCVd) y al viroide del moteado clorótico del crisantemo (*Chrysanthemum chlorotic mottle viroid,* CChMVd), aunque se han detectado algunas variantes de tamaño superior (Flores *et al.,* 2003) (Tabla 1).

Los viroides fueron descubiertos en los primeros años de la década de 1970 cuando se identificaron y caracterizaron los agentes causales de dos enfermedades de importancia económica, el viroide del tubérculo fusiforme de la patata (*Potato spindle tuber viroid,* PSTVd) (Diener, 1971) y el CEVd (Semancik y Weathers, 1972). Inicialmente se pensó que estas enfermedades, transmisibles por injerto, tenían una etiología viral; sin embargo, los análisis posteriores revelaron que los agentes infecciosos presentaban propiedades bien distintas y se propuso el término “viroide” para designar a esta nueva clase de agentes fitopatógenos subvirales con propiedades físicas y químicas diferentes a las de los virus conocidos hasta entonces.

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 infectivo como la replicación, el movimiento o la encapsidación para eludir los sistemas de defensa de la planta.

El estudio de los viroides tiene dos 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 y hasta hoy se conocen una treintena de viroides, la mayoría de ellos asociados a algún tipo de patología (Tabla 1).

Desde esta perspectiva se abordan aspectos prácticos como la búsqueda de variedades y especies tolerantes o resistentes, la puesta a punto de técnicas de detección y diagnóstico o el desarrollo de programas de certificación de plantas libres de viroides. Por otro lado, se estudian aspectos básicos de la biología de los viroides, como la replicación, el movimiento, o las interacciones con sus huéspedes. Los viroides son las entidades biológicas más simples y de menor genoma conocidas hasta el momento y su capacidad para interactuar con el huésped es un magnifico modelo de estudio para la comprensión de las bases moleculares que rigen los mecanismos celulares.

2.1. Clasificación taxonómica y estructuras de los viroides

El primer viroide secuenciado fue el PSTVd, confirmándose su naturaleza circular previamente observada al microscopio electrónico (Sänger *et al.,* 1976). Desde entonces se han caracterizado biológica y molecularmente alrededor de treinta especies de viroides (Tabla 1). Los viroides se clasifican según la normativa establecida por el Comité Internacional de Taxonomía de Virus (ICTV) (Flores *et al*., 2005) que sigue un criterio de clasificación en el que se diferencian familias, géneros y especies.

Los análisis filogenéticos elaborados a partir de la secuencia completa de los viroides y de los motivos conservados de los mismos (Elena *et al.,* 2001) han llevado a clasificar los viroides en dos grandes familias: *Pospiviroidae* y *Avsunviroidae* (Tabla 1). La especie tipo de la primera familia es el PSTVd (Gross *et al.,* 1978) y los distintos miembros que la componen se caracterizan por compartir una serie de motivos de secuencia y estructura conservados, que incluyen la región central conservada ('central conserved region', CCR), la región terminal conservada ('terminal conserved region', TCR) y la horquilla terminal conservada ('terminal conserved hairpin', TCH) (Fig. 1), por replicarse y acumularse en el núcleo, y por la ausencia de ribozimas de cabeza de martillo. La replicación de los viroides de esta familia sigue el modelo asimétrico del círculo rodante (Fig. 2). La secuencia de la CCR, y la presencia o ausencia de TCR y TCH, permiten clasificar a los miembros de esta familia en cinco géneros (Tabla 1).

La especie tipo de la segunda familia es el viroide del manchado solar del aguacate (*Avocado sunblotch viroid*, ASBVd) (Symons, 1981) que, junto a los otros tres miembros que la integran, carece de motivos conservados, se replica y acumula en el cloroplasto, y sus RNAs de ambas polaridades se autocortan por medio de ribozimas de cabeza de martillo (Flores *et al*., 2005). La replicación de los viroides de esta familia sigue el modelo simétrico del círculo rodante (Fig. 2). Dentro de la familia

Avsunviroidae, los viroides se agrupan en base al contenido en bases guanina (G) y citosina (C), la estructura de mínima energía libre y las propiedades de su actividad ribozimática.

Tabla 1. Clasificación de los viroides caracterizados molecularmente según la ICTV (adaptada de Flores *et al.,* 2005).

Famila	Género	Especie[1]	Tamaño	N. acceso
Pospiviroidae	*Pospiviroide*	PSTVd (*viroide del tubérculo fusiforme de la patata*)	359 nt	V01465
		TCDVd (*viroide del enanismo clorótico del tomate*)	360 nt	AF162131
		MPVd (*viroide de la papita mexicana*)	360 nt	L78454
		TPMVd (*viroide de la planta macho del tomate*)	360 nt	K00817
		CEVd (*viroide de la exocortis de los cítricos*)	371 nt	M34917
		CSVd (*viroide del enanismo del crisantemo*)	356 nt	V01107
		TASVd (*viroide del enanismo apical del tomate*)	360 nt	K00818
		IrVd (*viroide de la iresine*)	370 nt	X95734
		CLVd (*viroide latente de la Columnea*)	370 nt	X15663
	Hostuviroide	HSVd (*viroide del enanismo del lúpulo*)	297 nt	X00009
	Cocadviroide	CCCVd (*viroide del cadang-cadang del cocotero*)	246 nt	J02049
		CTiVd (*viroide del tinangaja del cocotero*)	254 nt	M20731
		HLVd (*viroide latente del lúpulo*)	256 nt	X07397
		CBCVd (*viroide de de la corteza agrietada de los cítricos*)	284 nt	X14638
	Apscaviroide[2]	ASSVd (*viroide de la piel cicatrizada de la manzana*)	329 nt	M36646
		CDVd (*viroide del enanismo de los cítricos*)	294 nt	AF184147
		ADFVd (*viroide del fruto picado del manzano*)	306 nt	X99487
		GYSVd1 (*viroide 1 del moteado amarillo de la viña*)	367 nt	X06904
		GYSVd2 (*viroide 2 del moteado amarillo de la viña*)	363 nt	J04348
		CBLVd (*viroide de la hoja curvada de los cítricos*)	318 nt	M74065
		PBCVd (*viroide del chancro pustuloso del peral*)	315 nt	D12823
		AGVd (*viroide australiano de la viña*)	369 nt	X17101
	Coleviroide	CbVd1 (*viroide 1 de Coleus blumei*)	248 nt	X52960
		CbVd2 (*viroide 2 de Coleus blumei*)	301 nt	X95365
		CbVd3 (*viroide 3 de Coleus blumei*)	361 nt	X95364
Avsunviroidae	*Avsunviroide*	ASBVd (*viroide del manchado solar del aguacate*)	247 nt	J02020
	Pelamoviroide	PLMVd (*viroide del mosaico latente del melocotonero*)	337 nt	M83545
		CChMVd (*viroide del moteado clorótico del crisantemo*)	399 nt	Y14700
	Elaviroide	ELVd (*viroide latente de la berenjena*)	333 nt	AJ536613

[1] Las especies subrayadas corresponden a las especies tipo.
[2] Otros dos viroides de los cítricos, el viroide OS (CVd-OS, Ito *et al*., 2001) y el viroide V (CVd-V, Serra *et al*., 2008a) han sido propuestos como nuevos miembros del género *Apscaviroide*.

Las secuencias de mayoría de los viroides tienen una alta proporción (más del 50% del total de nucleótidos) de citosina y guanina. Esta propiedad les permite adoptar conformaciones estructurales muy estables originadas por la auto-complementariedad entre segmentos de sus genomas, lo que les otorga algunas características moleculares propias de las moléculas bicatenarias.

Los viroides no tienen capacidad de codificar proteínas y lógicamente sus funciones biológicas dependen de su propia secuencia que determina la conformación del RNA y/o de la adopción de determinadas estructuras secundarias. Mediante cálculos termodinámicos se ha determinado la estructura secundaria de mínima energía libre que pueden adoptar los viroides en condiciones no desnaturalizantes *in vitro*. La conformación más estable propuesta para la mayoría de los miembros de la familia *Pospiviroidae* es de tipo varilla (Fig. 1), que es la que se observó al microscopio electrónico con preparaciones purificadas del PSTVd (Sänger *et al*., 1976), aunque también se han descrito otras conformaciones posibles de cuasi-varilla (Steger y Riesner, 2003). En estas conformaciones de varilla o cuasi-varilla, formadas como consecuencia de la gran autocomplementariedad de las secuencias viroidales, se encuentran tramos de doble hélice que alternan con pequeños bucles de bases desapareadas (Fig. 1).

Este tipo de estructuras secundarias fueron inicialmente propuestas en base a análisis realizados *in vitro* con el PSTVd utilizando nucleasas específicas de cadena simple y la modificación con bisulfito para detectar las zonas desapareadas de la molécula (Gross *et al.,* 1978). Ello fue posteriormente apoyado por los resultados de otros análisis de tipo *in silico* realizados mediante programas teóricos de cálculo de las conformaciones termodinámicamente más estables (Zuker, 1989). Hoy, una vez determinada la secuencia de un viroide, se puede obtener la estructura secundaria de mínima energía libre mediante programas informáticas que calculan la conformación termodinámica más estable en unas condiciones estandarizadas. Por lo tanto, aunque la determinación teórica de estas estructuras no tiene por qué reflejar necesariamente lo que sucede *in vivo,* estos datos experimentales indican que los viroides pueden realmente adoptar, e incluso preservar, estas conformaciones.

En 1985, Keese y Symons propusieron un modelo que divide la estructura de varilla de los viroides de la familia *Pospoviroidae* en cinco dominios (Fig. 1). En este modelo se distinguen un dominio central (C) flanqueado por los dominios patogénico (P) y variable (V), y dos dominios terminales derecho (T_R) e izquierdo (T_L) situados en los extremos de la molécula. En un principio se trató de asignar a estos dominios una implicación funcional concreta, aunque estudios posteriores indican la correlación estructura-función es mucho más compleja (Ding y Itaya, 2007).

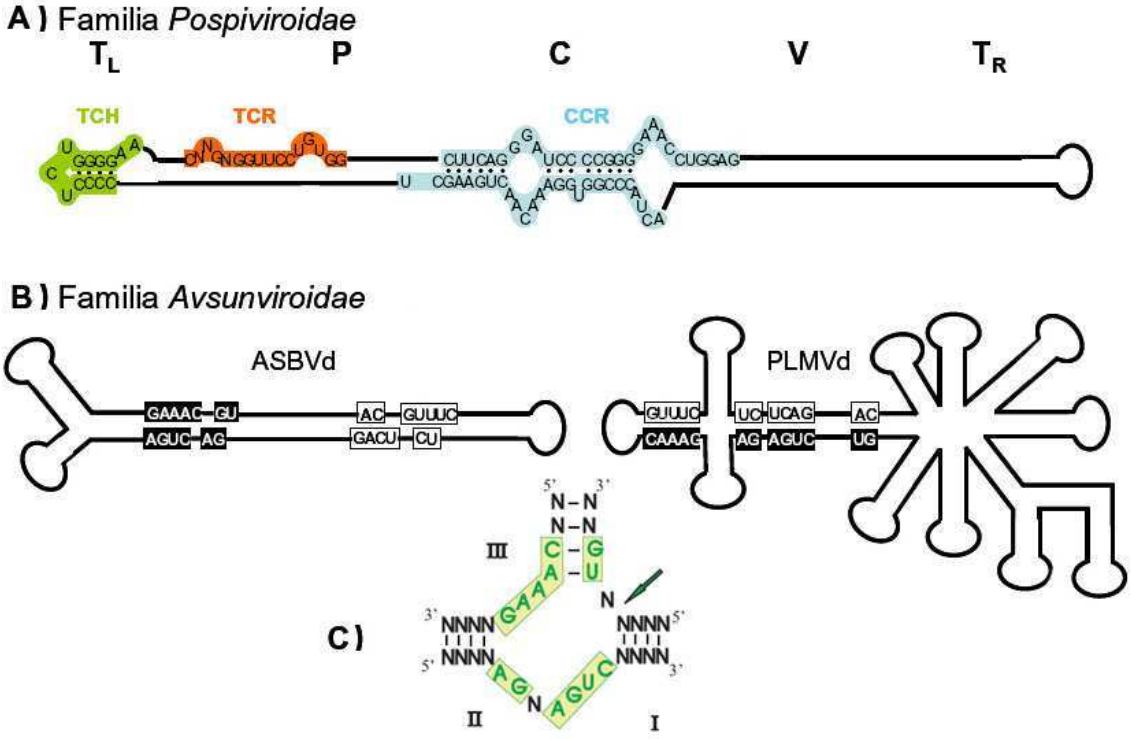

Fig. 1. Estructura de los viroides. (A) Representación esquemática de la estructura secundaria de tipo varilla propuesta para los miembros de la familia *Pospiviroidae*. La localización aproximada de los dominios C, P, V, T_L y T_R se indica en la parte superior de la figura. Los nucleótidos que forman las regiones conservadas CCR, TCR y TCH se representan en negro sobre un fondo azul, naranja y verde, respectivamente. N indica cualquier nucleótido. (B) Representación esquemática de las estructuras secundarias de tipo cuasi-varilla y ramificada del ASBVd y del PLMVd, respectivamente, de la familia *Avsunviroidae*. Los nucleótidos estricta o altamente conservados en las ribozimas de cabeza de martillo naturales se representan en recuadros con fondo negro y blanco para las polaridades positiva y negativa respectivamente. (C) Consenso de la estructura de cabeza de martillo con nucleótidos conservados se indicada en las casillas. N indica los nucleótidos no conservados. El núcleo central está flanqueado por tres hélices, I, II y III. El sitio de auto-corte está indicado por una flecha (adaptado de Flores *et al.,* 2005 y Góra-Sochacka, 2004).

Como se ha mencionado anteriormente, los viroides de la familia *Pospiviroidae* se caracterizan por la existencia de una serie de motivos de secuencia conservados que se localizan en el dominio C y en los dominios terminales. El motivo conservado más estudiado es la CCR que consiste en dos series de nucleótidos conservados en ambas hebras del dominio C (Fig. 1). La serie de nucleótidos de la hebra superior consta de una zona central flanqueada por dos repeticiones invertidas o palindrómicas. Existen cinco tipos de CCR en función de las series nucleotídicas conservadas y es por ello que esta información se utiliza como criterio de clasificación de viroides (Koltunow y Rezaian, 1988). Este motivo además parece estar involucrado en el ciclo replicativo de los viroides (Schrader *et al.,* 2003).

Localizados en el dominio T_L se encuentran la TCR y la TCH. La TCR es un motivo conservado de 13 a 16 nucleótidos localizados en la hebra superior de este

dominio (Flores *et al.*, 1997) mientras que la TCH compuesta por 13 nucleótidos, forma un elemento de estructura secundaria en forma de horquilla en el extremo del dominio T_L (Flores *et al.,* 1997). La función de dichas regiones se desconoce hasta el momento.

Dentro de la familia *Avsunviroidae*, el ASBVd y el viroide latente de la berenjena (*Eggplant latent viroid*, ELVd) adoptan estructuras secundarias de cuasi-varilla (Fig. 1) (Symons, 1981; Fadda *et al*., 2003). Sin embargo, el viroide del mosaico latente del melocotonero (*Peach latent mosaic viroid*, PLMVd) y el CChMVd presentan una conformación ramificada (Hernández y Flores, 1992; Navarro y Flores, 1997) (Fig. 1).

Los viroides pertenecientes a la familia *Avsunviroidae* no poseen ninguno de los motivos conservados mencionados, pero en cambio se caracterizan por presentar estructuras secundarias metaestables en forma de cabeza de martillo llamadas estructuras ribozimáticas que les confieren capacidad de autocorte. Se ha demostrado que estas estructuras ribozimáticas son capaces de catalizar una reacción de autocorte *in vitro* en las cadenas de ambas polaridades del RNA viroidal (Hutchins *et al.,* 1986; Forster *et al.,* 1988; Hernández y Flores, 1992; Navarro y Flores, 1997; Fadda *et al.,* 2003). Estas ribozimas están constituidas por tres hélices, denominadas I, II y III, que se disponen alrededor de una región central de cadena simple donde se sitúan una serie de nucleótidos de secuencia conservada que, junto con el nucleótido que precede al sitio de corte, forman un bolsillo catalítico. El centro catalítico posee 13 bases distribuidas en cuatro segmentos y las hélices pueden estar abiertas o cerradas por bucles terminales (Fig. 1). En la mayoría de los casos, los cambios de secuencia observados en variantes naturales son compensatorios o se encuentran en los bucles, lo que apoya la función de dichos ribozimas (Navarro y Flores, 1997; Ambrós *et al.,* 1998). Los trabajos realizados con CChMVd han mostrado la implicación de determinadas secuencias periféricas a la estructura ribozimática sobre el incremento de la actividad catalítica (De la Peña *et al.,* 2003; Khvorova *et al.,* 2003).

2.2. Ciclo infectivo de los viroides

A partir de mínimas cantidades de inóculo, una vez transcurrido el periodo de incubación necesario, los viroides son detectables en tejidos distales de la planta. Este hecho conlleva que en este periodo de tiempo el viroide se multiplica, se mueve y se acumula en el huésped. Para alcanzar una infección sistémica los viroides deben superar las distintas etapas de su ciclo infectivo. En primer lugar, el viroide debe introducirse en la célula huésped y localizarse en el orgánulo subcelular específico para su replicación y así, utilizar la maquinaria replicativa celular para multiplicarse. La nueva progenie debe salir al citoplasma e invadir las células vecinas mediante un movimiento denominado ‘’movimiento célula a célula’’ hasta alcanzar el sistema

vascular y así, difundirse a larga distancia para finalmente invadir otros tejidos no vasculares.

Mientras que los virus codifican proteínas que intervienen en distintas etapas de su ciclo como la replicación y el movimiento, el ciclo infectivo de los viroides está exclusivamente mediado por factores del huésped. A pesar que el genoma de los viroides no codifica proteínas, su estructura primaria y secundaria determinan motivos moleculares que le otorgan la capacidad de interactuar con la maquinaria celular del huésped y así llevar a cabo todas las funciones necesarias de su ciclo.

2.2.1. Localización subcelular y replicación de los viroides

Un criterio importante en la clasificación de los viroides es el sitio donde se replican y se acumulan en la célula del huésped. Como se ha mencionado antes, la replicación y acumulación de los viroides de las familias *Pospiviroidae* y *Avsunviroidae* tiene lugar en el núcleo y en el cloroplasto, respectivamente.

Los estudios dirigidos a determinar la localización de los viroides dentro de la célula se han llevado a cabo utilizando principalmente técnicas de fraccionamiento subcelular mediante centrifugación, hibridación *in situ* y microscopía electrónica. A partir de los resultados obtenidos se estableció que algunos viroides se localizaban en el núcleo mientras que otros lo hacían en el cloroplasto.

Los viroides de la familia *Pospiviroidae*, se localizaron en el núcleo de las células infectadas (Harders *et al.*, 1989; Bonfiglioli *et al.*, 1994 y 1996; Qi y Ding, 2003). Entre los viroides de esta familia se observaron ciertas diferencias, ya que mientras el CEVd parece distribuirse esencialmente en el núcleo en general (Bonfiglioli *et al.*, 1996), el CCCVd y el PSTVd parece que se concentran en el nucleolo (Bonfiglioli *et al.*, 1994 y 1996; Schumacher *et al.*, 1983). Además, en este orgánulo, la molécula viroidal se presenta en diferentes formas, siendo la más abundante la unidad monomérica de polaridad positiva (Branch y Robertson, 1984). También se encuentran, aunque en menor cantidad, oligómeros de polaridad negativa (Grill y Semancik, 1978). Estudios posteriores apuntan que ambos tipos de moléculas se localizan en el nucleoplasma mientras que las de polaridad positiva se acumulan en mayor medida en el núcleo (Qi y Ding, 2003).

Por otro lado, los miembros de la familia *Avsunviroidae* no se encuentran en el núcleo (Marcos y Flores, 1992) sino en el cloroplasto, donde se han identificado 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.,* 1999). También se han detectado bajos niveles de viroides en el

citoplasma (Lima *et al.*, 1994), pero sin embargo no han sido asociados a otros orgánulos (Marcos y Flores, 1992).

Aunque la acumulación de los viroides en un determinado orgánulo no significa que necesariamente su replicación tenga lugar en ese mismo lugar, la localización de cadenas de polaridad negativa, que se sintetizan durante la replicación, apuntan a que el PSTVd se replica en el núcleo (Spiesmacher *et al.*, 1983) y el ASBVd y el PLMVd lo hacen en el cloroplasto (Bonfiglioli *et al.*, 1994; Navarro *et al.*, 1999; Bussière *et al.*, 1999).

Los mecanismos propuestos para la replicación de los viroides se basan en el modelo del círculo rodante (Fig. 2) debido, por una parte, a su naturaleza circular y, por otra, a la presencia y características de los RNAs identificados en tejidos infectados (Owens y Diener, 1982; Branch y Robertson, 1984; Hutchins *et al.*, 1985). El RNA circular monomérico infectivo más abundante se designa como de polaridad positiva (+), siendo el RNA de polaridad negativa (-) el intermediario replicativo de cadena complementaria. El viroide de polaridad positiva (+) sirve como molde y es transcrito por una RNA polimerasa como un oligómero de polaridad negativa (-). Éste a su vez sirve de molde para la síntesis de nuevas cadenas (+), que después de ser cortadas y ligadas mediante ribonucleasas y RNA ligasas, dan lugar al producto final del ciclo rodante, un monómero (+) circular de RNA.

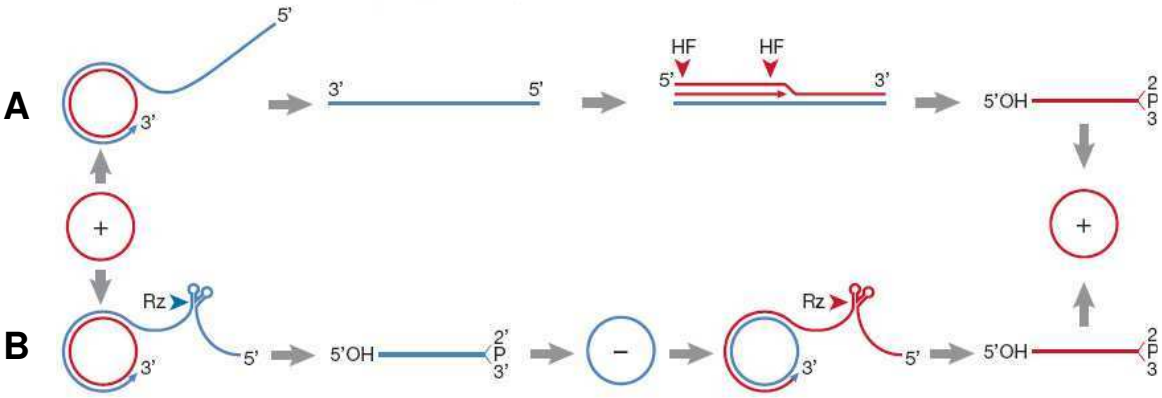

Fig. 2. Mecanismo del círculo rodante propuesto para la replicación de los viroides. (A) La variante asimétrica con un único círculo rodante seguido por los miembros de la familia *Pospiviroidae* en el núcleo, donde una RNasa y una RNA ligasa del huésped median el corte de los intermediarios oligoméricos y la ligación de los monómeros resultantes. (B) La variante simétrica con dos círculos rodantes seguida por los miembros de la familia *Avsunviroidae* en el cloroplasto, donde los oligómeros viroidales se autocortan mediante ribozimas generándose RNAs lineales de longitud completa con extremos 5'-OH y 2',3'-fosfodiéster que presumiblemente son ligados por una RNA ligasa del huésped o autocatalíticamente. Las líneas rojas y azules se refieren a las cadenas positivas y negativas, respectivamente. Las flechas indican los sitios de corte mediante un factor del huésped (HF) o las ribozimas (RZ) (adaptado de Daròs *et al.*, 2006).

Se han propuesto dos variantes del modelo del círculo rodante, la simétrica y la asimétrica (Fig. 2). En la variante simétrica, los oligómeros (-) son procesados y ligados, obteniéndose monómeros circulares (-) que actúan como molde en un segundo círculo rodante por el que se producen monómeros circulares de polaridad positiva (+). De esta forma, si se detectan monómeros circulares de polaridad (-) se puede afirmar que el viroide se replica siguiendo la variante simétrica. En el caso de la variante asimétrica, los oligómeros de polaridad negativa (-) sirven como molde para la síntesis de oligómeros de polaridad positiva (+), que se procesan posteriormente en moléculas monoméricas lineales y después de ser ligadas dan lugar a las moléculas circulares maduras de polaridad positiva.

Los estudios realizados al respecto han permitido determinar que los viroides de la familia *Pospiviroidae* se replican siguiendo la variante asimétrica, mientras que los viroides de la familia *Avsunviroidae* lo hacen siguiendo la variante simétrica (Branch y Robertson, 1984; Daròs *et al.,* 2006). Los mecanismos implicados en la replicación de los viroides requieren de la actividad de tres tipos de enzimas: una RNA polimerasa que sintetice las moléculas oligoméricas, una RNasa que genere los monómeros viroidales y una RNA ligasa que los circularice.

La RNA polimerasa II es una RNA polimerasa dependiente de DNA y ha sido identificada como la responsable de la transcripción de los viroides de la familia *Pospiviroidae*. Esta afirmación se estableció tras ensayos realizados con distintos inhibidores de polimerasas en PSTVd (Mühlbach y Sänger, 1979; Schnidler y Mühlbach, 1992), así como de HSVd y CEVd (revisado por Ding e Itaya, 2007). Los trabajos realizados con viroides de la familia *Avsunviroidae* mostraron que éstos se replican mediante una RNA polimerasa dependiente de DNA y diferente a la RNA polimerasa II, y se ha propuesto que una polimerasa tipo NEP (Nuclear-encoded polymerase) que es una enzima codificada en el núcleo pero con actividad en el cloroplasto sea la responsable de la transcripción de estos viroides (Delgado *et al.,* 2005). Sin embargo, no puede descártarse la posibilidad que sean las polimerasas codificadas en el cloroplasto (PEP) las implicadas en la replicación de viroides de esta familia. Apuntando a esta última hipótesis, estudios *in vitro* han mostrado que las RNA polimerasas de *Escherichia coli*, enzimas más próximas a las polimerasas codificadas por el genoma cloroplástico que a las nucleares, son capaces de transcribir el PLMVd (Pechalt *et al*., 2002).

La replicación de los RNAs viroidales conlleva la intervención de otras dos enzimas. La primera es una RNasa capaz de procesar los oligómeros y generar las correspondientes formas monoméricas lineales. El procesamiento de oligómeros de polaridad positiva en viroides de la familia *Pospiviroidae* parece estar mediado por

enzimas del huésped. En el PSTVd se ha propuesto la existencia de una estructura específica del oligómero viroidal que sería reconocida por una RNasa nuclear que determinaría un corte específico sobre el mismo (Tsagris *et al*., 1991; Baumstark *et al*., 1997). En el caso de los miembros de la familia *Avsunviroidae*, la actividad RNAsa no parece estar mediada por factores del huésped ya que puede realizarse mediante la actividad de los ribozimas en forma de cabeza de martillo que pueden formar las moléculas oligoméricas de ambas polaridades (Hutchins *et al*., 1986; Hernández y Flores, 1992; Navarro y Flores, 1997; Darós *et al*., 2006). En plantas infectadas con ASBVd se ha identificado una proteína del cloroplasto, la PARBP 33, que se puede unir al viroide y facilitar su autocorte *in vitro* (Darós y Flores, 2002).

La otra actividad enzimática implicada es una RNA ligasa que cataliza la circularización de los monómeros lineales generados en la etapa anterior. 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). Este tipo de actividad también se da en plantas transgénicas de *Arabidopsis thaliana* que expresan dímeros de secuencias de viroides representativos de los distintos géneros de la familia *Pospiviroidae.* A pesar de que esta planta no es huésped de los viroides, la identificación 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 una enzima distinto a las tRNA ligasas (Gas *et al*., 2007). En los viroides de la familia *Avsunviroidae,* la actividad ligasa puede ser autocatalítica ya que *in vitro* los monómeros del PLMVd provenientes del autocorte ribozimático son capaces de circularizarse en ausencia de proteínas. Sin embargo, no puede descartarse que esta actividad ligasa esté mediada por algún factor cloroplástico del huésped (Côté y Perreault, 1997).

2.2.2. Movimiento de los viroides

Una infección sistémica implica el desplazamiento del viroide a lo largo de toda la planta. En este desplazamiento se pueden distinguir tres tipos de movimientos; el movimiento intracelular, el movimiento intercelular y el movimiento a larga distancia (Fig. 3). A pesar de que con estos tipos de movimiento el viroide podría acceder a cualquier tejido del huésped, se han identificado tejidos excluidos de la infección así como barreras que inhiben o regulan estratégicamente el tráfico viroidal en algunos puntos de la planta.

Movimiento intracelular

La localización de los viroides en orgánulos celulares y la capacidad que tienen para invadir células vecinas indican que existe un desplazamiento intracelular. Los mecanismos que rigen este tipo de desplazamiento están muy poco elucidados. Se han realizado estudios acerca del mecanismo de entrada del PSTVd al núcleo mediante protoplastos permeabilizados, en los cuales se observó que dicho proceso parecía ser específico y mediado por factores del huésped no identificados (Woo *et al*., 1999). Por otro lado, el proceso involucrado en la exportación de los viroides en el núcleo es por el momento desconocido (revisado por Ding, 2009). Los viroides de la familia *Avsunviroidae*, se acumulan y replican en el cloroplasto. No se conoce si son capaces de replicarse en otros tipos de plastidios, como protoplastidios, amiloplastos, etioplastos y cromoplastos. Los mecanismos de entrada y salida a este orgánulo no han sido esclarecidos hasta el momento (Ding e Itaya, 2007).

Es probable que el tráfico viroidal en el citoplasma esté también mediado por factores del huésped. En el citoplasma el viroide debe coexistir con RNAsas y mecanismos de silenciamiento post-transcripcional que pueden degradarlo. La unión del viroide con proteínas del huésped podría influir tanto en el movimiento como en la protección del viroide frente a este tipo de ataques (Ding, 2009).

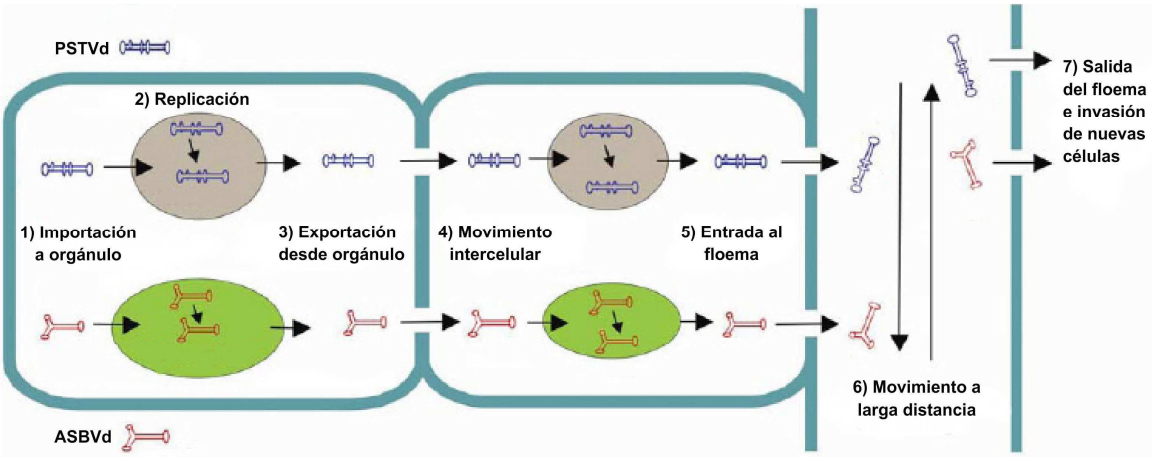

Fig. 3. Distintas etapas de la infección sistémica del PSTVd y ASBVd, las especies tipo de las familias *Pospiviroidae* y *Avsunviroidae*, respectivamente (adaptado de Ding e Itaya, 2007).

Movimiento intercelular

Una vez infectadas las primeras células, el viroide debe colonizar las células adyacentes antes de llegar a partes distales de la planta. Tanto los virus como los 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 del PSTVd marcados con fluorescencia sugieren que los viroides también utilizan estas vías al transitar entre células (Ding *et al*., 1997).

Mientras que los virus pueden codificar una o varias proteínas de movimiento capaces de interactuar con factores celulares para mediar su desplazamiento intercelular, el movimiento de los viroides probablemente dependa de otro tipo de mecanismos. Es probable que los viroides se unan a algún factor del huésped para utilizarlo como medio de transporte (Ding, 2009).

No todas las uniones entre células son iguales ya que 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 el PSTVd queda excluido de los ápices meristemáticos de plantas infectadas de tomate y *Nicotiana benthamiana* (Zhu *et al*., 2001), mientras que el PLMVd sí que parece invadir células muy cercanas a dichos ápices meristemáticos de plantas de melocotonero infectadas (Rodio *et al*., 2007). Estas observaciones sugieren que existen distintos mecanismos que rigen esta clase de movimiento en los miembros de las familias *Pospiviroidae* y *Avsunviroidae.*

Movimiento a larga distancia

El tráfico entre el mesófilo y el sistema vascular se encuentra también regulado. Se han identificado motivos de secuencia en el PSTVd que comprometen diferencialmente su entrada y salida del floema. Los resultados disponibles 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).

El PSTVd se distribuye en la planta siguiendo las mismas pautas que el transporte de fotoasimilados, lo que sugiere que el viroide se transporta a larga distancia a través del floema (Palukaitis, 1987). Dichas observaciones se confirmaron mediante hibridaciones *in situ,* que mostraron el PSTVd efectivamente se encontraba en tejidos vasculares de los tallos y raíces de plantas de tomate infectadas (Zhu *et al.,* 2007). El movimiento viroidal a través del floema parece estar regulado por elementos presentes en él, así como por la interacción del viroide con proteínas del huésped (Gómez y Pallas, 2001, 2004; Zhu *et al.,* 2007; Ding e Itaya, 2007). Por otro lado, como el floema carece de cloroplastos, cabe preguntarse cómo los viroides de la

familia *Avsunviroidae* que se replican en dicho orgánulo, son capaces de moverse a larga distancia. La presencia de otro tipo de plastidios en células acompañantes y en elementos del floema podría ser la clave.

La descarga a distintos órganos 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, el 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).

2.3. Sintomatología y patogénesis

Los síntomas ocasionados por los viroides en sus huéspedes son similares a los provocados por los virus. La expresión de síntomas inducidos por viroides es el resultado de la interacción entre el propio RNA viroidal y factores celulares del huésped que conducen a cambiar el desarrollo y la fisiología de la planta infectada (Flores *et al.,* 2005). La variedad de síntomas es muy amplia e incluye enanismo, epinastia, distorsiones, clorosis, rugosidad y necrosis de las hojas, necrosis, distorsiones y decoloraciones de los frutos, agrietamientos del tallo y de la corteza, disminución del tamaño y estriados en los pétalos, malformaciones en semillas y órganos de reserva. Algunos viroides producen efectos letales como el CCCVd en las plantaciones de cocoteros de Filipinas (Hanold y Randles, 1991), mientras que otros inducen síntomas suaves o son asintomáticas como el viroide latente de la Columnea (*Columnea latent viroid,* CLVd) (Hammond *et al*., 1989). Esta última situación suele ser común tanto en plantas silvestres como cultivadas que al infectarse de forma natural, pueden actuar como reservorios de viroides (Flores *et al*., 2005).

La expresión de síntomas está determinada por la interacción entre tres factores: el huésped, la variante del viroide y las condiciones ambientales en las que se desarrolla el huésped infectado. Un mismo aislado puede actuar como infección latente en determinadas especies y ser agresivo en huéspedes sensibles, como en el caso de un aislado natural del CEVd al ser transmitido de haba a tomate (Fagoaga *et al*., 1995). También se han encontrado aislados del 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 intra-específico. En virología, el término tolerante se refiere a aquellas plantas capaces de infectarse y multiplicar el patógeno sin manifestar síntomas, mientras que el término resistente se utiliza para referirse a aquellas situaciones en las que el patógeno o no infecta a la planta o no es capaz de replicarse en ella.

Al igual que el huésped, el papel del viroide es determinante en la manifestación e intensidad de los síntomas que provoca. En el caso del HSVd en mandarinos (*C. reticulata*) existen variantes patogénicas que provocan la enfermedad de la cachexia y variantes no patogénicas que no la provocan (Reanwarakorn y Semancik, 1998). También existen variantes de un mismo viroide que producen varias intensidades de síntomas como en el caso de las variantes tipo A y tipo B del CEVd, las cuales inducen síntomas agresivos y suaves, respectivamente en tomate (Visvader y Symons, 1985).

Las condiciones ambientales son un factor determinante en la expresión de síntomas. A diferencia de lo que ocurre con la mayoría de los virus, la replicación y acumulación de los viroides y la aparición de síntomas que inducen se ven favorecidos por temperaturas elevadas (25-35 ºC) y por altas intensidades luminosas (Sänger y Ramm, 1975; Harris y Browning, 1980). Por ello, la mayoría de enfermedades viroidales se presentan principalmente en zonas tropicales y subtropicales, en áreas continentales con veranos cálidos y en cultivos de invernadero.

A nivel ultra-estructural, mediante microscopía electrónica se han observado alteraciones citológicas inducidas por miembros de la familia *Pospiviroidae* consistentes en malformaciones de la pared celular y acumulación de depósitos electrodensos (Lawson y Hearon, 1971; Semancik y Vanderwoude, 1976; Wahn *et al*., 1980) así como alteraciones de la estructura de los cloroplastos (Hari, 1980; Semancik y Conejero, 1987). Estudios paralelos con miembros de la familia *Avsunviroidae* han revelado distorsiones en cloroplastos y presencia de cuerpos membranosos en las regiones cloróticas de hojas infectadas con el ASBVd, mientras que en hojas totalmente cloróticas se observan lo que parecen ser proplastidios alterados en vez de cloroplastos (Desjardins, 1987). Esta última observación ha sido reproducida en hojas infectadas con el PLMVd, algunas de cuyas variantes inducen una clorosis extrema (cálico), que en los casos más agudos cubre toda la superficie foliar (Malfitano *et al*., 2003; Rodio *et al*., 2006 y 2007).

También existen importantes diferencias en cuanto a la gama de huéspedes. Un caso extremo lo constituyen los viroides de la familia *Avsunviroidae* cuyo espectro se restringe a una sola o a un grupo reducido de especies próximas, mientras que otros viroides como el HSVd colonizan multitud de especies distintas, produciendo tanto enfermedades como infecciones latentes (Shikata, 1990; Astruc *et al*., 1996).

La patogénesis de los viroides está relacionada con sus propias secuencias y estructuras secundarias, ya que pequeñas variaciones en el genoma pueden tener profundos efectos en la gravedad de los síntomas. Cepas patogénicas y no patogénicas de HSVd difieren en un motivo de 5-6 nucleótidos situado en dominio V de su estructura secundaria. Incluso, un único cambio nucleotídico en este motivo

puede tener un efecto importante en la expresión de los síntomas de cachexia (Serra, *et al.,* 2008b).

En relación con el concepto estructura-función, también se han identificado posibles determinantes patogénicos en la molécula viroidal. Estudios iniciales llevados a cabo con el PSTVd y con el CEVd (Gross *et al*., 1982; Visvader y Symons, 1985), mostraron que mutaciones en el dominio P provocaban efectos drásticos en la patogénesis viroidal (Gross *et al*., 1982). Sin embargo, estudios posteriores sugieren también la participación de otros dominios en el proceso patogénico (Sano *et al.,* 1992; Reanwarakorn y Semancik, 1998; Qi y Ding, 2003). Por otro lado, aunque se han reportado excepciones (Sano *et al*., 1992; Visvader y Symons, 1985), no se ha encontrado una relación directa entre los niveles de acumulación de los viroides y la expresión de síntomas (Schnölzer *et al.,* 1985; Visvader y Symons 1986; Gruner *et al.,* 1995; Góra *et al.,* 1996; Rodio *et al.,* 2006). Así, se ha propuesto que la patogénesis viroidal en miembros de la familia *Pospiviroidae* podría depender de interacciones moleculares específicas entre secuencias o motivos de estructura secundaria y/o terciaria y factores celulares que resultarían en la manifestación de síntomas, pero los mecanismos específicos siguen siendo poco conocidos. En los viroides de la familia *Avsunviroidae* también se han descrito motivos que podrían estar involucrados en la patogénesis y por tanto ser responsables de la expresión de síntomas (Semancik y Szychowski, 1994; De la Peña y Flores, 2001; Maltifano *et al*., 2003 y Rodio *et al*., 2007).

Los datos disponibles indican 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 los motivos de unión o cambios conformacionales en las estructuras secundaria y terciaria de la molécula.

En los últimos años, ha emergido la existencia de mecanismos de silenciamiento génico mediado por pequeñas moléculas de RNA que actúan 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 pequeños RNAs (sRNAs de “small RNAs”), que actuando tanto a nivel pre-transcripcional como post-transcripcional, pueden redirigir la expresión génica mediada por promotores y factores de transcripción. Estos mecanismos que también actúan en la defensa frente a RNAs exógenos (Baulcombe, 2004), han llevado a plantear hipótesis alternativas sobre la patogénesis basadas en el silenciamiento mediado por RNAs. Estas

hipótesis están apoyadas por el descubrimiento en los tejidos de plantas infectadas por miembros de las familias *Pospiviroidae* y *Avsunviroidae,* de pequeños RNAs viroidales de 21-25 nucleótidos, indistinguibles de los pequeños RNAs interferentes (siRNAs), que son los marcadores típicos del silenciamiento mediado por RNA (Itaya *et al.,* 2001; Papaefthimiou *et al.,* 2001; Martínez de Alba *et al.,* 2002).

3. LOS VIROIDES DE LOS CÍTRICOS

Los cítricos son huéspedes naturales de siete viroides pertenecientes a cuatro de los cinco géneros de la familia *Pospiviroidae* (Tabla 1), pero solamente el CEVd y variantes específicas del HSVd generan los síntomas específicos de dos enfermedades que afectan a especies sensibles, la exocortis y la cachexia, respectivamente. La mayoría de cítricos son tolerantes a las infecciones viroidales salvo algunas especies que son sensibles y expresan diversos síntomas.

Todos los viroides inducen síntomas específicos en la planta indicadora cidro Etrog. Dado que el bioensayo basado en la utilización del cidro Etrog se desarrolló para el diagnóstico de la exocortis, la observación de una amplia gama de síntomas se atribuyó a la existencia de razas agresivas, moderadas y suaves del agente causal de la exocortis. Sin embargo, con la posterior identificación y caracterización molecular y biológica de otros viroides que infectan a los cítricos se demostró que la gama de síntomas era consecuencia de la infección y/o coinfección con viroides distintos al CEVd (Duran-Vila, 2000). En huéspedes susceptibles a la infección con distintos viroides, como los cítricos y la vid, es frecuente encontrar infecciones múltiples (Flores *et al.,* 1985; Semancik *et al.,* 1988; Duran-Vila *et al.,* 1988a; Szychowski *et al.,* 1991). En la actualidad se sabe que en cítricos, los viroides suelen presentarse en infecciones naturales como coinfecciones con varios viroides (Duran-Vila *et. al.,* 1988b; La Rosa *et al.,* 1988; Gillings *et al.,* 1991), pudiendo existir interferencias y sinergismos entre ellos (Garnsey y Randles, 1987; Vernière *et al.,* 2004 y 2006).

Dado que los cítricos comerciales se propagan vegetativamente, su cultivo puede sufrir pérdidas económicas importantes por estas enfermedades, ya que la propia propagación asegura la transmisión de viroides y otros patógenos transmisibles por injerto, que se encuentran ampliamente dispersos, generalmente de forma latente, en los países productores en los que no se han implementado programas de saneamiento y certificación. En muchos casos la necesidad de sustituir patrones tolerantes por otros susceptibles, ha obligado a los países productores de cítricos a

tener en cuenta estas enfermedades como un posible problema en las nuevas plantaciones.

