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FINAL REPORT
CONTRACT 90/093
FAO
ARGENTINA

INFORME ANUAL DEL PROYECTO "RESISTENCIA A VIROSIS EN PAPA"

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Junio 1991.

ORGANIZACION DEL INFORME:

I>- Descripción de lo realizado en los servicios comprometidos en el contrato con ONUDI para el segundo año)

Servicio A.- Firma del contrato

Servicio B.- Búsqueda de genes de resistencia. Infección "in vitro" de protoplastos obtenidos de genotipos resistentes de papa y evaluación de la replicación viral.

Servicio C.- Búsqueda y selección de clones codificantes para la cápside viral de PLRV.

Servicio D.- Construcción de genes quiméricos de resistencia a virosis
- D.1- PVX
- D.2- PVY
- D.3- PLRV

Servicio E.- Establecimiento de las técnicas de transformación de papa de interés para Argentina.

Servicio F.- Transformación de plantas de papa
- F.1- PVX
- F.2- PVY
- F.3- PLRV

F.4- Entrenamiento de una estudiante graduada en Mexico.
Servicio G.- Presentación de informes semestral y anual.

II>- Presentaciones a congresos y publicaciones.

III>- Intercambios e interacciones con otros países participantes del proyecto.

IV>- Evaluaciones de cumplimiento de Argentina y del proyecto en su conjunto.

I>- Desarrollo de actividades:

Servicio B.- Búsqueda de genes de resistencia. Infección "in vitro" de protoplastos obtenidos de genotipos resistentes de papa y evaluación de la replicación viral.

RESUMEN DE LA ACTIVIDAD PREVIA YA REALIZADA

1- Se obtuvieron protoplastos de papa de variedades locales (Huinkul, susceptible a PVX; Serrana-INTA, inmune a PVX) y extranjeras (Spunta, de amplia difusión en la Argentina y susceptible a PVX), obteniéndose un promedio de 106 protoplastos viables por gramo de tejido (hojas) de partida.
2-El PVX se aisló, purificó y cuantificó en base a su absorbancia. Protoplastos obtenidos de cultivares de papa susceptibles al PVX se infectaron con PVX en alta multiplicidad de infección en presencia de fusógenos químicos (PEG). Un 60 % de protoplastos sobreviven al tratamiento y de estos, más de un 70 % queda infectado con virus.
3- La multiplicación viral a lo largo del tiempo se analizó tomando alícuotas a distintos tiempos post infección hasta completar 40 horas mediante los siguientes parámetros: a) Incorporación de aminoácidos marcados (3H-Leu) a proteína de cubierta por inmunoprecipitación. b) Desaparición y posterior reaparición (síntesis) de proteína de cubierta por ELISA con anticuerpos policlonales y conjugados monoclonales anti-PVX. c) Síntesis de RNA genómico viral por hibridación molecular mediante la técnica del "dot-blot" con la sonda pX1 (cDNA de PVX). d) Inmunofluorescencia de los protoplastos.
4- Los resultados obtenidos nasta ahora indican que tras un período de aproximadamente 15 horas (valle del ELISA) se dispara la síntesis de proteína de cubierta en forma continua alcanzando un plateau a las 30 hs. La síntesis de RNA genómico empieza unas 5 hs. antes siguiendo una tendencia similar a la de la proteína de cubierta.
5- Por otro lado, se probó la infección en paralelo de protoplastos de variedades de papa susceptible e inmune al PVX a las siguientes densidades de infección: 0.01 ng PVX/protoplasto viable/ul; 0.1 ng PVX/protoplasto viable/ul y 1 ng PVX/protoplasto viable/ul; los resultados indican que en condiciones de bajo inóculo (densidades de infección de 0.01 y 0.1) la concentración de antígeno de cápside viral en los protoplastos de la variedad inmune, monitoreada mediante la técnica de ELISA, muestra un 90 % de reducción respecto a la de protoplastos de variedades susceptibles; sin embargo, cuando el inóculo es alto (densidad de infección de 1 ng), la cinética de replicación viral en los protoplastos de la variedad inmune medida a través de la producción de antígeno es análoga a la de la variedad susceptible control.
6- Experimentos de "slot-blot" realizados con infecciones de protoplastos de las variedades susceptibles e inmunes en paralelo, a densidades de infección bajas (0.1 ng) confirmaron una menor producción de RNA viral por protoplasto viable en la variedad inmune respecto a la producción de RNA viral observada en la variedad susceptible, lo cual sugeriría que la disminución relativa de producción de antígeno de cápside observada en protoplastos de la variedad inmune Serrana se vería acompañada por una disminución en la cantidad de RNA genóico viral.

7- Experimentos de inmunofluorescencia realizados con protoplastos de las variedades susceptibles e inmunes infectados a una densidad de infección baja (0.1 ng), muestran una menor acumulación de antígeno (cápside viral) por protoplasto (fluorescencia más débil) en la variedad inmune respecto a la observada en los protoplastos de la variedad susceptible control; estos resultados estarían indicando que la diferencia en la producción viral entre ambos tipos de protoplastos susceptibles e inmunes no se debería a una infección y replicación diferencial del virus en una sub-población celular de Serrana sino a una inhibición de la multiplicación viral que existiría en todas las células de esta variedad.
8- Además de estos experimentos realizados con protoplastos de ambos tipos de cultivares de papa, se realizaron Westerns y Northern blots a partir de hoja de cultivares de papa (susceptible e inmune) infectados y no infectados con PVX (controles), cuyos resultados confirmaron que in vivo la inhibición de la replicación viral en el cultivar inmune es practicamente del 100 %, dentro del marco de sensibilidad que estos sistemas de detección permiten.
9- Gran parte de estos resultados se publicaron el año pasado y durante el presente año (Saladrigas et al. 1990a y 1990b, Ceriani et al. 1990 y Tozzini et al. 1991).

ESTUDIO DE LA EXPRESION DEL GEN DE RESISTENCIA Rx acl A NIVEL CELULAR Y TISULAR.

En la foto A se muestran estacas de plántulas de los cultivares Huinkul-MAG y Serrana INTA, recién micropropagadas.

En la foto B se muestran plántulas del cultivar susceptible Huinkul MAG inoculadas con PVX y buffer (control) de un mes de crecimiento in vitro. La inoculación se realiza en las hojas medias de la plántula (*) y al cabo de 1 mes, las hojas inoculadas con virus o con buffer no presentan diferencias entre sí.

Un mes después de la infección, RNA aislado de hojas infectadas con PVX o buffer (control) de los cultivares susceptibles Huinkul MAG y Spunta, y resistente Serrana INTA, se corrió electroforéticamente en gel desnaturalizante de agarosa al 1.2 % . El Northern blot, revelado con una sonda dirigida contra genes tempranos y tardíos de PVX, el clon de cDNA de doble cadena 5x41, mostró hibridación positiva contra RNA genómico y otros RNAs de menor peso molecular, en el caso de las muestras susceptibles, pero no así en el caso de la muestra proveniente del cultivar inmune (foto C).
En paralelo a los RNAs mencionados se corrió el virión entero, que en las condiciones electroforéticas mencionadas, corre como una nucleoproteína de 8.7 kb. Se incluyó además en la corrida un escalera de RNA ("ladder", BRL) para la cuantificación del peso molecular en Kb de los RNAs subgenómicos. RNAs controles provenientes de hoja pseudoinoculada de 1 mes, con buffer y sin virus, muestra hibridación negativa.
Como se puede observar en la foto, además de la banda correspondiente a RNA genómico de 6.4 Kb (marcado con g en la foto) y RNA-proteína asociada de 8.7 Kb (marcado np), existen numerosas bandas correspondientes a posibles RNAs subgenómicos virales (sg) Algunas de esas bandas son netas y claras (bandas de 4, 3, 2.5, 2.2 y 2 Kb) pero otras son difusas, sugiriendo heterogeneidad de tamaño en los RNAs componentes, quizás debido en parte a degradación diferencial de esas especies respecto a las otras (relacionado con la estructura de los mismas, banda ancha y bien marcada de alrededor de 5.2 Kb, y bandas anchas y difusas de rango de peso molecular 1.1-1.6 y 0.1-0.9 Kb).

La intensidad autoradiográfica relativa de las bandas depende de la cantidad de especies de RNAs por un lado, y de la calidad de estos RNAs, por el otro, (por ejemplo largo de la especie subgenómica). Parecería que las especies mayoritarias son las correspondientes a los RNAs de rango de peso molecular de alrededor de 5.2 Kb y 4 Kb. Le seguirían en orden, RNAs de rango de peso molecular bajo: 1.1-1.6 y 0.1-0.9, y por último, los RNAs de peso molecular intermedio 3, 2.5, 2.2 y 2.

La estrategia de replicación a través de RNAs subgenómicos intermediarios no es novedosa y de hecho, con respecto a PVX, ya existen trabajos publicados en donde se muestran Northern blots realizados con RNAs extraídos de hoja o células infectadas que hibridan con distintas especies de RNAs de menor tamaño que el genómico (Dolja, 1987; Karasev,1987 ; Adams,1985). Sin embargo, los pesos moleculares asignados a esos RNAs subgenómicos varían según el grupo de trabajo (Dolja, 1987;Karasev A.V,1987).

Se han informado por lo menos 6 tipos de RNAs subgenómicos, todos 3' co-terminales y con análogos de doble cadena (Dolja, 1987), los pesos moleculares para estos RNAs en el trabajo de Dolja son: 3.6, 3, 2.1, 1.8, 1.4 y 0.9 Kb. A continuación, construimos una tabla para la visualización de diferencias y coincidencias en los resultados con este grupo:

Especie subgenómica PM (Kb)		Material de partida	
Dolja	Nosotros	Dolja	Nosotros
(*)	5.2a	Hoja	Hoja
3.6	4	Id	Hoja
3	3	Id	Hoja
--	2.5	Id	Hoja
2.1	2.2	Id	Hoja
1.8	2	Id	Hoja
1.4	(1.6-1.1)a	Id	Hoja
0.9	(0.9-0.1)a	Id	Hoja

a= determinación aproximada del peso molecular.

(*)= Aunque los autores no lo mencionan como subgenómico posible, en la figura 1 del trabajo aparece una banda subgenómica de alto peso molecular, que podría corresponder a esta especie.

El subgenómico de menor peso molecular, 0.9 Kb, es la expresión <u>in vivo</u> del gen de la cápside viral; a través de este subgenómico el virus produce proteína de cápside necesaria para la formación de viriones y aunque de su función ya no quedan dudas, existen diferencias en cuanto al peso molecular asignado al mismo: mientras un grupo informa 0.9 Kb (Dolja, 1987) otro informa 1 a 1.3 Kb (Karasev, 1987). Nuestros resultados estarían más a favor de los del primer grupo (0.9-0.1 Kb). En cuanto a los otros, pueden encontrarse similitudes en el peso molecular para algunos de ellos (3.6/4, 3/3, 2.1/2.2, 1.8/2, 1.4/(1.6-1.1) y 0.9/(0.9-0.1)), pero nuestros resultados arrojan claras diferencias en lo que respecta a por lo menos dos: Una banda mayoritaria de alto peso molecular (alrededor de 5.2 Kb) y otra banda menor pero también clara de 2.5 Kb.

Se atribuye a cada especie subgenómica simple cadena (de igual polaridad que el RNA genómico viral, por definición) su correspondiente de doble cadena (Dolja, 1987). Si esto es cierto, el aislamiento de RNA bicatenario a partir de una preparación de RNA total de hoja infectada por columnas de CF-11 y posterior corrida en gel de agarosa desnaturalizante debería mostrar un patrón electroforético de bandas equivalente al de la figura-foto 1 (ver HUIi).

En la foto D, se muestra una corrida electroforética de RNA total de

hoja infectada, RNAs poliA+ aislados a partir de la muestra de RNA total anterior, y un aislado de RNA bicatenario a partir de la misma muestra, hibridados contra la misma sonda anterior. En el gel del cual se obtuvo el northern, no fue posible sembrar una misma cantidad de RNAs por well, de modo que solo se sacarán conclusiones cualitativas y no cuantitativas.
Se vislumbran dos bandas muy tenues en la autoradiografía, de peso molecular igual a 6.4 y 5.7 aproximadamente, presentes en la muestra de RNA bicatenario. La no aparición de otras bandas, no es prueba de la no existencia de otros posibles intermediarios bicatenarios, ya que es posible que utilizando cantidades mayores a 100 ug de RNA total (en este experimento se utilizaron 100) en el aislamiento de RNAs bicatenarios por columna CF-11, puedan visualizarse.
Lo interesante de estos resultados es que las bandas que aparecen como mayoritarias en la foto C (ver Huii), es decir, las especies de 6.4 y la de 5.2 (aproximado) Kb, tendrían su contraparte bicatenaria, a juzgar por las bandas que aparecen en la foto D (6.4 y 5.7 Kb). En la primer figura, la especie de aproximadamente 5.2 Kb hibrida fuertemente con la sonda, produciendo una banda muy gruesa y el peso molecular estimado de 5.2 Kb está tomado considerando el centro del grosor de la banda, por lo cual discutir diferencias de peso molecular de 0.5 Kb (5.7-5.2) no tiene sentido.
Más interesante es comprobar que en el trabajo de Dolja (1987) también se han encontrado especies subgenómicas bicatenarias de alto peso molecular (5.0 Kpb, corridas en condiciones no desnaturalizantes en gel de agarosa teñido con bromuro de etidio), esta especie, si bien no es mayoritaria, también se vislumbra en la autoradiografía del Northern blot de RNA total de planta infectada, aunque los autores no la marcan como probable subgenómico.
En la Foto D, se vislumbra en la foto de la autoradiografía un intenso chorreado en la calle correspondiente a RNAs poli A+, pero como la muestra de poli A+ corrida en gel no mostró una intensa degradación en las bandas observadas, no podemos atribuir el chorreado a la degradación de la muestra original. Más bien, podría decirse que el chorreado correspondería a la existencia de cadenas genómicas y subgenómicas incompletamente sintetizadas y en formación. Se vislumbra sí, una banda poli A+ de peso molecular correspondiente al del RNA virión (ver Hipa+).

Protoplastos infectados y no infectados se resuspenden en medio de incubación A y se llevan a cámara de 210C. A distintos tiempos post infección se toman alícuotas celulares para la extracción proteica. Dos horas antes del tomado de las muestras, se agrega al medio el precursor radioactivo según se detalla en materiales y métodos.
Luego de extraer las proteínas y de resuspenderlas en el buffer de corrida electroforética, estas se corren en un gel discontínuo de poliacrilamida al 12 %, sembrándose por well una misma cantidad de cuentas TCA precipitables (ver materiales y métodos). Como marcadores de peso molecular se utilizaron stándares comerciales de rango 200-14.6 Kdaltons, corridos en una calle en paralelo con las muestras de proteínas marcadas; finalizada la corrida la banda correspondientes a estos marcadores se separa del gel y se tiñe con azul de Coomasie. El resto del gel se procesa para autoradiografía.
La foto E, muestra un gel al 12 %, con muestras de proteínas de células infectadas y no infectadas de los cultivares inmunes y resistentes, cuyo orden de siembra fue:

PVX* M@ Hinf 2 4 6 8 22 46 Hcon 4 6 8 22 SEinf 4 6 8 22 SEcon 4 6 8 22

En cada calle se sembró aproximadamente 100.000 cpm.

*= Proteína de cápside viral.
@= Marcadores de peso molecular.
Los números indican el tiempo post-infección al que se sacaron las muestras. La cinética de replicación viral alcanza su plateau a las 40 hs.
Acompañando a la foto, incluímos un gráfico de migración de los estándares de peso molecular. La línea que está marcando PVX, muestra que la proteína de la cápside viral, migra como una proteína de peso molecular igual a 28 Kda, en concordancia con otros trabajos.

En base a su interacción con los genes de hipersensibilidad Nb y Nx, o con el gen de resistencia extrema Rx, las cepas de PVX pueden clasificarse en 5 grupos:

Genotipo	grupo de cepa viral				
	1	2	3	4	HB
nx nb	sa	s	s	s	s
Nx nb	R	s	R	s	s
nx Nb	R	R	s	s	s
Nx Nb	R	R	R	s	s
Rx(acl)	R	R	R	R	s
Rx(adg)	R	R	R	R	s

aR=Resistente; s=susceptible. La resistencia está asociada a un alelo dominante, mientras que la susceptibilidad a un alelo recesivo (Tavantzis 1990; modificado de Cockerham, 1955) El primer tipo de resistencia extrema al virus fue informada por Schultz y Raleigh (1933). La resistencia parecía estar codificada por un único gen dominante (Cockerham,1970).
La resistencia provenía de un cultivar salvaje Solanum andígena y fue transferida al clon Solanum tuberosum USDA 41956. Este gen, llamado Rx(adg), fue incorporado a cultivares comerciales norteamericanos.
Otro caso de resistencia extrema fue informado por Ross en 1954, y fue encontrado en Solanum acaule. Este gen se denominó Rx(acl), y ha sido incorporado a algunos cultivares europeos. La relación entre Rx(adg) y Rx(acl) es desconocida.
Una cepa o grupo de cepas de PVX designada PVX-HB, común en el 7 % de los clones bolivianos, se comporta como un aislado virulento frente a este gen, es decir, es capaz de replicar en aquellos cultivares que lo poseen. También replica en cultivares que poseen los genes de hipersensibilidad, sin embargo, no lo hace frente a Solanum sucrense, un cultivar que posee un gen dominante denominado Rx(scr), que le confiere "inmunidad" extrema contra PVX-HB.
Hasta hoy, varias décadas después de la incorporación de los genes Rx(adg) y Rx(acl) a cultivares comerciales, no existen evidencias que cepas del grupo HB constituyan una amenaza importante para aquellos cultivares norteamericanos o europeos que poseen esos genes. Por lo tanto, siendo estos genes de resistencia extrema de alto valor agronómico, es muy deseable su caracterización.

Caracterización de los genes de virulencia de los aislamientos argentinos y extranjeros de PVX. Se infectaron razas fisiológicas indicadoras de papa a fin de clasificar dos aislamientos realizados por la Fac. Cs. Agrarias de Córdoba y compararlos con otras razas caracterizadas. Los resultados preliminares indican que en condiciones de laboratorio, son capaces de superar el gen Rxacl presente en Serrana INTA y en entradas de S. acaule manifestando un comportamiento que recuerda a la raza HB. Sin embargo, análisis inmunológicos realizados en el CIP indicaron que ambos aislamientos no tienen relación con el llamado grupo andino de razas de PVX al que pertenece HB.

