DEPARTAMENTO DE PROTECCIÓN VEGETAL Y BIOTECNOLOGÍA

ANÁLISIS GENÉTICO DE LA INTERACCIÓN ENTRE EL VIRUS DE LA TRISTEZA DE LOS CÍTRICOS (CTU) Y LAS CITRADIAS. OBTENCIÓN Y SELECCIÓN DE GENES CANDIDATOS.

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Análisis genético de la interacción entre el Virus de la Tristeza de los cítricos (CTV) y las citradias. Obtención y selección de genes candidatos

TESIS DOCTORAL

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Antecedentes

1.- Los cítricos

El término cítricos (del griego kitrón, que significa limón) se utiliza genéricamente en referencia a todo un conjunto de especies e híbridos enclavados taxonómicamente dentro de la familia *Rutaceae*. Las especies con mayor importancia podrían clasificarse en los siguientes grandes grupos: las naranjas dulces (*Citrus sinensis* (L.) Osb.); las mandarinas, incluyendo satsumas (*C. unshiu* (Mak.) Marc.) y clementinas (*C. clementina* Hort. ex Tan.); los limones (*C. limon* (L.) Burm. f.) y limas (*C. aurantifolia* (Christm.) Swing.); y los pomelos (*C. paradisi* Macf.). Además de *Citrus* (naranjos, mandarinos, limoneros y pomelos), *Fortunella* (Kumquat) y *Poncirus* (naranjo trifoliado con frutos no comestibles) son otros dos géneros con cierta importancia.

Las numerosas especies de cítricos provienen fundamentalmente de las zonas tropicales y subtropicales de Asia y del archipiélago Malayo, existiendo unos pocos géneros localizados en el Africa tropical. La dispersión hacia otras regiones del mundo está ligada a la actividad humana, siendo los primeros viajeros y comerciantes los que fueron distribuyendo los cítricos por el resto de zonas actuales de cultivo. En general el cultivo de los cítricos es viable en una franja entre los 40° de latitud al norte y sur ecuatoriales, si bien la mayor producción comercial se concentra entre los 20° y 40° a ambos lados del Ecuador.

1.1.- Importancia económica

Con una superficie cultivada superior a los 7 millones de hectáreas y una producción de más de 102 millones de toneladas anuales, los cítricos son en la actualidad el cultivo frutal de mayor importancia (FAO, 2002). España, siendo el quinto país productor con casi 6 millones de toneladas, es el principal exportador de cítricos para consumo en fresco del mundo (más del 60% de la producción). El mantenimiento de esta posición depende en gran medida de la capacidad de mantener una elevada producción sin perder la calidad que demanda el mercado.

1.2.- Variedades y propagación

Dentro de un mismo grupo de cítricos, el número de variedades existentes es muy alto, sin embargo, la variabilidad genética entre ellas es muy baja (Herrero y col., 1996a). Las variedades se distinguen unas de otras en función de determinadas diferencias en caracteres de interés agronómico como pueden ser variaciones en la época de floración y maduración, en el calibre de los frutos o en la cantidad de zumo. La aparición de mutaciones en brotes es habitual en los cítricos (Raghuvanshi, 1962; Cameron y Frost, 1968) de tal modo que, en algunos casos, la rama resultante presenta caracteres agronómicos novedosos e interesantes y es seleccionada como origen de una nueva variedad. La propagación vegetativa de variedades restringe su diversificación genética al eludirse los procesos sexuales.

Para el cultivo de los cítricos la propagación mediante semillas presenta una serie de inconvenientes como son el largo período juvenil hasta la obtención de frutos y la heterogeneidad de la cosecha. En la actualidad la propagación se efectúa mayoritariamente injertando yemas de una variedad sobre un patrón cultivado a partir de semilla, lo cual evita los inconvenientes antes mencionados y permite seleccionar el patrón más adecuado a las condiciones específicas de cada zona de cultivo. Los patrones son una parte esencial del cultivo de los cítricos pues repercuten en el vigor y tamaño de la variedad, en la producción (uniformidad, cantidad y calidad) y en la tolerancia o resistencia a estreses tanto bióticos como abióticos.

Se tiende a utilizar mayoritariamente el patrón que mejor rendimiento proporciona. Sin embargo, la utilización de un único genotipo como patrón supone un riesgo potencial ya que la aparición de cualquier patógeno (nuevo, importado o latente) provocaría la práctica destrucción del cultivo. De hecho, los cambios en la utilización de patrones en España ha venido marcada por la aparición de enfermedades. Así, a mediados de siglo XIX la llegada de la "gomosis" producida por el hongo *Phytophthora* provocó la masiva introducción del patrón naranjo amargo (*C. aurantium* L.), resistente al hongo, pero también tolerante a otros muchos patógenos y condiciones del suelo. Parecía el patrón ideal y de hecho permitió un importante crecimiento de la citricultura española hasta que en los años 1930-35 irrumpió en la península el virus de la tristeza de los cítricos (*citrus tristeza virus*, CTV) provocando graves perjuicios económicos. Naranjos, mandarinos y pomelos injertados sobre naranjo amargo quedaban seriamente dañados (40 millones de árboles muertos), lo cual obligó a un nuevo cambio de patrones, esta vez hacia aquellos tolerantes a CTV, fundamentalmente los citranges Troyer y Carrizo (*C. sinensis* (L.) Osb. x *P. trifoliata* (L.) Raf.) y el mandarino Cleopatra (*C. reshni* Hort. *ex* Tan.). Estos patrones, sin embargo, no proporcionan las excelentes cualidades agronómicas del naranjo amargo (Moreno y col., 1993) y son más vulnerables a patógenos. La práctica totalidad de cultivares españoles de cítricos estaban infectados con patógenos transmisibles por injerto, los cuales eran tolerados por el naranjo amargo, pero no por los patrones tolerantes a tristeza. Gracias a programas de saneamiento, cuarentena y certificación de plantas y yemas (Navarro, 1993), se pudo disponer de material vegetal libre de patógenos, lo que ha hecho medianamente viable la utilización de patrones tolerantes a CTV. En la actualidad, el 75% de la citricultura española está injertada sobre citrange Carrizo (Cambra, 2000), con lo que sigue latente el peligro que supondría la llegada de aislados virulentos de CTV u otros patógenos que afectasen al citrange Carrizo.

1.3.- Combinaciones patrón-injerto

El injerto de una determinada variedad de cítrico sobre un patrón concreto no siempre resulta viable. Se han descrito incompatibilidades para diferentes combinaciones patrón-injerto

que pueden obedecer a distintas causas. Algunas son debidas a patógenos que sólo afectan a ciertas plantas de una determinada combinación variedad-patrón. El caso más grave es la incompatibilidad provocada por el virus de la hoja rasgada de los cítricos (*Citrus tatter leaf virus*, CTLV) que afecta a la mayoría de híbridos de cítricos injertados sobre los patrones resistentes al virus *P. trifoliata* y algunos de sus híbridos. Otras incompatibilidades son de origen genético y afectan a todas las plantas de una combinación específica variedad-patrón. En las variedades de limonero son especialmente comunes este tipo de incompatibilidades (Schneider, 1978). Naranjo amargo, *P. trifoliata* y citrange Troyer son ejemplos de patrones que pueden presentar problemas de incompatibilidad con según qué variedades de limonero.

1.4.- Estreses abióticos y bióticos

Existen factores abióticos y bióticos que pueden afectar negativamente la cantidad y calidad de la producción citrícola. Entre los estreses abióticos cabría destacar la salinidad de los suelos, las inundaciones y las heladas. En cuanto a los factores de estrés biótico, los cítricos son susceptibles a la infección por múltiples patógenos (hongos, bacterias, virus, viroides...). En la Tabla 1 se muestran algunos ejemplos de enfermedades que han causado y/o causan importantes pérdidas. La utilización de plantones sanos de alta calidad (programas de saneamiento, cuarentena y certificación) ha demostrado ser eficaz para controlar las enfermedades que se dispersan únicamente a partir de yemas infectadas, pero para aquellas infecciones que pueden propagarse de forma natural en campo (por ejemplo, a través de insectos) es necesario adoptar medidas adicionales como la utilización de genotipos resistentes, el control de los vectores o la protección cruzada (Navarro, 2000).

Enfermedad	Agente causal
Blight	Desconocido
Cancrosis	Bacteria – *Xanthomonas axonopodis pv. citri*
Caquexia	Viroide - CVd-II (viroide II de los cítricos)
Clorosis variegada	Bacteria – *Xylella fastidiosa*
Decaimiento lento de los cítricos	Nematodo – *Tylenchulus semipenetrans*
Exocortis	Viroide – CEVd (Viroide de la exocortis de los cítricos)
Gomosis, aguado, podredumbre de la raíz	Hongo – *Phytophthora*
Huanglongbing (ex-greening)	Bacteria – "Candidatus" *Liberobacter*
Muerte súbita	Probablemente un virus muy similar a CTV
Psoriasis	Virus – *Citrus psorosis virus* (CPsV)
Tristeza	Virus – *Citrus tristeza virus* (CTV)

Tabla 1.- Estreses bióticos en los cítricos.

Ante esta variedad de organismos patógenos, alguno de los cuales como el CTV, caracterizado por una gran variabilidad poblacional, cabe preguntarse qué mecanismos defensivos presentan los cítricos. Aparte de la reproducción sexual, ¿tienen los cítricos otros mecanismos especialmente importantes en la diversificación de genes de resistencia?.

2.- Elementos transponibles

Gran parte del genoma de los organismos eucariotas y sobre todo de las plantas está compuesto por elementos móviles denominados genéricamente elementos transponibles. Se trata de fragmentos de ADN capaces de insertarse en diferentes posiciones cromosómicas a través de un mecanismo de transposición (movilización e integración) que requiere de una serie de actividades enzimáticas codificadas en el propio elemento transponible. En algunos casos las regiones codificantes para la transposición están degeneradas o incluso delecionadas (elementos no autónomos). Se han establecido dos clases de elementos transponibles en función de su mecanismo de transposición, la primera, retrotransposones, se caracteriza por utilizar un intermediario de ARN que es retrotranscrito y entonces integrado, es decir, tras la retrotransposición se obtienen dos copias del elemento en diferentes posiciones del genoma; la otra clase, transposones, no utiliza intermediarios en su movilización, es el propio fragmento el que se escinde de una región del genoma y se inserta en otra.

2.1.- Retrotransposones

Los retrotransposones se subdividen en función de la presencia o ausencia de repeticiones terminales directas largas (LTRs).

Los retrotransposones sin LTRs son un grupo muy heterogéneo y numeroso de elementos móviles. Se les denomina secuencias repetitivas interdispersas y, al tener pocas características generales, se diferencian dos tipos en función de su tamaño:

+ SINEs: secuencias no autónomas de hasta 500 nucleótidos que se caracterizan por la presencia de un promotor para la RNA polimerasa III implicado en la transposición.
+ LINEs: pueden alcanzar hasta 7 Kb. y llevan codificadas las funciones necesarias para la transposición autónoma.

Los retrotransposones con LTRs comparten muchas características estructurales y funcionales con los retrovirus. Los retrovirus son virus icosaédricos con envoltura membranosa de origen celular que incluye proteínas del propio virus. Cuando un retrovirus infecta una célula puede insertarse en el genoma del huésped quedando en forma de provirus. El genoma de los retrovirus suele presentar tres pautas de lectura abierta (ORFs) denominadas *gag*, *pol* y *env* así como señales de corte y empalme para el procesado de los transcritos. La estructura de un retrovirus integrado y un retrotransposón con LTRs es altamente homóloga, y así, en un retroelemento con LTRs podemos encontrar:

+ LTRs (long terminal direct repeats): se pueden diferenciar las tres regiones típicas de retrovirus (U3-R-U5) implicadas en la regulación de transcripción y replicación.
+ PBS (primer binding site) y PPT (polypurine tract) necesarios para la retrotranscripción

+ GAG (group-specific antigen): dominio con alta homología a la región codificante *gag* de retrovirus que codifica para las proteínas estructurales de la cápsida.
+ POL (polyprotein): dominio con alta homología a la región codificante *pol* de retrovirus que codifica para una poliproteína con cuatro dominios estructurales, que son *pro* (proteasa), *rt* (transcriptasa reversa), *int* (integrasa) y *rh* (Rnasa H).
+ ENV (envelope): dominio con homología a la región codificante *env* de retrovirus que codifica para las glicoproteínas de la envoltura. Los retrotransposones generalmente carecen de este dominio o en todo caso está muy degenerado. Es el dominio responsable de la infectividad ya que las proteínas de la envoltura del virus reaccionan específicamente con receptores de membrana de la célula huésped permitiendo la entrada del virus. En realidad la mayor diferencia entre retrovirus y retrotransposones radica en la capacidad infectiva de los virus y, de hecho, a los retrotransposones con reminiscencias del dominio *env* se les ha catalogado como virus (pseudovirus y metavirus).

Se han establecido dos grandes grupos de retrotransposones con LTRs en función del orden de los dominios en la región *pol* (Figura 1):

+ tipo *Ty1/copia*: Engloba a todos aquellos elementos que, al igual que *Ty1* de *Saccharomyces cerevisae* y *copia* de *Drosophila melanogaster*, tienen una estructura $^{5'}$*pro-int-rt-rh*$^{3'}$ en la región *pol*.
+ tipo *Ty3/gypsy*: Agrupa retroelementos con una estructura $^{5'}$*pro-rt-rh-int*$^{3'}$ en la región *pol* como es el caso de Ty3 de *S. cerevisae* y *gypsy* de *D. melanogaster*. Esta organización es idéntica a la de los retrovirus.

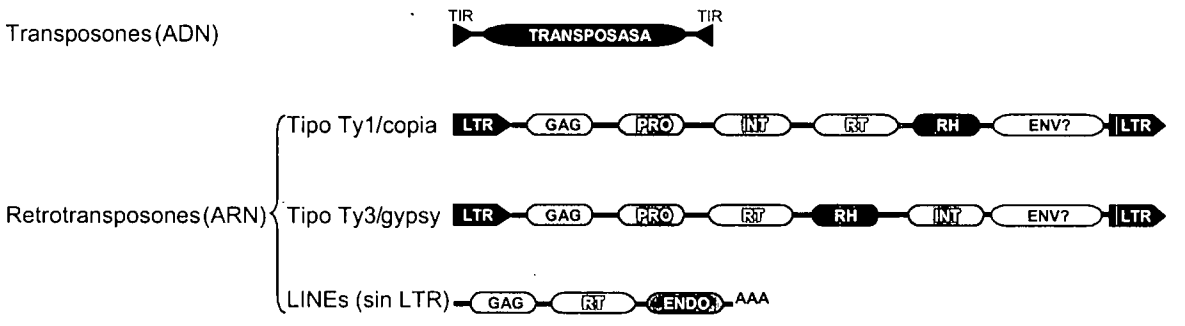


Figura 1.- Clasificación y características estructurales de los elementos transponibles. (Endo = endonucleasa; TIR = repeticiones terminales invertidas)

2.2.- Efectos de la transposición en el genoma

Los elementos transponibles en general y los retrotransposones en particular debido a su capacidad de movilización e inserción autónoma actúan como agentes mutagénicos. Así, por

ejemplo, el 80% de las mutaciones detectadas en *Drosophila* han sido atribuidas a la transposición (Capy, 1998). Las inserciones pueden producirse tanto en regiones codificantes (poco habitual como luego veremos) como reguladoras, pudiendo en éste último caso modificar el patrón de expresión de los genes (Weil y Wessler, 1990; Kidwel y Lisch, 1997) e incluso propiciar la aparición de nuevas funciones génicas (White y col., 1994). Se han descrito numerosos casos que muestran que la inserción de un elemento no es necesariamente deletérea, sino que puede desempeñar importantes funciones dando lugar a un nuevo patrón de expresión génica que podría ofrecer una ventaja evolutiva (López y col., 1999). Aparte de ser responsables de reestructuraciones genómicas, los retrotransposones favorecen la recombinación, en general, y la recombinación asimétrica, en particular, dando lugar a duplicaciones (Zhang y Peterson, 1999; Fedoroff, 2000). Por todo ello, los elementos transponibles constituyen un factor importante en la generación de variabilidad (Heslop-Harrison, 2000) y su actividad tiene importantes implicaciones en la evolución del genoma de los organismos (Fedoroff, 2000).

2.3.- Control de la transposición

Los elementos transponibles deben mantener un cierto nivel de actividad que les permita asegurar su propagación y supervivencia en el genoma pero, a su vez, una movilización indiscriminada de elementos resultaría en mutaciones deletéreas por lo que la propia célula presenta mecanismos para reprimir la transposición. Gracias a estos mecanismos, la transposición es un fenómeno poco habitual en condiciones normales, si bien, bajo condiciones de estrés se han detectado niveles elevados de movilización de elementos transponibles (Hirochika, 1995; Wessler, 1996).

Existe un mecanismo general de control de la transposición a partir de la metilación del ADN, mecanismo que utiliza la célula para regular la expresión del resto de genes. Una secuencia metilada tiene bloqueada la transcripción y dificultada la recombinación, además de tener mayor probabilidad de mutación (Fedoroff, 2000). La célula parece reconocer las secuencias transposónicas y las metila impidiendo que se movilicen. Por otro lado, parece existir por parte de los elementos transponibles un cierto grado de especificidad de inserción en zonas no codificantes lo cual evitaría efectos deletéreos. En levadura, por ejemplo, sólo el 3% de inserciones del elemento Ty1 estudiadas fueron en ORFs (Ji y col., 1993). Asimismo, las células parecen tener mecanismos de eliminación de elementos durante el procesado de los ARNm (Fridell y col., 1990; Purugganan y Wessler, 1992; Rushforth y Anderson, 1996) lo que evitaría la pérdida de función de un gen en el que se hubiese insertado un elemento transponible.

La mayoría de los elementos transponibles que se pueden identificar en el genoma de un individuo son inactivos debido a mutaciones en su secuencia que provocan interrupciones en la

pauta de lectura por acumulación de codones de paro y mutaciones que cambian de pauta de lectura correcta (Flavell y col., 1992). Sin embargo, los mecanismos de control de la transposición son imperfectos y existen evidencias de elementos transponibles transcripcional e insercionalmente activos incluso en ausencia de condiciones de estrés (Echenique y col., 2002) lo cual significa que, aunque lentamente, siguen actuando en la generación de variabilidad y reestructuración de los genomas.

El grado de implicación de los retrotransposones (elementos transponibles en general) en la diversificación de genes de resistencia es un tema todavía en estudio que trataremos en apartados posteriores.

3.- Resistencia a enfermedades en plantas

Los organismos vivos están constantemente sometidos a ataques externos tanto bióticos (depredadores, competidores, patógenos...) como abióticos (frío, calor, intensidad de luz, condiciones del suelo...). La supervivencia frente a dichos ataques depende de la capacidad del individuo de generar una respuesta defensiva efectiva. En este apartado nos centraremos en los mecanismos que las plantas han desarrollado para protegerse de los patógenos (todo aquel organismo que puede causar una enfermedad).

Las plantas no pueden escapar por lo que han tenido que crear dispositivos defensivos para responder activamente a la amenaza. La mayoría de veces la reacción defensiva es eficaz siendo una excepción la aparición de enfermedades. Mecanismos pasivos de defensa tales como la presencia de paredes celulares, ceras y otras barreras químicas constituyen una primera muralla defensiva que confiere a las plantas resistencia frente a multitud de patógenos (Osbourn, 1996). Igualmente, la ausencia en la planta de determinados componentes necesarios para el desarrollo del patógeno provocaría el fracaso de la infección (resistencia pasiva). Si el patógeno es capaz de sobrepasar esta primera línea defensiva, las plantas disponen de una segunda barrera de protección consistente en toda una batería de proteínas codificadas en el genoma (resistencia activa). En función del número de genes implicados en la generación de la respuesta resistente se habla de resistencia horizontal (poligénica) o resistencia vertical (monogénica).

En este apartado nos centraremos en la resistencia vertical que es la más estudiada y de la que más conocimientos se tiene acerca de su funcionamiento general. Como hemos comentado se trata de un tipo de resistencia activa en la que participa inicialmente un sólo gen (gen de resistencia o gen R) capaz de "descubrir" la presencia del patógeno y directamente paralizar su desarrollo (resistencia constitutiva) o desencadenar una serie de procesos encaminados a detener la infección (resistencia inducida).

3.1.- Resistencia

En un sentido amplio podría definirse la resistencia como la capacidad (determinada genéticamente) para reducir o eliminar un determinado patógeno (Andrivon, 1993). Se trata pues de un término relativo pudiéndose establecer diversos grados de resistencia. En general, para un mismo patógeno podemos encontrarnos plantas con:

+ respuestas resistentes de inmunidad en las que el patógeno está totalmente ausente de la planta, y ésta no muestra ningún signo de infección (resistencia extrema) o, en todo caso, pequeñas regiones necrosadas en torno a la zona de infección (respuesta hipersensible, HR).
+ respuestas resistentes en las que el patógeno está presente en la planta pero no causa enfermedad (tolerancia)
+ respuestas que no pueden impedir la infección y acaban desarrollando los síntomas de la enfermedad (susceptibilidad).

En función del patógeno, se pueden distinguir otros tipos de respuesta, como por ejemplo frente a virus se han definido diferentes tipos de resistencia (Solomon-Blackburn y Barker, 2001) en función del posible mecanismo que la está generando:

+ resistencia a la infección: la planta resistente tiene menos probabilidad de ser infectada. El mecanismo que utiliza la planta para evitar la infección en este caso suele estar relacionado con repeler el vector que transporta al virus.
+ resistencia a la acumulación del virus: la planta resistente se infecta pero el virus nunca llega a alcanzar altas concentraciones. La planta es capaz de bloquear algún paso del ciclo vital del virus evitando su multiplicación. Este tipo de resistencia puede ser evaluada por inmunoensayos (ELISA) capaces de cuantificar la carga viral de una planta.
+ resistencia al movimiento del virus: la planta resistente es infectada pero la diseminación del virus por toda la planta está bloqueada. En este caso, el mecanismo defensivo utilizado es diferente según estemos hablando de bloquear el movimiento a corta distancia del virus (célula a célula) que se produce a través de los plasmodesmos o del movimiento a larga distancia que requiere del paso de virus al sistema vascular de la planta y su posterior descarga en los tejidos.

Igualmente, para virus, cabe distinguir entre resistencia de no-huésped en la que todos los individuos de una misma especie o categoría taxonómica son resistentes, y resistencia de huésped en la que la resistencia es una característica individual (Mansky and Hill, 1993).

En definitiva, la resistencia es un concepto relativo y comparativo que debe ser establecido *a priori* para evaluar la resistencia a un patógeno en un determinado grupo de

plantas. Hay que definir un umbral de cantidad de patógeno, evolución del patógeno en la planta o tiempo tras la infección para diferenciar una planta resistente de una susceptible.

3.2.- Respuesta resistente frente a patógenos

La capacidad de resistencia activa de la planta viene determinada por la presencia o ausencia de todos aquellos genes implicados en la generación de una protección eficaz. El inicio de la respuesta defensiva requiere que la planta detecte determinadas moléculas indicadoras de infección (elicitores) que pueden ser productos de la degradación de su pared celular o productos sintetizados por el propio patógeno (factores de avirulencia). Los factores de avirulencia del patógeno (codificados en los denominados genes de avirulencia, *Avr genes*) pueden ser muy variados, desde toxinas bacterianas hasta proteínas de la cápsida vírica o enzimas hidrolíticos fúngicos. En cualquier caso el elicitor es reconocido, directa o indirectamente, por los productos de los genes R de la planta y la reacción elicitor-producto del gen R provoca la respuesta defensiva, bien sea interrumpiendo directamente la acción agresiva del patógeno (inactivar toxina, bloquear la replicación vírica) o bien activando simultáneamente distintas vías metabólicas que generalmente acaban en la muerte de la célula (HR) y en la inducción de respuestas defensivas inespecíficas en otras células de la planta (resistencia sistémica adquirida, SAR).

Se ha denominado resistencia "gen a gen" (Flor, 1942 y 1971) al caso de reconocimiento específico entre los productos del gen R de la planta y del gen Avr del patógeno y presupone la existencia de multitud de genes R cada uno con una especificidad distinta. Tras la interacción planta-patógeno se genera una fuerte y rápida respuesta defensiva que incluye (Hammond-Kossack y Jones, 1996; Baker y col., 1997):

+ fortalecimiento de la pared celular: síntesis lignina y calosa (implicada en bloquear plasmodesmos)
+ cambios en la permeabilidad de la membrana (canales de Ca^{2+}, H^+, K^+)
+ fosforilación de proteínas (implicada en activación de vías metabólicas)
+ acumulación de metabolitos secundarios con propiedades antimicrobianas (fitoalexinas)
+ generación de radicales reactivos de oxigeno (ROS): O_2^-, H_2O_2
+ incremento de determinadas proteínas (proteínas relacionadas con patogénesis, PRP): quitinasas, β-1,3 glucanasas, inhibidores de proteinasas
+ producción de Acido Salicílico (responsable de SAR). Translocado vía floema a otras regiones de la planta actúa como indicador de infección en ausencia de patógeno de tal modo que toda la planta activa sus defensas inespecíficas y queda preparada para responder rápidamente a una posible infección incluso por parte de otro patógeno.

La activación de todos estos procesos acaba dañando las membranas celulares y provocando la muerte celular tanto en la célula donde el patógeno fue detectado como en las adyacentes (necrosis en la zona de infección). En consecuencia el patógeno queda aislado.