Los viroides identificados en cítricos incialmente se clasificaron en cinco grupos, basándose en su movilidad en electroforesis secuencial en geles de poliacrilamida (sPAGE), similitud de secuencia estimada por hibridación molecular con sondas de cDNA, síntomas inducidos en la planta indicadora cidro Etrog y gama de huéspedes (Semancik y Duran-Vila, 1991). Con la secuenciación de distintas fuentes de estos viroides se demostró que los cinco grupos se correspondían a las cinco especies de viroides que han sido ya reconocidas por la ICTV (Tabla 1) (Flores *et al.,* 2005). Posteriormente se describieron otros dos viroides (Ito *et al.,* 2001 y Serra *et al.,* 2008a). Las distintas especies de viroides conocidas hasta hoy son:

El viroide de la exocortis de los cítricos (CEVd)

El CEVd tiene un tamaño que oscila entre 370 y 375 nucleótidos y pertenece al género *Pospiviroide*. Se ha demostrado que el CEVd es el agente causal de la exocortis al verificar los postulados de Koch inicialmente en *Gynura aurantiaca* y cidro Etrog (Semancik y Weathers, 1972) y posteriormente en *P. trifoliata* (Vernière *et al.,* 2004). Se han descrito numerosas variantes de secuencia del CEVd (Gross *et al*., 1982; Visvader y Symons, 1985), entre las cuales cabe destacar las variantes de clase A y clase B (Visvader y Symons, 1985). Estas variantes, inductoras de síntomas acusados y suaves en tomate, respectivamente, se diferenciaban en una serie de cambios nucleotídicos característicos localizados en los dominios P, C y V de su estructura secundaria (Visvader y Symons, 1985). Se han descrito numerosas variantes de secuencia que se suelen hallarse como poblaciones heterogéneas en las plantas infectadas (Visvader y Symons, 1985; Gandía *et al.,* 2000; Bernard *et al.,* 2009). Estas observaciones que inicialmente se consideraban como el resultado de sucesivas inoculaciones, se ha demostrado que al menos son en parte, el resultado de la habilidad de este viroide para incorporar mutaciones como consecuencia de su replicación en ausencia de mecanismos de prueba de lectura (Gandía *et al.,* 2000).

El CEVd como agente causal de la exocortis se describió inicialmente como un desorden que afectaba a árboles injertados en *P. trifoliata* y que se caracterizaba por la presencia de grietas y escamas en la corteza de este patrón (Fig. 4A), asociadas a enanismo más o menos acusado, el cual se traducía en una disminución de la cosecha como consecuencia del menor tamaño de los árboles afectados (Fawcett y Klotz, 1948; Benton *et al.,* 1949). En Australia se describió una enfermedad similar también en árboles injertados en *P. trifoliota* que fue denominada "scally butt" y cuya naturaleza biótica se demostró mediante la transmisión por injerto (Benton *et al.,*

1950). Posteriormente, se relató la presencia de síntomas similares en lima Rangpur *(Citrus limonia*) en Texas (USA) y Brasil (Olson, 1952; Moreira, 1959; Montenegro y Salibe, 1957). Montenegro y Salibe (1957) asociaron la "falsa gomose" o "doença do limoneiro Cravo", caracterizada por la descamación y exudación de goma en lima Rangpur, con el mismo agente infeccioso que afectaba al *P. trifoliata* en otros países. Actualmente se considera que se trata de la misma enfermedad a la que se denomina exocortis.

El CEVd se halla muy disperso en todos los países citrícolas. Hasta la aparición de la tristeza, la amplia utilización del naranjo amargo como patrón permitió el desarrollo de plantaciones con árboles asintomáticas pero portadores de éste y otros viroides. Sin embargo, la necesidad de introducir patrones tolerantes a tristeza como la lima Rangpur, el *P. trifoliata* o los híbridos tipo citrange, todos ellos sensibles a exocortis, puso de manifiesto que el agente causal de la enfermedad se hallaba difundido en las variedades comerciales cultivadas en la mayoría de países citrícolas. Las pérdidas económicas que causa la exocortis son variables y dependen de la estirpe del patógeno, la edad del árbol en el momento que tiene lugar la infección y las condiciones climáticas de la zona en la que se desarolla el cultivo (Duran-Vila, 2000).

El CEVd produce en las especies sensibles, grietas verticales y escamas en la corteza (Fig. 4A), manchas amarillas y grietas en los brotes tiernos y enanismo. El síntoma clásico inducido por el CEVd es la descamación del *P. trifoliata*, sus híbridos los citranges (*C. sinensis* X *P. trifoliata*) Troyer y Carrizo y la lima Rangpur (*C. limonia*), todos ellos de interés comercial como patrones (Duran-Vila, 2000). El CEVd induce un fuerte enanismo y una epinastia muy marcada acompañada de necrosis en el nervio central de la hoja en cidro Etrog, la especie utilizada como indicadora en la detección biológica.

El CEVd tiene una amplia gama de huéspedes cítricos siendo la mayoría de ellos portadores asintomáticos (Duran-Vila *et al.,* 1986). Además, son también huéspedes naturales la vid (Flores *et al.,* 1985; Garcia-Arenal *et al.,* 1987), el tomate (Mishra *et al.,* 1991; Fagoaga y Duran-Vila, 1996), el haba (Fagoaga *et al.,* 1995), el nabo, la zanahoria, la berenjena (Fagoaga y Duran-Vila, 1996) y una serie de plantas ornamentales (Bostan *et al.,* 2004; Nie *et al.,* 2005; Singh *et al.,* 2006). La gama de huéspedes experimentales también es amplia y se han identificado tanto especies sensibles que desarrollan síntomas de enanismo, epinastia y distorsión foliar, como tolerantes.

En los cítricos, los viroides suelen presentarse como infecciones múltiples, lo que impidió durante muchos años conocer el efecto de cada uno de ellos en la expresión de síntomas en especies de cítricos sensibles. El primer estudio exhaustivo en tal

sentido se llevó a cabo en una parcela experimental de Córcega donde se estudió el efecto de cada uno de los viroides en clementinos injertados sobre *P. trifoliata* (Vernière *et al*., 2004). Con los resultados obtenidos se confirmó que el CEVd era el único viroide capaz de inducir la exocortis y que, además, limitaba el crecimiento de árbol y el rendimiento de la cosecha. Por el momento se desconoce el efecto de cada uno de los viroides en otras especies sensibles como el citrange Troyer, el citrange Carrizo y la lima Rangpur. La mayor o menor gravedad de los síntomas depende de numerosos factores como la estirpe del patógeno, la edad del árbol en el momento de la infección y las condiciones climáticas en las que se desarrolla el cultivo.

El viroide del enanismo del lúpulo (HSVd)

El HSVd es el único miembro del género *Hostuviroide* y las variantes de cítricos tienen un tamaño que oscila entre 296 y 301 nucleótidos. Todas las variantes de este viroide inducen síntomas de enanismo y rugosidad foliar en pepino, y pueden presentar diferentes grados de agresividad (Sano *et al.,* 1988). En cidro Etrog induce síntomas muy suaves y se encuentra en concentraciones mucho más bajas que los otros viroides de cítricos (Duran-Vila *et al.,* 1993).

El HSVd es el viroide con mayor gama de huéspedes naturales ya que se ha descrito en pepino (Sano *et al.,* 1988), vid (Rezaian *et al.,* 1988), almendro (Cañizares *et al.,* 1999), ciruelo y melocotonero (Sano *et al.,* 1989), peral (Shikata, 1990) y albaricoquero (Amari *et al.,* 2001).

El HSVd se describió 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 frente a la infección (Sano *et al.,* 1986). Algunas variantes del HSVd son agentes causales de la cachexia de los cítricos, uno de las dos importantes enfermedades producidas por viroides en cítricos. La cachexia afecta tanto a especies que se utilizan como patrón como a especies que se utilizan como variedad. Son especialmente sensibles *Citrus macrophylla,* tangelos, mandarinos, clementinos, satsumas e híbridos de los mismos. Estas especies manifiestan acanaladuras en la madera, que se corresponden con proyecciones de la corteza, e impregnaciones de goma en la zona próxima a la línea de injerto (Fig. 4B) (Duran-Vila, 2000). Roistacher *et al.* (1983) propusieron la hipótesis de que el agente causal de la cachexia fuera un viroide debido a su forma de transmisión, resistencia a la termoterapia y la facilidad de eliminarlo mediante el microinjerto de ápices caulinares *in vitro.* Posteriormente Semancik *et al.* (1988) consiguieron aislar y caracterizar el patógeno como un viroide al que tentativamente denominaron viroide de la cachexia de los cítricos (CCaV) (Semancik *et al.,* 1988).

Muchos años antes, se había descrito una enfermedad de la lima dulce de Palestina (*Citrus limettioides*) conocida como xiloporosis (Reichert y Perlberger, 1934). Las plantas afectadas presentaban hendiduras en la madera con las correspondientes proyecciones de la corteza en la zona del injerto, síntomas que estaban asociados a decoloración de la madera e impregnación de goma. Las plantas presentaban hojas pequeñas y amarillas, mostrándose deprimidas, y muchas morían. En estadíos más avanzados, las plantas afectadas presentaban chancros en la corteza del tronco por encima de la zona de unión del injerto y también en las ramas, clorosis foliar y enanismo. En Brasil, también se observaron síntomas similares en lima dulce y lima Rangpur (Moreira, 1938). Las síntomas de las enfermedades descritas inicialmente como xiloporosis y cachexia eran muy similares, y por tanto podía tratarse de la misma enfermedad, pero éste ha sido un tema controvertido que ha perdurado durante varios años, hasta que se ha demostrado que ambas afecciones están causadas por el mismo patógeno (Reanwarakom y Semancik, 1999).

En cítricos, el HSVd se identificó por primera vez en 1986 como un RNA de movilidad superior al CEVd, y se le denominó viroide II de los cítricos (CVd-II) (Duran-Vila *et al.,* 1986). El análisis de una serie de aislados de campo permitió identificar en la región donde migra este viroide, otra dos bandas de movilidades algo superiores pero con una homología elevada con la anterior determinada mediante hibridación molecular con sondas de cDNA (Duran-Vila *et al.,* 1988b), por lo que estos viroides se denominaron CVd-IIa, CVd-IIb y CVd-IIc). La caracterización biológica de diversas fuentes de estos viroides mostró que los aislados que migraban como CVd-IIb o como CVd-IIc eran patogénicos e inducían la enfermedad de la cachexia, mientras que los aislados que migraban como el CVd-IIa no eran patogénicos (Semancik *et al.,* 1988). Las variantes no patogénicas no están asociadas a ninguna enfermedad de importancia económica pero inducen grietas en la corteza del *P. trifoliata* (Roistacher *et al.,* 1993; Vernière *et al.,* 2004 y 2006) y enanismo en árboles de naranjo dulce injertados en este patrón (Semancik *et al.,* 1997). Las variantes patogénicas inducen síntomas de cachexia en clementino (Vernière *et al.,* 2004), tangelo Orlando y *C. macrophylla* (Reanwarakorn y Semancik, 1998 y 1999). Mediante hibridación molecular se demostró que existía una elevada similitud de secuencia entre los aislados patogénicos y no patogénicos (Semancik *et al.,* 1988; Semancik y Duran-Vila, 1991) y también con el HSVd (Diener *et al.,* 1988; Albanese *et al.,* 1991; Davino *et al.,* 1991). Con la posterior secuenciación de estos tipos de aislados se demostró que todos eran variantes del HSVd (Levy y Hadidi, 1993) y que las variantes patogénicas y no patogénicas diferían en sólo 5-6 nucleótidos localizados en el dominio V (Reanwarakorn y Semancik, 1999; Palacio-Bielsa *et al.,* 2004).

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

El CBLVd fue descubierto por primera vez como una banda de movilidad superior al CEVd a partir de aislados que se consideraban erróneamente como razas suaves de exocortis, y se le denominó viroide I de los cítricos (CVd-I) (Duran-Vila *et al.,* 1986). Posteriormente se identificó otra banda de movilidad algo superior pero con una homología elevada con la anterior, determinada mediante hibridación molecular con sondas de cDNA (Duran-Vila *et al*., 1988b). Estos viroides se denominaron CVd-Ia y CVd-Ib y su posterior secuenciación demostró que se trataba de variantes de un mismo viroide al que se le dió el nombre de *Citrus bent leaf viroid* (CBLVd) (Ashulin *et al*., 1991). Es probable que la variante CVd-Ia sea el resultado de una duplicación de parte de la secuencia de la variante CVd-Ib de menor tamaño (Hataya *et al*., 1998). Estos dos tipos de variantes presentan tamaños de 327 y 318 nucleótidos, respectivamente (Duran-Vila *et al*., 1988b; Ashulin *et al*., 1991).

El CBLVd pertenece al género *Apscaviroide* y tiene una gama de huéspedes restringida a la familia Rutáceas, aunque se ha transmitido experimentalmente a aguacate mediante injerto heterólogo (Hadas *et al.,* 1992). Dado que ambas variantes tienen una gama de huéspedes muy restringida y no se han encontrado co-infectando una misma planta, se ha propuesto que podrían haberse generado de forma independiente por sucesos recombinatorios similares en plantas que estaban infectadas con otros viroides (Foissac y Duran-Vila, 2000). Todos las especies de cítricos analizados se muestran tolerantes frente a este viroide (Vernière *et al*., 2004), salvo el cidro Etrog cuya sintomatología se caracteriza por la aparición de necrosis puntual del nervio central que provoca una epinastia característica, a veces acompañada de exudaciones gomosas en el tallo y ramificaciones debidas a la pérdida de la dominancia apical (Duran-Vila *et al.,* 1988a; Serra *et al.,* 2008a). Estos mismos síntomas han sido descritos en una variante descubierta en Japón y denominada CVd-I-LSS, que presenta una similitud de secuencia del 82-85% con las variantes previamente descritas del CBLVd (Ito *et al.,* 2000).

El CBLVd se ha asociado a la presencia de hendiduras en la corteza del patrón *P. trifoliata* (Roistacher *et al.,* 1993), una observación que no ha sido corroborada en estudios posteriores (Vernière *et al.,* 2004). Este viroide induce también enanismo leve en árboles injertados en *P. trifoliata* (Semancik *et al.,* 1997; Vernière *et al.,* 2004).

El Viroide del enanismo de los cítricos (CDVd)

El CDVd pertenece al mismo género que el apscaviroide CBLVd, y su gama de huéspedes está restringida a las rutáceas. Este viroide fue descubierto como una banda de movilidad superior al CEVd también a partir de aislados que se consideraban

erróneamente como razas suaves de exocortis, y se le denominó viroide III de los cítricos (CVd-III) (Duran-Vila *et al.,* 1988b). A partir de patrones electroforéticos y de hibridación se descubrió la existencia de cuatro tipos de variantes designadas CVd-IIIa, CVd-IIIb, CVd-IIIc y CVd-IIId. Con la secuenciación de una serie de aislados se constató la existencia de variantes con tamaños que oscilaban entre 291 y 297 nucleótidos y alta similitud de secuencia entre ellos (Semancik y Duran-Vila, 1991; Rakowski *et al.,* 1994; Semancik *et al.,* 1997; Murcia *et al.,* 2009b). La secuenciación de fuentes de CDVd procedentes de distintos países ha mostrado que poseen un genoma muy conservado, siendo las variantes tipo CVd-IIIb mucho más frecuentes que las de tipo CVd-IIIa o CVd-IIIc (Owens *et al.,* 1999).

El CDVd tiene el cidro Etrog como único huésped experimental, en el que induce síntomas de enanismo acompañado de un epinastia leve de las hojas debido a necrosis y anillamiento del peciolo. En especies de interés comercial se ha asociado a enanismo en plantas injertadas en *P. trifoliata* y citranges Troyer y Carrizo (Gillings *et al.,* 1991; Semancik *et al.,* 1997; Owens *et al.,* 2000). Se ha demostrado que la variante más abundante (de secuencia muy similar a CVd-IIIb) causa una significativa reducción del volumen de la copa y del tamaño del árbol en cultivares de naranjo y clementino injertados sobre *P. trifoliata* (Roistacher *et al.,* 1993; Semancik *et al.,* 1997; Vernière *et al.,* 2004). Dado que las variantes ensayadas en condiciones de campo no inducen síntomas específicos, el efecto enanizante inducido por este viroide puede tener interés en algunos países productores ya que la reducción de la altura del árbol y el volumen de la copa puede facilitar las labores del cultivo (Semancik *et al.,* 1997; Hutton *et al.,* 2000).

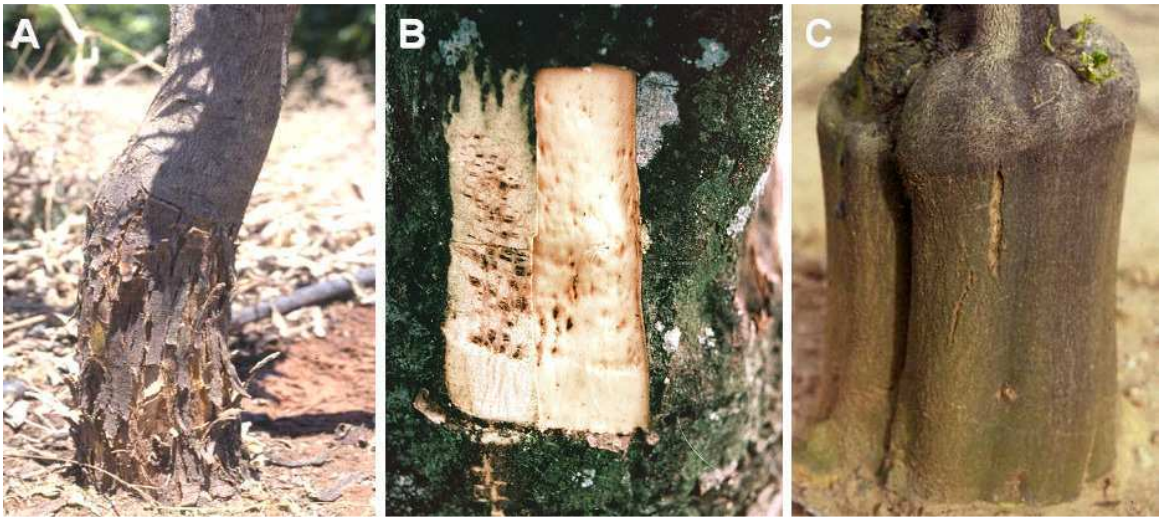

Fig. 4. (A) Descamaciones características de exocortis en el patrón *Poncirus trifoliata*. (B) Acanaladuras en la madera que se corresponden con proyecciones de la corteza e impregnaciones de goma en la zona próxima a la línea de injerto en un árbol de mandarino infectado con cachexia. (C) Grietas verticales producidas por el CBCVd en el patron *P. trifoliata.*

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

El CBCVd fue descubierto por primera vez como una banda de movilidad superior a la de los demás viroides a partir de aislados que se consideraban razas suaves de exocortis, y se le denominó viroide IV de los cítricos (Duran-Vila *et al.,* 1988b). Parece estar menos disperso que otros viroides y se describió inicialmente en California (USA) e Israel, y posteriormente en Turquía (Duran-Vila *et al.,* 1988a; Hadas *et al*., 1989; Önelge *et al.,* 1996) como un viroide asociado al enanismo en cítricos. Su caracterización molecular mostró que tenía una secuencia de 284 nucleótidos y una gran similitud de secuencia con los dominios V y T_R del CEVd (Puchta *et al.,* 1991), lo que explica la reacción positiva en hibridación molecular con sondas específicas del CEVd (Duran-Vila *et al.,* 1988b). El CBCVd pertenece al género *Cocadviroide* debido a la secuencia de su CCR y TCH. La inoculación en cidro Etrog produce enanismo y epinastia con necrosis en el nervio central. Los resultados de los ensayos de campo realizados en clementinos injertados en *P. trifoliata* han mostrado que este viroide induce grietas en el patrón (Fig. 4C) pero el porte y la altura de los árboles, el tamaño de copa y la cosecha son similares a los de los árboles libres de viroides (Vernière *et al.,* 2004).

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

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

El viroide ''V'' de los cítricos (CVd-V)

El CVd-V, recientemente descrito como una nueva especie del género *Apscaviroide* dentro de la familia *Pospiviroidae.* Este viroide con tamaño de 293-294 nucleótidos, se identificó inicialmente en *Atalantia citroides,* un genero afín a los cítricos, y posteriormente en variedades comerciales de Estados Unidos, España, Nepal y el Sultanato de Omán (Serra *et al.,* 2008c). Se han descrito diversas variantes de este viroide (Serra *et al.,* 2008a) y por el momento se desconoce su efecto sobre el cultivo en campo.

3.1. Enfermedades de posible etiología viroidal en los cítricos

El CEVd y el HSVd son agentes causales de dos enfermedades para las que se han verificado los postulados de Koch. Sin embargo, existen algunas enfermedades

descritas en cítricos cuyos agentes causales no han sido todavía identificados. Entre ellas se encuentran: el "Gummy bark", el "Gum pocket-Gummy pitting" y la enfermedad de Kassala (Duran-Vila y Semancik, 2003). Las síntomas de estas enfermedades son similares a los de cachexia (aunque en huéspedes diferentes), por lo que se ha propuesto que pudieran estar causadas por determinados viroides de los cítricos, o quizá alguna variante de éstos (Bernard *et al.,* 2005).

El "Gummy bark" se manifiesta en variedades de naranjo dulce, donde se desarrollan síntomas de acanaladuras en la madera y depósitos de goma en la corteza del tronco (Fig. 5A), así como un decaimiento general del árbol (Önelge *et al.,* 1996). Estos síntomas han sido también observados en limón rugoso (Bové, 1995). Se ha demostrado que esta enfermedad es transmisible por injerto, lo que indica la implicación de un agente biótico. El "Gummy bark" se halla disperso en determinados países de la cuenca Mediterránea y Oriente Próximo (Bové, 1995).

El "Gummy pitting" o "Gum pocket" se manifiesta en el patrón *P. trifoliata*, donde se observa la presencia de acanaladuras en la madera y depósitos de goma tanto en la madera como en la corteza en el sitio de unión injerto-patrón (Fig. 5B) (Fernández-Valiela *et al.,* 1965). Esta enfermedad ha sido descrita en Argentina, Australia, Italia y Sudáfrica. Es transmisible por injerto y, aunque la etiología sigue siendo una incógnita, se especula con la posible implicación de una variante inusual del CDVd o de un viroide de tamaño similar no caracterizado hasta el momento (Marais *et al.,* 1996).

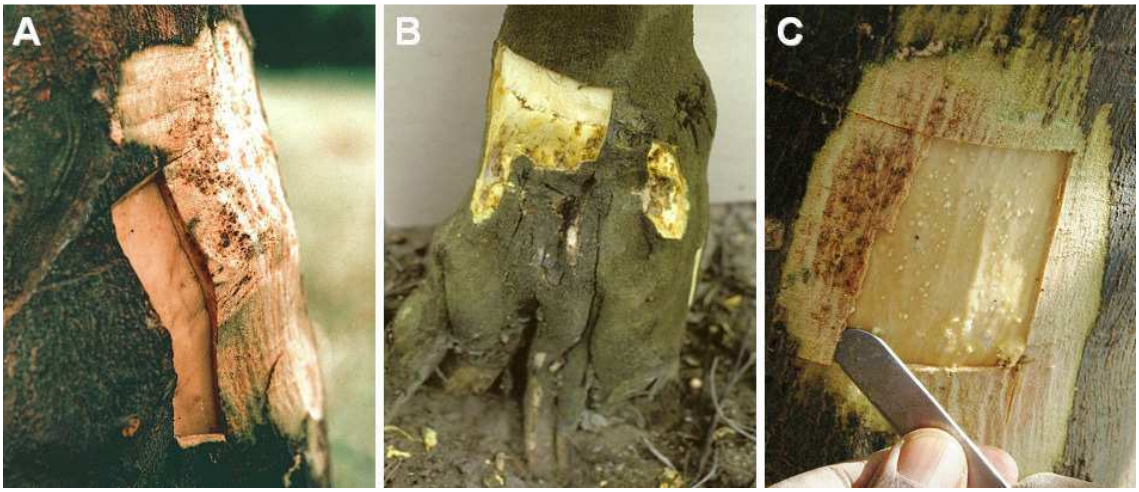

Fig. 5. (A) Acanaladuras en la madera y depósitos de goma en la corteza del tronco de un árbole de naranjo dulce características de la Gummy bark (Libia, J. M. Bové). (B) Acanaladuras en la madera y depósitos de goma tanto en la madera como en la corteza del patron *P. trifoliata* características de Gummy pitting (L. R. Fraser y P. Broadbent). (C) Depósitos de goma en la corteza del pomelo características de la enfermedad de Kassala (Sudán, N. Duran-Vila). www.ivia.es/iocv/enfermedades.

La enfermedad de Kassala se ha descrito en pomelos de la localidad de Kassala (Sudán) y en Yemen. Se caracteriza por la presencia de acanaladuras en la

madera y depósitos de goma en la corteza en árboles de pomelo (Fig. 5C) (Bové, 1995). Los síntomas son similares a los de la cachexia en mandarino o a los de "Gummy bark" en naranjo dulce. Sin embargo, se desconoce si la enfermedad es transmisible por injerto, por lo que la hipótesis de que su agente causal esté relacionado con el de la cachexia y/o el del "Gummy bark" debe ser tomada con precaución (Semancik y Duran-Vila, 1991).

3.2. Métodos de diagnóstico y detección de los viroides de los cítricos

Hasta hace muy poco tiempo, la detección se ha realizado exclusivamente mediante métodos biológicos, pero la caracterización de los agentes causales de las enfermedades producidas por viroides ha permitido el desarrollo de métodos de detección basados en la detección del RNA viroidal. Los métodos serológicos comúnmente utilizados en la detección de otros patógenos, como los virus, no son adecuados, ya que los viroides carecen de proteínas que son las que confieren la capacidad antigénica para producir anticuerpos específicos. Los ensayos biológicos se llevan a cabo mediante inoculación de plantas indicadoras seleccionadas por su sensibilidad y la observación de síntomas después de un periodo de incubación determinado empíricamente.

El diagnóstico y detección de la exocortis y la cachexia se realizaba inicialmente mediante pruebas de infectividad que se habían desarrollado antes de ser establecida su etiología. El *P. trifoliata* fue la primera especie que se utilizó como indicadora de exocortis. El periodo de incubación en esta indicadora era largo, ya que las plantas manifiestan síntomas tras 4-8 años después de la inoculación (Benton *et al.,* 1949 y 1950). En Brasil, se utiiizó como indicadora la lima Rangpur, que permitía la visualización de síntomas entre 5 y 18 meses, y por tanto una reducción considerable del tiempo necesario para realizar el diagnóstico (Moreira, 1961).

Posteriormente se observó que el cidro manifestaba síntomas entre 1-5 meses tras la inoculación y fue adoptada como la indicadora más idónea (Calavan *et al.,* 1964). Debido a que el cidro es monoembriónico y la progenie obtenida por vía sexual puede no ser uniforme, este indicador se multiplica vegetativamente para así poder evaluar los síntomas en un mismo genotipo. La selección 861-S1 de cidro Etrog fue obtenida al evaluar la sensibilidad de varias plantas de semilla, lo que permitió seleccionar la más sensible, que posteriormente fue saneada por microinjerto y propagada vegetativamente (Roistacher *et al.,* 1977).

El tangelo Orlando fue la primera planta indicadora que se utilizó para el diagnóstico y detección de la cachexia (Childs, 1950). Esta especie, a pesar de su sensibilidad, tarda cerca de 4 años en exhibir síntomas en respuesta a la infección con

los aislados menos agresivos y los resultados suelen ser erráticos y poco reproducibles (Roistacher, 1988). Fue a partir de 1973, cuando Roistacher y colaboradores seleccionaron el mandarino Parson's Special como un genotipo sensible y capaz de exhibir síntomas en poco menos de un año tras la inoculación. El mandarino Parson's Special injertado sobre limonero rugoso u otro patrón vigoroso es la indicadora utilizada para el diagnóstico y detección de cachexia. Los síntomas de la enfermedad se manifiestan como depósitos de goma en la línea de unión del injerto y para su expresión es preciso mantener las plantas a 28-32ºC durante un período de 12 a 18 meses (Roistacher *et al.,* 1973). El método, aunque es sensible, presenta inconvenientes, como el largo periodo de incubación y la interferencia con otros viroides de los cítricos que pueden impedir o retrasar la expresión de síntomas (Pina *et al.,* 1991).

El ensayo biológico utilizando el cidro Etrog, aunque fue desarrollado para la detección de la exocortis, no es específico para detectar el CEVd y puede ser utilizado para todos los viroides descritos en cítricos. La selección 861-S1 de cidro Etrog sigue siendo la indicadora por excelencia pero presenta limitaciones debidas al coste, el tiempo de incubación necesario y la baja especificidad. Sin embargo, cuando se utiliza para el diagnóstico de aislados de campo, normalmente portadores de varios viroides, los síntomas observados son complejos debido a las interacciones entre ellos (Duran-Vila y Semancik, 1990; Duran-Vila *et al.,*1991), y es imposible determinar qué viroides están presentes en el material analizado. Actualmente, la detección y diagnóstico de los viroides de los cítricos se realiza combinando métodos biológicos y moleculares, basados éstos últimos en la detección del RNA viroidal a partir de los cidros inoculados, lo que permite acortar el periodo de incubación necesario y aumentar la especificidad (Duran-Vila *et al.,* 1993).

También se han propuesto y utilizado distintos sistemas de doble electroforesis como la electroforesis secuencial en geles de poliacrilamida (sPAGE) (Semancik y Harper, 1984), que permite distinguir los viroides presentes en un aislado por su movilidad electroforética. Este método resulta sensible y satisfactorio cuando se emplean extractos de ácidos nucleicos procedentes de cidros infectados, incluso antes que manifiesten síntomas. La detección de viroides a partir de extractos de especies y cultivares comerciales también puede realizarse por dicho método (Barksh *et al.,* 1984; Boccardo *et al.,* 1984), pero su fiabilidad y precisión no ha sido comprobada de forma sistemática para todas las especies y variedades comerciales ni en distintas condiciones de cultivo. Estudios realizados utilizando especies y variedades comerciales cultivadas en las condiciones de la Comunidad Valenciana, indican que el

método no es fiable debido a fluctuaciones interanuales en el título de los viroides (Palacio, 1999).

Otro método utilizado es la hibridación molecular, que permite detectar los viroides mediante el empleo de sondas específicas. La hibridación dot-blot con sondas cDNA marcadas radiactivamente se ha propuesto para detectar el CEVd a partir de plantas de cidro (Flores, 1988). También se ha propuesto la hibridación "northern" con sondas de cDNA y cRNA marcadas radiactivamente para la detección del CEVd y el HSVd a partir de extractos de especies cultivadas en campo (Albanese *et al.,*1991) u oligómeros también marcados radiactivamente (La Rosa *et al.,* 1993). Actualmente, la disponibilidad de formas de marcaje no radioactivo de las sondas abre nuevas posibilidades que facilitan la detección rutinaria mediante este tipo de técnicas. Sin embargo, al igual que en el caso de análisis por sPAGE, las fluctuaciones interanuales no permiten detectar viroides en materiales de campo, incluso cuando el muestreo se había realizado en la época más idónea (Palacio, 1999).

Estas limitaciones se han paliado cambinando este método con las excelentes propiedades del indicador cidro Etrog, en el que todos los viroides alcanzan titulos que permiten su detección mediante sPAGE o hibridación molecular (Duran-Vila *et al.,* 1993). Así pues, el análisis de los cidros inoculados permite acortar el periodo de incubación y detectar todos los viroides con niveles adecuados de sensibilidad y especificidad. Estos métodos permiten la detección específica de los distintos viroides incluso antes de que el cidro Etrog manifieste síntomas. Dado que incluso puede detectarse el HSVd, puede obviarse el bioensayo con el mandarino Parson's Special como indicadora de cachexia que plantea problemas en el caso de aislados portadores de variantes patogénicas y no patogénicas de este viroide. También se ha propuesto analizar los cidros inoculados mediante un sistema de hibridación de impresiones (Palacio-Bielsa *et al.,* 1999) que evita la necesidad de realizar extracciones de ácidos nucléicos, y por tanto analizar simultáneamente un gran número de muestras. Recientemente, se ha puesto a punto un sistema de hibridación "northern" con sondas marcadas con digoxigenina que permite detectar todos los viroides en extractos obtenidos directamente de muestras de campo (Murcia *et al.,* 2009a).

La retrotranscripción y amplificación mediante la reacción en cadena de la DNA polimerasa (RT-PCR), que es un método muy sensible, ha sido propuesto también para la detección de los viroides (Yang *et al.,* 1992; Tessitori *et al.,* 1996; Bernard *et al.,* 2006). Se han establecido distintas aproximaciones, protocolos e incluso combinaciones de cebadores que permiten la detección de todos los viroides mediante un sistema de multiplex RT-PCR (Ito *et al.,* 2002). A pesar de las ventajas de este método, la detección rutinaria mediante RT-PCR como metodología única en

programas de certificación no ha llegado a generalizarse (Sieburth *et al.,* 2002), ya que con cierta frecuencia se obtienen falsos negativos en muestras que se sabe que están infectadas, así como amplificaciones inespecíficas que dificultan la interpretación de los resultados obtenidos (Garnsey *et al.,* 2002).

3.3. Dispersión y control de los viroides de los cítricos

La transmisión por yemas infectadas es el principal método de dispersión de estos patógenos. De hecho, el intercambio internacional de material propagativo de cítricos es el responsable de la presencia de estos patógenos en las diferentes zonas de cultivo del mundo. El uso rutinario de métodos de diagnóstico y detección, y la implementación de programas de certificación han proporcionado información adicional sobre la dispersion de las diferentes especies de viroides en el mundo. Se ha constatado que en todos los países en los que existen cultivos comerciales de cítricos, éstos son portadores de viroides, a no ser que se haya producido y/o distribuido material vegetal procedente de programas de saneamiento (Randles, 2003). La información disponible en la actualidad, indica que el HSVd y el CDVd se encuentran muy dispersos generalmente como mezclas. El CBLVd y el CBCVd son los menos frecuentes, mientras que el CEVd se encuentra con una frecuencia intermedia.

La transmisión mecánica del agente de la exocortis por herramientas de corte se reportó ya antes de que se identificara el CEVd como su agente causal (Garnsey y Jones, 1967) y posteriormente se demostró también para el agente causal de la cachexia (Roistacher *et al.,* 1980). Estudios recientes han demostrado que, además del CEVd y HSVd, todos los viroides descritos también se transmiten mecánicamente en campo y en invernadero mediante las herramientas de trabajo (Barbosa *et al.,* 2005). La transmisión de viroides por semilla y por vectores no se ha demostrado.

Los métodos de control de las enfermedades producidas por viroides son de tipo preventivo, y se basan en la utilización de plantas libres de viroides en las nuevas plantaciones (plantas certificadas) y en evitar su dispersión desde plantas infectadas a plantas sanas. Los programas de cuarentena, saneamiento y certificación permiten ofrecer a los viveristas material vegetal libre de viroides a partir del cual suministrar al agricultor plantones sanos y yemas para injerto o sobre-injerto en campo. Para la prevención de la transmisión mecánica se deben desinfectar las herramientas de corte con hipoclorito sódico ya que el RNA de los viroides es muy resistente a la degradación por calor o por otros agentes químicos comúnmente utilizados para la inactivación de virus (Roistacher *et al.,* 1969).

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OBJETIVOS

Objetivos

Existen numerosos trabajos que describen síntomas de exocortis y cachexia en distintas especies y variedades de cítricos, pero parte de esta información se obtuvo antes de que se conocieran los agentes causales de estas enfermedades y la existencia de otros viroides que infectan a los cítricos. Por otra parte, dado que los cítricos suelen estar co-infectados con varios viroides que pueden manifestar efectos sinérgicos o de interferencia, la información acerca del efecto de cada uno de los viroides es muy limitada.

Teniendo en cuenta estos antecedentes, el objetivo general de este trabajo ha sido generar información acerca de la respuesta de distintos genotipos de cítricos y géneros afines a la infección con viroides.

En el primer capítulo se aborda un estudio para determinar el efecto de un aislado de viroides en árboles de clementino de Nules y naranjo 'Navelina' injertados sobre citrange Carrizo y mantenidos en una parcela experimental durante más de diez años. Los objetivos del primer capítulo son:

1. Caracterizar molecularmente las cuatro especies de viroides, *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd) y *Citrus dwarfing viroid* (CDVd) identificados en el aislado objeto de estudio.
2. Comparar y analizar estadísticamente los parámetros de crecimiento, cosecha y calidad de la fruta de árboles sanos e infectados.
3. Identificar los síntomas inducidos por este aislado profundizando en aspectos relacionados con el desarrollo del sistema radicular.

Estudios preliminares mostraron que *Citrus karna*, una especie de origen desconocido y utilizada como patrón comercial en la India, es susceptible a la infección. Aunque los viroides inoculados alcanzan en *C. karna* concentraciones tan elevadas como en cidro Etrog (*Citrus medica*), los intentos realizados para utilizarlo como huésped experimental dieron resultados contradictorios. En el segundo capítulo se aborda un estudio dirigido a determinar la distribución de los viroides en plantas de *C. karna* inoculadas con CEVd, HSVd, CBLVd, CDVd, *Citrus bark cracking viroid* (CBCVd) o *Citrus viroid V* (CVd-V). Los objetivos del segundo capítulo son:

4. Comparar los perfiles de distribución de los viroides en *C. karna* y cidro Etrog que se alcanzan en una infección sistémica.

5. Determinar la capacidad de los viroides inoculados para moverse desde el floema a las células del mesófilo, e identificar estrategias biológicas que favorecen dicho movimiento.
6. Comparar los perfiles de distribución de los viroides en *C. karna* y cidro Etrog con los de naranjo amargo (*C. aurantium*) y naranjo trifoliado (*Poncirus trifoliata*).

Los resultados de estudios preliminares permitieron identificar *Eremocitrus glauca* y *Microcitrus australis*, dos especies de géneros afines a los cítricos, como genotipos aparentemente resistentes a la infección con viroides. El tercer objetivo aborda estudios para determinar si estos genotipos son efectivamente resistentes a la infección. Los objetivos del tercer capítulo son:

7. Analizar la infección, movimiento y acumulación de CEVd, HSVd, CBLVd, CDVd, CBCVd y CVd-V en plantas auto-enraizadas, en plantas injertadas sobre el patrón limón rugoso y en plantas sobreinjertadas con cidro Etrog.
8. Determinar si estos genotipos permiten o no el movimiento a larga distancia desde el punto de inoculación hacia el patrón y hacia el cidro Etrog sobreinjertado.

La reciente disponibilidad de métodos que permiten el diagnóstico de viroides en especies y variedades comerciales cultivadas en campo nos llevó a estudiar el estado sanitario de plantaciones en la citricultura de tres países distintos. Los objetivos de este cuarto capítulo son:

9. Identificar viroides en plantaciones comerciales de Sudán y su relación con dos enfermedades atribuidas a viroides, la enfermedad de la corteza gomosa del naranjo dulce (del inglés “Gummy bark”) y la enfermedad de Kasala del pomelo.
10. Identificar viroides en colecciones de material vegetal y plantaciones comerciales de Irán y analizar su repercusión ante la necesidad de sustituir los patrones predominantes en este país, el naranjo amargo y la lima mexicana, por patrones tolerantes al virus de la tristeza de los cítricos.
11. Identificar viroides en parcelas comerciales de la Comunidad Valenciana y acotar el origen y el tipo de transmisión responsables de la distribución de árboles infectados en dichas parcelas.

Durante el periodo de desarrollo de estos trabajos he tenido la oportunidad de colaborar en otros trabajos de investigación que han dado lugar a las publicaciones que se recogen en los anejos de esta memoria.

CAPÍTULO 1

Efecto de una mezcla de viroides procedente de un aislado de campo en el comportamiento de árboles de clementino Nules y naranjo Navelino injertados sobre citrange Carrizo

Effect of a field-source mixture of citrus viroids on the performance of 'Nules' clementine and 'Navelina' sweet orange trees grafted on Carrizo citrange

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ABSTRACT

A field-source mixture of citrus viroids was characterized and shown to contain CEVd, HSVd, CBLVd and CDVd. Sequencing results showed that: (i) CEVd contained the P_L and P_R characteristic of class A variants; (ii) HSVd was a non-cachexia variant; (iii) CBLVd was related to CVd-Ia variants; (iv) CDVd was a mixture of two types (CVd-IIIa and CVd-IIIb) of variants.

The presence of the same type of variants in inoculated 'Nules' clementine and 'Navelina' sweet orange trees on Carrizo citrange rootstocks was confirmed. The effect of infection was determined by assessing the performance of infected and non-infected trees growing in the field. Infection resulted in small trees with reduced canopy, yielding a reduced crop. Fruit characteristics were also affected: (i) clementine and sweet orange fruits from infected trees were larger than those from non-infected trees; (ii) clementine fruits from infected trees differed in shape from those of non-infected trees; (iii) sweet orange fruits from infected trees had maturity indexes and juice contents higher than those from non-infected trees; (iv) in both species, the density of the juice, the amount of soluble solids and the acidity of the fruits from infected trees were lower than those of fruits from non-infected trees. Infected trees had a poorly developed root system with fibrous roots containing fewer amyloplasts than non-infected trees. The results of an *in vitro* assay on the induction and development of roots in cultured explants are discussed.

INTRODUCTION

Viroids are unencapsidated, small, single-stranded, circular RNAs that replicate autonomously when inoculated in their plant hosts, where they may elicit diseases. Citrus species are natural hosts of several viroids, all of which are in the family *Pospiviroidae* and which are characterized by the presence of a central conserved region (CCR) and the absence of RNA self-cleavage mediated by hammerhead ribozymes (Flores *et al.,* 2005). In citrus, two viroid-induced diseases, exocortis and cachexia, were described (Fawcet and Klotz, 1948; Childs, 1950) before viroids were identified as plant pathogens (Diener, 1971; Semancik and Weathers, 1972). In addition to *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd), the respective agents of exocortis and cachexia, additional citrus viroids have been described (Duran-Vila *et al.,* 1986, 1988; Ito *et al.,* 2001; Serra *et al.,* 2008). Three of them, *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) (former *Citrus viroid III*, CVd-III), and

Citrus bark cracking viroid (CBCVd) (former *Citrus viroid IV*, CVd-IV), have been included as distinct species in the Virus Taxonomy Scheme established by the International Committee on Taxonomy of Viruses (ICTV) (Flores *et al.,* 2005). Even though these viroids were initially erroneously considered to be associated with the exocortis syndrome (Duran-Vila *et al.,* 1986), recent studies have elucidated their effect on field grown trees grafted on viroid-sensitive rootstocks (Semancik *et al.,* 1997; Vernière *et al.,* 2004; Vidalakis *et al.,* 2004).

Except for countries in which sanitation programs have been implemented, viroids are widespread in commercial citrus plantations where they are perpetuated with the propagation of infected, symptomless budwood. Usually field trees are co-infected with several viroids, a situation that for many years impaired the understanding of the effect of single or multiple infections on the performance of the host. When several viroids co-infect a single plant, the effects of some may be masked by others, resulting in either attenuation or enhancement of the expected symptoms. Attenuation or "cross protection" is observed when two strains of the same viroid or two closely related viroids co-infect the same plant. This phenomenon has been documented to occur between strains of HSVd (Semancik *et al.,* 1992), between strains of CEVd (Duran-Vila and Semancik, 1990) as well as between CEVd and CBCVd, two viroids that share sequence homology (Vernière *et al.,* 2006). On the contrary, when unrelated viroid pairs such as CEVd and CDVd, CBLVd and CDVd, CVd-V and CBLVd, and CVd-V and CDVd co-infect the same plant, the symptoms may be much more severe than expected if the effect of the symptoms induced by each viroid were only additive (Vernière *et al.,* 2006; Serra *et al.,* 2008). This phenomenon, initially described on the Etrog citron (*Citrus medica* L.) indicator plant (Duran-Vila *et al.,* 1993; Semancik and Duran-Vila, 1991; Serra *et al.,* 2008), also has been shown to occur in field grown trees (Vernière *et al.,* 2006).

A field trial was initiated in 1988 to determine the response of clementine (*C. clementina* Hort. ex Tan.) and sweet orange (*C. sinensis,* L) trees grafted on Carrizo citrange (*Poncirus trifoliata* × *C. sinensis*) to infection with a field source of citrus viroid(s). Here we report: (i) the characterization of the viroids present in the source; (ii) the performance of the experimentally infected trees; and (iii) the morphological traits associated with symptom expression.

MATERIALS AND METHODS

Viroid source

The original source of citrus viroid(s) used in this study was a 'Nules' clementine field tree. This inoculum has been maintained in 'Washington navel' sweet orange as part of the "virus" collection of the Instituto Valenciano de Investigaciones Agrarias (IVIA). Biological indexing was positive on Etrog citron 861-S1 (Calavan, 1968; Roistacher, *et al.,* 1977) which developed severe stunting and leaf epinasty, and negative on Parson's special mandarin, the indicator for cachexia (Roistacher *et al.,* 1973).