FOTO A

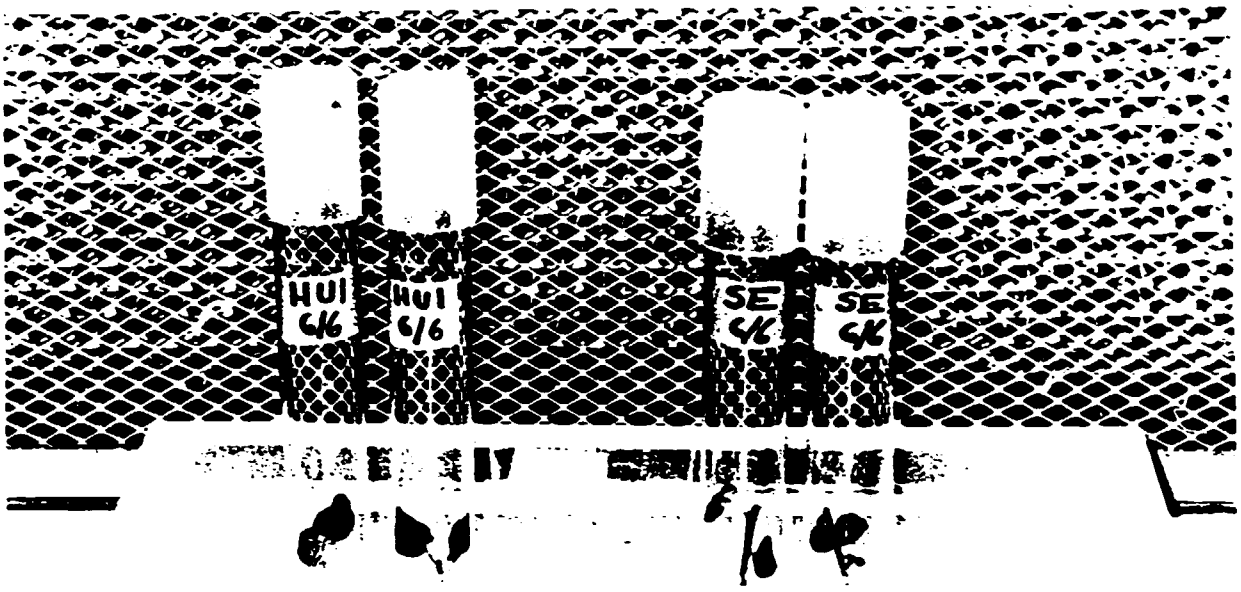


FOTO B

FOTO C

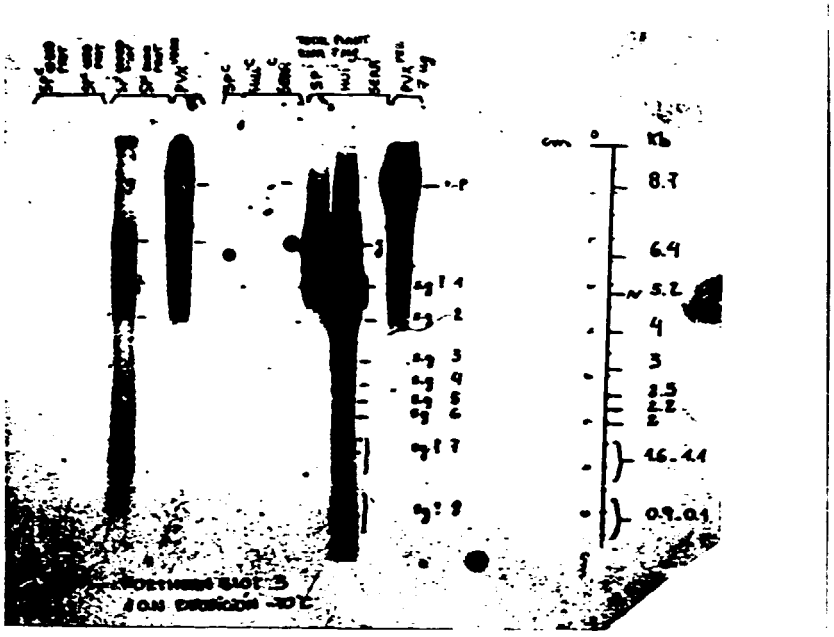


FOTO D

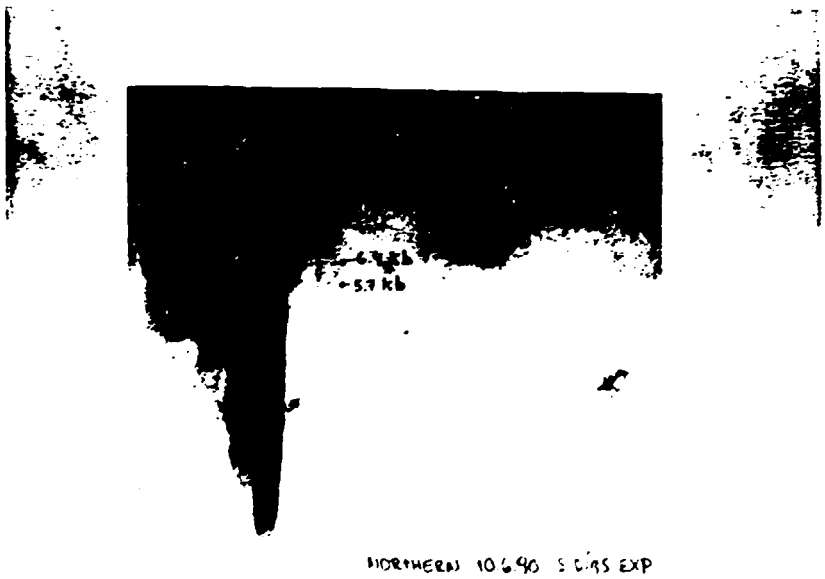


FOTO E

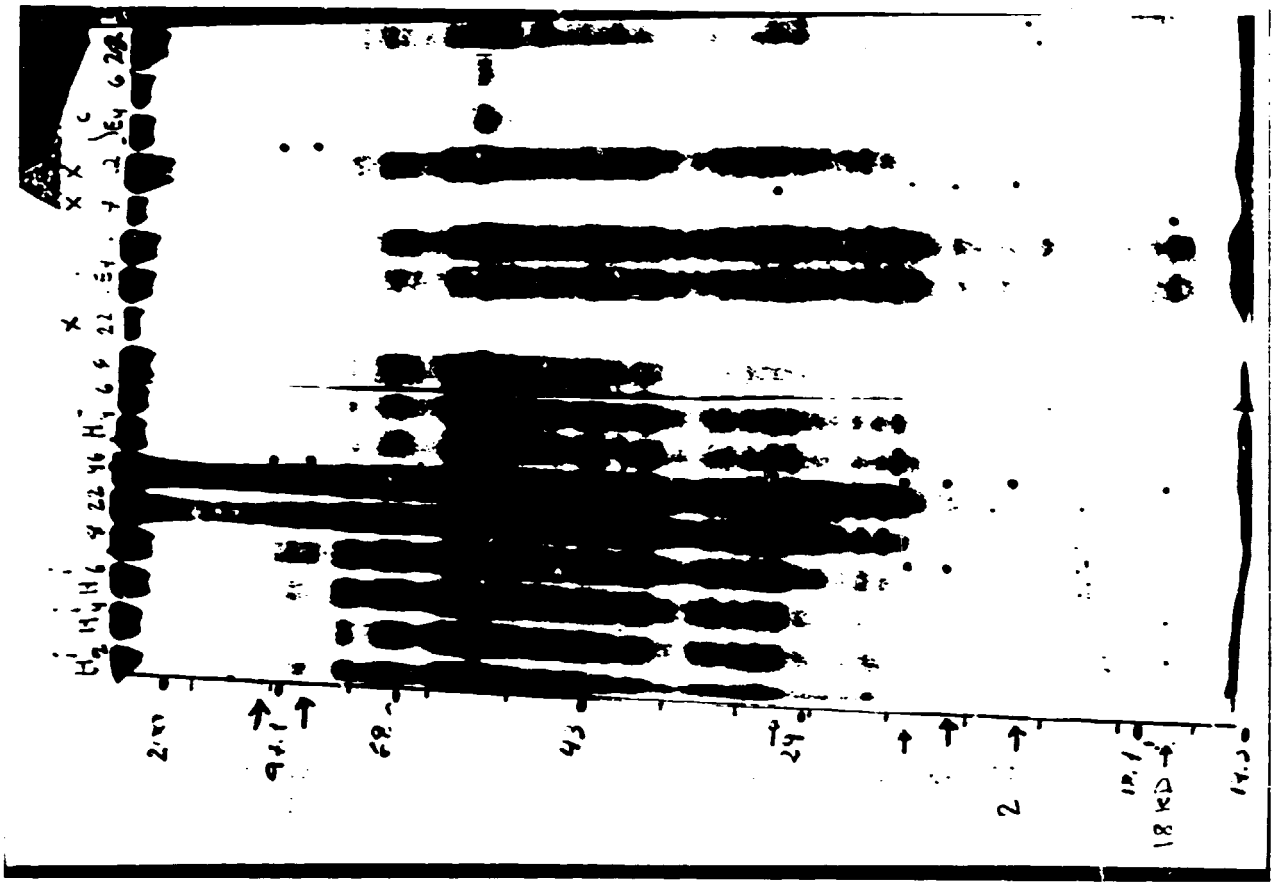
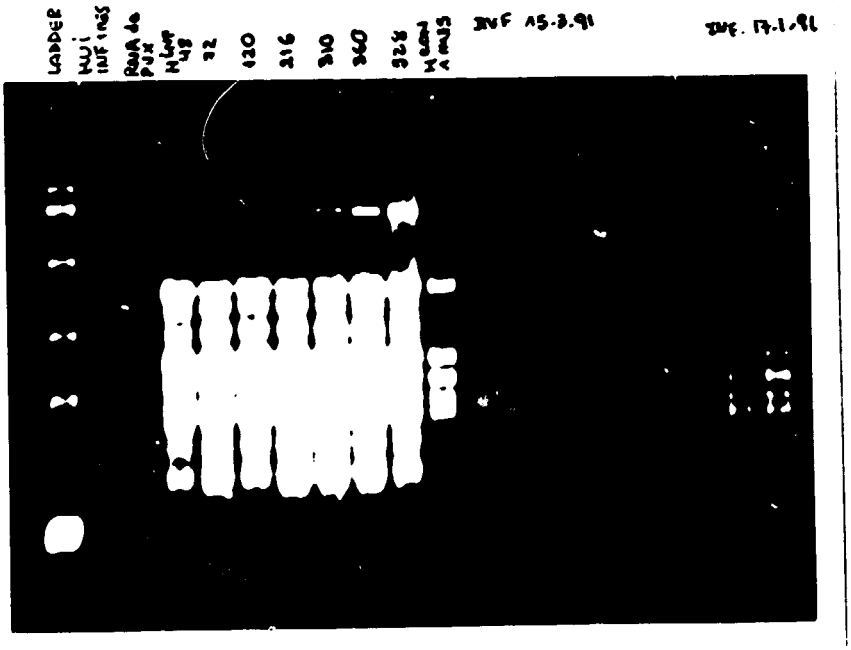


FOTO F



BEPSA-TGE Resultados y primeras conclusiones

Los resultados, por causas desconocidas, mostraron una gran variación dentro de cada tratamiento (combinación genotipo hospedante/genotipo patógeno). Los valores promedios presentaron una gran variancia por ello es difícil obtener conclusiones estadísticamente fundamentadas. La primera técnica utilizada para el análisis de estos resultados fue realizar un estudio de máxima; esto es tomar el mayor valor obtenido para cada tratamiento. En el presente estudio, donde lo que se esta evaluando es la resistencia ofrecida por cada genotipo a la replicación de cada raza viral, esta técnica de análisis implica situarse en la peor situación desde el punto de vista de la resistencia. Los resultados y conclusiones que a continuación se presentan estan basados en el análisis de máxima, sin embargo, cabe aclarar que los histogramas obtenidos mediante esta técnica son equivalentes a los histogramas promedio presentando estos últimos valores proporcionalmente menores.

ANALISIS POR RAZA VIRAL

PVX cp

Resultados

Muestra la máxima concentración en los tejidos del hospedante susceptible (cv.Maris Bard - nxnb). El cv. King Edward con el gen de resistencia Nx, el cual es inefectivo frente a la raza cp, mostró una replicación del virus del mismo orden que Maris Bard. En ambos cv. no hubo diferencias entre los títulos observados en las hojas inoculadas (hi) y sistémicas (hs).

El cv. Maris Piper, portados de los genes de resistencia Nx y Nb, presentó concentraciones un orden menor que los cultivares susceptibles. Tambien se evidencia una disminución en el título observado en las hojas sistémicas con respecto a las inoculadas del mismo cv.

Frente al gen de inmunidad Rx (Serrana INTA) el virus practicamente no replicó, observandose concentraciones practicamente iguales a cero, tanto en las hojas inoculadas como en las sistémicas (ver tabla 1).

Conclusiones

La raza cp mostró el patrón replicativo que se esperaba según el comportamiento descripto en la bibliografía frente a los genes Nx, Nb y Rx. Sin embargo no se conoce ninguna publicación que caracterice este comportamiento cuantificando las concentraciones mediante ELISA u otra técnica.

PVX HB

PVX HB

Resultados

Este virus mostró concentraciones similares en todos los genotipos (de 30 a 120 ng/gr). No se observaron diferencias entre los títulos observados entre hojas inoculadas y sistémicas con excepción las concentraciones observadas en las hojas sitemicas de los genotipos portadores del gen Rx (Serrana INTA y Ale-S. acaule), las cuales fueron dos ordenes de magnitud menor.
Los niveles registrados de PVX HB son inferiores a los observados para la raza cp, no alcanza los altos títulos que PVX cp desarrolla en Maris Bard (nxnb) y King Edward (Nx) (ver tabla 1).

Conclusiones

La raza HB infectó a todos los genotipos por igual, siendo todos ellos igualmente "susceptibles". El gen Rx (Serrana y Ale) inhibio la dispersión sistémica del virus, dato que contradice lo publicado. La caracterización existente de esta raza esta basada en la sintomatología y en la presencia del virus evaluada por back-inoculation, por ello, es posible que las concentraciones observadas sean las normales y suficientes para lograr los síntomas descriptos por otros investigadores.

PVX HF

Resultados

Alcanzó su máxima concentración en hi (200 ng/gr) en el genotipo Maris Piper (NxNb) y la mínima (6ng/gr) en el genotipo K. Edward (Nx). Los valores fueron intermedios en Serrana (Rx - 37 ng/gr) y M. Bard (nxnb - 52 ng/gr). Con respecto a las hojas sistémicas, las concentraciones fueron similares (30ng/gr) en los cv. M. Bard, M. Piper y K. Edward; Serrana mostró el menor valor (7 ng/gr) (ver tabla 1).

Conclusiones

La máxima concentración en hi la alcanza frente a los genes NxNb mostrando que esta raza es capaz de superar ambos genes, sin embargo el valor mínimo se obtiene frente al gen Nx. Esto podría indicar que la raza HF estaría reaccionando con otro gen de resistencia presente en el background de K. Edward. Para las h. sistémicas las concentraciones son iguales.
Los títulos observados frente al gen Rx indican que esta raza supera a este gen, mostrando un comportamiento similar a la raza HB.

PVX MS

Resultados

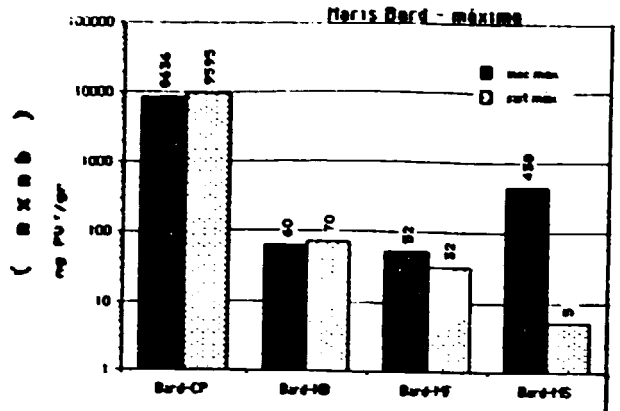
Alcanza su valor máximo en M. Bard y su mínimo en Serrana. En Piper y K. Edward los valores son del mismo orden, siendo menor en este último cultivar (ver tabla 1).

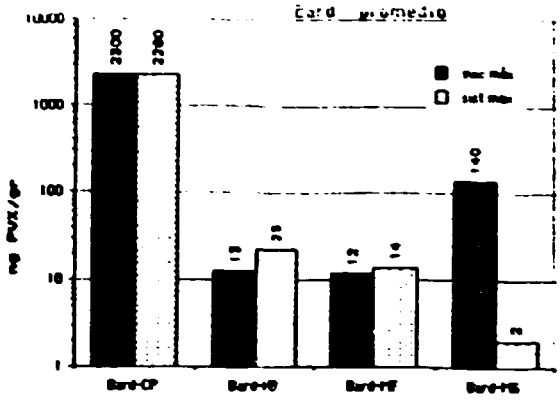
Conclusiones

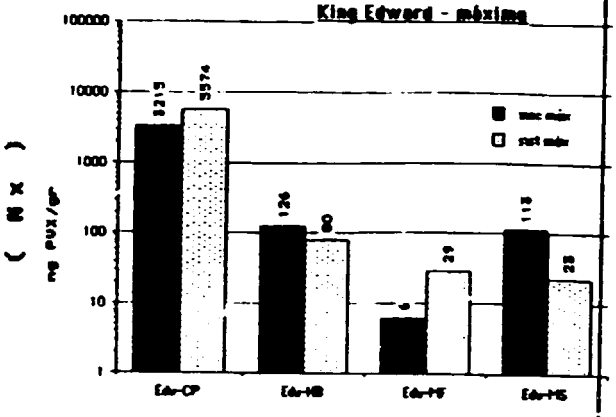
Los títulos alcanzados tanto en el cv. susceptible (M. Bard) como en los portadores de los genes Nx y NxNb (K. Ed. y M. Piper respec.) son similares indicando que esta raza superaría a estos genes. De igual manera, el análisis del comportamiento frente al gen Rx muestra que es capaz de superar a este gen también; sobretodo teniendo en cuenta los resultados de las razas cp y HB. El gen Rx no sería tan efectivo para impedir la dispersión sistémica de esta raza como lo es con respecto a la raza HB.