3.3.- Genes R

Dada la definición de resistencia que se ha planteado en este apartado podríamos considerar gen de resistencia todo aquel gen cuya presencia diferencial en la planta genera resistencia frente a un patógeno. En la actualidad, se han identificado y caracterizado una treintena de genes de resistencia para distintos tipos de patógenos y en diversas plantas (Tabla 2). La gran mayoría de ellos codifican para proteínas de transducción de señal y el análisis de su secuencia ha permitido detectar diversas características y dominios estructurales conservados, lo cual hace pensar que todas las plantas utilizan similares mecanismos en respuesta a la presencia de un patógeno independientemente de cual sea la naturaleza de éste. Existe especificidad para la detección del agente infeccioso pero, una vez identificado, la estrategia defensiva parece ser la misma independientemente del patógeno (y de la planta).

A los dominios proteicos conservados codificados en los genes R se les supone un papel fundamental en la generación de la respuesta resistente. En un gen R suelen encontrarse codificados:

+ repeticiones ricas en Leucina (LRR): presente en todos los genes R caracterizados hasta el momento, parece ser el dominio responsable de la interacción directa con el elicitor y se ha postulado como responsable de la especificidad de reconocimiento. Los dominios LRR median interacciones proteína-proteína y mutaciones puntuales en esta región de la proteína provocan la pérdida de función del gen R. Paradójicamente el primer gen mayor de resistencia que fue aislado (*Pto*, Martin y col., 1993) es el único gen R que no presenta LRR. Estudios posteriores demostraron que para que *Pto* sea funcional es imprescindible la presencia de otro gen con dominio LRR (*Prf*) que es el que realmente se considera gen R.
+ sitio de unión a nucleótidos (NBS): presente en todas las proteínas R que se localizan intracelularmente, su función es todavía desconocida, pero parece un dominio implicado en la señalización, es decir, en desencadenar la transducción de señal probablemente activando kinasas o proteínas G a partir de su unión a nucleótidos trifosfato. Así, por ejemplo mutaciones en la región NBS del gen *RPS2* de *Arabidopsis* provocan que no se desencadene la HR.
+ cremallera de Leucinas (LZ): también llamado "*coiled-coil*" (CC) parece igualmente implicado en la señalización, aunque poco se conoce de su modo de acción más allá de que puede favorecer interacciones entre proteínas al ser un dominio que favorece la dimerización homóloga o heteróloga de proteínas.

+ dominio con similitud al receptor para Toll/interleucina-1 (TIR): por su similitud con los dominios Toll de mosca e interleucina-1 de mamíferos, se le supone la misma función, es decir, señalización para la activación de determinadas rutas metabólicas generalmente con ayuda de kinasas.
+ región transmembrana (TM): presente en todas los genes R cuyo producto se localiza al menos en parte extracelularmente, sirve para anclar la proteína a la membrana.

Los genes R parecen ser pues los encargados de detectar la presencia del patógeno (dominio LRR) e iniciar las vías de transducción de señal que provoquen la respuesta defensiva (dominios NBS, LZ y/o TIR)

Especie vegetal	Genes R	Estructura
Tomate	Prf, Mi	LZ-NBS-LRR
	I2	NBS-LRR
	Cf-2, Cf-4, Cf,5, Cf-9	LRR-TM
Arabidopsis	*RPS2, RPS5, RPP8, RPM1*	LZ-NBS-LRR
	RPS4,RPP1, RPP5, RPP10;RPP14	TIR-NBS-LRR
Arroz	*Xa1, Pib, Pi-ta*	NBS-LRR
	Xa21	LRR-TM-PK
Lino	*L, M*	TIR-NBS-LRR
Patata	*Rx, Gpa2*	NBS-LRR
Cebada	*Mla*	NBS-LRR
Maíz	*Rp1-D*	NBS-LRR
Pimiento	*Bs2*	NBS-LRR
Tabaco	*N*	TIR-NBS-LRR
Trigo	*Cre3*	NBS-LRR

Tabla 2.- Genes de resistencia caracterizados en plantas y dominios proteicos conservados que se han identificado en cada uno de ellos.(PK= dominio protein-kinasa). Modificado de Takken y Joosten (2000).

3.4.- Origen y evolución de genes R

Dadas las características de los genes R vegetales lo más probable es que hayan surgido a partir de secuencias codificantes de sistemas de reconocimiento y señalización endógeno implicadas en el crecimiento y desarrollo normal de la planta (Hammond-Kossack y Jones, 1997). Además, su similitud funcional y estructural con genes involucrados en el sistema inmunitario de los mamíferos hace suponer que ambos sistemas defensivos presentan un origen evolutivo común.

La mayoría de patógenos se caracterizan por su elevada tasa de mutación, lo cual les permite evitar los sistemas defensivos específicos del huésped. Basar la resistencia contra estos patógenos en un único gen R resultaría poco efectivo y, como hemos dicho, la susceptibilidad de las plantas a un patógeno es la excepción. Todo esto sugiere la existencia de coevolución entre patógeno (genes *Avr*) y planta (genes R) y, en consecuencia, un amplio

rango de especificidades codificadas de algún modo en los genes R debe estar a disposición de la maquinaria defensiva vegetal de modo análogo al sistema inmunitario de los mamíferos.

Los mecanismos de que dispone la planta para generar nuevas especificidades de reconocimiento pueden ser muy variados. Se han detectado casos de un solo gen R con múltiples alelos, cada uno con una especificidad distinta (gen *L* del lino), e incluso se especula con la posibilidad de que la variabilidad se genere a partir del procesado ("*splicing*") alternativo de los mensajeros de un sólo gen (Whitham y col., 1994; Lawrence y col., 1995). En cualquier caso, la variabilidad de especificidades que generan estos procesos es limitada y, de hecho, a partir del estudio de la organización genómica de los genes de resistencia se ha deducido cuál debe ser el principal mecanismo involucrado en la aparición de nuevas capacidades de reconocimiento. La mayoría de genes R se encuentran estrechamente ligados a otros genes R y secuencias homologas de función desconocida formando grupos organizados en tándem ("*clusters*") de genes de resistencia con distintas especificidades incluso frente a diversos patógenos (Bent, 1996). Esto hace pensar que se originaron por duplicación génica debida a sobrecruzamientos asimétricos facilitados por la presencia de secuencias repetitivas (como por ejemplo las LRR). Sucesivas rondas de sobrecruzamiento irían generando nuevos genes, incluso genes parcialmente delecionados o quiméricos, con nuevas especificidades tanto frente al mismo como a otros patógenos (Richter y col., 1995; Michelmore y Meyers, 1998). En este sentido, merece especial atención la actividad de los retrotransposones que, como se apuntó en el capítulo anterior, por un lado favorecen la recombinación y por otro generan mutaciones por lo que parecen especialmente asociados a la evolución de los genes de resistencia (Richter y Ronald, 2000).

Resulta aparentemente paradójico que elementos genéticos similares a virus (retrovirus) estén implicados en la generación de nuevas especificidades para los genes de resistencia que utiliza la planta para defenderse de los propios virus (y otros patógenos). Más aún, si consideramos que uno de los principales estímulos que promueven la movilización de transposones son los estreses, tanto bióticos como abióticos (Hirochika, 1995; Wessler, 1996), podría decirse que la presencia en la planta de virus (o cualquier otro patógeno) estimularía la generación de nuevas especificidades de resistencia contra patógenos.

4.- Resistencia a CTV

El virus de la tristeza de los cítricos (CTV) es el causante de la enfermedad viral más grave de los cítricos, conocida como tristeza. A partir de 1930 comenzaron a aparecer epidemias por todo el mundo causando importantes pérdidas y en la actualidad sigue siendo un serio problema para la citricultura mundial a pesar del extenso conocimiento que se está

acumulando acerca de la naturaleza del virus y de la resistencia natural existente en algunos géneros relacionados con los cítricos.

4.1.- CTV

El virus de la tristeza de los cítricos pertenece a la familia de los closterovirus y se caracteriza por tener partículas filamentosas flexuosas de 2000 nm (nanómetros) de longitud y 12 nm de diámetro. Es un virus asociado al floema que se disemina en la naturaleza mediante áfidos pudiéndose transmitir también por injerto. El genoma es un ARN de cadena sencilla con polaridad positiva de aproximadamente 20 Kb (el más grande entre los virus de plantas). De su secuencia nucleotídica (Karasev y col., 1995) se deducen 12 ORFs (Figura 2), teniendo capacidad para codificar hasta 19 proteínas. En la organización del genoma se aprecian dos rasgos característicos de los closterovirus (Dolja et al., 1994): dos ORFs codificantes de proteínas capsídicas (p25 y p27, que cubren el 95 y 5% de la partícula respectivamente adoptando una estructura de "serpiente de cascabel") y ORFs codificantes de homólogos de proteínas de choque térmico (p65 y p61).

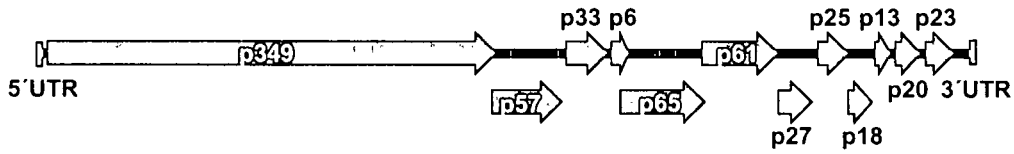


Figura 2.- Estructura genómica de CTV. Las flechas representan las ORFs: p349, poliproteína de 349 kilodaltons (kDa) con dominios para dos proteasas tipo papaína, una metiltransferasa y una helicasa; p57, ARN polimerasa ARN-dependiente (RdRp) de 57 kDa; p33, proteína de 33 kDa de función desconocida; p6, proteína hidrofóbica; p65, homólogo a la proteína de choque térmico HSP70; p61, homólogo a la proteína de choque térmico HSP90; p27, proteína menor de la cápsida; p25, proteína mayor de la cápsida; p18, proteína de 18 kDa de función desconocida; p13, proteína de 13 kDa de función desconocida; p20, proteína de 20 kDa de función desconocida pero se ha hallado en los cuerpos de inclusión; p23, proteína de unión a ARN asociada a la acumulación de ARN; 5' UTR y 3' UTR, regiones no traducidas indispensables para la replicación. (Modificado de Dolja y col., 1994).

Existen gran cantidad de aislados de CTV que varían en secuencia, transmisibilidad y síntomas que producen en los huéspedes. En la planta infectada se pueden detectar un conjunto de variantes de secuencia (con una o dos mayoritarias) las cuales probablemente determinan las características de la infección. Así pues, existen aislados de CTV más o menos virulentos pero de momento no es posible establecer una relación clara entre alguna región del genoma y la virulencia del virus.

4.2.- Síntomas de tristeza en plantas infectadas

CTV tiene como huéspedes naturales a las especies e híbridos de los géneros *Citrus* y *Fortunella* a los cuales infecta generando síntomas variables en función de la combinación variedad/patrón y de la cepa del virus. En algunas especies se han detectado cultivares con resistencia a ciertos aislados del virus como es el caso de *F. crassifolia* Swing. (Mestre y col., 1997) o *C. grandis* (L.) Osb. (Garnsey y col., 1997; Fang y Roose, 1999), pero la resistencia a la mayoría de aislados sólo se ha encontrado en otros géneros afines como es el caso de *Severina buxifolia* (Poir) Tenore, *Swinglea glutinosa* (Blanco) Merr. y *P.trifoliata.* (Bar-Joseph y col., 1989; Garnsey y col., 1987; Mestre y col., 1997).

En general, la mayoría de aislados de CTV producen sobre las especies de cítricos sensibles al menos uno de los siguientes síntomas:

+ decaimiento de la planta injertada sobre naranjo amargo ("*decline*"). Excepto limoneros, cualquier cultivar o híbrido injertado sobre amargo sufre decaimiento debido a la muerte de las células del floema del patrón justo por debajo de la línea de injerto, causando un déficit hídrico en la parte aérea y una falta de nutrientes en la zona radicular. El árbol sufre amarilleamiento y pérdida de hojas y en general va perdiendo vigor hasta prácticamente anular su producción.
+ clorosis nervial y acanaladuras en la madera. Independientemente del patrón sobre el que estén injertadas, son muchas las especies que sufren el síntoma, pero las más afectadas son lima mejicana (*C. aurantifolia* (Christm.) Swing.) y *C. macrophylla* Wester. Se observa una amarillez en los nervios secundarios de las hojas llegándose en casos extremos a la suberización generalizada de las nerviaciones ("*vein corcking*"). Por otro lado, aparecen estriaciones en tronco y ramas ("*stem pitting*") que hace que sean quebradizas y que la planta reduzca su crecimiento.
+ amarilleamiento de plantas de semilla ("*seedling yellows*"). Aparece en plántulas de semilla de limonero, pomelo o naranjo amargo cultivadas en invernadero, raramente se detecta en campo. Aparece clorosis intensa en las hojas y la planta deja de crecer.

4.3.- Epidemiología, diagnóstico y control de la tristeza

El virus de la tristeza de los cítricos se transmite por injerto al utilizar yemas infectadas lo cual permitió a principios del siglo XX la dispersión del virus por prácticamente todo el mundo a causa del tráfico de material vegetal sin control sanitario. Dentro de una determinada región, el virus puede ser transmitido de forma semipersistente por diversas especies de pulgones. La eficiencia de transmisión depende del pulgón, pero también de la cepa del virus y del cítrico donante y receptor. El vector más eficiente es *Toxoptera citricida* que vive casi exclusivamente en los cítricos por lo que a partir de un sólo árbol infectado, el virus se transmite rápidamente a, prácticamente, todos los vecinos. Otro vector importante aunque menos eficiente es *Aphis*

gossypii, especie polífaga por lo que las picaduras en cítricos son esporádicas diseminando la enfermedad por árboles dispersos. *T. citricida* no ha sido detectado (por el momento) en España por lo que el vector principal de CTV es *A. gossypi*. También existen otros vectores de menor eficiencia como *A. spiraecola* y *T. aurantii*.

Debido a la variabilidad de síntomas en función de la cepa de CTV y del cítrico infectado, el diagnóstico visual de la enfermedad es muy difícil. El método clásico de diagnóstico ha sido la inoculación por injerto en plantas indicadoras como lima mejicana la cual revela síntomas de infección bastante evidentes (clorosis nervial y acanaladuras en la madera). Los principales inconvenientes de éste método son la lentitud (de 3 a 8 meses hasta la aparición de síntomas) y la imposibilidad práctica de diagnóstico a gran escala. En la actualidad existe un método rápido de detección serológica de CTV, sensible y aplicable de modo rutinario basada en técnicas inmunoenzimáticas (ELISA). A partir de la detección con anticuerpos monoclonales específicos (3DF1 y 3CA5, Vela y col., 1986) se puede detectar prácticamente cualquier cepa del virus tanto de modo cualitativo (inmunoimpresión-ELISA) como semicuantitativo (DAS-ELISA). Se han desarrollado otras pruebas diagnósticas basadas en la detección del ARN viral por hibridación molecular o PCR (reacción en cadena de la polimerasa) pero de momento resultan más costosas e inabarcables para la detección a gran escala del virus.

El control de una enfermedad vírica transmisible por pulgones es difícil, de modo que la medida más eficaz para evitarla es impedir la entrada del virus mediante programas de cuarentena y certificación de yemas, lo cual, en zonas como España donde el virus ha llegado pero los aislados son en general poco agresivos, al menos evita la entrada de nuevas cepas virulentas. Otra medida de control es la eliminación de los árboles infectados, pero sólo sería eficaz en zonas de baja incidencia. En zonas donde CTV esté bien establecido, la única solución (de momento) es convivir con la enfermedad tratando de disminuir los daños a partir de la utilización de patrones resistentes o tolerantes como Poncirus, Troyer, Carrizo, Cleopatra, lima Rangpur (*C .limonia* Osb.) o limonero rugoso (*C. jambhiri* Lush.). En algunas zonas donde existen cepas de CTV virulentas se está complementando la utilización de patrones tolerantes con la protección cruzada, es decir, la inoculación de las plantas deliberadamente con aislados suaves del virus lo cual, aunque con limitaciones (Fulton, 1986), parece evitar en cierto grado el establecimiento de cepas agresivas (Costa y Muller, 1980; Van Vuuren y col., 1993).

4.4.- Biotecnología para la resistencia a CTV

La obtención de cítricos resistentes al virus a partir de métodos tradicionales de hibridación-selección (mejora clásica) utilizando como donante de la resistencia a *P. trifoliata* (o cualquier otro genotipo resistente) requiere más tiempo y dinero que si se tratara de una especie herbácea. Al igual que en otras especies, la principal dificultad radica en transferir la capacidad de resistencia sin transferir caracteres de la especie silvestre que provoquen la

pérdida de cualidades agronómicas en el cultivar. En general, la mejora clásica de cítricos se ve dificultada por las propias características del cultivo, existiendo muchos factores que influyen negativamente en la posibilidad de éxito. Por un lado, el largo periodo juvenil impide la evaluación de la calidad de producción hasta pasados 5-8 años retrasando enormemente la selección de genotipos interesantes. Por otro lado, la peculiar biología reproductiva de los cítricos también afecta negativamente (Cameron y Frost, 1968), así, la apomixis de la mayoría de especies dificulta la obtención de híbridos y la esterilidad o la incompatibilidad de algunos genotipos interesantes condiciona la elección de parentales. Todos estos inconvenientes junto con los elevados costes de cultivo explicarían la escasez de estudios genéticos y de programas de mejora en cítricos.

El creciente progreso de la biotecnología supone una de las mayores esperanzas para la mejora genética vegetal en general y para el control y erradicación de la tristeza de los cítricos en particular. El desarrollo de marcadores moleculares cada vez más fiables e informativos está resultando de gran utilidad en múltiples campos como estudios de variabilidad, estudios filogenéticos, análisis de estructura y organización genómicas, construcción de mapas genéticos, disección de caracteres cuantitativos (QTLs), o localización y clonación de genes de interés. También los marcadores tienen aplicaciones interesantes para los programas de mejora como pueden ser la distinción temprana de individuos zigóticos y nucelares en plantas con apomixis (Torres y col., 1982; Ruiz y col., 2000), o la selección temprana de individuos con marcadores ligados a caracteres interesantes (selección asistida por marcadores). Igualmente, las técnicas de transformación genética e hibridación somática mediante fusión de protoplastos han permitido superar las restricciones que planteaba a la mejora clásica la necesidad de compatibilidad sexual.

Así, actualmente se está trabajando en la obtención de cítricos resistentes a CTV a partir de la transformación genética. Plantas transgénicas expresando genes del propio virus (PDR, resistencia derivada del patógeno) o genes naturales de resistencia han resultado eficaces en distintos sistemas virus-planta (Baulcombe, 1996; Thilmony y col., 1995). En cítricos también se están explorando las posibilidades de la PDR y ya se han descrito plantas transgénicas de lima mejicana con un nivel moderado de protección a CTV a partir de la incorporación del gen p25 del virus (Domínguez y col., 2002). En el caso de la transformación con genes naturales de resistencia a CTV, el primer paso es clonar dicho gen (o genes), lo cual se está persiguiendo actualmente en diversos laboratorios de Estados Unidos (Deng y col., 2000; Yang y col., 2001, Yang y col., 2003).

4.5.- Clonación del gen de resistencia a CTV de *P. trifoliata*

P. trifoliata es el genotipo resistente a CTV más estudiado habiéndose encontrado resistencia para todos los aislados del virus ensayados (Garnsey y col., 1987; Mestre y col.,

1997). Diversos estudios genéticos sobre la naturaleza de la resistencia de Poncirus apuntan a que se trata de un caracter controlado por un único gen llamado *Ctv-R* (Gmitier y col., 1996; Fang y col., 1998) si bien otros autores han presentado evidencias en favor de la implicación de al menos otro gen (*Ctv-M*, Mestre y col., 1997). La resistencia de *C. grandis* a ciertos aislados de CTV también ha sido estudiada y atribuida a un gen (*Ctv-R2*) no alélico de *Ctv-R* (Fang y Roose, 1999). Por el momento se desconocen los mecanismos que provocan que las respuestas resistentes desencadenadas por los distintos genes sean más o menos generales y duraderas. La introducción de un gen de resistencia (*Ctv-R* en este caso) en una variedad susceptible al virus permitirá la protección de dicha variedad sólo si el gen es funcionalmente compatible con el fondo genético de la planta a transformar. De hecho, esta es la principal limitación de la transformación genética y no puede esperarse que la transferencia de genes de resistencia entre especies resulte efectiva en todos los casos (Bent, 1996). Por ello, el conocimiento de la naturaleza de la resistencia a CTV proporcionada por el gen es de especial interés a la hora de plantearse la transferencia exitosa de la resistencia a otras especies por transformación genética.

En cualquier caso, varios grupos han acometido la clonación de *Ctv-R*. La estrategia utilizada, clonación basada en el mapeado, podría dividirse en tres pasos (Figura 3):

+ desarrollo de mapas genéticos de alta resolución en torno al gen de interés: El caracter derivado del gen a clonar es evaluado en la descendencia de un cruce (familia segregante) en el que al menos uno de los parentales presenta dicho gen. Igualmente se realiza el análisis genético (con marcadores moleculares) de la familia con el objetivo de establecer un orden relativo de marcadores (mapa de ligamiento). En el mapa resultante deben conseguirse marcadores estrechamente ligados al gen (distanciados por frecuencias de recombinación lo más pequeñas posibles). Dos aspectos son de especial importancia en este paso, disponer de familias segregantes amplias y desarrollar marcadores moleculares fiables e informativos. Diversos grupos han construido mapas de cítricos con marcadores ligados a *Ctv-R* (Gmitter y col., 1996; Deng y col., 1997; Mestre y col., 1997; Fang y col., 1998).
+ construcción de mapas físicos por paseo cromosómico: A partir del desarrollo de genotecas de ADN genómico se secuencia el inserto con el marcador molecular flanqueante al gen de interés y se van secuenciando sucesivamente clones solapantes hasta llegar al gen. La principal dificultad del proceso radica en conseguir una ordenación fidedigna de clones solapantes que lleve a la obtención de una secuencia lineal de clones. Yang y col. (2001 y 2003) realizaron este paseo cromosómico en la región donde se había ubicado *Ctv-R* y delimitaron una región de aproximadamente 300 Kb donde debe encontrarse el gen y en la cual se han encontrado hasta 7 ORFs

con homología a genes de resistencia. Como se apuntó en apartados anteriores los genes de resistencia suelen encontrarse agrupados en clusters probablemente originados por duplicaciones en las que los retrotransposones podrían estar involucrados. El análisis de la región donde se sospecha debe estar *Ctv-R* coincide con estas características y junto con varios genes de resistencia podemos encontrar también diversos tipos de elementos transponibles (Figura 3).

+ determinación del gen diana: A partir de la transformación de una variedad sin la característica deseada (susceptible en este caso) con el gen de interés se puede demostrar la función de éste si se detecta complementación genética (aparición de resistencia) en el individuo transgénico. En el caso de Ctv-R, al haberse encontrado un mínimo de 7 candidatos, en estos momentos se están ensayando plantas transformadas con varios de estos potenciales genes de resistencia a tristeza (Yang y col., 2001 y 2003).

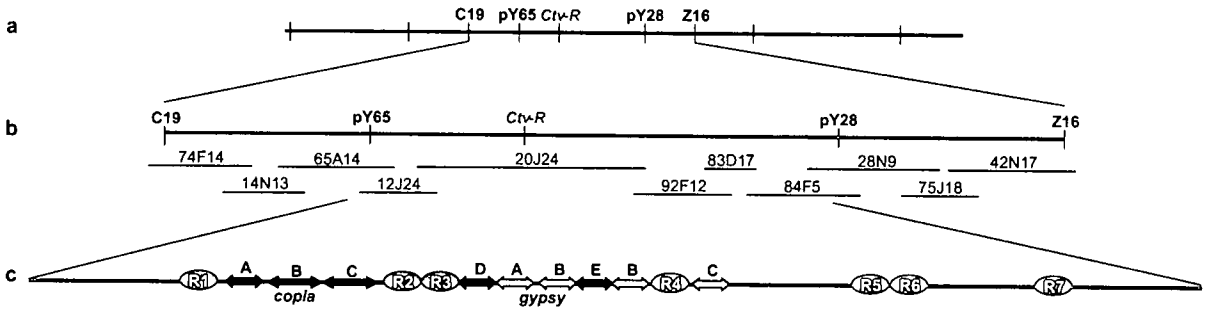


Figura 3.- Mapa genético (a) y físico (b) de la región cromosómica donde se ha mapeado *Ctv-R*. La secuenciación de los clones correspondientes a dicha región (c) ha permitido identificar 7 ORFs (R1-R7) con homología a genes de resistencia de otras plantas así como 9 secuencias retrotransposónicas (tanto de tipo *copia* como de tipo *gypsy*) y otros elementos transponibles (no indicados en la figura). Modificado de Yang y col. (2001 y 2003).

Todo este trabajo requiere de mucho tiempo y un enorme gasto económico asociado por lo que habría que comprobar si Ctv-R es el único gen responsable de la resistencia, si esta bien localizado, independientemente de la progenie utilizada, si existe alguna estrategia para aislar su producto génico en forma de cDNA y si no necesita de otros genes para funcionar y evitar así la muerte por tristeza.

Objetivos

Los programas de mejora genética de cítricos son muy largos y costosos. Largos por el tiempo que tardan en producir frutos (periodo juvenil) y costosos por los gastos derivados de su mantenimiento durante los años de juvenilidad y evaluación. La selección asistida por marcadores (MAS) y la transformación genética son dos importantes herramientas que usadas convenientemente permitirían disminuir estos gastos. Un primer paso hacia la aplicación de estas herramientas en la mejora de la resistencia a CTV sería el estudio genético de dicha resistencia así como la obtención de mapas genéticos detallados de la región cromosómica donde se encuentra el gen (genes) de resistencia. El desarrollo de ambos puntos constituye el principal propósito de la presente Tesis.