Plant materials and inoculation

Certified budwood of 'Nules' clementine and 'Navelina' sweet orange, that tested negative for graft transmissible diseases, was grafted on Carrizo citrange seedling rootstocks. One year later, a set of 18 trees of each species was graft-inoculated on the scion with the viroid source. Another set of 18 trees of each species was kept as non-inoculated controls. Three months after inoculation, the trees of both species were planted in a randomized block arrangement, each block consisting of two trees (one inoculated and one non-inoculated control), in a calcareous, alkaline (pH around 8.0), sandy-loam soil suitable for Carrizo citrange. The field plot was located at the Instituto Valenciano de Investigaciones Agrarias, Moncada (Valencia, Spain), and the trees were subjected to the standard pruning and harvesting operations of the region. In order to shape the canopy of the trees, two major prunings were performed during two consecutive years after planting, and suckers were removed in all subsequent years. Tools were disinfested with a sodium hypochlorite solution between trees. All the trees were indexed for viroid content ten years after planting.

Viroid indexing

Biological indexing was performed by graft inoculation on the sensitive 861-S1 selection of Etrog citron grafted on rough lemon (*C. jambhiri* Lush.) rootstock (Calavan, 1968; Roistacher *et al.,* 1977). The inoculated indicators were maintained at 28 to 32ºC for 6 months and were further analyzed as follows. Tissue samples (5 g of young leaves and stems) were homogenized (Virtis Cyclone IQ2 homogenizer) in 20 ml of extraction medium containing 15 ml phenol and 5 ml buffer (0.4 M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA, pH 7.0; 4% (v/v) mercaptoethanol). The 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 $MgCl_2$). Aliquots of these preparations (20 μl was equivalent to 300 mg fresh weight) were subjected to two consecutive rounds of polyacrylamide gel electrophoresis in 5% gels (sequential PAGE or sPAGE), the first under non-denaturing and the second under denaturing conditions (Rivera-Bustamante *et al.,* 1986). The circular forms of the viroids were viewed by silver staining (Igloi, 1983).

Viroid characterization

Tissue samples (500 mg) were placed in sealed plastic bags with a heavy net (Plant Print Diagnostics®, Valencia, Spain) containing 5 ml of extraction buffer (0.1 M Tris-HCl, pH 8.0; 50 mM EDTA; 0.5 M NaCl; 10 mM mercaptoethanol), and gently crushed with a manual roller (Plant Print Diagnostics®, Valencia, Spain). The homogenate was subjected to alkaline denaturation (Astruc *et al.,* 1996; Cañizares *et al.,* 1998), followed by reverse transcription and PCR amplification (RT-PCR) using viroid specific primer pairs. First-strand cDNA was synthesized at 60ºC using 27-mer primers specific for each viroid and thermoscript reverse transcriptase (Invitrogen®, Carlsbad, CA, USA) as described (Bernad and Duran-Vila, 2006). In order to recover full-length viroid DNA, second strand synthesis and DNA amplification were performed using a set of two contiguous forward and reverse primers specific for each viroid (Bernad and Duran-Vila, 2006). Electrophoresis in 2% agarose gels was used to confirm the synthesis of the expected DNA products. The amplified DNAs were sequenced with an ABI PRISM DNA sequencer 377 (Perkin-Elmer®, Wellesley, MA, USA).

When the amplified sequence showed heterogeneity, the amplified product was cloned in the pGEM-T vector (Promega®, Madison, WI, USA) and a consensus sequence was obtained by sequencing several clones representing the most frequent profiles revealed by single-strand conformation polymorphism (SSCP) analysis (Palacio and Duran-Vila, 1999). Briefly, the cloned sequences were amplified from the cloning vector by PCR, partially denatured and analyzed by PAGE in 14% gels and the DNA bands were visualized by silver staining (Igloi, 1983). Multiple sequence alignments were performed with Clustal W (Thompson *et al.,* 1994). The most stable secondary structure was determined with the MFOLD program (circular version) from the GCG package (Zuker *et al.,* 1989), and viewed with the RNAviz program (De Rijk and De Wachter, 1997).

Northern-blot hybridization

RNAs separated by sPAGE were electro-blotted (400 mA for 2 h) to positively charged nylon membranes (Roche Applied Science®, Basel, Switzerland) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.0) and immobilized by UV cross-linking. Viroid-specific DIG-labeled DNA probes were synthesized by PCR from plasmids containing the full-length viroid sequence (Palacio-Bielsa *et al.,* 1999). Prehybridization (60ºC for 2-4 h) and hybridization (60ºC overnight) were performed in 50% formamide and 6X SSPE (Sambrook *et al.,* 1989). The DIG label was detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and visualized with the chemiluminiscence substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (CSPD) (Roche Applied Science®, Basel, Switzerland).

Field symptom evaluation

Bark symptoms were recorded annually. In 2005, when the experiment was terminated, the trees were decapitated 30 cm above the bud union and the bark of the stumps was removed to verify symptoms on the wood above and below the bud union. The stumps were pulled out from the soil and symptoms below the soil level were also recorded.

Tree growth, fruit yield and quality.

Tree height, canopy size and trunk circumferences of the scion and the rootstock (10 cm above and below the bud-union line) were measured in 2003. Fruits were harvested manually and the yield of each tree was recorded from 1997 to 2002. Given the spherical shape of the canopy, its size was calculated as the volume of a sphere: $Vc = 4/3\pi R^3$, R being the radius of the canopy. The value of R was estimated as ½ of the average of the canopy height measurement and two perpendicular width measurements made in the centre of the canopy (Vernière *et al.,* 2004 y 2006). In 2003, 12 fruits were randomly collected from each tree and evaluated for fruit weight, fruit size, rind thickness, and juice volume, acidity, soluble solids, color and maturity index. All data from each citrus species were analyzed by ANOVA using the Statgraphics Plus 5.1. software.

Light microscopy

Root tips were fixed in formalin-acetic acid-alcohol (18:1:1), dehydrated through a graded ethanol series and embedded in paraffin. The embedded tissues were cut serially every 10 μm, stained with safranin and counter-stained with fast-green. To

identify the presence of carbohydrates in the cortex cells, the embedded tissues were also stained in Schiff's reagent (Jensen, 1962).

In vitro root formation

Four one-year-old Carrizo citrange seedlings were subjected to microsatellite analysis or SSRs (Simple Sequence Repeat Polymorphism) (Dellaporta *et al.,* 1983; Benbouza *et al*., 2006; Ruiz *et al.,* 2000; Froelicher *et al.,* 2007) using seven primer sets (TAA1, TAA15, TAA27, TAA33, TAA41, TAA45 and CAGG9) (Kijas *et al.,* 1997) to confirm their nucellar origin. Individual sets of two seedlings, each set composed of one tree graft inoculated from the Washington navel viroid source and one non-inoculated, were maintained at 23-25ºC or at 28-32ºC. Six months after inoculation the plants were decapitated and infection was confirmed by sPAGE analysis as described. The second flush of tissue was used as a source of material for *in vitro* root formation assay. Explants consisted of 30 stem internodes (1 cm long) per treatment prepared and disinfested as described previously (Duran-Vila *et al.,* 1989). Root initiation medium contained inorganic salts (Murashige and Skoog, 1962), 100 mg l^{-1} *i*-inositol, 0.2 mg l^{-1} thiamine hydrochloride, 1 mg l^{-1} pyridoxine hydrochloride, 1 mg l^{-1} nicotinic acid, 30g/L sucrose, 10 mg l^{-1} naphthalene acetic acid (NAA) and 10 g l^{-1} agar (Difto Bacto®, Detroit, MI, USA). After culturing the explants in root initiation medium for 45 days, they were transferred to root elongation medium (the same composition as root initiation medium but without NAA) for 15 additional days. The cultures were always maintained in the culture room at 26±1ºC and 60% relative humidity and exposed 16 h per day to an illumination of 40 μE m^{-2} s^{-1}.

RESULTS

Characterization of citrus viroids from the field source

The original source of the citrus viroid(s) had been maintained in 'Washington navel' sweet orange and budwood from this source was graft inoculated on Etrog citron. The sPAGE analysis of the inoculated citron plants demonstrated that the source contained several viroids with the mobilities of CEVd, CBLVd, HSVd and CDVd (data not shown). A nucleic acid preparation obtained from the Washington navel sweet orange tree was subjected to RT-PCR using primers specific for CEVd, HSVd, CBLVd, CDVd, CBCVd and CVd-V (Bernard and Duran-Vila, 2006; Serra *et al.,* 2008). The results confirmed that the source contained CEVd, HSVd, CBLVd and CDVd (data not shown).

The consensus sequence of the CEVd amplicon (GenBank Accession EU872276) had 371 nt (Fig. 1A) and 98.1% identity with a reference sequence (M30868) representative of class A, characterized as severe on the basis of its virulence in tomato (Visvader and Symons, 1986). The sequence of the P_L region located within the pathogenicity (P) domain possessed the characteristics of class A, while the P_R region located in the variable (V) domain had an insertion (+U129) and a transversion (U234→A). These changes resulted in a larger loop in the predicted rod-like secondary structure of the CEVd molecule. The consensus sequence of HSVd (EU872277) was 301 nt (Fig. 1B) with 99.0 % identity with the reference sequence of HSVd (variant IIa) (AF213503) (Reanwarakorn and Semancik, 1998; Palacio-Bielsa *et al.,* 2004), and in the V domain showed the six-nucleotide motif characteristic of the non-pathogenic HSVd sequence citrus variants (Reanwarakorn and Semancik, 1998). The consensus sequence of CBLVd (EU872278) was 328 nt (Fig. 1C), with 98.5% identity with CBLVd (variant Ia) (AF040721) (Semancik *et al.,* 1997; Foissac and Duran-Vila, 2000).

Since attempts to sequence the CDVd amplicon failed, cDNA clones derived from the amplicon were subjected to SSCP analysis (Fig. 1F). The migration of the ssDNAs suggested the existence of at least two subpopulations, with the size of two CDVd variants (variants IIIa and IIIb) (Fig. 1D, E). Sequencing of four clones confirmed the presence of at least two sequence variants (EU872279 and EU872280) of 297 and 294 nt, which had 99.3% and 99.7 % identity with the two CDVd variants (variants IIIa and IIIb) (S76452 and AF184147) (Rakowski *et al.,* 1994), respectively.

Assessment of viroid infection in inoculated 'Nules' clementine and 'Navelina' sweet orange trees

Inoculated and non-inoculated clementine and sweet orange trees grafted on Carrizo citrange rootstocks were established in the field in 1988. Ten years after planting, the four viroids (CEVd, HSVd, CBLVd and CDVd) present in the inoculum source were detected in all the inoculated trees whereas the non-inoculated trees had remained viroid-free. Samples from three randomly chosen infected clementine trees and three randomly chosen infected sweet orange trees were subjected to RT-PCR using primers specific for CEVd, HSVd, CBLVd, and CDVd. The consensus sequences of CEVd, HSVd and CBLVd obtained by sequencing the amplicons revealed only minor nucleotide changes in comparison with the consensus sequences of the viroid source maintained in Washington navel (data not shown).

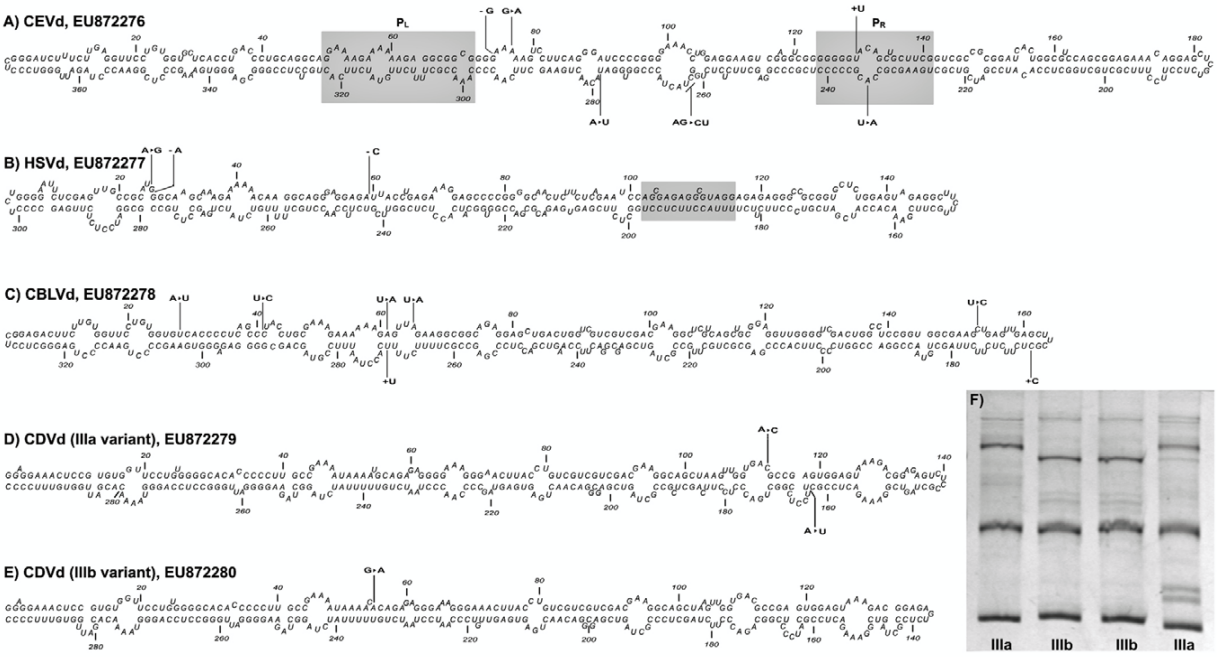

Fig. 1. Primary and secondary structure of the consensus sequences of CEVd (A), HSVd (B), and CBLVd (C) were obtained by sequencing the RT-PCR amplicons. Sequences of two CDVd variants (IIIa) (D) and (IIIb) (E) were generated from the sequences of four independent clones selected according to the SSCP profiles (F). The sequences were compared with the reference sequences of CEVd (type A) (Visvader and Symons, 1986), HSVd (variant IIa) (Palacio-Bielsa *et al.,* 2004), CBLVd (Foissac and Duran-Vila, 2000) and CDVd (variants IIIa and IIIb) (Rakowski *et al.,* 1994) (GenBank Accessions M30868, AF213503, AF040721, S76452 and AF184147 respectively). The P_L and P_R regions of CEVd and the "cachexia expression motif" of HSVd are shown shaded. Positions at which the derived sequences differ from the reference sequences are indicated by connecting lines above and below the sequence showing the variant nucleotide at that position.

When the nucleic acid preparations obtained from three sweet orange trees and three clementine trees were separated by sPAGE and subjected to northern hybridization with a CDVd specific probe, two bands were observed, with the slow and fast migrating viroid bands corresponding to the mobilities of variants IIIa and IIIb of CDVd, respectively (Fig. 2). The sweet orange trees had similar titers of IIIa and IIIb variants (Fig. 2, lanes 3-5), whereas in clementine trees (Fig. 2, lanes 6-8) the titers of variant IIIa were higher than those of variant IIIb.

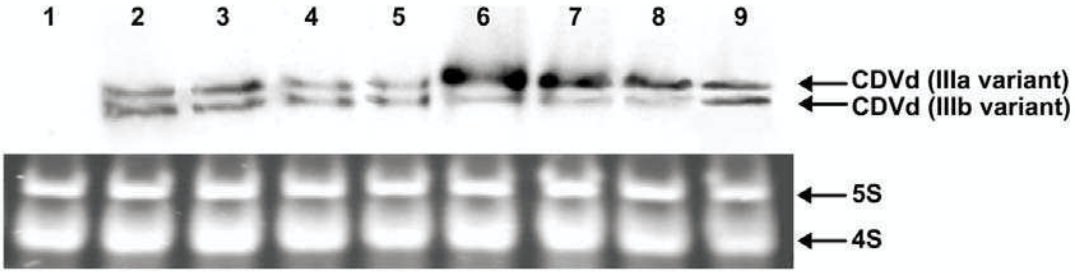

Fig. 2. Results of Northern-blot hybridization analysis with a CDVd specific probe. Lanes contain extracts from: (1) Non-infected Washington navel sweet orange; (2) Infected Washington navel sweet orange used as a source of inoculum; (3-5) Infected Navelina sweet orange trees; (6-8) Infected clementine trees; (9) Mixture of CDVd variants IIIa and IIIb from infected Etrog citron. Each variant is identified in the right margin. The 5S and 4S RNAs visualized in the ethidium bromide stained gel are shown to illustrate comparative amounts of material in each lane.

Effect of infection on tree size, yield and fruit quality

In general, infected trees were smaller than the non-infected controls (Figs. 3A and 3F), the difference being more important in clementine trees. As shown in Table 1, statistical analysis of tree size parameters (height, canopy volume, rootstock and scion circumferences) recorded in 2005 when the experiment was terminated, showed that measurements for all parameters on infected clementine trees were significantly smaller than on the non-infected trees, whereas in the case of sweet orange the differences were significant only for rootstock and scion circumferences.

Table 1. Effect of a field-source mixture of CEVd, HSVd, CBLVd, and CDVd on the size of infected 'Nules clementine and 'Navelina' sweet orange trees grafted on Carrizo citrange[z]

	'Nules' clementine			'Navelina' sweet orange		
Tree size parameters	**Non-infected**	**Infected**	**P-value**	**Non-infected**	**Infected**	**P-value**
Tree height (m)	2.0±0.1	1.6±0.1	0.0011	1.4±0.1	1.3±0.1	0.1157
Canopy volume (m^3)	3.2±0.3	1.4±0.1	0.0000	1.4±0.1	1.2±0.2	0.3462
Rootstock circumference (cm)	40.9±1.3	33.0±1.1	0.0001	36.1±0.9	29.7±1.2	0.0002
Scion circumference (cm)	36.4±1.3	29.9±0.7	0.0010	35.4±1.6	29.5±1.4	0.0098

[z]Data were subjected to ANOVA. Numbers are the mean ± standard error.

As shown in Table 2, except for 1997 for 'Nules' Clementine, the fruit yield of infected trees of both species was significantly smaller each year than that of the non-infected controls. Over six seasons (1997-2002), cumulative yields of infected clementine and sweet orange trees were only 57.4% and 67.0%, respectively, of those of the corresponding non-infected controls.

Table 2. Effect of a field-source mixture of CEVd, HSVd, CBLVd and CDVd on fruit yield of infected 'Nules clementine and 'Navelina' sweet orange trees grafted on Carrizo citrange[z]

	'Nules' clementine			'Navelina' sweet orange		
Fruit yield (kg) / year	**Non-infected**	**Infected**	**P-value**	**Non-infected**	**Infected**	**P-value**
1997	3.8±0.9	2.9±0.6	0.4056	17.9±1.8	11.9±1.9	0.0285
1998	8.6±1. 7	4.6±0.9	0.0440	14.9±2.1	9.4±1.5	0.0432
1999	26.0±3.7	14.0±1.6	0.0053	15.6±2.1	10.0±1.4	0.0337
2000	26.7±4.1	9.4±1.8	0.0005	7.7±1.1	4.9±0.8	0.0468
2001	48.6±4.2	27.9±2.5	0.0002	29.7±3.3	19.1±1.9	0.0089
2002	27.0±2.8	19.1±1.5	0.0175	21.1±3.3	15.5±1.9	0.1513
Cumulative yield (kg)	**135.6±12.6**	**77.9±6.5**	**0.0001**	**105.8±10.4**	**70.9±7.8**	**0.0111**

[z]Data were subjected to ANOVA. Numbers are the mean ± standard error.

As summarized in Table 3, viroid infection affected the characteristics of fruits. For Nules clememtine, rind thickness, maturity index, juice volume, fruit height and color index values were not significantly different between treatments (Table 3).

Table 3. Effect of a field-source mixture of CEVd, HSVd, CBLVd and CDVd on the quality of fruit from infected 'Nules' clementine and 'Navelina' sweet orange trees grafted on Carrizo citrange[z]

	'Nules' clementine			'Navelina' sweet orange		
Fruit quality parameters	**Non-infected**	**Infected**	**P- value**	**Non-infected**	**Infected**	**P- value**
Average fruit weight (g)	104.7±3.0	114.0±2.5	0.0222	199.6±5.0	233.9±6.1	0.0001
Fruit density (g ml^{-1})	0.9±0.0	0.8±0.0	0.0016	0.9±0.0	0.9±0.0	0.5151
Fruit height (mm)	50.6±0.4	51.7±0.6	0.1219	71.9±0.8	76.7±1.1	0.0011
Fruit diameter (mm)	63.3±0.6	66.2±0.6	0.0025	73.8±0.6	77.5±0.7	0.0005
Fruit shape (diameter/height)	1.4±0.0	1.3±0.0	0.0161	1.0±0.0	1.0±0.0	0.2213
Color index	15.9±0.5	14.8±0.4	0.1026	14.3±0.4	14.7±0.4	0.4628
Rind thickness (mm)	2.5±0.1	2.5±0.1	0.7165	4.8±0.1	5.3±0.4	0.1733
Volume of juice (ml)	541.7±17.6	577.8±11.9	0.0020	986.9±58.2	1245.6±36.7	0.0006
Density of juice (g ml^{-1})	1.1±0.0	1.0±0.0	0.0000	1.052±0.0	1.047±0.0	0.0247
Soluble solids (%)	14.9±0.1	13.0±0.1	0.0000	14.1±0.2	13.3±0.2	0.0120
Acidity	9.0±0.2	7.6±0.1	0.0000	12.0±0.4	10.3±0.2	0.0011
Maturity index	17.7±0.3	17.1±0.4	0.4430	11.9±0.4	13.0±0.3	0.0420

[z]Data were subjected to ANOVA. Numbers are the mean ± standard error.

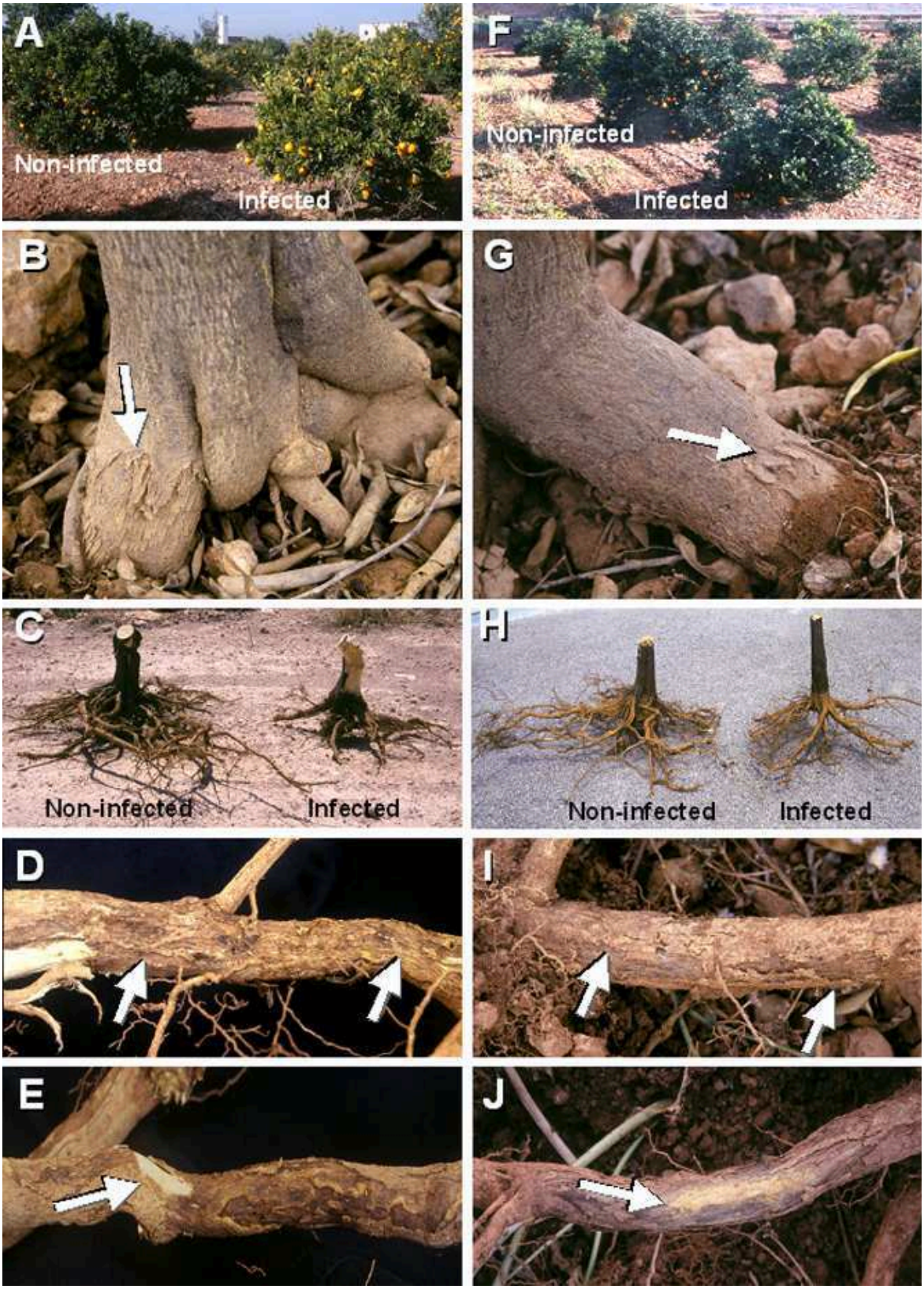

Fig. 3. Symptoms of 'Nules' clementine trees (A-E) and 'Navelina' sweet orange trees (F-J) grafted on Carrizo citrange. (A, F) Comparison of overall growth of infected and non-infected trees. (B, G) Small scales (indicated by arrows) observed below the soil level on the Carrizo citrange rootstock of infected trees. (C,H) Comparison of the root systems from infected trees and non-infected tree. (D,I) Scales and dark lesions (indicated by arrows) on infected roots. (E,J) Arrows point to the absence of gum deposits in the wood of roots after scraping away the bark.

Average weight and diameter of fruits from infected trees were significantly larger, whereas values for fruit density, fruit shape, juice density, soluble solids and acidity were larger for fruits from uninfected trees. For Navelina sweet orange, values for fruit density, shape, and color index and rind thickness were not significantly different between treatments. Values for average fruit weight, height, diameter, juice volume maturity were significantly greater for fruits from infected tress, whereas values for juice density, soluble solids and acidity were significantly greater for fruits from uninfected trees.

Symptoms

No bark scaling symptoms were observed in the trunk of the Carrizo citrange rootstock of the clementine and sweet orange trees. However, after removing the soil around the trees, the infected trees of both species showed small scales on the main roots of the Carrizo citrange rootstock (Figs. 3B, 3G). After decapitating the canopy at about 30 cm above the bud-union line and removing the bark from the remaining trunk, no symptoms were observed in the wood below or above the bud-union. Observations of the root system after the stumps were pulled out from the soil showed that infected trees of both scions had a visibly smaller root system than their non-infected counterparts (Figs. 3C, 3H). Additionally, the roots of infected trees presented scales and dark lesions (Figs. 3D, 3I) never observed on the roots of non-infected trees. Such lesions resembled those of *Phytophthora* root rot, however unlike *Phytophthora* lesions, they were dry and devoid of gum exudates. No gum deposits in the wood were observed after scrapping away the bark (Figs. 3E, 3J).

No histological abnormalities were observed in mature fibrous roots from infected trees, which showed the arrangement of four primary phloem strands alternating with xylem ridges characteristic of roots from non-infected trees (Figs. 4A, B left panels). However, the storage parenchyma cells of the root cortex of non-infected trees (Figs. 4A, B, indicated by c in the left panels) contained numerous dark-stained bodies that were very scarce in the cortex of infected trees (Figs. 4A, B right panels). As shown by the Periodic Acid–Schiff's (PAS) reaction, which is a specific staining for carbohydrates, these bodies had an intense purplish-red stain, confirming that they were amyloplasts accumulating in the storage parenchyma (Fig. 4, left inset). The number of amyloplasts in the root cortex was estimated in a random selection of three sections from six independent roots. Statistical analysis showed significant differences (Pearson Gi-dos, $P \leq 0.01$) between infected (average: 12.5 cells with amyloplasts per section) and non-infected (average: 26.0 cells with amyloplasts per section) sweet orange trees grafted on Carrizo citrange, and between infected (average: 7.3 cells with

amyloplasts per section) and non-infected (average: 29.6 cells with amyloplasts per section) clementine trees grafted on Carrizo citrange.

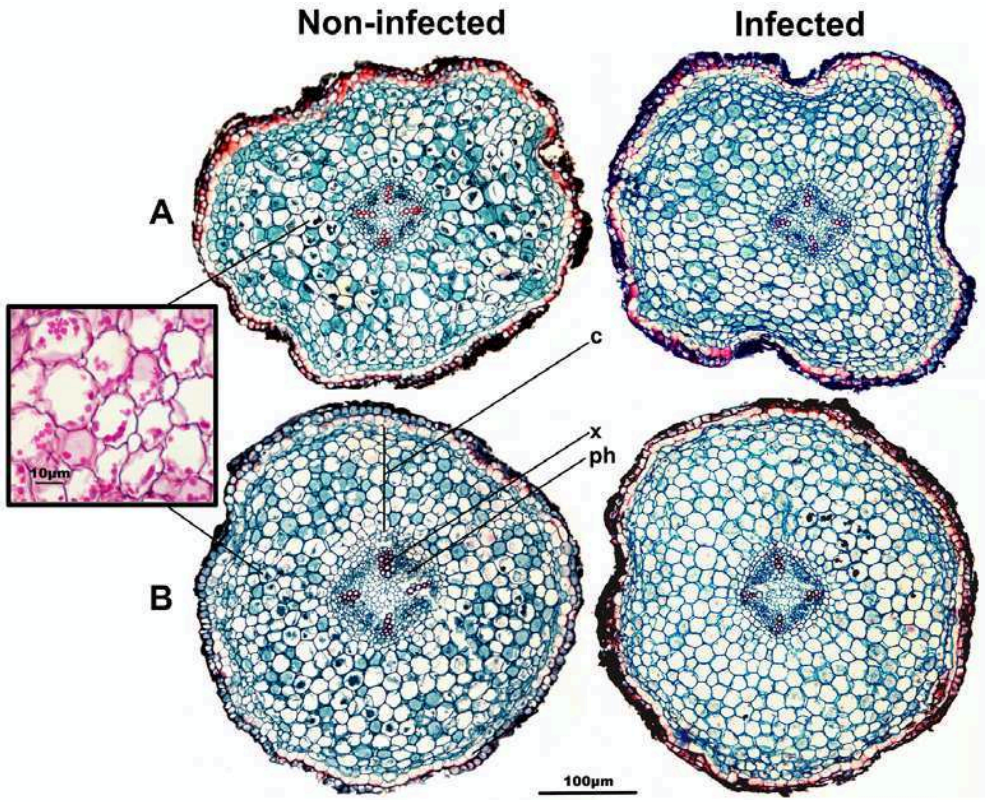

Fig. 4. Histological sections of fibrous roots of Carrizo citrange rootstocks from non-infected (left) and infected (right) 'Nules' clementine trees (A) and Navelina' sweet orange trees (B) showing the characteristic arrangement of phloem strands (ph) alternating with xylem ridges (x). The cortex cells (c) of non-infected trees (left) contain dark stained bodies that are very scarce in roots of infected trees (right). The left inset shows these bodies stained an intense purplish red after Schiff's (PAS) reaction. Scale-bar at the bottom indicates 100 µm increments.

Root formation *in vitro*

An *in vitro* assay was designed to estimate the effect of viroid infection on the capacity of Carrizo citrange explants to regenerate roots. In order to avoid the use of seedling plants that were not true-to-type, leaf samples were subjected to SSR analysis to confirm their nucellar origin. Ploidy analysis confirmed that none of the plants were autotetraploid (data not shown), an abnormality that is not infrequent in the progeny of diploid Carrizo citrange trees (Schwarz, 2001). Two sets of two seedlings each (one graft inoculated and one non-inoculated control) were maintained at 22-25ºC or at 28-32ºC, and six months after inoculation the nucleic acid extracts from these plants were subjected to sPAGE analysis to confirm infection (Fig. 5A, lanes 1, 3). Based on visual assessment of a comparison to a dilutions series of viroid extract from seedlings maintained at 28-32ºC (Fig. 5B, lanes 2-7), the viroid titers in the Carrizo citrange

seedlings maintained at 22-25ºC were estimated to be one-hundred and five-fold less for CEVd and CBLVd, respectively and two-fold less for HSVd and CDVd compared to titers in seedlings maintained at 28-32ºC (Fig. 5B).

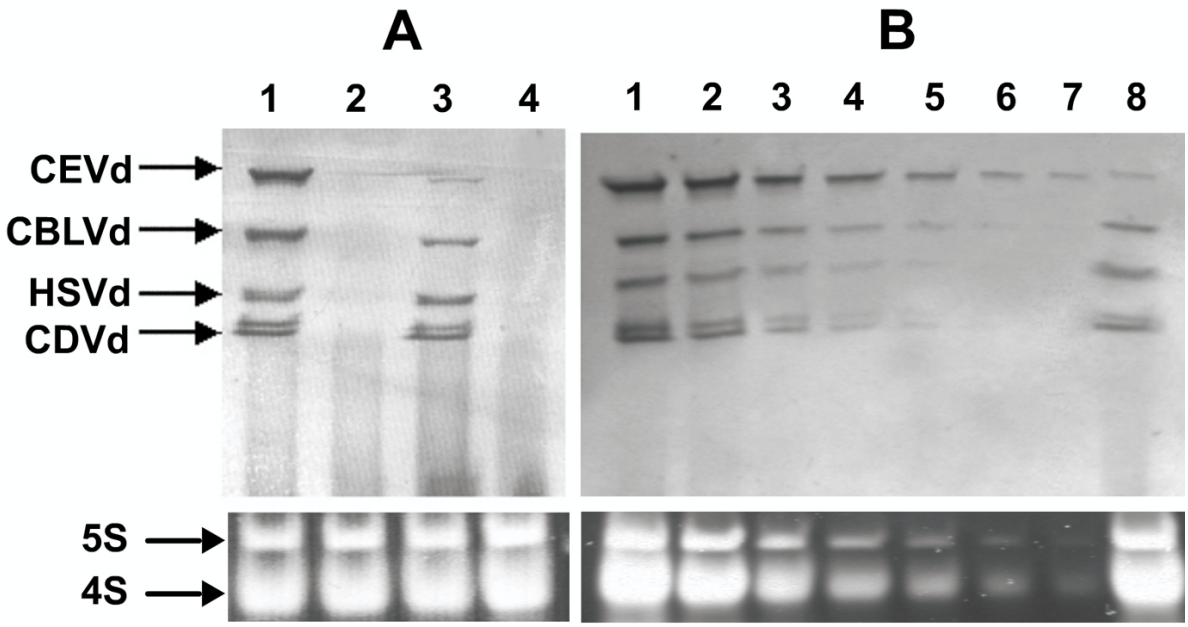

Fig. 5. (A) Sequential PAGE analysis of nucleic acid extracts from inoculated (lanes 1, 3) and non-inoculated (lanes 2, 4) Carrizo citrange seedlings grown at 28-32ºC (lanes 1, 2) and at 22-25ºC (lanes 3, 4). (B) Sequential PAGE analysis of nucleic acid extracts from inoculated Carrizo citrange seedlings grown at 28-32ºC; undiluted extract (lane 1) and extracts diluted 1/2, 1/5, 1/10, 1/20, 1/50, 1/100 (lanes 2-7, respectively) or undiluted extract from inoculated seedlings grown at 22-25ºC (lane 8). The 4S RNAs (A) and 5S RNAs (B, diluted as indicated for each respective lane) visualized in the ethidium bromide stained gel are shown to illustrate comparative amounts of nucleic acid loaded in each lane.

The *in vitro* cultured stem-segment explants (30 explants per treatment in two separate assays) developed root primordia after 4 wk in culture. After 45 days, they were transferred to elongation medium to assess root growth. For explants from seedlings grown at 22-25ºC there was no significant difference in percentage of explants with roots between infected and non-infected seedlings (Table 4). For explants collected from seedlings grown at 28-32ºC, the number of infected explants that produced roots was significantly lower (42.3%) than in the case of non-infected explants (89.2%) (Table 4, Fig. 6).

Multifactor ANOVA of the number of roots per explant and average root length revealed that the number and length of the roots were affected as a result of infection ($F=22.12$; $P \leq 0.0001$) as well as by the temperature at which the Carrizo citrange seedlings used as the source of explants were grown ($F=53.77$; $P \leq 0.0001$), with a significant reduction in root production in infected plants grown at 28-32ºC and in root length due to infection regardless of the temperature at which seedlings were grown

(Table 4). In addition, a synergistic interaction was found between the two factors (infection and temperature) (F=28.74; P ≤0.0001), a result that is in full agreement with the higher titers reached by CEVd and CBLVd in plants growing at 28-32ºC rather than at 22-25ºC (Fig. 5). Table 4 illustrates how infection affected the three rooting parameters studied.

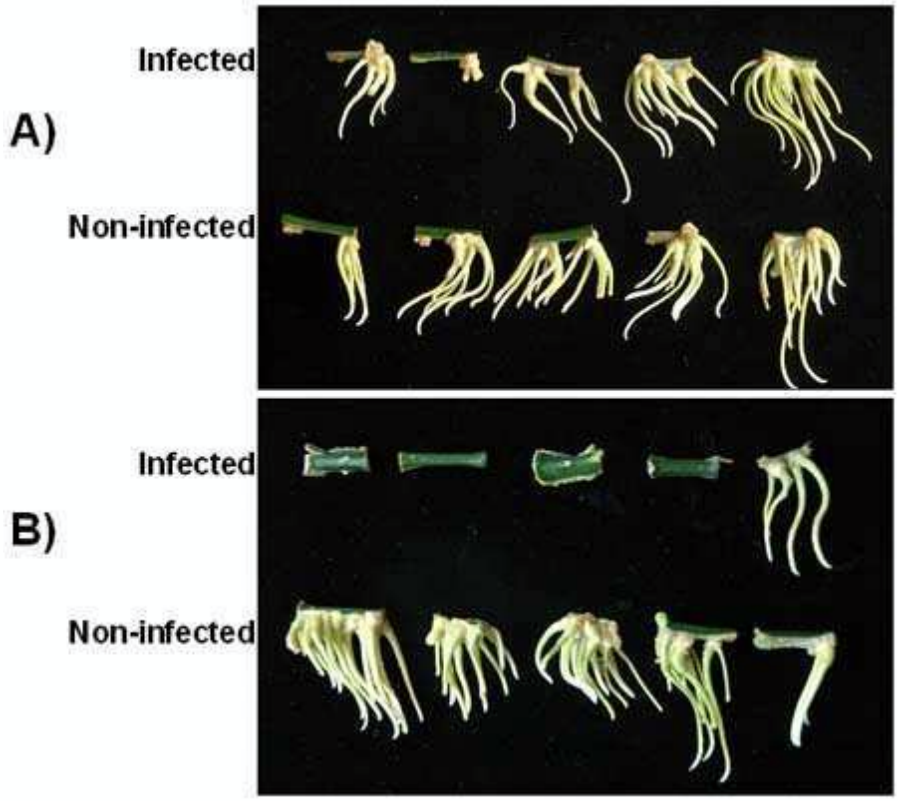

Fig. 6. Root formation on *in vitro* cultured explants from infected and non-infected Carrizo citrange seedlings grown at 22-25ºC (A) or at 28-32ºC (B).

Table 4. Effect of a field-source mixture of CEVd, HSVd, CBLVd and CDVd on root formation of infected Carrizo citrange explants cultured *in vitro*[z]

	Incubation of Carrizo citrange plants					
	22-25ºC			28-32ºC		
Rooting parameters	**Non-infected**	**Infected**	**P- value**	**Non-infected**	**Infected**	**P- value**
Explants with roots (%)	95.0	96.3	0.8528	89.2	42.3	0.0086
Number of roots / explant	9.9±0.6	10.3±0.7	0.6587	8.7±0.7	2.6±0.5	0.0000
Average root length (cm)	2.0±0.0	1.8±0.0	0.0000	1.7±0.0	1.0±0.1	0.0000

[z]Data are the average of two separate assays in which 30 explants per treatment were cultured. Data were subjected to ANOVA. Numbers are the mean ± standard error

DISCUSSION

Citrus exocortis disease, caused by CEVd, was described in 1948 as a "bark shelling or scaling" disorder of the trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock of grafted trees, which also showed marked dwarfing (Fawcett and Klotz, 1948). When Etrog citron (*Citrus medica*) was later used as an indicator for exocortis disease indexing, a variety of symptoms, ranging from severe to very mild, were erroneously considered, for many years, as evidence for the occurrence of CEVd strains. However, the identification and biological characterization of additional viroids, naturally occurring in citrus, has shown that each viroid induces specific symptoms not only on Etrog citron (Duran-Vila *et al.,* 1988), but also on trees grafted on trifoliate orange and trifoliate orange hybrid rootstocks (Semancik *et al.,* 1997; Vernière *et al.,* 2004; Vidalakis *et al.,* 2004). Except for orchards established with certified viroid-free nursery plants, field trees are usually co-infected with several viroids, a situation that prevented identification of symptoms expressed by specific viroids in commercial species and cultivars. Only recently, the attenuation or enhancement of viroid-induced effects due to inoculum of known composition could be studied and elucidated in field grown clementine trees grafted on trifoliate orange rootstocks (Vernière *et al.,* 2006). The viroid field source characterized in the present study was found to contain CEVd, HSVd, CBLVd and two distinct variants of CDVd, a combination of viroids that previoulsy had been found to result in synergistic viroid interactions, and which caused in addition to the expected severe rootstock bark-scaling, a most dramatic effect on tree size and fruit yield of clementine trees grafted on trifoliate orange rootstock (Vernière *et al.,* 2006).

In the present study, infected trees did not show the severe bark-scaling symptoms characteristic of exocortis disease, but the roots were affected by small lesions and scales never reported before. Even though the CEVd variant in the inoculum source was characterized as a class A member with the pathogenic determinants of CEVd variants that induce severe symptoms in tomato (Visvader and Symons, 1985, 1986) and in trifoliate orange (Vernière *et al.,* 2004), the observed symptoms were found to be mild in Carrizo citrange. These unexpectedly mild symptoms could be because Carrizo citrange is less sensitive to the effects of CEVd than trifoliate orange, as well as to the relatively mild climatic conditions where the trials were conducted. In fact, in the same region, we have noticed similar mild symptoms in CEVd infected field trees on Carrizo or Troyer citrange rootstocks (unpublished data). However, and more importantly, in spite of the mild symptoms observed in the rootstock, the infected trees were generally smaller, produced reduced yields with

larger fruit size but poorer fruit quality in comparison with non-infected trees. Earlier studies on using viroids to control tree size in high-density plantings showed a similar marked effect of viroid infection in tree growth, canopy size and yield (Polizzi *et al.,* 1991; Semancik *et al.,* 1997; Hutton, *et al.,* 2000). Fruit quality was affected in lemon trees (Broadbent *et al.,* 1988) but not in sweet orange (Polizzi *et al.,* 1991; Hutton, *et al.,* 2000). The discrepancy observed in this and past studies regarding the effect on sweet orange might be due to the specific viroid combination used in the present study that was not used in previous dwarfing assays.

The performance of a normal healthy tree is based on an adequate balance between the canopy manufacturing, carbohydrates and other photosynthesis products, and the root system supplying water and mineral nutrients. In other words, the size of the canopy reflects the size of the root system and vice versa. In the case of grafted trees, this balance is also affected by the degree of compatibility between the rootstock and the scion. In the case of viroid infected trees made of viroid-tolerant cultivars such as sweet orange or clementine on viroid-sensitive rootstocks such as Carrizo citrange, the results of the present study have shown that these trees have an underdeveloped root system as well as a smaller canopy than non-infected trees. The cortex of fibrous roots from infected trees had a very smaller number of parenchyma cells containing stored carbohydrates compared to non-infected trees. The absence of stored carbohydrates very probably reflects the poor performance and scion/rootstock imbalance of viroid-infected trees.

Early studies from *in vitro* cultures of CEVd-infected tomato explants showed that root formation was impaired (Duran-Vila and Semancik, 1982). Similar results were obtained in the present study when explants from viroid-infected Carrizo citrange seedlings were cultured in root induction medium. Most explants collected from seedlings growing at 22-25ºC produced roots regardless of whether the plants were infected or not. However, in the case of explants that had been collected from seedlings growing at 28-32ºC, the number of infected explants that produced roots was twice as low as in the case of non-infected explants. In addition, root development (number of roots per explant and average root length) was lower in viroid infected explants, in particular when they came from the infected seedlings grown at the higher temperature. Interestingly, in these “high temperature” seedlings, the titers of CEVd and CBLVd, as judged by the intensity of the viroid bands in the sPAGE analysis, were higher than those in the “low temperature” seedlings. Therefore, the hindered root development characteristic of the explants from the “high temperature” seedlings could be due to the higher viroid titers.