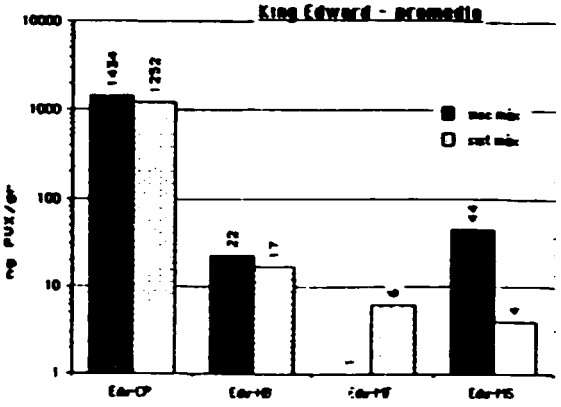
Tabla 1: Análisis de Máxima. Concentraciones de PVX expresadas mediante su orden de magnitud.

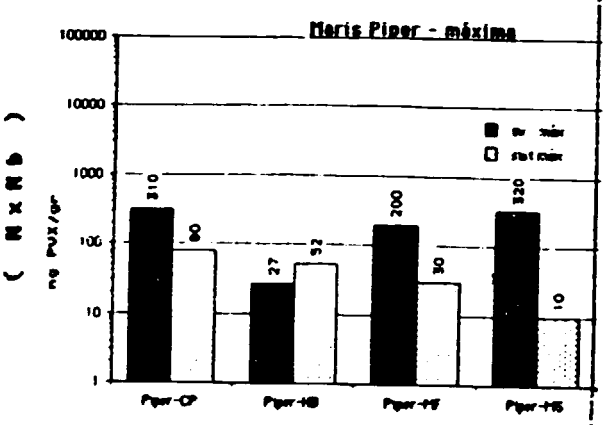
Genes	cp in.	cp sis.	HB in.	HB sis.	MF in.	MF sis.	MS in.	MS sis.
nx nb	4	4	2	2	2	2	3	1
Nx	4	4	3	2	1	2	3	2
Nx Nb	3	2	2	2	3	2	3	1
Rx	0	0	2	1	2	1	2	2

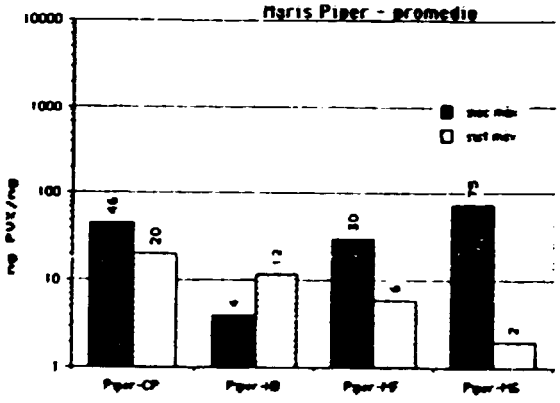

Maris Bard - máxima
(M x m b)
ng PUX/gr
Bard-CP
Bard-HB
Bard-HF
Bard-HS

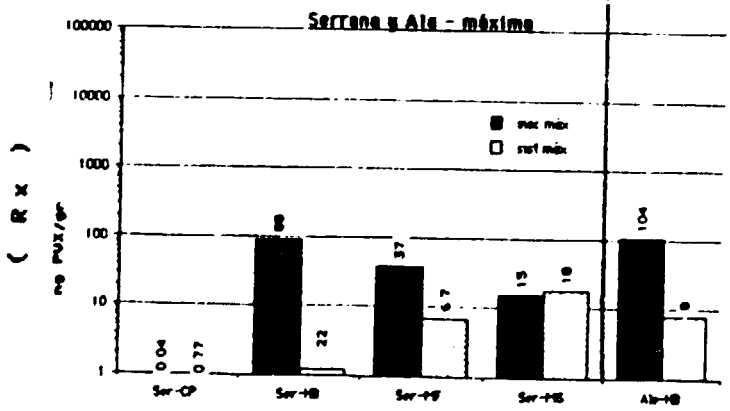

ng PUX/gr

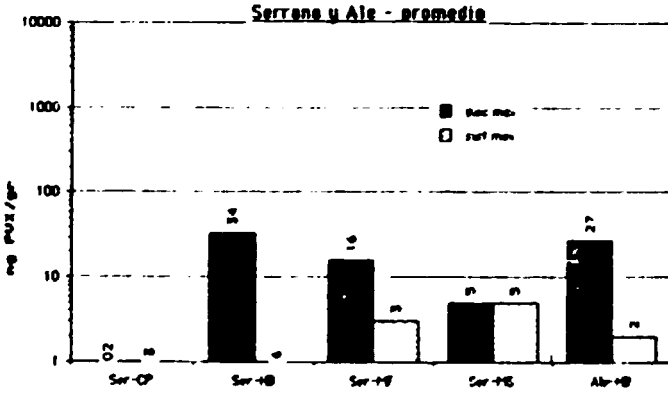

King Edward - máxima
(M x)
ng PUX/gr
Edw-CP
Edw-HB
Edw-HF
Edw-HS


King Edward - promedio
ng PUX/gr


Maris Piper - máxima
(M x M b)
ng PUX/gr
Piper-CP
Piper-HB
Piper-HF
Piper-HS


Maris Piper - promedio
ng PUX/gr


Serrana y Ale - máxima
(R x)
ng PUX/gr
Ser-CP
Ser-HB
Ser-HF
Ser-HS
Ale-HB


Serrana y Ale - promedio
ng PUX/gr

PUESTA A PUNTO Y UTILIZACION DE LIPOFECTINA PARA LA TRANSFORMACION DE PAPA E INOCULACION ARTIFICIAL DE PROTOPLASTOS DE PAPA CON VIRUS:

1) Inóculo adsorvido:
El Inóculo (PVX) adsorvido por lipofectina es menor al adsorvido por PEG (en general del 3 al 6 % del adsorvido por PEG).
El virión en contacto con los protoplastos parece tener una entrada espontánea a lo largo de las 2 hs de incubación. La presencia de lipofectina no incrementa demasiado esta entrada de virión. La producción de viriones progenie a partir de un inóculo viral con o sin lipofectina parece ser equivalente a las 48 hs.
La presencia de PEG y no de lipofectina es lo que determina la entrada eficiente de virion a la célula. Esto se desprende de los controles de infección viral realizados según el trabajo del Dr.Chua para transfección estable con DNA en protoplastos de arroz. En este paper, donde se dice lograr la transfección estable utilizando una combinación de PEG-lipofectina no se incluye el control correspondiente de transfección con la misma densidad de PEG sin lipofectina. Cabe uno preguntarse si la transfección positiva lograda en dicho trabajo se debió o no a la lipofectina.

2) Toxicidad de lipofectina:
La temperatura de incubación con lipofectina es crítica en lo que respecta al cultivar utilizado: mientras que para algunos temperaturas de hasta 37 oC y no lavado de la lipofectina luego de la incubación pueden ser inocuas (SPUNTA), para otros la mejor viabilidad se obtiene con bajas temperaturas y lavado de lipofectina luego de la incubación con la misma (HUINKUL,SERRANA). En estos últimos casos se observa incluso un mejoramiento y mantenimiento de la fluorescencia en general tanto en controles no infectados como en celulas infectadas con el virus a lo largo del ciclo viral. A horas más allá de las 48, se han observado figuras en división celular (otra indicación de la inocuidad de los liposomas).
En comparación con el método del PEG, la utilización de los liposomas permite utilizar de partida un menor número de células (usualmente un orden de magnitud inferior) por la poca toxicidad que estos poseen,manteniendose una buena viabilidad a lo largo de todo el ciclo de replicación viral. Aquellos protoplastos infectados o pseudoinfectados por el método del PEG, muestran una mayor tasa de mortalidad a lo largo del ciclo viral que los tratados con lipofectina, poseyendo además, ya desde el tiempo 0 del ciclo viral, una viabilidad también menor con mayor preponderancia de fluorescencia del tipo verde pálida a rojiza.
Una cantidad óptima de lipofectina a utilizar parece estar en el rango de 30 a 45 ug/millón de protoplastos viables. Dado que la utilización de cualquiera de estos dos valores produjo resultados equivalentes en lo que respecta a la viabilidad celular y a la producción de virión, se tomó como concentración de elección la menor. No se probaron concentraciones menores o mayores de liposomas para infección o transfección vía RNA.

3) Transfección vía lipofectina:
El método de la lipofectina permite la transfección y expresión de RNA viral en protoplastos.
Utilizando una misma densidad de infección (30 ng RNA/ 300 pv/ul) e independientemente del método de preparación de RNA (guanidina o TAE/SDS), la elección de la temperatura es crítica: bajas temperaturas permiten una mayor entrada de RNA y su expresión (producción de antígeno viral) puede detectarse ya a las 20 hs por ELISA. En cambio,

temperaturas mayores (ambiente: 25-30oC) parecen hacer menos eficiente la captura de RNA por la célula, de modo que la producción de antígeno solo es detectable a partir de las 40 hs (fin del ciclo viral).
Un corto período de preincubación a 0 oC (usualmente 10 min) del complejo liposomas/RNA parece contribuir a la eficiencia de entrada del ácido nucleico.
Se probaron dos densidades de transfección vía lipofectina diferentes: Una de bajo inóculo dió negativa la producción de antígeno viral por ELISA a las 40 hs (10 pg RNA viral/pv/ul), en cambio un inóculo un orden mayor (100 pg RNA viral/pv/ul) dió infección positiva.

Se comparó este protocolo de transfección vía lipofectina con otro de transfección vía PEG (sacado de Maule y Boulton, 1980, J.Gen.Virol. 47:199-200). Este último protocolo también se probó a dos densidades de infección distintas (las mismas que para lipofectina) resultando no sólo negativa la producción de antígeno de cápside por ELISA a 40 hs post infección, sino también un efecto notablemente deletéreo sobre la viabilidad y la tasa de mortalidad en general, luego del tratamiento con el PEG.
Es de notar que en el trabajo original de Maule et al. se parte de una cantidad de células a infectar dos órdenes de magnitud mayor de la que se parte en forma estándard en nuestro caso (1x107 y 1x105 respectivamente) y de una cantidad también notablemente mayor de RNA (0.5 mg RNA).
Otros protocolos similares de transfección de RNA vía PEG, si bien pueden ser efectivos utilizando densidades de transfección menores que las nuestras con lipofectina (entre 8 a 50 pg RNA/pv) necesitan también partir de una cantidad de células del orden de 107 por la alta mortalidad que provoca el tratamiento, lo que también implica poder contar con cantidades de RNA considerablemente mayores (del orden del miligramo en muchos casos).
En nuestra experiencia de transfección de RNA viral con lipofectina, la alta viabilidad post-transfección obtenida permite partir de cantidades celulares y de RNA una o dos ordenes de magnitud menores a las utilizadas en protocolos convencionales de transfección vía PEG.

-<u>CARACTERIZACION DE NUEVOS GENES DE INMUNIDAD DEL GERMOPLASMA DE PAPA:</u>

Durante 1990 se caracterizó la cinética de replicación viral mediante ELISA, inmunoprecipitación, inmunofluorescencia, hibridación de ácidos nucleicos, etc. en protoplastos de entradas del banco de germoplasma de S.acaule que tienen el gen de reacción a PVX Xi de Ross, también llamado Rxacl (ver Saladrigas et al. 1990, Ceriani et al. 1990, Hopp et al. 1991); así como de otras entradas con genes de resistencia extrema que estamos caracterizando (Tozzini et al. 1991) de las especies S.sparsipillum, S.oplocense, S.tuberosum andigena, etc. observandose una clara inhibición de la replicación viral que estaría afectando la transcripción o replicación del genoma viral. El detalle de todo lo hecho en este punto está claramente expresado en el trabajo de Tozzini et al. aceptado para publicarse que se adjunta a este informe. Actualmente se continúa la caracterización molecular y se están implementando distintas estrategias para aislar los genes de resistencia.

Servicio C.- Búsqueda y selección de clones codificantes para la cápside viral de PLRV.

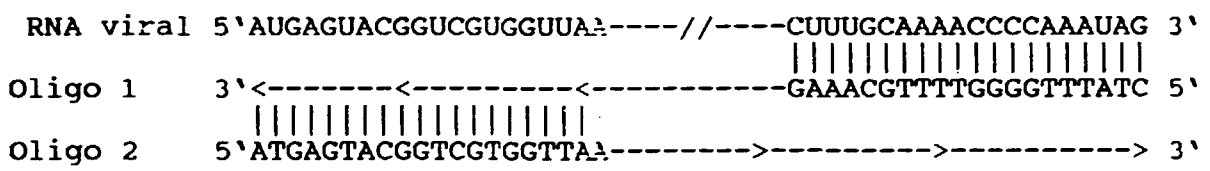
Se localizaron y caracterizaron las secuencias nucleotídicas virales de los extremos 5' genómicos y codificantes de la proteína de cápside del PLRV con el propósito de construir los primeros genes de resistencia

que interfieran la estrategia de replicación viral. En este contexto, se logró aislar clones conteniendo parte de la cápside viral del PLRV a partir de una genoteca construida a partir de un aislamiento uruguayo y se construyeron nuevas genotecas específicamente del gen de la cápside por la técnica de PCR (polymerase chain reaction) con oligonucleótidos sintetizados "ad hoc".

Obtención, búsqueda e identificación del gen de la subunidad de cápside viral del PLRV:
Se cuenta (gracias a la colaboración con el Instituto de Investigaciones Biológicas Clemente Estable del Uruguay) con una genoteca de cDNA (hecha en el fásmido Bluescript) sintetizado mediante la técnica de iniciación al azar a partir de RNA viral purificado.
Esta genoteca fue rastreada mediante hibridación con oligonucleótidos sintetizados "ad hoc".

2. El oligo 1 hibrida con el extremo 3' de la zona de RNA viral que codifica para cápside y el oligo 2 hibrida con el cDNA sintetizado con la transcriptasa inversa usando el oligo 1 como primer, en la zona correspondiente al extremo 5' de cápside viral. Esto se muestra esquemáticamente en la Figura No1:

Figura No1:



Se fijó DNA correspondiente a 25 clones de la genoteca a una membrana y se realizó una hibridación con ambos oligos a la vez, marcados con σ32P-ATP, como sondas. Se detectaron dos clones positivos, el 69 y el 55. La idea original era usar estos clones como templados alterntivos para, mediante el método de PCR, lograr la amplificación del gen de la proteína de la cápside. Para evaluar si ésto es posible, se realizó un mapeo de restricción de estos dos clones, utilizando las enzimas Hinf I, Bam HI, Dde I, Hha I y Pvu II. En ningún caso se obtuvieron bandas del tamaño esperado si el inserto de estos clones contuviera información para la cápside viral.
Esto último hizo que fuera imprescindible la repetición del relevamiento de la genoteca, marcando, esta vez, a los dos oligos por separado (de esta manera si un mismo clon hibrida con los dos oligos estaríamos seguros que contiene el gen de la proteína de la cápside completo) e hibridando en paralelo con dos membranas idénticas.
Esta vez se utilizaron membranas de Nylon (Z-probe, Bio Rad), y se siguió las instrucciones del fabricante tanto para el fijado del DNA a la membrana como para la hibridación en sí (ver Materiales y Métodos).
Para el marcado de los oligonucleótidos se utilizó un kit comercial para marcado de extremos 5' romos de IBI. La actividad específica de ambas sondas fue del orden de 107cpm/μg DNA. Otra diferencia con el experimento antes informado es que se separaron los oligonucleótidos marcados del ATP no incorporado mediante el uso de columnas de Sephadex comerciales (Nu-clean D25 de IBI) y no mediante precipitación con acetato de amonio y etanol, lo cual disminuyó el fondo inespecífico.
En este caso, se sembró en las membranas 100 ng de DNA de 22 clones que no se habían incluído antes (además de los anteriores 25 clones y, por supuesto del control negativo: DNA del plásmido Bluescript sin inserto).
El resultado de la hibridación usando el oligo 2 como sonda, indicó un

sólo clon positivo, el número 32.
La hibridación usando el oligo 1 como sonda no arrojó ningún positivo.
A continuación se analizó mediante el uso de enzimas de restricción al DNA del clon 32. Por un lado se liberó el inserto cortando DNA purificado del clon 32 con dos enzimas, Bam HI y Pst I. Por otro lado, se cortó DNA de este clon y del plásmido Bluescript por separado con la enzima Hinf I, lo cual libera siete fragmentos; uno de ellos aumenta su tamaño proporcionalmente al tamaño del inserto. Se corrió un gel de poliacrilamida al 5% sembrando el resultado de todas estas restricciones y un marcador de peso molecular. De esta manera se determinó que el inserto del clon 32 es muy corto, tiene unas 50 pb.
En el informe anterior, se reportó la realización de una reacción de PCR, utilizando los oligonucleótidos como iniciadores y la primer cadena de cDNA (obtenida utilizando RNA de planta infectada como templado y transctiptasa inversa) como templado. Esta reacción no dio resultados, es decir, a pesar de que se comprobó que se sintetizaba cDNA, no hubo amplificación del gen de la proteína de la cápside. Como también se explicó en ese informe, ésto pudo haber ocurrido porque el templado para la reacción de PCR, si bien era suficiente para detectarlo, marcado con α-32P-CTP en un gel de agarosa, no era suficiente para comenzar la reacción de amplificación. Un problema importante es que, normalmente, para una reacción de síntesis de primer cadena de cDNA hace falta partir de 1 μg de RNA templado. Como se contaba con RNA total de planta infectada para usar como templado, para poner 1 μg de RNA viral, habría que utilizar unos 200 μg de RNA total, lo cual es metodológicamente dificultoso.
Es por ésto que se comenzó la búsqueda de templados alternativos en los clones de la genoteca. Como no se detectó algún clon que contuviera el gen de la proteína de la cápside completo, estos clones no sirvieron como templados alternativos.
Fue importante entonces, volver al punto de partida, el RNA extraído de planta infectada, y tratar de enriquecerlo en RNA viral. Para ello se aplicó la técnica de liberación del híbrido.
Se fijaron 5 μg de DNA de c/u de los clones 55, 69, y 32 en una membrana de nylon según el protocolo descripto en Materiales y Métodos. Se eligieron los clones 55 y 69 porque tienen insertos muy grandes (aprox.2 kpb), y el clon 32 porque contiene un inserto correspondiente al de cápside según se desprende del experimento descripto anteriormente.
Una vez finalizados los lavados (ver Materiales y Métodos), se liberó de la membrana el RNA que hibridó con alguno de esos clones por calentamiento. Se precipitó el RNA, se lo resuspendió en agua y se lo corrió en un gel desnaturalizante. Se obtuvo una sóla banda de RNA, a diferencia de las varias bandas que se ven cuando se corre RNA total de planta infectada (correspondientes a RNA ribosomal de citoplasma, cloroplastos y mitocondrias). En total se obtuvieron 3 μg de RNA.
Identificación del RNA viral rescatado con clones de cDNA por la técnica de liberación del híbrido:
Se fijó DNA y RNA a un filtro de nylon, y realizó una hibridación de este filtro usando el clon 32 marcado por iniciación al azar como sonda. Lo que interesa demostrar es que el RNA liberado en el experimento anteriormente descripto hibrida con el clon 32 que, ya se sabe, contiene parte del gen de la proteína de la cápside. Se observó una fuerte hibridación de la sonda con el RNA liberado y con el control positivo, y algo de hibridación inespecífica con el tRNA. No hay hibridación con el RNA total, lo cual era esperable ya que, durante el experimento de liberación del híbrido, de 200 μg de RNA total se obtuvieron 3 μg de RNA liberado. Este resultado nos indica que el RNA es de origen viral y puede ser usado como molde (templado) para generar una genoteca de cDNA.