Dicho objetivo ha sido acometido a partir del establecimiento de cinco objetivos parciales:

1.- Obtención de un nuevo grupo de marcadores moleculares derivados de retrotransposones del tipo *gypsy* que podrían estar involucrados en la diversificación de los genes de resistencia y permitirían mejorar los mapas genéticos actuales de cítricos.

2.- Saturación de la región donde previamente se había situado el gen de resistencia a CTV (*Ctv-R*) con distintos marcadores moleculares y secuencias expresadas localizadas (LES). Para la obtención de LES se desarrollaron dos estrategias novedosas que combinan el análisis de segregación masal (*bulk segregant analysis*) con el de *differential display* o con la construcción de una genoteca de sustracción de cDNA.

3.- Comparación de los mapas de ligamiento resultantes en *C. aurantium* y en *P. trifoliata*.

4.- Mapeo fino de *Ctv-R* bajo la hipótesis de herencia monogénica a partir del seguimiento de la presencia de CTV durante más de dos años en una progenie de citradias (resultante del cruce *C. aurantium* x *P. trifoliata*).

5.- Detección de QTLs e interacciones epistáticas relacionadas con la acumulación de CTV en la anterior familia segregante y análisis de genes candidatos.

Los resultados correspondientes a estos objetivos parciales serán discutidos en tres capítulos. En el primero de ellos nos centraremos en el objetivo 1, mientras que en el segundo se acometerán los objetivos 2, 3 y 4, y en el tercero se abordará el objetivo 5. Los capitulos están redactados en inglés tal y como han sido enviados a publicar a una revista científica.

Identification and genomic distribution of *gypsy*-like retrotransposons in *Citrus* and *Poncirus*

Chapter I

1.- Introduction

Citrus is one of the most important fruit crops in the world. Citrus species are diploid (2n=18) trees with hesperidium fruits, and seeds often with two or more nucellar embryos which are genetically identical to the seed parent. Nucellar embryony (a type of apomictic reproduction) has very important consequences for evolution, breeding, and culture of citrus fruit trees (Asíns et al. 2002). Citrus are almost universally propagated by budding onto (nucellar) rootstocks to ensure yield uniformity. Bud mutations arise often in citrus (Raghuvanshi 1962; Cameron and Frost 1968) and are generally detected by the growers themselves in branches of trees showing altered horticultural traits, such as maturity and flowering time or fruit characteristics. Transposons can clearly inactivate genes by integration or by becoming methylated the region where they are located. They might also contribute to agronomic variation (maturation date, flesh color) by increasing allelic diversity or by changing the regulation of gene expression.

Based on the domain structure in the POL region, LTR retrotransposons are divided into two groups, the Ty1/*copia* type (*pro-int-rt-rh* from 5' to 3') and the Ty3/*gypsy* type (*pro-rt-rh-int*). Asíns et al. (1999) investigated the presence of *copia*-like retrotranposons in citrus. They found that these elements were quite abundant throughout the citrus genome and very heterogeneous for the *rt* domain. Polymorphisms based on *copia*-like elements (RFLPs and IRAPs) have been found distinguishing groups of varieties within *C. sinensis* (Asins et al. 1999), *C. clementina* (Bretó et al. 2002) and *C. limon* (Bernet et al. 2003). Moreover, polymorphisms based on these elements are more abundant than those based on primers of random sequence or simple sequence repeats (Bretó et al. 2002).

Gypsy-type retrotransposons are the most similar elements to retroviruses. Both of them are organized in the same manner differing mainly in the infective capability of retroviruses which critically depends on a third open reading frame (ENV) encoding envelope glycoproteins (Frankel and Young 1998). *Gypsy*-like retrotransposons containing ENV-like domains have been already reported even in the plant kingdom (Vicient et al. 2001) where retroviruses are thought to be lacking. Until recently, little was known about the Ty3/*gypsy* group of elements in plants (Smyth et al. 1989; Purugganan and Wessler 1994). Nowadays, it seems clear that Ty3/*gypsy*-like retrotransposons appear to be broadly distributed among plants in multiple families like the Ty1/*copia* group (Sueniemi et al. 1998; Chavanne et al. 1998; Friesen et al. 2001; Shcherban et al. 2001; Feschotte et al. 2002). There seems to be no difference for activity between both types, either. This activity is low (Gradbastien 1998; Vicient et al. 2001; Echenique et al. 2002; Feschotte et al. 2002) and low to middle repetitive LTR retrotransposons are more frequently found in EST collections of maize than the very high copy number elements

(Meyers et al. 2001). More importantly, there are evidences that biotic and abiotic stresses are related to an increment of their activity (Hirochika et al. 1996; Echenique et al. 2002; Feschotte et al. 2002). Although integration sites for most mammalian and *Drosophila* retroelements appear to be distributed more or less randomly in the genome, a clear bias in the site selection choice has also been observed, at least for some yeast retrotranposons. Thus, Ty3 elements integrate almost exclusively upstream of genes transcribed by RNA polymerase III. Therefore, the distribution and implications of Ty1/*copia*- and Ty3/*gypsy*-like retrotransposons on the citrus genome evolution might be different. The investigation on their heterogeneity, activity and genomic distribution in apomictic perennial species might contribute to our understanding about the evolution of their genomes. This knowledge might enlighten also new ways to improve cultivated citrus. Hence, the objectives of the present paper are to investigate the presence of *gypsy*-like retrotransposons, in *Citrus* and *Poncirus*, their heterogeneity and their genomic distribution.

2.- Materials and methods

2.1.- Plant materials

All citrus plants analyzed belong to the Citrus germplasm bank at IVIA. Varieties "Fino", "Doblefina" and "Loretina" from *C. limon* (L.) Burm f., *C. sinensis* (L.) Osb. and *C. clementina* Hort. ex Tan., respectively, were selected for amplification of gypsy-like elements. Similarly, DNA from *C. limon* "Verna", *C. sinensis* "Ricolate", *C. clementina* "Marisol" and *Poncirus trifoliata* (L.) Raf. "Flying Dragon" were used for Southern blot analysis. A segregating population derived from the cross between *C. aurantium* L. "Afín Verna" and *P. trifoliata* "Flying Dragon" (AxPa) consisting in 66 hybrids was genotyped for IRAPs based on four *gypsy*-like elements (the most different ones among each other) to study their genomic distribution. This progeny had been previously used to obtain the genetic linkage maps of the parental species (Ruiz and Asins 2003)

2.2.- Isolation of gypsy-like fragments

Citrus genomic DNA extractions were carried out from 1g of leaf tissue according to Dellaporta et al. (1983) with minor modifications (Ruiz et al. 2000). DNA concentration was estimated using a 6105 spectrophotometer (Jenway).

In order to investigate the presence of *gypsy*-like retrotransposons in Citrus spp. the PCR-based strategy described by Suoniemi et al. (1998) was used. Degenerate primers, designed to match conserved residues from *rt* (forward) and *int* (reverse) domains of gypsy-like retoelements and DNA from three different *Citrus* species (*C. limon, C. clementina* and *C. sinensis*) were used for PCR. The amplified fragments containing *gypsy*-like fragments were

expected to be of approximately 1.6 Kb, but according to the results obtained for different members of the plant kingdom it might range from 2.0 to 0.8 Kb. (Suoniemi et al., 1998). Diverse amplification conditions were attempted in order to reduce PCR artifacts. Amplification reactions with the best results consisted of 300 ng of template DNA, 1x supplied reaction buffer, 1.5 mM $MgCl_2$, 100 µM each dNTP, 0.5 µM each degenerate primer, 1.25 u Taq (EcoTaq, Ecogen) and sterile water up to 25 µl. Each reaction was overlaid with 25 µl of mineral oil and amplified in a PTC-100 thermal cycler (MJ Research) under the following conditions: an initial step at 94°C for 5 min; 30 cycles of 1min at 94°C, 1 min at 47°C and 2 min at 72°C; and a final step at 72°C for 10 min. PCR products were mixed with 6 µl of 5x loading buffer (50% v/v glycerol, 1x TAE, 10% v/v saturated bromophenol blue, 0.2% w/v xylene cyanole) and visualized with ethydium bromide staining after electrophoresis in 0.8% agarose-TAE gels. In some cases, electrophoresis using 10% polyacrylamide sequencing-type gels followed by silver staining according to Ruiz et al (2000) were also carried out to improve banding resolution. Amplification products were extracted from gels, eluted in sterile water and re-amplified to verify its isolation. Purified PCR products using ADN from *C. clememtina* "Loretina" were cloned into the pGEM-T Easy Vector System (Promega). Searching for diversity, inserts from white colonies were analyzed by digestion with 3 restriction endonucleases (HindIII, XbaI and EcoRV).

2.3.- DNA sequencing and sequence analysis

Selected clones were sequenced in both strands at the IBMCP (UPV-CSIC) sequencing service. Given that the size of the selected clones was too large to reach the entire sequence of the inserts, two sequences per clone (RT-side and INT-side), corresponding to the forward and reverse primers, were obtained and analyzed separately. Sequence analysis, alignments, and putative translations were performed using SEQUENCHER (Gene Codes Corporation) and OMIGA (Accelrys Inc.) computer programs. Homology searches were done using the online service of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and TBLASTX and BLASTP searching tools. Statistical significance is represented by the E-value (p-value and E-value are nearly identical when $E<0.01$). Sequence similarities were graphically represented by dendograms using the p-distance and the neighbor-joining aggregation method, and bootstrapping with 500 replicates implemented in MEGA 2.1 (Kumar et al. 2001). Primer design to obtain probes and IRAPs by PCR was conducted using the PRIME program of the University of Wisconsin Genetics Computer Group (GCG) software package. GCG and SEQUENCHER programs were accessed through the Bioinformatics Service-CSCI at the University of Valencia.

2.4.- Southern blot analysis

Genomic DNA (15 µg) from the above specified *Citrus* species and *P. trifoliata* was digested with four different 6-cutter restriction enzymes (EcoRI, HindIII, DraI and BamHI). The

fragments were size fractionated by 0.8% agarose gel electrophoresis and transferred onto a positively charged nylon membrane (Hybond-N+, Amersham pharmacia biotech). To check probe specificity, approximately 5 ng DNA from four representative *Citrus gypsy*-like fragments (each representing one nucleotide sequence homology group) were denatured and dot-blotted onto another nylon filter. These same fragments were digoxigenin labeled using the DIG DNA labeling kit (Roche) and used as probes for hybridization, which was carried out under high stringency conditions achieved at 68ºC for 14h and by washing the membranes twice in 2x SSPE, 0.1% SDS for 10 min at 68ºC and twice again in 0.5x SSPE, 0.1% SDS for 10 min at 68ºC. The hybridization signals were subsequently detected with the ECF chemifluorescent substrate (Amersham pharmacia biotech), and analyzed on a STORM 860 optical scanner (Molecular Dynamics).

2.5.- Development of IRAP markers and linkage analysis

Four primer pairs for IPAP markers (Kalendar et al., 1999) were designed from four distinct *Citrus gypsy*-like sequences (C1, C2, C8 and C11). Each pair is based on the RT and INT regions of the sequence, facing outward. Primers are available from IVIA, upon request. Amplification reactions for 25 µl, final volume, contained 300 ng of template DNA, 1x supplied reaction buffer, 100 µM each dNTP, 0.12 µM each primer and 1u Taq (Netzyme, N.E.E.D.). Reaction mixtures were amplified under the following conditions: an initial step at 95ºC for 5 min; 45 cycles of 1min at 95ºC, 1 min at 44ºC, a slope of +0.3ºC per second to 72ºC and 3 min at 72ºC; and a final step at 72ºC for 8 min. Amplification products were resolved by polyacrylamide gel electrophoresis and visualized by silver staining as above. IRAP segregation data in AxPa family was analyzed using JOINMAP 3.0 (Van Ooijen and Voorrips 2001) with a linkage criterion of LOD generally above 4.0, recombination fraction of 0.5 and Kosambi mapping function was used for linkage analysis. The population was analyzed as the "Cross pollinator" population type with no previous knowledge of the linkage phase of the markers. Nomenclature of the linkage groups follows that described by Ruiz and Asins (2003). New IRAPs were named as the *gypsy*-like clone (C1, C2, C8 or C11) followed by a number that indicates the size in base pairs of the segregating band.

3.- Results

3.1.- Isolation and characterization of *Gypsy*-like sequences in *Citrus*

Every citrus species tested yielded the same weak banding pattern consisting in three bands of approximately 2200 pb, 1650 pb, and 1550 pb (coded as A, B and C respectively in Figure 1). *C. clementina* bands were more intense, so we tried to isolate them from agarose and polyacrylamide gels but the re-amplification of the largest fragment (A) was unsuccessful while

B and C fragments were easily purified and subsequently used for cloning experiments. Only one clone from fragment B was obtained while those from C fragment were abundant. Polyacrylamide gel electrophoresis showed slight size differences among C clones. Such differences were tested and confirmed by endonuclease restriction analysis since four different restriction patterns were found for C clones. All C clones showed the same restriction patterns except for C1, C8 and C11 that had a particular one. The only B positive clone (B9) showed the same restriction patterns as C1. Along with four clones representative of the common restriction pattern (C2, C12, C13 and C14), clones C1, C8, C11 and the B clone were also sequenced.

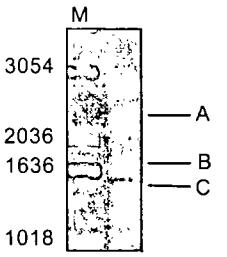
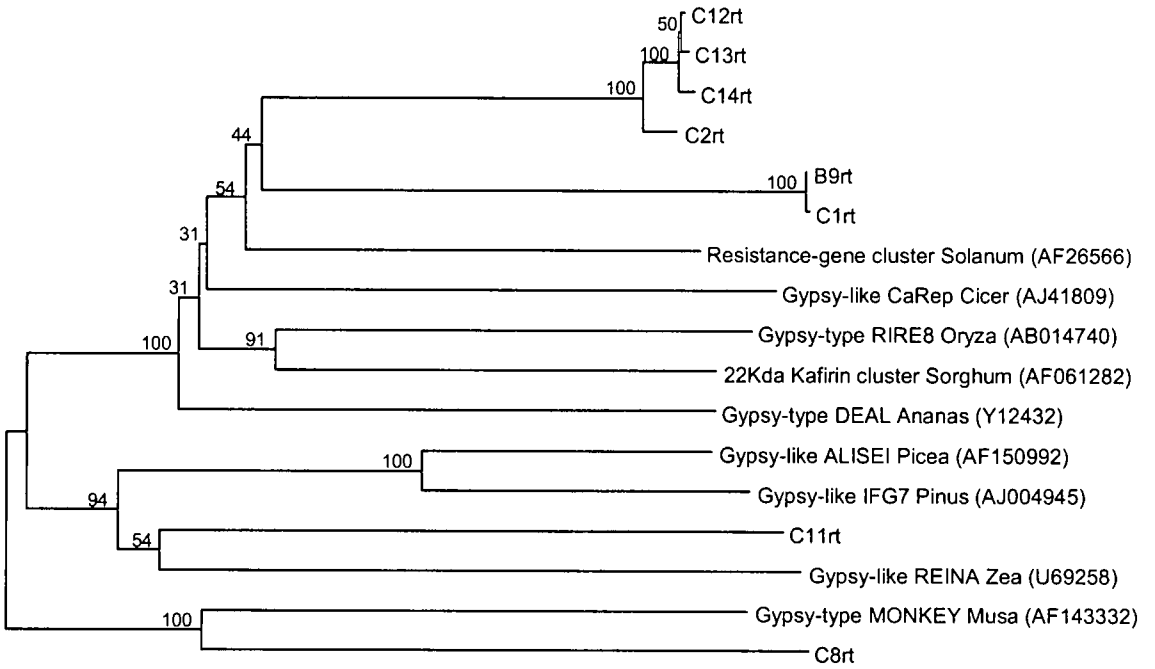


Figure 1.- Agarose gel electrophoresis of amplification products using Genomic DNA from *C. clementina* "Loretina" and *gypsy* degenerated primers. M: Molecular weight markers in base pairs.

Sequence searches at NCBI databases revealed, in every case, high significant similarities (E-values ranging from e^{-98} to e^{-42}) to Ty3/*gypsy*-type retrotransposons from different plant species. Additionally, some of our sequences strongly matched with a resistance-gene cluster from potato and several kafirin cluster from *Sorghum*. A representative pool of closely related sequences were aligned with our Citrus putative *gypsy* clones. A neighbor-joining tree based on nucleotide sequence alignment was constructed for both RT-side and INT-side sequences resulting in very similar representations of relationships (Figure 2). As expected, Citrus RT and INT nucleotide sequences were closely related to different members of the Ty3/*gypsy* class of retrotransposons, suggesting they are part of the POL coding region of citrus *gypsy* elements. Cluster analysis clearly reflected the same four groups obtained by restriction analysis, one including C2, C12, C13 and C14, another with C1 and B9, and the other two groups with only one sequence each (C8 and C11). Percentages of nucleotide identities between clones were also almost identical for RT and INT sides. Identities above 95% were found within groups, while between groups, it ranged from 63% (C2 and C1 groups) down to 45%, between C2 and C8 groups.

The potential correct frame for translation of both *rt* and *int* domains was inferred using the TBLASTX search tool. The resulting amino-acid sequences revealed several stop codons for B9, C1, C8 and C11 while no stop codon was found interrupting the putative coding region at

RT side:



INT side:

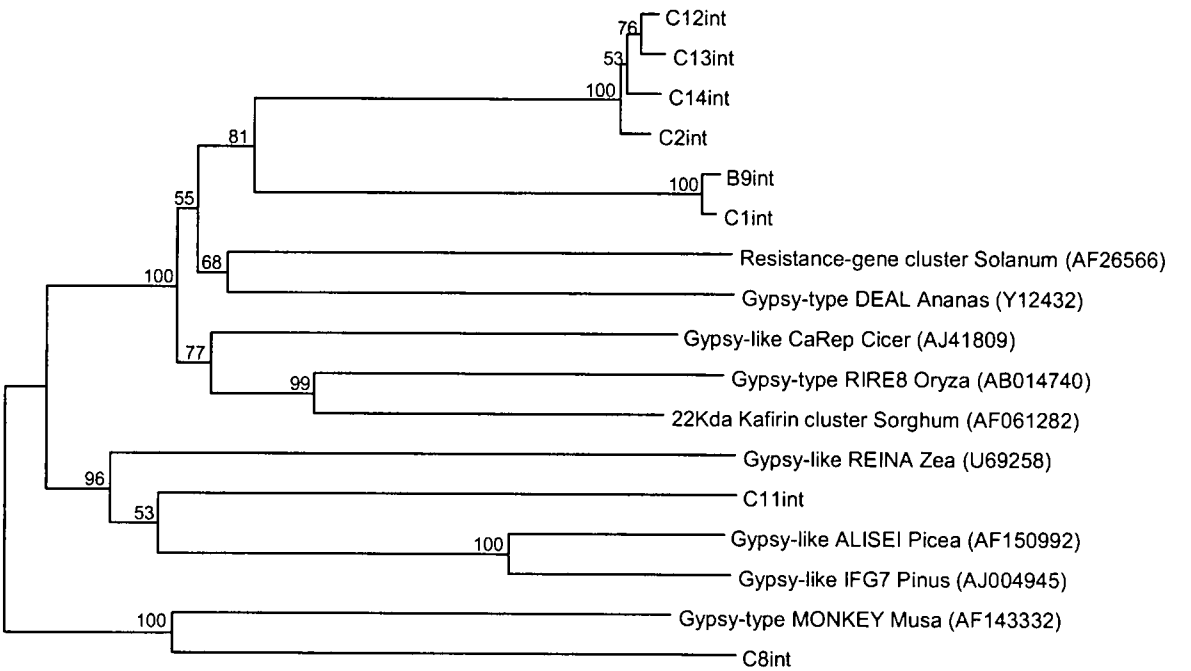


Figure 2.- Dendogram of Citrus gypsy-like sequences and related sequences from NCBI databases. The tree is based on nucleotide sequences alignment. Numbers adjoining the branches indicate bootstrap values (percentages from 500 replicates) based on p-distance estimate of sequence distance and neighbor-joining aggregation method. Numbers into brackets correspond to sequence accession numbers at NCBI databases.

C2, C12, C13 or C14 clones. Again, the predicted translation products of RT and INT domains showed high similarities to Ty3/*gypsy* polyproteins from the NCBI protein databases. Matches with plant disease resistance polyproteins and Sorghum kafirin cluster polyproteins were also found. The alignment of some of these proteins with our *Citrus* putative translation products is shown in Figure 3. RT and INT translated sequences for B9, C1, C8 and C11 present not only stop codons but also frame-shift mutations (insertions and deletions). The putative amino acid sequences from C2, C12, C13 and C14 show the blocks of residues widely described as universal in the Ty3/*gypsy* group and no stop codon. For these clones the translation of the RT domain begin with the invariant DD motif of the reverse transcriptase active site and, approximately 40 residues downstream, the highly conserved SKCEF block, including the invariant Lysine (K) of RNA-dependent polymerases, is found (Suoniemi et al., 1998, citing Barber et al., 1990). Characteristic residues of the Rnase H domain are also present in these citrus clones since the conserved DAS motif containing a key Aspartate (D) active-site could be identified (Springer and Britten, 1993; Chavanne et el., 1998) followed by a Glutamate (E) residue which is essential for RNAse H catalysis.

Two out of the three distinct conserved motives within the integrase domain of Ty3/*gypsy* retrotransposons are also present (Malik and Eickbush 1999). The N terminal and the central region could be identified in the *Citrus* clones (Figure 3). The N-terminal integrase subdamain contains an $H_6H_{29}C_2C$ motif forming a zinc-finger structure implicated in binding to LTR retrotransposon sequences (Khan et al. 1991). The integrase central core subdomain begins with the highly conserved GLLQPLPI motif (Suoniemi et al. 1998) and continues with the catalytic $D_{60}D_{35}E$ motif essential for enzymatic activity (Kulkosky et al. 1992). The C-terminal subdomain lays out of the region we have cloned.

Given that C2, C12, C13 and C14 *Citrus* clones (from now on named C2 group) present all the conserved motifs described in the literature as essential for *gypsy*-like retrotransposon activity, they might correspond to a family of active *gypsy* elements. On the other hand, the sequence analysis of B9, C1, C8 and C11 clones shows several mutations disrupting the appropriate reading frame suggesting that these elements, if functional, are non-autonomous.

3.2.- Genomic distribution of Citrus *gypsy*-like elements

Internal specific primers were designed for C1, C2, C8 and C11 sequences, each representing one group of citrus *gypsy*-like elements. The resulting dig-labeled PCR amplification products were used as probes for genomic southern blot hybridizations under high stringency conditions. RFLPs obtained using C1 probe are shown in Figure 4. Similar results were achieved when dig-labeled C2, C8 and C11 were used as probes. A very simple banding pattern was observed in all cases, suggesting a small number of copies of each clone in the *Citrus* and *Poncirus* genomes. *C. sinensis* and *C. clementina* hybridization patterns are almost

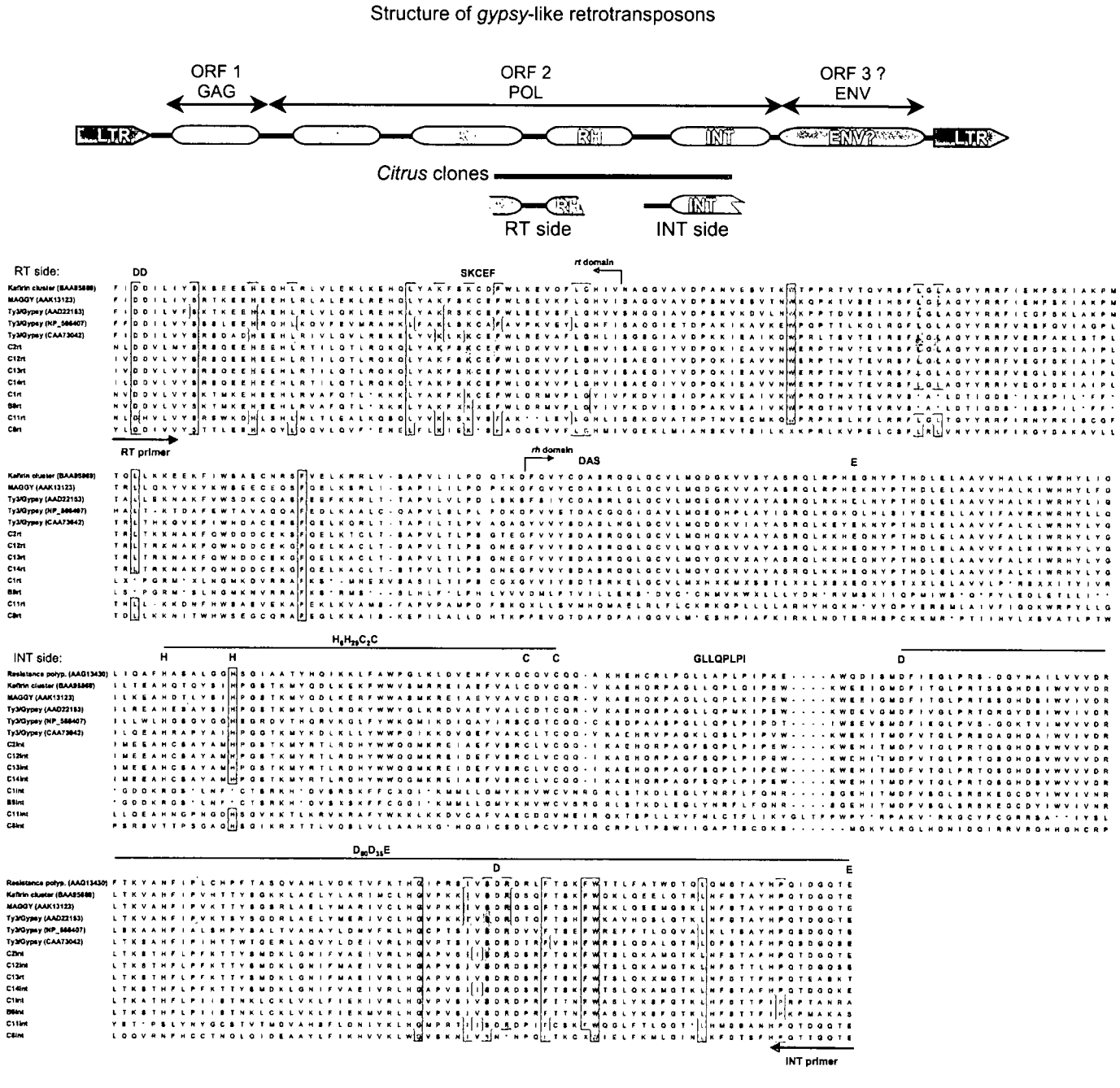


Figure 3.- Typical structure of *gypsy*-like retrotransposons and predicted translation products of *Citrus gypsy*-like sequences aligned with different proteins from NCBI databases. Gaps and stop codons are indicated as – and * respectively.

identical. A few differences arise when genomic DNA from *C. limon* is hybridized. The most distinct pattern corresponds to the farthest related species, *P. trifoliata*.