The hindered root development shown by *in vitro* grown explants from the "high temperature" Carrizo citrange seedlings, mimics the poor root development shown by the viroid infected clementine and sweet orange field trees grafted on Carrizo citrange rootstocks, as reported here. Therefore *in vitro* assays on root development, similar to that reported here, might be a quick aid to evaluate rootstock sensitivity to viroid infection.

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CAPÍTULO 2

Los viroides de los cítricos están restringidos al floema en *Citrus karna*, *Poncirus trifoliata* y *Citrus aurantium*

Citrus viroids are phloem restricted in *Citrus karna, Poncirus trifoliata and Citrus aurantium*

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ABSTRACT

Viroid systemic infection involves long distance movement through the phloem as well as cell-to-cell movement through the plasmodesmata. All the viroids (CEVd, CBLVd, HSVd, CVd-V, CDVd or CBCVd) inoculated in Etrog citron were consistently detected by northern blot hybridization in bark, leaf midribs and leaf blades devoid of the midrib, and mesophyll protoplasts. In contrast, the same analysis performed in *Citrus karna*, sour orange and trifoliate orange plants infected with the same viroids failed to detect the viroids in the mesophyll protoplasts. These results suggest that in *C. karna,* sour orange and trifoliate orange, and probably other citrus species but not Etrog citron, viroid infection is essentially phloem restricted and that a barrier preventing trafficking from the bundle sheath to adjacent tissues must exist in certain citrus species. Using *C. karna* infected with CEVd as an experimental model, the viroid was able to overpass this barrier when *C. karna* was top-worked with Etrog citron or when it was co-infected with CTV. The putative mechanisms involved are discussed.

INTRODUCTION

Viroids are unencapsidated, small, single-stranded, circular RNAs that replicate autonomously when inoculated in their plant hosts where they may elicit diseases. Citrus are natural hosts of several viroids, all of them belonging to the family *Pospiviroidae* characterized by the presence of a central conserved region (CCR) and the absence of RNA self-cleavage mediated by hammerhead ribozymes (Flores *et al.,* 2005). Several viroids, *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) (former *Citrus viroid III,* CVd-III) and *Citrus bark cracking viroid* (CBCVd) (former *Citrus viroid IV*, CVd-IV) have been included as distinct species in the Virus Taxonomy Scheme established by the Committee on Taxonomy of Viruses (www.ictvonline.org/virusTaxonomy.asp). Two additional viroids, *Citrus viroid original sample* (CVd-OS) reported in Japan (Ito *et al*., 2001) and the recently characterized *Citrus viroid V* (CVd-V) (Serra *et al.,* 2008) are still considered as tentative new species of the genus *Apscaviroid.* All these viroids became systemic upon inoculation to their citrus hosts, which may develop disease symptoms (sensitive hosts) or replicate the viroid(s) as latent infections (tolerant hosts).

Most citrus species are tolerant hosts and act as viroid reservoirs, but when they are transmitted to sensitive species by mechanical transmission (Barbosa *et al.,* 2005) or by graft propagation of infected tolerant scions to sensitive rootstocks, disease symptoms may develop. Among the viroid sensitive hosts, Etrog citron (*Citrus medica*) and in particular the sensitive selection 861-S1 has been widely used for infectivity purposes because it develops viroid specific symptoms and accumulates easily detectable viroid titers.

The first evidence indicating that viroids infect their hosts in a systemic manner were provided by Palukaitis (1987), who established that phloem was the pathway for long distance movement of *Potato spindle tuber viroid* (PSTVd), and was latter confirmed by *in situ* hybridization studies (Zhu *et al.,* 2001). Studies also conducted with PSTVd indicate that cell-to-cell movement occurs through plasmodesmata and it is mediated by the viroid structural motifs (Ding *et al.,* 1997). With these and other evidences (reviewed by Flores *et al*., 2005; Ding and Wang, 2009) the infection process followed by viroids upon inoculation involves: (i) intracellular movement to reach their replication site followed by the release of the progeny to the cytoplasm; (ii) cell-to-cell movement to infect adjacent cells via the plasmodesmata and entry into the phloem; (iii) long-distant movement within the phloem to reach upper leaves and organs and to the roots; (iv) exit from the phloem to invade non-vascular cells.

The results of previous studies conducted to compare the response of diverse hosts to infection with different citrus viroids showed that their replication/accumulation was species dependent and *Citrus karna* was identified as a host in which upon inoculation, citrus viroids were present at unusually high concentrations (Barbosa *et al.,* 2002). *C. karna* is an old Indian species of unknown origin, probably a natural hybrid. Both tree and fruit are distinctive and exhibit characters of both Rough lemon (*C. jambhiri*) and sour orange (*C. aurantium*) (Hodgson, 1967) and its commercial value arises from the fact that has been extensively employed in India as a rootstock. Even though, the recognition of *C. karna* as a true species is controversial, data on genetic diversity suggest that it belongs to the lemon-lime group and that it arose through interspecific hybridization (Herrero *et al.,* 1996).

The present study reports evidences demonstrating that viroid systemic infection in *C. karna* does not follow the proposed scheme because viroids in this especies are phloem restricted, and that the same apply to trifoliate orange (*Poncirus trifoliata*) and sour orange, both widely used as rootstocks in commercial orchards.

MATERIALS AND METHODS

Viroid sources

The viroid sources used were CEVd (CEVd-117) (Gandía *et al.,* 2005), HSVd-1 (non-cachexia inducing strain CVd-IIa-117) and HSVd-2 (cachexia inducing strain X-707) (Palacio-Bielsa *et al.,* 2004), CBLVd (CVd-Ia-117) (Foissac and Duran-Vila, 2000), CDVd (CVd-IIId) (Foissac and Duran-Vila, 2000), CVd-V (Serra *et al.,* 2008) and CBCVd (CVd-IV-Ca) (Francis *et al.,* 1995). Etrog citron plants infected with these viroids were used as a source of viroids for graft-transmission to different hosts.

Plant sources and inoculation

Seedling plants were produced from seeds collected from a *C. karna* tree (IVIA-242) maintained at the Citrus Germplasm Bank of the Instituto Valenciano de Investigaciones Agrarias (www.ivia.es/germo/). When the plants reached a height of 20 cm, they were inoculated using two graft patches from viroid infected Etrog citron. Three seedlings were inoculated with each of the viroid sources and were grown under greenhouse conditions at 28-32ºC.

Protoplast isolation

Protoplast isolation was essentially performed as described by Grosser and Gmitter (1990) and subsequently modified by Chen *et al.* (2008). Selected leaves from greenhouse grown plants were surface disinfested by immersion in 20% commercial bleach for 10 min and rinsed three times with autoclaved water. The leaves were cut into thin strips with a sharp scalpel and incubated in 60 mm plates in a mixture of 2.5 ml of 0.6 M BH3 protoplast culture medium and 1.5 ml enzyme solution containing 0.7 manitol, 12.0 mM $CaCl_2$, 6.0 mM MES, 1.4 mM $NaHPO_4$, 2% (w/v) Onozuka RS cellulase and 2% (w/v) Macerozyme R-10. The plates were kept in a rotatory shaker (35 rpm) at 25ºC in the dark for at least 16 h. The protoplasts were purified by passage through a 45-µm stainless steel mesh screen and then by centrifugation on 25% sucrose and 13% manitol gradient (Fig. 1). Protoplast yields were evaluated after the gradient purification step by using a Fuchs-Rosenthal hematocytometer chamber and the suspension was adjusted to 2 x 10^6 protoplasts/ml.

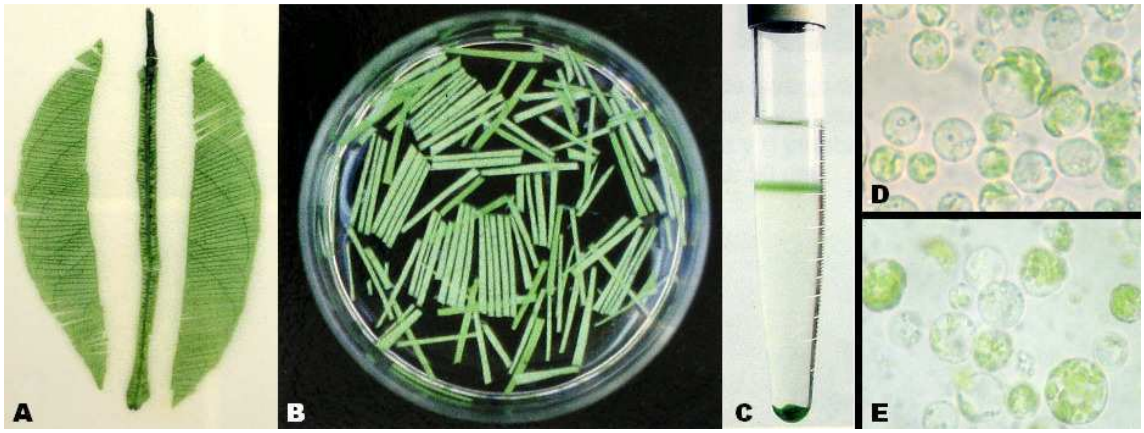

Fig. 1. Process of protoplast isolation. (A) Leaf blades cut into thin strips with a sharp scalpel. (B) Incubation of leaf strips in a 60 mm plates containing a mixture of 2.5 ml of 0.6 M BH3 protoplast culture medium and 1.5 ml enzyme solution. (C) Purification of protoplasts through gradient step. Purified protoplasts of Etrog citron (D), and *C.karna* (E).

Viroid analysis

Tissue samples (bark, leaf blade or midrib) were powdered in liquid nitrogen with mortar and pestle and homogenized by vortexing in extraction medium (0.4 M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA, pH 7.0; 4% (v/v) β-mercaptoethanol) containing water-saturated phenol (Semancik *et al.,* 1975). The 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 $MgCl_2$). Protoplast samples (2 x 10^6 protoplasts/ml) were centrifugated and eluted in 300 µl of extraction medium with 300 µl of phenol:cloroform:isoamilic alcohol (48:48:2). Nucleic acids were concentrated by ethanol precipitation and resuspended in 20 µl TKM buffer.

Northern blot hybridization analyses were performed as described by Murcia *et al.* (2009). Aliquots of the nucleic acid preparations (20 µl equivalent to 300 mg fresh weight, or 2 x 10^6 protoplasts) were subjected to 5% non denaturing PAGE and stained with ethidium bromide. The RNAs separated by 5% PAGE 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), immobilized by UV cross-linking and hybridized with viroid specific probes. Digoxigenin (DIG)-labeled DNA probes were synthesized by PCR using as a template a cloned plasmid containing full-length viroid monomeric DNA, as described earlier (Palacio-Bielsa *et al.,* 1999). Prehybridization (at 60ºC for 2-4 h) and hybridization (at 60ºC overnight) were performed in 50% formamide and and 5X SSC buffer containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking reagent. 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). The hybridization signals were visualized with the Luminiscent Image Analyzer LAS-3000 (Fujifilm) and quantified with the Multi Gauge V3.0 package (FujiFilm).

RESULTS

Viroid detection in tissues and cells of graft inoculated *C. karna* and Etrog citron plants

Preliminary attempts to use *C. karna* seedlings as an experimental host for viroid infectivity assays were rather contradictory. Briefly, seedlings were inoculated with CEVd, HSVd, CBLVd, CDVd or CBCVd (three plant each) and infection was assessed by northern hybridization using leaf samples, at monthly intervals over a six-month period. Unexpectedly, positive detection of the inoculated viroids was always erratic (data not shown). A study was then initiated to compare the viroid distribution in tissues of these inoculated *C. karna* seedlings with those of infected Etrog citron plants. Northern hybridization analysis of nucleic acid preparations from bark samples recovered from young shoots yielded strong hybridization signals in both hosts, regardless of the inoculated viroid (Fig. 2A, 2C). Analysis of preparations from fully expanded leaves devoid of petioles and midribs yielded strong hybridization signals in Etrog citron but not generally in *C. karna* (Fig. 2B, 2D). Quantification of the hybridization signals obtained from Etrog citron samples showed that with the exception of CBLVd, the estimated viroid titers were 1.4 (CVd-V) to 4.5 (HSVd-1) times higher in the bark than in the leaf blade samples (Fig. 2A, 2B). The same trend was found in the case of infected *C. karna* seedlings, with estimated viroid titers being 5.5 (CBCVd) to 1.7 (CBLVd) higher in the bark than in the leaf blade samples (Fig. 2C, 2D). The two strains of HSVd and CDVd were undected in the leaf blade samples of *C. karma* (Fig. 2D). These results altogether suggest that in *C. karna* viroid downloading from the phloem to adjacent cells is somewhat impaired.

These observations were further confirmed through the analysis of protoplast preparations isolated from young expanded leaves sampled from all these viroid/host combinations. In all cases, consistent protoplasts yields were obtained after purification in a sucrose/manitol gradient. Northern hybridization analysis of equalized protoplast

preparations (2X10^6 protoplasts) revealed that all the viroids were detected in the Etrog citron mesophyll protoplasts whereas they were undetected in the *C. karna* protoplasts (Fig. 2E, 2F). These results demonstrate that viroids are efficiently downloaded from the phloem to adjacent cells in Etrog citron but are phloem restricted in *C. karna.*

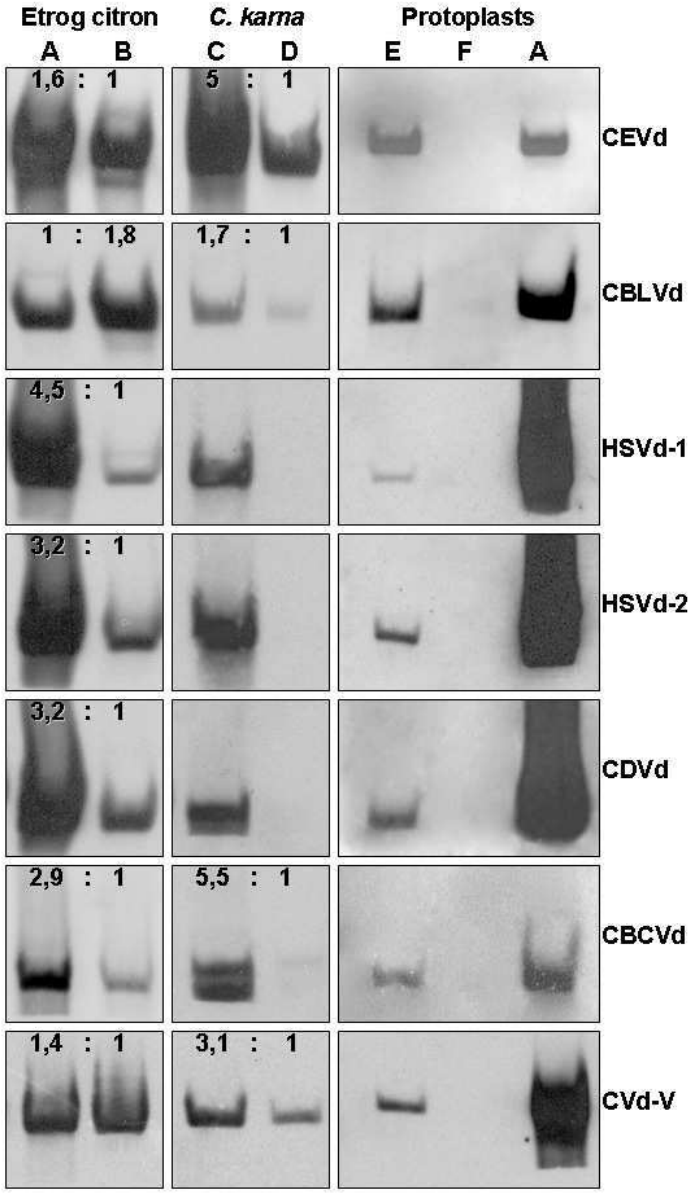

Fig. 2. Northern hybridization signals of nucleic acid preparations from bark samples recovered from young shoots (A) and fully expanded leaves devoid of petioles and midribs (B) of Etrog citron, and from bark (C) and leaves devoid of petioles and midribs (D) of *C. karna* infected with different viroids. Northern hybridization signals of equalized protoplast preparations (2X10^6 protoplasts) of Etrog citron (E) and *C. karna* (F).

Viroid is also phloem restricted in *Poncirus trifoliata* and *Citrus aurantium*

In order to assess if the finding that citrus viroids are phloem restricted in *C. karna* is an unusual characteristic of this genotype or a general behaviour of citrus

hosts, an additional study was performed using *P. trifoliata*, a viroid sensitive species that like Etrog citron develop symptoms as a result of viroid infection, and with *C. aurantium*, which is tolerant to viroid infection. Seedlings of both species were graft-inoculated with the same viroid sources of CEVd, HSVd, CBLVd, CDVd, CVd-V and CBCVd, and infection was confirmed by northern blot hybridization six months after inoculation. Samples of bark, midribs and leaves (devoid of petioles and midribs) were viroid tested by northern blot hybridization. Bark and midrib samples were clearly positive (Fig. 3A, 3A', 3B, 3B'), whereas leaf samples devoid of petioles and midribs were negative or yielded weak hybridization signals (Fig. 3C, 3 C'). These results were further confirmed with analysis of equalized protoplast preparations ($2X10^6$ protoplasts) of both species, in which viroids were undetected in these two viroid/host combinations (Fig. 3D, 3D', 3E, 3E').

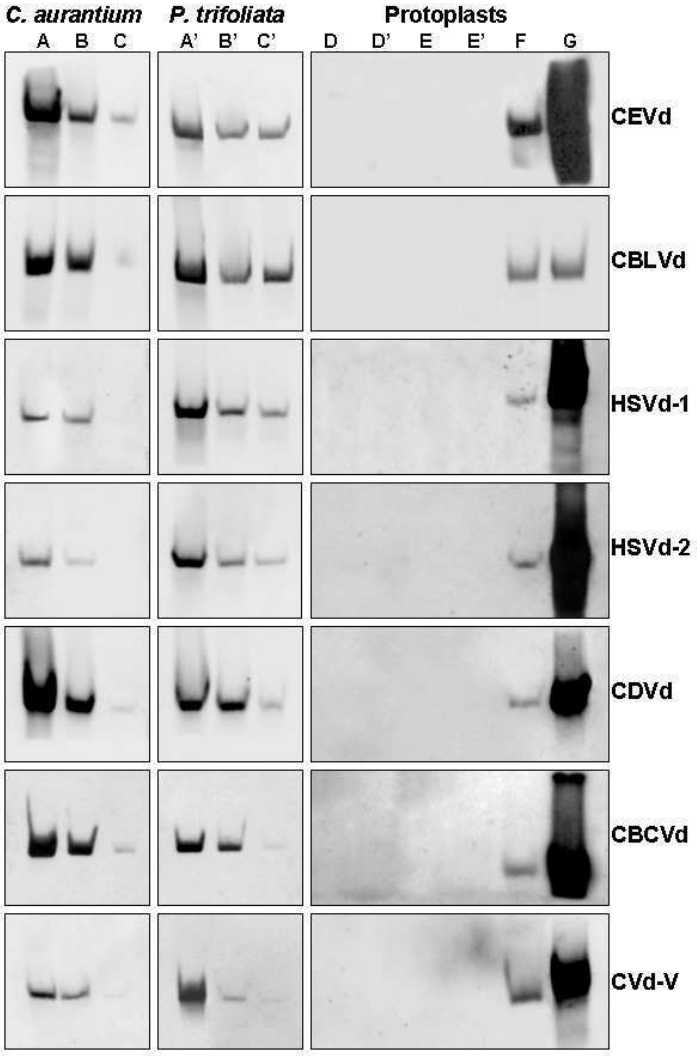

Fig. 3. Northern hybridization signals of nucleic acid preparations from bark samples of young shoots (A and A'), Leaf midveins (B and B') and fully expanded leaves devoid of petioles and midribs (C and C') of *C. aurantium* and *P. trifoliata* infected with different viroids. Northern hybridization signals of equalized protoplast preparations ($2X10^6$ protoplasts) of two *C. aurantium* (D and D') and two *P. trifoliata* (E and E'). Northern hybridization signals of protoplast (F) and bark (G) preparations of infected Etrog citron analyzed as positive controls.

Identification of the putative mechanisms involved in CEVd traffic from the phloem to adjacent cells

In order to gain some insight on the nature of the putative barrier that affects viroid trafficking in *C. karna*, the effect of the phloem restricted *Citrus tristeza virus* (CTV) known to act in suppressing silencing (Lu *et al.,* 2004) was addressed by inoculating two CEVd infected *C. karna* seedings with the CTV isolate T318A (Virus collection, IVIA). These co-inoculated plants and two CEVd-infected plants used as controls were maintained at 23-25ºC. Three months after CTV inoculation, infection was assessed by Immunoprinting-ELISA (Cambra *et al.,* 2000) using 3DF1 and 3CA5 CTV-specific monoclonal antibodies according to the EPPO protocol (EPPO, 2004). After four additional months, small titers of CEVd were detected by northern hybridization analysis of equalized protoplast preparations ($2X10^6$ protoplasts) from *C. karna* seedlings co-inefected with CEVd and CTV (Fig. 4D, 4D') whereas the viroid remained undetectable in the corresponding controls singly infected with CEVd (Fig. 4B, 4B').

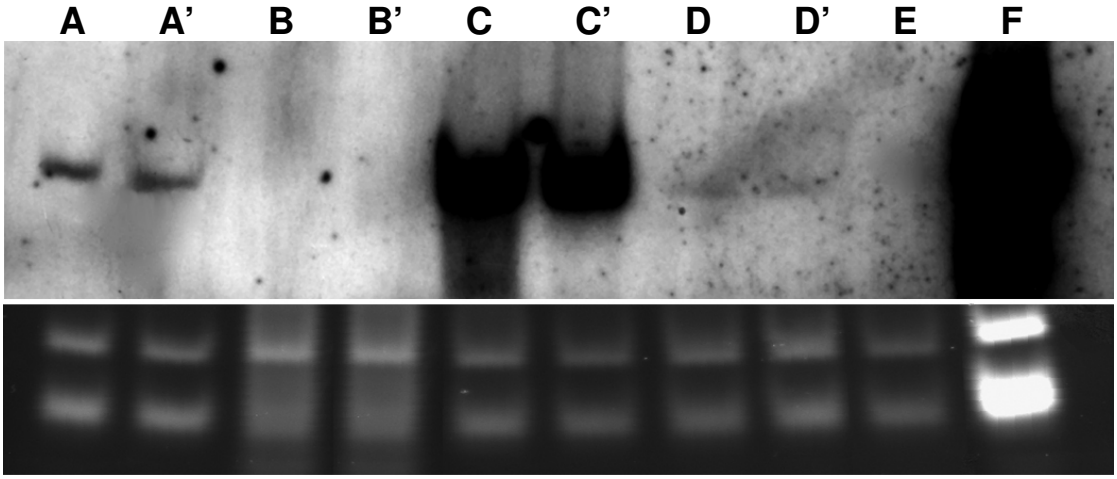

Fig. 4. Northern hybridization signals of equalized protoplast preparations ($2X10^6$ protoplasts) of: two CEVd-infected *C. karna* plants topworked with Etrog citron (A and A'); two CEVd-infected *C. Karna* plants (B and B'); two CEVd-infected Etrog citron plants (C and C') and two *C. karna* plants co-infected with CEVd and CTV (D and D'). Preparations from protoplast of a non-infected Etrog citron (E) and from bark of an infected Etrog citron plant (F) were included as negative and positive controls. The below inset showing 5S and 4S RNAs visualized in the ethidium bromide stained gel, illustrates the nucleic acid amounts in each lane.

Furthermore, since Etrog citron appears to be viroid trafficking competent, an additional assay was performed to elucidate if Etrog citron contains a translocatable factor mediating the movement of viroids across the messophyl/phloem barrier. Two CEVd infected *C. karna* seedlings were decapitated and top grafted with an Etrog citron bud-stick (3 cm-long and containing 3-4 buds) (Fig. 5). These two top-worked

plants, now composed of a *C. karna* stock and an Etrog citron top, and two CEVd infected *C. karna* seedlings used as controls were maintained for three months at 23-25ºC. Northern hybridization analysis of equalized protoplast preparations ($2X10^6$ protoplasts) of each plant revealed that CEVd was fully detected in the *C. karna* mesophyll protoplasts from the top-worked plants (Fig. 4A, 4A') whereas it remained undetectable in the corresponding seedling controls (Fig. 4B, 4B').

Fig. 5. CEVd infected *C. karna* seedlings top grafted with an Etrog citron bud-stick.

DISCUSSION

Studies regarding viroid systemic infection in herbaceous hosts lead to the proposal that they follow a general and characteristic pattern of movement within their hosts involving intracellular movement, cell-to-cell movement, entry into, and long-distant movement within the phloem to reach other plant organs (reviewed by Flores *et al.,* 2005; Ding and Wang, 2009). The results of the present study show that viroid movement/accumulation in Etrog citron follows this proposal. Indeed, northern blot hybridization analysis detected all the inoculated viroids in the bark, which contains the vascular bundles, as well as in mesophyll protoplasts. In graft-inoculated Etrog citron plants, viroids quickly move from the grafted bark inoculum into the bark and vascular system of the inoculated host, from where they are distributed throughout the plant including the distant leaves. However, other citrus species such as *C. karna,* sour orange and trifoliate orange do not seem to follow this model. In fact, the inoculated viroids were easily detected in preparations from tissues containing vascular bundles

(bark, leaf midveins and leaf blades devoid of midribs) but, in contrast to Etrog citron, in these three species none of the inoculated viroids were detected in the mesophyll protoplasts. It should be noted that even the viroid titers in the leaf blades of *C. karna,* sour orange and trifoliate orange were rather low, suggesting that the viroids were probably only present in the secondary veins of the leaf blades, from where they were unable to reach other cell types, including the mesophyll cells, explaining why they could not be detected in the protoplasts. In contrast, relatively high viroid titers were consistently detected in all the Etrog citron tissues analyzed, including leaf blades and mesophyll protoplasts.

Etrog citron is a citrus species that has been widely used as an experimental host for biological indexing, and has been shown to act as a bioamplification host in which all viroids reach titers easily detectable by molecular methods. In contrast, attempts to use molecular approaches, such as electrophoresis and hybridization techniques, to detect viroids from other citrus species other than Etrog citron always gave inconsistent and/or erratic results. The finding that in Etrog citron viroids are present not only in vascular tissues but also in mesophyll cells explains the desirable properties of this species for detection purposes. The results presented here indicate that in *C. karna,* sour orange and trifoliate orange, and probably other citrus species but not Etrog citron, viroid infection is essentially phloem restricted.

Phloem restricted systemic infection is not exceptional and has been reported in the case of viruses such as CTV, which is able to replicate and cause a range of disease symptoms in sensitive citrus hosts. The mature phloem contains: (i) the sieve tubes, which are composed of a vertical row of elongated cells devoid of nuclei and are responsible for the long distance movement of viruses and viroids, and (ii) the companion cells, which contain nuclei and are therefore competent for virus and viroid replication. The high viroid titers found in the bark samples of *C. karna* indicate that viroid replication occurs in an efficient manner probably only in the phloem, with companion cells playing a key role. Even though *C. karna* and Etrog citron contain similarly high titers of viroids in the bark tissue, the most relevant finding of the present work is the fact that, in spite of these high titers, viroids cannot be detected in *C. karna* protoplasts, whereas appreciable viroid titers can be detected in Etron citron protoplasts. These results indicate that a barrier preventing trafficking from the bundle sheath to adjacent tissues actually exists in certain citrus species, such as *C. karna*. The existence of a regulatory point for viroid trafficking between the phloem and nonvascular tissues has been demonstrated for *Potato spindle tuber viroid* (PSTVd) in two herbaceous hosts, *Nicotiana tabacum* and *N. benthamiana* (Qi *et al.,* 2004; Zhong *et al.,* 2007). In these viroid/host model systems it has been shown that the viroid has

motifs that mediate trafficking across specific cellular boundaries in a defined direction, suggesting the existence of protein factors that recognize these RNA motif-directed trafficking (Ding and Wang, 2009).

In order to investigate the properties of the barrier that prevents viroid trafficking from the bundle sheath to adjacent tissues in citrus, two approaches have been undertaken using *C. karna* infected with CEVd as an experimental model. Following a first approach, CEVd was detected in protoplasts from infected *C. karna* after having been top-worked with Etrog citron, indicating: (i) that the Etrog citron partner is able to transfer its viroid trafficking ability to *C. karna,* suggesting that a translocatable factor mediating the viroid movement across the bundle sheath-mesophyll boundary indeed exists in Etrog citron, and (ii) that once CEVd traverses this boundary is able to replicate in the *C. karna* mesophyll cells. Following a second approach, low CEVd titers were detected in protoplasts of *C. karna* co-infected with CTV. Since CTV encodes three distinct suppressors of RNA silencing (Lu *et al.,* 2004), its effect on the CEVd movement across the bundle sheath-mesophyll boundary suggests the implication of silencing mechanisms in this barrier.

The results achieved in the case of *C. karna* top worked with Etrog citron suggest the existence of a protein factor in Etrog citron that must recognize and interact with a putative viroid motif. In order to advance in the understanding of this type of interaction, work is in progress to characterize the populations of CEVd variants present in different tissue of *C. karna* and Etrog citron.

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CAPÍTULO 3

Acerca de la resistencia de *Eremocitrus glauca* y *Microcitrus australis* a la infección con viroides: La limitada replicación/acumulación no afecta al movimiento a larga distancia

On the resistance of *Eremocitrus glauca* and *Microcitrus australis* to viroid infection: Impaired replication/accumulation does not affect long distance movement

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ABSTRACT

In studies addressed to identify genotypes resistant to infection with citrus viroids, *Eremocitrus glauca* and *Microcitrus australis* were selected because their evolution in the peculiar habitat in Australia and New Guinea probably led to the selection of unusual traits. The movement and accumulation of *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd), *Citrus bark cracking viroid* (CBCVd) and *Citrus viroid V* (CVd-V) in self rooted as well as in graft propagated *E. glauca* and *M. australis* plants was assessed by northern hybridization, RT-PCR and by top-working to the sensitive selection 861-S1 of Etrog citron. In both species the inoculated viroids were undetectable unless these plants were grafted to a susceptible *Citrus* partner, the Rough lemon rootstock and/or the top-worked Etrog citron, acting as viroid sources. The results obtained indicate that *M. australis* and in particular *E. glauca* are poor viroid hosts in which viroid replication/accumulation does not occur or it is extremely inefficient. However, the viroid downward and upward movement to grafted *Citrus* partners, in which viroid replication and accumulation occurs efficiently was not impaired. *E. glauca* and *M. australis* present differences regarding their properties as viroid hosts, with CEVd having the lowest affinity and CVd-V the highest one. Even though *E. glauca* and *M. australis* do not appear to be truly resistant to viroid infection, they are interesting genotypes for further characterization of mechanisms involved in viroid infection.

INTRODUCTION

Two genera of true citrus fruits, *Eremocitrus* and *Microcitrus*, both of which are members of the sub-family *Aurantioideae* in the family *Rutaceae*, are native of Australia and New Guinea (Swingle and Reece, 1967). The monotypic *Eremocitrus glauca*, also known as limebush, is endemic to the semi-arid regions of Queensland, New South Wales and South Australia. It is the only pronounced xerophyte in the subfamily *Aurantioideae*, and because it is extremely drought tolerant and able to withstand extreme temperatures (up to 45ºC) (Swingle and Reece, 1967), it is an interesting genotype for citrus breeding. Five of the six known species of the genus *Microcitrus* are endemic to rainforest habitats on the east coast and their distribution originally extending from the Cape York Peninsula to the Clarence River on the north coast of New South Wales. *M. australis*, also called Dooja or Gympie lime, is the most vigorous of the native Australian rutaceous species, and it is found in lowland sub-tropical

rainforests of south-eastern Queensland. *E. glauca* and the six known species of *Microcitrus* are believed to be the result of slow evolution, over millions of years, from a primitive ancestral type that underwent speciation in Australia and New Guinea once these islands were separated from the Asiatic mainland. In the framework of studies that were initiated to determine the effect of viroid infection in citrus and citrus relatives, *E. glauca* and *M. australis* were selected for this work because their peculiar habitat probably led to the selection of unusual traits.

Viroids are unencapsidated, small, single-stranded, circular RNAs that replicate autonomously when inoculated in their plant hosts. Citrus are natural hosts of several viroids, all of them belonging to the family *Pospiviroidae,* characterized by the presence of a central conserved region (CCR) and the absence of RNA self-cleavage mediated by hammerhead ribozymes (Flores *et al*., 2005). Five viroids, *Citrus exocortis viroid* (CEVd, genus *Pospiviroid*), *Hop stunt viroid* (HSVd, genus *Hostuviroid*), *Citrus bent leaf viroid* (CBLVd, genus *Apscaviroid*), *Citrus dwarfing viroid* (CDVd, genus *Apscaviroid*) (former *Citrus viroid III*, CVd-III), *Citrus bark cracking viroid* (CBCVd, genus Cocadviroid) (former *Citrus viroid IV*, CVd-IV) have been included as distinct species in the Virus Taxonomy Scheme (www.ictvonline.org/virusTaxonomy.asp). Two additional viroids, *Citrus viroid original sample* (CVd-OS) only reported in Japan (Ito *et al*., 2001) and the recently characterized *Citrus viroid V* (CVd-V) (Serra *et al*., 2008) are still considered as tentative new species of the genus *Apscaviroid.*

Each citrus viroid induces specific symptoms on Etrog citron (*Citrus medica*), an indicator plant (Duran-Vila *et al*., 1988; Ito *et al*., 2001; Serra *et al*., 2008) that replicates and accumulates high viroid titers. However, only two viroids, CEVd and HSVd have been demonstrated to cause specific diseases in citrus: exocortis and cachexia, respectively (Semancik *et al*., 1975; Reanwarakorn and Semancik, 1998). Other citrus viroids produce more subtle effects on commercial species used as scion or rootstock (Vidalakis *et al.,* 2004; Semancik *et al.,* 1997; Vernière *et al.,* 2004). Early studies conducted by Salibe (1961) at a time when viroids were not yet known as distinct biological entities acting as plant pathogens, showed that the so-called "exocortis" agent had a wide host range. Further studies using well-characterized sources of citrus viroids confirmed that commercial and non-commercial species of the genus *Citrus* were hosts of all the viroids tested, whereas some non-*Citrus* species in the *Rutaceae* were likely to be viroid resistant (Barbosa *et al.,* 2002).

Citrus viroids are not seed born, and natural transmission occurs by graft-propagation of infected scions and to a lesser extent by mechanical transmission with naturally or experimentally contaminated pruning and cutting tools (Barbosa *et al.,* 2005a). Graft-inoculation of bark patches from infected sources is the usual procedure

for viroid transmission assays and biological indexing purposes (Roistacher *et al.,* 1991). The infection process involves: (i) long-distance movement within the phloem to reach leaves and organs, including roots; (ii) exit from the phloem to invade non-vascular cells; (iii) intracellular movement to reach their replication site (the nucleus in members of the family *Pospiviroidae*), followed by release of the viroid progeny into the cytoplasm; and (iv) cell-to-cell movement to infect adjacent cells via plasmodesmata (reviewed by Flores *et al.,* 2005; Ding and Wang, 2009).

Here we report results demonstrating that *E. glauca* and *M. australis* are unusual viroid hosts in that they maintain inoculated viroids at unusually low titers without, however, impairing their downward and upward movements.

MATERIALS AND METHODS

Propagation of *E. glauca* and *M. australis*

The source of plant material for propagation of the two citrus relatives was pathogen free *E. glauca* (IVIA-346) and *M. australis* (IVIA-313) from the IVIA germplasm bank (www.ivia.es/germo/). Plants were graft-propagated on Rough lemon seedlings (*Citrus jambhiri*) (Fig. 1A). Self-rooted plants of *E. glauca* were obtained by *in vitro* micropropagation. Stem pieces (10 cm long) were stripped of leaves and disinfected in 70% (v/v) ethanol for 3 min and 1.5% (w/v) sodium hypochlorite for 10 min and rinsed in autoclaved water. Stem segments (1 cm long) with a single bud were split longitudinally and cultured with the longitudinal cut surface in contact with culture medium containing basic nutrient solution (BNS) (Duran-Vila *et al.,* 1989) and 1 mg L^{-1} benzylaminopurine, pH 5.7. Four months later, shoots with a minimal length of 0.5 cm originating from the cultured explants were transferred to rooting medium containing BNS and 3 mg L^{-1} naphthalene acetic acid, pH 5.7. After six weeks, shoots showing root primordia were transferred to elongation medium containing BNS, pH 5.7 and transferred to soil 4 months later. Self-rooted plants of *M. australis* were propagated as seedlings (seeds were kindly provided by R. Krueger, University of California at Riverside, CA, USA).

Viroid sources

The viroid sources used were CEVd (CEVd-117) (Gandía *et al.,* 2005), HSVd (a mixture of cachexia and non-cachexia inducing strains, X-707 and CVd-IIa-117, repectively) (Palacio-Bielsa *et al.,* 2004), CBLVd (CVd-Ia-117) (Foissac and Duran-Vila, 2000), CDVd (CVd-IIId) (Foissac and Duran-Vila, 2000), CBCVd (CVd-IV-Ca) (Francis

et al., 1995) and CVd-V (Serra *et al.,* 2008). CEVd-117 had been characterized as a severe strain (Gandía *et al.,* 2005) highly homologous to the CEVd sequences defined by Visvader and Symons (Visvader and Symons, 1985, 1986) as class A. Etrog citron plants, each one infected with one of the above six viroids, were used to graft-inoculate the viroids into a 'Fino' lemon (*C. limon*) tree grafted on Rough lemon rootstock. Indexing confirmed that the 'Fino' lemon tree was infected with all six viroids. The 'Fino' lemon tree was used as a source of all six viroids for graft-transmission to *E. glauca* and *M. australis* plants.

Viroid inoculation

E. glauca and *M. australis* scions propagated on Rough lemon were graft-inoculated with bark patches of the 'Fino' lemon tree co-infected with all six viroids. A shoot from the Rough lemon rootstock was allowed to grow for further analyses. Similarly, self-rooted *E. glauca* and *M. australis* plants were graft-inoculated with the same viroid source. In all instances non-inoculated plants were kept as negative controls.

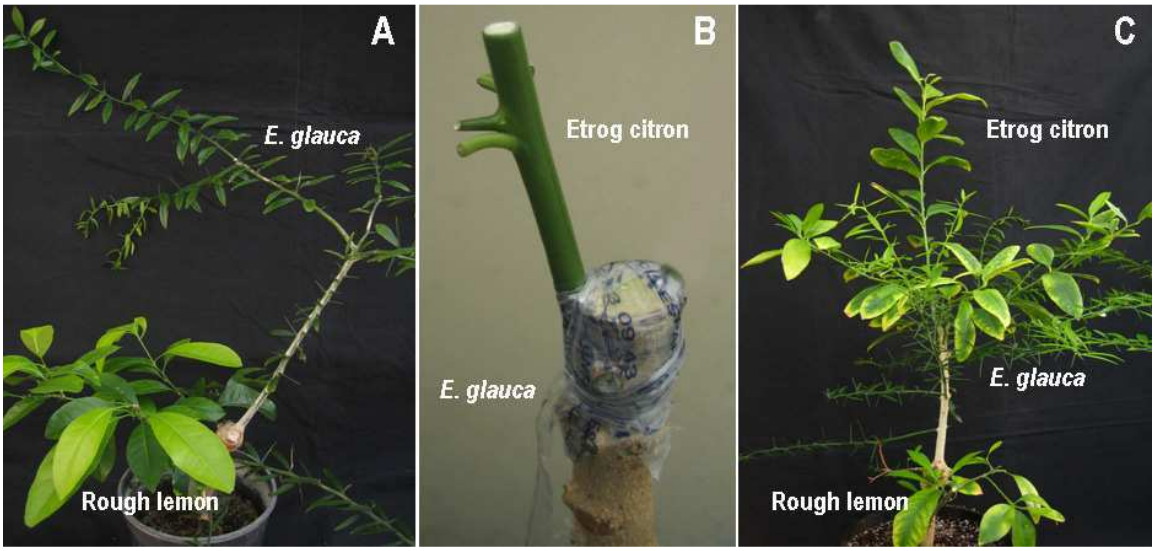

Fig. 1. (A) *E. glauca* graft propagated on Rough lemon. (B) *E. glauca* plant decapitated and top-worked with an Etrog citron budstick. (C) Resulting plant composed of a Rough lemon rootstock, an *E. glauca* interstock and an Etrog citron scion.

Top-working

Inoculated and non-inoculated *E. glauca* and *M. australis* plants were top-worked with the 861-S1 selection of Etrog citron, well known as a viroid "amplification host" because of its capacity to replicate and accumulate all citrus viroids at high titers. For

top-working, the plants were decapitated and side-grafted with Etrog citron bud-sticks (3 cm-long and containing 2-3 buds) (Fig. 1B).

Viroid analysis

Samples of bark tissue were powdered in liquid nitrogen and homogenized in 5 ml of extraction medium (0.4 M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA, pH 7.0; 4% (v/v) β-mercaptoethanol) and 15 ml of water-saturated phenol (Semancik *et al.,* 1975). 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 $MgCl_2$).

For northern blot hybridization, aliquots (20 µl equivalent to 300 mg fresh weight) were subjected to 5% non-denaturing PAGE and stained with ethidium bromide. The RNAs separated by 5% PAGE 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), immobilized by UV cross-linking and hybridized with viroid specific probes (Murcia *et al.,* 2009). Digoxigenin (DIG)-labeled DNA probes were synthesized by PCR using as template a cloned plasmid containing full-length viroid monomeric DNA, as described by Palacio-Bielsa *et al.* (1999). Prehybridization (at 60ºC for 2-4 h) and hybridization (at 60ºC overnight) were performed in 50% formamide and 5X SSC buffer containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking reagent. 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®).

RT-PCR was performed as described by Bernad and Duran-Vila (2006). First-strand cDNA was synthesized at 60ºC using 27-mer primers specific for each viroid and Thermoscript reverse transcriptase (Invitrogen®). In order to recover full-length viroid DNA, second strand synthesis and DNA amplification were performed using a set of two contiguous 18-mer forward and reverse primers specific for each viroid in 50 µl reactions containing 1.5 mM $MgCl_2$, 0.12 mM dNTPs, 0.5 µM of each primer and 1 U of *Taq* DNA polymerase (Roche Applied Science®). PCR parameters consisted of a 5 min denaturation at 94°C followed by 35 cycles of 94°C (30 s), 60°C (30 s), 72°C (1 min) and finishing with a 5 min extension step at 72°C. Electrophoretic analysis in 2% agarose gels confirmed the synthesis of the expected DNA products.

RESULTS

Viroid movement and accumulation in *E. glauca* plants grafted on Rough lemon rootstock

Two *E. glauca* plants graft-propagated on Rough lemon rootstock were graft-inoculated in the scion with bark from the 'Fino' lemon source containing CEVd, HSVd, CBLVd, CDVd, CBCVd and CVd-V. Nine months after inoculation (Fig. 2), when CEVd was detected in the Rough lemon rootstock but not in the *E. glauca* scion (data not shown), these plants were cut back below the graft-inoculation site and the remaining *E. glauca* scion was allowed to grow. After four additional months, all the viroids present in the inoculum source were detected in the rootstocks (Fig. 2A, 2A': lanes R1, R2) but not in the *E. glauca* scions (Fig. 2A, 2A': lanes E1, E2). CBLVd was detected in the rootstocks by RT-PCR but not by northern hybridization, indicating that this viroid was present at very low titers. These results indicate that either viroid replication does not occur in *E. glauca* or that their replication/accumulation is so inefficient that they cannot be detected. However, since the viroids were detected in the Rough lemon rootstock, it means that *E. glauca* allowed the long distance downward movement from the inoculated scion to the viroid susceptible rootstock in which they were able to reach detectable titers.