Para su amplificación y clonado se utilizó un kit de Invitro-gen utilizando los oligonucleótidos arriba detallados. El fragmento amplificado mostró un comportamiento electroforético coherente con lo esperado. A su vez, al ser transferido mediante Southern blot a una membrana de nitrocelulosa e hibridizado con sondas del clon arriba mencionado, mostró una alta señal de hibridación. Después de ser subclonado e introducido en un fásmido vector en E.coli, se preparó cantidad suficiente para secuenciación, la cual se encuentra actualmente en curso.

Servicio D.- Construcción de genes quiméricos de resistencia a virosis

-D.1 y D.2- PVX y PVY:

Como se informó anteriormente, a partir del conocimiento y de los clones de cDNA obtenidos para PVX y PVY se realizaron distintas construcciones quiméricas con el objeto de introducirlas en plantas de papa y obtener resistencia. Se prosiguen dos estrategias paralelas:
1) Por un lado se intenta introducir y expresar las respectivas cápsides virales en papa (cross protection). Se dispone ya de tres construcciones constituidas por los genes de cápside de PVX y PVY bajo el control del promotor constitutivo doble del transcripto 35S del CaMV.
2) Por otro lado se intenta inhibir la replicación viral por expresión constitutiva en la planta de transcriptos de polaridad negativa que corresponden a regiones del genoma consideradas clave en el ciclo de replicación. Se dispone de tres construcciones "antisentido" (bajo la dirección del promotor doble arriba mencionado) de la región 5' no codificante del genoma de PVX y PVYn y de la región 5' del mensajero subgenómico de la cápside viral de PVX. Para ello se realizó el subclonado en vectores de tipo Ri de agrobacterias.

- D . 3 - P L R V
Aún no iniciada.

Servicio E.- Establecimiento de las técnicas de transformación de papa de interés para Argentina.

Puesta a punto de tecnología de regeneración y transformación de cultivares de papa de uso local:
Paralelamente se realizaron experimentos de puesta a punto de transformación y regeneración de los cultivares de papa Huinkul MAG y Spunta (de uso en Argentina) y Alfa (de uso en México).
Ya han sido determinadas las condiciones que permiten regenerar plantas a partir de discos de hojas y minitubérculos. Se realizaron ensayos de transformación de discos de hoja y, en menor medida, de cortes de minitubérculo variando los métodos de infección y utilizando distintas cepas de Agrobacterias capaces de transferir a la planta marcadores genéticos de fácil identificación (resistencia a antibióticos y herbicidas y expresión de glucuronidasa). Estas condiciones se utilizarán para la transferencia de las quimeras descriptas y de otros genes de interés.
Ensayo de regeneración a partir de discos de tubérculo: Se realizó un ensayo de regeneración utilizando mini tubérculos libres de virus obtenidos en invernadero, del cultivar de mayor utilización en la Argentina, Spunta.
El primer paso fue la determinación de las condiciones de esterilización superficial del tubérculo. Para ello se ensayaron tres tratamientos. En todos ellos, primero se lavan los tubérculos con detergente y cepillo. Se pelan y se tratan con alcohol 70% durante un

minuto. Luego se tratan con:
a) lavandina 0,6%, tween 20 0,1% durante 20'
b) lavandina 1,2%, tween 20 0,1% durante 20'
c) lavandina 1,2%, tween 20 0,1% durante 35'.
Se toma la parte central con un sacabocados estéril, se cortan las rodajas y se colocan en un medio con las sales y vitaminas MS, sacarosa 3%, agar 5,5 g/l pH 5,7.
El tratamiento c) fue el que dió mejores resultados, ya que no hubo contaminación con bacterias y hongos sobre las rodajas de papa como se observó en los tratamientos a) y b).
Una vez que se determinaron las condiciones de esterilización, se realizó un experimento que consistió en determinar las condiciones óptimas de regeneración del cultivar Spunta a partir de rodajas de tubérculo.
En 1989 Cornelissen realizó una tarea similar para las variedades europeas Bintje, Désiree y Escort, en donde determinó, luego de probar distintas hormonas (kinetina, ac. naftalenacético, ac. indolacético, ribósido de zeatina) que las hormonas y concentraciones adecuadas eran: 10 μM Zeatina/1μM IAA para Escort y Bintje, y 5 μM Zeatina/0,3 μM IAA para Désiree.
Luego de una esterilización superficial (según el tratamiento c)) de tubérculos de Spunta, se colocaron las distintas rodajas de unos 0,9 cm de diámetro y 2 mm de espesor en 7 medios diferentes según las concentraciones de hormonas utilizadas. Todos los medios contenían los macro y micronutrientes y vitaminas de MS, sacarosa 3%, agar 5,5 gr/lt.
Ver la Tabla No1:
Tabla No1:

IAA(μM) \ ZEA(μM)	0	5	10
0	10*	-**	15
5	-	15	15
10	15	15	15

* indica número de rodajas de tubérculo por tratamiento.
** indica concentraciones no ensayadas.

A los 14 días de iniciado el tratamiento, se observó la aparición de callos en las concentraciones ZEA 10/IAA 10, ZEA 10/IAA 5, ZEA 5/IAA 10, y ZEA 5/IAA 5. No se observó aparición de callos en las concentraciones ZEA 0/IAA 0, ZEA 0/IAA 10, ZEA 10/ IAA 0.
A los 35 días los callos eran verdes y de un diámetro de entre 1,5 y 2,5 cm. No se diferenció ningún brote a partir de estos callos.
A los 35 días se observó la aparición de microcallos en el 100% de las rodajas de tubérculos que estaban bajo el tratamiento de ZEA 10/IAA 0. Una semana después, surgieron brotes a partir de todos estos microcallos. Cuando los brotes alcanzaron un tamaño de unos 10 cm se los separaró de las rodajas de tubérculo con un bisturí y se los transfirió a medio de enraizamiento. A los 15 días, todos los brotes enraizaron. El tratamiento con zeatina 10 μM/IAA 0 fue, por lo tanto, el de elección para el ensayo de transformación que se describe a continuación.
Ensayo de transformación de discos de tubérculo: Al igual que el ensayo anterior se utilizaron mini tubérculos de Spunta y las mismas condiciones de esterilización superficial y manipuleo.
Para la transformación se utilizó la cepa de Agrobacterium tumefaciens C58C1 rifr (pGSFR1280). Como control se utilizó la cepa de Agrobacterium tumefaciens C58C1 rifr (pGV2260). Se realizó la coinfección de 60 rodajas control y 60 rodajas con la cepa que contiene el plásmido pGSFR1280 que confiere resistencia a kanamicina.

A continuación se transfirieron a tubos sin presión de selección (20 rodajas) o con presión de selección. Se ensayaron dos concentraciones de kanamicina (kan): 25 µg/ml y 50 µg/ml (20 rodajas en cada concentración).
El relevamiento realizado al mes arrojó los siguientes resultados (ver tabla No2).
Tabla No2:

		km 0	km 25	km50
pGFSR1280	A	12 (60%)	15 (75%)	18 (90%)
	B	4 (20%)	2 (10%)	0
	C	4	4	2
	D	15 (20%)	8 (15%)	2 (10%)
pGV2260	A	7 (35%)	16 (80%)	20 (100%)
	B	6 (30%)	0	0
	C	7	4	0
	D	15 (35%)	16 (20%)	0

A: rodajas necrosadas.
B: rodajas verdes; tejido vivo.
C: rodajas que dieron lugar a callos.
D: rodajas que dieron lugar a brotes.

Como se observa en la tabla, en ambos casos hay una mayor proporción de necrosis a medida que la presión de selección aumenta. Además un 10% de rodajas coinfectadas con las bacterias que contienen el vector que confiere resistencia a kanamicina son capaces de generar callos y/o brotes a altas concentraciones de kanamicina.
Las rodajas coinfectadas con el vector control no fueron capaces de dar lugar a callos y/o brotes en 50 µg/ml de kan, por lo que se usará en lo sucesivo ésta concentración de kan. como presión de selección.
Todos los brotes fueron transferidos a medio de enraizamiento con 50 µg/ml de kan. Si alguno logra a enraizar en esta concentración de kan, se ensayarán concentraciones mayores y se harán las pruebas necesarias para confirmar transformación.

Ensayo de transformación de discos de hojas de papa: Se siguió un protocolo proporcionado por la Dra. Aileen O'Connor del Centro de Investigación y de Estudios Avanzados del IPN (Instituto Politécnico Nacional), Irapuato, México, con el que tenemos colaboración. Se trabajó primero con la variedad europea Desirée y luego con las variedades Huinkul MAG y Alfa. Para la transformación se utilizó la cepa de Agrobacterium tumefaciens C58C1 rifr(pGSFR1280).
Se hicieron dos tipos de controles. Por un lado se hizo una infección simulada siguiendo todos los pasos detallados en el protocolo pero sin agregar el Agrobacterium. Por otro lado se realizaron coinfecciones con la cepa de Agrobacterium tumefaciens C58C1 Rifr (pGV2260) Se utilizaron 36 discos de hoja en cada uno de los tres tratamientos (transformación y dos controles). Luego de dos días de coinfección, se colocaron 18 discos de cada tratamiento en tubos con un medio sin kanamicina (sin presión de selección) y 18 discos en tubos con medio al que se le agregó 50 µg/ml de sulfato de kanamicina (con presión de selección).
Es importante subcultivar los explantos cada semana.
A las tres semanas se observó aparición de pequeños callos sobre los bordes de los explantos de todos los tratamientos que se encontraban sin presión de selección, así como aquellos con presión de selección previamente coinfectados con Agrobacterium, mientras que no se

observaron callos y sí un leve amarillamiento en los explantos controles sin inocular que se encuentran en un medio con kanamicina.
Para las rutinas de transformación se utilizó el sistema de cocultivo de Agrobacterium sp. con hojas de plántulas crecidas "in vitro".
Se lograron eficiencias de regeneración de brotes a partir de hojas del 70% para el cultivar argentino Huinkul, del 80% para el cultivar europeo Desirée y del 100% para el cultivar mexicano Alfa utilizando 2 mg/lt de zeatina.
El ensayo de glucouronidasa (cuando se utilizó un vector conteniendo este gen marcador) dio positivo en el 60% de las plántulas regeneradas indicando su transformación. Por este motivo se eligió esta metodología para los ensayos posteriores.

Servicio F.- Transformación de plantas de papa

F.1- PVX

En la actualidad se cuenta con dos plantas regeneradas en medio selectivo (km 50 µg/ml) obtenidas a partir de hojas de Alfa coinfectadas con el vector pGSH163 que lleva el gen de la toxina de Bacillus turingiensis (BT 884) bajo el control del promotor PTR2 y el gen de la neomicina fosfotransferasa (NPTII) bajo el promotor PTR1.
Se realizaron ensayos para detectar tanto actividad enzimática de NPTII (por detección en fase sólida de neomicina fosforilada por un extracto de proteínas totales y gamma ATP) como la presencia de este gen por la técnica de PCR (polymerase chain reaction).
Se están micropropagando plantas de Huikul MAG y de Alfa conteniendo construcciones con el gen Bt de Bacillus thuringiensis y de Bintje con el gen de cápside de PVX.

F.2- PVY

A punto de iniciarse.

F.3- PLRV

Aún no iniciada

F.4- Entrenamiento

Se informa en el punto III (intercambios e interacciones).

II>- Presentaciones a congresos y publicaciones.

a) Congresos

- HOPP HE, A ARESE, BE ORMAN, F BRAVO ALMONACID, L HAIM y AN MENTABERRY (1989) Diagnóstico de virosis de papa por hibridación molecular. XIV Reunión de la Asociación Latinoamericana de la Papa, Comun. 19, p.15 del Libbro de Resúmenes, 5-11 marzo 1989, Mar del Plata.
- ARESE AI y HE HOPP (1989) Uso del sistema biotina-estreptoavidina para la inmunodetección de virus de papa por ELISA. Ibid, comun. 18, p.14 Libro de resúmenes, Mar del Plata.
- SALADRIGAS MV, MF CERIANI, A TOZZINI y HE HOPP (1989) Replicación de PVX en protoplastos de papa. Ibid. Comun. 17, p.14, Mar del Plata.
- HOPP HE, AI ARESE, BE ORMAN, F BRAVO ALMONACID y AN MENTABERRY (1989) Diagnosis of potato viruses by molecular hybridization using PVX, PVY and PLRV cDNA clones. Ibid, Comun. S12-033 Libro de Resumenes, La Habana, Cuba.
- SALADRIGAS MV, MF CERIANI, A TOZZINI y HE HOPP (1989) Replication of PVX in potato protoplasts. Ibid, Comun. S12-032 Libro de Resumenes, La Habana, Cuba.
- SALADRIGAS MV, MF CERIANI, AC TOZZINI y HE HOPP (1989) Efecto del gen Rx en la inhibición de la replicación de PVX en protoplastos de papa. Ibid, Res. 239 p. 96, Buenos Aires.
- TOZZINI AC, MF CERIANI, MV SALADRIGAS, AI ARESE y HE HOPP (1989) Genes de resistencia contra PVX en distintos genotipos del género Solanum. 12a Reunión Cientifica Anual de la SAV (Sociedad Argentina de Virología), Res. 4.6 p. 28, Buenos Aires.
- SALADRIGAS V, F CERIANI, A TOZZINI y E HOPP (1990) Expression of resistance gene Xi in Solanum tuberosum protoplasts infected with PVX.

Ibid, Res. No. 941.
- DEL VAS M, A ESCANDON y E HOPP (1990) Regeneración de plantas de papa del cultivar Spunta para utilización en experimentos de transformación genética. Presentado a la XXVI Reunión Anual de la Sociedad Argentina de Investigación Bioquímica, Resumen No263, Mar del Plata 25-27 noviembre.
- BRAVO ALMONACID F, A ARESE, E HOPP y A MENTABERRY (1990) Obtención de anticuerpos policlonales contra la proteína de fusión proteína A de S.aureus/cápside de PVY. Ibid, resumen No 74, Mar del Plata 25-27 noviembre 1990.
- CERIANI MF, MV SALADRIGAS, AC TOZZINI y HE HOPP (1990) "Resistance to replication of PVX in protoplasts of a Solanum acaule clone" Actas del Simposio Internacional en Virosis Vegetales, organizado por la IFS (International Foundation for Science, Suecia) y el Instituto de Fitovirología INTA- Córdoba, Villa Carlos Paz, Córdoba, Revista de Investigaciones Agropecuarias INTA (en prensa).
- DEL VAS M, MF CERIANI, AS ESCANDON y HE HOPP (1991) Puesta a punto de la transformación genética de papa. Ibid p.82, Vaquerías, Córdoba.
- SALADRIGAS MV, P CRAMER y HE HOPP (1991) Uso de protoplastos para el estudio de la expresión de genes de resistencia a virus a nivel celular. Ibid p.85, Vaquerías, Córdoba.
- BRAVO ALMONACID F, A ARESE, E HOPP y A MENTABERRY (1991) Caracterización de antisuero contra la proteína de fusión proteína A de S.aureus\cápside de PVY. Ibid p.89, Vaquerías, Córdoba.
- TOZZINI AC, MF CERIANI, MV SALADRIGAS y HE HOPP (1991) Identificación y caracterización de genotipos resistentes a PVX en germoplasma de Solanum utilizando plantas micropropagadas y protoplastos, Ibid p.98, Vaquerías, Córdoba.

PUBLICACIONES

- BRAVO ALMONACID F y AN MENTABERRY (1989) Nucleotide sequence for PVYo 3'end genomic RNA. Nucl. Acids Res. 17: 4401
- SALADRIGAS V, F CERIANI, A TOZZINI y HE HOPP (1990) Expression of resistance gene Xi (Rxacl) in Solanum tuberosum protoplasts infected in vitro with PVX (potato virus X). Plant Physiol. (Suppl.) 93: 161.
- CERIANI MF, MV SALADRIGAS, AC TOZZINI y HE HOPP (1990) Resistance to replication of PVX in protoplasts of a Solanum acaule clone. Proceedings of the International Foundation for Science, Sweden and INTA, Argentina: Seminar on Plant Viruses. Rev. Investig. Agropec. 22(1): 22-30.
- SALADRIGAS MV, MF CERIANI, A TOZZINI, AI ARESE y HE HOPP (1990) Potato gene Xi confers inoculum dependent resistance to potato virus X replication in protoplasts. Plant Cell Physiol. 31(6): 749-755.
- TOZZINI A, MF CERIANI, MV SALADRIGAS y HE HOPP (1991) Extreme resistance to potato virus X infection in wild tuber bearing Solanum species. Potato Research (en prensa).
- HOPP HE, L HAIM, F BRAVO ALMONACID, AI ARESE, A TOZZINI, MF CERIANI, MV SALADRIGAS, B ORMAN, R CELNIK, M del VAS y AN MENTABERRY (1991) Development and application of a non-radioactive nucleic acid hybridization system for the simultaneous detection of four potato pathogens. J. Virol. Meth. 31 (1): 11-30.
- SALADRIGAS MV, P CRAMER y HE HOPP (1991) Use of cationic liposomes for infection of potato protoplasts with virus particles or RNA. Enviado para publicación a J. Virol. Meth.

III>- Intercambios e interacciones con otros países participantes del proyecto.

- Se envió al Dr. Luis Herrera Estrella varios clones bacterianos conteniendo un plásmidos recombinantes con construcciones capaces potencialmente de conferir resistencia a PVY.
- Viaje y estadía de Mariana del Vas a México, estudiante de doctorado

argentina, para realización de experimentos de transformación de papa, subclonado y caracterización del genoma de PLRV en el marco del proyecto conjunto y en cumplimiento del contrato con ONUDI.