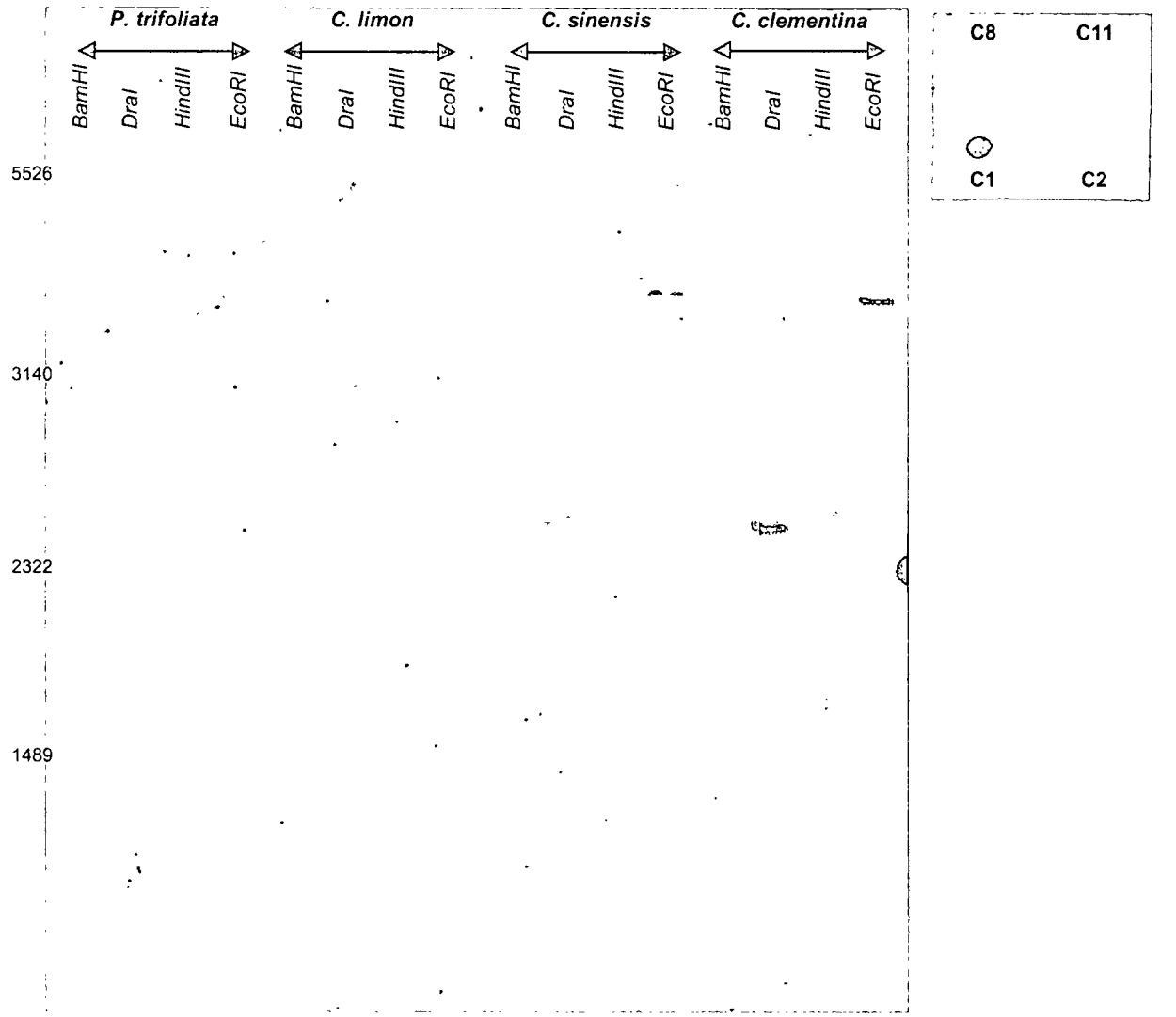


Figure 4.- Southern blot analysis of digested genomic DNA from different species probed with C1. Numbers to the left correspond to molecular weight markers (MWM IV, Roche) in base pairs. To the right, dot blot of clones also probed with C1 to check probe specificity.

IRAP markers based on these *gypsy*-like sequences (C1, C2, C8 and C11) were also developed (Figure 5). Not all IRAPs segregated as dominant markers (presence *versus* absence of band). Two different fragments segregate in *C. aurantium* for C11_410, C11_900, C2_800, C2_170 and C1_200 indicating they behave as codominant markers. In *Poncirus trifoliata*, only C2_800 behaves similarly. Bands at C2_800, segregating at both parental species, behave as alleles at the same locus. Four dominant IRAPs segregating at *C. aurantium* presented distorted segregation ratios: C11_510 (χ^2=5.3), C1_250 (χ^2=4.6), C11_875

(χ^2 =12.5) and C8_200 (χ^2=22.3). The direction of the distortion is towards absence of band for both C11 IRAPs while for the others the direction is towards their presence.

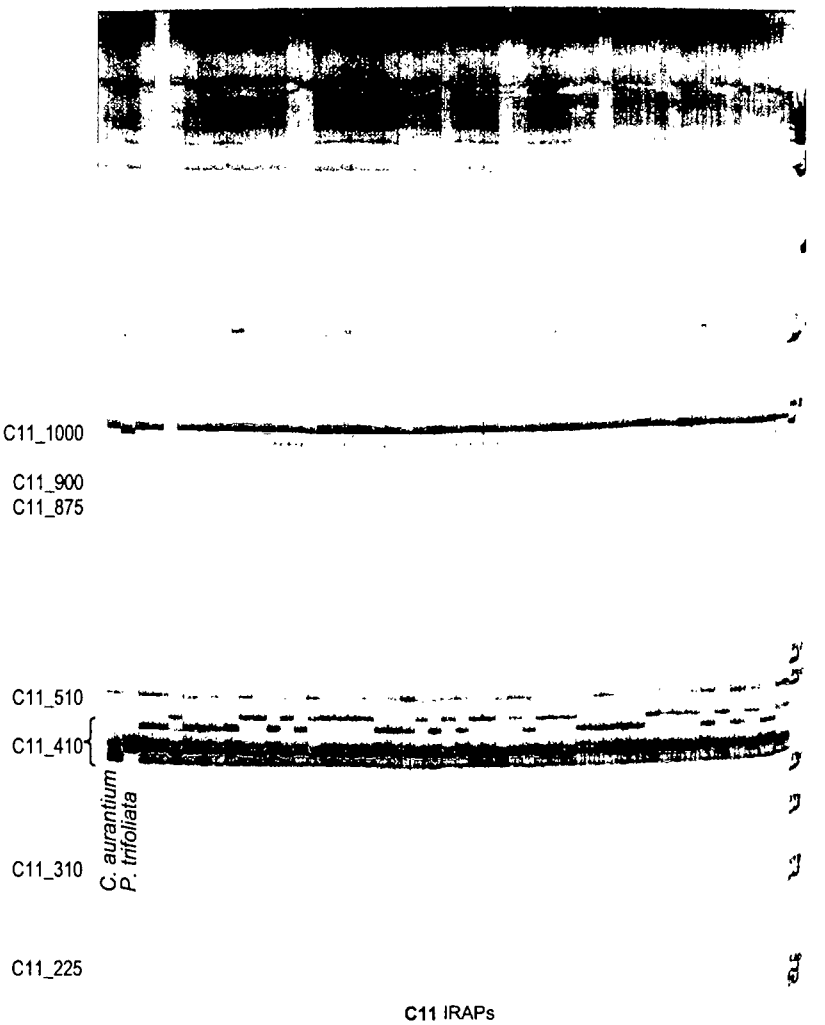


Figure 5.- IRAP banding patterns derived from the gypsy sequence C11 for hybrids belonging to AxPa family. Segregating IRAP loci are shown at the left side.

All IRAP markers were mapped using a progeny derived from the cross *C. aurantium* x *P. trifoliata* that had been already genotyped for 120 markers (Ruiz and Asins 2003). Linkage groups where these new IRAPs map are shown in Figure 6. Only five IRAPs were found to segregate in *P. trifoliata*, two of them cosegregate and do not join to any linkage group. On the other hand, all twenty IRAP markers segregating at *C. aurantium* gametes could be added to an existing linkage group, resulting in an extended and better-resolved linkage map. Most of the *gypsy* retrotransposon-based markers (15 out of 25) map without clustering to other markers, seven of them map at positions where ESTs are located (CR markers are microsetallites

derived from EST sequences) and only one (C11_1000) joins a cluster of *copia* retrotransposon-based markers. Therefore, IRAPs based on both types of retrotransposons seem to distribute differently throughout the genome providing a new, complementary set of molecular markers. Data related to the number of bands and heterozygosity percentage per sequence and parental species is presented on Table 1. Similarly to *copia* derived IRAPs, *P. trifoliata* presents lower heterozygosis percentages than *C. aurantium*. Noteworthy, average heterozygosity for *gypsy*-derived IRAPs was two fold greater than that obtained from *copia*-derived IRAPs.

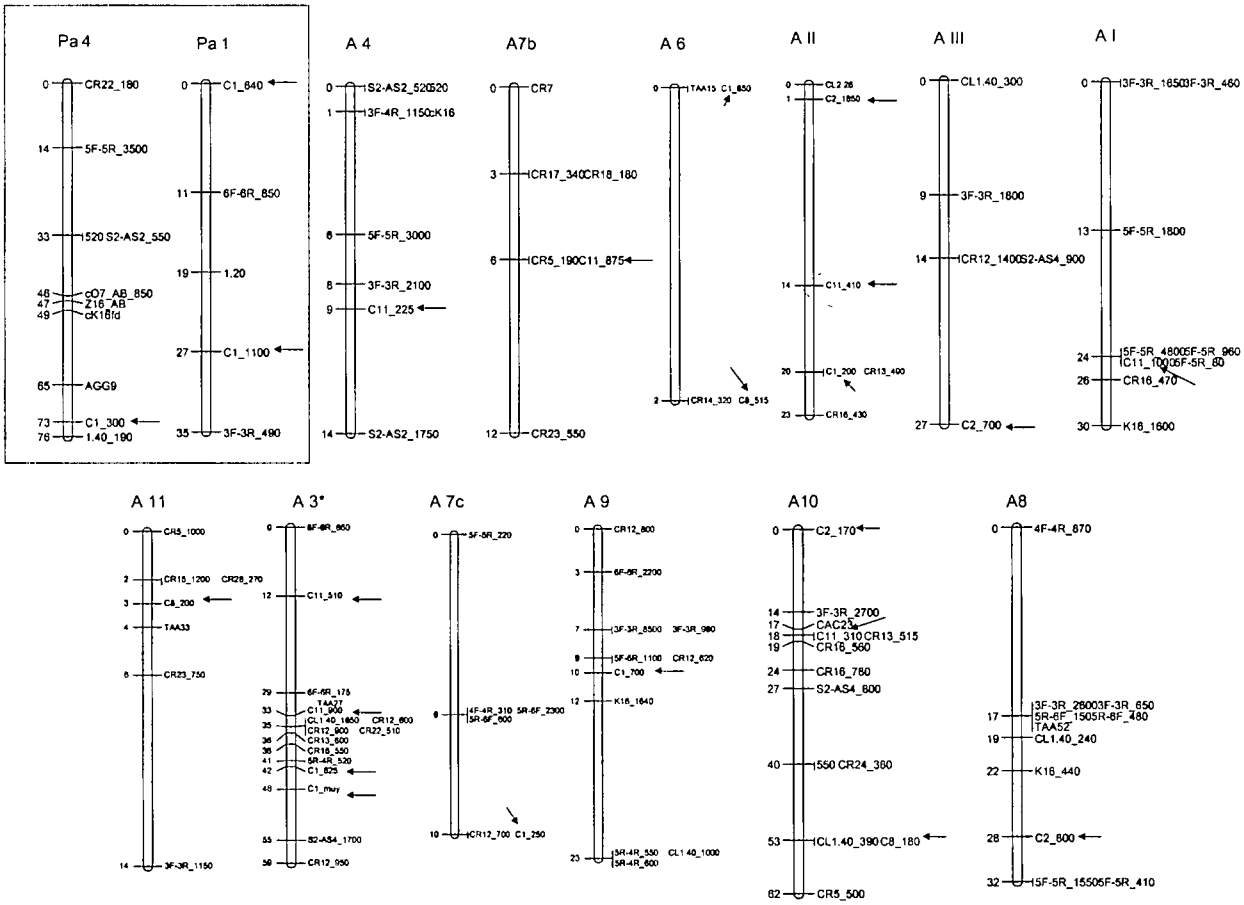


Figure 6.- Location of gypsy derived IRAPs on the *C. aurantium* and *P. trifoliata* maps (Ruiz and Asins 2003). Framed linkage groups belong to P. trifoliata map. Linkage groups are not scaled. Asterisk at linkage group A3 means that marker TAA27 is included but its position is not reliable.

Primers based on clones	Parental	Bands	Polymorphisms	Heterozygosity (%)	
C1	*C. aurantium*	13	8	61.5	52.4
	P. trifoliata	8	3	37.5	
C2	*C. aurantium*	5	5	100.0	75.0
	P. trifoliata	3	1	33.3	
C8	*C. aurantium*	3	3	100.0	66.7
	P. trifoliata	3	1	33.3	
C11	*C. aurantium*	12	9	75.0	60.0
	P. trifoliata	3	0	0.0	
Total for *gypsy*-like elements	*C. aurantium*	33	25	75.8	60.0
	P. trifoliata	17	5	29.4	
Total for *copia*-like elements	*C. aurantium*	74	33	44.6	30.8
	P. trifoliata	69	11	15.9	
Average over *Gypsy*-based IRAPs	*C. aurantium*	8.25	6.25		
	P. trifoliata	4.25	1.25		
Average over *Copia* -based IRAPs	*C. aurantium*	18.5	8.25		
	P. trifoliata	17.25	2.75		

Table 1.- Number of *gypsy* based amplification products (bands), number of (polymorphic) IRAP bands and heterozygosity values for each parent of the AxPa family. Heterozygosity has been estimated as the percentage of polymorphic bands. Data from *copia* derived IRAPs was reported by Ruiz and Asins (2003).

4.- Discussion

We have described the isolation, analysis and genomic location of eight representative sequences isolated from *C. clementina* that show homology to retroelements of the Ty3/*gypsy* group. Four of them (C2 group) might correspond to an active element since they carry no in frame stop codon nor frame-shift mutations.

Nucleotide sequence of the cloned fragments revealed high homology to diverse well-characterized plant *gypsy*-like retrotransposons (figures 2 and 3). The *in silica* predicted translation peptides were useful to detect a group of clones with no frame disruption and conserved motifs essential for autonomous retrotransposition activity (Figure 3). The sequences of the other group of clones carried several stop codons and frame-shift mutations suggesting that these elements, if functional, are non-autonomous. Copies with frame-shifts and in-frame stop codons within the POL coding region have been reported for several retroelements of the gypsy-like family (*Micropia*, Lankenau et al. 1988; *Cyclops*, Chavanne et al. 1998; *yoyo*, Zhou and Haymer 1998), and have been explained by the gradual accumulation of mutations during relatively long periods of silence after bursts of high transpositional activity (Chavanne et al. 1998). Since most isolated clones belong to the C2 group, a very homogeneous group, this could support the hypothesis that they have been amplified more recently than the others

(Feschotte et al. 2002). Therefore, the *Citrus clementina* genome seems to contain potentially active *gypsy*-like elements (the C2 group).

Cluster analysis of *C. clementina* gypsy-like sequences (Figure 2) show their heterogeneity. Although all of them were isolated from the same clementine variety, 3 main groups are distinguished: C8 related to the *Monkey gypsy*-like element from *Musa* (Balint-Kurti et al. 2000), C11 related to the *Reina* and *IFG7 gypsy*–like elements from maize and *Pinus radiata*, respectively, and the rest, the C2 group plus C1 and B9, related to the *gypsy*-like retrotransposons *CaRep* and *Dea1* (Thomsom et al. 1998) from *Cicer* and *Ananas*, respectively. Given that *Reina, Dea1* and *IFG7* are *gypsy*-like plant elements lacking *env* (envelope) domain (Vicient et al. 2001) it could be assumed that our clementine elements lack it too, in spite of the indirect evidences contributed by these authors supporting the presence of such domain in *C. sinensis* elements.

Surprisingly, the sequences of the C2 group, C1 and B9 also showed high homology to a resistance-gene cluster in potato that confers resistance to a virus and a nematode (van der Vossen et al. 2000). Detailed analysis of this cluster showed conserved domains such as those corresponding to the GAG protein, reverse transcriptase and integrase core domains in the proper frames and order to deduce the presence of a *gypsy*-like retroelement between resistance genes. Recently, the complete sequence of the *Poncirus trifoliata* citrus tristeza virus resistance gene locus (282,699 nucleotides) has become available (AF 506028). This contig contains several putative disease-resistance genes similar to the rice *Xa21* gene, the tomato *Cf-2* gene and the *Arabidopsis thaliana Rps2* gene (Yang et al. 2001). Between resistance genes, it also contains conserved domains corresponding to 4 gypsy-like elements; the sequence of two of them presents high homology (over 80 %) to our C2 clone, another presents 79 % homology to our C8 clone and the third one is not related to ours. Not as easy to explain is the case of two proteins directly submitted to NCBI databases (without reference paper) and described as "putative plant disease resistance polyprotein" from *Oryza sativa* (database accessions AAM51835 and AAG13430). Both sequences showed high similarities to the putative translations of our *Citrus* fragments and the analysis of polyprotein sequences yielded regions with strong homologies to conserved (total or partial) *gypsy* domains such as the integrase core domain and the CHROMO (chromatin organization modifier) domain (Malik and Eickbush, 1999).

Many transposable elements have been found at resistance-gene clusters (Richter and Ronald 2000; Wicker et al. 2001). Members of a resistance gene family are often arranged as tandem direct repeat, which is consistent with their origin through gene duplication and their continued evolution through unequal exchange. In plants, it has long been hypothesized that transposable elements play a role in the reconstruction of genomes in response to

environmental stresses such as tissue culture, irradiation or pathogen infection (McClintock 1984; Wessler et al. 1995). Transposable element insertion into and excision from regulatory and coding regions can change the coding capacity and expression patterns of the gene (Marionette and Wessler 1997; McDonald 1995; Wessler et al. 1995). Additionally, movement of transposable elements may result in further allelic diversity, either by disrupting genes, or by influencing recombination or chromosomal rearrangements. Under the gene-for-gene model proposed by Flor (1956) plant and pathogen genes involved in the interaction are subject to different evolutionary forces. Since virulence is recessive, a simple loss-of-function mutation in the avirulence gene of the pathogen allows it to become virulent on the host, while the plant must gain a new resistance function to counter new pathogen biotypes or species (Richter and Ronald 2000). Therefore, the presence of factors that increase allelic diversity at resistance genes must be selectively advantageous for the plant. In support of this hypothesis, studies of the maize resistance locus *rp1* revealed that recombination of flanking markers was associated with the creation of novel resistance phenotypes (Richter et al. 1995).

As suggested by Fedoroff (2000), a genetic mechanism, in addition to polyploidization, transposition and duplication, that drive plant genome expansion might be preferential transmission of tandemly repeated sequences (including retrolements) through the gametes. Our data on the direction of the distortions does not support this hypothesis since only half the number of IRAP with distorted segration ratios showed a bias towards the presence of band.

Polymorphisms based on C2 sequence are proportionally the most abundant. This makes heterozygosity percentage reach the highest value in *C. aurantium* for IRAPs based on this element. Addionally, it shows the highest proportion of codominant IRAPs. Most IRAPs in *Citrus* are dominant, i.e. segregation corresponds to presence versus absence of band (Ruiz and Asíns 2003). An IRAP is codominant when the amplified inter-retrotransposon fragments from both homologous chromosomes in an heterozygote differ in size or sequence. Since the allelic differentiation (mutation ratio) should be slower for non-methylated (potentially active) retrotransposons than for methylated (inactivated) retrotransposons, the high proportion of codominant C2-IRAPs might be considered as another indirect evidence reinforcing the hypothesis that C2 sequence correspond to an active (not methylated in certain genomic positions, at least) *gypsy*-like retrotransposon.

Both southern hybridization analysis and mapping of *gypsy*-based IRAPs indicate that nested copies of these elements are scattered along the *Citrus* and *Poncirus* genomes. It is also clear that these elements were introduced before the divergence of both genera and evolved separately thereafter. All IRAPs map in different positions when linkage maps of both genomes are compared. There is only a doubt, C2_800 segregating bands from *Poncirus* and *C. aurantium* correspond to the same locus. It maps at A8 in *C. arantium* map, while it does not

join any linkage group at the *Poncirus* map; it is just closely linked, in repulsion phase, to C8_850, another IRAP, both of them might belong to a putative Pa8 group, homeologous to A8.

Southern hybridization analysis and mapping IRAPs based on both *copia*- (Asíns et al. 1999; Ruiz and Asíns 2003) and *gypsy*-like retrotransposons also show that they are more abundant and polymorphic through the *Citrus* genome than through the *Poncirus* genome. If the thesis that the mechanisms that control transposition are a reflection of the more general capacity of eukariotic organisms to detect, mark and retain duplicated DNA through repressive chromatin structures (Fedoroff 2000) is true, then we could say that this control capacity is greater in *Poncirus trifoliata* than in *Citrus aurantium*. Another non-excluding possibility is related to their reproductive system. Hickey (1982) predicted that loss of sex would result in a population free of transposable elements by preventing their spread. Recently, Arkhipova and Meselson (2000) have reported results on the presence of *LINE*-like and *gypsy*-like retrotransposons in sexual and ancient asexual taxa supporting Hickey´s thesis. In our case, both species are highly apomictic. Nevertheless, it could be said that the degree of apomixis evaluated as a function of the mean percentage of zygotic (sexual) seedlings (Asíns et al. 2002) is higher for *P. trifoliata* than for *C. aurantium*. It is important to point out again that all *gypsy*-like retrotransposons we have isolated come from one of the few sexual species in *Citrus, C. clementina*. Clementines are much closer related to *C. aurantium* than to *Poncirus trifoliata* and they were mostly grafted on *C. aurantium* trees until a few decades ago. Therefore, the abundance of retrotransposons in *Citrus* could be due not only to a more recent common sexual ancestor (vertical transmission) but also to horizontal transmission, or even infection of the rootstock by the grafted variety given that, at least, some *Citrus gypsy*-like retroelements might bear not only active domains for retrotransposition but also ENV-like domains, conferring infective capability, as Vicient et al (2001) indirectly deduced. Concerning horizontal transmission, a *gypsy*-like retrotransposon named *yoyo* has been isolated from the Mediterranean fruit fly, *Ceratitis capitata* (Zhou and Haymer 1998) but it shows no significant homology to any of the clementine *gypsy*-like elements.

In conclusion, a largely heterogenous set of *gypsy*-like elements has been isolated from the clementine cultivar 'Loretina' that has recently originated as the product of somatic natural mutation. Since *gypsy*-based IRAPs present a different genomic distribution compared to that of *copia*-based IRAPs, they constitute a new, complementary set of molecular markers that are available to study and follow the variation of agronomic traits in segregant progenies derived from *Citrus*. Among these traits, their application to locate disease resistance gene clusters seems specially promising.

.....Expressed sequence enrichment for candidate gene analysis of Citrus Tristeza Virus resistance

Chapter II

1.- Introduction

Citrus Tristeza Virus (CTV) is one of the most important pathogens affecting citrus. There is a wide range of CTV isolates. Some isolates are very mild and cause little damage. Others may cause a severe decline of trees grafted on sour orange (*Citrus aurantium*). Some isolates also cause a serious stem-pitting disease in limes (*C. aurantifolia*), grapefruit (*C. paradisi*), and sweet orange (*C. sinensis*) that limits their commercial production (Garnsey 1999).