The above plants were top-worked after eight months to the sensitive selection 861-S1 of Etrog citron and the resulting plants were composed of the viroid susceptible Rough lemon rootstock, the *E. glauca* interstock and an Etrog citron scion (Fig. 1C). Eight months after top-working, all the viroids were detected by northern hybridization on the Etrog citron scion (Fig. 2B: lanes C1, C2) but not in the *E. glauca* interestock (Fig. 2B: lanes E1, E2). These results indicate that *E. glauca* allowed the long distance upward movement of viroids from the infected rootstock to the top-worked Etrog citron, in which they replicated and accumulated to high titers. In contrast to the negative RT-PCR results obtained with bark samples of *E. glauca* growing as scions on Rough lemon rootstock (Fig. 2A': lanes E1, E2), positive RT-PCR results were obtained with most of the bark samples collected on the same *E. glauca* material, but growing now as an interstock between a citron top and a Rough lemon stock (Fig. 2B': lanes E1, E2). These results suggest that it is the high viroid titer present in the Etrog citron top, associated with a downward movement of the viroids, which is responsible for the presence of detectable amounts of viroids in the *E. glauca* interstock.

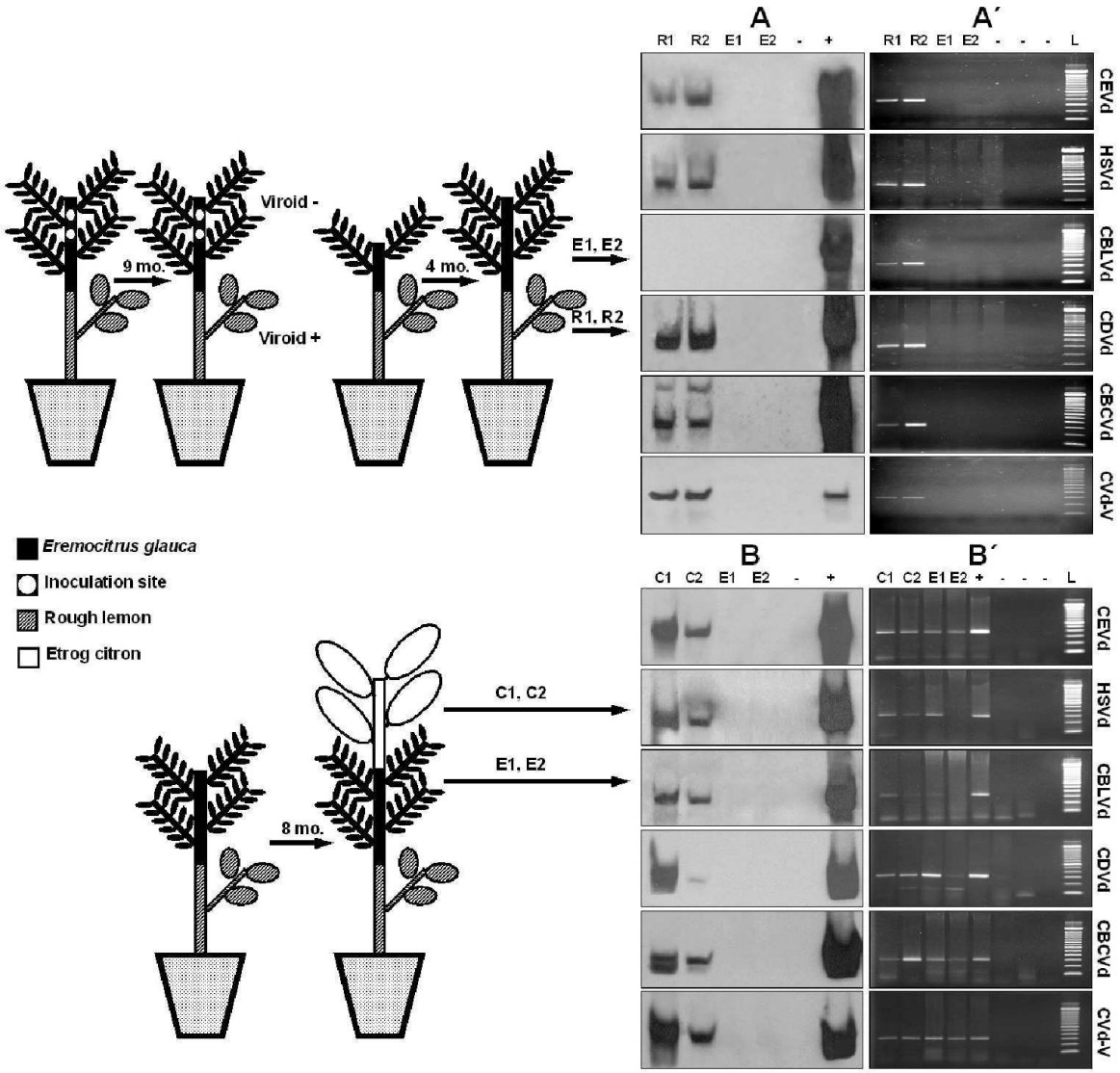

Fig. 2. Analysis of two *E. glauca* plants graft-propagated on Rough lemon rootstock and co-inoculated with citrus viroids. The graft-inoculated plants were cut back below the graft-inoculation site and the remaining *E. glauca* scion was allowed to grow and, after four months, samples collected from the Rough lemon rootstock (R1, R2) and from the *E. glauca* scion (E1, E2) were viroid tested by northern hybridization (A) and RT-PCR (A'). Eight months after, these plants were top-worked to the sensitive selection 861-S1 of Etrog citron and samples collected from the Etrog citron top (C1, C2) and from the *E. glauca* interstock (E1, E2) were viroid tested by northern hybridization (B) and RT-PCR (B'). Samples from viroid-free (-) and viroid-infected (+) Etrog citron plants were included as controls in the northern hybridization tests. In RT-PCR tests, negative controls (-) included RT-PCR analysis of viroid free samples, RT-PCR control without RNA template and PCR control without cDNA.

Viroid movement and accumulation in self-rooted *E. glauca* plants

Two self-rooted *E. glauca* plants were graft-inoculated with the 'Fino' lemon viroid source. Over a period of 18-month, samples collected above and below the inoculation site and analyzed by northern hybridization and RT-PCR tested negative (Fig. 3A, 3A').

Since the 'Fino' lemon bark-patch grafted at the inoculation site is capable of viroid replication and can act as a source of viroids, all inoculated plants were cut back below the inoculation site. Eight months later, they were top-worked to the 861-S1 selection of Etrog citron in order to assess if traces of the inoculated viroids remained

in these *E. glauca* plants. The resulting plants were now composed of an *E. glauca* stock and an Etrog citron top. Eight months after top-working, none of the viroids were detected in the *E. glauca* stock by northern hybridization (Fig. 3B: lanes E1, E2), but unexpectedly, CVd-V was detected in one of the Etrog citron tops (Fig. 3B, 3B': lane C2). Also, the more sensitive RT-PCR detected CBCVd in one of the Etrog citron samples (Fig. 3B': lane C2).

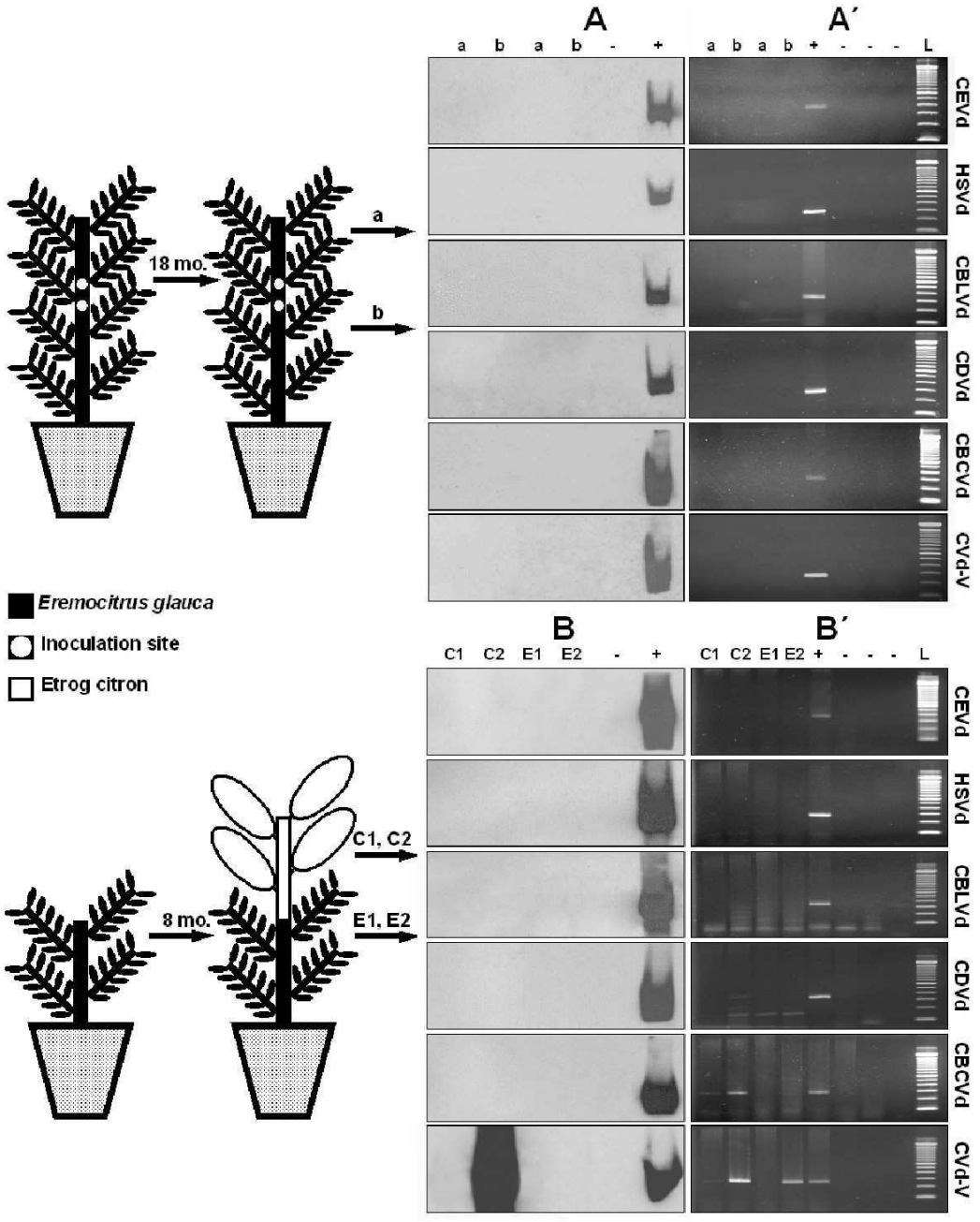

Fig. 3. Analysis of two self-rooted *E. glauca* plants co-inoculated with citrus viroids. Eighteen months after inoculation, samples collected above (a) and below (b) the inoculation site were viroid tested by northern hybridization (A) and RT-PCR (A'). These plants were top-worked to the sensitive selection 861-S1 of Etrog citron and samples collected from the Etrog citron top (C1, C2) and from the *E. glauca* stock (E1, E2) were viroid tested by northern hybridization (B) and RT-PCR (B'). Samples from viroid-free (-) and viroid-infected (+) Etrog citron plants were included as controls in the northern hybridization tests. In RT-PCR tests negative controls (-) included RT-PCR analysis of viroid free samples, RT-PCR control without RNA template and PCR control without cDNA.

Failure to detect the inoculated viroids in the *E. glauca* plants before as well as after removal of the inoculum bark patch suggests that *E. glauca* was either resistant to viroid infection/replication or else that *E. glauca* did replicate the viroids but at such low levels that neither northern hybridization nor RT-PCR were able to detect them. However, surprisingly, CBCVd and CVd-V were detected in one of the two the Etrog citron tops (Fig. 3B, 3B': lane C2). This indicates that these two viroids were still present but undetectable in *E. glauca* (Fig. 3B, 3B': lane E2) eight months after removal of the Fino lemon bark patch, and that they were able to move upwards into the Etrog citron top (Fig. 3B, 3B': lane C2), a sensitive host in which they were able to replicate to detectable levels. Finally, viroid CVd-V was also detected in the top-worked *E. glauca* tissue (Fig. 3B': lane E2) and results probably from the downward movement of viroid produced to high titers in the Etrog citron top.

Viroid movement and accumulation in *M. australis* plants grafted on Rough lemon rootstock

Two *M. australis* plants propagated on Rough lemon rootstock were graft-inoculated in the scion with bark patches from the 'Fino' lemon viroid-source. Twelve months after inoculation, when CDVd was detected in the Rough lemon rootstock but not in the *M. australis* scion (data not shown), these plants were decapitated below the graft-inoculation site. The remaining *M. australis* scions were allowed to grow for four months before being top-worked with the sensitive selection 861-S1 of Etrog citron. After five additional months these plants (composed of a viroid susceptible Rough lemon rootstock, the *M. australis* interstock and an Etrog citron scion top) were viroid tested by northern hybridization and RT-PCR. All six viroids present in the 'Fino' lemon inoculum source were detected in the Rough lemon rootstock (Fig. 4A, 4B: lanes R1, R2) and in the Etrog citron top (Fig. 4A, 4B: lanes C1, C2). Viroid detection in the *M. australis* interstock by northern hybridization was either negative or showed very weak signals (Fig. 4A: lanes M1, M2), but in almost all instances positive detection was achieved by RT-PCR (Fig. 4B: lanes M1, M2). These results indicate that *M. australis* is a poor host for replication of viroids and in particular CEVd and CBLVd, but allows long distance movement of viroids from the inoculated *M. australis* scion downwards to the susceptible Rough lemon rootstock and upwards to the susceptible Etrog citron, both of which replicate and accumulate all viroids to appreciable titers.

Viroid movement and accumulation in self-rooted *M. australis* plants.

Two self-rooted *M. australis* plants were graft-inoculated with the 'Fino' lemon viroid-mixture. Twelve months after inoculation, these plants were decapitated below

the graft-inoculation site and the remaining *M. australis* plants were allowed to grow for four months before being top-worked with the sensitive selection 861-S1 of Etrog citron. After five additional months these plants (composed of the *M. australis* stock and an Etrog citron top) were viroid tested by northern hybridization and RT-PCR. Samples from the Etrog citron tops tested positive for HSVd, CBLVd, CDVd, CBCVd and CVd-V but not for CEVd (Fig. 5A, 5B: lanes C1, C2).

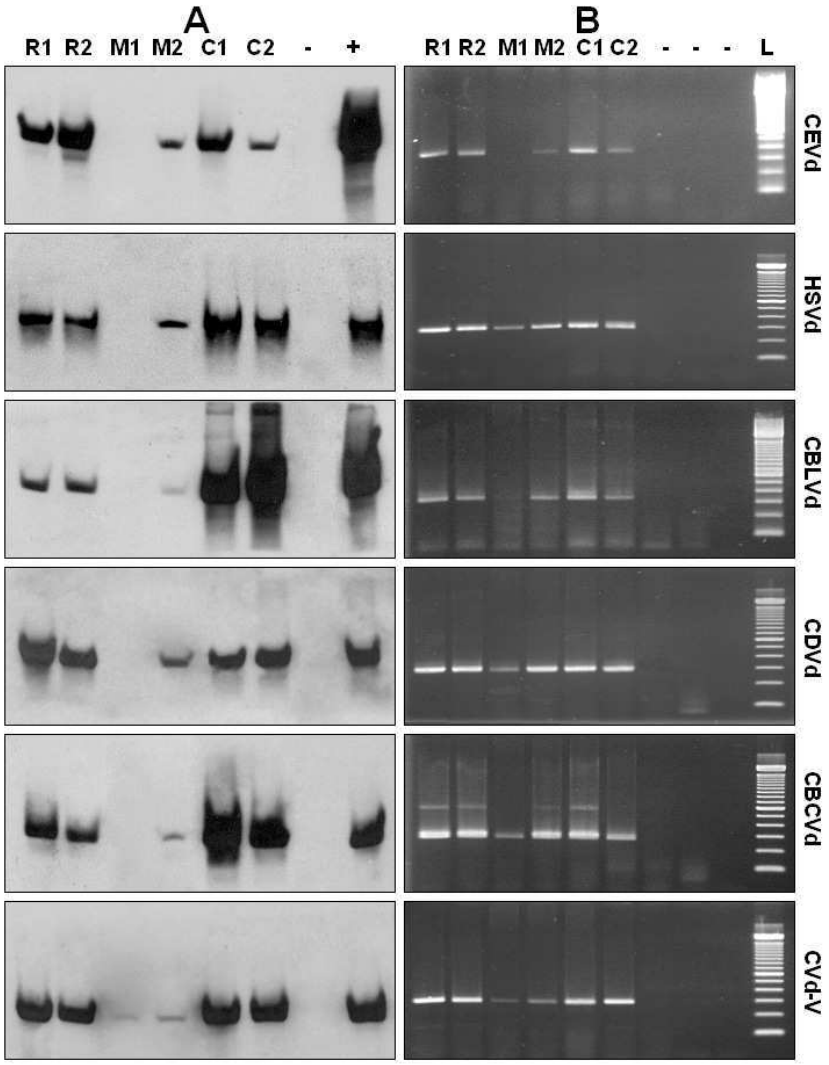

Fig. 4. Analysis of two *M. australis* plants graft-propagated on Rough lemon rootstock and co-inoculated with citrus viroids. The graft-inoculated plants were cut back below the graft-inoculation site and the remaining *M. australis* scion was allowed to grow for four months before being top-worked with the sensitive selection 861-S1 of Etrog citron. Five months after, samples collected from the Rough lemon rootstock (R1, R2), from the *M. australis* interstock (M1, M2) and from the Etrog citron top (C1, C2) were viroid tested by northern hybridization (A) and RT-PCR (B). Samples from viroid-free (-) and viroid-infected (+) Etrog citron plants were included as controls in the northern hybridization tests. In RT-PCR tests negative controls (-) included RT-PCR analysis of viroid free samples, RT-PCR control without RNA template and PCR control without cDNA.

Samples from the *M. australis* stock also tested positive for HSVd, CDVd, CBCVd and CVd-V by RT-PCR but not by northern hybridization analysis (Fig. 5B: lanes M1, M2), whereas CEVd and CBLVd were undetectable by both methods. However, as

seen above, CBLVd but not CEVd was present in the Etrog citron top, probably because *M. australis* is able to replicate CBLVd but not CEVd, and allows upward movement of CBLVd into Etrog citron where active replication occurs. What is true for CBLVd in *M. australis* is also true for HSVd, CDVd, CBCVd and CVd-V, but not CEVd, apparently the only viroid unable to infect *M. australis.* In comparison, *E. glauca* seems to be a much poorer host than *M. australis.*

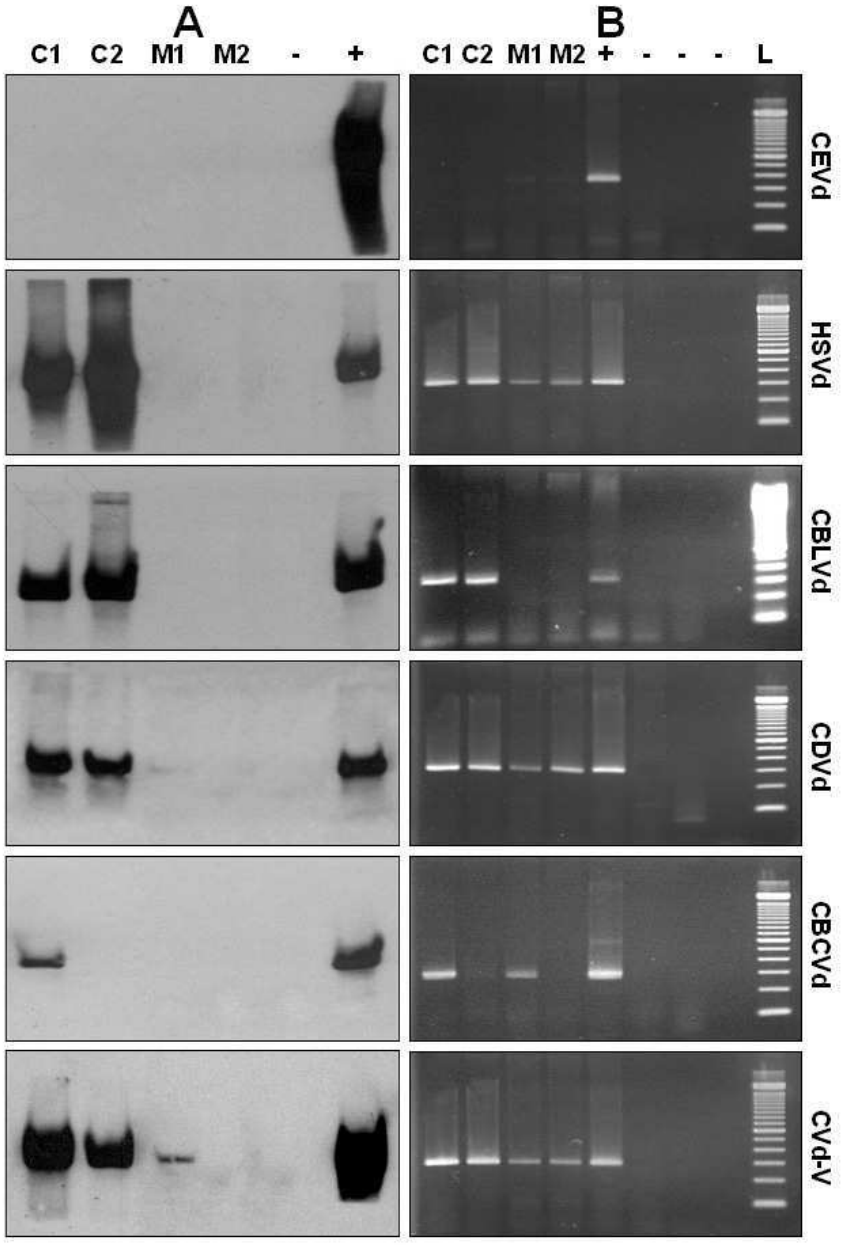

Fig. 5. Analysis of two self-rooted *M. australis* plants co-inoculated with citrus viroids. The graft-inoculated plants were cut back below the graft-inoculation site and the remaining *M. australis* scion was allowed to grow for four months before being top-worked with the sensitive selection 861-S1 of Etrog citron. Five months after, samples collected from the *M. australis* stock (M1, M2) and from the Etrog citron top (C1, C2) were viroid tested by northern hybridization (A) and RT-PCR (B). Samples from viroid-free (-) and viroid-infected (+) Etrog citron plants were included as controls in the northern hybridization tests. In RT-PCR tests negative controls (-) included RT-PCR analysis of viroid free samples, RT-PCR control without RNA template and PCR control without cDNA.

DISCUSSION

Earlier studies showed that commercial and non-commercial species of the genus *Citrus* were hosts of all viroids tested, whereas in some rutaceous species of non-*Citrus* genera inoculated viroids were undetectable even after transmission to Etrog citron which is considered the best "viroid amplificacion" host (Barbosa *et al.,* 2002). Studies addressed to identify resistant genotypes in woody species are lacking and pertinent assays are difficult to perform. In particular, citrus and citrus relatives are usually graft-propagated and the resulting plant is composed of two species, which may have different responses to viroid infection. The present study reports the unusual reaction of graft propagated and self-rooted *E. glauca* and *M. australis,* two non-*Citrus* species in the *Rutaceae* that present certain levels of resistance to viroid infection/replication. In both species the inoculated viroids were undetectable unless these plants were grafted on a susceptible *Citrus* partner: a Rough lemon rootstock and/or a top-worked Etrog citron, acting as viroid sources. These results indicate that *M. australis* and in particular *E. glauca* are poor viroid hosts in which viroid replication/accumulation is extremely inefficient. However, in spite of the fact that, because of their low titers, the viroids remained essentially undetected in the two non-*Citrus* species, their downward and upward movement to grafted *Citrus* partners in which viroid replication and accumulation occurs efficiently, was not impaired. In addition, our results also show that *E. glauca* and *M. australis* present differences regarding their properties as viroid hosts. In this regard, CEVd seems to have the lowest affinity for the two non-*Citrus* hosts, while CVd-V presents the highest one. This latter observation is in line with earlier results that lead to the discovery of CVd-V in *Atalantia citroides,* another non-*Citrus* species, which turned out to be a poor host for all citrus viroids, except CVd-V (Barbosa *et al.,* 2002, 2005b, Serra *et al.,* 2008).

The mechanisms involved in viroid infection and host resistance are poorly understood. A resistant genotype may affect one or several of the four steps involved in the viroid infection process (long-distant movement, invasion of non-vascular tissue, replication and cell-to-cell movement), which are mediated by host factors. The search for resistant genotypes is of interest for breeding purposes and, as shown here, the identification of the specific steps involved in resistance are crucial to demonstrate whether or not a given genotype is truly resistant or not. *E. glauca* and *M. australis* do not appear to be truly resistant to viroid infection but they remain the most interesting genotypes for further characterization of mechanisms involved in viroid infection.

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CAPÍTULO 4

Caracterización de aislados naturales de viroides procedentes de distintos huéspedes comerciales:

- **Incidencia e identificación de viroides de cítricos en Sudán**
- **Identificación de viroides en la citricultura de Irán**
- **El mal comportamiento de cítricos injertados sobre citrange Carrizo está asociado a la infección con viroides**

Short communication

Occurrence and identification of citrus viroids from sudan

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ABSTRACT

The citrus industry in Sudan cultivates mainly old-line cultivars of grapefruit, sweet orange and Willow leaf mandarin, for which sour orange is the almost exclusive rootstock. Infection with graft-transmissible agents, viroids in particular, may have deleterious effects on the productivity of these citrus species and, more importantly, limits the choice of rootstocks if sour orange needs to be replaced by alternative rootstocks because of tristeza disease. Three Foster pink grapefruit sources and one local selection of Nuri-16 sweet orange were found to be infected with several viroids, including CEVd and HSVd, the causal agents of exocortis and cachexia, respectively. Cloning and sequencing of four HSVd isolates showed that in addition to the characteristic variants containing the "cachexia expression motif" or the "non-cachexia expression motif", other HSVd variants were also present in Sudan. To extend these results, samples from 24 citrus species and cultivars were collected in several citrus growing regions and analyzed for viroids. The results confirmed that CEVd, HSVd, CBLVd, CDVd, and CBCVd, but not CVd-V, were widely spread throughout the surveyed regions. None of the collected samples was found to be viroid-free. The relationships between viroid infection and "gummy bark" and "Kassala disease", two affections of unknown etiology, but with cachexia-like gum impregnations in the bark, are discussed.

The citrus industry in Sudan is based mainly on the cultivation of old-line cultivars of grapefruit (*Citrus paradisi*), sweet orange (*C. sinensis*) and Willow leaf mandarin (*C. reticulata*), with sour orange (*C. aurantium*) being the almost exclusive rootstock. Discovery of stunted mandarin trees with leaf yellowing and showing characteristic cachexia symptoms of gum impregnations in the bark strongly suggested that at least *Hop stunt viroid* (HSVd), the causal agent of cachexia disease was present in Sudan. Symptoms of Gummy bark (Nour-Eldin, 1956, 1968) and Kassala disease (Bové, 1995), on sweet orange and grapefruit respectively, have also been observed in Sudan (Bové, 1995). The symptoms of these two diseases of unknown etiology resemble those of cachexia on mandarin and, as a consequence, the possibility of a viroid as their causal agent has been proposed but not demonstrated (Bové, 1995; Önelge *et al.,* 1996, 2004).

Because of its dependence on sour orange, the Sudanese citrus industry would be seriously endangered if presence and spread of *Citrus tristeza virus* (CTV)

(Abubaker, unpublished information) is confirmed in Sudan. Since the control of the tristeza disease is based on replacing sour orange by other species giving rootstock/scion combinations tolerant to CTV, the choice of rootstock species to replace sour orange will strongly depend on the sanitary status of the commercial cultivars available in Sudan. For instance, cultivars which carry the viroids inducing cachexia and exocortis cannot be grafted on such rootstocks as tangelo, alemow, Rangpur lime, trifoliate orange and citrange. The objective of the present work was to investigate the occurrence of citrus viroids in commercial citrus cultivars in Sudan.

Samples from three Foster pink grapefruit sources collected from three locations, Shambat, El-Bagair and El-Halfaya (Khartoum State) and from Nuri-16, a local sweet orange selection (Kassala State), were biologically indexed by graft-transmission to two plants each of the sensitive selection 861-S1 of Etrog citron (*C. medica*). Three months after inoculation, the citron plants presented stunting and leaf curling symptoms characteristic of viroid infection (Duran-Vila *et al.,* 1988). Analysis of nucleic acid preparations of the inoculated citrons by sequential polyacrylamide gel electrophoresis (sPAGE) and silver staining (Rivera-Bustamante *et al.,* 1986; Duran-Vila *et al.,* 1993) revealed the presence of four, five or six bands with the mobilities of the circular forms of viroids (Fig. 1A). When following sPAGE, the RNAs of these samples and the corresponding controls were electroblotted (400 mA for 2 h) to positively-charged nylon membranes using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA), and hybridized with digoxigenin (DIG)-labeled viroid specific probes generated by PCR (Palacio *et al*., 1999), the presumed viroid nature of the bands viewed in sPAGE analysis was confirmed. The citrus viroids identified were: *Citrus exocortis viroid* (CEVd) (in Foster pink grapefruit from Shambat, El-Halfaya and Nuri-16 sweet orange); *Citrus bent leaf viroid* (CBLVd) (in Foster pink grapefruit from Shambat, El-Bagair and El-Halfaya); HSVd; *Citrus viroid III* (CVd-III), recently renamed as *Citrus dwarfing viroid* (CDVd) and *Citrus viroid IV* (CVd-IV), recently renamed as *Citrus bark cracking viroid* (CBCVd) (in all four sources) (Fig. 1B). The newly described *Citrus viroid V* (CVd-V) (Serra *et al.,* 2008a) was not detected in any of the four samples.

Northern blot hybridization showed that two of the grapefruit samples (from El-Bagair and El-Halfaya) contained two distinct HSVd bands whereas the other two samples presented a single HSVd band (Fig. 1B). Reverse transcription and PCR amplification (RT-PCR) using the HSVd-specific primers (HSVd-RT, HSVd-F1 and HSVd-R1) (Bernad and Duran-Vila, 2006) yielded amplicons of the expected HSVd size. HSVd amplicons were sequenced or cloned into the pGEM-T vector (Promega) and the cloned viroid-cDNA inserts sequenced with an ABI PRISM DNA sequencer 377 (Perkin Elmer).

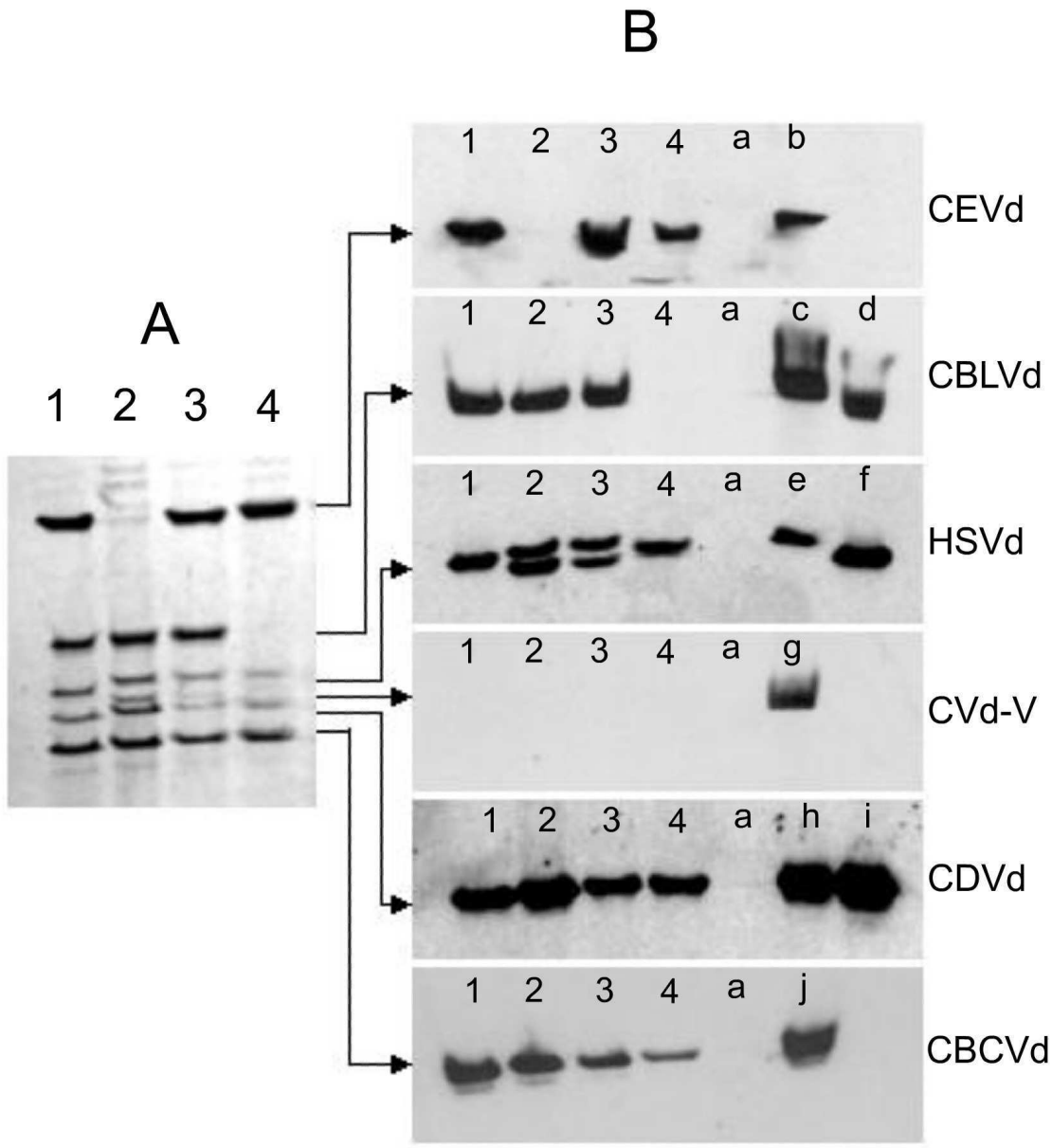


Fig. 1. (A) Sequential PAGE analysis and (B) Northern-blot hybridization with viroid-specific probes of Etrog citron plants that have been graft-inoculated with three sources of Foster pink grapefruit sources collected in Shambat (1), El-Bagair (2) and El-Halfaya (3) and from the sweet orange selection Nuri-16 (4). Samples from healthy and viroid-infected Etrog citron plants were included as negative and positive controls: (a) healthy; (b) CEVd; (c, d) CBLVd, variants CVd-Ia and CVd-Ib respectively; (e, f) HSVd, variants CVd-IIa and CVd-IIc, respectively; (g) CVd-V; (h, i) CDVd, variants CVd-IIIa and CVd-IIIb, respectively; (j) CBCVd.

Since the pathogenic properties of HSVd in citrus depend on the presence of either the "cachexia expression motif" in strains that induce the cachexia disease, or the "non-cachexia expression motif" in strains that do not induce the cachexia disease (Reanwarakorn and Semancik, 1998), multiple alignments with the reference sequences of CVd-IIa-117 (non-cachexia inducing variant) (Palacio-Bielsa *et al.,* 2004), CVd-IIb, and CVd-IIc (cachexia inducing variants) (Reanwarakorn and Semancik, 1998, 1999) were conducted using the Clustal W program (Thompson, *et al.,* 1994). As

shown below (Fig. 2), the presence of one or the other of these motifs was detected together with some unusual variants with changes affecting their secondary structure. The pathogenicity of some of these unusual HSVd variants has not been tested and the biological significance of the changes identified is still unknown.

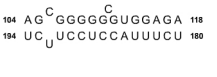
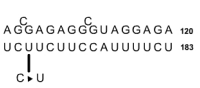
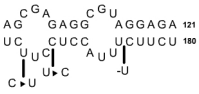
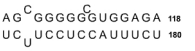
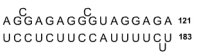
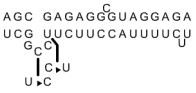
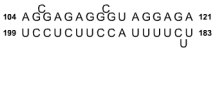
	HSVd source	NCBI Genebank accession	Structure of expression motif	Reference expression motif	
A)	HSVd (Shambat)	EU931255			CVd-IIb
B)	HSVd (El-Bagair)	EU931256 EU931257			CVd-IIa-11
C)	HSVd (El-Bagair)	FJ004236			CVd-IIa-11
D)	HSVd (El-Halfaya)	EU931259			CVd-IIc
E)	HSVd (El-Halfaya)	EU931258			CVd-IIa-11
F)	HSVd (El-Halfaya)	FJ004237			
G)	HSVd (Nuri-16)	EU931260			CVd-IIa-11

Fig. 2. Nucleotide changes and structural deviations of the cachexia and non-cachexia expression motifs found in four HSVd sources from Sudan. HSVd (Shambat) shows structural changes from the cachexia reference variant CVd-IIb. HSVd (El-Bagair and Nuri-16) show structural changes from the non-cachexia reference variant CVd-IIa-117. HSVd (El-Halfaya) did not show deviations from the cachexia reference variant CVd-IIc (EU931259) and from the non-cachexia reference variant CVd-IIa-117 (EU931258).

In the case of HSVd (Shambat) (Fig. 2A), the consensus sequence (NCBI GenBank EU931255) obtained by sequencing four independent clones showed that it had 301 nt and 98.3% identity with the CVd-IIb reference sequence. All four clones presented two modifications: (i) a substitution (U195→C), recently demonstrated to be responsible for the modulation of cachexia symptoms (Serra *et al*., 2008b), and (ii) an

insertion (+G107). These two changes resulted in a stretch of six ‘’G’’ between the two unpaired ‘’C’’, instead of the five ‘’G’’ in the CVd-IIb reference sequence.

With HSVd (El-Bagair), the sequences of several clones revealed variants of 300, 301 and 303 nt. The 300 and 301 nt variants presented three contiguous deletions in the lower strand of the pathogenicity domain (not shown on Fig. 2), also reported in the cachexia variant CVd-IIc (Reanwarakorn and Semancik, 1999). The consensus sequences of these two variants (NCBI GenBank EU931256 and EU931257) (Fig. 2B) had identities of 99.0% and 97.0% with the CVd-IIa-117 reference sequence, and both contained five of the six nucleotides characteristic of the “non-cachexia expression motif”. The substitution C196→U converted a canonic G-C pair into a wobble G-U pair. However, two additional changes (-U185 and U192→C), only present in the 303 nt variant (NCBI GenBank FJ004236) (Fig. 2C) resulted in a reorganization of the predicted base pairing.

The sequences of several clones of HSVd (El-Halfaya) revealed that this isolate contained variants of 297, 300 and 303 nt. The 297 nt variant (NCBI GenBank EU931259) (Fig. 2D) presented 99.3% identity with the CVd-IIc reference sequence, and both sequence and structure of its “cachexia expression motif” were identical to those of the reference motif. The 303 nt variant (NCBI GenBank EU931258) (Fig. 2E) presented 98.3% identity with the CVd-IIa-117 reference sequence, and the “non-cachexia expression motif” of the variant and the reference motif were identical. The 300 nt variant (NCBI GenBank FJ004237) (Fig. 2F) was rather unusual because it presented in the upper strand the sequence of the “cachexia expression motif” and in the lower strand the sequence of the “non-cachexia expression motif”. This unusual composition was identical to that of the synthetic chimera SM1 reported to be infectious but non-pathogenic when bioassayed on the Parson’s Special indicator (Reawankaron and Semancik, 1998).

The consensus sequence (NCBI GenBank EU931260) of HSVd (Nuri-16) (Fig. 2G), obtained by sequencing the RT-PCR amplicon, had 303 nt, with 96.7% identity with the CVd-IIa-117 reference sequence, and the sequence of its “non-cachexia motif” was the same than that of the reference motif. As illustrated on Fig. 2G, two changes (C197→U and U199→C) resulted in a reorganization of the predicted base pairing.

In April 2008, a survey was conducted in several citrus growing areas of Sudan and 24 samples (18 sweet orange, 4 grapefruit and 2 mandarin) were collected for viroid testing (Table 1).

Table 1. Viroids detected in samples collected in different locations of Sudan.

Sample	Location	Cultivar	Symptoms[1]	Viroids					
				CEVd	HSVd	CBLVd	CDVd	CBCVd	CVd-V
1	Dongola	Valencia sweet orange		+	+	+	+	+	-
2	Dongola	Willowleaf mandarin	CX	+	+	+	+	+	-
3	Dongola	Sweet orange		+	+	-	+	+	-
4	Borgage	Sweet orange	GB	+	+	+	+	+	-
5	Borgage West	Sweet orange	GB	+	+	+	+	+	-
6	Borgage West	Sweet orange	GB	+	-	+	+	+	-
7	Argo	Sweet orange		+	+	+	+	+	-
8	Al-Golid	Sweet orange		+	+	+	+	+	-
9	Al-Golid	Sweet orange	GB	+	+	-	-	+	-
10	Al-Golid	Sweet orange	GB	+	-	-	+	+	-
11	Al-Golid	Sweet orange	GB	+	+	+	+	+	-
12	Gushabi	Sweet orange	GB	+	+	+	+	+	-
13	Kulud	Sweet orange	GB	+	+	-	+	+	-
14	Kulud	Sweet orange	GB	+	+	+	+	+	-
15	Kitiab	Grapefruit	KD	+	+	+	+	+	-
16	Kitiab	Sinnari sweet orange	GB	+	+	+	+	+	-
17	Kitiab	Sinnari sweet orange	GB	+	+	-	+	+	-
18	Hudeiba	Red Blush grapefruit		-	+	-	+	+	-
19	Kassala	Sweet orange	GB	+	+	+	+	+	-
20	Kassala	Sweet orange	GB	+	+	+	+	+	-
21	Kassala	Mandarin	CX	-	+	+	+	+	-
22	Kassala	Grapefruit	KD	+	+	+	+	+	-
23	Kassala	Foster pink grapefruit	KD	+	+	+	+	+	-
24	Kassala	Nuri-16 sweet orange	GB	-	+	-	+	+	-

[1]Symptoms observed in the trees from which samples were collected: CX (cachexia), GB (gummy bark), KD (Kassala disease).

Since all analyzed species are grown on sour orange rootstocks, no symptoms of bark scaling or bark cracking (Vernière *et al.,* 2004) due to viroid infection were to be expected. The bark of all the surveyed trees was scraped to identify symptoms of cachexia on mandarin (Fig. 3A), gummy bark on sweet orange (Fig. 3B) and Kassala disease on grapefruit (Fig. 3C) (Table 1).

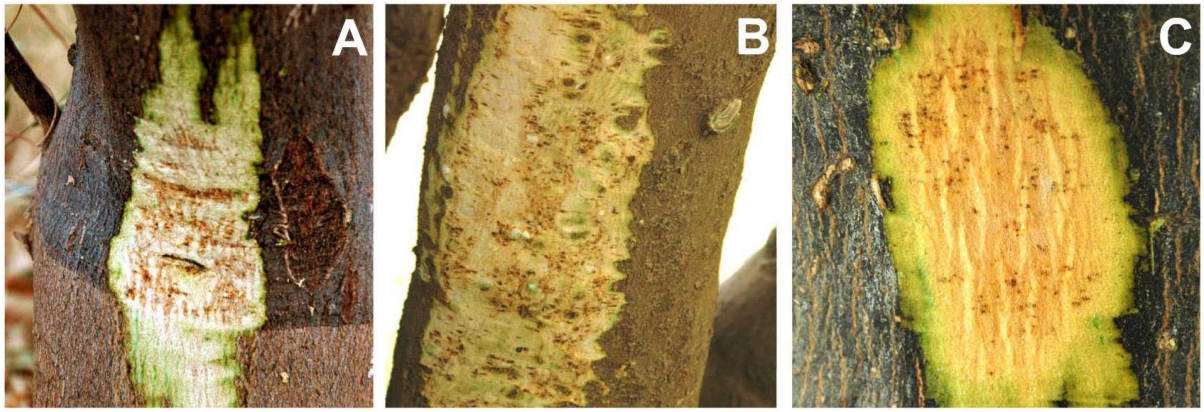


Fig. 3. (A) Cachexia symptoms on a Willowleaf mandarin grafted on an unidentified rootstock in Dongola (sample 2). (B) Gummy bark symptoms on a Nuri-16 sweet orange tree (sample 24) in Kassala; (C) Kassala disease symptoms on a Foster grapefruit tree in Kassala (sample 23).