IV>- Evaluaciones de cumplimiento de Argentina y del proyecto en su conjunto.

En lo referente al cumplimiento de Argentina puede desprenderse de la lectura del punto I que se cumplió con creces la gran mayoría de lo comprometido, con excepción de partes de los puntos D y F (el D.3 y el F.2 recién se inician, y el F.3 aún no se inició). En ambos casos se previó (ver actividades 3.3. PLRV, 3.4.2. PVY y PLRV del anexo I, proyecto global) que debían continuar en el tercer año del proyecto, aunque se supuso que partiendo de un grado mayor de avance de lo realizado en el segundo año).

A N E X O I

CALENDARIO GLOBAL

A C T I V I D A D	PAIS RESPONSABLE	A N O S 1	2	3	4	Grado de Cumplimiento de lo planificado para el segundo año.
1. Búsqueda de genes de resistencia.						
1.1 Caracterización de genotipos (al menos 3 para cada virus) con resistencia a virus previamente seleccionados a partir de cultivares de papa locales o colecciones de germoplasma. Se inocularán artificialmente plantas liberadas de virus cuantificando la concentración viral resultante y la reacción específica de resistencia:						
PVX	A, Ch	XXX				Cumplidos
PVY	A, Ch	XXX				
PRLV	A	XXX				
1.2 Construcción de genotecas de papa con resistencia a virus (de cDNA y genómicas)	U	XXX	XXX			Cumplidos
1.3 Recuperación y caracterización de clones de cDNA y genómicos para ser testados como marcadores genéticos del tipo RFLP. Asignación de grupos de ligamiento y asociación a resistencia viral	U		XXX	XXX		Cumplidos
1.4 Infección <u>in vitro</u> de protoplastos obtenidos de plantas resistentes y evaluación de la replicación						

ACTIVIDAD	PAIS RESPONSABLE	AÑOS 1	2	3	4	
- PVX (mediante ELISA, hibridación molecular, inmunofluorescencia e hibridación <u>in situ</u> combinada con microscopia electrónica)	A, Ch, U	XXX	XXX			
- PVY (ELISA, hibridación molecular, imunofluorescencia)	A, Cu	XXX	XXX			Cumplidos
2. Obtención de plantas resistentes mediante variación somaclonal y/o genética.						
2.1 Búsqueda y selección de cultivares con alta frecuencia de regeneración:						
- a partir de callos	Ch, E	XXX				
- a partir de tejidos (hojas)	Ch, E	XXX				Cumplidos
2.2 Empleo de mutagénesis y/o agentes que incrementan la variación somaclonal y/o genética a callos o tejidos						
- infectadas con PVX+PVY o	Ch, E		XXX	XXX		Cumplidos por Chile
- infectadas con PLRV	Ch, E		XXX	XXX		
- no infectadas	Ch, E		XXX	XXX		no se de Ecuador
2.3 Evaluación de las plantas regeneradas	Ch, E			XXX	XXX	Cumplido por Chile
3. Obtención de plantas resistentes por ingeniería genética.						

ACTIVIDAD	PAIS RESPONSABLE	AÑOS 1	2	3	4	
3.1 Obtención de genotecas de cDNA virales:						
PVX	A	XX				
PVY	A, Cu	XXX				
PLRV	A, Cu,	XX	XX			Cumplidos
PVS	Ch		XXX			
3.2 Localización y caracterización de secuencias nucleotídicas relevantes.						
3.2.1 Secuenciación nucleotídica de clones seleccionados:						
PVX	A,	XX				
PVY	A,	XX				
PLRV	Cu	X	XXX			Cumplidos
3.2.2 Búsqueda y selección de clones codificantes para fragmentos subgenómicos:						
PVX	A	XX				Cumplidos
3.3.3 Búsqueda y selección de clones codificantes para la cápside viral:						
PVX	A, Cu	XX				
PVY	A, Cu	XX				
PLRV	A, Cu		XXX			
PVS	Ch		XX	XXX		Cumplidos no sé
3.3 Construcción de genes quiméricos de resistencia a virus:						
PVX	A, Cu, M	X	XXX			
PVY	A, Cu, M		XXX			
PLRV	A, Cu, M		XX	XX		Cumplidos
3.4 Transformación vegetal.						

ACTIVIDAD	PAIS RESPONSABLE	AÑOS 1	2	3	4	
3.4.1 Adaptación de las técnicas de transformación a cultivares locales de papa	Todos los países		XXX			Cumplidos para México, Chile, Argentina y Cuba en su inicio.
3.4.2 Transformación de plantas de papa:						
PVX	A, Cu, M		XXX	XXX		
PVY	A, Cu, M		XX	XXX		
PLRV	A, Cu, M			XXX		
3.5 Evaluación final	Todos los países				XX	

A = Argentina; Cu = Cuba; Ch = Chile; E = Ecuador; M = México; U = Uruguay.

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Development and application of a nonradioactive nucleic acid hybridization system for simultaneous detection of four potato pathogens

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Summary

cDNA clones of potato virus X (PVXcp strain), potato virus Y (PVYo strain), potato leaf roll virus (PLRV) and potato spindle tuber viroid (PSTV) were used separately or combined for the detection of the corresponding RNAs in extracts of infected plants. A general method for the rapid preparation of RNA extracts without use of organic solvents (i.e. phenol) was developed for this purpose. Plant extracts from a range of field, artificially inoculated germplasm genotypes, micro-propagated and protoplast samples, as well as vector insect extracts, were dot–blotted onto nylon or nitrocellulose membranes, subjected to sandwich nucleic acid hybridization with non-labelled specific single-stranded DNA probes followed by a biotin-labelled second step hybridization probe. Each probe was virus-specific but not strain-specific. Healthy or non-related plant extracts developed very faint or no signals. Sensitivity was tested by slot–blot hybridization. Detection levels were between 1.5 to 6 pg of viral nucleic acids and between 20 to 50 times more sensitive than standard double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The assay developed was tested with material that was prepared for processing in the field (combination of fresh sap with extraction solution) and tested under simple laboratory conditions for detection. It was also successfully employed for screening of germplasm for virus resistance, detection of pathogens

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in vector insects, plantlets grown in vitro and in more sophisticated quantitative determinations of viral replication in artificially inoculated plants and protoplasts.

Introduction

Development of nucleic acid hybridization techniques has resulted in extremely reliable and sensitive diagnostic procedures for the detection of infectious agents due to the sequence specificity provided by the recognition of complementary nucleic acid strands (see Tenover, 1988, for a review). Production of diagnostic kits based on this technology will become a major industry in human, animal and plant infectious disease control due to the flexibility of designing probes to the specific problem (Van Brunt and Klausner, 1987; Tenover, 1988; Chu et al., 1989). For example, in the case of quarantine or eradication programs, it is possible to design a broad spectrum probe, while for epidemiologic studies a narrow spectrum one could be developed (Boulton et al., 1987).

Considerable progress has been made in the development of DNA probes for detection of many phytopathogens such as viruses, particle-deficient viruses, viroids, bacteria, spiroplasma- and mycoplasma-like organisms and fungi (for example see Boulton et al., 1987; Chu et al., 1989; Harrison et al., 1983; Hull, 1987; Maule et al., 1983b; Palukaitis et al., 1985; Owens and Diener, 1984; Roy et al., 1989; Symons, 1984). In the case of potato, nucleic acid hybridization is starting to be used routinely for screening purposes in breeding programs for resistance, for indexing material to ensure maintenance of freedom from virus infection (Boulton et al., 1987; Karjalainen, 1988), and for routine detection of viroids in seed potato certification programs (International Potato Center, 1985). As mentioned above, specifically designed diagnostic probes could be useful in pathogen control programs. For example, a combination of different probes able to simultaneously detect many different pathogens could prove useful in the selection of pathogen-free material (i.e. seed potato production programs) where identification of an individual pathogen usually is not so important because all infected plants are discarded.

Even though hybridization methods are relatively simple, their application is still restricted to specialized laboratories due to logistic problems regarding standardization of sample preparation and of non-radioactive probe labelling and detection. Recently, we and several other laboratories developed non-radioactive DNA probes for routine diagnosis of potato pathogens in plant extracts (see for example Bruning and Wenzel, 1988; Boulton et al., 1987; Eweida et al., 1988; Hopp et al., 1988; Owens and Diener, 1984, Roy et al., 1989).

This paper describes a general non-radioactive dot-blot hybridization protocol for specific or general detection of four important potato pathogens in plant extracts together with a single and rapid standardized protocol for the preparation

of samples. The detection sensitivity and specificity of these probes is comparable to standard ^{32}P-labelled probes. These techniques are currently starting to be implemented in simply equipped laboratories to test for presence of viruses in the in vitro production program of seed potato in the Province of Córdoba, Argentina, and for indexing in the Balcarce Experimental Station of INTA, Argentina.

Materials and Methods

Plant material and virus strains

The following *Solanum tuberosum* L. ssp *tuberosum* cultivars were used: *Spunta*, *Huinkul MAG*, and *Serrana INTA* (the last one is resistant to the three viruses studied here and was used as negative control in field experiments). *Solanum acaule* clone PI 320277 is from the Potato Introduction Station, Sturgeon Bay, U.S.A. This Station generously sent us sib seeds of this clone. Virus-free seedlings were handled in sterile conditions and micropropagated as described by Masson et al. (1987). Leaf protoplasts from shoot cultures were obtained as reported by Masson et al. (1987). In vitro infection was carried out essentially as in Maule et al. (1983a) modified by Ceriani et al. (1990). Protoplasts were incubated for 40 h and concentrated by centrifugation for estimation of PVX concentration by ELISA and slot-blot hybridization (Ceriani et al., 1990).

PVX from South American isolate cp (Fribourg, 1975; Orman et al., 1990) was grown in *Nicotiana tabacum* or *N. glutinosa* by Ing. Agr. S. F. Nome (Instituto de Fitovirología-INTA-Córdoba) and purified as described (Orman et al., 1990). Virus concentrations were estimated spectrophotometrically using A_{260} 1% = 2.97 (Bercks, 1970). Leaves infected with two Argentine isolates (PVXnormal and PVXfuerte) were sent by Ing. Agr. Muñoz (Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba). PVY and PLRV were grown in *N. glutinosa* and *Physalis floridana*, respectively, in Ing. Agr. S. F. Nome's research institute (INTA-Córdoba) and kindly donated for this work. PVY and PLRV were purified according to Gugerli (1978, 1979). Samples of PVX, PVYn and PSTV infected leaves were sent by Ing. Agr. I. Butzonich (INTA-Balcarce). Virus-free and PLRV-infected *Myzus persicae* were donated by Ing. Agr. Muñoz.

Sample preparation

About 0.2 g of potato tissue were introduced into 1.5 ml Eppendorf tubes containing 0.5 ml extraction buffer (4 M guanidinium isothiocyanate, 0.4 M NaCl, 50 mM 2-mercaptoethanol, 50 mM MOPS, 15% ethanol, pH 7) and homogenized with a plastic pestle fitting the Eppendorf tube bottom (PolyLabo Paul Block, France). Alternatively, tissues were sealed and then squashed inside a plastic bag in the presence of the same extraction buffer (Hopp et al., 1988). About 0.3 ml

of the sap were then squeezed into Eppendorf tubes. Tissue debris was removed by centrifugation for 1 min in a microfuge (Eppendorf, F.R.G.).

Total cellular RNA was purified following a basic protocol from Diagen (1988) which was modified as follows. Micropipette tips containing anion exchange resin with capacity to adsorb 5 μg of nucleic acids (Qiagen-tip 5, Diagen, F.R.G.) were pre-equilibrated by pipetting in and out 300 μl of 0.4 M NaCl, 50 mM MOPS and 15% ethanol pH 7 as recommended by the supplier. Nucleic acids were adsorbed onto the resin by slowly pipetting about 150 μl of the clarified supernatant in and out four times. Residual contaminants were washed by forcing through 2 ml of buffer 0.5 M NaCl, 50 mM MOPS and 15% ethanol, pH 7. Finally, total cellular RNA was eluted from the resin with 3 x 200 μl buffer 2 M urea, 1.05 M NaCl, 50 mM MOPS, 15% ethanol and 7% formaldehyde, pH 7. Standard extractions yielded about 1.5 μg of total RNA per sample (depending on extraction procedure and tissue source) as estimated by ethidium bromide staining and comparison with known amounts of pure RNA standards.

In parallel, 0.20 μm-nitrocellulose membranes (BA 83, Schleicher & Schüll, F.R.G.) were soaked first in double distilled autoclaved H_2O for 10 min and then in 20 x SSC (3 M NaCl, 0.3 M sodium citrate) for 10 min. Alternatively, 0.45 μm-nylon membranes (Nytran, Schleicher & Schüll, F.R.G.) were presoaked in autoclaved double distilled H_2O just before use. Samples containing about 0.2 μg total cellular RNA from healthy or infected plants were serially diluted in a solution containing 50 ng total cellular RNA from healthy potato leaves or yeast tRNA (Boehringer Mannheim, F.R.G.) in 1.05 M NaCl, 50 mM MOPS, 15% ethanol, 7% formaldehyde pH 7 and heated for 10 min at 60°C followed by quick chilling on ice. RNA extracts were loaded onto nylon or nitrocellulose membranes with the aid of a dot- or slot-blot manifold (Schleicher & Schüll, F.R.G.) and negative pressure. RNA was fixed to membranes by 3 min exposure to UV light (Khandjian, 1986) followed by 15 min heating at 80°C in a slab gel dryer (Hoefer, U.S.A.). Filters were stored dry until hybridization.

Probes

PVX and PVY cDNA libraries were constructed as published previously (Hopp et al., 1988; Mandel et al., 1989; Bravo Almonacid and Mentaberry, 1989). The PVX cDNA clone phX used in the present paper is a 3555 nucleotide insert corresponding to the 3′ end of the PVXcp genome (Orman et al., 1990), cloned in the *Sma*I site of pBluescript SK M13(+) phagemid (Stratagene, U.S.A.). Similarly, PVYo cDNA clone phY carries a 1182 nucleotide insert corresponding to the viral genome cloned in the *Eco*RI site of the same vector (Bravo Almonacid and Mentaberry, 1989). The 3′ region of both PVX and PVY genomes was chosen because of the high level of sequence conservation in this region in both Potexviruses and Potyviruses, respectively. The aim was to obtain virus-specific but not strain-specific probes. Cloned PSTV cDNA (Van Wezenbeek et al., 1982) was kindly supplied by Dr Van Kammen (Wageningen, The Netherlands). The cDNA insert, containing the full length PSTV genomic sequence was excised

and subcloned in the *Bam*HI site of pBluescript SK M13(
was synthesized by the procedure of Gubler and Hoffman (
from Amersham (U.K.). Template RNA was obtained by ph
purified PLRV virions (isolate XIV; kindly donated by Dr
Rennes, France) as described for PVX (Hopp et al., 1988).
inserted by blunt end ligation into the *Sma*I site of pBlues
Recombinant clone phLR was selected on the basis of its st
with extracts of PLRV-infected *Physalis floridana* and large
1600 nucleotides).

Preparation of probes

Single-stranded DNA of recombinant phagemids phX, phY
as well as the non-recombinant phagemid used by the seco
hybridizations [pBluescript SK M13(−)] were prepared by pr
in detail by vector DNA supplier (Stratagene, 1988). Resulti
pBluescript SK M13(−) DNA (fully complementary to sing
script SK M13(+) DNA except for the intergenic region of F1
packaging and replication) was labelled with photobiotin as
et al., 1983) or sulfonated using the Chemiprobe kit (Orgenic
stranded pBluescript DNA was prepared by a standard plasm
cedure (Birnboim, 1983) and labelled by incorporation of biot
U.S.A.) according to Leary et al. (1983). DNAse concentratio
incubation time was 2 h at 15°C. Under these conditions t
cleotide substituted about 25% of the A residues and the a
resulting nick-translated DNA fragments was approximatel
Biotin-labelled and sulfonated DNAs were stored at −20°C
The 'polyprobe' was prepared by mixing equal proportions
stranded phagemid DNAs of phX, phY, phLR and phST.
Individual or combined ('polyprobe') cDNA probes were
boiling followed by quick chilling on ice until pouring in th
of plastic bags containing the previously prehybridized filter

Sandwich nucleic acid hybridizations

Hybridizations were performed in plastic bags containin
hybridization solution (0.45 M NaCl, 25 mM NaH_2PO_4, 2
dextran sulfate, 1% sodium dodecyl sulfate, 0.1% polyane
(sodium salt, Sigma, U.S.A.) and 0.001% sodium pyropho
prehybridization step was carried out for at least 15 min at 6
1 μg/ml heat denatured non-labelled 'polyprobe' or individua
through a cut corner. The sequence-specific hybridization s
proceed for one hour or more at the same temperature. After th
out all the solution and washing with 0.2 × SSC and 0.1% SD
fresh hybridization solution was poured together with 1 μg/m

or double-stranded pBluescript SK M13(−) phagemid DNA. Af
of hybridization at 60°C (universal post-hybridization step, com
sequences), membranes were extensively washed with at least
SSC – 0.1% SDS for 10 min at room temperature, 2 × SSC
30 min at 60°C, followed by three washes with 0.2 × SSC for
Probes in hybridization or post hybridization solutions could b
at −20°C, boiled and reused several times. Filters were trans
plastic bag and sealed together with 50 μl/cm^2 of a blocking so
0.1 M NaCl, 0.1 M Tris-HCl, 3 mM $MgCl_2$, 3% skimmed milk
Argentina) and 0.2% polyanethole sulfonic acid pH 7.5 for
rocking-type shaker. Visualization of nucleic acid hybridizati
modified from recommended conditions by the kit suppliers (B
U.S.A. for biotin-labelled probes and Chemiprobe, Orgenics, Isr
probes). After the blocking step, either covalently coupled stre
phosphatase conjugate (1 μg/μl) or specific monoclonal antib
were added in fresh blocking buffer (without polyanethole s
further incubated at 37°C for 30 min. The membranes were w
with washing buffer (0.1 M NaCl, 0.1 M Tris-HCl, 3 mM Mg
20, pH 7.5) at room temperature for 10 min, followed by addit
antibodies in the Chemiprobe detection system. After a new
developing buffer (0.1 M Tris-HCl, 0.1 M NaCl and 50 mM Mg
added. Development was carried out after 30 min at room te
dim light or in the dark, by addition of 3.3 μl nitroblue tetrazol
70% *NN*-dimethyl formamide) and 2.5 μl 5-bromo-4-chloro-3
(50 μg/ml in *NN*-dimethyl formamide) per ml of fresh d
Alkaline phosphatase reaction was stopped when filter backgro
to become visible (30 min to 3 h). Nylon membranes were
95% ethanol and transferred to distilled water for final rinsi
was rinsed directly in distilled water.