CTV is a member of the genus *Closterovirus;* it has a genome of 19.2 Kb, which is the largest among RNA plant viruses (Karasev et al 1995). CTV probably originated in Asia, which is also the centre of origin of Citrus, and has been disseminated to many countries by movement of infected plant material. Subsequent spread by aphid vectors has created major epidemics. RNA viruses are extremely adaptable and are capable of rapid change. There are several factors contributing to the great variability of CTV: RNA polymerase errors in replication building up large viral populations, recombination and the fact of being hosted by a perennial crop where multiple CTV genotypes (by repeated aphid inoculations) and other viruses may evolve together for long time increasing the probabilities of reassorment (Roosnick 1997). On the other hand, the vector acts as an evolutionary bottleneck (Karasev 2000).

Most citrus species are hosts of CTV. Up to now, The citrus and related genotypes where CTV resistance has been found are: the trifoliate orange (*Poncirus trifoliata*) (Yoshida et al. 1983), the Meiwa kumquat (*Fortunella crassifolia*) (Mestre et al 1997c) and the pummelo 'Chandler' (*C. grandis*) (Fang and Roose 1999). All cultivars of *P. trifoliata* tested have been found resistant to most isolates (Mestre et al. 1997c); however a New Zealander CTV isolate has been reported to break such horizontal resistance (Dawson and Mooney 2000). The evolutionary diversification of CTV and its quicker spread by efficient vectors such as *Toxoptera citricida* (Garnsey 1999) force farmers to change sour orange as rootstock for other rootstocks sometimes not very well adapted to local areas. The number of rootstock cultivars that are now available from breeding programs is very low and trifoliate orange is the only source of CTV resistance used up to now. Citrus breeding programs takes long time due to the long juvenility period of these species, and are very expensive because the long time needed and the huge cultivation costs for maintaining and evaluating the large segregating progenies needed. To alleviate such limitations, marker assisted selection within the progenies and genetic transformation of outstanding cultivars to introduce resistance genes are valuable tools. There are several groups pursuing the isolation of *Ctv-R* by chromosome walking (Yang et al. 2001, Deng et al. 2001). A first step towards obtaining those tools is the genetic dissection and mapping of the resistance gene(s). Several studies have reported markers linked to a putative resistance gene in *P. trifoliata,* named *Ctv-R* (Gmitter et al. 1996, Mestre et al. 1997a, Fang et

al. 1998). Fang et al. (1998) reported a marker that cosegregated with the resistance gene but later on, the same group (Yang et al. 2001) reported it was not so due to some scoring errors. In addition to scoring errors, the estimated genetic distance depends on the family, the software for linkage analysis and how CTV resistance is defined (Mestre et al. 1997a). There are also other features that might be relevant: is CTV resistance a monogenic trait? Mestre et al. (1997b) presented evidences supporting the involvement of more than one gene. How the time after inoculation affects the classification of plants into resistant and susceptible phenotypes? Would *Ctv-R* be effective within any genetic background, including sour orange, the well-adapted rootstock to arid and semi-arid regions?

Resistance genes controlling extreme resistance are thought to be constitutively expressed and usually are distributed in clusters along of the genome (Hammond-Kosack and Jones 1997; Grube et al. 2000). Besides, some of these gene clusters are involved in the resistance to very different types of pathogens (Van der Vossen et al. 2000). Therefore, whenever a region is known to contain a resistance gene it would be very useful to enrich this region with markers involving not only anonymous but mainly expressed sequences (localized expressed sequences, LES). Methodologies for LES enrichment would be also important at locations where QTL of large effect map. Selection of candidate genes by QTL analysis might be a useful strategy to connect research on functional genomics with the metabolic pathways of traits (Asins 2002). However, for most traits biochemical information on metabolic pathways is extremely limited and so, one of the main limitations of candidate gene analysis is the low number of genes to be used as candidates.

The objectives of this work are: 1) the enrichment of the region containing *Ctv-R* with anonymous markers and localized expressed sequences by using two new strategies that combine bulk segregant analysis and differential display analysis or the construction of a cDNA subtraction library; 2) Comparison between the new linkage maps of sour orange and *P. trifoliata*; and 3) to fine map *Ctv-R* under the hypothesis of monogenic inheritance using a progeny of citradias derived from the cross between sour orange and *P. trifoliata* for which the virus presence has been followed for more than 2 years after inoculation.

2.- Materials and methods

2.1.- Plants and segregating populations

Two segregating populations were used for mapping. One was derived from the self-pollination of *P. trifoliata* (L.) Raf. var 'Flying Dragon' (PpxPp) and had been previously used for mapping *Ctv-R* by Mestre et al. (1997a). It consists of 66 plants. The other segregating population (104 AxPa hybrids) derives from the cross between *C. aurantium* L. var 'Afin Verna'

(A) and the trifoliate orange 'Flying Dragon' (FD). A sample of 63 hybrids had been used previously to build up a map for *C. aurantium* and another for FD (Ruiz and Asins 2003).

Enrichment strategies based on pools of resistant and susceptible plants consisted of 7 individuals of each type (*RR* versus *rr*) from the PpxPp population, homozygotes for the region between two RFLP markers (cG18 and cK16) at Pp4 linkage group.

Presence of *Ctv-R* was inferred from the presence of FD alleles at pY65 and pY28 marker loci (Yang et al. 2001). To do this, specific primers were designed to develop the corresponding SCAR markers (see Figure 4).

2.2.- Evaluation of CTV resistance and accumulation

Data on CTV resistance of PpxPp population was taken from Mestre et al. (1997a and 1977b).

Before inoculation, AxPa hybrids and parents (sour orange and FD) were propagated on sweet orange rootstocks. Three months after this propagation, the rootstocks were inoculated by grafting patches of infected sweet orange. CTV isolate was T-346, a common Spanish isolate kept at the bank of CTV isolates at IVIA.

A first lot of 66 AxPa hybrids was inoculated at the beginning of 1999 and the rest, two years later. Control and inoculated plant were grown in a greenhouse with temperature control (25 ± 10° C). The presence of the virus was checked every 6 months at the shoots and at the inocula, as a positive control of each challenged plant, by Direct Tissue Blot Immuno-Assay (DTBIA) following the procedure described in Garnsey et al. (1993) (Figure 1). CTV accumulation was also evaluated by a semiquantitative a double-antibody sandwich ELISA (DAS-ELISA) using monoclonal antibodies 3CA5 and 3DFI together as described in Cambra et al. (1993).

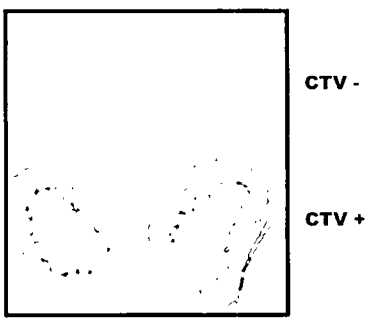


Figure 1.- Direct tissue blot immuno-assays of susceptible (CTV+) and resistant (CTV-) plants.

At spring of both 2001 and 2002, at least 4 branches of each inoculated plant were analyzed by DTBIA and DAS-ELISA. Those hybrids where the virus was detected in at least one branch but not in others were declared susceptible with an irregular distribution of the virus.

Following Mestre et al (1997a) two different criteria were used to classify plants into susceptible and resistant classes: a plant was considered susceptible (a) when the virus was detected at least once along the experiment and (b) when the virus was detected at the end of the experiment (year 2002). Therefore, some few resistant plants for criterion b might be considered susceptible for criterion (a).

2.3.- Enrichment strategies and isolation of candidate resistance genes

DNA from the parents and members of AxPa family including CTV resistant and susceptible trees was extracted according to Dellaporta et al. (1983) with minor modifications. Total RNA was isolated from shoots or leaves of all plant materials using the MPG Total RNA Isolation Kit (CPG inc.). For accurate mapping, those amplification products that were of interest were extracted from gels, eluted in sterile water and reamplified to verify its presence and purity. Purified PCR products were then cloned into the pGEM-T Easy vector system (Promega) and sequenced.

Five methodologies to enrich *Citrus* and *Poncirus* linkage groups 4 with markers and LES (Table 1) were followed using bulk segregant analysis (Michelmore et al 1991):

1.- Primers of random sequence: Operon primers O7, A15, D7, G9, G19, K16, O10, W16, B10, O16, I1, E4 and Z16. ISSR primers 840 $(GA)_8YT$, 835 $(AG)_8YC$ and 857 $(AC)_8YG$ were also used and correspond to USB set # 9 from the Biothechnology Laboratory, University British Columbia, Vancouver, British Columbia (Canada). At least one of the corresponding PCR products was specific of the *RR* or *rr* DNA pools. PCR reactions, electophoresis and staining conditions are described in Bretó et al. (2001). Segregating bands were mapped in the PpxPp progeny.

2.- Degenerated primers described by Mago et al (1999) that were designed on the basis of nucleotide-binding-site (NBS) motifs conserved among resistance genes. Amplified fragments were mapped previously by Ruiz and Asins (2003). One of them showing linkage to *Ctv-R* and others of similar size were cloned and sequenced for accurate mapping. Electrophoresis and staining conditions were as mentioned before. Segregating bands were mapped in the PpxPp and AxPa progenies.

3.- RAPDs using degenerated primers designed from conserved regions of known resistance genes; CTVA: RAGAYRAGCATTGNTA and CTVB: WTNCTTTGCAAAATRH. These primers were used separately as primers of random sequence. Segregating bands were mapped in the PpxPp progeny.

4.- LESs that correspond to differentially expressed sequences obtained through differential display analysis (Hieroglyph mRNA profile kit, Genomyx corporation) of RNA pools from *RR* vs. *rr* plants. In brief, first strand cDNA synthesis from RNA samples was accomplished with different 3' oligo(dT) anchored primers (AP1 to AP12) and then double-stranded cDNA fragments labeled with α^{33}P-dATP were synthesized by pairwise combinations of AP and 5' arbitrary primers (ARP). As arbitrary primers we used from ARP5 to ARP8, primers Z16, CTVA CTVB, K16 O7, 857 and primers derived from cloned bands that map closely to *Ctv-R* in PpxPp family (cK16, cZ16 and D857). Resulting fragments were electrophoresed using the Genomix LR sequencer (Genomix corporation) in a 200 micron thick denaturing 5% polyacrylamide - 7M urea gel. After a 40°C, 800V, 14hr electrophoresis, gels were rinsed, dried and exposed to Biomax MR film (Kodak) for two days at room temperature. Bands differentially present in one of the two pools analyzed were recovered from gels and PCR-reamplified. In most cases, an additional purifying step (10% polyacrylamide gel electrophoresis and band excision) was necessary to isolate the desired fragment.

5.- LESs from the construction of a cDNA subtraction library using RNAm pools from *RR* vs. *rr* plants (PCR-select cDNA subtraction kit, Clontech laboratories). RNAm pools from 5 resistant (*RR*) and 5 susceptible (*rr*) plants of the PpxPp family were used for tester and driver cDNA preparation respectively. Poly A^+ RNA was extracted from five CTV resistant (*RR*) and five CTV susceptible (*rr*) members of the PpxPp family. These RNA samples were pooled separately and SMART PCR cDNA Synthesis Kit (Clontech) was used for cDNA synthesis in order to avoid excess of ribosomal RNA and low concentration of cDNA corresponding to the poly A^+ fraction which results in inefficient subtractive hybridization. Once ds cDNAs were obtained, column chromatography, RsaI digestion and purification of digested cDNAs were done according to the manufacturer's instructions resulting in cDNAs suitable for subtractive hybridization with PCR-Select cDNA subtraction kit (Clontech). Subtracted cDNA library was established by cloning the resulting subtracted mixture into the pGEM-T Easy vector system (Promega). A total of 135 clones were randomly chosen, PCR amplified with universal primers and blotted onto two identical nylon membranes (Hybond-N+, Amersham pharmacia biotech). For 54 clones, two replicates were blotted on both membranes. Total cDNA from resistant and susceptible pools was then digoxigenin labeled using the DIG DNA labeling kit (Roche) and hybridized to the filters separatedly. The blots were pre-hybridized for 2h and hybridized for 14h in 1% blocking reagent, 0.1% N-Lauroylsarcosine, 0.2% SDS and 5x SSPE at 68°C. Filters were finally washed twice in 2x SSPE, 0.1% SDS for 10 min at 68°C and twice again in 0.5x SSPE, 0.1% SDS for 10 min at 68°C. The hybridization signals were subsequently detected with CSPD chemiluminescent substrate (Roche). 38 clones including all the combinations of hybridization signals for both filters (++, +-, -+, --) were selected for sequencing. According to

their abundance in the subtracted library and sequence homologies, 10 sequences were finally chosen for specific primer designing and mapping in the AxPa family. In some cases where no heterozygosis at the parents could be detected, the SCAR bands corresponding to both resistant and susceptible individuals was excised from the gel, purified and sequenced to search for single nucleotide polymorphisms.

The AxPa hybrids a110 and a19 presented 'vein corking' (Figure 2) in less than 3 months after inoculation but not when they were just propagated on sweet orange. RNA from a110 mother plant, a101 propagated on sweet orange but not inoculated and a110 propagated on sweet orange and inoculated (showing 'vein conking' and 'stunting' symptoms) were used separately for differential display analysis. Methodology was as described in strategy 4.

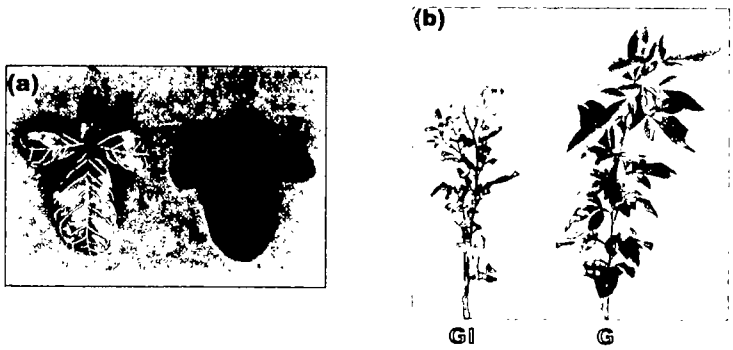


Figure 2.- 'Vein corking' (a) and 'stunting' (b) symptoms of some inoculated resistant hybrids. GI: inoculated, propagated plant, G: non-inoculated, propagated plant.

JOINMAP 3.0 (Van Ooijen and Voorrips 2001) with a linkage criterion above LOD 4, recombination fraction of 0.5 and Kosambi mapping function was used for linkage. The PpxPp population was analyzed as "self pollination" population type and the AxPa population as a "cross pollinator" population type with no previous knowledge of the linkage phase of the markers. The nomenclature used for linkage groups is described in Ruiz and Asins (2003). Some inter-retrotranposon amplified polymorphisms (IRAP) derived from gypsy-like retrotransposons sequences (C1, C2, C8, C11) had been also included in the map (Bernet and Asins, submited).

3.- Results

3.1.- Enrichment strategies

Five strategies have been followed to enrich linkage group 4 of *P. trifoliata* (where *Ctv-R* is located) with new markers and expressed sequences: "random" DNA markers, analogues of

resistance and cDNA clones from differential display analysis and a subtraction library between *RR* and *rr* plants (Table 1). Most problems encountered using the differential display strategy were related to the cloning of differential bands while in the case of the subtraction library, searching for polymorphism (heterozygosis) at the parents for the cDNA clones was the limiting step. The most profitable enrichment strategies were those based on primers for analogues of resistance by Mago et al (1999) and the new one to get LES from the subtraction library.

Code	Strategy	Population	Marker/genes at LG 4
1	RAPD, ISSR	PpxPp	13/59
2	Mago et al., 1999	AxPa	3 out of 6
3	CTV primers	PpxPp	4/23
4	Differential display	PpxPp	2/3 (12)
5	CDNA subtraction library	PpxPp	3/6 (10)

Table 1.- Marker types used to saturate linkage group 4 (LG4). Selection of bands was carried out by comparing *RR* versus *rr* pools of individuals. Between brackets, number of differential bands at strategy 4 and initially selected clones at strategy 5.

Each DNA sequence, or its translation into amino acid sequence, was used to search for orthologous genes at the NCBI (Tables 2, 3 and 4) and some might correspond to genes involved in the plant defence response. Three bands of similar size, two specific of *P. trifoliala* (S2AS2_550 and S2AS2_519) and one specific of *C. aurantium* (S2AS2_520) were cloned. No sequence of significant similarity was found using the sequence of S2AS2_550 as query sequence however, the sequence of the others (S2AS2_519 and S2AS2_520) were highly similar to NBS-LRR type disease resistance gene analogues of apple tree (AAM77246, 5e-34), *P. trifoliata* (CTV.4 AAN62348, 3e-33; CTV.11 AAN62352 8e-29; CTV.17 AAN62353, 6e-28) and *C. grandis x P. trifoliata* (Pt8 AAN08176, 3e-30; 16R1-19 AAN08160, 5e-27; 16R1-13 AAN08159, 3e-26, Pt11 AAN08178, 3e-26; Pt9 AAN08177, 4e-26).

Clone	Specific of	D.D. primers	Homologues – E value	LG
ANER	Inoculated	AP4-ARP5	Wound-induced protein (WIN1) CAA31851 (*Solanum tuberosum*) - 3e-09 Pathogen and wound-inducible antifungal protein CBP20 precursor AAB29959—4e-09	Pa,un
NER	Inoculated	AP1-ARP7	Suppressor of Sulfoxyde Ethionine resistance NP_009333 (*Saccharomyces cerevisiae*) - 1.0	np
B	Not inoculated	AP4-ARP5	No significant similarity found	-
2-1	Mother plant	AP1-ARP7	No significant similarity found	-

Table 2.- Cloned bands from differential display analysis of citradia a101 mother plant (not grafted) and propagations on sweet orange that were inoculated ('vein corking' and 'stunting' symptoms) or not. LG: linkage group; un: unlinked; np: non-polymorphic.

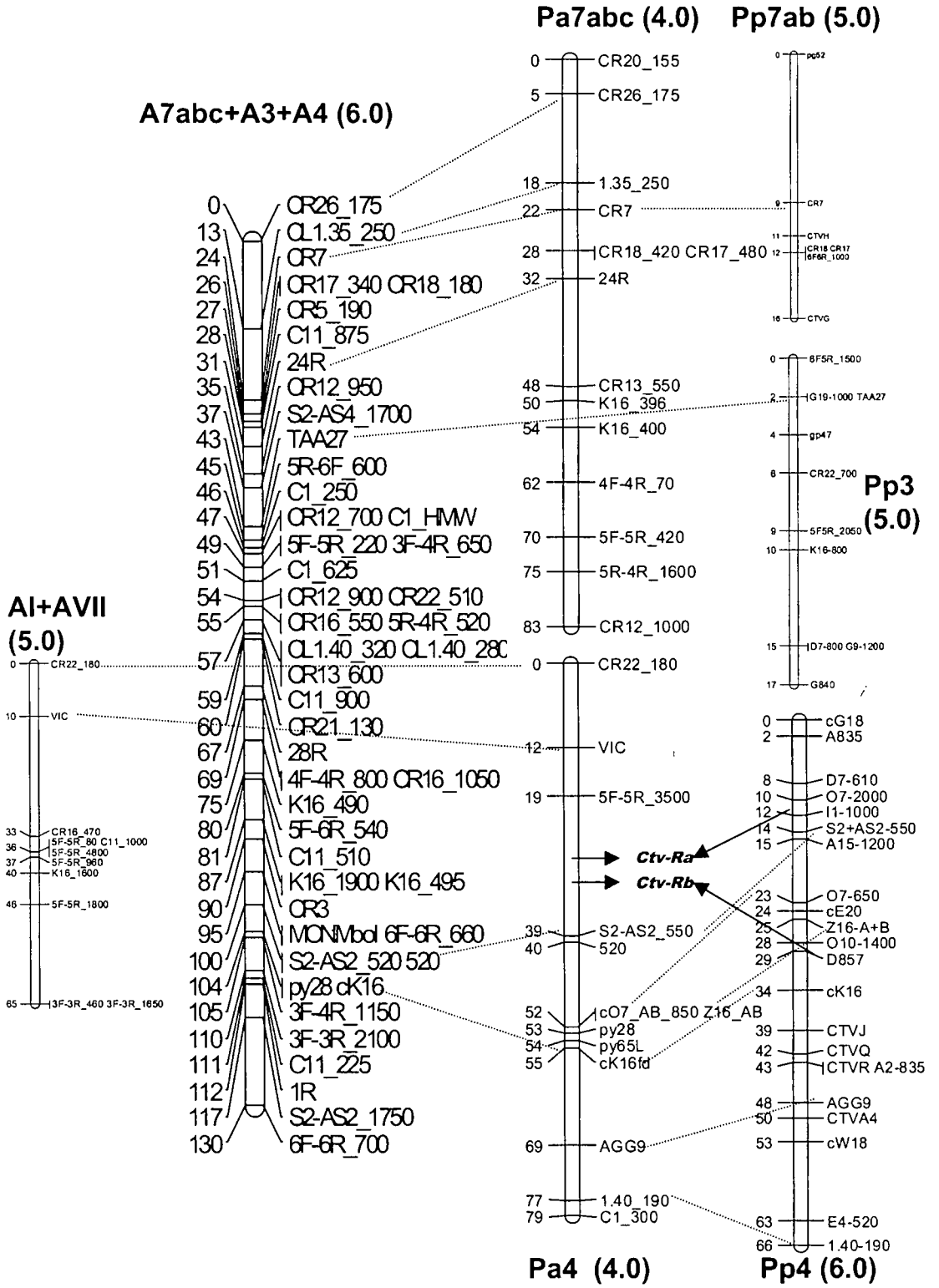
Clone	Specific of	Primers	Homologues – E value	LG
VIC	rr	AP10-**DO857**	Hypothetical protein NP_179106 (*Arabidopsis thaliana*) - 8e-35	Pa4, A(I+VII)
MON	RR	AP11-**DO857**	Polygalacturonase NP_194113 (*A. thaliana*) - 4e-27	A(7+3+4)
SCAB	rr	AP3-**ck16A**	SCARECROW gene regulator-like NP_199626 (*A. thaliana*) - 6e-16	Pa5b, A(10+5b)
WIL	rr	AP11-**UO857**	Phosphate/PEP translocator protein-like NP_196201(*A. thaliana*) - 6e-11	np

Table 3.- Cloned bands from differential display analysis of *RR* versus *rr* pools from PpxPp segregating plants (enrichment strategy 4). Primers derived from cloned markers closely linked to *Ctv-R* in PpxPp family are indicated in bold.

Clone	N° clones	Homologies - E value	LG	Dot-blot
1R	3 (818)	Actin T51182 (*Malva pusilla*) - e-113	A(7+3+4)	*rr>RR*
15R*	1 (1400)	Calcium/proton exchanger CAX1-like protein AAF91349 (*A.thaliana*) - 3e-27	A11	*RR>rr*
24R*	1 (450)	Putative GDSL-motif lipase/acylhydrolase AAM64916 (*A. thaliana*) - 1e-60	Pa7, A(7+3+4)	*RR>rr*
25RL	2 (238)	Lipid transfer protein NP_188456 (*A. thaliana*) - 2e-12	A2	*RR*
27R*	1 (300)	Putative protein AAM97138 (*A. thaliana*) - 9e-33	A11	ns
28R*	1 (379)	No significant similarity found	Pa un, A(7+3+4)	*RR=rr*
4R	4 (373)	Glutamate decarboxylase isozyme 4 AAK38667 (*Nicotiana tabacum*) - 4e-58	np	*rr*
6R	3 (168)	bis(5'-adenosyl)-triphosphatase-like; fHIT NP_200632 (*A. thaliana*) - 7e-18	np	*rr*
18R*	1 (545)	Tubulin alpha-6 chain (TUA6) NP_193232 (*A. thaliana*) - 3e-81	np	ns
22R	1 (316)	Disease resistance protein (TIR-NBS-LRR) NP_199438 (*A. thaliana*) - 0.22	np	*RR>rr*

Table 4.- Initially selected cDNA clones from the subtraction library for mapping analysis (enrichment strategy 5). LG: Linkage group. An asterisk indicates the presence of introns. Number of clones with the same sequence is also indicated. Between brackets the size in base pairs. np: not polymorphic. Last column summarizes results on dot blot analysis using cDNA probes from RR and rr pools. *RR* stands for hybridization signal only with *RR* probe. *RR>rr* means brighter signal with *RR* than with *rr* probe, and so on. ns: not signal.

The marker and expressed sequence (ES) enrichment of linkage group 4 resulted in the fusion of several *Citrus aurantium* linkage groups. Thus, previous groups A7abc, A3 and A4 joins at LOD 6 in the new linkage map (Figure 3). Marker correspondences with *P. trifoliata* "Flying Dragon" maps from AxPa and PpxPp families are also shown in this figure. Unfortunately, the high density of markers at A(7abc+3+4) might make gene ordering not very reliable in some regions.