These samples were analyzed by northern hybridizations, using a procedure sensitive enough to detect viroids directly in commercial citrus species and cultivars, without the need of an amplification step in citron (Murcia *et al.,* 2009). Briefly, bark samples were powdered in liquid nitrogen, and the nucleic acids were recovered in the aqueous phase of the phenol-containing extraction medium. The nucleic acids were partitioned in LiCl 2M and concentrated by ethanol precipitation. The RNAs separated in 5% PAGE (60 mA, 2 h) were electroblotted to Nylon membranes and hybridized with viroid-specific DIG-labelled DNA probes. The results showed that citrus viroids are widely spread in all species and cultivars tested (Fig. 4). All samples were found to be infected with three, four or five viroids: CEVd was detected in 21 samples, HSVd in 22 samples, CBLVd in 17 samples, CDVd in 23 samples, CBCVd in all the samples, and CVd-V in none of the samples (Table 1).

Attempts to disclose a relationship between viroid infection and the “gummy bark” disease of sweet orange gave the following results: (i) Only CBCVd has been found in all the “gummy bark” sources analyzed in this survey (Table 1, samples 4 to 6, 9 to 14, 16, 17, 19, 20, and 24) as well as in all the sources from Turkey (Önelge *et al.,* 1996)

and from the Sultanate of Oman (Bernad *et al.*, 2005) previously tested; (ii) HSVd, considered the most likely candidate to be the casual agent of the disease (Önelge *et al.,* 2004) should be ruled out because two samples that presented mild (sample 10) and severe (sample 6) gummy bark symptoms were found to be free of HSVd (Fig. 4, Table 1); (iii) Even though CEVd and CDVd were found in all the tested sources from Turkey and the Sultanate of Oman (Önelge *et al.,* 2004; Bernad *et al.*, 2005), they were not detected in two sources from Sudan: a sweet orange tree (sample 24), which presented the most severe symptoms of gummy bark (Fig. 3B), was found free of CEVd, while another sweet orange tree (sample 9) with mild symptoms was found to be free of CDVd as well as of CBLVd. Therefore, CBCVd remains the only candidate for a viroid etiology of gummy bark, assuming that the disease is indeed caused by a single, known viroid species.

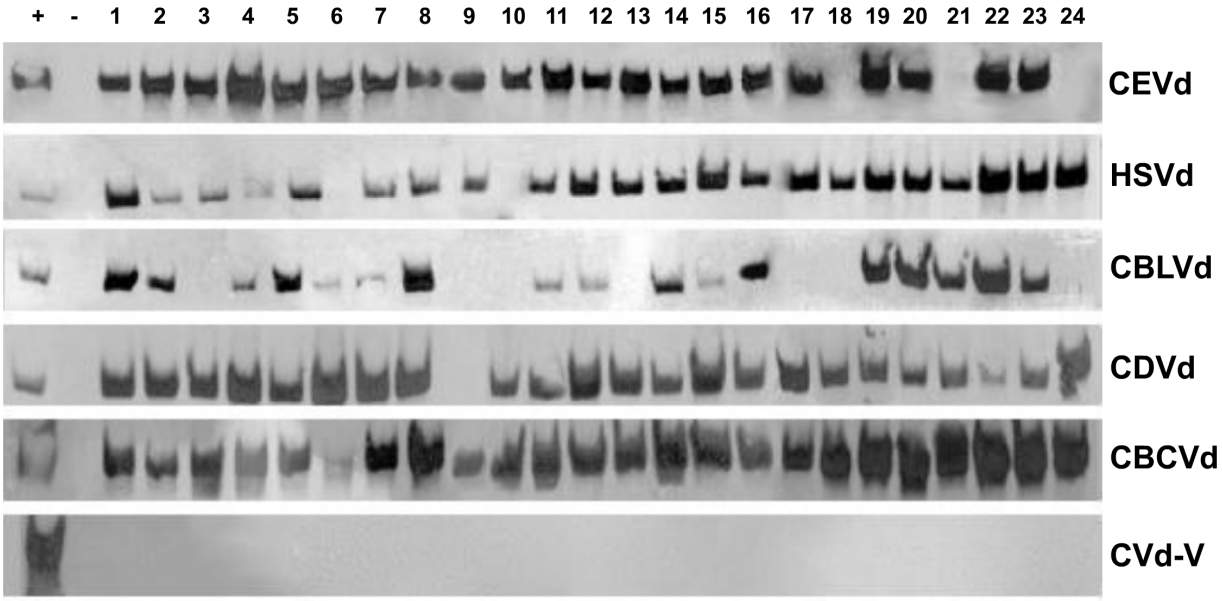


Fig. 4. Northern hybridization, with viroid-specific probes, of samples collected in different location of Sudan. Sample numbers are those listed in Table 1.

However, the possibility cannot be ruled out that the Sudanese gummy bark might be (i) the result of a specific synergistic interaction between two or several viroids co-infecting the same tree, as described by Vernière *et al.,* (2006) in the case of bark scaling symptoms in trifoliate orange (*Poncirus trifoliata* (L,) Raf.); or (ii) a consequence of the high temperatures and the very low relative humidities characteristic of the regions where the disease has been described; or (iii) even both (i) and (ii) together, should not be ruled out. The same considerations also apply to the “Kassala disease” of grapefruit for which, however, no single, known viroid seems to be the etiological

agent, as the three grapefruit sources tested (Table 1, samples 15, 22, 23) were found to be infected with CEVd, HSVd, CBLVd, CDVd and CBCVd.

Given the present situation of the citrus industry in Sudan, where old-line cultivars are grafted on sour orange, citrus viroids represent a real threat not only today in case mandarin scions infected with cachexia-inducing variants of HSVd, but also in the future, when rootstocks like trifoliate orange, Troyer and Carrizo citranges, alemow (*C. macrophylla*) or Rangpur lime (*C. limonia*), sensitive to exocortis and/or cachexia, will have to be used to control tristeza.

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Short communication

Identification of viroids in citrus grown in Iran

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ABSTRACT

The citrus industry in Iran is based on graft propagation of local and imported cultivars on a limited number of rootstocks such as sour orange (*C. aurantium*), Mexican lime (*C. aurantifolia*) and Bakravi, a local natural hybrid. Infection of the available cultivars with graft-transmissible agents, viroids in particular, may have deleterious effects on the productivity of citrus and, more importantly, limits the choice of rootstocks. Rootstocks other than sour orange and Mexican lime will be needed as a consequence of the introduction and spread of CTV in the country. In the present study, two 'Sanguinella" sweet orange and two 'Duncan' grapefruit trees were selected according to the severe and mild bark cracking symptoms observed in the trifoliate orange and Troyer citrange rootstocks. Analysis of nucleic acid preparations of Etrog citrons graft-inoculated with these sources revealed the presence of several viroids, including CEVd and HSVd, the causal agents of exocortis and cachexia, respectively. These and three other viroids, CBLVd, CDVd and CBCVd, detected in these samples were sequenced and compared to the corresponding reference sequences. To extend these results, samples from diferent citrus cultivars were collected in the two main citrus growing regions and analyzed for viroids. From the 49 samples tested, 27 were viroid-free and the existence of cultivars infected with CEVd, HSVd, CBLVd, CDVd, and CBCVd as mixed infections was confirmed in the surveyed regions. Also one sample was found to be co-infected with the newly described CVd-V.

The Islamic Republic of Iran has been a citrus growing country since about 2500 years ago with citron (*Citrus medica*) being the first species cultivated in the country. The Arabs introduced other citrus species such as sour orange, lemon and lime in the tenth century, and much later many other species reached the country probably introduced initially from India and between 1933 and 1977 from Turkey, Italy, Lebanon, USA, Australia, Japan, Marocco, France and the former USSR, without much concern about the sanitary status of the imported budwood. For many centuries, citrus were seed propagated and, since most virus and virus-like pathogens are not seed born, the citrus industry remained disease-free. However, the heavy losses due to *Phytophthora* root rot prompted the growers to switch from seedling trees to graft-propagated trees (Bové, 1995).

Presently there are two major citrus growing areas: (i) The Caspian Sea belt which includes Golestan, Mazadaran and Guilan provinces, with Mediterranean climate mainly producing early maturing cultivars; (ii) The Southern region which includes Fars,

Kerman, Ilam, Khuzestan, Kohkiluieh and Boyerahmad provinces and the Southern Coastal belt of Persian Gulf and Oman Sea, which includes Hormozgan and Bushehr provinces with tropical and subtropical climate. Citrus are graft-propagated on a limited number of rootstocks, sour orange (*C. aurantium*) being the major rootstock in the Caspian Sea area and Mexican lime (*C. aurantifolia*) and Bakravi, a local natural hybrid between mandarin and lime, both drought tolerant are the major rootstocks in the southern areas.

Citrus tristeza virus (CTV) was introduced in 1969 in Mazadaran province through the importation of over 40,000 satsuma trees grafted on trifoliate orange (*Poncirus trifoliata*) from Japan, where tristeza is endemic (Ebrahim-Nesbat and Nienhaus, 1978). Even though the natural spread of CTV was initially low, it eventually spread with the vector *Aphis gossipii* (Rahimian *et al.,* 2000) through the Caspian Sea belt where sour orange is the major rootstock. The Southern region were assumed to be tristeza-free, however surveys conducted in 1996 in Fars and Bushehr provinces showed the presence of CTV (Shafiee and Izadpanah, 1996). Further characterization of the CTV strains in these southern areas indicated that these were of different origin that those in the north (Izadpanah *et al.,* 2002).

CTV infection and spread on trees grafted on sour orange, Mexican lime and Bakravi eventually will cause tree decline, and alternative rootstocks will be needed. The choice of rootstock to replace the existing ones will depend on the presence of graft-transmissible agents, and viroids in particular, in the species and cultivars available. Here we report the identification of citrus viroids in a selection of cultivars collected in different areas.

Two ‘Sanguinella” sweet orange and two ‘Duncan’ grapefruit trees were selected according to the severe (Fig. 1) and mild bark cracking symptoms observed in the trifoliate orange and Troyer citrange rootstocks. These cultivars had been introduced from Italy in the 1930s and are available at the facilities of Citrus Research Institute at Kotra Station.

Fig. 1. Severe bark cracking symptoms in the rootstock part of the ‘’Sanguinella’’ sweet orange tree grafted on *Poncirus trifoliate,* Kotra Station, Caspian sea belt.

Samples from these sources were biologically indexed by graft-transmission to the sensitive selection 861-S1 of Etrog citron (*C. medica* L). Analysis of nucleic acid preparations of the inoculated citrons by sequential polyacrylamide gel electrophoresis (sPAGE) and silver staining (Rivera-Bustamante *et al.,* 1986; Duran-Vila *et al.,* 1993) revealed the presence of three up to seven bands with the mobilities of the circular forms of viroids (Fig. 2A). When the RNAs of these samples and the corresponding controls were separated by sPAGE, electroblotted (400 mA for 2 h) to positively-charged nylon membranes using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA), and hybridized with digoxigenin (DIG)-labeled viroid specific probes generated by PCR (Palacio *et al*., 1999), the viroid nature of the bands viewed in sPAGE analysis was confirmed. The citrus viroids identified were: *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) and *Citrus dwarfing viroid* (CDVd) (former *Citrus viroid III*, CVd-III) in the four samples tested (Fig. 2A, 2B: lanes 1 to 4); *Citrus bent leaf viroid* (CBLVd) in the two 'Sanguinella" sweet orange sources and in one of the 'Duncan' grapefruit sources (Fig. 2A, 2B: lanes 1, 2 and 4); and *Citrus bark cracking viroid* (CBCVd) (former *Citrus viroid IV*, CVd-IV) in the two 'Sanguinella" sweet orange sources (Fig. 2A, 2B: lanes 1 and 2). All plants tested negative for *Citrus viroid OS* (CVd-OS) (Ito *et al.,* 2001) and *Citrus viroid V* (CVd-V) (Serra *et al.,* 2008) (Fig. 2A, 2B: lanes 1 to 4). sPAGE and northern blot hybridization analysis showed that two of the samples (Fig. 2A, 2B, lanes 2 and 4) contained two distinct HSVd bands whereas the other two samples presented a single HSVd band (Fig. 2A, 2B: lanes 1 and 3). Similarly, two of the samples contained several CDVd bands (Fig. 2A, 2B: lanes 1 and 2).

The nucleic acid preparations from these four citron plants were subjected to reverse transcription and PCR amplification (RT-PCR) using viroid-specific primers (Bernad and Duran-Vila, 2006) and yielded amplicons of the expected sizes of CEVd, CBLVd, HSVd, CDVd and CBCVd. The amplicons were sequenced either directly or after cloning into the pGEM-T vector (Promega), using a ABI PRISM DNA sequencer 377 (Perkin Elmer). The sequences were aligned with reference sequences of the corresponding viroids using the Clustal W program (Thompson *et al.,* 1994).

The consensus sequences of two CEVd isolates from samples 1 and 3 obtained by sequencing the RT-PCR amplicons, showed identities of 99.7% and 100% with the predominant sequence variant of our type strain (Gandía *et al.,* 2005) (Table 1) and had a composition of the P (Pathogenicity) domain characteristic of severe strains of CEVd as defined by Visvader and Symons (1986). Most of the nucleotide changes identified in these two isolates were clustered in a loop of the V (Variable) domain and did not affect the predicted viroid secondary structure (data not shown). The two CEVd

isolates from samples 2 and 4 presented high identities with a new class of CEVd variants identified originally in the Sultanate of Oman (Bernad *et al.,* 2005) (Table 1).

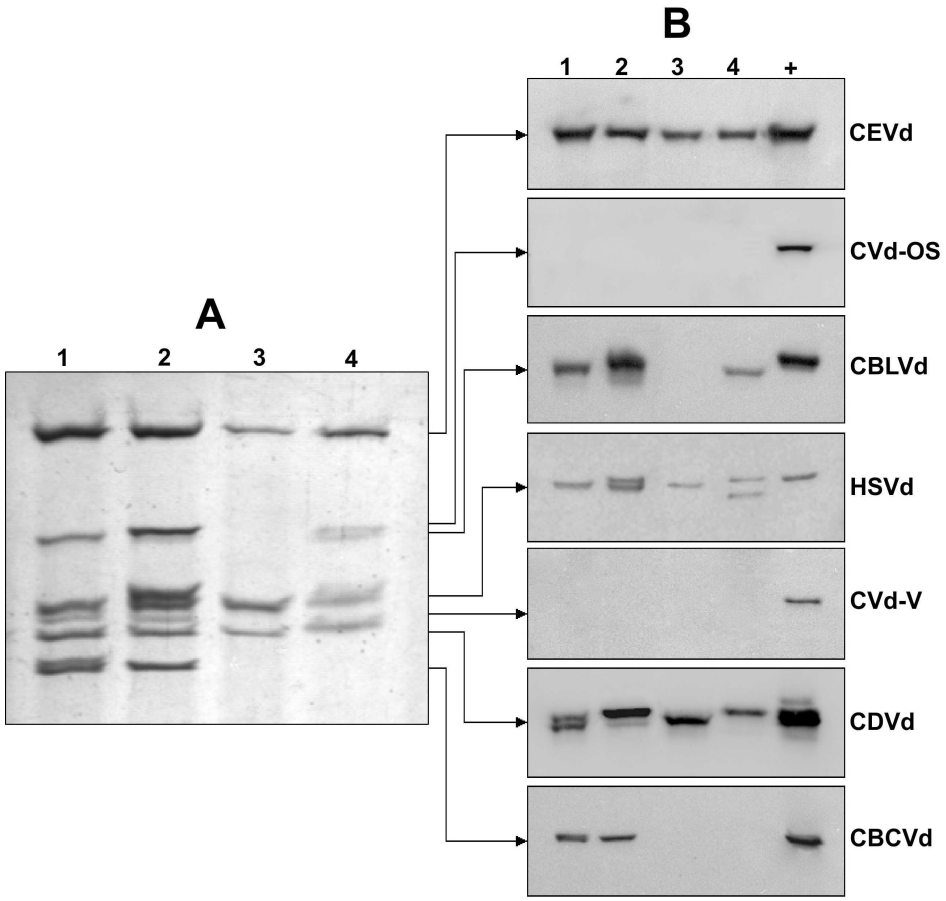

Fig. 2. (A) Sequential PAGE analysis and (B) Northern-blot hybridization with viroid-specific probes of extracts from Etrog citron plants that have been graft-inoculated with two ‘’Sanguinella’’ sweet orange (lines 1 and 2) and two Doncan grapefruit (lines 3 and 4) sources collected in Kotra Station, Caspian sea region. Samples from viroid-infected Etrog citron plants were included as positive controls (+).

The consensus sequences of the three CBLVd isolates (samples 1, 2 and 4) obtained by sequencing the RT-PCR amplicons, had a size of 318 nt and presented the highest identities with CBLVd (variant CVd-Ib) (Hataya *et al.,*1998) (Table 1).

Since the pathogenic properties of HSVd in citrus depends on the presence of either the “cachexia expression motif” in strains that induce the cachexia disease or the “non-cachexia expression motif” in strains that do not induce the cachexia disease (Reanwarakorn and Semancik, 1998), the four HSVd isolates were characterized by cloning, sequencing and multiple alignments with the reference sequences of CVd-IIa,117 (non-cachexia inducing variant), CVd-IIb and CVd-IIc (cachexia inducing variants) (Reanwarakorn and Semancik, 1999; Palacio-Bielsa *et al.,* 2004). The HSVd isolate in sample 1 contained a mixture of variants of 300 and 299 nt. The 300 nt variant contained the sequence and structure of the “non-cachexia expression motif”

and presented a high identity (98.3%) with the CVd-IIa-117 reference sequence, whereas de 299 nt variant contained the sequence and structure of the "cachexia expression motif" and presented the highest identity with the CVd-IIb reference sequence (Table 1). The relatively low sequence identity (94.3%) was consequence of 13 changes, two deletions and an addition that affected the upper and lower strands of the regions flanking the Central Conserved Region (CCR), and affecting the HSVd secondary structure. This variant had a high identity (99.7%) with a cachexia inducing variant previously described in Japan (Ito *et al.,* 2002).

Table 1. Molecular characteristics of citrus viroids identified in four isolates from Iran.

Sample	Viroid	NCBI GenBank	Size (nt)	Sequence identity	Reference
1	CEVd	GQ260196	371	99.7% (CEVd, E117V1)	Gandía *et al.,* 2005
	CBLVd	GQ260200	318	98.1% (CVd-Ib)	Hataya *et al.,* 1998
	HSVd	GQ260207	300	98.3% (CVd-IIa, 117)	Palacio-Bielsa *et al.,* 2004
	HSVd	GQ260206	299	94.3% (CVd-IIb, X-715-1)	Palacio-Bielsa *et al.,* 2004
	CDVd	GQ260212	294	100% (CVd-IIIb)	Rakowski *et al.,* 1994
	CDVd	GQ260214	292	98.6% (CVd-IIIb)	Rakowski *et al.,* 1994
	CBCVd	GQ260216	284	100% (CVd-IV)	Puchta *et al.,* 1991
2	CEVd	GQ260199	370	100% (CEVd, gb4)	Bernard *et al.,* 2005
	CBLVd	GQ260201	318	99.4% (CVd-Ib)	Hataya *et al.,* 1998
	HSVd	GQ260203	302	99.0% (CVd-IIa-117)	Palacio-Bielsa *et al.,* 2004
	HSVd	GQ260205	298	97.3% (CVd-IIb, X-715-1)	Palacio-Bielsa *et al.,* 2004
	CDVd	GQ260210	297	96.0% (CVd-IIIa)	Rakowski *et al.,* 1994
	CDVd	GQ260215	293	98.6% ($CDVd^{SO}$)	Murcia *et al.,* 2009a
	CBCVd	GQ260216	284	100% (CVd-IV)	Puchta *et al.,* 1991
3	CEVd	GQ260197	371	100% (CEVd, E117V1)	Gandía *et al.,* 2005
	HSVd	GQ260208	300	98.7% (CVd-IIa, 117)	Palacio-Bielsa *et al.,* 2004
	CDVd	GQ260213	294	99.7% (CVd-IIIb)	Rakowski *et al.,* 1994
4	CEVd	GQ260198	370	99.7% (CEVd, gb4)	Bernard *et al.,* 2005
	CBLVd	GQ260202	318	100% (CVd-Ib)	Hataya *et al.,* 1998
	HSVd	GQ260204	302	99.0% (CVd-IIa, 117)	Palacio-Bielsa *et al.,* 2004
	HSVd	GQ260209	295	100% (CVd-IIc)	Reanwarakorn and Semancik, 1999
	CDVd	GQ260211	297	99.7% (CVd-IIIa)	Rakowski *et al.,* 1994

The HSVd isolate in sample 2 contained a mixture of variants of 302 and 298 nt. The 302 nt variant contained the sequence and structure of the "non-cachexia expression motif" and presented the highest identity with the CVd-IIa-117 reference sequence, whereas the 298 nt variant contained the sequence and structure of the "cachexia expression motif" and presented the highest identity with CVd-IIb reference sequence (Table 1). HSVd isolate in sample 3 contained a 300 nt variant with the sequence and structure of the "non-cachexia expression motif" and a high identity with the CVd-IIa-117 reference sequence. HSVd isolate in sample 4 contained a mixture of variants of 302 and 295 nt. The 302 nt variant contained the sequence and structure of

the “non-cachexia expression motif” and presented a high identity with the CVd-IIa-117 reference sequence, whereas the 295 nt variant contained the sequence and structure of the “cachexia expression motif” and presented 100% identity with CVd-IIc reference sequence (Table 1) .

Given the large number of variants with different pathogenic properties described (Murcia *et al.,* 2009a), the four CDVd isolates were characterized by cloning, sequencing and multiple alignments with the reference sequences of CVd-IIIa, CVd-IIIb and CVd-IIIc (Rakowsky *et al.,* 1994; Semancik *et al.,* 1997). The CDVd isolate in sample 1 contained a mixture of variants of 294 and 292 nt, both with high identity with the reference variant CVd-IIIb (Table 1). The 292 nt variant differed in three changes that affected the organization of the secondary structure of the T_L domain as described for other isolates (Murcia *et al.,* 2009a). The CDVd isolate in sample 2 contained a mixture of variants of 297 and 293 nt. The 297 nt variant presented the highest identity with the reference sequence CVd-IIIa (Table 1), whereas the 293 nt presented the highest identity (98.6%) with an usual CDVd variant ($CDVd^{SO}$) from the Sultanate of Oman (Murcia *et al.,* 2009a), and contained the three changes that affected the organization of the secondary structure of the T_L domain found in the 292 nt variant from sample 1. The CDVd isolate from sample 3 contained a 294 nt variant with a consensus sequence that presented a high identity with the reference sequence CVd-IIIb, whereas the isolate from sample 4 contained a 297 variant with a consensus sequence with the highest identity with the reference sequence CVd-IIIa.

The consensus sequences of the two CBCVd isolates (samples 1 and 2) obtained by sequencing the RT-PCR amplicons, had a size of 284 nt and both presented 100% identity with the CBLVd reference sequence (Puchta *et al.,* 1991).

The analysis of these four samples provided an indication that at least five of the seven citrus viroids known to infect citrus are present in the country. With the availability of a procedure sensitive enough to detect viroids directly in commercial citrus species and cultivars, without the need of an amplification step in citron (Murcia *et al.,* 2009b), samples from the field collection maintained at the Iran Citrus Research Institute in Ramsar as well as from Research Stations, nurseries and commercial field plots located in the Caspian Sea belt and in the Southern region, were viroid tested. Briefly, bark samples were powdered in liquid nitrogen, and the nucleic acids were recovered in the aqueous phase of the phenol-containing extraction medium. The nucleic acids were partitioned in 2M LiCl and concentrated by ethanol precipitation. The RNAs separated in 5% PAGE (60 mA, 2 h) were electroblotted to Nylon membranes and hybridized with viroid-specific DIG-labelled DNA probes. The results (Table 2) can be summarized as follows:

Table 2. Viroid detected in citrus samples collected in different locations of Iran.

Sample	Scion	Rootstock	Location	Region	Original Source	Symptom[1]	Viroids						
							CEVd	HSVd	CBLVd	CDVd	CBCVd	CVd-V	CVd-OS
1	Marrs navel sweet orange	Sour orange	Ramsar station	Caspian sea	California		-	-	-	-	-	-	-
2	Hamlin sweet orange	Trifoliate orange	Ramsar station	Caspian sea	California		-	-	-	-	-	-	-
3	Persian lime	Sour orange	Ramsar station	Caspian sea	France		-	-	-	-	-	-	-
4	Okitsu satsuma	Sour orange	Ramsar station	Caspian sea	France		-	-	-	-	-	-	-
5	Clausellina satsuma	Sour orange	Ramsar station	Caspian sea	France		-	-	-	-	-	-	-
6	Sugiyama satsuma	Sour orange	Ramsar station	Caspian sea	France		-	-	-	-	-	-	-
7	Nules clementine	Sour orange	Ramsar station	Caspian sea	France		-	-	-	-	-	-	-
8	Marisol clementine	Sour orange	Ramsar station	Caspian sea	France		-	-	-	-	-	-	-
9	Newhall navel orange	Sour orange	Ramsar station	Caspian sea	France		-	-	-	-	-	-	-
10	Valencia sweet orange	Sour orange	Ramsar station	Caspian sea	France		-	-	-	-	-	-	-
11	Palestine sweet lime	Swingle citrumelo	Ramsar station	Caspian sea	Unknown		-	-	-	-	-	-	-
12	Owari Satsuma	Trifoliate orange	Ramsar station	Caspian sea	Morocco		-	-	-	-	-	-	-
13	Thompson navel sweet orange	Troyer citrange	Ramsar station	Caspian sea	Unknown		-	-	-	-	-	-	-
14	Thompson navel sweet orange	Trifoliate orange	Ramsar station	Caspian sea	Unknown		-	-	-	-	-	-	-
15	Ponkan mandarin	Swingle citrumelo	Ramsar station	Caspian sea	California		-	-	-	-	-	-	-
16	Redblush grapefruit	Sour orange	Ramsar station	Caspian sea	Unknown		-	-	-	-	-	-	-
17	Page mandarin	Trifoliate orange	Ramsar station	Caspian sea	Unknown	A	+	+	+	+	+	-	-
18	Duncan Grapefruit	Trifoliate orange	Khoram Abad station	Caspian sea	Unknown	A	+	+	+	+	+	-	-
19	Thompson navel sweet orange	Trifoliate orange	Khoram Abad station	Caspian sea	Unknown		-	-	-	-	-	-	-
20	Moro blood orange	Trifoliate orange	Khalatbari orchard, Ramsar	Caspian sea	Unknown	A	+	+	+	+	+	-	-
21	Page mandarin	Trifoliate orange	Nazerian orchard, Tonekabon	Caspian sea	Unknown		-	-	-	-	-	-	-
22	Thompson navel sweet orange	Trifoliate orange	M.A. nursery,Tonekabon	Caspian sea	Unknown	A	+	+	+	+	-	-	-
23	Thompson navel sweet orange	Sour orange	M.A. nursery,Tonekabon	Caspian sea	Unknown		-	+	-	-	-	-	-
24	Redblush grapefruit	Sour orange	M.A. nursery,Tonekabon	Caspian sea	Unknown		+	+	+	+	+	-	-
25	Moro blood orange	Sour orange	M.A. nursery,Tonekabon	Caspian sea	Unknown		+	+	+	+	+	-	-

[1] Symptoms observed in the rootstock of the trees from which samples were collected: A (sever bark cracking), B (mild bark cracking).

Table 2. (Continue). Viroid detected in citrus samples collected in different locations of Iran.

Sample	Scion	Rootstock	Location	Region	Original Source	Symptom[1]	Viroids						
							CEVd	HSVd	CBLVd	CDVd	CBCVd	CVd-V	CVd-OS
26	Persian lime	Sour orange	M.A. nursery,Tonekabon	Caspian sea	Unknown		-	-	-	-	-	-	-
27	Duncan Grapefruit	Trifoliate orange	Kotra station	Caspian sea	Italy	B	+	+	-	+	-	-	-
28	Thompson navel sweet orange	Trifoliate orange	Kotra station	Caspian sea	Unknown		-	+	-	+	-	-	-
29	Valencia sweet orange	Trifoliate orange	Kotra station	Caspian sea	California		-	-	-	-	-	-	-
30	Grapefruit (unknown)	Trifoliate orange	Kotra station	Caspian sea	California		-	-	-	-	-	-	-
31	Orlando tangelo	Sour orange	Kotra station	Caspian sea	California		-	-	-	-	-	-	-
32	Minneola tangelo	Sour orange	Kotra station	Caspian sea	California		-	+	-	+	-	-	-
33	Eureka lemon	Sour orange	Kotra station	Caspian sea	California		+	+	+	+	+	-	-
34	Lisbon lemon	Sour Orange	Kotra station	Caspian sea	California		-	-	-	-	-	-	-
35	Sanguigno blood orange	Trifoliate orange	Kotra station	Caspian sea	Italy	A	+	+	-	+	+	-	-
36	Sanguinella blood orange	Trifoliate orange	Kotra station	Caspian sea	Italy	A	+	+	+	+	+	-	-
37	Nagami kumquat	Sour Orange	Kotra station	Caspian sea	Morroco		-	+	-	+	-	-	-
38	Kinnow mandarin	Mexican lime	Khafr	Southern	Unknown		-	-	-	-	-	-	-
39	Thompson navel sweet orange	Mexican lime	Darab	Southern	Unknown		+	+	-	-	-	-	-
40	Persian lime	Mexican lime	Bakhtajerd station	Southern	Unknown		-	-	-	-	-	-	-
41	Valencia sweet orange	Mexican lime	Brentin	Southern	Unknown		-	-	-	+	-	-	-
42	Moro blood orange	Mexican lime	Brentin	Southern	Unknown		+	+	+	+	-	+	-
43	Local sweet orange	Mexican lime	Brentin	Southern	Unknown		+	+	+	+	+	-	-
44	Palestine sweet lime	Mexican lime	Shahy plane	Southern	Unknown		-	-	-	-	-	-	-
45	Local sweet orange	Mexican lime	Farjab, Rudan	Southern	Unknown		+	+	-	+	-	-	-
46	Valencia sweet orange	Mexican lime	Farjab, Rudan	Southern	Unknown		+	+	-	+	-	-	-
47	Pera shape blood orange	Mexican lime	Farjab, Rudan	Southern	Unknown		+	+	+	+	+	-	-
48	Lemon (unknown)	Mexican lime	Berentin, Rudan	Southern	Unknown		-	+	+	-	+	-	-
49	Red blush grapefruit	Mexican lime	Berentin, Rudan	Southern	Unknown		-	-	-	-	-	-	-

[1] Symptoms observed in the rootstock of the trees from which samples were collected: A (sever bark cracking), B (mild bark cracking).

From the 49 samples tested, 27 were viroid-free. The viroid-free samples covered a range of cultivars of sweet orange, satsuma, clementine, mandarin, tangelo, grapefruit, lemon, Persian lime and Palestine sweet lime that had been introduced from California (USA), Corsica (France) and Morocco (Table 2). The remaining 22 samples were all co-infected with several viroids, with 16 of them containing CEVd, the causal agent of the exocortis disease. Six of the CEVd containing samples (samples 17, 18, 20, 22, 35 and 36) were collected from trees grafted on trifoliate orange rootstock that showed the characteristic exocortis bark scaling symptoms in the rootstock. HSVd, CDVd, CEVd, CBLVd, and CBCVd are widely spread in the co-infected samples (found respectively in 21, 19, 16, 12 and 11 out of the 49 samples tested). The newly described CVd-V has been identified in a single Moro blood orange tree of unknown origin (Sample 42, Table 2).

Given the present situation of the citrus industry in Iran, with sour orange and Mexican lime as the major rootsocks, the spread of CTV (Izadpanah *et al.,* 2002 Barzegar *et al.,* 2006; Ahmadi *et al.,* 2007) will endanger citrus production unless these two rootstocks are replaced by other CTV-tolerant rootstock species. Therefore, cultivars, which carry viroids cannot be grafted on viroid-sensitive rootstocks such as tangelo (*C. reticulata* X *C. paradisi*), alemow (*C. macrophylla*), Rangpur lime (*C. limonia*), trifoliate orange and Troyer and Carrizo citrange (*P. trifoliata* X *C. sinensis*). One should be also be aware that infection with cachexia inducing variants of HSVd also induces a deleterious effect in cultivars of mandarin, satsuma, clementine and their hybrids, regardless of the rootstock due to their own sensitivity to cachexia. Since the control of the tristeza disease is based on planting rootstock/scion combinations tolerant to CTV, the choice of rootstock species will strongly depend on the sanitary status of the commercial cultivars available in Iran.

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Short communication

Low performance of citrus trees grafted on Carrizo citrange is associated with viroid infection

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ABSTRACT

After the implementation of a Citrus Variety Improvement Program in Spain, citrus cultivars free of virus and virus-like pathogens have become available and cover today about 70% of the citrus growing regions. In addition, because of the need to control tristeza, the main rootstock used in commercial plantings in Spain is Carrizo citrange. In spite of these developments, low performing orchards can still be found in certain areas in which tree vigor and fruit production are poor. Infection with viroids may have deleterious effects on the productivity of citrus species grafted on the viroid sensitive Carrizo citrange. Viroid infected trees were indeed found in four low performing orchards of 'Clemenules' and 'Afourer/Nadorcor' clementines and 'Ortanique' tangor trees grafted on Carrizo citrange. Bark scaling symptoms in the Carrizo citrange rootstock were always associated with CEVd infection but CBLVd, HSVd and CDVd were also consistently detected. The presence of viroid-infected and viroid-free trees in these orchards is discussed.

The tristeza outbreak in Spain in 1957 prompted important changes in the citrus industry through the establishment of a Citrus Variety Improvement Program with the following objectives: (i) recovery of plants free of virus and virus-like pathogens of local cultivars; (ii) importation of additional cultivars through strict quarantine measures; (iii) establishment of a germplasm bank to maintain citrus plants free of virus and virus-like pathogens; and (iv) release of budwood to nurseries through a certification program. With these measures, the Spanish citrus industry has reached a leading position, and about 70% of the citrus growing areas are now planted with certified plant material (Navarro *et al.,* 2002). However, in spite of these efforts, growers who do not follow the established guidelines are facing problems due to low performing trees.

In the municipality of Picassent (Valencia, Spain) low performing orchards, established in the early 1990s, were identified and suspected of being infected with viroids. Orchard #1 had been planted with 'Clemenules' clementine (*Citrus clementina*) grafted on Carrizo citrange (*Poncirus trifoliata* X *C. sinensis*) to replace an old plantation of the same cultivar grafted on Cleopatra mandarin (*C. reshni*). Most of the trees are now declining and present poor canopy growth (Fig. 1A, 1B). Orchard #2 had been planted with 'Ortanique' tangor (*C. reticulata* X *C. sinensis*) grafted on Carrizo citrange. The trees were not uniform; small trees showing bark scaling symptoms were scattered among larger symptomless trees (Fig. 1C, 1D). Orchard #3 had been planted with 'Clemenules' clementine grafted on Carrizo citrange. About half of the trees

located in one side of the orchard presented mild bark scaling symptoms on the Carrizo citrange rootstock, whereas the other half, located on the other side of the orchard, were symptomless, suggesting that the trees were not planted at the same time and were probably originated from different budwood sources. Orchard #4 had been planted with 'Afourer/Nadorcor' clementine grafted on Carrizo citrange and did not present any symptoms.

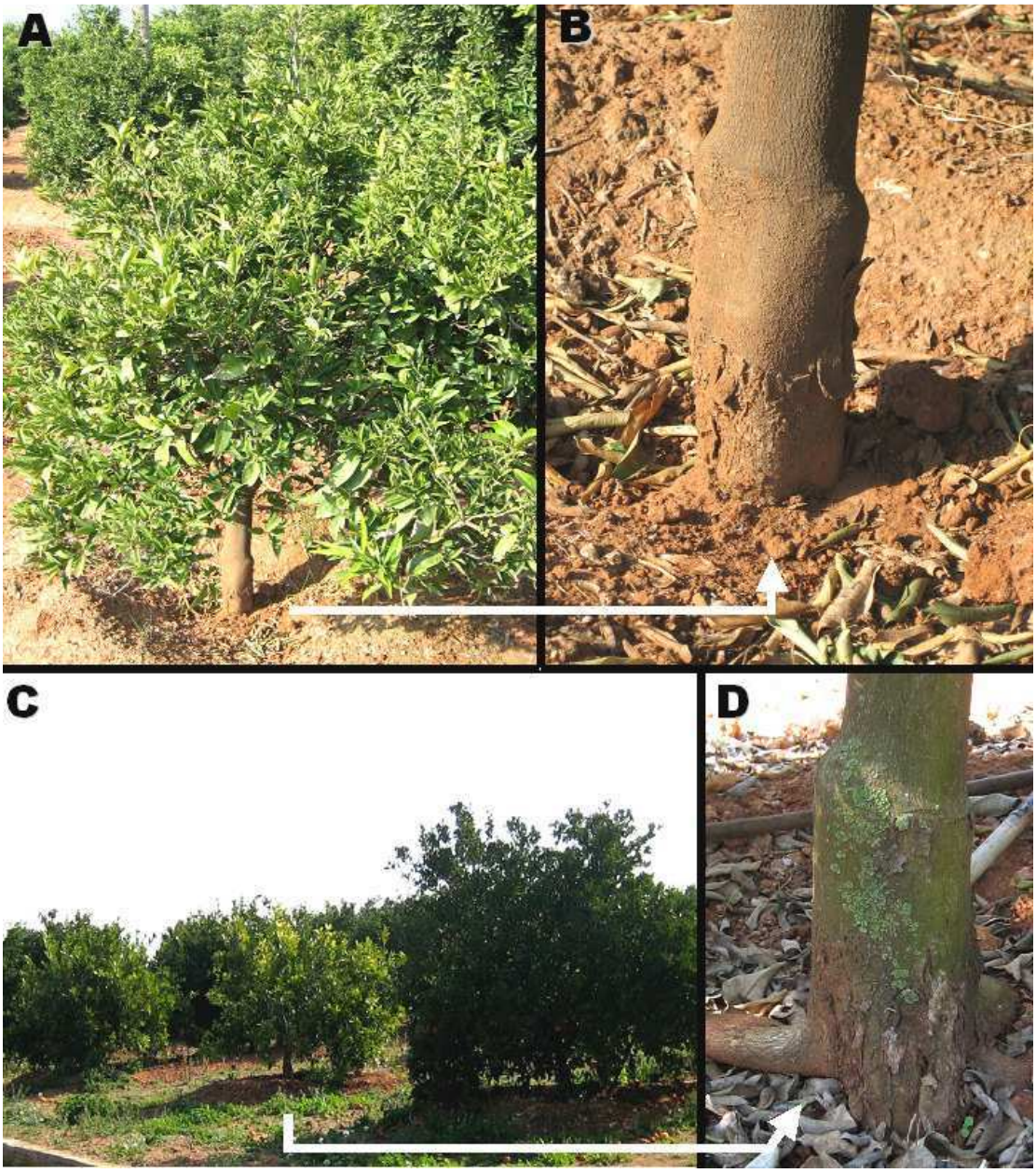

Fig. 1. Symptoms of infected trees in the field. (A) Declining 'Clemenules' clementine tree grafted on Carrizo citrange in orchard #1. (B) Bark cracking in the rootstock of the same tree. (C) Small infected trees scattered among larger symptomless trees in orchard #2. (D) Bark scaling symptoms in the rootstock of an infected tree of orchard #2.

In March 2006, a tree, considered as the most representative, was selected in each one of the four orchards. Samples from the four trees were biologically indexed by graft-transmission to the sensitive selection 861-S1 of Etrog citron (*C. medica*). Three months after inoculation, the citron plants presented symptoms characteristic of viroid infection. Analysis of nucleic acid preparations of the inoculated citrons by sequential polyacrylamide gel electrophoresis (sPAGE) and silver staining (Rivera-Bustamante *et al.,* 1986; Duran-Vila *et al.,* 1993) revealed the presence of several bands with the mobilities of the circular forms of viroids (Fig. 2A). When the RNAs of these samples and the corresponding controls were separated by sPAGE, electroblotted (400 mA for 2 h) to positively-charged nylon membranes using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA), and hybridized with digoxigenin (DIG)-labeled viroid specific probes generated by PCR (Palacio *et al*., 1999), the viroid nature of the bands viewed in sPAGE was confirmed. The identified citrus viroids were: *Citrus exocortis viroid* (CEVd) and *Citrus bent leaf viroid* (CBLVd), present in the samples from orchards #1, #2 and #3 (Fig. 2B, 2C); *Hop stunt viroid* (HSVd) and *Citrus dwarfing viroid* (CDVd) (former *Citrus viroid III*), present in the samples from the four orchards (Fig. 2E, 2G). All plants tested negative for *Citrus viroid OS* (CVd-OS) (Ito *et al.,* 2001) (Fig. 2D), *Citrus viroid V* (CVd-V) (Serra *et al.,* 2008a) and *Citrus bark cracking viroid* (CBCVd) (former *Citrus viroid IV*) (Duran-Vila *et al.,* 1988) (Fig. 2F, 2H). Reverse transcription and PCR amplification (RT-PCR) using viroid-specific primers (Bernad and Duran-Vila, 2006) yielded amplicons of the expected sizes that were sequenced either directly or after cloning into the pGEM-T vector (Promega), using a ABI PRISM DNA sequencer 377 (Perkin Elmer). The sequences were aligned with reference sequences of the corresponding viroids using the Clustal W program (Thompson *et al.,* 1994).

The consensus sequences of the CEVd isolates from orchards #1, #2 and #3 (NCBI GenBank GQ246192, GQ246194 and GQ246191, respectively), obtained by sequencing the RT-PCR amplicons, showed identities of 97.3%, 99.5% and 97.8% with the predominant sequence variant of our type strain (Gandía *et al.,* 2005) and had a composition of the P (Pathogenicity) domain characteristic of severe strains of CEVd as defined by Visvader and Symons (1986). Most of the nucleotide changes identified in the isolates from orchards #1 and #3 were clustered in a loop of the V (Variable) domain and did not affect the predicted viroid secondary structure.

The consensus sequences of the CBLVd isolates from orchards #1 and #3 (NCBI GenBank GQ246193 and GQ246196, respectively), obtained by sequencing the RT-PCR amplicons, presented identities of 97.9% each with the predominant sequence variant of our type strain (Foissac and Duran-Vila, 2000). Both sequences had 328

instead of 327 nt as a result of an insertion (+C in position 167) located in terminal right loop of the viroid secondary structure. The sPAGE and northern hybridization results (Fig. 2A and 2C: lane 2) indicated that the sample from orchard #2 contained two CBLVd related variants of different sizes, a fact that was confirmed by cloning and sequencing. The large variant (NCBI GenBank GQ246195) also had 328 nt as a result of an insertion (+C in position 167) and presented a 98.8% identity with the predominant sequence variant of our type strain, whereas the small variant of 318 nt (NCBI GenBank GQ246197) presented 98.1% identity with CBLVd (CVd-Ib) (Hataya *et al.,*1998).

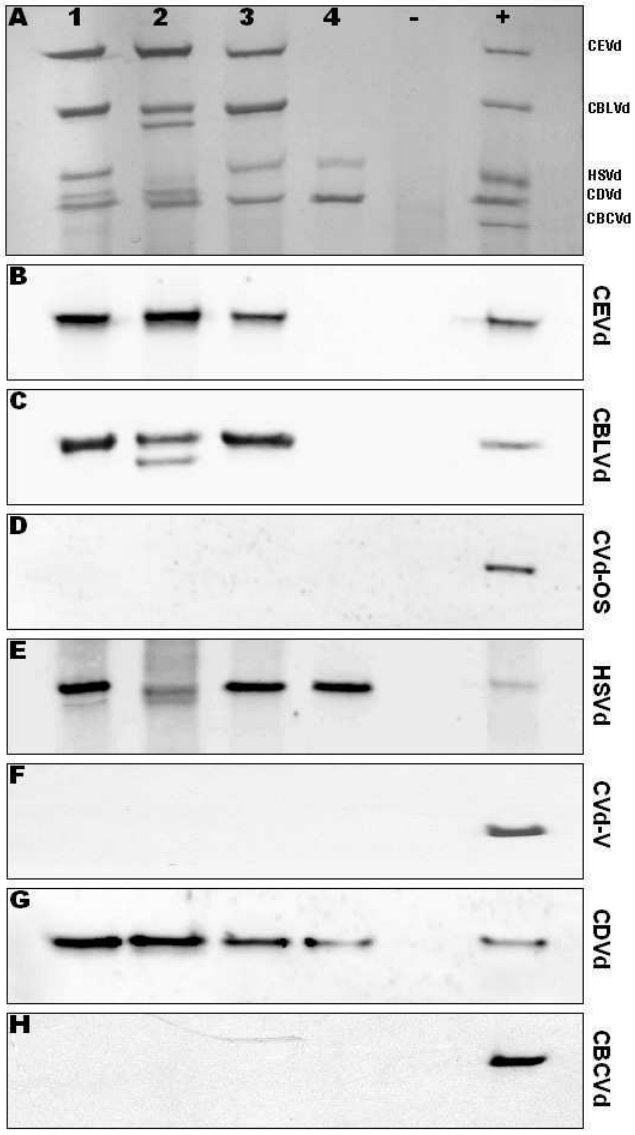

Fig. 2. Sequential PAGE analysis (A) and northern-blot hybridization with viroid-specific probes (B to H) of Etrog citron plants graft-inoculated with 'Clemenules' clementine collected in orchard #1 (1), 'Ortanique' tangor collected in orchard #2 (2), 'Clemenules' clementine collected in orchard #3 (3) and 'Afourer/Nadorcor' clementine collected in orchard #4 (4). Samples from healthy (-) and viroid-infected plants (+) were included as negative and positive controls.