ELISA

Assays were performed in microtitre plates (Nunc Immuno
and were analyzed using a Titertek Multiscan MC eight-chann
trophotometer (Flow Laboratory). Double antibody sandwich E
out using polyclonal antibodies against capsid proteins as
and alkaline phosphatase conjugated monoclonal antibodies ge
by Dr P. Gugerli (Station Fédérale de Recherches Agronomi
Switzerland). PVX was detected with polyclonal antibodies p
mended (Gugerli, 1979) or kindly provided by Dr S. Pérez
de Ingeniería Genética y Biotecnología de La Habana, Cuba).
. ere coupled to biotin-N-succinimide ester derivative (Ame
used as recommended by Zrein et al. (1988). PVY polyclonal
pared and conjugated to alkaline phosphatase as described (Gu
was detected with a commercial ELISA kit from Boehringer M

For estimation of virus content, aliquots of potato tissue or protoplasts were resuspended in 200 μl of 8 g/l NaCl, 0.2 g/l KH_2PO_4, 2.9 g/l $Na_2HPO_4 \cdot 12\ H_2O$, 0.2 g/l KCl, 0.5 ml/l Tween 20 and 20 g/l polyvinylpirrolidone (PVP) 10000, added to wells precoated with polyclonal immunoglobulin G and incubated overnight (Gugerli, 1980). After reaction with specific biotinylated immunoglobulin G, wells were incubated with a streptavidin-alkaline phosphatase complex as recommended by Amersham (U.K.). *p*-Nitrophenyl phosphate was added and the absorbance at 405 nm was recorded and compared to a plot of values obtained from wells containing known amounts of purified PVX virions simultaneously assayed in the same ELISA plate.

Results

Sample preparation

Alternative sample preparation methods (most of them based on organic extraction and nucleic acid precipitations) were tested in order to devise optimal extraction protocols for each pathogen infection compatible with a standard scaled up procedure of detection. Small pieces of tissue were introduced into Eppendorf tubes and homogenized in the presence of a highly denaturing extraction buffer. Guanidinium isothiocyanate and other components of this buffer protected RNA from degradation for several hours allowing samples to be sent at room temperature from the field to the laboratory, except for tuber extracts which had to be stored on ice to avoid swelling caused by starch. Total cellular RNA was isolated by adsorbing to an anion exchange resin fixed to a pipet tip. Agarose gel electrophoresis indicated the presence of small amounts (less than 5%) of contaminating plant DNA as well as some pigments when tissues were not fresh (particularly when they were stored frozen for long time before processing). These contaminants did not significantly affect detection capacity of the assay.

Detection conditions

Hybridization time is another limiting factor for rapid diagnosis of pathogens by nucleic acid hybridization. Hybridization rate largely depends on nucleic acids concentration. Overloading with RNA extracts from virus infected tissue (more than 0.5 μg) was avoided since sensitivity did not improve significantly and background hybridization became important. Since target RNA is a rather fixed parameter, probe concentration was increased up to 1 μg/ml. The fact that probe is single-stranded DNA prevents the typical self rehybridization that occurs with denatured double-stranded probes. Thus, effective probe concentration becomes fully available for hybridization with viral RNA. At this concentration it was found that 1 h was enough for sensitive detection of PVX RNA without significative loss of sensitivity (not shown).

To test whether indirect hybridization advantages (Wolf et al., 1986) could

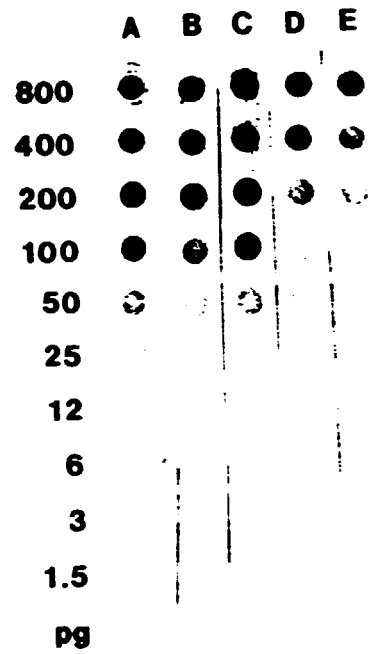

Fig. 1. Sensitivity of variants of sandwich hybridization assay. 800 pg purified PVX genomic RNA were serially diluted with a solution containing 50 ng tRNA and loaded onto nylon membranes as stated in Materials and Methods section. Hybridization (sequence specific step) was conducted with 1 μg/ml phX probe, followed (or not in columns D and E) by a post-hybridization step. (A) Post-hybridization was performed in presence of double-stranded pBluescript SK M13(−) DNA, previously labelled with biotin by nick-translation (standard procedure). (B) Post-hybridization probe was single-stranded pBluescript SK M13(−) DNA chemically labelled with photobiotin. (C) Post-hybridization probe was sulfonated single-stranded pBluescript SK M13(−) DNA. (D) Non-labelled phX probe was combined with equimolecular amounts of photobiotin-labelled single-stranded pBluescript SK M13(−) DNA (see lane B) and used together in a single step hybridization protocol. (E) Double-stranded phX DNA was labelled with biotin by nick translation and used directly for detection as in Hopp et al. (1988).

be applied to potato pathogen detection, its relative sensitivity was compared to the traditional direct hybridization protocol. Fig. 1 shows that the indirect hybridization protocol was eight times more sensitive than direct detection for detecting PVX genomic RNA. (Fig. 1, column A versus column E). Fig. 1 also compares the relative sensitivity of some variations on the sandwich hybridization procedure. Sandwich hybridization could be carried out in a single step, combining sequence specific and universal probes (Fig. 1, column D) or as a two-step reaction with a sequence specific hybridization step followed by a general post-hybridization step (Fig. 1, columns A, B and C). The post-hybridization step could be performed with a universal probe labelled either with biotin (Fig. 1, columns A and B), or with antigenic epitopes introduced by sulfonation (Fig. 1, column C). Single step sandwich hybridization was less sensitive than two-step hybridization, probably because most of the biotinylated

probe hybridized to the excess of non-labelled complement. unbound. Use of sulfonated probes, as part of an alterna detection system, resulted in similar levels of detection than suggesting that either method could be used.

Specificity of the cDNA probes

In order to examine the specificity of the cDNA probes, h iments were carried out using extracts of healthy or infecte of the viruses or viroid analyzed in this paper as target. T

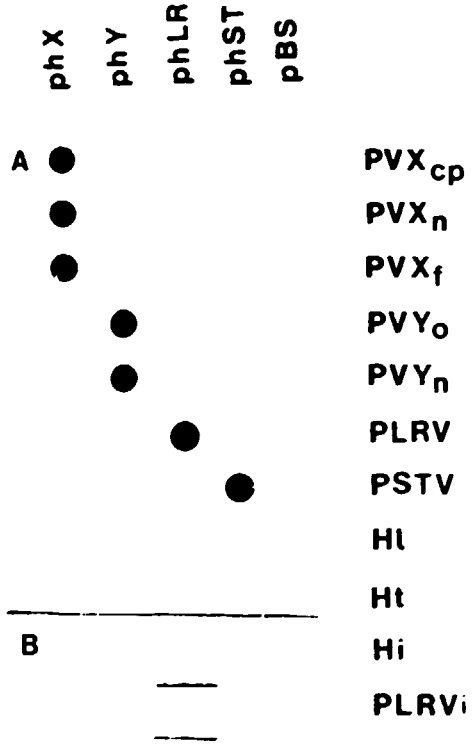

Fig. 2. Specificity of individual probes. RNA extracts were prepared from (A) or *Myzus persicae* (B) and spotted onto nylon membranes. Each spo total RNA (calibrated by relative intensity of ethidium bromide staining). H in the presence of the individual cDNA probes (indicated at the top) spec and PSTV respectively, and with a control 'non-recombinant' vector DNA in the case of Fig. 1A (standard procedure). In the specific case of aphid derived from a single individual insect and hybridization was carried out was detected with the Orgenics kit. Samples: PVXcp, PVXn and PVXf= P and 'fuerte', respectively. Hl= healthy potato leaf RNA extract. Ht= healthy Hi= healthy insect extract. PLRVi= PLRV infected insect

indicate that each of the different probes correctly detected the
fected with the specific pathogen. No background reaction wa
healthy plant extracts nor in extracts from plants infected with nc
or viruses, suggesting that neither plant nucleic acids nor non-re
cause significant interference with specific hybridization.

Fig. 2A also shows that even though each cDNA probe is
specific, they are not strain specific. Nucleotide sequence analys
the PVX probe comprises a rather conserved region of the P
comparison to other sequenced PVX isolates (i.e. 78% homolo
and Potexvirus genomes (Orman et al., 1990). PVX cDNA pr
plementary to the PVXcp genome) also detects two other PV
widely distributed in Argentina, having very different behavio
to virulence and symptom development in potato and tobacco
(Ing. Agr. Muñoz, personal communication). This confirms wh
from nucleotide sequence comparisons. The PVY cDNA probe,
ing PVYo genomic RNA as template, also detects PVYn (Fig.
from the 98% nucleotide sequence homology between PVYo a
3′ region used as probe (Bravo Almonacid and Mentaberry, 19
probe made with a French virus strain detects a field isolate fr
extracts of infected leaves or insects; and the PSTV cDNA probe
bridizes to viroid infected potato leaf extracts (Fig. 2). 'Non-re
type' pBluescript SK(+) phagemid DNA control probe failed to
of the samples indicating lack of homology between vector D
plant or pathogen RNA (Fig. 2A).

Sensitivity of the sandwich polyprobe nucleic acid hybridization

Sensitivity was assessed by slot-blotting known amounts of pa
RNA and 'polyprobe' hybridization. Viral RNA (PVX and PV
script (PSTV) or RNA extracts from infected plants (PLRV) w
a solution containing healthy potato leaf RNA and slotted onto
membrane. After sequence specific hybridization with a 'poly
of non-labelled single-stranded DNA probes specific to PVX, I
PSTV), post-hybridization was performed usi..g a 'non recombir
SK M13(−) labelled with biotin. Fig. 3 shows that as low as 1.5
genomic RNA (equivalent to about 0.025 ng of virions) could b
represents a minimum detection ratio of about 1:30000 betwee
total RNA and the amount found in approximately 1.5 mg of inf
tivar Huinkul, see Table 2). Assuming the A_{260} 1% of PVX to
1970) and the molecular weight of a PVX particle to be 3.5 ×
1959), the detection limit is 4 × 10^4 viral particles. The same
was tested by a highly sensitive ELISA based on the use of b
clonal antibodies. Maximum sensitivity achieved with this mod
0.5 ng of purified PVX particles in our hands; this is still twenty
tive than with sandwich hybridization. Fig. 3 also shows that ab

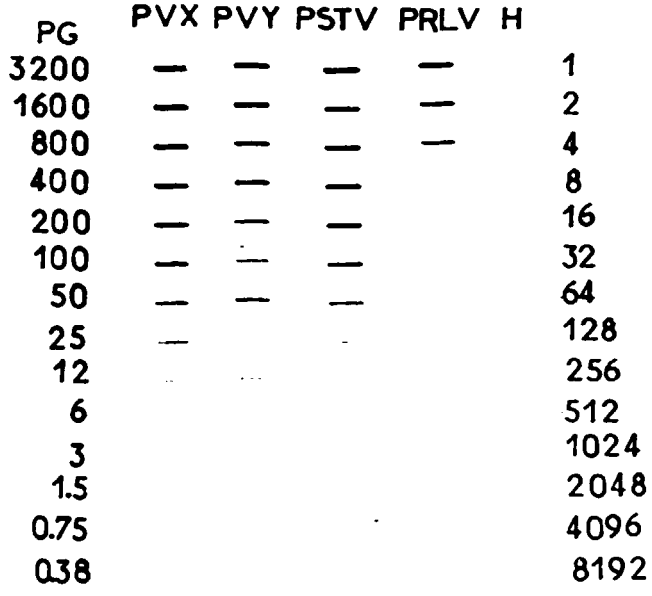

Fig. 3. Sensitivity of detection for each of the pathogens. Purified PVX and PVY genomic RNAs from 3200 to 0.75 pg were serially diluted with a solution containing 50 ng of potato leaf RNA as stated in Materials and Methods and loaded in the first two columns of a 0.20 μm-nitrocellulose membrane. PSTV cDNA cloned in pBluescript SK M13(+) was transcribed in vitro with a kit from Stratagene (U.S.A.), quantitated by comparative ethidium bromide staining and serially diluted as for PVX and PVY in the third column of the filter. About 0.2 μg of PLRV infected *Physalis floridana* RNA extract was serially diluted as for samples above and slotted in the fourth column of the manifold. Finally, 0.2 μg of potato tuber RNA (extracted as stated in 'sample preparation') were serially diluted as for samples above, loaded in the fifth column (indicated with H) and hybridized together with the rest. Hybridization was conducted with a 'polyprobe' combination of phX, phY, phLR and phST DNAs. Post-hybridization was carried out with a double-stranded biotinylated pBluescript SK(−) DNA. Numbers at the left indicate the amount of RNA of PVX, PVY and PSTV loaded into the slots. Numbers at the right indicate dilution factor for PLRV infected leaf and healthy tuber RNA extracts.

RNA could be detected by slot-blot hybridization. Translated into virion terms this means a limit of 0.05 ng PVY particles which is 50 times more sensitive than the corresponding limit detected by currently used standard ELISA.

Samples derived from PLRV infected *Physalis floridana* leaves showed positive color reaction up to a dilution of 1/64 in potato leaf extracts. Although an absolute lower limit of detection of PLRV by this slot-blot hybridization experiment could not be determined, the degree of sensitivity was directly comparable to that obtained with standard nick-translated ^{32}P-labelled probes (overnight exposure) and was 32 times more sensitive than a commercial ELISA kit (not shown). Under the same conditions, mixtures of healthy leaf RNA extract and dilution series of PSTV cDNA transcript were blotted on the same membrane (Fig. 3). The lower limit for positive signals was observed in the slot containing

1.5 pg of viroid cDNA transcript. Although it is difficult to compare the detection limit of linear RNA directly to that of circular RNA, the difference would probably not increase this value significantly if true genomic PSTV RNA is present instead of its cDNA transcript. Neither samples from healthy plants nor dilution of pathogen nucleic acid in healthy plant RNA extracts interfered the detection sensitivity (compare Fig. 1, column A and Fig. 3, column PVX).

Field trials

In order to study the feasibility of using the hybridization assay for tuber indexing, field potato tubers belonging to the five potato cultivars of major production in Argentina (representative of a wide geographic region of potato production) were collected in the central wholesale vegetable market of Buenos

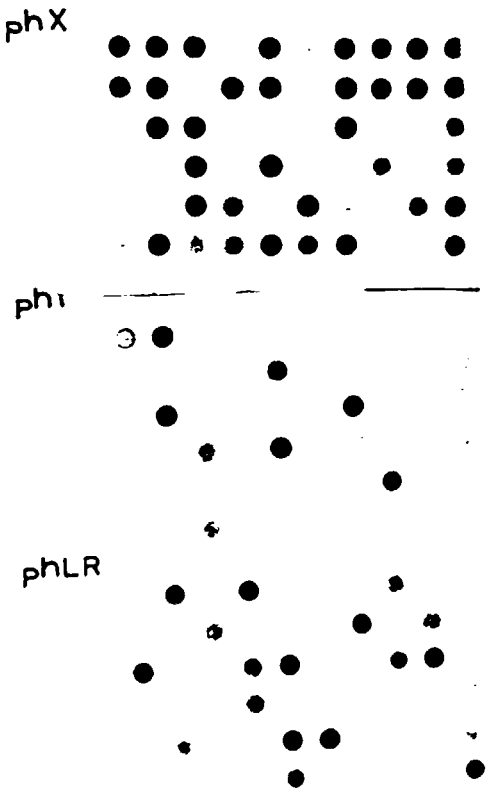

Fig. 4. Potato indexing with individual probes. Potato tuber sprout RNA extracts were prepared from field production tubers for consumption belonging to cultivars *Hankul MAG*, *Ballenera*, *Serrana INTA*, *Spunta* and *Kennebec*. Samples were dotted with a manifold in three nylon membrane replicates. Each replica was subjected to hybridization with individual probes phX (for PVX), phY (for PVY) and phLR (for PLRV) as in Fig. 2.

TABLE I
Comparison of results obtained by dot blot hybridization and ELISA in field tests for PVX, PVY and PLRV in potato tubers

	ELISA(+) SDBH(+)	ELISA(−) SDBH(−)	ELISA(+) SDBH(−)	ELISA(−) SDBH(+)
PVX	43	15	1	1
PVY	7	51	0	2
PLRV	15	29	1	5

Potato tissue extracts, assayed as indicated in Fig. 5, were tested in parallel by ELISA. Results were grouped into four categories. SDBH= sandwich dot-blot hybridization. (+)= positive detection. (−)= negative detection.

Aires for analysis. After sprout development, presence of PVX, PVY and PLRV was tested in tissues expanding out from eye plugs, using both ELISA and nucleic acid hybridization for each individual virus. Fig. 5 shows the dot–blot hybridization filters resulting from this experiment. Positive signals can be clearly distinguished from the background. Non-specific coloration of rings around some of the samples in the figure are easily distinguished from true positives and might be attributed to problems in handling the filters. Although the sample is too small to make epidemiological conclusions, it is clear that PVX is widespread in Argentine potato tubers for consumption, while PVY and PLRV are of more limited distribution. When the results from ELISA and dot–blot hybridization were compared, there was more than 93% agreement (Table 1). It is concluded from the good agreement between ELISA and dot–blot hybridization that either method could be used for indexing purposes.

Pathogen control in potato explants and vector insects

Forecast systems based on the frequency of virus-infected insects is a means of potato disease control through vector elimination. In order to start an epidemiological survey of virus-infected insect population dynamics, the method was assayed with virus-free *Myzus persicae* that were fed (or not) with PLRV infected plants to induce persistent infection. After few weeks, aphid extracts were dot–blotted and hybridized to a PLRV cDNA probe (Fig. 2B). Infected insects developed clear hybridization signals compared to virus-free controls.