Pa7abc (4.0)
Pp7ab (5.0)
A7abc+A3+A4 (6.0)
AI+AVII (5.0)
Pp3 (5.0)
Ctv-Ra
Ctv-Rb
Pa4 (4.0)
Pp4 (6.0)

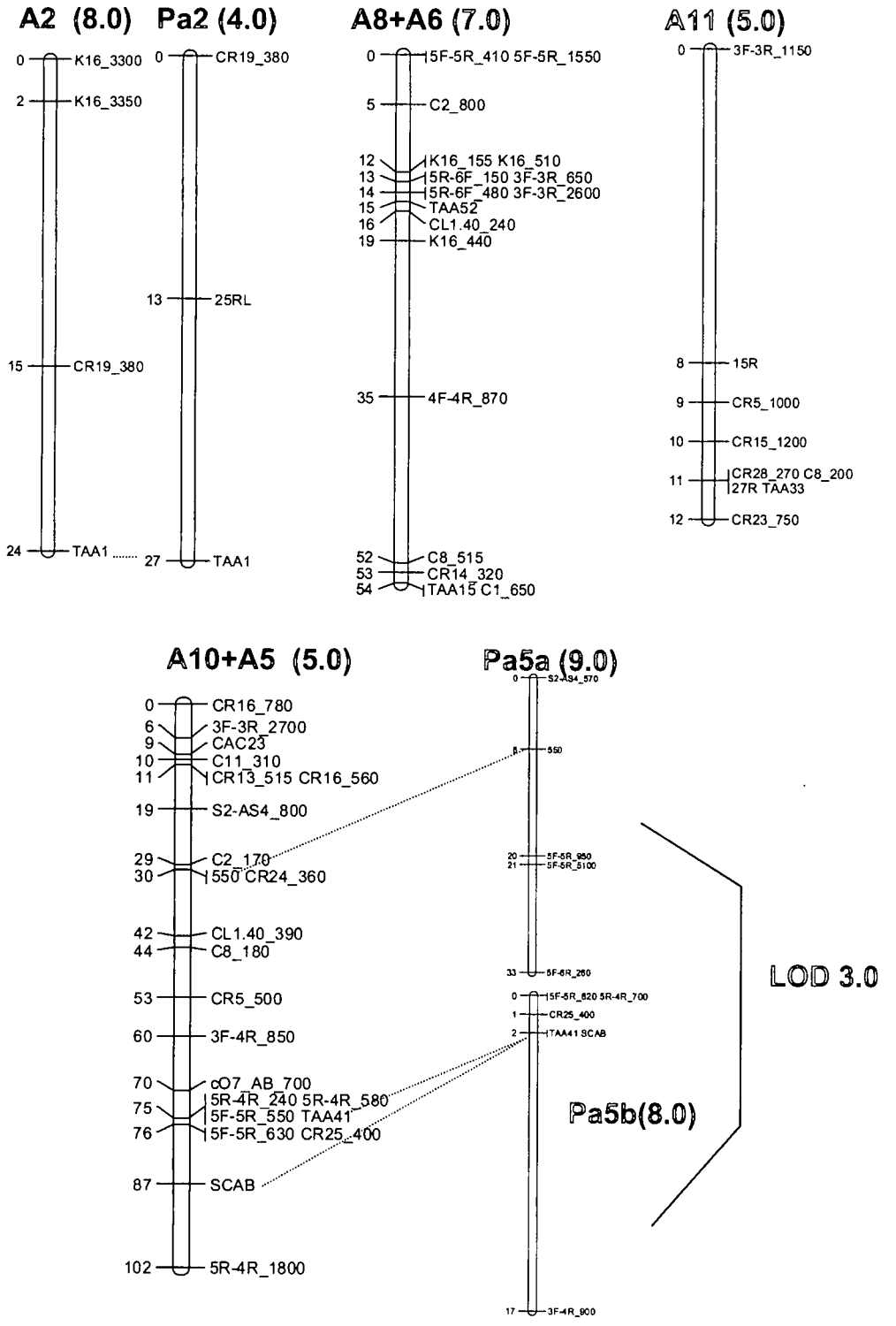


Figure 4.- Enriched linkage groups from AxPa and PpxPp families. LOD scores are indicated between brackets for each linkage group. Marker and linkage group nomenclature follow that reported by Ruiz and Asins (2003). Location of Ctv-R following criterion (a) or (b) is indicated at both Pp4 (from PpxPp family) and Pa4 (from AxPa family).

Although the corresponding homeologous *P. trifoliata* linkage groups do not join in a single one, the order of common markers is quite conserved except for CR22_180 and VIC that are in other *C. aurantium* linkage group (AI+AVII).

3.2.- Evaluation and mapping of CTV resistance

Seven AxPa plants (7.28 %) died before the first CTV evaluation. DTBIA evaluation agreed with DAS-ELISA data, in general. Only one hybrid showed low DAS-ELISA means but the presence of the virus had been detected by DTBIA. This plant also showed irregular distribution of CTV among branches, which would explain the disagreement.

Both parental genotypes and 64 AxPa hybrids were resistant; i.e. CTV was not detected at the end of the fourth/second year of evaluation. Eight of them were evaluated as susceptible (by both methods) three/six months after inoculation but later on became resistant, four of which do not carry *Ctv-R* as inferred by pY65 and pY28 markers (Figure 4). This putative resistance gene was not present in 15 resistant hybrids.

Thirty-three AxPa hybrids were susceptible. In five of these plants, the virus was not detected 3/6 months after inoculation but later on they became susceptible although the distribution of the virus was irregular within each of these plants. Heterogeneous distribution of CTV was present in 23 out of the 33 susceptible hybrids. Three of them carry *Ctv-R* as inferred by pY65 and pY28 markers. Mortality along the experiment, after inoculation, was higher in the group of resistant hybrids (13), especially those without *Ctv-R,* than in the group of susceptible ones (1).

A certain percentage of hybrids showed 'vein corking' some time after CTV inoculation. To avoid confounded effects between CTV inoculation and grafting on sweet orange, two of those hybrids showing 'vein corking' (a110 and a19) were chosen. From 4 virus-free propagations per hybrid on sweet orange, 2 were inoculated and 2 were kept virus-free. 'Vein corking' never appeared in non-inoculated propagations of both hybrids however, inoculated propagations of a19 stopped showing 'vein corking' some time after, at the new development of leaves. It is important to point out that a19 changed from resistant to susceptible at the second year of CTV evaluation. The resistant hybrid a110 quickly developed 'vein corking' and 'stunting' as a consequence of inoculation (Figure 2). Inoculated and non-inoculated propagations of this hybrid, and its mother plant were used for differential display analysis (Figure 5). Four differential bands were cloned, sequenced and, one of them mapped (Table 2). The segregation of ANER clone (whose translation product is highly similar to a pathogenic-related protein) could be studied because its digestion with NlaIII yielded products that segregated at *P. trifoliata* gametes. Its segregation ratio was highly distorted and linkage analysis did no allow its inclusion in a linkage group, remaining unlinked. The hybrid used in the differential display

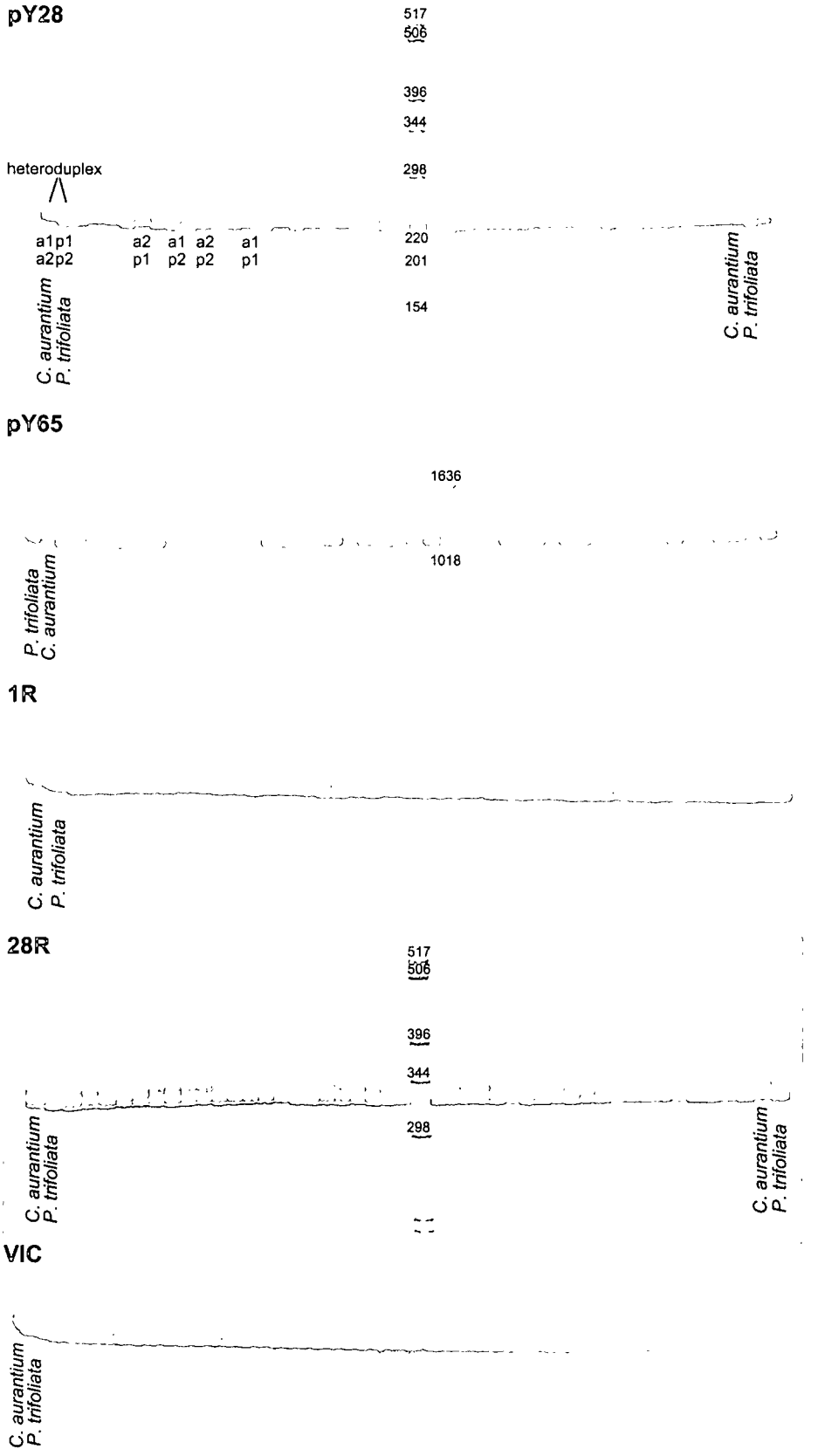


Figure 4.- Silver-stained acrylamide gels showing SCARs for clones pY28, pY65, 1R, 28R and VIC. Differences in heteroduplex mobility allowed the genetic interpretation (Ruiz and Asins 2003) of genotypes at pY28, 1R, 28R and VIC loci.

analysis is homozygous for the highly frequent allele. The other allele is present in only 7 plants, three of which (all from the first lot of inoculated plants) died along the experiment.

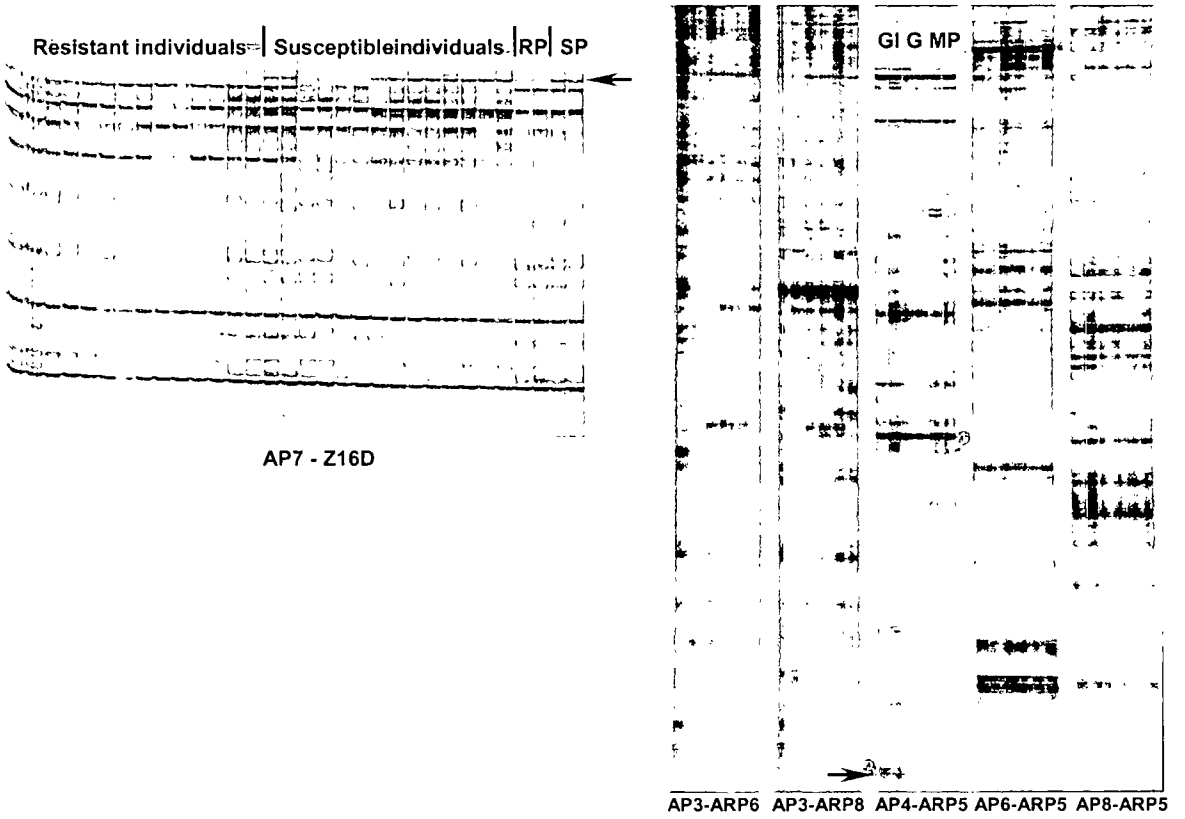


Figure 5.- Examples of differential display analysis for the enrichment strategy 4 (to the left) and for the obtaining of ESTs from the resistant AxPa hybrid a110. Arrows point differential bands. RP: resistant pool; SP: susceptible pool. MP: Mother plant: GI: inoculated, propagated plant, G: non-inoculated, propagated plant.

Considering CTV resistance as a monogenic trait, the responsible gene is placed only in the *P. trifoliata* map, at group Pa4, between 5F-5R_3500 and the *Rps2* homologue '520', not between pY28 and pY65L (Figure 3). On reanalyzing PpxPp family, including the new markers, the resistance gene is placed depending on the criteria at O7_2000 (a) or at D857 (b). *Ctm* (Mestre et al. 1997b) is now included in Pp4 at LOD 4.0 but its precise position depends on including or not *Ctv-R*.

Therefore, considering CTV resistance (T-346 isolate) as a monogeneic trait, as evaluated at the end of, at least, 2 years after inoculation, in a progeny derived from the cross between two resistant genotypes of *C. arantium* and *P. trifoliata,* the putative unique responsible gene *Ctv-R* does not map between pY65L and pY28. Only two hypothesis might explain it, the

progeny (sour orange background) or CTV resistance is not monogenically inherited in this family.

4.- Discussion

Poncirus resistance gene to CTV (*Ctv-R*) has been located in the *Poncirus* genome by means of molecular markers (Gmitter et al. 1996; Mestre et al. 1997a; Fang et al.; 1998). Nowadays it seems apparent that the genomic region where *Ctv-R* is located corresponds to a disease resistance gene cluster that includes many resistance gene analogues and a putative QTL for nematode resistance (Deng et al. 2001; Ling et al. 2000). Chromosome walking along this region (Yang et al. 2001) allowed to enclose *Ctv-R* gene location in a region between two resistance gene analogs (pY65 and pY28) spanning 322 Kb approximately. Molecular markers linked to *Ctv-R* have been included in linkage group 4 of *Poncirus trifoliata* (Ruiz and Asins 2003). Given that this region is so rich in genes involved in the resistance response to a broad spectrum of pathogens, its enrichment for markers, resistance gene analogs and expressed genes, in general, has been approached for candidate resistance gene analysis of CTV and other pathogens in the future.

4.1.- Marker and expressed enrichment strategies

Five strategies to enrich *Citrus* and *Poncirus* linkage maps with markers, resistance analogues and ES linked to *Ctv-R* were attempted. A certain degree of success was reached with any of them, ranging from 16.7 % (strategy 4) to 50 % (strategy 2). Two of the five strategies are new and combine bulk segregant analysis (Michelmore et al. 1991) (*RR* versus *rr* pools of PpxPp individuals) with differential display analysis or with the construction of a cDNA subtraction library. These strategies were thought to identify genes of constitutive expression whenever those genes are present in a portion of a segregating family and lacking in other part. For plants that respond rapidly to microbial attack it was anticipated that R proteins should be present in healthy plants throughout life. RNA gel blot analyses using, *RPS2*, *RPM1, Pto, Cf-9*, and *Cf-2* as gene probes have revealed the presence of low abundance transcripts in unchallenged plants, indicating that at least the *RPM1* gene and some members of multigenic *R* families are expressed in the absence of the corresponding Avr-expressing pathogen (Hammond-Kosack 1997). Since CTV resistance seems to be a case of extreme resistance, a constitutive expression of *Ctv-R* was hypothesized and strategies 4 and 5 were especially designed to isolate *Ctv-R* or at least closely linked expressed sequences to *Ctv-R*. Although the proportion of these genes that could be mapped was low (between 16.7 and 30 %), fifty per cent of them, mapped at linkage group 4, where *Ctv-R* had been previously mapped. The ES 28R locates at group A(7+3+4) but remains unlinked in the FD map. Another clone, ANER, only

segregates in FD but also remains unlinked. Both presents distorted segregation ratios, which makes their mapping difficult. An important inconvenience of ES is the lack of polymorphism some of them present, even at the nucleotide level. Why do methodologies exploiting differential expression result in non-segregant sequences? There are two possible explanations: although the amplified fragment does not contain any polymorphism, it may exist upstream or downstream. Alternatively, the segregating gene might be a cis-acting regulatory element of the ES corresponding gene.

The ES enrichment strategies we have presented are methodologically complex but at least part of the resulting clones that have been sequenced present high identities with genes that might be associated with the plant defense response. Further investigations are needed to verify the associated expression of the selected clones with the citrus defense against CTV or other pathogens but, according to their sequence homologies (kinases, phosphatases, ion channel regulators, lipases), some of them could be components of the downstream R protein-mediated signaling events (Hammond-Kosack and Jones, 1997; Glazebrook 2001). For instance, concerning strategy 4, MON presents high identity with a polygalacturonase (Table 3). Both, pathogen invasion and aphid feeding involve physical trauma that might provoke the release of, not only external but also internal elicitors. The release of internal polygalacturonase (that is also an external elicitor from the aphid) and other hydrolytic enzymes might degrade cell wall polysaccharides producing oligosaccharides. These products may be possible signals initiating plant hypersensitive responses (Ma et al. 1990; Campbell and Dreyer 1990; Hammerschmidt 1991) or activating the expression of proteinase inhibitor genes (Ryan and Farmer 1991). SCAB (Table 3) shows high homology to a SCARECROW gene regulator. The first class of plant proteins to be ascribed a cell-to-cell transport function were transcription factors involved in plant meristem identity (Oparka and Santa Cruz 2000). Moreover, microinjected transcription factor KNOTTED 1 increase the plasmodesmatal size exclusion limit in mesophyll cell and also mediated the selective plasmodesmal trafficking of its sense RNA (Lucas et al. 1995). Concerning strategy 5 (Table 4), the translated product of 22R presents similarity to a disease resistant protein of *A. thaliana*; 24 R seems to code for a lipase. *EDS1* and *PAD4,* which code for lipases, are known to mediate *R*-gene signal transduction (Falk et al 1999; Jirage et al. 1999). 1R represents a highly abundant clone in the subtraction library and seems to code for an actin (Table 4). Kobayashi et al. (1997) reported that actin microfilaments are required for the expression of non-host resistance against fungus in higher plants. Microinjection experiments have shown that viral movement proteins (MPs) alone or complexed with nucleic acid can be transported by leaf plasmodesmata. The nucleic acid binding activity of the MPs presumably shape viral genomes into a thin structure that is compatible with the narrow dimensions of plasmodesmata channels. Transiently expressed tobacco mosaic virus

MP appears as filaments that colocalize primarily with microtubules and to a lesser extent with actin filaments. Whether this cytoskeletal association plays a role in active intracelular transport of the MP (or the MP-viral genome complexes) or in anchoring the MP in the cytoplasm for nucleoprotein complex formation remains to be determined. Cytoskeleton-mediated transport of viral nucleoprotein complexes to plasmodemata would be more effective than diffusion of such elongated complexes through viscous cytoplasm (Baker et al. 1997).

The molecular markers generated have allowed the saturation of the linkage group Pa4 of *P. trifoliata* where *Ctv-R* had been previously positioned. Increasing the number of markers and the progeny size of AxPa family has made several linkage groups to merge along with group A4 of *C. aurantium*, the Pa4 homeologous linkage group. Resultant linkage group A(7+3+4) is now saturated with 48 molecular markers. This saturation that is not balanced with the progeny size has caused the misdetection or detection of very few recombinants between some markers making their ordering not very reliable. Lower marker heterozygosity and strong segregation distortions, from both gametic and zygotic origin, in *P. trifoliata* (Ruiz and Asins 2003) might be responsible of Pa4, Pa3 and Pa7 remaining separate linkage groups, although synteny of common markers is evident. Pa4 linkage group includes molecular markers pY28 and pY65, separated by 1 cM, that were reported to be flanking the *Ctv-R* resistance gene (Yang et al. 2001). Therefore, an approximated equivalence of 322 Kb to 1 cM may be established at this mapping position.

Utilization of sequence homology of conserved domains from known resistance genes to amplify analogues of resistance has proven to be useful in many plant taxa including citrus (Deng et al. 2000). It is a relatively easy technique that has provided excellent results (Table 1) and has allowed the cloning of '520' which according to its sequence homology and map position seems to be part of the previously mapped resistance QTL with major gene effects, *Tyr1*, against the citrus nematode (*Tylenchulus semipenetrans*) (Ling et al. 2000). Besides, '520' present high similarity to *Rps2*, the resistance gene of *A. thaliana* against *Pseudomonas syringe* pv. tomato strains that carry *avrRpt2* (in Baker et al. 1997). *Pseudomonas syringe* is also a pathogen of citrus and causes two diseases, blast and black pit, that are important in some areas (Menge 2000). Although resistance genes and their analogous do not usually mark regions of synteny (Richter and Ronald 2000; Grube et al. 2000), it seems to be conserved between *P. trifoliata* and *C. aurantium* at linkage group 4.

4.2.- Resistance to CTV

For breeding purposes, there are several types of responses that occurs in resistant plants that have been reviewed by Solomon-Blackburn and Barker in potato (2001): extreme resistance (ER) and hypersensitive resistance (HR); resistance to infection (i.e. from vector inoculation); resistance to virus accumulation (RVA) and resistance to virus movement (RVM).

ER and HR correspond to the gene-for-gene resistance pathways (Glazebrook et al. 1997). Resistant virus-host interactions have been extensively reviewed. In contrast, little is known about the factors involved in susceptibility. Virus of plants have limited and specific host ranges. Plants the virus can infect and systemically invade under laboratory conditions are considered the host range. For Dawson and Hilf (1992) susceptibility and resistance occur in various degrees. They describe 7 types of virus-host interaction: (1) total susceptibility; (2) initial susceptibility (HR); (3) limited susceptibility due to a reduced level of virus replication; (4) limited susceptibility in which the virus can spread but not systemically infect the plant; (5) limited susceptibility in which the virus replicates but is limited to the initially infected cells. (6) resistance to infection (ER) and (7) true immunity, the genotype is a non-host even at the cellular level. Since CTV can replicate in *P. trifoliata* protoplasts (Albiac-Martí et al. 1999), this species can not be considered a non-host of CTV. No hypersensitive response has been observed in *Ctv-R*-mediated resistance to virus infection (Yang et al. 2001). Mestre et al. (1997b) speculated that resistance provided by *Ctv-R* would be a constitutive response interrupting the virus infection cycle at initial steps, so three-four months after inoculation no virus is detected. Within the resistant PpxPp plants, some of them, heterozygous at *Ctv-R* were graft inoculated to test CTV spread (Mestre et al. 1997b). One year after, the virus had spreaded around the inoculum at some of them, suggesting that the resistance of *P. trifoliata* was due to both lack of CTV unloading from sieve elements (type 4) and lack of cell-to-cell movement (type 5). No type 2 was observed in PpxPp plants.

Analysing the evolution of CTV infection in the AxPa family along 2-4 years after inoculation has shown the appearance by segregation of different types of virus-host interactions. Thus, 15 % of the hybrids reverted their capability to control CTV infection during the first year after inoculation and 69.7% of susceptible plants showed irregular distribution of virus among branches. Since 60 % of hybrids that reverted to susceptible, presented irregular distribution of virus, it can be said that all these hybrids present a certain degree of resistance to systemic infection (type 4). Within the susceptible group, 30.3 % of the hybrids presented uniform distribution of the virus. A few of them showed low titres which might be considered a certain degree of RVA or type 3 interaction.

Within the resistant group of AxPa hybrids, most of them carry *Ctv-R* (76.6 %) but there is a proportion (23.4 %) that lacks it. Unexpectedly, sour orange is as resistant to CTV (isolate T-346) as FD, therefore resistance could have been inherited from sour orange in these hybrids. Sour orange resistance to CTV is now under study and had been observed for certain isolates in the past (P. Moreno, personal communication). It is also important to point out that CTV was detected in 12.5 % of resistant hybrids, three to six months after inoculation, becoming resistant afterwards. This behavior is common to hybrids containing or lacking *Ctv-R* and a similar

proportion of both show it. The cure from CTV might agree with the existence of a silencing mechanism for becoming resistant (Vance and Vaucheret 2001), genetically different from *Ctv-R* (type 3).