Since the pathogenic properties of HSVd in citrus depend on the presence of either the "cachexia expression motif" in strains that induce the cachexia disease, or the "non-cachexia expression motif" in strains that do not induce the cachexia disease in sensitive species (Reanwarakorn and Semancik, 1998), the four HSVd isolates were characterized by cloning, sequencing and multiple alignments with the reference sequences of CVd-IIa-117 (non-cachexia inducing variant), CVd-IIb and CVd-IIc (cachexia inducing variants) (Reanwarakorn and Semancik, 1999; Palacio-Bielsa *et al.,* 2004). The HSVd isolate from orchard #1 was found to contain a mixture of variants of 296 and 302 nt. The 296 nt variant (NCBI GenBank GQ254645) contained the sequence and structure of the "cachexia expression motif" and presented 99.3% identity with the CVd-IIc reference sequence, whereas the 302 variant (NCBI GenBank GQ246198) contained the sequence and structure of the "non-cachexia expression motif" and presented 99.3% identity with the CVd-IIa-117 reference sequence. The HSVd isolate from orchard #2 contained a mixture of variants of 296 and 300 nt. Both variants (NCBI GenBank GQ246200 and GQ246201, respectively) contained the sequence and structure of the "cachexia expression motif". The 296 nt variant presented the highest identity (99.6%) with CVd-IIc whereas the 300 nt variant presented the highest identity (98.3%) with CVd-IIb with a U→C change in position 194, which is characteristic of variants that induce mild cachexia symptoms (Serra *et al.,* 2008b). The consensus sequences of the HSVd isolates from orchards #3 and #4 (NCBI GenBank GQ246198 and GQ246199, respectively) had 302 nt, contained the sequence and structure of the "non-cachexia expression motif" and presented 99.3% and 98.6% identities with the CVd-IIa-117 reference sequence.

The four CDVd isolates were characterized by cloning, sequencing and multiple alignments with the reference sequences of CVd-IIIa, CVd-IIIb, CVd-IIIc (Rakowsky *et al.,* 1994; Semancik *et al.,* 1997). The CDVd isolate from orchard #1 (NCBI GenBank GQ254646) was found to contain a 294 nt variant with the highest identity (99.0%) with CVd-IIIb. The CDVd isolate from orchard #2 was found to contain a mixture of variants of 293 and 294 nt. The 293 nt variant (NCBI GenBank GQ246202) presented the highest identity (98.6%) with an usual CDVd variant ($CDVd^{SO}$) from the Sultanate of Oman (Murcia *et al.,* 2009a) whereas the 294 nt (NCBI GenBank GQ254647) presented the highest identity (99.7%) with CVd-IIIb. The CDVd isolate from orchard #3 contained a 294 nt variant (NCBI GenBank GQ254648) that was found be identical to CVd-IIIb. The CDVd isolate from orchard #4 also contained a 294 nt variant (NCBI GenBank GQ254647) with the highest identity (99.7%) with CVd-IIIb.

Table 1. Viroids detected in samples collected in four low performing orchards.

Orchard	Tree	CEVd	CBLVd	CVd-OS	HSVd	CVd-V	CDVd	CBCVd	Symptoms[1]
Orchard #1	1	+	+	-	+	-	+	-	+
Orchard #1	2	+	+	-	+	-	+	-	+
Orchard #1	3	+	+	-	+	-	+	-	+
Orchard #1	4	+	+	-	+	-	+	-	+
Orchard #1	5	+	+	-	+	-	+	-	+
Orchard #1	6	+	+	-	+	-	+	-	+
Orchard #1	7	-	-	-	-	-	-	-	-
Orchard #1	8	+	+	-	+	-	+	-	+
Orchard #1	9	+	+	-	+	-	+	-	+
Orchard #1	10	+	+	-	+	-	+	-	+
Orchard #2	11	+	+	-	+	-	+	-	+
Orchard #2	12	+	+	-	+	-	+	-	+
Orchard #2	13	+	+	-	+	-	+	-	+
Orchard #2	14	+	+	-	+	-	+	-	+
Orchard #2	15	+	+	-	+	-	+	-	+
Orchard #2	16	+	+	-	+	-	+	-	+
Orchard #2	17	+	+	-	+	-	+	-	+
Orchard #2	18	+	+	-	+	-	+	-	+
Orchard #2	19	+	+	-	+	-	+	-	+
Orchard #2	20	+	+	-	+	-	+	-	+
Orchard #2	21	-	+	-	+	-	-	-	-
Orchard #2	22	-	-	-	-	-	-	-	-
Orchard #2	23	-	+	-	+	-	+	-	-
Orchard #2	24	-	+	-	-	-	-	-	-
Orchard #2	25	-	-	-	-	-	-	-	-
Orchard #2	26	-	+	-	-	-	-	-	-
Orchard #2	27	-	-	-	-	-	+	-	-
Orchard #2	28	-	+	-	-	-	-	-	-
Orchard #2	29	-	-	-	-	-	-	-	-
Orchard #2	30	-	-	-	-	-	-	-	-
Orchard #3	31	+	+	-	+	-	+	-	+
Orchard #3	32	+	+	-	+	-	+	-	+
Orchard #3	33	+	+	-	+	-	+	-	+
Orchard #3	34	+	+	-	+	-	+	-	+
Orchard #3	35	+	+	-	+	-	+	-	+
Orchard #3	36	+	+	-	+	-	+	-	+
Orchard #3	37	-	-	-	-	-	-	-	-
Orchard #3	38	-	-	-	-	-	-	-	-
Orchard #3	39	-	-	-	-	-	-	-	-
Orchard #3	40	-	-	-	-	-	-	-	-
Orchard #4	41	-	-	-	+	-	+	-	-
Orchard #4	42	-	-	-	+	-	+	-	-
Orchard #4	43	-	-	-	+	-	+	-	-
Orchard #4	44	-	-	-	+	-	+	-	-
Orchard #4	45	-	-	-	-	-	-	-	-
Orchard #4	46	-	-	-	+	-	+	-	-
Orchard #4	47	-	-	-	+	-	+	-	-
Orchard #4	48	-	-	-	-	-	+	-	-
Orchard #4	49	-	-	-	+	-	+	-	-
Orchard #4	50	-	-	-	+	-	+	-	-

[1]Presence (+) or absence (-) of bark scaling symptoms on the Carrizo citrange rootstock.

In October 2008, additional samples were collected and analyzed by northern hybridization, using a procedure sensitive enough to detect viroids directly in commercial citrus species and cultivars, without the need of an amplification step in citron (Murcia *et al.,* 2009b). Briefly, bark samples were powdered in liquid nitrogen, and the nucleic acids were recovered in the aqueous phase of the phenol-containing extraction medium. The nucleic acids were partitioned in 2M LiCl and concentrated by ethanol precipitation. The RNAs separated in 5% PAGE (60 mA, 2h) were electroblotted to Nylon membranes and hybridized with viroid-specific DIG-labelled DNA probes. The results (Table 1) can be summarized as follows:

Orchard #1: Nine out the ten samples collected were infected with CEVd, CBLVd, HSVd and CDVd, whereas one of the samples was viroid-free. These results are in agreement with the earlier detection of the same viroids using Etrog citron as an amplification host, and indicate that the orchard was initially established with propagations of infected budwood. The single sample found to be viroid-free (Table 1: sample 7) suggests that plants that did not take were probably replaced with viroid-free plants from a different source. Since Carrizo citrange is viroid sensitive and clementine is cachexia sensitive, the poor performance of the orchard illustrates the effect of viroid infection in a specific scion/rootstock combination in which both partners are affected.

Orchard #2: The ten samples collected from trees showing bark scaling symptoms on the rootstock were infected with CEVd, CBLVd, HSVd and CDVd (Table 1: samples 11 to 20), whereas the ten samples collected from symptomless trees were either viroid-free (Table 1: samples 22, 25, 29 and 30) or infected with viroid/s other than CEVd (Table 1: samples 21, 23, 24, 26, 27 and 28). These observations suggest that the planting material consisted of viroid-infected as well as viroid-free plants. Viroids are known to be mechanically transmissible but, as demonstrated in experimental and commercial plots, when donor plants are co-infected with several viroids, the viroids are not necessarily co-transmitted (Barbosa *et al.,* 2005). Therefore, the lack of uniformity regarding viroid infection in the symptomless trees suggests that these trees were probably viroid-free at the time of planting and they became mechanically infected by cutting tools during pruning and harvesting operations. As indicated above, the poor performance of the symptomatic trees illustrates the effect of viroid infection in a specific scion/rootstock combination in which both partners are affected.

Orchard #3: Six out the ten samples collected were infected with CEVd, CBLVd, HSVd and CDVd (Table 1: samples 31 to 36), whereas the remaining four samples were viroid-free (Table 1: samples 37 to 40). The four viroids detected in the infected samples are the same than those detected earlier using Etrog citron as an amplification

host. This indicates that the orchard was initially established with citrus material from two different sources: a source of infected budwood and a source of viroid-free budwood. The location of affected trees on one side of the orchard and non-affected trees on the other side did not favor mechanical transmission, as in the case of orchard #2 where symptomless plants were mixed with infected ones.

Orchard #4: Eight out of the ten samples tested were infected with HSVd and CDVd, whereas one plant was viroid-free and another one was infected with only CDVd (Table 1: samples 45 and 48). When this orchard was established in the early 1990s, plant materials with a reliable sanitary status were not yet available, however given the popularity of the 'Afourer/Nadorcor' clementine, many growers made their own propagations or purchased the trees from illegal sources.

The poor performance of orchards #1, #2 and #3 can be partially or entirely attributed to the effect of viroid infection. Indeed, a long-term field experiment demonstrated that clementine trees co-infected with CEVd, CBLVd, HSVd (non cachexia variant), and CDVd had a small canopy and yielded a reduced crop, reaching only 57.4% of the production of viroid-free trees (Bani Hashemian *et al.,* 2009). In fact, the above combination of viroids was found to be very damaging for clementine trees grafted on trifoliate orange (*Poncirus trifoliata*) in a study addressed to understand how interactions among viroids co-infecting the same tree could affect symptom expression and field performance (Vernière *et al.,* 2006). Even though the effect of viroid infection on viroid-susceptible rootstocks and even whole trees grafted on these rootstocks have been well documented (Polizzi *et al.,* 1991; Vernière *et al.,* 2004), growers are not aware of its overall importance. It is not uncommon to hear complains about trees lacking vigor and over-fertilization as the solution. Furthermore, under our climatic conditions, CEVd-induced bark scaling symptoms in the Carrizo citrange are not very prominent, and growers tend to get them confused with *Phytophthora* root rot. Last, but not least, in orchards #1 and #2, the performance of the trees is not only affected by the effect of CEVd on the susceptible Carrizo citrange rootstock, but perhaps even more so by the effect of cachexia inducing variants of HSVd on the cachexia-susceptible clementine and tangor scions.

HSVd and CDVd have been shown to affect tree size without any apparent symptoms (Semancik *et al.,* 1997; Hutton *et al.,* 2000). The identification of these two viroids in the 'Afourer/Nadorcor' clementine trees of orchard #4 could not be clearly associated with the performance of the trees. However, this orchard illustrates how the demand for new clementine cultivars prompted malpractices such as the uncontrolled introduction and propagation of the 'Afourer/Nadorcor' cultivar without taking into consideration its sanitary status.

Finally, large numbers of samples could be tested in this study because detection procedures have become available, which are sensitive enough to detect viroids directly in commercial citrus species and cultivars, without the need of an amplification step in citron (Murcia *et al.,* 2009b). The method has been demonstrated to be suitable for the detection of all the citrus viroids described so far, and will undoubtedly facilitate additional field studies.

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CONCLUSIONES

CONCLUSIONES

Los trabajos desarrollados y que se hallan incluidos en esta memoria de tesis doctoral permiten llegar a las siguientes conclusiones:

1. La caracterización molecular del aislado natural de viroides elegido para determinar su efecto en clementino de 'Nules' y naranjo 'Navelina', ambos injertados sobre el patrón citrange Carrizo, ha permitido determinar que contiene: CEVd (viroide de la exocortis de los cítricos) con los motivos de patogenicidad P_L y P_R característicos de las razas agresivas (clase A); HSVd (viroide del enanismo del lúpulo) con el determinante de patogenicidad característico de las variantes que no inducen la cachexia de los cítricos; CBLVd (viroide de la hoja curvada de los cítricos) relacionado con las variantes de tipo CVd-Ia; y CDVd (viroide del enanismo de los cítricos) con una mezcla de variantes de los tipos CVd-IIIa y CVd-IIIb.

2. El análisis de una serie de parámetros relacionados con el crecimiento, cosecha y calidad de la fruta han permitido establecer que la infección con este aislado afecta las características de los árboles infectados, que son de menor tamaño (altura y volumen de copa), y en consecuencia producen una menor cosecha de frutos de menor calidad (forma, tamaño, índice de madurez y cantidad y calidad del zumo), que los controles no inoculados.

3. A pesar de que el patrón de los árboles infectados presentaba síntomas muy suaves, un estudio histológico ha permitido relacionar el escaso desarrollo del sistema radicular con un bajo contenido en amiloplastos.

4. Se ha comprobado que el escaso desarrollo radicular puede correlacionarse con la capacidad rizogénica de explantes de citrange Carrizo cultivados *in vitro.*

5. Se ha comprobado que la infección sistémica de cidro Etrog (*C. medica*) con CEVd, CBLVd, HSVd, CVd-V, CDVd o CBCVd sigue el patrón característico de movimiento intracelular, movimiento célula a célula y movimiento a larga distancia a través del floema, determinado en estudios con especies herbáceas experimentales. Sin embargo, los resultados de nuestro trabajo demuestran que en *C. karna*, naranjo amargo (*C. aurantium*) y naranjo trifoliado (*P. trifoliata*) la infección sistémica se halla restringida al floema.
6. Utilizando plantas de *C. karna* infectadas con CEVd se ha conseguido desbloquear la barrera que impide que el viroide alcance las células del mesófilo mediante dos estrategias distintas: (i) la co-infección con el virus de la tristeza de los cítricos (CTV); y (ii) el sobreinjerto con cidro Etrog.

7. La caracterización de dos genotipos, *Eremocitrus glauca* y *Microcitrus australis*, aparentemente resistentes a la infección con viroides ha permitido concluir que los viroides no se replican en dichos genotipos o lo hacen con una eficiencia muy baja.

8. Los análisis realizados en plantas de *Eremocitrus glauca* y *Microcitrus australis* injertadas sobre el patrón limón rugoso (*C. jambhiri*) y en plantas sobreinjertadas con cidro Etrog, demuestran que ambos genotipos permiten el movimiento a larga distancia tanto hacia el patrón como hacia el cidro Etrog sobreinjertado.

9. La caracterización de varios aislados de campo recuperados de plantaciones comerciales de Sudán ha mostrado que CEVd, HSVd, CBLVd, CDVd y CVd-IV se hallan muy difundidos en Sudán. La caracterización molecular de cuatro aislados de HSVd ha permitido identificar variantes en las que el motivo de patogenicidad no se ajusta a los modelos previamente establecidos. No se ha identificado una asociación clara entre la infección con viroides y la manifestación de síntomas de dos enfermedades descritas en el país, “gummy bark” y “Kasala disease”.

10. La caracterización de varios aislados recuperados tanto de colecciones como de plantaciones comerciales de las principales zonas citrícolas de Irán ha permitido identificar 27 fuentes de material vegetal libre de viroides, así como plantas co-infectadas con varios viroides. CEVd, HSVd, CBLVd, CDVd y CBCVd son muy frecuentes como infecciones múltiples, mientras que CVd-V, se ha detectado en una única fuente de naranja sanguina procedente de Brentin en el sur del país. Ninguno de los materiales analizados era portador de CVd-OS.

11. El análisis de cuatro parcelas comerciales de la Comunidad Valenciana ha permitido establecer que habían sido plantadas con material no certificado. La distribución en dichas parcelas de árboles libres de viroides y de árboles infectados ha permitido determinar que tanto el material de origen como la transmisión mecánica son responsables de la dispersión de los viroides en dichas parcelas.

ANEJOS

- *Citrus viroid V*: Occurrence, host range, diagnosis and identification of new variants (P. Serra, M. Eiras, **S. M. Bani Hashemian**, N. Murcia, E. W. Kitajima, J. A. Daròs, R. Flores, N. Duran-Vila)

- An artificial chimeric derivative of *Citrus viroid V* involves the terminal left domain in pathogenicity (P. Serra, **S. M. Bani Hashemian**, G. Pensabene-Bellavia, S. Gago, N. Duran-Vila)

- Molecular and biological characterization of natural variants of *Citrus dwarfing viroid* (N. Murcia, L. Bernard, P. Serra, **S. M. Bani Hashemian**, N. Duran-Vila)

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

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ABSTRACT

Serra, P., Eiras, M., Bani-Hashemian, S. M., Murcia, N., Kitajima, E. W., Daròs, J. A., Flores, R., and Duran-Vila, N. 2008. Citrus viroid V: Occurrence, host range, diagnosis, and identification of new variants. Phytopathology 98:1199-1204.

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 United States, 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 it 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 reverse transcription-polymerase chain reaction, have been evaluated for detecting CVd-V, either using Etrog citron as an amplification host or directly from commercial species and cultivars.

Viroids are nonencapsidated, small, circular, single-stranded RNAs that replicate autonomously when inoculated in their host plants. *Citrus* spp. 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) (5). 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* (7,20).

CVd-V has a GC-rich genome of 293 to 294 nucleotides (nts) and its predicted secondary structure of minimum free energy is a rodlike 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 coinfections it interacts synergistically with either CBLVd or CDVd producing enhanced leaf symptoms and very pronounced dwarfing (20).

Most citrus viroids (CEVd, HSVd, CBLVd, CDVd, and CBCVd) are widespread, usually occurring as complex mixtures coinfecting the same plant. CVd-OS has only been reported in Japan (7). 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 (2). 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) (2,4). The recent identification of a new variant of CVd-V in a tangelo Orlando grown in Spain (21) 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 (1). Even though citrus viroids elicit diseases in sensitive hosts such as exocortis and cachexia (17–19) or affect tree size and crop (23), 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 new variants of CVd-V in sources from California and Nepal, (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.

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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 32°C for at least 6 months before nucleic acid extraction. One of the viroid sources, 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* × *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% [wt/vol] sodium dodecyl sulfate [SDS]; 5 mM EDTA, pH 7.0; and 4% [vol/vol] 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; and 0.1 mM $MgCl_2$) (17).

PAGE. Aliquots (20 µl) of the nucleic acid preparations (equivalent to 333 mg of fresh weight tissue) were fractionated by nondenaturing PAGE in a 5% gel that was stained with ethidium bromide (8). For 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 (13,16), which following electrophoresis was stained with silver (6).

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 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 8% formaldehyde for 15 min at 60°C and blotted onto the membrane. For imprint hybridization, freshly-cut stem sections were firmly pressed onto the membrane (14). In all instances, RNAs were immobilized by UV cross-linking and hybridized with ^{32}P-RNA or digoxigenin (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 (10) with minor modifications (20). DIG-labeled DNA probes were PCR-synthesized as described by Palacio et al. (11) using a plasmid containing the full-length CVd-V sequence and a pair of specific primers.

Prehybridization (at 50 or 60°C for 2 to 4 h) and hybridization (at 50 or 60°C overnight) were performed in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.4]) containing 50% formamide as described by Sambrook et al. (15). After hybridization the membranes were washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min, once in 0.1× SSC, 0.1% SDS at 60°C 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 (9,20). 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*HI and *Xba*I were separated by nondenaturing 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. Reverse transcription-polymerase chain reaction (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 (22). Secondary structure analyses were obtained with the MFOLD program (circular version) from the GCG package (25) and the RNAviz program (3).

Host range and detection studies. A selection of 18 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* × *C. tangerina*), 'Nova' mandarin (*C. clementina* × [*C. paradisi* × *C. tangerina*]), 'Page' mandarin ((*C. paradisi* × *C. tangerina*) × *C. clementina*), and 'Nagami' kumquat (*Fortunella margarita* (Lour.) Swing.).

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 (9). The cDNAs obtained were mostly in the range of 150 to 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 (20) (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, arrows), were selected for sequencing. The consensus sequence of 88 nts contained a segment of 16 nts identical to the upper central conserved region (CCR) 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 and 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′-GACGA-

AGGCCGGTGAGCAGTAAGCC-3′) and (5′-GACGACGACAG-GTGAGTACTTTC-3′) corresponding to CVd-V positions 90 to 114 and 69 to 89, respectively. The cDNA product obtained by RT-PCR amplification of the purified circular forms of the viroid-like RNA exhibited in nondenaturing 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 (224C→U, 225C→U) in the lower strand of the rodlike 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.

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* × *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. (21). Its complete primary structure was

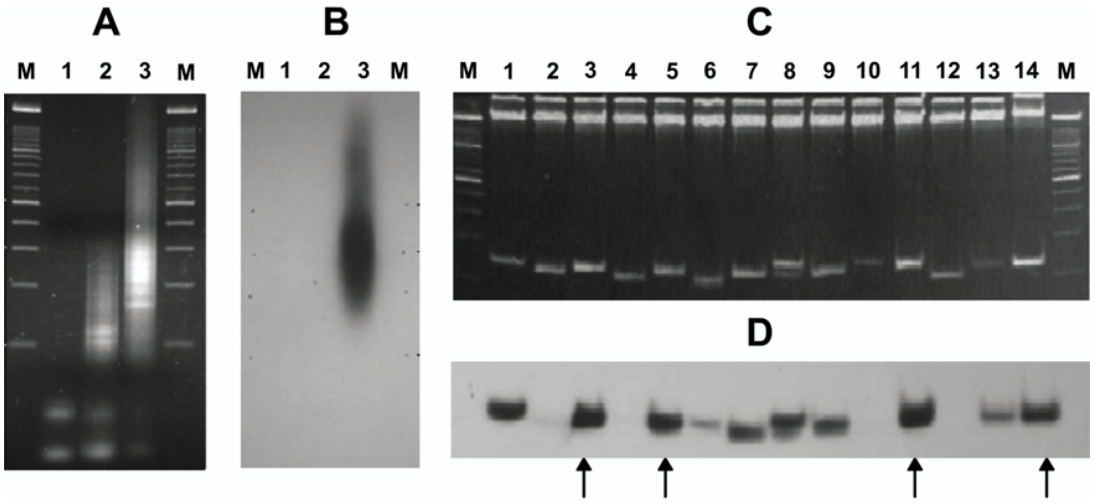

Fig. 1. A, Analysis by nondenaturing polyacrylamide gel electrophoresis and ethidium bromide staining of viroid-like cDNAs obtained by reverse transcription-polymerase chain reaction (RT-PCR) with a primer degenerated in its six 3′-terminal positions. Lane 1, PCR control mixture without template; lane 2, RT-PCR control mixture without template; lane 3, complete RT-PCR mixture; and lane M, markers of 100-bp multimers. **B,** Southern blot analysis of the viroid-like cDNAs with a specific radioactive probe. **C,** Restriction analysis by nondenaturing polyacrylamide gel electrophoresis and ethidium bromide staining of recombinant plasmids digested with *Bam*HI and *Xba*I (lanes 1 to 14); and lane 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.

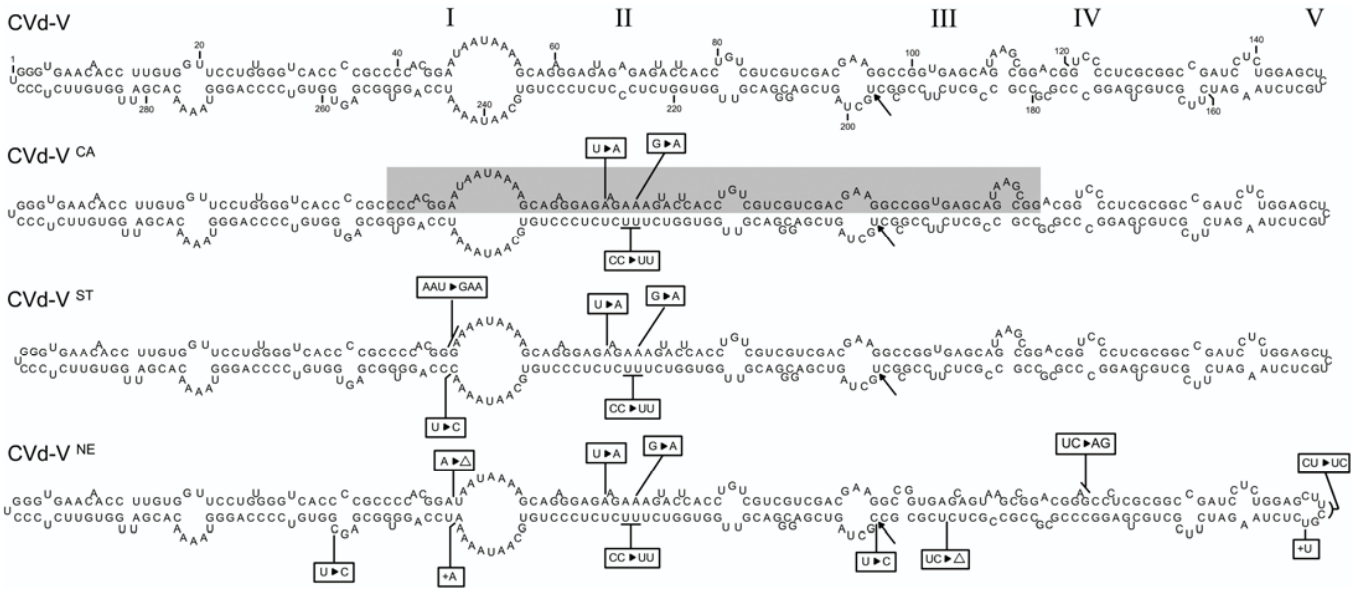

Fig. 2. Primary and predicted secondary structure of minimum free energy of Citrus viroid V (CVd-V) showing the changes identified in the variants obtained from an Etrog citron from California (CVd-V^{CA}), a Seminole tangelo (CVd-VST) from Spain, and a sweet orange from Nepal (CVd-V^{NE}). The fragment 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-VST, instead of C as in CVd-V^{NE} and in all other members of the genus *Apscaviroid.*

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 (21). 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 rodlike 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.

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 to 16 changes (CVd-V^{NE}) from the reference CVd-V variant (20). These three variants have

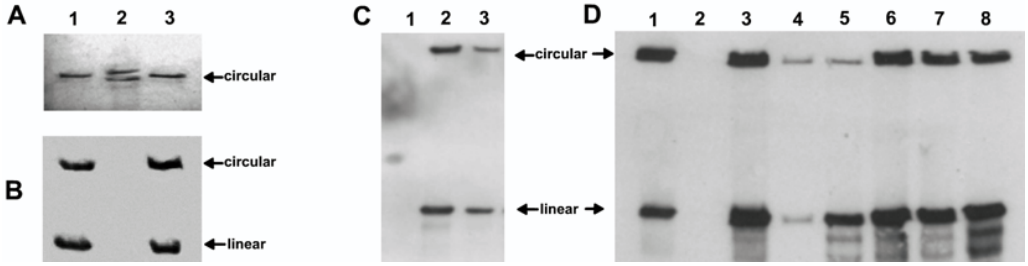

Fig. 3. Detection of Citrus viroid V (CVd-V) in samples from **A and B,** California, **C,** Nepal, and **D** the Sultanate of Oman. The sample from California was analyzed by **A,** sequential polyacrylamide gel electrophoresis and silver staining and **B,** northern blot hybridization with a CVd-V-specific probe: (lane 1) Etrog citron infected with the viroid-like RNA from California; (lane 2) Etrog citron coinfected with *Hop stunt viroid* and *Citrus dwarfing viroid* used as a negative control; and (lane 3) Etrog citron infected with CVd-V used as a positive control. **C,** Samples from Nepal were analyzed by northern blot hybridization with a CVd-V-specific probe: (lane 1) noninoculated Etrog citron used as a negative control; (lane 2) Etrog citron infected with CVd-V used as a positive control; (lane 3) sweet orange collected in Nepal. **D,** Samples from the Sultanate of Oman were analyzed by northern blot hybridization with a CVd-V-specific probe: (lane 1) Etrog citron infected with CVd-V used as a positive control; (lane 2) noninoculated Etrog citron used as a negative control; (lanes 3 to 8) Etrog citrons graft-inoculated with 'Baladi' (lanes 3 to 4), Washington navel (lane 5), Succari (lanes 6 to 7), and Valencia (lane 8) sweet orange sources collected in the Sultanate of Oman.

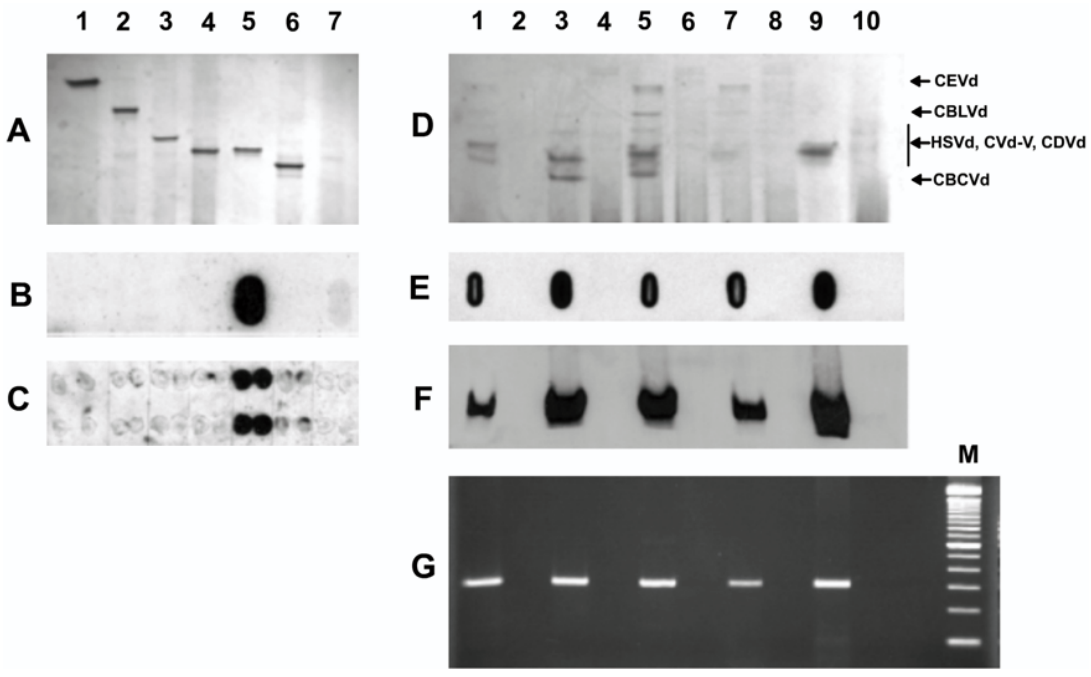

Fig. 4. Detection of Citrus viroid V (CVd-V) in Etrog citron by **A,** sequential polyacrylamide gel electrophoresis (sPAGE), **B,** dot blot hybridization, and **C,** imprint hybridization using a CVd-V-specific probe. Samples were from Etrog citrons infected with (lane 1) *Citrus exocortis viroid* (CEVd), (lane 2) *Citrus bent leaf viroid* (CBLVd), (lane 3) *Hop stunt viroid* (HSVd), (lane 4) *Citrus dwarfing viroid* (CDVd), (lane 5) CVd-V, (lane 6) *Citrus bark cracking viroid* (CBCVd), and (lane 7) from a noninfected control. Detection of CVd-V in sweet orange, clementine, Tahiti lime, and lemon by **D,** sPAGE, **E,** dot, and **F,** northern blot hybridization with a CVd-V-specific probe, and **G,** reverse transcription-polymerase chain reaction with CVd-V-specific primers. Samples were collected from sweet orange, clementine, Tahiti lime and lemon plants coinoculated with CEVd, CBLVd, HSVd, CDVd, CBCVd, and CVd-V (lanes 1, 3, 5, and 7) and from noninoculated controls of the same species (lanes 2, 4, 6, and 8). Samples from Etrog citrons infected with CVd-V (lane 9) and noninoculated (lane 10) were included as controls. Lane M, markers of 100-bp multimers.

a predicted rodlike 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 and 69G→A) and lower (224C→U, 225C→U) strands of the rodlike secondary structure resulting in an extended base pairing (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} (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 rodlike secondary structure with less conspicuous loops (regions III and IV in Fig. 2) and a prominent terminal right loop (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 (arrows in Fig. 2).

Indexing methods for detection of CVd-V. As shown for other citrus viroids (4,12), 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 coinoculated 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).

Host range of CVd-V. RNA preparations from 20 citrus genotypes that had been graft-inoculated with the original CVd-V source (20) 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 noninoculated controls (Fig. 5B).

DISCUSSION

Transmission studies using *Atalantia citroides* revealed the existence of a new member of the genus *Apscaviroid*, which induces mild, but characteristic symptoms on Etrog citron and displays synergistic effects when coinoculated with other members of the same genus (2,20). 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, United States, 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 that differ from CVd-V in four (CVd-V^{CA}), eight (CVd-V^{ST}), and 15 to 16 (CVd-V^{NE}) changes, four of which (U65→A, G69→A, C224→U, and C225→U) 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.

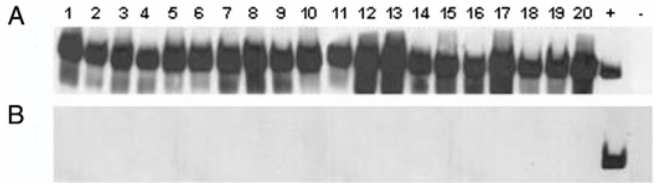

Fig. 5. A, Detection of Citrus viroid V (CVd-V) by northern blot hybridization with a CVd-V-specific probe in (lane 1) 'Hernandina' clementine, (lane 2) 'Ricart navelina' sweet orange, (lane 3) 'Nagami' kumquat, (lane 4) 'Cajel' sour orange, (lane 5) 'Oroval' clementine, (lane 6) 'Orlando' tangelo, (lane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

In conclusion, the recently described CVd-V (20,21) 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.

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Short communication

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

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SUMMARY

The recently described *Citrus viroid V* (CVd-V) induces, in Etrog citron, mild stunting and very small necrotic lesions and cracks, sometimes filled with gum. As Etrog citron plants co-infected with *Citrus dwarfing viroid* (CDVd) and CVd-V show synergistic interactions, these host–viroid combinations provide a convenient model to identify the pathogenicity determinant(s). The biological effects of replacing limited portions of the rod-like structure of CVd-V with the corresponding portions of CDVd are reported. Chimeric constructs were synthesized using a novel polymerase chain reaction-based approach, much more flexible than those based on restriction enzymes used in previous studies. Of the seven chimeras (Ch) tested, only one (Ch5) proved to be infectious. Plants infected with Ch5 showed no symptoms and, although this novel chimera was able to replicate to relatively high titres in singly infected plants, it was rapidly displaced by either CVd-V or CDVd in doubly infected plants. The results demonstrate that direct interaction(s) between structural elements in the viroid RNA (in this case, the terminal left domain) and as yet unidentified host factors play an important role in modulating viroid pathogenicity. This is the first pathogenic determinant mapped in species of the genus *Apscaviroid*.

Viroids are infectious, small, single-stranded, circular RNAs (246–401 nucleotides) that fold into compact secondary structures as a result of the high degree of self-complementarity of their sequences. In spite of their apparent simplicity, the sequence of the viroid genome ensures maintenance of the secondary and tertiary structures, and provides specific motifs for interaction with the host. Species of the family *Pospiviroidae* have a central conserved region (CCR) and lack hammerhead ribozymes. Citrus are natural hosts of several viroid species, all of which belong to the family *Pospiviroidae* (Duran-Vila *et al*., 1988; Flores *et al*., 2004) and adopt *in vitro* rod-like or quasi-rod-like secondary structures of minimal free energy. On denaturation, the viroid RNA may acquire metastable branched structures resulting from base pairing of inverted repeats that lead to the formation of stem loop structures (hairpins I and II) (Loss *et al*., 1991; Riesner *et al*., 1979). Following the model proposed by Keese and Symons (1985), the rod-like secondary structure is divided into five structural–functional domains: P (pathogenicity), C (central), V (variable), T_L (terminal left) and T_R (terminal right). Although members of the genus *Pospiviroid* appear to be well adjusted to this model, evidence shows that the P domain is not the sole determinant of symptom development, and suggests a more complex relation between different structural domains and pathogenicity. Studies with artificial chimeras derived from variants of the same viroid (Góra *et al*. 1996; Reanwarakorn and Semancik, 1998; Visvader and Symons, 1986), from closely related viroids of the same genus (Sano *et al*., 1992) and, to a lesser extent, from viroids of different genera (Sano and Ishiguro, 1998) have provided relevant information on the relationship between specific regions of the viroid molecule and symptom expression.

In members of the genus *Apscaviroid*, all restricted to woody species, studies to determine the relationship between different portions of the RNA secondary structure and the biological properties are very limited. Although a CCR and terminal conserved region (TCR) have been clearly identified, the presence and role of the P, V, T_L and T_R domains remain unknown. *Citrus viroid III* (CVd-III), recently renamed *Citrus dwarfing viroid* (CDVd) (http://www.ictvonline.org/virusTaxonomy.asp), is a member of the genus *Apscaviroid* that induces, in Etrog citron (*Citrus medica* L.), stunting and a characteristic 'leaf dropping pattern' caused by the moderate epinasty resulting from petiole and mid-vein necrosis (Rakowski *et al*., 1994; Semancik *et al*., 1997). The recently described *Citrus viroid V* (CVd-V) also has a rod-like conformation and induces, in Etrog citron, mild stunting and very small necrotic lesions and cracks, sometimes filled with gum (Serra *et al*., 2008a, b). Moreover, as Etrog citron plants

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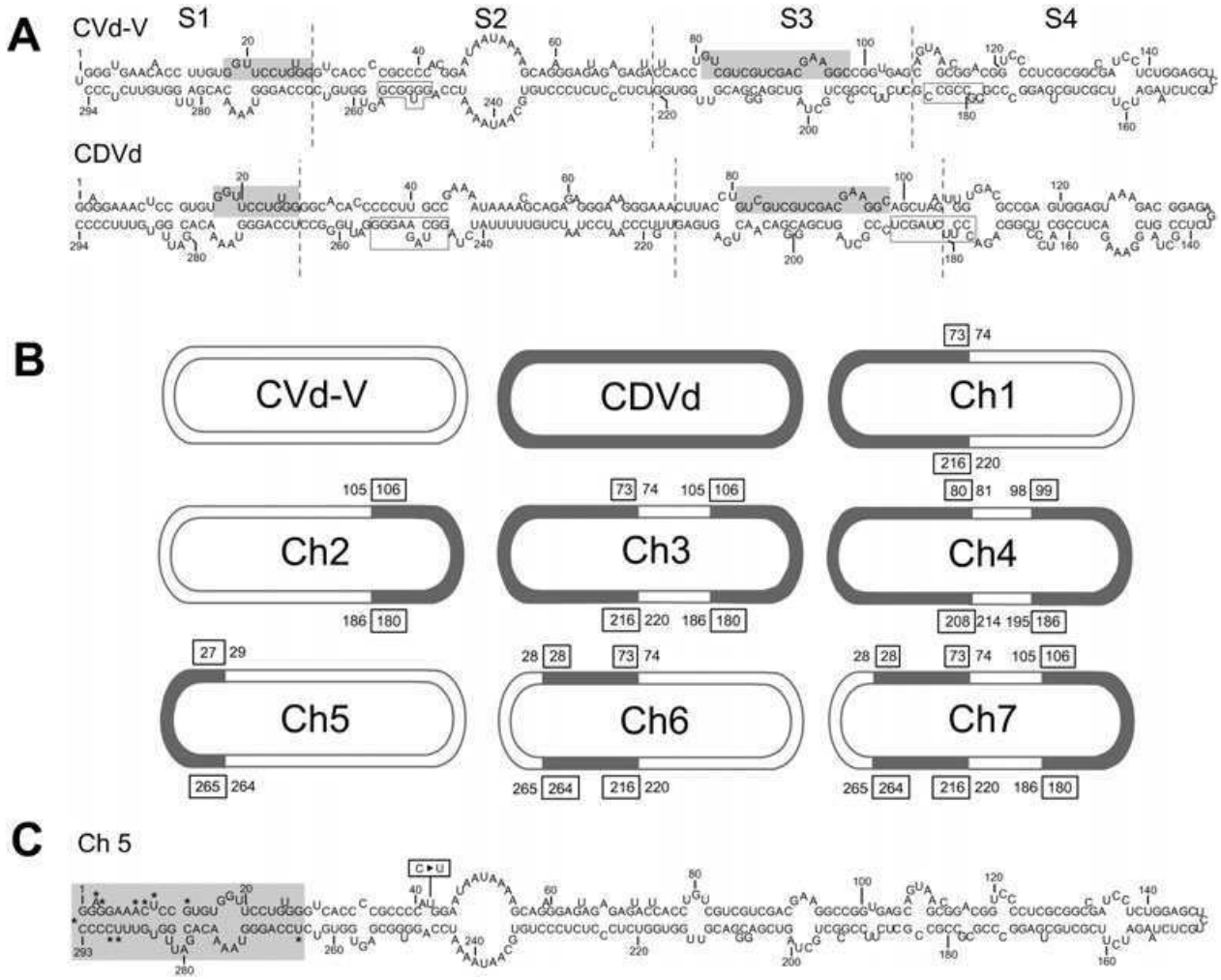

Fig. 1 (A) Proposed secondary structures of minimum free energy of *Citrus viroid V* (CVd-V) and *Citrus dwarfing viroid* (CDVd). Broken lines divide the secondary structures into four segments (S1, S2, S3 and S4). Conserved regions [terminal conserved region (TCR) within S1 and central conserved region (CCR) within S3] of members of the genus *Apscaviroid* are shaded. Boxed nucleotides show the putative motifs 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 indicate the first and last nucleotides of the segments from CDVd and CVd-V, respectively. (C) Proposed secondary structure of minimum free energy of Ch5. Shaded region shows the S1 segment from CDVd that replaces the S1 segment of CVd-V. Nucleotide changes between CVd-V and Ch5 are indicated by asterisks. The change C → U found in the progeny of Ch5 is shown boxed.

co-infected with CDVd and CVd-V show synergistic interactions manifested in enhanced leaf symptoms and very pronounced dwarfing (Serra *et al*., 2008a), these host–viroid combinations provide a convenient model to identify the pathogenicity determinant(s) in members of the genus *Apscaviroid*. The aim of this study was to evaluate the effect on symptom expression of exchanging discrete portions of the CVd-V rod-like structure for their corresponding counterparts of CDVd.

The compositions of the seven chimeras designed by the interchange of discrete portions of the secondary structure of CVd-V and CDVd are illustrated in Fig. 1. Unlike the strategy followed previously for the construction of chimeric viroids (Góra *et al*., 1996; Owens *et al*., 1990; Reanwarakorn and Semancik, 1998; Sano *et al*., 1992; Sano and Ishiguro, 1998; Spieker, 1996; Visvader and Symons, 1986), a polymerase chain reaction (PCR)-based novel approach, which does not rely on the availability of shared restriction sites and permits the construction of any choice of chimeric viroids, was developed. Briefly, the segments of CDVd used to replace the corresponding segments of CVd-V were obtained by PCR, employing a cDNA insert cloned into a recombinant plasmid (Bluescript KS+, Promega®, Madison, WI, USA), pCDVd, containing the full-length CDVd sequence (GenBank accession number EU934004) as a template and different primer sets phosphorylated at their 5′ termini (Table S1,

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see Supporting Information). The CVd-V sequences were also obtained by PCR using the recombinant Bluescript KS+ plasmid, pCVd-V, containing the full-length CVd-V sequence (GENBANK accession number NC010165) as a template and different primer sets (Table S2, see Supporting Information). This PCR results in the amplification of the full plasmid sequence plus the CVd-V insert devoid of the segment to be replaced. Ligation of CVd-V and CDVd segments generated the designed chimeric variants.