Pathogen-free potato production programs are based on micro-propagation of plantlets produced in vitro by meristem or shoot tip culture. Screening for the presence of pathogens is performed at the earliest stage of development with the most sensitive and reliable diagnostic system available to avoid discarding large numbers of diseased plants at a later stage. In order to test the performance of the hybridization system, virus-free potato plantlets from Argentine cultivars were grown in micro-propagation medium and artificially infected with individual potato viruses. After four weeks, small pieces of leaves were cut and tested by slot–blot hybridization and ELISA. RNA extracts from infected plantlets developed hybridization signals that could be quantitated by densitometry when

TABLE 2
Quantitation of resistance to viral replication in in vitro plantlets and protoplasts

Plant material	RNA concentration	% inhibition	
		SSBH	ELISA
Plantlets			
S. acaule	78	92	94
Resistant control	112	89	90
Susceptible cont.	1005	–	–
Protoplasts			
S. acaule	89	95	95
Resistant control	306	84	93
Susceptible cont.	1912	–	–

Wild potato plants (*S. acaule* P.I.320277) of Argentine origin, as well as resistant and susceptible controls (*S. tuberosum tuberosum* cv. *Serrana INTA*, carrying immunity gene X^{n}, and cv. *Huinkul MAG*, respectively) were used for establishing in vitro lines that were multiplied by micro-propagation of internode sections. These virus-free in vitro micropropagated plants were mechanically inoculated with a purified preparation of the South American strain PVXcp, to analyze resistance gene effect on viral multiplication. Plantlets, or protoplasts derived from their leaves, were assayed one month or 30 h later (respectively) by slot-blot hybridization in the presence of standards with known amounts of virion RNA. Levels of PVX RNA were quantified by densitometry and represented in the first column as ng RNA/g leaf weight or as pg RNA/viable protoplast (Ceriani et al., 1990). The second column indicates the percentage of inhibition of virus replication respect to susceptible control. For comparison, the percentage of inhibition determined in parallel by ELISA is shown in the third column. SSBH = sandwich slot-blot hybridization.

compared to data of virus-free control background signals (see cv. Huinkul MAG in Table 2).

Argentine potato germplasm is being screened for resistance to infection with potato viruses in order to use them as new genetic sources in breeding programs. Seeds were germinated in sterile conditions and artificially infected with individual potato viruses. After four weeks, small pieces of leaves were cut and tested by slot–blot hybridization and ELISA (Table 2). Resistant genotypes were further micro-propagated and protoplasts prepared from their leaves for in vitro infection (Saladrigas et al., 1990). Virus replication was followed in resistant protoplasts, as well as in susceptible controls, by slot–blot hybridization and ELISA (Table 2). Hybridization assay results were quantitated by densitometry and ELISA by spectrophotometry, indicating that (within a range of virus concentrations) slot–blot hybridization compares well with ELISA in this respect. However, linear relation of densitometry readings to virus concentration was restricted to a rather narrow range (0–100 pg) as compared to ELISA, and varied from experiment to experiment. Densitometry of hybridization assay may be used to complement immunological quantification data when pathogen concentrations are below the limits of ELISA detection by including known standards in each assay.

Discussion

Well-characterized PVX, PVY, PLRV and PSTV cDNA clones were used separately or combined for the development of a general diagnostic kit for potato pathogens. For routine diagnostic purposes it is desirable for each probe to be strictly pathogen-specific while being able to recognize the polymorphic variants, strains or quasispecies occurring in pathogenic groups. This was achieved by choosing highly conserved sequences for Potexviruses and Potyviruses located in the 3′ region of the genomes of PVX and PVY, respectively. While all four cDNA probes showed lack of cross hybridization to non-related pathogens (Fig. 2), PVX cDNA probe hybridized to three different isolates of this virus and showed 96% agreement with polyclonal antibody-based ELISA when heterogeneous field material was tested. A similar situation was true for PVY which is supported by the comparatively higher degree of genomic nucleotide sequence conservation among different isolates of PVY and members of the potyvirus group in general. Lack of strain specificity for the PLRV probe was partially assured by selecting a clone containing a rather large cDNA insert and tested indirectly by comparison with field ELISA trials. However, as is true for ELISA, lack of detection of some polymorphic variants cannot be ruled out. Problems in the availability of virus variants did not allow us to perform a more complete assessment.

In establishing a general diagnostic assay it is essential to standardize rapid and easy sample preparation procedures for sensitive detection of all pathogens. The recent availability of commercial anion-exchange systems for purification of nucleic acids in presence of highly chaotropic agents that denature and expose viral genomes while protecting them from nuclease action, helped us to design such a sample preparation protocol. This purification step introduces additional processing time (1 min centrifugation plus approximately 10 min per sample) as compared to direct sap spotting used when radioactive-labelled probes are employed (Boulton et al., 1987; Chu et al., 1989; Maule et al., 1983b). However, this inconvenience is compensated by the elimination of host components that reduce sensitivity in crude plant homogenates (Maule et al., 1983) and eliminate background due to interfering plant pigments.

The use of phagemids as cloning, in vitro transcription and sequencing vectors is of common practice in molecular biology laboratories. Single-stranded recombinant DNA can be cheaply obtained in large amounts without the use of sophisticated equipment. Sandwich hybridization using a universal-labelled probe is only slightly more complex than standard direct hybridization, but offers some logistic advantages with higher sensitivity (Wolf et al., 1986 and this paper). The sequence-specific hybridization step can be performed in short time periods by increasing concentration of non-labelled specific probes. The more costly biotin-labelled probe can then be universally applied in a post-hybridization step amplifying the signal generated from pathogen nucleic acid. This universal probe can be produced and labelled on large scale using chemical labelling, such as photobiotinylation or sulfonation, providing advantages for simply-equipped laboratories, like reduced hazards, lowered costs and longer shelf life.

Non-radioactive sandwich hybridization allowed the detection of a few pg of viral genomic RNA. These values compare well with previous reports on detection of the mentioned pathogens using either radioactive or non-radioactive probes (Boulton et al., 1987; Eweida et al., 1988; Hopp et al., 1988; Owens and Diener, 1984; Van Wezenbeek et al., 1982) and with standard or modified ELISA (Gugerly, 1978, 1979, 1980 and this paper), with exception of PSTV where better sensitivity was obtained using RNA probes (Roy et al., 1989).

The assay described here was tested with field material that was prepared in situ for processing (combination of fresh sap with extraction solution) and tested using simple laboratory conditions. It was also successfully employed for a) germplasm screening in relation to virus tolerance, b) detection of pathogenic genome in vector insects and plantlets derived from in vitro explants and c) in more sophisticated quantitative determinations of viral replication in artificially inoculated plants and protoplasts, indicating that this method could be useful as a diagnosis test for viral infections in the field, indexing potato seed tubers and for screening germplasm gene sources in the context of breeding programs.

Diagnosis of potato pathogens is currently carried out by different methods, i.e. ELISA for viruses in most cases, nucleic acid hybridization for PSTV and particle-deficient viruses such as tobacco rattle virus (Harrison et al., 1983). As more pathogen genomes are cloned, adoption of hybridization methods will eventually help to standardize diagnostic procedures. We have demonstrated here that sensitivity of detection for each individual pathogen was not impaired by the simultaneous presence of all four probes, thus showing the feasibility of implementing a routine diagnostic test for potato based in the combination of several probes for different pathogens. Such a 'polyprobe' diagnostic test could prove to be useful in selecting pathogen-free material (i.e. seed production programs) and is currently being implemented in Argentina for this purpose.

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References

Birnboim, H.C (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. 100, 243–270.

Boulton, R.E., Jellis, G.J., Baulcombe, D.C. and Squire, A.M. (1986) The application of complementary DNA probes to routine virus detection, with particular reference to potato viruses. In: R.A.C. Jones and L. Torrance (Eds) Developments and applications in virus testing. A.A.B., pp. 41–53. Lavenham Press, Wellesbourne, U.K.

Bercks, R. (1970) Potato virus X. Commonwealth Mycol. Institute/Assoc. Appl. Biol. Descript. Plant Viruses, No. 4.

Bravo Almonacid, F. and Mentaberry, A.N. (1989) Nucleotide cDNA sequence coding for the PVYo coat protein. Nucl. Acids Res. 17, 4401.

Bruning, H. and Wenzel, G. (1988) Gensonden zur Diagnose der Erreger von Schwartzbeinigkeit und Knollennassfaule. Molekulare Hybridisierung als Nachweismethode von *Erwinia carotovora*. Nachrichtenbl. D. Pflanzenschutzdienstes 40, 17–19.

Cerian, M.F., Saladrigas, M.V., Tozzini, A.C. and Hopp, H.E. (1990) Resistance to replication of PVX in protoplasts of a *Solanum acaule* clone. Proceedings Internatl. Found. Science-INTA Seminar on Plant Viruses. Carlos Paz, Córdoba. Rev. Investig. Agrop. (in press).

Chu, P.W.G., Waterhouse, P.M., Martin, R.R. and Gerlach, W.L. (1989) New approaches to the detection of microbial plant pathogens. In: G.E. Russell (Ed.). Biotechnology and Genetic Engineering Reviews, Vol. 7, pp. 45–111. Intercept Ltd., Andover, U.K.

Diagen (1988) The Qiagenologist. Application Protocols. Dusseldorf, F.R.G., 48 pp.

Eweida, M., Sit, T.L., Sira, S. and Abbou Haidar, M.G. (1989) Highly sensitive and specific non-radioactive biotinylated probes for dot-blot, Southern and colony hybridizations. J. Virol. Methods 26, 35–44.

Forster, A.C., Mc Innes, J., Skingle, D.C. and Symons, R. (1985) Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent: photobiotin. Nucl. Acids Res. 13, 745–763.

Fribourg, C.E. (1975) Studies on potato virus strains isolated from Peruvian potatoes. Potato Res. 18, 216–226.

Gubler, U. and Hoffman, B.J. (1983) A simple and very efficient method for generating cDNA libraries. Gene 25, 263–269.

Gugerly, P. (1978) The detection of two potato viruses by enzyme-linked immunosorbent assay (ELISA). Phytopathol. Z. 92, 51–56.

Gugerly, P. (1979) Le test immuno-enzymatique (ELISA) et son application peur le diagnostic rapide des virus de la pomme de terre. Rev. Suisse Agric. 11, 253–260.

Gugerly, P. and Gehriger, W. (1980) Enzyme-linked immunosorbent assay (ELISA) for the detection of potato leafroll virus and potato virus Y in potato tubers after artificial break of dormancy. Potato Res. 23, 353–359.

Harrison, B.D., Robinson, D.J., Mowat, W.P. and Duncan, C.H. (1983) Comparison of nucleic acid hybridization and other tests for detecting tobacco rattle virus in narcissus plants and potato tubers. Ann. Appl. Biol. 102, 331–338.

Hopp, H.E., Giavedoni, L., Mandel, A., Arese, A., Orman, B., Bravo Almonacid, F., Torres, H.N. and Mentaberry, A.N. (1988) Biotinylated nucleic acid hybridization probes for potato virus detection. Arch. Virol. 103, 231–241.

Huarte, M.A., Mendiburu, A.O., Monti, M.C. and Butzonich, I.P. (1986) Serrana INTA: a widely adapted virus resistant potato cultivar from Argentina. Am. Potato J. 63, 695–699.

Hull, R. (1986) The potential for using dot-blot hybridisation in the detection of plant viruses. In: R.A.C. Jones and L. Torrance (Eds.), Developments and Applications in Virus Testing, pp. 3–12. A.A.B., Lavenham Press, Wellesbourne, U.K.

International Potato Center (1985) Annual Report CIP 1984, Lima, Peru, 169 pp.

Karjalainen, R. (1988) Prospects for using DNA hybridization as a means of screening plant virus resistance. Nord. Jordbruks-Forskning 4, 516–517.

Khandjian, E.W. (1987) Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. Biotechnology 5, 165–167.

Leary, J.J., Brigati, D.J. and Ward, D.C. (1983) Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. Proc. Natl. Acad. Sci. USA 80, 4045–4049.

Mandel, A.M., Orman, B.E., Celnik, R., Torres, H.N. and Mentaberry, A.N. (1989) Nucleotide cDNA sequence coding for the PVXc coat protein. Nucl. Acids Res. 17, 1265.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York, U.S.A.

Masson, J., Lecerf, M., Tousselle, P., Perennec, P. and Pelletier, G. (1987) Plant regeneration from protoplasts of diploid potato derived from crosses of *Solanum tuberosum* with wild *Solanum* species. Plant Sci. 53, 167–176.

Maule, A.J., Boulton, M.I. and Wood, K.R. (1983a) Infection of protoplasts from several *Brassica* species with cauliflower mosaic virus following inoculation with polyethylene glycol. J. Gen. Virol. 64, 2655–2660.

Maule, A.J., Hull, R. and Donson, J. (1983b) The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. J. Virol. Methods 6, 215–224.

Nur, I., Bove, J.M., Saillard, C., Rottem, S., Whitcomb, R.M. and Razin, S. (1986) DNA probes in detection of spiroplasmas and mycoplasma-like organisms in plants and insects. FEMS Microbiol. Lett. 35, 157–162.

Orman, B., Celnik, R.M., Mandel, A.M., Torres, H.N. and Mentaberry, A.N. (1990) Complete cDNA sequence of a South American isolate of potato virus X. Virus Res. 16, 293–306.

Owens, R.A. and Diener, T.O. (1984) Spot hybridization for the detection of viroids and viruses. Methods Virol. 3, 173–187.

Palukaitis, P., Cotts, S. and Zaitlin, M. (1985) Detection and identification of viroids and viral nucleic acids by dot-blot hybridization. Acta Horticult. 164, 109–118.

Reichmann, M.E. (1959) Potato virus X. Part III. Light scattering studies. Can. J. Chem. 37, 384–388.

Roy, B.P., AbouHaidar, M.G. and Alexander, A. (1989) Biotinylated RNA probes for the detection of potato spindle tuber viroid (PSTV) in plants. J. Virol. Methods 23, 149–156.

Saladrigas, M.V., Ceriani, M.F., Tozzini, A.C., Arese, A.I. and Hopp, H.E. (1990) Potato gene X^1 confers inoculum dependent resistance to potato virus X replication in protoplasts. Plant Cell Physiol. 31, 749–755.

Stratagene (1988) Bluescript Exo/mung DNA Sequencing System Instruction Manual. San Diego, California U.S.A., 60 pp.

Symons, R.H. (1984) Diagnostic approaches for the rapid and specific detection of plant viruses and viroids. In: T. Kosuge and E.W. Vester (Eds.), Plant Microbe Interactions Vol. I, pp. 93–124. MacMillan Publishing Co., New York, U.S.A.

Tenover, F.C. (1988) Diagnostic deoxyribonucleic acid probes for infectious diseases. Clin. Microbiol. Rev. 1, 82–101.

Van Brunt, J. and Klausner, A. (1987) Pushing probes to market. Biotechnology 5, 211–221.

Van Wezenbeek, P., Vos, F., Van Boom, J. and Van Kammen, A. (1982) Molecular cloning and characterization of a complete DNA copy of potato spindle tuber viroid RNA. Nucl. Acids Res. 10, 7947–7957.

Wolf, H., Leser, U., Haus, M., Gu, S.Y. and Pathmanathan, R. (1986) Sandwich nucleic acid hybridization: a method with a universally usable labeled probe for various specific tests. J. Virol. Methods 13, 1–8.

Zrein, M., Burckard, J. and Van Regenmortel, M.H.V. (1986) Use of the biotin-avidin system for detecting a broad range of serologically related plant viruses by ELISA. J. Virol. Methods 13, 121–128.

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Potato Gene X^1 Confers Inoculum Dependent Resistance to Potato Virus X Replication in Protoplasts

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Protoplasts were obtained from genetically related Argentine potato (*Solanum tuberosum tuberosum*) cultivars: *Serrana INTA* and *Huinkul MAG* and from an European cultivar: *Spunta*. *Serrana INTA* is resistant to potato virus X (PVX) infection because it carries the major reaction gene X^1 (believed to be the same as Rx_{acl}) while *Huinkul MAG* and *Spunta* are susceptible. Protoplasts were inoculated with a purified preparation of PVXcp (a South American isolate) and then assayed for PVX concentration at different times postinfection by enzyme-linked immunosorbent assay (ELISA), immunofluorescence and nucleic acid hybridization. PVX multiplication rates in *Serrana INTA* were about fifteen times slower than in susceptible genotypes when cell-bound virus levels just after infection were in the range of 0.01 to 0.1 ng PVX per viable protoplast. However, when inoculum levels were raised to 1 ng PVX per viable protoplast, PVX multiplication was about the same in all three genotypes. To rule out genetic background effects in this behaviour, protoplasts of an Argentine *S. acaule* clone (PI: 320277) likely carrying the same resistance gene, were infected with PVX in similar conditions, reproducing those results obtained with *Serrana INTA*. The comparison of PVX replication in protoplasts and whole plants indicate that although X^1 gene confers resistance at the cell level it necessitates of tissue structure to fully express immunity.

Key words: Potexviruses — PVX — *Solanum acaule* — *Solanum tuberosum tuberosum* — Virus-host relationship — Virus resistance gene expression.

Incorporation of genes that decrease the extent of pathogen multiplication and the severity of symptom expression is of general practice in potato breeding programs. In the case of potato virus X, three characterized reaction genes have been used. The hypersensitive reaction genes Nx and Nb (Cockerham 1955, 1970) are present in many commercial cultivars (Cardman 1942, Cockerham 1943, 1958). A third gene, X^1, conferring extreme resistance to most PVX strains (Cockerham 1958, Ross 1954a, b, Solomon 1985) is present in many *Solanum acaule* genotypes and in some few *S. tuberosum* clones (i.e. clones 44/1016/10 and G5399[4]). This gene is likely to be identical to Rx_{acl} and independent from Rx_{adg} (Cockerham 1970). However it is not so extensively distributed in commercial cultivars, yet. The potato breeding program of INTA-Balcarce recently released the cultivar *Serrana INTA* carrying this gene together with genes for resistance to potato virus Y (PVY) and potato leaf roll virus (PLRV) (Huarte et al. 1986). Although incorporated into potato cultivars, little is known about the mechanism of resistance. The use of artificially inoculated protoplasts provides a controlled system for studying the action of resistance genes on virus replication at the single cell level. In vitro infection of protoplasts with PVX was pioneered by Otsuki et al. (1974) and Shalla and Petersen (1973) using tobacco as plant source. Much later Prakash and Foxe (1985) were able to reproduce these experiments with protoplasts derived from potato. It was shown that resistance to PVX could be analyzed under these conditions, with conflicting results for gene Nx (Foxe and Prakash 1986, Adams et al. 1985, 1986). Protoplasts obtained from potato cultivar *Cara* carrying immunity gene Rx, supported only

Abbreviations: ELISA, enzyme-linked immunosorbent assay; PVX, potato virus X; PI, plant introduction number from the Potato Introduction Station, Sturgeon Bay, U.S.A.

limited multiplication of PVX (Adams et al. 1986).