Some CTV isolates may cause a severe decline of trees grafted on sour orange. In fact, more than 80 million trees on sour orange have been destroyed to date. The decline results from viral effects on the phloem of the sour orange rootstock just below the bud union. The decline may occur over a period of several years or very rapidly (quick decline). T-346 was isolated from a 'stunted' satsuma grafted on sour orange that declined (P. Moreno, personal communication). Although the resistance of sour orange to T-346 has been surprising, it will not be the first case that the infection of a scion grafted on a resistant rootstock provokes bud union incompatibility, leading to the death of the tree (i.e., citrus tatter leaf virus and *P. trifoliata*). Therefore, it is tempting to speculate that CTV-sour orange presents a type 2 interaction resulting in a hypersensitive response (HR). Then, the continuous defence response of sour orange when CTV is highly accumulated in the scion might irreversibly damage its vascular tissues (or their development) under the bud union leading to the death of the tree. The hypersensitive response is an acting form of defence that is characterized by the appearance of necrosis at the site of infection and inhibition of further pathogen multiplication and spread. Hypersensitive response includes an oxidative burst leading to production of reactive oxygen intermediates, alteration of membrane potentials, an increase in lipoxygenase activity, cell wall modifications, lignin deposition, production of antimicrobial compounds (Baker et al. 1997) and the expression of a characteristic set of genes including pathogenesis-related protein 1 (Glazebrook 2001). Three observations in our data might support the hypothesis of sour orange death through a continuous HR. One corresponds to the differences in mortality index between resistant (8.3 %) and susceptible hybrids (0.33 %). Even more, within resistant hybrids, mortality was higher in hybrids without *Ctv-R* (10.5 %) than in those carrying it (2.9 %). Therefore, mortality is more related to CTV resistance, especially the resistance not derived from the *Ctv-R* gene from *P. trifoliata*. The other result concerns the resistant hybrid a110 that showed 'vein corking' as a consequence of CTV inoculation. The third supporting data corresponds to the differential expression of a gene coding for a putative pathogenesis related protein in CTV inoculated propagations of a110 (Table 2). The translated sequence of this differential EST (ANER) presents high identity with wound induced protein (WIN 1) in potato (Stanford et al. 1989) and a pathogen- and wound –inducible antifungal protein CBP20 precursor in tobacco (Ponstein et al. 1994). CBP20 protein was purified from tobacco leaves inoculated with tobacco mosaic virus and contains an N-terminal chitin-binding domain. Sour orange and a110 are homozygous for ANER while FD is heterozygous. It has not been possible to place ANER within any *Poncirus* linkage group due to its strong segregation distortion. The other allele is very rare

in the progeny and all hybrids that carried it and were inoculated in the first lot, died along the experiment contrarily to FD. Not all pathogenesis-related proteins might be harmless plant defence enzymes depending on the genetic background.

It has been speculated in potato that *R* genes initiate responses that could result in HR, but some *R* gene responses may prevent disease so effectively that the cell death is not activated (Hammond-kosack and Jones 1997). However, Bendahmane et al. (1999) showed in both *Nicotiana* spp and potato that ER is separate and epistatic to necrosis (HR). This would explain why in spite *P. trifoliata* presents extreme resistance to CTV and not HR, under a certain sour orange genetic background, HR might appear at the location of phloem unloading giving rise to 'vein corking'. If sour orange (as rootstock) triggers a HR where CTV is unload, below the graft union, irreversibly damaging its vascular tissue then, receiving *Ctv-R* from *P. trifoliata* might not avoid decline of sweet orange grafted trees after CTV infection.

The genetic analysis of virus-plant interaction in the AxPa family after a CTV chronic infection has shown the segregation of five types of interaction which is not compatible with the hypothesis of a single gene controlling resistance. This would explain why *Ctv-R* does not map between pY28 and pY65, but 15 cM far away. Another type of genetic analysis of CTV resistance is needed as alternative to assume monogenic inheritance. QTL analysis of CTV accumulation has allowed such approach and will be presented in a separate paper (Asins et al. submitted). In brief, a major resistance QTL that presents maximum LOD score between pY65 and pY28 and five QTLs controlling CTV accumulation have been identified.

QTL analysis of Citrus Tristeza virus-citradia interaction

Chapter III

1.- Introduction

Citrus tristeza virus (CTV) is the causal agent of one of the most important diseases of citrus (Bar-Joseph et al. 1989). This phloem-associated closterovirus exists in a large variety of isolates that differ in biological properties such as symptoms in the field, reaction on indicator plants and aphid transmissibility. Since its outbreak in the early 1930s, tristeza has caused the death of million of trees grafted on sour orange all around the world. The use of only tristeza-resistant rootstock cultivars, worse adapted to semi-arid conditions than sour orange, has greatly narrowed the genetic diversity of rootstock cultivars increasing the vulnerability of citriculture in some areas. A sad example is 'Citrus Sudden Death', a new tristeza-like disease (Bassanezi et al. 2003) that affects sweet orange grafted on Rangpur lime. It has recently appeared in Brazil, in huge areas where only this rootstock is used for sweet orange production. The ability of viruses to expand their host range is an important practical effect of virus evolution (Roossnick 1997). Virus emergence is caused by several factors, including expanded host and vector range, human population increases, and rapid global movement of humans and their domesticated plants and animals. On the other hand, there are several factors contributing to the great variability of CTV: RNA polymerase errors in replication building up large viral populations, recombination and the fact of being hosted by a perennial crop where multiple CTV genotypes and other viruses may evolve together for long time increasing the probabilities of reassorment.

Resistance or tolerance to CTV is a major component of most breeding programmes. Several studies have reported markers linked to a putative resistance gene against citrus tristeza virus (CTV) in *P. trifoliata,* named *Ctv-R* (Gmitter et al. 1996; Mestre et al. 1997a; Fang et al. 1998). Nowadays it seems apparent that the genomic region where *Ctv-R* is located corresponds to a disease resistance gene cluster that includes other resistance genes as a putative QTL for nematode resistance (Deng et al. 2001; Ling et al. 2000). Chromosome walking along this region (Yang et al. 2001) allowed to enclose *Ctv-R* gene location in a region between two resistance gene analogs (pY65 and pY28) spanning 322 Kb approximately. However, all previous linkage studies trying to locate *Ctv-R* assume the hypothesis of a monogenic inheritance (resistant versus susceptible) and resistance was evaluated, at most, a year after inoculation and not after a prolonged challenge. In a companion paper, Bernet et al. (submitted) mapped *Ctv-R* in a different position within Pa4 linkage group using a population of citradias (derived from the cross between sour orange and *P. trifoliata)* after a prolonged challenge. The change in positioning was interpreted as a deviation from the hypothesis of monogenic control for CTV resistance. In fact, Mestre et al. (1997b) had previously presented evidences supporting the involvement of more than one gene after prolonged challenge.

Dawson and Hilf (1992) reviewed numerous results supporting the quantitative nature of susceptibility and resistance. Moreover, at the molecular level, the defence response triggered by many gene-for-gene systems also presents a quantitative nature due to the number of genes involved (Bent 1996). QTL analysis has provided geneticists with an opportunity to genetically analyse the plant-pathogen interaction; not only allowing the study of factors related to the resistance response but also factors involved in susceptibility, which are much more scarcely reported. Some aspects of plant-pathogen interaction complicate QTL dissection of disease resistance. For example, disease incidence or severity may be rated on an ordinal scale rather than a truly quantitative evaluation that has the normal distribution required for most statistical approaches. Genetic differences between strains or isolates may also result in different QTL profiles. Furthermore, the timing and method of inoculation may alter the QTL detected. In spite of all these complications, QTL analysis avoids the need of assuming monogenic inheritance and provides information on the number, position and relative contribution of putative genes involved which is very relevant for the success of future marker assisted selection and genetic transformation experiments. Of course, it is impossible to unveil all genes just using a single segregating population because not all QTLs are segregating, or segregating alleles may not be different enough at all QTLs to allow their detection. Therefore, the larger the number of independent progenies the better the estimation of the number of QTLs or genes involved.

In this paper we describe the detection of QTLs involved in CTV accumulation in a family of citradias (104 *C.aurantium* x *P. trifoliata* hybrids), for more than two years after inoculation with a common CTV isolate. Epistatic interacions were also studied. A saturated linkage map for each parent was used that contains not only anonymous markers but also expressed sequences and analogues of resistance (Bernet et al. submitted).

2.- Materials and methods

A segregating population (AxPa) consisting of 104 hybrids was used for QTL analysis of CTV accumulation. It was derived from the cross between sour orange (*C. aurantium* L. var 'Afin Verna') and the trifoliate orange 'Flying Dragon' (FD). A sample of 63 hybrids had been used previously to build up a map for *C. aurantium* and another for FD (Ruiz and Asins 2003).

Presence of *Ctv-R* was inferred from the presence of FD alleles at pY65 and pY28 marker loci (Yang et al. 2001). To do this, specific primers were designed to develop the corresponding SCARs (Bernet et al. submitted).

2.1.- Evaluation of CTV resistance and accumulation

AxPa hybrids and parents (sour orange and FD) were propagated on sweet orange rootstocks. After this propagation, the rootstocks were inoculated by grafting patches of infected

sweet orange with CTV isolate T-346, a common Spanish isolate kept at the bank of CTV isolates at IVIA.

A first lot of 66 AxPa hybrids was inoculated at the beginning of 1999 and the rest, two years later. The presence of the virus was checked every 6 months at the shoots and at the inocula, as a positive control of each challenged plant, by direct tissue blot immuno-assay (DTBIA) following the procedure described in Garnsey et al. (1993). CTV amount was also evaluated by DAS-ELISA (Double Antibody Sandwich Enzyme-Linked Immuno Sorbent Assay) using monoclonal antibodies 3CA5 and 3DFI together as described in Cambra et al. (1993).

At Spring of both 2001 and 2002, at least 4 branches of each inoculated plant were analysed by DTBIA and DAS-ELISA. Those hybrids where the virus was detected in all branches were considered susceptible (with uniform spatial distribution of the virus) and those where the virus was detected in at least one branch but not in others were declared susceptible with an irregular distribution of the virus.

2.2.- QTL analysis

The mean optical density of DAS-ELISA reactions through years for the whole tree was used as variable (CTV titre or CTV accumulation) for the QTL analysis.

Putative QTLs were identified using several statistical methodologies. Since CTV accumulation heavily departs from normality, a non-parametric test based on the Kruskal-Wallis (KW) methodology was considered for both *C. aurantium* and *P. trifoliata* linkage maps, separately, using the MapQTL 3.0 computer program (Van Ooijen and Maliepaard, 1996). These linkage maps were first reported by Ruiz and Asins (2003) and enriched with new markers, analogues or resistance, expressed sequence tag (EST) and expressed sequences by Bernet et al. (submitted). For markers segregating as a backcross (like in the pseudo-test-cross design described by Grattapaglia et al. 1996; M_iM_j x M_iM_i for the *C. aurantium* genetic map, or M_iM_i x M_iM_j for the *P. trifoliata* one), log transformed ELISA means were used for QTL analysis by interval mapping (IM) and composite interval mapping (CIM) using the QTL Cartographer computer program (Basten et al. 2002). All these analysis were repeated omitting resistant hybrids; i.e., using data on CTV accumulation only from susceptible individuals.

Epistatic interactions affecting CTV accumulation were analyzed by two-way ANOVA for pairwise combinations of significant marker loci. Only epistatic interactions between pY65 and candidate resistance genes (strategies 4 and 5 in Bernet et al. submitted) or *P. trifoliata* markers where a QTL had been detected, were tested. The same study was carried out in sour orange but using as reference marker pY28. Bonferroni adjustment was used to obtain an overall protection level of 0.05.

3.- Results

The distribution of mean DAS-ELISA values although continuous, is not normally distributed. A little improvement was obtained by using the logarithm-transformed values (Figure 1). Mean DAS-ELISA values for the resistant parents were 0.375 and 0.379 for *C. aurantium* and trifoliate orange, respectively. Some hybrids showed important changes in DAS-ELISA values through time (time-irregular hybrids). In some of these hybrids the distribution of virus was also irregular among branches. In general, all hybrids showing high levels of CTV presented also a uniform distribution among branches. In a hybrid with very low amount of CTV (similar to the titre of resistant genotypes), the distribution of the virus was highly irregular among branches. This hybrid is one of those 3 susceptible ones carrying the putative *Ctv-R* gene from *Poncirus trifoliala* (Bernet et al. submitted). Given that this result and others previously reported (Mestre et al. 1997b) disagree with the hypothesis of one single gene controlling the abundance of CTV in inoculated citrus plants, the involvement of other genomic regions was investigated by QTL analysis.

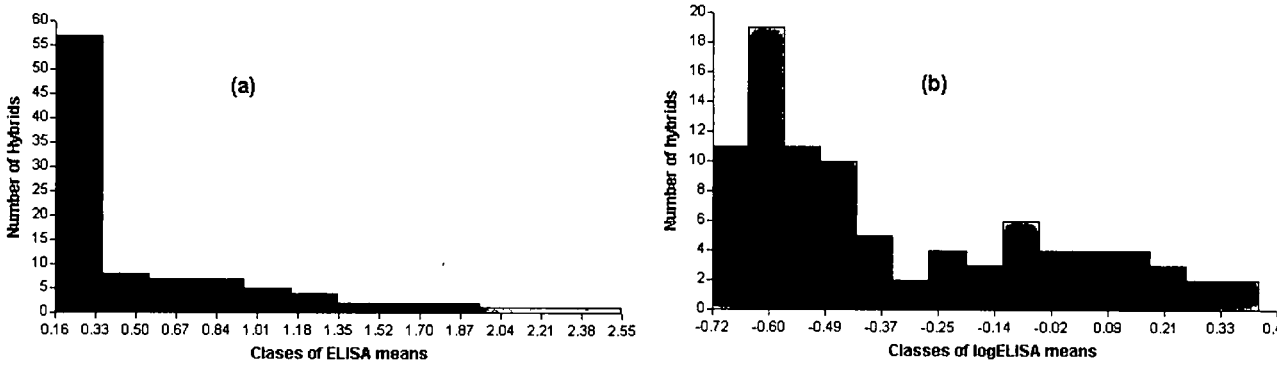


Figure 1. Histogram of the distribution of ELISA means (a) and ELISA log transformed means (b).

Although some differences in QTL detection were obtained depending on the statistical method, a general agreement was observed between QTLs detected by the Kruskal-Wallis (KW) and the Interval Mapping (IM) or Composite Interval Mapping (CIM) methodologies. The nearby marker, its linkage group and the statistical significance of each QTL is presented in Table 1. QTLs detected by KW for DAS-ELISA means in the whole population or only for the susceptible hybrids are located on linkage groups Pa4, A(7+3+4), A(8+6) and A(10+5b). They are presented in Figure 2.

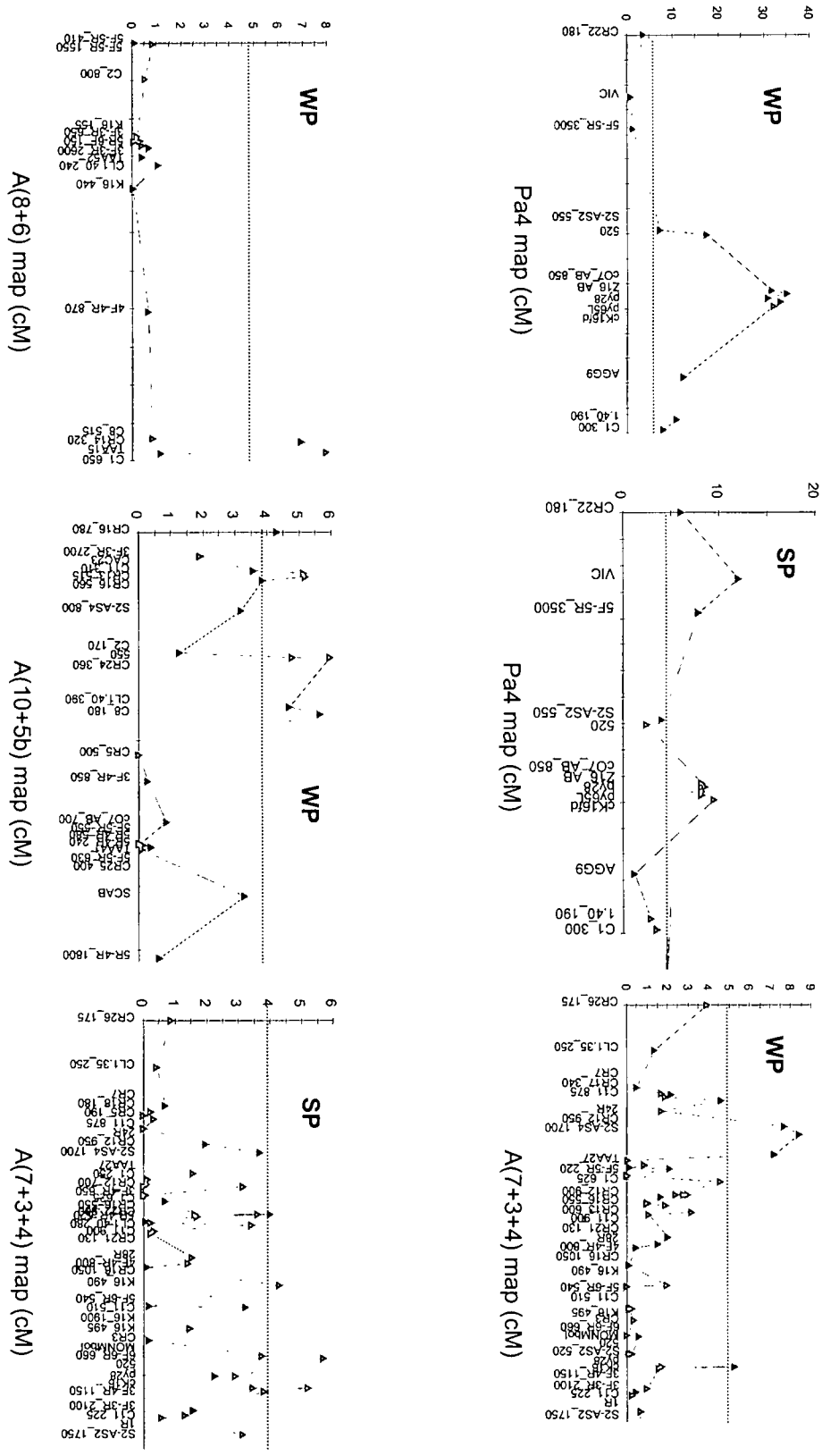


Figure 2. Value of the Kruskal-Wallis statistic for ELISA at markers of relevant linkage groups. Linkage groups as in Bernet et al. (submitted). WP: Whole population; SP: susceptible subpopulation.

	Whole population						Susceptible sub-population				
Linkage group	Marker/clone	ELISAM (KW)	LELISAM (CIM)	a	R^2	QTL	Marker/clone	Suscept (KW)	LSuscept (CIM)	a	R^2
Pa4	py65L	33	62.89	0.547	0.674	***Ctv-R$_1$***	ck16	12.5	15.4	0.275	0.167
Pa4	Z16	35	61.22	0.458	0.461	***Ctv-R$_2$***					
Pa4						***Ctv-A$_1$***	VIC	12	11.59	0.229	0.148
Pa						***Ctv-A$_2$***	TAA15	3.97	ns		
A(7+3+4)	ck16	5	28.39	0.563	0.598	***Ctv-R$_a$***	ck16	5	16.87	0.314	0.203
A(7+3+4)	S2-AS4_1700	8	14.67	0.248	0.144	***Ctv-A$_3$***					
A(7+3+4)						***Ctv-A$_4$***	6F-6R_660	5.7	12.98	0.294	0.191
A(8+6)	TAA15	8	ns			***Ctv-A$_2$***					
A(10+5b)	CR16_780	4.3	ns			***Ctv-A$_5$***	CR16_780	4.6	21.86	0.239	0.282
A(10+5b)	CR13_515	5.2	ns								
A(10+5b)	CR14_360	6	ns								

Table 1.- Map closest marker/clone, Kruscal Wallis statistic (KW), Likelihood ratio statistic under composite interval mapping (CIM), additive effects (a) and contribution to the total variance (R^2) of QTLs for CTV resistance (*Ctv-R*) and CTV accumulation (*Ctv-A*). Ns: non-significant.

QTLs with the greatest contributions, *Ctv-R$_1$*, *-R$_2$* and *-R$_a$* are located on linkage group 4 where *Ctv-R* had been previously mapped (Gmitter et al. 1996; Mestre et al. 1997a; Fang et al. 1998), and in disagreement with the previous study where monogenic inheritance of CTV resistance had been assumed (Bernet et al. submitted). However, two maxima instead just one is observed, therefore, two closely linked genes at pY65 and Z16 (Ctv-R$_1$ and Ctv-R$_2$, respectively) are hypothesized. Interestingly, another QTL is also detected within this small region at sour orange linkage group 4. It has been named *Ctv-R$_a$*.

A QTL with smaller gene effect (*Ctv-A$_3$*) is also involved in CTV accumulation, at the position of a putative analogue of resistance in sour orange linkage map (S2-AS4_1700).

Some QTLs are detected using KW but not with CIM in the whole population. However, when analysing only susceptible hybrids some of those QTLs, and *Ctv-A$_5$*, are also detected by CIM. In susceptible hybrids, an important QTL, *Ctv-A$_1$*, seems to be involved in CTV accumulation. Its position (Figure 3a) coincides with a cDNA clone obtained by diffential display analysis (Bernet et al. submitted) and it is specific of (always) susceptible (*rr*) PpxPp plants (derived from self-pollination of *P. trifoliata*).

Also some QTLs were detected using CIM but not KW; for instance, a peak at the '520' marker (an *Rps2* analogue) in Figure 3, and another between markers CL140_300 and CR12_1400 in linkage group AIII (data not shown). We think they might be artefacts due to the deviation of the trait from the normal distribution and we rather be conservative in this context.

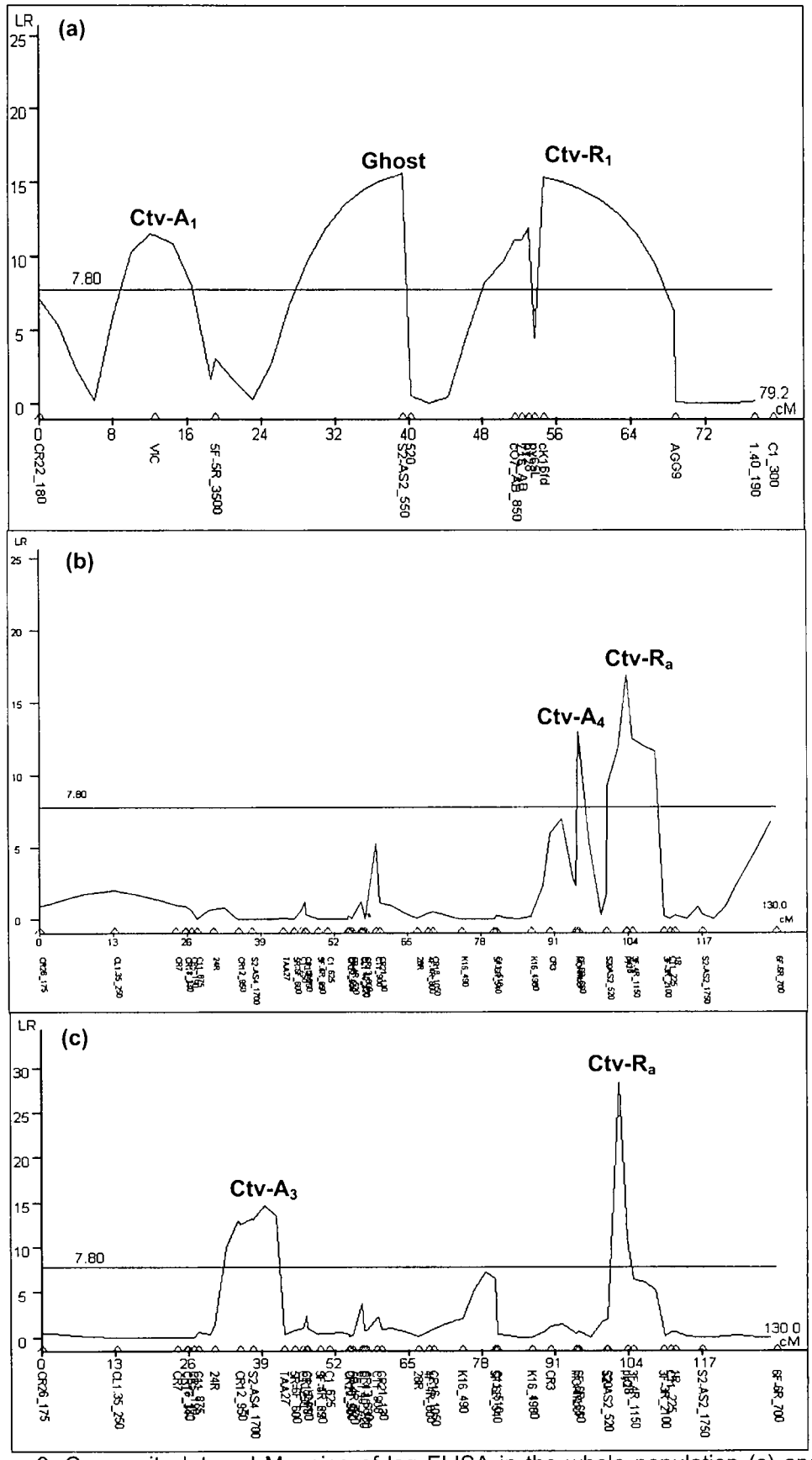


Figure 3. Composite Interval Mapping of log ELISA in the whole population (c) and in the susceptible subpopulation (a and b) for linkage groups Pa4 (a) and A(7+3+4) (b and c). LR: Likelihood ratio test value.