Considering that structural–functional domains have not been clearly defined in members of the genus *Apscaviroid*, the segments to be replaced in the present work were chosen to preserve the CCR and the flanking inverted repeats responsible for the formation of hairpin I and the TCR, both located in the upper strand of the viroid secondary structure. Four discrete segments were then defined in the secondary structure of the two viroids (Fig. 1A): (S1) segment containing the T_L domain and delimited in the upper strand by the 3′ end of the TCR (positions 28 and 27 of CVd-V and CDVd, respectively), and the corresponding fragment of the lower strand; (S2) segment delimited by the 3′ 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, respectively), and the corresponding fragment of the lower strand; (S3) segment containing the CCR and both flanking repeats, and the corresponding fragment of the lower strand; (S4) segment containing the T_R domain and delimited in the upper strand by the end of the right flanking repeat of the CCR, and the corresponding fragment of the lower strand.

Although hairpin II has only been observed in *Potato spindle tuber viroid* (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 (CDVd) (boxed in Fig. 1A) that could form a stable hairpin II-like structure with a GC-rich stem (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, was also considered. The utilization of S3′ required concurrent modifications of the adjacent segments S2 and S4 into S2′ (S2 plus the left inverted repeat of the CCR and the corresponding fragment of the lower strand) and S4′ (S4 plus the right inverted repeat of the CCR and the corresponding fragment of the lower strand), respectively.

The resulting chimeric viroids generated using these criteria were as follows (Fig. 1B): 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).

To obtain infectious preparations of the seven chimeric viroids, head-to-tail dimeric cDNAs were generated and used as templates to produce the corresponding *in vitro* transcripts homologous to the seven chimeric constructions employing T7 RNA Polymerase (Roche Applied Science®, Basel, Switzerland). These preparations were inoculated mechanically (50 ng of transcript per plant) into three citron seedling plants each. Inoculated plants were kept in the glasshouse at 28–32 °C for 9 months. Dimeric *in vitro* transcripts of CVd-V and CDVd were 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 CDVd developed symptoms, whereas plants inoculated with chimeric variants remained symptomless and indistinguishable from the negative controls over a 9-month period. However, analysis by northern blot hybridization with viroid-specific DNA probes (Serra *et al*., 2008a) and reverse transcriptase-polymerase chain reaction (RT-PCR) (Bernad and Duran-Vila, 2006) showed that a single plant from the three inoculated with the chimeric variant Ch5 was infected. RT-PCR analysis using P1 and P2 primers (Table S1, see Supporting Information) and amplicon sequencing confirmed that the chimeric variant Ch5 was stable, except for a single 42C → U substitution in the S2 segment (Fig. 1C).

In contrast with the success achieved with chimeras between *Citrus exocortis viroid* (CEVd) and *Tomato apical stunt viroid* (TASVd), two closely related viroids of the same genus (Sano *et al*., 1992), and, to a lesser extent, between CEVd and *Hop stunt viroid* (HSVd), belonging to different genera (Sano and Ishiguro, 1998), only one (Ch5) of the seven chimeric constructs between CVd-V and CDVd was infectious. Unexpectedly, Ch4, which differs from CDVd in only four nucleotides in the lower strand of CCR, was not infectious. These changes that appear to annul Ch4 infectivity were also present in all the other chimeric constructs, which, with the exception of Ch5, were not infectious. The infectivity of Ch5 is probably because it is essentially identical to CVd-V, except in T_L, which was replaced with that of CDVd. It should be noted that replication of Ch5 in only one of the three inoculated plants was associated with a 42C → U substitution in the S2 segment, which seems essential for viability. Thus, this is the first report of an artificial chimeric viroid within the genus *Apscaviroid* that replicates, becomes systemic and is readily transmissible. Overall, Ch5 differs with regard to 11 changes with respect to CVd-V, leading to a secondary structure of T_L identical to that of CDVd. In contrast with previous studies conducted with PSTVd, in which mutations in the loops of the T_L domain resulted in low replication and impaired or defective trafficking (Zhong *et al*., 2008), the replication of Ch5 was comparable with that of CVd-V and was trafficking competent.

To confirm the absence of symptoms in plants infected with Ch5, buds from sensitive selection 861-S1 of Etrog citron were graft propagated onto rough lemon seedlings and, concurrently, graft inoculated (three plants each) with bark from Etrog citron seedlings infected with the chimeric variant Ch5 or with CVd-V

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or 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 at 28–32 °C, and infection was confirmed by sequential polyacrylamide gel electrophoresis (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 displayed the characteristic 'dropping leaf pattern' caused by moderate epinasty resulting from petiole and mid-vein necrosis (Fig. 3A, C). By contrast, Ch5-infected citron plants were symptomless (Fig. 3A, D). The titres of the three viroids were comparable (Fig. 2A) and RT-PCR analysis and amplicon sequencing confirmed the stability of the inoculated sequences. Unlike plants infected with CVd-V or 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: necrotic lesions and cracks, sometimes filled with gum, characteristic of CVd-V, and stunting and moderate epinasty resulting from petiole and mid-vein necrosis, characteristic of CDVd. This observation suggests that the T_L domain is involved in blocking symptom expression. A different situation was found by Sano *et al*. (1992) with chimeras between CEVd and TASVd, both belonging to the genus *Pospiviroid*, in which the T_L domain did not block, but modulated, pathogenesis.

As CDVd and CVd-V co-infecting the same plant show synergistic interactions, specifically enhanced leaf epinasty and very pronounced dwarfing (Serra *et al*., 2008a), two additional treatments were carried out to examine the effect of co-infections of Ch5 with CDVd or CVd-V. Six buds from Ch5-infected citron were each graft propagated onto rough lemon seedlings; concurrently, three of these plants were graft inoculated with bark from CDVd-infected citron, and the other three with bark from CVd-V-infected citron. Three additional buds from CVd-V-infected citron were also graft propagated on rough lemon seedlings and, concurrently, graft-inoculated with bark from CDVd-infected citron as a synergistic control. Three buds each from CDVd-, CVd-V- and Ch5-infected plants, and three from non-inoculated plants, were graft propagated as controls. Inoculated plants were kept in the glasshouse at 28–32 °C. Data on plant height and number of internodes collected 4 months after inoculation were analysed using one-way analysis of variance (ANOVA) and the Student–Newman–Keuls post-test, which compares all pairs of means (Table 1). The corresponding results showed that: (i) there were significant differences among treatments; (ii) no significant differences were found among plants singly infected with CDVd or CVd-V, or co-inoculated with Ch5, which, however, were significantly smaller than non-infected controls; (iii) plants co-inoculated with CDVd and CVd-V were significantly smaller than those of the other treatments; and (iv) plants infected with Ch5 were significantly taller than the non-infected controls.

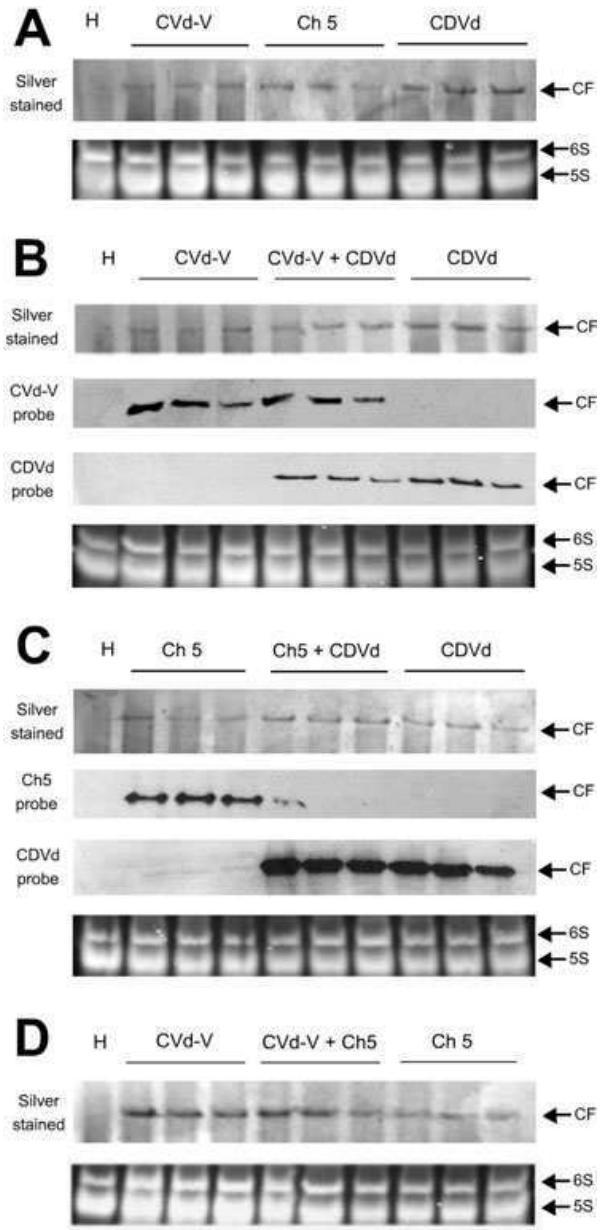

Fig. 2 (A) Analysis by sequential polyacrylamide gel electrophoresis (sPAGE) and silver staining of citron plants infected with *Citrus viroid V* (CVd-V), chimera 5 (Ch5) or *Citrus dwarfing viroid* (CDVd). (B) Analysis by sPAGE and northern hybridization of citron plants singly infected with CVd-V or CDVd, or co-infected with both viroids. (C) Analysis by sPAGE and northern hybridization of citron plants singly infected with Ch5 or CDVd, or co-infected with both. (D) Analysis by sPAGE of citron plants singly infected with CVd-V or Ch5, or co-infected with both. Silver staining of the second denaturing gel of sPAGE and northern hybridization show the viroid circular forms (CF). H (mock-inoculated control) is shown in all the analyses. Ethidium bromide staining of the first non-denaturing gel of sPAGE shows that RNA levels (6S and 5S RNAs) in all preparations are comparable.

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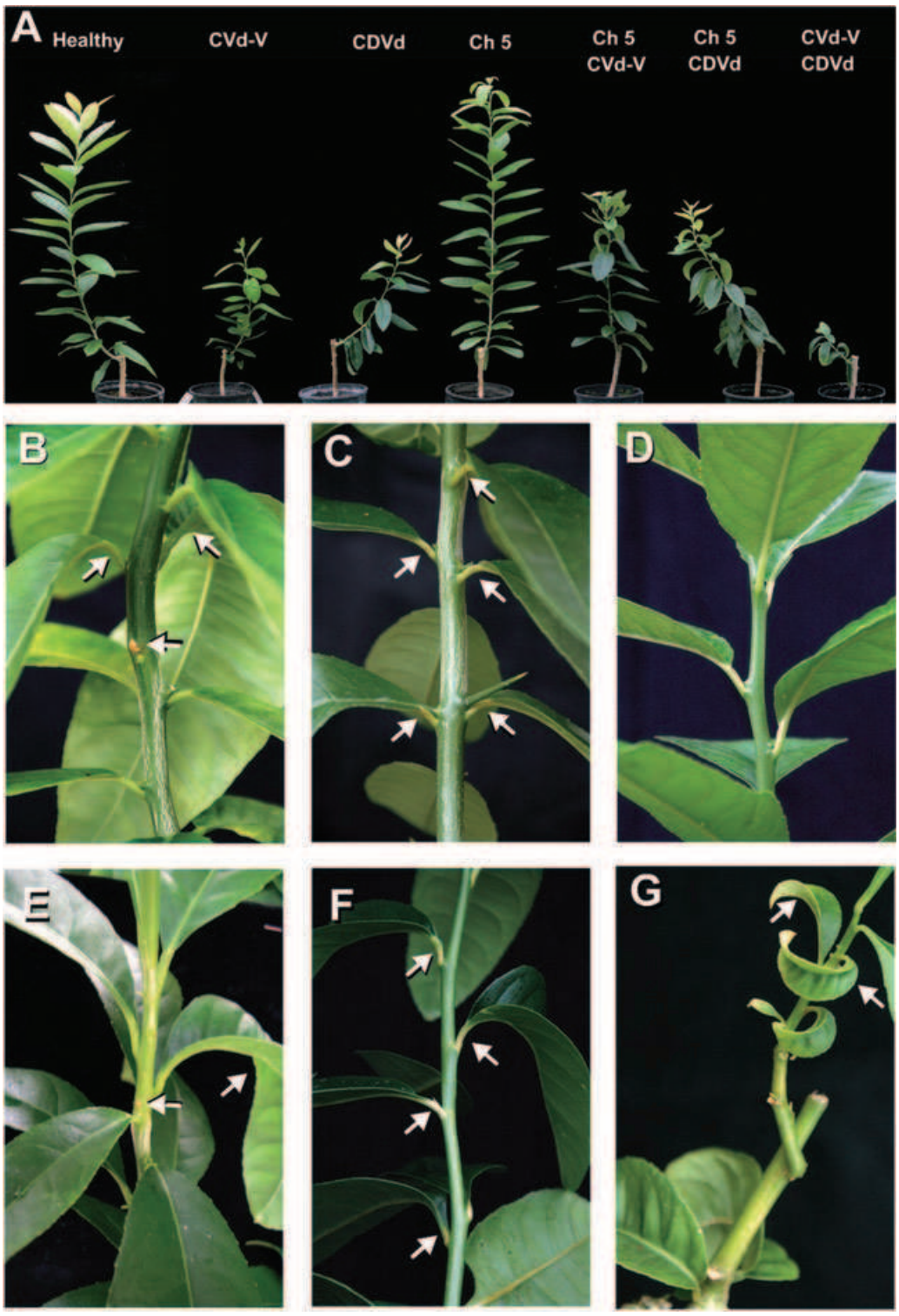

Fig. 3 Symptoms of viroid infection in Etrog citron plants at 4 months post-inoculation. (A) General aspect of plants non-inoculated (healthy), infected with a single viroid [*Citrus viroid V* (CVd-V), *Citrus dwarfing viroid* (CDVd) or chimera 5 (Ch5)] or co-infected with two viroids (Ch5 and CVd-V, Ch5 and CDVd or CVd-V and CDVd). (B) Leaf epinasty and cracks and gum exudate 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 epinasty and cracks and gum exudate 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.

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Table 1 Size of Etrog citron plants infected with single viroids or co-infected with two viroids.

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

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

CDVd, *Citrus dwarfing viroid*; Ch5, chimera 5; CVd-V, *Citrus viroid V*.

Plants co-infected by two viroids or two strains of the same viroid may display two distinct types of interaction, termed 'cross-protection' (Duran-Vila and Semancik, 1990; Pallás and Flores, 1989) and 'synergism' (Serra *et al*., 2008a). Given the high sequence similarity 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. On the other hand, as plants co-infected with CVd-V and CDVd displayed a synergistic interaction, a similar interaction was anticipated in plants co-infected with Ch5 and CDVd. Unexpectedly, the results of the present study showed that, in contrast with the synergistic effect observed in plants 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 CDVd (Fig. 3A, C), respectively. This suggests that no interaction exists between Ch5 and these two related viroids.

The synergistic effect in plants co-infected with CVd-V and CDVd is not associated with changes in the titre of these two viroids (Serra *et al*., 2008a). Northern blot hybridization analysis using CVd-V- and CDVd-specific probes confirmed this observation (Fig. 2B) but, unexpectedly, analysis of plants co-infected with Ch5 and CDVd showed very low or even undetectable accumulation of the former (Fig. 2C). The low titre of Ch5 was corroborated using a more sensitive hybridization test (Murcia *et al*., 2009) and by RT-PCR with the primer pair P1 and P2 (Fig. 4A). Analysis by sPAGE and silver staining showed that plants co-infected with Ch5 and CVd-V presented similar viroid titres as plants singly infected with Ch5 or CVd-V (Fig. 2D). Given the high sequence identity (96%) between CVd-V and Ch5, these viroids could not be differentiated by northern blot hybridization and required RT-PCR analysis with discriminating primer pairs C1 and V7 for CVd-V or C2 and V7 for Ch5 (Table S1, see Supporting Information) (Fig. 4B). 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 in the generation of cDNAs from CVd-V and Ch5 with primers P1 and P2 was evaluated by PCR using, as templates, serially diluted (1 : 2, 1 : 20, 1 : 200) cDNAs generated from equalized preparations of CVd-V or Ch5 (Fig. 4C); (ii) the suitability of this approach was confirmed using mixtures (1 : 1, 1 : 9 and 9 : 1) of initially equalized preparations of CVd-V and Ch5 that were subjected to RT-PCR. The resulting amplicons were ligated into the pGem-T vector, and PCR analysis using discriminating primer pairs of 11 clones from each ligation resulted in the same ratios of CVd-V and Ch5 as in the original mixtures. Using this approach, preparations from each of the three plants co-infected with CVd-V and Ch5 were examined: PCR analysis of 15 clones from each preparation revealed the presence of CVd-V in all. These results show that, as in plants co-infected with CDVd and Ch5, the titre of Ch5 was also very low in plants co-infected with CVd-V and Ch5.

Although co-infected plants were produced by graft propagation of Ch5-infected buds, which were further inoculated with either CVd-V or CDVd, analysis of the co-infected plants showed that infection with CVd-V or CDVd reduced the concentration of Ch5 to extremely low titres. These results explain the lack of interaction regarding symptom expression. Similar results have been found in *Gynura aurantiaca* and tomato plants co-infected with PSTVd and CEVd, where CEVd displaced PSTVd in *G. aurantiaca*, whereas PSTVd displaced CEVd in tomato (Pallás and Flores, 1989). These observations suggest that a limiting host factor that was initially used by one of the viroids was later monopolized by the other because of its higher affinity. Similarly, Ch5 could be displaced by either CVd-V or CDVd. Ch5 is an artificial viroid with primary and secondary structures of T_L different from those of its CVd-V progenitor, which appears to be well adapted to the Etrog citron host. However, in contrast with the naturally occurring CDVd and CVd-V, which have been subjected for a long time to natural selection under different hosts and environmental pressures, Ch5 has not suffered similar evolutionary pressures.

The mechanisms involved in viroid-induced symptoms are poorly understood. As sequence variants with changes affecting specific domains of the viroid secondary structure are responsible for the induction of certain diseases (Malfitano *et al*., 2003; Palacio *et al*., 2000; Reanwarakorn and Semancik, 1998) or for pronounced changes in symptom severity (De la Peña *et al*., 1999; Serra *et al*., 2008c), 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. Undoubtedly, symptom development is a complex process that could involve additional viroid-

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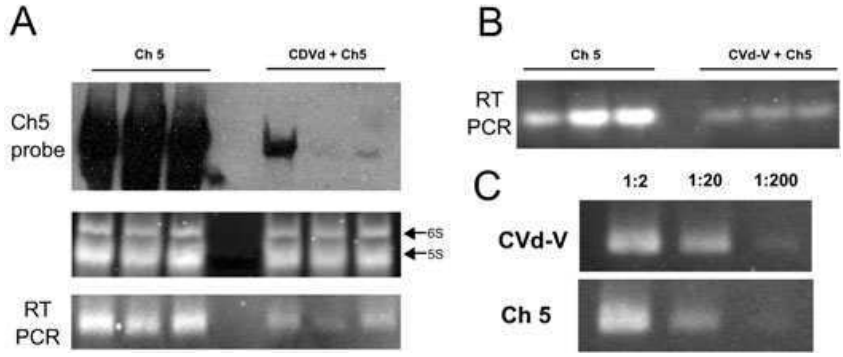

Fig. 4 (A) Top panel: northern hybridization analyses of samples electrotransferred from a non-denaturing gel using a digoxigenin (DIG)-labelled probe specific for chimera 5 (Ch5) of citron plants singly infected with Ch5 or co-infected with Ch5 and *Citrus dwarfing viroid* (CDVd). Ethidium bromide staining of the first non-denaturing gel of sequential polyacrylamide gel electrophoresis (sPAGE) shows that RNA levels (6S and 5S RNAs) in all preparations are comparable. Bottom panel: reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of citron plants singly infected with Ch5 or co-infected with Ch5 and CDVd using primers P1 and P2. (B) RT-PCR analysis of citron plants singly infected with Ch5 or co-infected with Ch5 and *Citrus viroid V* (CVd-V) using primers C2 and V7. (C) Efficiency of reverse transcription for the generation of cDNAs from CVd-V and Ch5 using primer P2 was evaluated by PCR using, as templates, serially diluted (1 : 2, 1 : 20, 1 : 200) cDNAs generated from equalized preparations of CVd-V or Ch5.

related RNA molecules, such as small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Baulcombe, 2004; Carrington and Ambros, 2003). The replacement of the T_L domain of CVd-V by that of CDVd would alter the structure of the viroid RNA and its affinity for a host factor, or the generation of certain specific viroid-derived siRNAs. Recently, it has been shown that viroid infection can downregulate or upregulate gene expression (Itaya *et al*., 2002; Tessitori *et al*., 2007), ultimately leading to symptoms. This cascade of events must be mediated by specific elicitors. The range of symptoms associated with CVd-V, which are not induced by Ch5, suggests that the latter does not act as an appropriate elicitor.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Primers used to amplify monomeric viroid sequences, specific viroid segments and plasmids containing partial viroid sequences.

Table S2 Strategies for construction of chimeric viroids.

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BRIEF REPORT

Molecular and biological characterization of natural variants of *Citrus dwarfing viroid*

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Abstract Citrus dwarfing viroid has been proposed as an agent to control tree size in high-density plantations. Thirty-three field isolates have been characterized, and the most frequent sequence/s have been identified. Five distinct variants were selected for biological characterization. Symptom expression analysis demonstrated a good correlation between leaf/stem symptoms and plant growth. The discriminating nucleotide sequence differences included two deletions and an insertion resulting in a reorganization of the base pairing of the terminal left loop, two (G42 → A and C52 → U) changes found in one of the variants, and as many as thirteen changes located in the right and left regions flanking the CCR.

The beneficial effects of high-density planting in citrus orchards has been the subject of many studies addressing improved early returns, reduced labor costs and efficient land use [7]. Pioneering work conducted in Australia lead to the identification of "graft-transmissible dwarfing factors" [6]. Following the discovery of citrus exocortis viroid (CEVd) [17], additional viroids were identified [4] and subsequently found to induce different levels of stunting in field-grown trees [7, 18, 21]. Among the viroids tested in long-term field assays, citrus dwarfing viroid (CDVd) (formerly CVd-III), has been recognized as the most promising viroid to control tree size without undesirable effects.

Viroids are small, infectious, single-stranded, circular RNAs. CDVd is a member of the genus *Apscaviroid* of the family *Pospiviroidae* with a "central conserved region" (CCR) and lacking RNA self-cleavage activity [5]. The rod-like secondary structure of viroids can be divided into five structural-functional domains: P (pathogenicity), C (central), V (variable), T_L (terminal left) and T_R (terminal right) [9]. In viroids of the genus *Apscaviroid*, all of which are restricted to woody species, the CCR and the "terminal conserved region" (TCR) have been clearly identified, whereas the presence and role of the P, V, T_L and T_R domains remain uncertain. The host range of CDVd is restricted to citrus and citrus relatives, and no specific symptoms have been associated with infection in commercial species [21]. In the Etrog citron (*Citrus medica*) indicator, CDVd causes mild stunting and a "leaf-dropping pattern" due to a moderate epinasty resulting from petiole and mid-vein necrosis.

Several CDVd variants were initially recognized by their distinct mobilities in sequential polyacrylamide gel electrophoresis (sPAGE) [4], and these were later characterized as three distinct sequence variants (CVd-IIIa, CVd-IIIb and CVd-IIIc) [15, 18]. These variants differ in size by as much as 18 nucleotides located in the left and right regions flanking the CCR, but limited information is available regarding whether or not these changes are associated with distinct biological properties. The objective of the present work is the identification of additional sequence variants and the characterization of their biological properties.

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Thirty-three field isolates recovered from different hosts and different locations, and containing RNAs with the mobility of CDVd were used in this study. To avoid the identification of variants that had accumulated in a given host as a result of host-driven selection [2], Etrog citron plants infected by graft transmission from the original sources were used as the source of tissue. Nucleic acid extracts were subjected to RT-PCR using CDVd-specific primers [1]. SSCP analysis of the amplicons revealed variations in their conformation and, therefore, in their nucleotide sequences [14] (data not shown). The purified DNAs were ligated in the pGEM-T vector (Promega), and the presence of the desired inserts was confirmed by PCR.

Since previous studies [12, 13] showed that natural CDVd isolates propagate in their hosts as populations of closely related sequence variants with predominant variant/s representing single or multiple fitness peaks, a strategy to identify the predominant variant present in the infected plants was followed. Briefly, for each cloning assay, the inserts of at least ten clones recovered by PCR were subjected to SSCP analysis, and the number of electrophoretic profiles provided an indication of the heterogeneity of the isolates. The populations of variants was found to be homogeneous in 32 of the 33 isolates tested, and a single sequence variant was identified by sequencing three clones of each, whereas in one isolate recovered from a 'Nules' clementine tree, two distinct sequence variants were found to be equally representative. The nucleotide sequences of the 34 variants are available in GenBank (see accession numbers in Fig. 1).

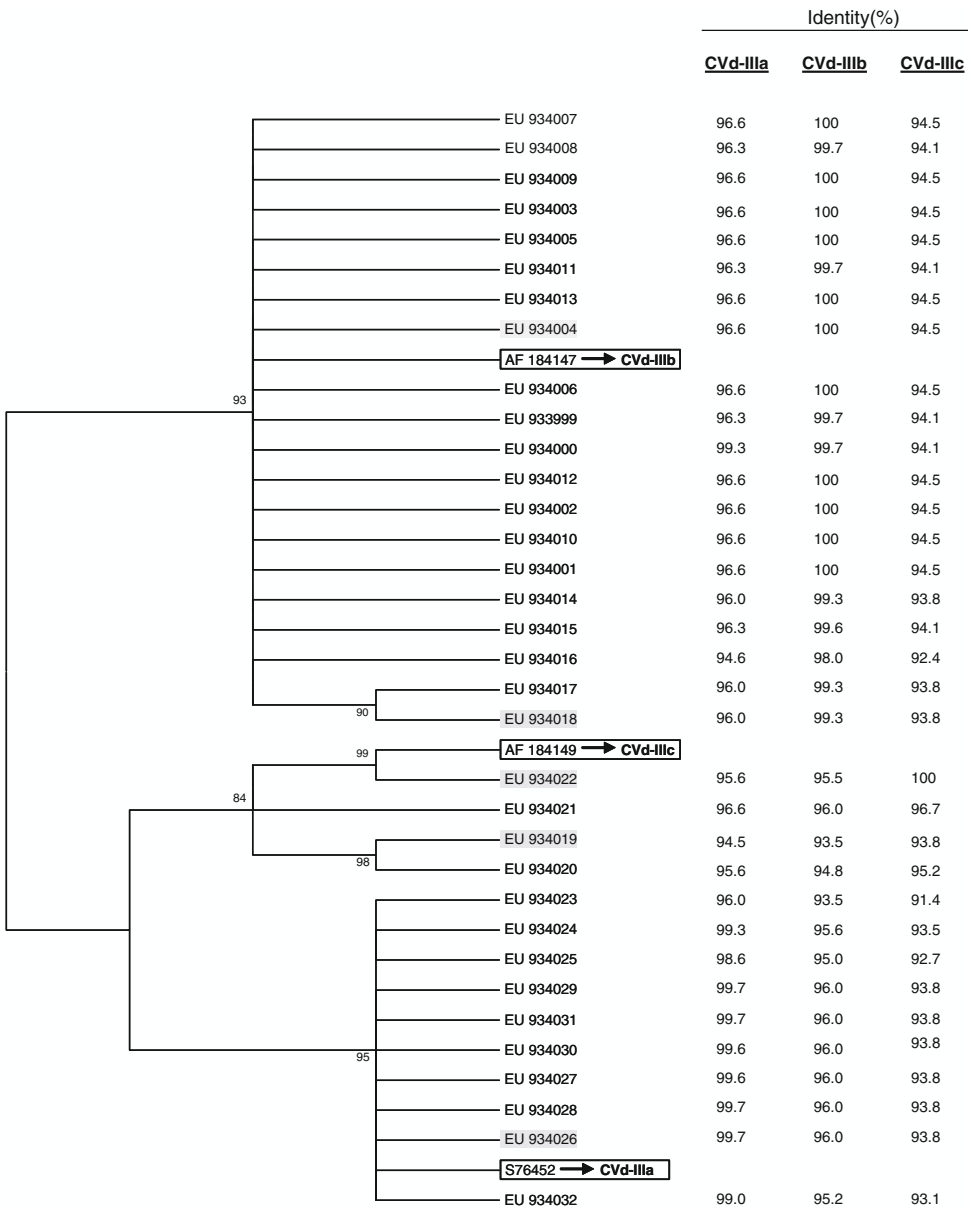

Fig. 1 Condensed neighbor-joining [16] phylogenetic tree (80% bootstrap value level) of 34 variants and the three CDVd reference sequences (CVd-IIIa, CVd-IIIb and CVd-IIIc). Nucleotide distances were estimated using the Jukes and Cantor method [8] after manual adjustment to maximize sequence similarities. These analyses were conducted with the MEGA 3.1 program [10]. Booststrap values (%) based in 10,000 replicates are indicated at the nodes

Sequence alignment of the 34 sequences with the three reference variants (CVd-IIIa, CVd-IIIb and CVd-IIIc) performed using the Clustal W program [20] revealed that ten were closely related to CVd-IIIa, twenty were related to CVd-IIIb, and one was identical to CVd-IIIc (Fig. 1). The remaining three variants presented similar identities with CVd-IIIa, CVd-IIIb and CVd-IIIc. These observations were in accordance with the neighbor-joining phylogenetic tree obtained with these 34 variants and the three reference sequences (Fig. 1).

Based on the above results, five variants were selected for biological characterization (shown shaded in Fig. 1): (1) EU934026 ($CDVd^{IIIa\ it}$), recovered from a 'Comune' clementine tree from Italy, differed by a single nucleotide change from CVd-IIIa (Supplementary Fig. 1). (2) EU934004 and EU934018 (referred to as $CDVd^{IIIb}$ and $CDVd^{IIIb\ sp}$, respectively) were recovered from a single 'Nules' clementine tree from Spain. $CDVd^{IIIb}$ was identical to CVd-IIIb, and $CDVd^{IIIb\ sp}$ differed by three changes that affected the organization of the secondary structure of the T_L domain (Supplementary Fig. 1). (3) EU934022 ($CDVd^{IIIc}$), recovered from a 'Nules' clementine tree from Spain, was identical to the reference sequence CVd-IIIc. (4) EU934019 ($CDVd^{so}$), recovered from a 'Valencia' sweet orange from Sohar (Sultanate of Oman), showed numerous changes relative to the closest variant, CVd-IIIa. Some of these changes affected the conformation of the T_L domain, which had the same organization as that of $CDVd^{IIIb\ sp}$. The primary structure and predicted rod-like secondary structure of these variants are shown in Supplementary Fig. 1.

To obtain infectious preparations of the five selected variants, head-to-tail dimeric cDNAs were synthesized and used as templates to produce the corresponding in vitro transcripts [19], which were slash inoculated (50 ng per plant) to three citron seedlings each. Infection was assessed by northern-blot hybridization [11] 14 months after inoculation. With the exception of one plant that became infected with $CDVd^{so}$ at an unusually low viroid titer, viroid concentrations of all the infected plants were high and comparable to those of the graft-inoculated controls (data not shown). RT-PCR analysis and amplicon sequencing showed that, in all instances, the sequences were identical to those of the inoculated variants.

To compare the performance of plants infected with these CDVd variants, five sets of three Swingle citrumello seedlings were graft-inoculated with two bark chips from infected citron seedlings, and three additional non-inoculated Swingle citrumello seedlings were kept as negative controls. Northern-blot hybridization and RT-PCR [1, 11] confirmed infection 4 months after inoculation. As found in the Etrog citron seedlings, the titer of variant $CDVd^{so}$ was considerably lower than those of the other variants (Fig. 2a). Amplicon sequencing revealed that $CDVd^{IIIa\ it}$, $CDVd^{IIIb}$ and $CDVd^{so}$ remained stable in the infected citrumello seedlings, whereas $CDVd^{IIIb\ sp}$ contained two changes (A221 → C, U223 → A) and $CDVd^{IIIc}$ one change (A254 → U). After four additional months, these citrumello seedlings were used as rootstocks to graft-propagate Etrog citron buds immediately above the inoculum site. One month later, the grafted buds started growing, and they were trained to grow as a single shoot. Northern-blot hybridization analysis of the citron scions showed that, similar to the citrumello seedling rootstocks, the titer of variant $CDVd^{so}$ remain considerably lower than those of the other variants (Fig. 2b). RT-PCR, cloning and sequencing (three clones from each plant) showed that $CDVd^{IIIa\ it}$, $CDVd^{IIIb}$ and $CDVd^{so}$ remained stable, whereas the changes identified in the citrumello seedlings infected with $CDVd^{IIIb\ sp}$ and $CDVd^{IIIc}$ reverted to the sequence inoculated to the original citron seedlings.

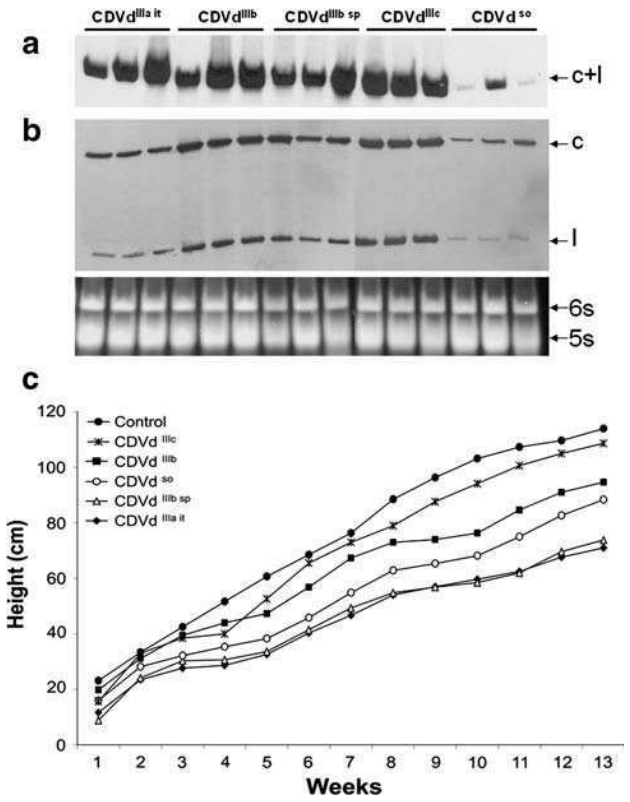

Fig. 2 Northern hybridization analysis of samples from Swingle citrumello seedlings inoculated with five CDVd variants (**a**) and from the Etrog citron scion graft-propagated on the infected Swingle citrumello seedlings (**b**). **a** Samples were subjected to non-denaturing PAGE, electroblotted to membranes and hybridized with a CDVd-specific probe. **b** Samples were subjected to sPAGE, and the nucleic acids separated in the second denaturing PAGE were electroblotted to membranes and hybridized with a CDVd-specific probe. **c** Growth curves of the second flush of growth of Etrog citron plants infected with five variants of CDVd. Data are the means of the height values determined over a 13-week interval

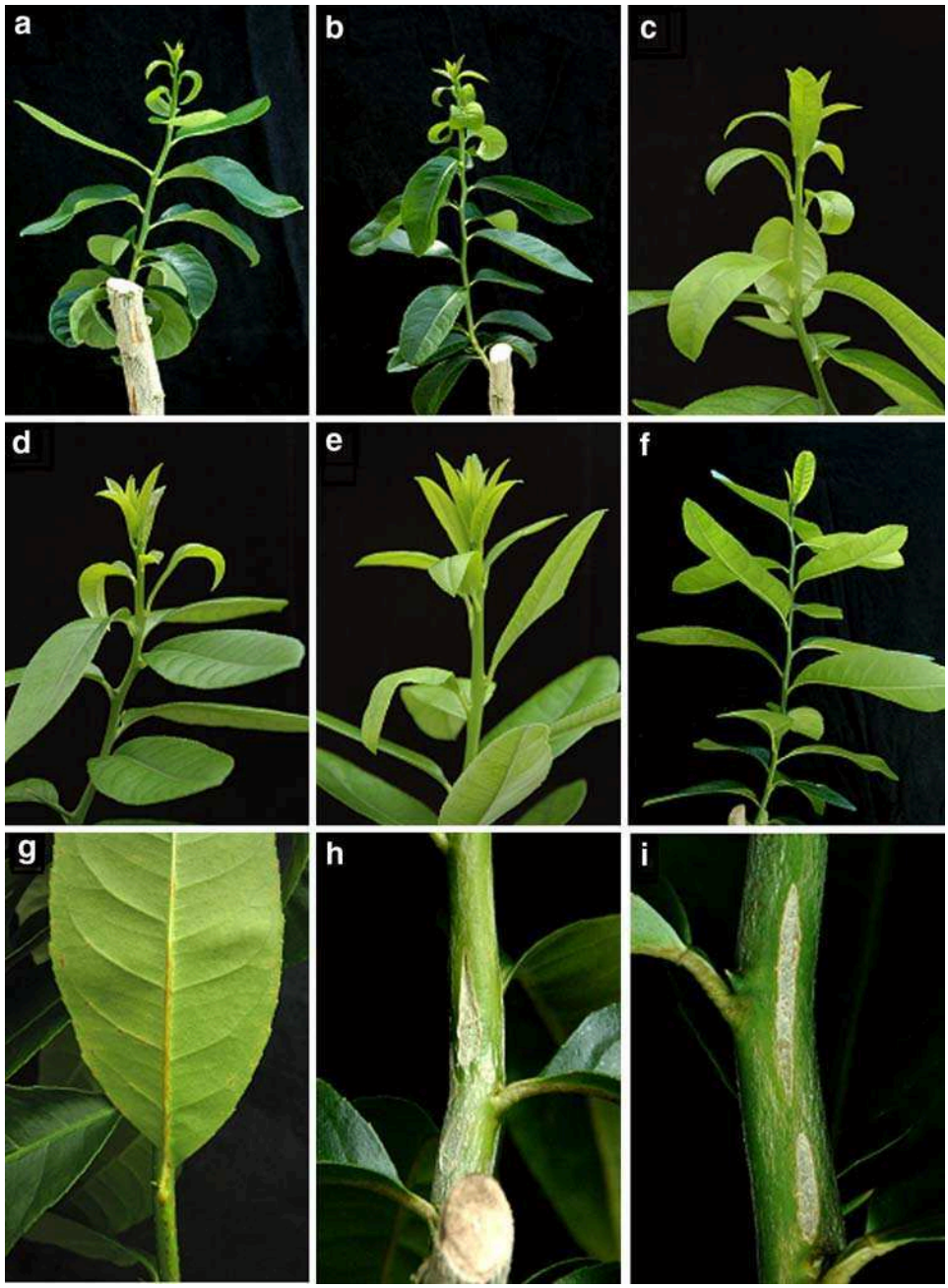

Fig. 3 Symptoms observed in Etrog citron plants infected with five CDVd variants. Epinasty on young leaves of Etrog citron infected with CDVd$^{IIIa\ it}$ (**a**), CDVd$^{IIIb\ sp}$ (**b**), CDVd^{IIIb} (**c**), CDVd^{so} (**d**) and CDVd^{IIIc} (**e**). Symptomless leaves of non-inoculated control (**f**). Petiole and midvein necrosis (**g**). Cracks at the basal end of the Etrog citron scion (**h**), usually extending along the stem (**i**) in plants infected with CDVd$^{IIIa\ it}$ or CDVd$^{IIIb\ sp}$

The young leaves of infected citron scions showed epinasty that was very prominent in plants infected with CDVd$^{IIIa\ it}$ or CDVd$^{IIIb\ sp}$ (Fig. 3a, b), mild in plants infected with CDVd^{IIIb} and CDVd$^{IIIc\ so}$ (Fig. 3c, d) and almost imperceptible in plants infected with CDVd^{IIIc} (Fig. 3e). In all instances, old leaves developed petiole necrosis extending along the midvein (Fig. 3g), causing the characteristic "leaf-dropping pattern". These symptoms were also severe in plants infected with CDVd$^{IIIa\ it}$ or CDVd$^{IIIb\ sp}$, moderate in plants infected with CDVd^{IIIb} or CDVd$^{IIIc\ so}$ and mild in plants infected with CDVd^{IIIc}. In addition, plants infected with CDVd$^{IIIa\ it}$ or CDVd$^{IIIb\ sp}$ developed cracks, not previously described, that started at the basal end of the scion (Fig. 3h) and usually extended along the stem (Fig. 3i).

In order to monitor viroid-induced stunting, the plants were cut at the level of the second citron internode, and the second flush of growth was measured weekly. Figure 2c displays data on citron growth over a thirteen-week period, illustrating the differences found in growth patterns. Comparisons of average height values (mean of weekly measurements) and the linear components that refer to the slope of the growth curve using a one-way ANOVA test showed significant differences in the average height

($P = 0.014 < 0.05$) and in the linear component ($P = 0.021 < 0.05$). It should be noted that whereas uninfected controls yielded a smooth growth curve, plants in the five CDVd-infected treatment groups followed unusual patterns, with periods of active growth alternating with periods of slow growth, and they therefore reached smaller sizes. Orthogonal contrast [3] confirmed that average heights ($P = 0.021 < 0.05$) and linear components ($P = 0.026 < 0.05$) of infected plants differed from the uninfected controls. A multiple range post-test using the 95% LSD method indicated that growth data (average height and linear component) clustered into three distinct groups: (1) plants infected with CDVd^{IIIc} that presented the mildest symptoms fell in the same group as the non-infected controls; (2) plants infected with CDVd$^{IIIa\ it}$ or CDVd$^{IIIb\ sp}$ that presented the most severe symptoms fell in the same group; (3) plants infected with CDVd^{IIIb} and CDVd$^{IIIc\ so}$ occupied an intermediate position (Figs. 2c, 3).

The present study demonstrates that naturally occurring variants of CDVd show differences in their biological properties. Infectivity assays with five variants selected as representative of the major clusters found in the phylogenetic tree showed that they were indeed infectious and stable in Etrog citron. However, after graft-transmission to Swingle citrumello, three of them remained stable, whereas CDVd^{IIIc} and CDVd$^{IIIb\ sp}$, contained one and two mutations, respectively, illustrating host-driven selection from the spectrum of mutants generated during viroid replication in the absence of proofreading activity of the RNA polymerases. As demonstrated in the case of CEVd [2], the mutations identified in citrumello reverted back to the original sequence once the viroid reached and replicated in the Etrog citron scion.

Symptoms and growth of infected citron plants demonstrate that the five variants studied behave as mild (CDVd^{IIIc}), moderate (CDVd^{IIIb} and CDVd^{so}) and severe strains (CDVd$^{IIIa\ it}$ and CDVd$^{IIIb\ sp}$), with a good correlation between leaf and stem symptoms and the final size reached by the infected plants suggesting that the effect of CDVd in symptom expression and plant growth are probably driven by the same viroid-host interaction mechanism. It should also be noted that in contrast to the mild CDVd^{IIIc} strain, which accumulated at high titers in infected plants, CDVd^{so}, characterized as a moderate strain, accumulated at very low viroid titers, demonstrating the lack of a relationship between viroid accumulation and symptom expression.

The attempts to identify structural domains involved in pathogenesis can be summarized as follows: (1) The type variants (CVd-IIIa, CVd-IIIb and CVd-IIIc) represented in this study by CDVd$^{IIIa\ it}$, CDVd^{IIIb} and CDVd^{IIIc}, which differ in changes in the two regions flanking the CCR, behave like distinct strains of CDVd. (2) CDVd$^{IIIb\ sp}$ which differs from CDVd^{IIIb} by two deletions and an insertion, resulting in a reorganization of the base pairing of the terminal left loop, shows an increase in symptom severity, therefore supporting the involvement of the T_L domain in the pathogenicity of CDVd [19]. (3) CDVd^{so}, which, in addition to the changes affecting the terminal left loop, contains additional changes that support its peculiar position in the phylogenetic tree as well as its somewhat low identity with the three reference variants.

In summary, this study demonstrates that strains with different levels of severity exist. Although the symptoms induced in Etrog citron show a good correlation with their stunting properties, such an effect should be further monitored before being used to control tree size in commercial rootstock/scion combinations.

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