The possible existence of epistatic interactions involved in the genetic control of CTV accumulation was investigated. Only epistatic interactions between pY65 and *P. trifoliata* markers where a QTL had been detected or candidate resistance genes (strategies 4 and 5 in Bernet et al. submitted) were tested. The same study was carried out in sour orange but using as reference marker pY28. Two significant epistatic interactions were detected involving *P. trifoliata* markers VIC ($p>0.01$) and TAA 15 ($p>0.02$) using the whole population (Figure 4). For sour orange, a significant epistatic interaction was found involving the differential cDNA clone 28R ($p>0.02$). Other interactions, near significance, involved 1R ($p>0.06$) and SCAB ($p>0.08$).

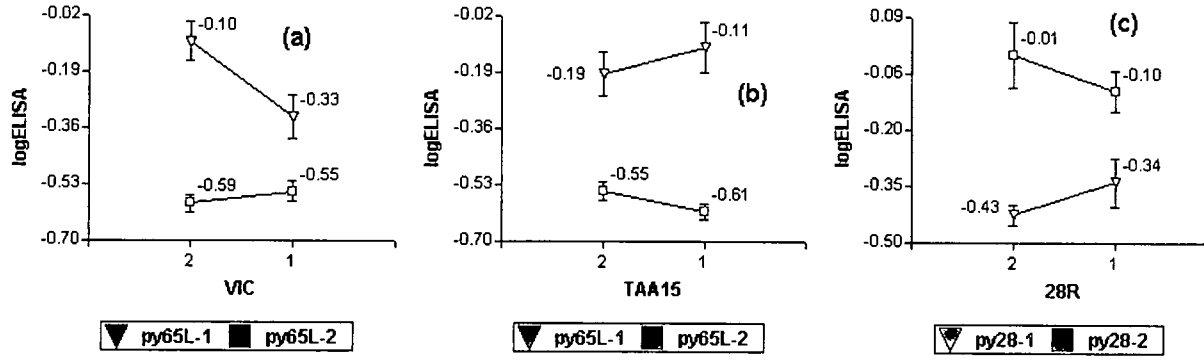


Figure 4. Means and standard errors of log ELISA in the whole population (a and b) and in the susceptible subpopulation (c) for pairs of interacting markers. 1: allele b (low molecular weight band); 2: allele a (high molecular weight band).

4.- Discussion

As expected, QTL analysis of CTV accumulation gives information that it is more complex but also more robust than that obtained under the assumption of a monogenic inheritance. One (or several) major closely linked QTL (*Ctv-R_1*, *Ctv-R_2*) are involved in the resistance coming from *P. trifoliata*. Their contribution is very large (67-47 %) and their position coincides with that reported by previous studies for *Ctv-R* using other progenies, after less prolonged challenges (Gmitter et al. 1996, Mestre et al. 1997a, Fang et al. 1998). Sour orange also presents a major QTL (*Ctv-R_a*), contributing almost a 60 % to the total variance. Its position on A(7+3+4) seems to coincide with that of *Ctv-R_1* and it would explain the CTV resistance of sour orange. Therefore, *Ctv-R_1* and *Ctv-R_a* might be allelic and their most likely position would be between pY28 and cK16. In addition to the resistant QTLs, two CTV accumulation QTLs are also located at linkage group 4, *Ctv-A_1* in *P. trifoliata* and *Ctv-A_3* in *C. aurantium* but they do not correspond to the same locus because VIC (the closest marker to *Ctv-A_1*) is in another linkage group

(AI+AVII) in the *C. aurantium* map. An analogue of resistance, S2-AS4-1700 is the candidate gene for *Ctv-A*$_3$, on linkage group A(7+3+4).

Sequencing data on chromosome walking throughout the region containing *Ctv-R*$_1$ and *Ctv-R*$_2$ has shown how rich in resistance gene analogues this region is (Yang et al. 2001, Deng et al. 2001). As with any QTL analyses, demonstration of a significant association between resistance and a chromosomal region does not distinguish between a single QTL of large effect or multiple QTLs of smaller effects. No resistance QTL has yet been dissected by recombination to distinguish between these possibilities. However some resistance genes have already been cloned (Hammond-Kosack and Jones 1997) and one of them, the *Pto* gene that confers resistance to races of *Psedomonas syringe* pv. tomato that carry the *AvrPto* gene, was cloned using the map-base approach (Martin et al. 1993). Even in this case, where the disease resistance gene has a very large effect, further mutagenesis experiments have revealed an additional gene, *Prf*, that is required for *Pto* function and is located 24 Kb from *Pto* (Salmeron et al. 1996). Clusters of disease-resistance genes are quite common and would explain their evolution (Hammond-Kosack and Jones 1997; Takken and Joosten 2000) and involvement in the resistance response to very different pathogens (van der Vossen et al. 2000). In *Arabidopsis*, five of these *R* gene-rich regions are now recognized and have been designed as "major resistance complexes". Linkage group 4 of *P. trifoliata* and *Citrus* definitively contain at least one major resistance complex. How many resistance genes of this complex are necessary to confer resistance to CTV? Will it depend on other loci at which *P. trifoliata* is homozygous and *C. aurantium* is heterozygous? A genetic approach based on mutagenesis to find interacting partners of R proteins (Required for Disease Resistance or RDR loci) has been fruitfully followed in *Arabidopsis*, tomato and barley (reviewed by Takken and Jooste, 2000). In species, like citrus, where this approach is not affordable, an alternative one might be QTL analysis of the virus-plant interaction using progenies derived from far related resistant parents which constitutes the objective of the present paper.

QTL analysis presents several limitations (Asins 2002) and most of them affect this study. CTV accumulation presents a skewed distribution, even after log-transforming data (Figure 1) and we do not know how this deviation from normality affects type errors I and II of IM and CIM methodologies. Consequently, we assume KW is the proper methodology for the QTL analysis of the trait. Of course, QTL detected by all methodologies are expected to be the most reliable ones. QTLs in linkage groups A(8+6) and mainly A(10-5b) are only detected by using the KW methodology while some QTLs were only detected by IM and CIM. Although CIM is expected to provide more accurate estimates of the QTL position than any other methodology (Zeng 1994) we think that, in the present situation, its % of false positives (error type II) might be greatly

affected. Thus, the QTL detected between *Ctv-A*$_1$ and *Ctv-R*$_1$ (Figure 3a) is considered a likely artefact or ghost QTL.

Since *Ctv-R*$_1$, *Ctv-R*$_2$ and *Ctv-R*$_a$ have large effects, another QTL analysis of CTV accumulation has been carried out using only susceptible hybrids (those where the virus was detected at least once along the experiment). Besides, it could be consider a way to unveil factors involved in susceptibility; i.e., host factors needed by CTV for replication, movement and systemic infection. The obvious limitation of this second study is the reduced progeny size, 43 hybrids. Nevertheless, this analysis has two advantages: the distribution of the trait improves towards normality and other minor QTL, localized at group Pa4 or A(3+7+4) can now be detected by reducing the variance associated with their phenotypic effects. This is the case of *Ctv-A*$_1$ in Pa4 and *Ctv-A*$_4$ which is closely linked to *Ctv-R*$_a$. However, why does *Ctv-A*$_3$ disappear then? We think this is due to its relative large distance from *Ctv-R*$_a$, its low contribution (14 %) and the reduction in progeny size.

The position of an expressed sequence (VIC) coincides with the position of maximum LOD for QTL *Ctv-A*$_1$ (Figure 3a). VIC also presents epistatic interactions with *Ctv-R*$_1$ using data on CTV accumulation from the whole progeny (Figure 4). Thus, in those hybrids that inherited *Ctv-r*$_1$ (pY65-1) the amount of CTV will be significantly different depending on the inherited allele at VIC. VIC is a good candidate gene for *Ctv-A*$_1$. Unfortunately, the search for homologous sequences resulted in a gene of *A. thaliana* that codes for an hypothetical protein of unknown function. These results support the need to deeply study the expression and function of the VIC protein. Another gene involved in cell-to-cell movement, *Ctm*, different from *Ctv-R* was reported to affect resistance of *P. trifoliata* against CTV (Mestre et al. 1997b). Because of its positioning, *Ctv-A*$_1$ could be it.

Another *P. trifoliata* epistatic QTL is *Ctv-A*$_2$. Its closest marker, TAA 15, is associated with CTV accumulation in susceptible hybrids. TAA 15 at the sour orange linkage map appears also associated with CTV accumulation. A genetic study on apomixis in citrus reported a QTL of large effect (*Apo 2*) linked to TAA 15 in the *C. volkameriana* map (Garcia et al. 1999). Asins et al (2002) suggested that the large negative effects of *Apo 2* on apomixis might be explained by its involvement in the synthesis or signal transduction pathway of an inhibitory "complex substance" (probably containing ethylene, abcisic acid, auxin, gibberellin and ethanol). By using the yeast-two-hybrid system a direct interaction between Pto and the trascription factors Pti4, Pti5 and Pti6 (Pto interacting) was found in absence of elicitor (AvrPto). Upon phosphorylation by Pto, these transcription factors bind to elements present in the promoter of ethylene-induced genes, including many basic pathogenesis-related proteins (Zhou et al. 1997). Recently, signal transduction pathways that involve jasmonates and ethylene as regulators of several defence-related genes have been identified and microarray analysis has demonstrated the existence of

a substantial network of regulatory interaction and coordination among plant defence pathways (Schnek et al. 2000). Therefore, as far as quantitative traits are concerned, pleiotropic effects on multiple traits are expected from QTLs at genomic regions that contain genes regulated by phytormones.

A QTL of moderate effect (28 %) has been detected in susceptible hybrids (Ctv-A_5). This QTL is also detected by using the KW methodology with the whole progeny. The maximum LOD score of this QTL coincides with the position of a microsatellite marker (CR16-780) that was derived from a EST (Ruiz and Asins 2003). This EST corresponds to a NADP-isocitrate dehydrogenase. Another microsatellite (CR13-515) derived from an EST (Miyagawa-wase maturation stage pcMA1MA1M0910-38) at linkage group A(10+5b) is associated with CTV accumulation. The *in silico* translated protein coded by this EST contains an RNA recognition motif characteristic of splicing factors. The expression profile of all these candidate genes need to be studied to assess their putative involvement in CTV accumulation as a previous step before confirmation by genetic transformation experiments.

The presence of two major types of disease resistance to plant pathogenes, vertical resistance and horizontal resistance, has long been recognized in interactions between plant hosts and their pathogens. Vertical resistance in many plant host-pathogen relationships is hypersensitive, race-specific, and governed by interactions between avirulence genes in pathogens and resistance genes in plant hosts. In contrast, horizontal resistance is quantitative, presumably non-race specific, and controlled by polygenes, though these assumptions have not been actively tested. In this context, an hypothesis is that resistance genes would correspond to QTL of large effect that would be responsible of vertical resistance, while QTL with moderate or small effect would be responsible of the horizontal resistance. Going further, Li et al al. (1999) have shown that a "defeated" resistance gene against a virulent strain of *Xanthomonas oryzae* acts as a QTL. If any QTL involved in the accumulation of CTV was a defeated resistance gene, then we should observe a change in the genetic composition of the CTV population hosted by citradias with low CTV titre in comparison to that of citradias with high CTV titre. The haplotype composition for two CTV genes (P20 and P25, coding for the coat protein) was studied by single strand conformation polymorphism analysis (Ayllon et al. 1999) in all susceptible citradias from the first inuculation lot at the fourth year after inoculation, and the original T-346 isolate maintained in a sweet orange tree. All samples, including sweet orange, presented the same haplotype of CTV. Therefore the QTLs of moderate and small effect we have detected do not seem to be defeated resistance genes.

Dawson and Hilf (1992) reviewed mounting evidence that systemic infection is an additive effect of replication, cell-to-cell movement, and long distance movement. Before a virus can invade a plant systemically it must accomplish a series of tasks, including infection, replication,

cell-to-cell movement, and long distance movement. The virus must be able to perform each of these functions above some threshold rate. Systemic infection appears to be a balance of these functions, which must occur in concert. Due to their parasitic nature, viral replication and movement throughout the plant is controlled by a combination of viral and plant genes. We do not know which citrus gene products are used by CTV for replication and movement but they have to exist since citrus is its natural host. Even *Poncirus trifoliata*, that is a "nonhost" species, seems to be a cellular host since CTV can replicate within its protoplasts (Albiach-Martí et al. 1999). Therefore, it is possible that some accumulation QTLs, at least, correspond to loci where different alleles correspond to proteins used by CTV with a varying degree of efficiency in replication and/or movement. Additionally, the resistance response of the gene-for-gene system seems to require more than one single gene product, not only the products of RDR genes but also a pathogenecity target, like in the 'guard' model proposed by Van der Biezen and Jones (1999). If the progenies used segregates for more than one of those genes, and gene effects of the different alleles can be detected, then the genetic analysis of the virus-plant interaction provides a mean to detect and locate them simultaneously.

It is the first time that the CTV-citrus interaction has been studied by QTL analysis of virus accumulation. It has allowed to study its genetic control without assuming the restrictive hypothesis of a monogenic inheritance. Several major QTLs have been detected at the position where *Ctv-R* had been previously located in other progenies. The detection of epistatic interactions with *Ctv-*R_1 and *Ctv-*R_a suggest that the response of the citrus plant to the infection with CTV involves the interaction between the resistance gene and other genes of the plant genome. Candidate genes have been selected that map where 3 QTLs locate (*Ctv-*A_1, *Ctv-*A_3 and *Ctv-*A_5) and will be the objective of future experiments on expression profiling.

Resumen

Los cítricos son las especies frutales más importantes a nivel mundial, siendo España el quinto país productor y el principal exportador de cítricos para consumo en fresco. Estos datos contrastan con la escasez de estudios para la mejora genética del cultivo, probablemente debida a la dificultad que conlleva el manejo de un cultivo leñoso de compleja biología reproductiva. En la presente tesis se ha pretendido avanzar en el conocimiento de la resistencia al virus de la tristeza de los cítricos (CTV) con el propósito de diseñar estrategias de mejora más eficientes.

El desarrollo de marcadores moleculares fiables e informativos supone una importante ayuda en los programas de mejora genética al ser útiles para la construcción de mapas genéticos que faciliten el análisis genético de caracteres agronómicos y así acometer con mayor eficacia la selección asistida por marcadores. Los retrotransposones son especialmente abundantes en los genomas de las plantas, están implicados en su evolución y parecen contribuir a la generación de nuevos genes de resistencia. El estudio de estos elementos móviles en cítricos resulta interesante tanto para el diseño de marcadores moleculares de alta repetibilidad, potencialmente abundantes y polimórficos como para progresar en el conocimiento de los mecanismos de evolución genómica de los cítricos. Para los objetivos de la presente tesis también resultan interesantes dada su posible relación con la diversificación de genes de resistencia.

En este trabajo se ha obtenido un nuevo grupo de marcadores moleculares derivados de retrotransposones del tipo *gypsy* y se ha analizado la presencia, heterogeneidad y distribución de dichos elementos en especies de los géneros *Citrus* y *Poncirus*. A partir de una variedad de mandarino clementino se han aislado ocho clones que contienen una parte de la región codificante POL de los retrotransposones tipo *gypsy*. El análisis de sus secuencias parece indicar que cuatro de estos clones corresponden a elementos potencialmente activos (con capacidad de retrotransposición) puesto que presentan intactos, sin codones de paro ni desplazamiento de la pauta de lectura correcta, todos los motivos conservados indispensables para la retrotransposición. Asimismo, algunos de los clones aislados presentan altas homologías tanto con secuencias incluidas en "*clusters*" de genes de resistencia caracterizados en *P. trifoliata* y patata, como con partes de posibles poliproteínas de resistencia encontradas en arroz.

Por otra parte, el análisis de la hibridación molecular de los fragmentos de retrotransposones aislados revela la existencia de copias de elementos *gypsy* en los genomas de *P. trifoliata* y de las especies de *Citrus* estudiadas (*C. limon*, *C. sinensis* y *C. clementina*). El desarrollo de marcadores moleculares a partir de fragmentos de retrotransposones (IRAPs) tipo *gypsy* proporciona nuevas herramientas para el análisis genético de los cítricos. En *P. trifoliata* se ha detectado un menor número de IRAPs y grado de heterozigosidad que en *C. aurantium*.

Los mapas genéticos obtenidos con IRAPs tipo *gypsy* confirman una distribución homogénea (y diferente respecto a los elementos tipo *copia*) de retrotransposones por el genoma de los cítricos. Los resultados también indican que la introducción de retrotransposones tipo *gypsy* en los genomas de *Citrus* y *Poncirus* tuvo lugar con anterioridad a la divergencia de ambos géneros para posteriormente evolucionar independientemente.

En la mejora genética de cítricos uno de los principales objetivos es la resistencia a CTV. La tristeza de los cítricos es la principal virosis que afecta al cultivo de los cítricos en el mundo. La obtención de plantas resistentes a su agente causal (CTV) sería la forma más adecuada de combatir la enfermedad. *P. trifoliata* es una especie resistente al virus y sexualmente compatible con las especies del género *Citrus*, por lo que tradicionalmente en los programas de mejora de cítricos se le ha considerado como la mejor candidata para transferir su resistencia a los cítricos cultivados. El enorme desarrollo de las técnicas de biología molecular ha permitido que en la actualidad sea posible la transferencia directa y única del gen responsable de la resistencia por transformación genética. Para que esto sea factible (y eficaz) es necesario conocer la naturaleza de la resistencia al virus.

Varios estudios anteriores han definido la resistencia de *P. trifoliata* a CTV como un carácter controlado por un único gen (*Ctv-R*). En la actualidad se conoce con bastante precisión cuál es la localización de *Ctv-R* y se está persiguiendo su clonación. El objetivo final es obtener cítricos comerciales resistentes al virus a partir de la transformación genética con el gen de resistencia a CTV. Existen, sin embargo, posibles inconvenientes en este proceso que deben ser considerados. La resistencia no es un termino absoluto ya que depende del aislado, del método de inoculación, del tiempo tras la inoculación y del método de detección, por lo que debería estudiarse la influencia de estos factores sobre la acumulación/multiplicación del virus. Por otro lado, el hecho de que *Ctv-R* sea eficaz en *P. trifoliata* no significa que vaya a serlo igualmente en el fondo genético de otro cítrico transformado con dicho gen.

En la presente tesis se ha saturado con distintos tipos de marcadores moleculares la región donde previamente se había situado *Ctv-R*. Para ello se han utilizado dos familias segregantes derivadas de *P. trifoliata*, una (PpxPp) por autopolinización y la otra (AxPa) por cruce con *C. aurantium* (naranjo amargo), un patrón bien adaptado a las condiciones semi-áridas típicas de la cuenca mediterránea. Para la obtención de marcadores de secuencias expresadas se han puesto a punto metodologías novedosas basadas en el análisis de segregación masal combinado con el de *differential display* y con la construcción de genotecas de sustracción de cDNA.

El seguimiento de la infección con CTV en la familia AxPa ha proporcionado resultados sorprendentes. Amargo resulta tan resistente al aislado del virus ensayado (T-346) como *P. trifoliata*, y tres de los híbridos susceptibles al virus presentan el gen *Ctv-R* tal y como se

deduce del genotipo de los marcadores que flanquean dicho gen. Los mapas saturados resultantes de este estudio se han utilizado para localizar la resistencia al virus bajo la hipótesis de transmisión (herencia) monogénica. Los resultados han sido igualmente inesperados ya que la posición de *Ctv-R* no coincide con la anteriormente descrita en otras progenies. El análisis genético de la interacción virus-planta en la familia AxPa tras la infección crónica con CTV ha revelado cinco tipos distintos de interacción lo cual es incompatible con la hipótesis de un sólo gen de resistencia. Los resultados obtenidos indican que la transferencia del gen *Ctv-R* de *P. trifoliata* al naranjo amargo podría no evitar los síntomas típicos de decaimiento ("*decline*") en naranjo dulce injertado sobre este nuevo patrón.

En la presente tesis también se ha analizado genéticamente la acumulación de CTV en la familia AxPa. Para ello, 104 híbridos fueron injertados sobre naranjo dulce libre de virus y tres meses después se infectó el patrón con un aislado común del virus (T-346) por inoculación por injerto. Transcurrido un año tras la inoculación (y durante al menos dos años consecutivos) se analizó por técnicas inmunológicas (inmunoimpresión-ELISA) la presencia del virus tanto en los híbridos como en los inóculos, y se evaluó la acumulación de viriones de CTV a partir de los valores de densidad óptica obtenidos por ELISA-DAS ("*double antibody sandwich*").

Los mapas saturados obtenidos con anterioridad en este trabajo se han utilizado para localizar la resistencia a CTV considerándola como carácter cuantitativo (acumulación de virus) mediante análisis de QTLs ("*quantitative trait loci*"), evitando así la asunción de control monogénico de la resistencia. Los resultados revelan la presencia de tres QTLs de resistencia principales (de gran efecto), dos en *P. trifoliata* (*Ctv-*R_1 y *Ctv-*R_2) y uno en naranjo amargo (*Ctv-*R_a), en la región donde se había localizado *Ctv-R* en otras progenies. Además, se han detectado otros QTLs de acumulación (*Ctv-*A_1, *Ctv-*A_2, *Ctv-*A_3, *Ctv-*A_4 *y Ctv-*A_5) donde se localizan genes de menor efecto. También se han detectado interacciones epistáticas implicadas en el control genético del carácter.

Ante la posibilidad de que alguno de estos QTLs de acumulación encontrados fuera un gen de resistencia superado por algún haplotipo del virus, se ha estudiado la variabilidad de haplotipos mediante análisis SSCP ("*single strand conformation polymorphism*") de dos genes del virus (p20 y p25) en los híbridos susceptibles de la familia AxPa. Se ha detectado un único haplotipo en todos los casos, incluida la fuente original de inóculo, de lo que se deduce que las diferencias en la cantidad de virus acumulado en el huésped no son debidas a la selección de haplotipos de CTV, sino a diferencias en la capacidad de replicación/movimiento del virus en función del genotipo del huésped.

En función de la posición (máximo valor de LOD) donde se localizan los QTLs en el mapa genético se puede inferir el gen candidato correspondiente, así, un análogo de resistencia es el candidato para *Ctv-*A_3, y dos secuencias expresadas son los candidatos para *Ctv-*A_1 *y Ctv-*A_5.

Conclusiones

1.- El genoma de los cítricos *C. clementina*, *C. aurantium*, *C. limon*, *C. sinensis* y *P. trifoliata* presenta secuencias con alta homología a retrotransposones del tipo *gypsy*.

2.- La traducción *in silico* de algunas de estas secuencias revela la presencia de dominios proteicos funcionales para la retrotransposición autónoma por lo que serán empleados para el estudio de su actividad como fuente de variabilidad genética en los citricos.

3.- La generación de marcadores IRAP a partir de estas secuencias y su mapeo en *C. aurantium* y *P. trifoliata* ha puesto de manifiesto su distribución diferente respecto a los IRAP derivados de secuencias tipo *copia*, por lo que constituyen un grupo complementario y nuevo de marcadores moleculares de gran repetibilidad y potencial polimórfico.

4.- Comparativamente, la actividad de retrotransposones que se ha manifestado como IRAPs es mayor en *C. aurantium* que en *P. trifoliata*, por lo que los mecanismos implicados en el control de su actividad pueden estar relacionadas con: homozigosis, ausencia de reproducción sexual (apomixis) y/o la variedad injertada sobre estos patrones.

5.- *C. aurantium* variedad "Afin Verna" es tan resistente como *P. trifoliata* al aislado T-346 de CTV. Puesto que se trata de un aislado procedente de un árbol de satsuma (injertado sobre naranjo amargo) con claros síntomas de tristeza, es posible que la enfermedad sea consecuencia de una respuesta hipersensible de resistencia ante una prolongada y abundante fuente de inóculo en la variedad. De ser así, la transferencia al naranjo amargo del gen de resistencia al virus presente en *Poncirus* (*Ctv-R*) podría no evitar la muerte de variedades susceptibles injertadas.

6.- *Las* nuevas estrategias diseñadas para enriquecer con secuencias expresadas la región genómica donde se localiza *Ctv-R* han resultado efectivas. Gracias a ellas ha sido posible la comparación de la organización de los grupos de ligamiento 4, 7 y 3 de *P. trifoliata* y *C. aurantium*, poniéndose de manifiesto la existencia de una translocación que afecta a la secuencia expresada VIC correspondiente a un gen candidato implicado en la acumulación de CTV.

7.- El análisis genético de la interacción CTV-citradia ha revelado la presencia de cinco tipos distintos de interacción, así como la existencia de citradias resistentes sin *Ctv-R* y citradias susceptibles con *Ctv-R*. Estos resultados son incompatibles con la hipótesis de control monogénico de la resistencia.

8.- Dado que la hipótesis monogénica no es asumible, la mejor metodología de análisis genético del carácter es mediante el análisis de QTLs a pesar de que la distribución del carácter es no normal.

9.- Es necesario el desarrollo de nuevas metodologías de análisis de QTLs para caracteres de distribución no normal y diseños experimentales de cítricos. Puesto que las disponibles actualmente no cuentan con estas características, desconocemos la magnitud de los errores tipo I y II.

10.- El análisis de QTLs de acumulación de CTV ha permitido seleccionar para su posterior estudio genes candidatos para los QTLs *Ctv-A*$_1$, *Ctv-A*$_3$ y *Ctv-A*$_5$.

11.- En la región homeóloga de Pa4 en el mapa de amargo se localiza también un QTL de resistencia a CTV (*Ctv-R*$_a$).

12.- Los genes implicados en la acumulación de CTV no actúan aisladamente sino que al menos algunas interacciones (epistasias) son importantes como por ejemplo la detectada entre *Ctv-R*$_{1,2}$ y VIC (*Ctv-A*$_1$).

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