In this paper we arrive to conclusions on the expression of gene X^1 similar to those obtained by Adams et al. (1986) with the independently segregating gene Rx, by analyzing X^1 gene expression in two different genetic backgrounds: the commercial cultivar *Serrana INTA*, which also contains reaction genes for other viruses (Huarte et al. 1986) and a *Solanum acaule* clone obtained from a germplasm collection. However, protoplasts derived from both resistant genotypes behaved identically to those derived from susceptible genotypes when the viral inoculum was raised to higher levels.

Materials and Methods

Plant material, virus strains, cDNA clones and antibodies—The following *Solanum tuberosum* L. ssp *tuberosum* cultivars were used: *Spunta, Huinkul MAG*, and *Serrana INTA*. Cultivar *Serrana INTA* was obtained after crossing *Huinkul MAG* with a PVX immune potato clone carrying X^1 gene (Huarte et al. 1986). Therefore, *Serrana INTA* and *Huinkul MAG* are genetically related. *Solanum acaule* clone PI 320277 (with resistance to PVX likely due to the effect of X^1 gene, Ceriani et al. 1990) and segregants of clone PI 175396 (used as susceptible control) are both of Argentine origin. They are kept and multiplied by the Potato Introduction Station, Sturgeon Bay, U.S.A. This Station generously sent us sib seeds of the mentioned clones. PVX virions from South American isolate cp (Fribourg 1975, Moreira et al. 1980, Jones 1986, Torrance et al. 1986, Orman et al. 1990) and from an Argentine isolate (Province of Córdoba) were purified as described (Orman et al. 1990). PVX_{cp} cDNA clone pX1 (Hopp et al. 1988, Orman et al. 1990) was labelled by oligonucleotide primed incorporation of α-^{32}P-dCTP with a specific activity of 10^9 dpm/μg DNA and used as hybridization probe (Hopp et al. 1988).

Polyclonal antibodies were prepared as described (Gugerli 1978) and biotinylated with a commercial kit from BRL (Bethesda Research Laboratories, Gaithersburg, MA, U.S.A.) as recommended by the supplier.

Protoplast manipulation—Virus-free seedlings were handled in sterile conditions and micropropagated as described by Masson et al. (1987). Leaf protoplasts from shoot cultures were obtained as reported by Masson et al. (1987). In vitro infection was carried out essentially as in Maule (1983) except that infection density (multiplicity) varied from 0.01 to 1 ng of PVX virions per viable protoplast per μl. Protoplast suspensions were incubated one minute and a half at a final polyethylene glycol 6000 concentration of 25% (w/v) (Sigma, St. Louis, MO, U.S.A.). Mock inoculations were performed in the same way in the absence of virus. After 3 washes, protoplasts were resuspended in 0.4 M mannitol (minimum volume) and 10 μl aliquots were taken in order to assess the number and viability of protoplasts and the amount of PVX incorporated into cells by ELISA and immunofluorescence. Viability of virus inoculated and mock inoculated potato protoplasts was determined by fluorescein diacetate staining (Widholm 1972). Viability of protoplasts usually decreased about one order of magnitude after inoculation. Although the rate of viability loss varied among experiments, no marked differences were observed between virus- or mock-inoculated *Serrana INTA* and *Spunta* or *Huinkul MAG* protoplasts.

Protoplasts were incubated at different times in an excess of medium containing nutrients, hormones, etc. (medium A) as stated in Masson et al. (1987) at 21°C under continuous fluorescent light for up to 50 h. At each post-infection time, protoplast samples were concentrated by centrifugation at $100 \times g$ for 2 min in a swinging bucket type rotor centrifuge (MSE Chilspin, U.K.) and resuspended in buffers either for viability testing, estimation of PVX concentration by ELISA, slot-blot hybridization and immunofluorescence.

For estimation of virus content by ELISA, aliquots of protoplasts were resuspended in 200 μl of 8 g/liter NaCl, 0.2 g/liter KH_2PO_4, 2.9 g/liter $Na_2HPO_4 \cdot 12H_2O$, 0.2 g/liter KCl, 0.5 ml/liter Tween 20, 20 g/liter polyvinylpyrrolidone (PVP, 10,000, added to wells precoated with polyclonal anti-PVX immunoglobulin G and incubated overnight (Gugerli 1978). After reaction with specific biotinylated immunoglobulin G, wells were incubated with a streptavidine-alkaline phosphatase complex as recommended by BRL. *p*-Nitrophenyl phosphate was added and the absorbance at 405 nm was recorded and compared to a plot of values obtained from wells containing known amounts of purified PVX virions that were assayed simultaneously in the same ELISA plate.

For immunofluorescence assays protoplasts were fixed in 3% glutaraldehyde and processed as in Shalla and Petersen (1973) except that rabbit anti-PVX polyclonal antibodies and fluorescein labelled goat anti-rabbit immunoglobulin G were used. These immunofluorescence tests indicate that more than 80% of the surviving protoplasts were effectively infected in the conditions used.

Nucleic acid hybridizations and Western blot analysis—For slot-blot assays, equal number of protoplasts belonging to cultivars *Huinkul MAG, Serrana INTA* or *S. acaule* (PI 320277 and PI 175396) were resuspended in a freshly prepared solution containing 10 U/ml RNasin (Promega, Madison, WI, U.S.A.), 6% formaldehyde, 1% sodium *N*-lauroylsarcosine, 2.4 M NaCl and 0.24 M sodium citrate pH 7 at 65°C and maintained at this temperature for 15 min. After rapid ice-cooling RNA was sampled into pre-wetted Nytran (Schleicher and Schuell, F.R.G.) membranes with the aid of a slot-blot manifold (Schleicher and Schuell, F.R.G.). After that membranes were washed twice

with 100 µl of 3 M NaCl, 0.3 M Na citrate, RNA was fixed by exposing the wet membrane to UV light (Khandjian 1987) followed by vacuum baking for 15 min at 80°C in a slab gel dryer.

For Northern blot hybridization, in vitro micropropagated potato seedlings (3–4 weeks old) were mechanically inoculated by hand rubbing 14 mg/ml of PVX in 8 g/liter NaCl, 0.2 g/liter KH_2PO_4, 2.9 g/liter $Na_2HPO_4 \cdot 12H_2O$, 0.2 g/liter KCl pH 7.4, on leaves using carborundum as abrassive. After 4 weeks, 1–3 g of leaf tissue were frozen in liquid nitrogen and disrupted with mortar and pestle, followed by addition of three volumes of guanidine isothiocianate buffer (4 M guanidine isothiocianate, 50 mM 2-mercaptoethanol, 20 mM EDTA and 20 mM MES pH 7.0) and 10 min of strong vortexing. Homogenate was centrifuged for 20 min at 7,000 rpm in a Sorvall SS34 rotor at 4°C. The resulting supernatant was phenol extracted and processed as described in Maniatis et al. (1982). After that, 0.6 volumes of absolute ethanol and potassium acetate to a final concentration of 0.1 M were added to the resulting aqueous phase. RNA was precipitated overnight at −20°C, centrifuged 15 min at 12,000 × *g* and resuspended in autoclaved double-distilled water. Approximately 7 µg of RNA were submitted to formaldehyde agarose gel electrophoresis (Maniatis et al. 1972) and blotted to Nytran membranes with 0.36 M NaCl, 0.02 M NaH_2PO_4, 2 mM EDTA and 6% formaldehyde (pH 7.4). RNA was fixed to the membrane as for slot-blot hybridization. Hybridization, washing and autoradiography were performed as in Hopp et al. (1988).

Western blotting was carried out with leaf extracts and rabbit polyclonal antibodies against PVX as described (Towbin et al. 1979). Liquid nitrogen-frozen tissue was homogenized in 60 mM Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol, 0.1% SDS, pH 6.8. The extracted proteins were quantified (Bradford 1976) and separated by discontinuous SDS-polyacrylamide gel electrophoresis in 12% gels (Altman et al. 1983). Then, proteins were transferred to Nytran membranes in the presence of 192 mM glycine, 20% methanol pH 8.3 (Towbin et al. 1979). Capsid proteins were detected using rabbit anti-PVX immunoglobulin G followed by an anti-rabbit antibody conjugated to alkaline phosphatase (Sigma, U.S.A.) as second antibody. The enzyme reaction was developed as in Harlow and Lane (1988).

Results

Expression of resistance at plant level—In order to analyze the X' gene action over the viral multiplication at the plant level, virus-free in vitro micropropagated plants were mechanically inoculated with a purified preparation of South American strain PVX_{cp}. Figure 1 shows a Western-blot experiment of total protein extracts of two genetically related potato cultivars (*Huinkul MAG* and *Serrana INTA*) and a third non-related cultivar (*Spunta*). Antibody reaction clearly indicated that a 30 kDa band corresponding to the capsid subunit protein was present in both susceptible cultivars (tracks 5 and 6) and absent in the immune cultivar (track 4). No bands were detected in non-inoculated controls. Similarly, Northern-blot experiments indicated positive hybridization to genomic and subgenomic RNAs in susceptible cultivars (Fig. 2, tracks 3 and 4) as well as no detectable hybridization to RNAs of *Serrana INTA* (Fig. 2, track 2) or RNAs corresponding to the non-inoculated controls (Fig. 2, tracks 5 to 7). Relative intensity of hybridization of genomic versus subgenomic bands was strongly dependent on alkali treatment before transfer. Gels shown in Figure 2 were not treated; therefore, hybridization signal of genomic RNA is clearly less intense than it should be. Different degrees of relative PVX RNA concentration were found when different susceptible genotypes were compared (Fig. 2, tracks 3 and 4) perhaps reflecting some difference in viral genome multiplication or degradation rates.

Fig. 1 Western-blot analysis of PVX inoculated susceptible and resistant plants. Ex vitro grown seedlings of cultivars *Spunta*, *Huinkul MAG* and *Serrana INTA* were inoculated with 14 mg/ml of PVX. One month later, virus-inoculated and non-inoculated leaves were collected and proteins extracted as described in Materials and Methods. 10 µg of total protein were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with antibodies against PVX capsid protein. Lanes 1 and 3: Non-inoculated leaves of cultivars *Sarrana INTA*, *Spunta* and *Huinkul MAG*, respectively. Lanes 4 to 6: PVX-inoculated leaves of *Serrana INTA*, *Spunta* and *Huinkul MAG*, respectively. Lane 7: Purified PVXcp virions.

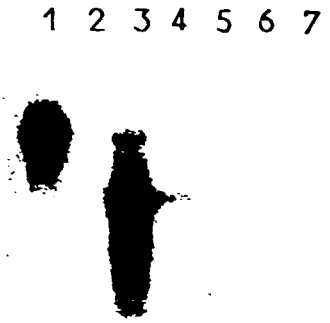

Fig. 2 Northern-blot analysis of PVX inoculated susceptible and resistant plants. Hybridizations of total RNA from PVX-inoculated and non inoculated seedlings of cultivars *Serrana INTA*, *Spunta* and *Huinkul MAG* with ^{32}P-labeled pX1 cDNA as probe. Lane 1: Purified PVX genomic RNA. Lane 2 to 4: Total RNA of PVX inoculated cultivars *Serrana INTA*, *Huinkul MAG* and *Spunta*, respectively. Lanes 5 to 7: Total RNA of the same non-inoculated cultivars.

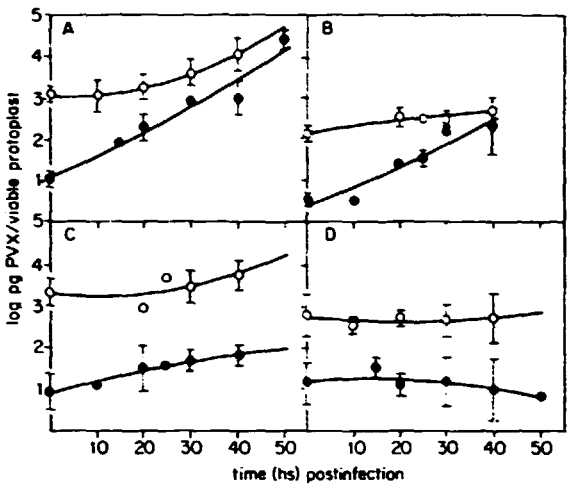

Fig. 3 Accumulation time course of PVX in susceptible and re- protoplasts. Potato leaf protoplasts prepared from *Huinkul MAG* (A), *Spunta* (B), *Serrana INTA* (C) and from *Solanum acaule* clone PI 320277 (D) were inoculated with 0.01 ng PVX/viable protoplast (-●-) or 1 ng PVX/viable protoplast (-○-) in the presence of polyethylene glycol. ELISA and viability analysis were carried out immediately after inoculation and at different times post-infection. Three representative independent experiments were chosen and graphicated. Best linear or quadratic functions fitting to experimental data were drawn. Bars indicate standard deviations for each of the analyzed times except in those cases where only one measurement was available.

Similar results to those obtained with *Serrana INTA* were obtained using *S. acaule* genotype PI 320277 (not shown) carrying the immunity gene in the context of a different genetic background. These results suggest that immunity conferred by gene X^1 in *Solanum* could be attributed to the lack of replication of PVX in leaf tissue.

PVX replication in potato protoplasts—Viral multiplication at the cellular level was monitored by ELISA (Fig. 3A and 3B) and slot-blot hybridization (Fig. 4A and 4C). Absolute PVX accumulation in inoculated protoplasts showed a great deal of variation from experiment to experiment. However, the trend was to greater yields of PVX with increasing concentrations of inoculum over the range from 0.01 to 0.1 ng cell-bound virus per viable protoplast. Figure 3A shows typical time course accumulation plots of PVX after in vitro infection of protoplasts obtained from an Argentine susceptible cultivar, *Huinkul MAG*. After a period of 15–20 h post-infection there was a logarithmic increase of capsid protein synthesis reaching maximum levels at 40–50 h post-infection. Nucleic acid hybridization was used to verify the presence of PVX RNA in the inoculated protoplasts. Similar plot shapes were observed when virus replication was scored either at capsid production level by ELISA or at the genomic level by slot blot hybridization densitometry in the same experiments. As stated above relative increase of virus concentration was largely dependent on inoculum levels. Best relative accumulations with good reproducibility were obtained with a multiplicity of 0.01 ng PVX/viable infected protoplast. Protoplasts were found to support PVX virion increase after 40 h of incubation that reach 94 times the concentration of cell-bound virion levels immediately after infection. At higher multiplicity levels (i.e. 1 ng PVX/viable protoplast) accumulation did not increase proportionally to inoculum levels and as a result, plots tend to a more horizontal shape (Fig. 3A), probably because the high number of virions per protoplast saturates the multiplication capacity of the cell.

Essentially similar results were obtained with protoplasts belonging to the susceptible European cultivar *Spunta* (Fig. 3B) or to the susceptible segregants of *S. acaule* clone PI 175396, as well as with a local PVX isolate different from PVX_{cp} when used as an inoculum source. This local isolate, used as an alternative virus strain, differs from PVX_{cp} in the structure of capsid subunit protein as assessed by difference in electrophoretic mobility and nucleotide sequence (unpublished results). These results suggest that, under the conditions used, the kinetics of PVX replication in *Solanum* protoplasts is not largely affected by differences in the general genetic background of the host-virus system.

Expression of resistance at the cellular level—As described above, absolute yields of virions in infected pro-

toplasts varied among experiments. This variation was also apparent when relative accumulation of PVX in protoplasts carrying X^1 gene was compared to susceptible protoplasts. Figure 3C illustrates a compilation of representative experiments where accumulation rates of PVX in protoplasts derived from the immune cultivar *Serrana INTA* showed significative difference to susceptible controls at multiplicity levels of 0.01 ng PVX/viable infected protoplast. The reproducibility of this behaviour was confirmed after repeating these experiments 10 times using either *Spunta* or *Huinkul MAG* as susceptible controls. The increase of protoplast-associated virions in *Serrana INTA* was not only slower but also smaller, since even after long-term incubations virus concentration remained low compared to susceptible controls.

In order to assess whether viral RNA production was correlatively affected by the effect of X^1 gene expression, equivalent number of PVX inoculated or mock inoculated protoplasts derived from susceptible or resistant plants were assayed by slot-blot hybridization. Densitometry analysis indicated that absolute increases of total viral RNA concentrations were smaller than those of the respective capsid protein concentrations in both genotypes. However, the reduction of virus RNA accumulation rates in *Serrana INTA* protoplasts comparing to those of the susceptible control, followed a sharp parallel to the overall relative inhibition measured by ELISA in the same experiment (data not shown).

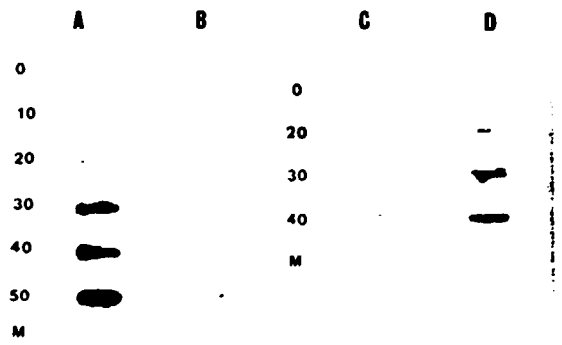

Fig. 4 Slot blot hybridization analysis of susceptible and resistant PVX-inoculated protoplasts. Slot blot hybridizations were carried out with total RNA from equal amounts of PVX-inoculated (multiplicity of infection: 0.025 ± 0.005 ng of cell-adsorbed PVX/protoplast) or mock-inoculated protoplasts of genotypes *Huinkul MAG* (A), *S. acaule* PI 320277 (B), *Serrana INTA* (C) and *Spunta* (D) with ^{32}P-labeled pX1 cDNA as a probe. At each post-infection time, cells were harvested, processed as indicated in Materials and Methods for total RNA isolation and sampled onto nylon membranes. Lanes 0 to 50: Viral RNA production at 0, 10, 20, 30, 40 and 50 h after infection, respectively. M: Mock-inoculated protoplasts (40 h after infection).

Immunofluorescence, ELISA and nucleic acid hybridization experiments indicated that consistently similar amounts of cell-bound viral antigen were detected in both types of protoplasts (susceptible and resistant) at 0 time after infection. Furthermore, when inoculation technique was changed using liposome mediated incorporation of PVX into protoplasts, no major differences in virus multiplication was observed. These results suggest that the lesser production of PVX RNA or capsid antigen in *Serrana INTA* protoplasts was not a function of the inoculation methodology or of a differential binding of virions to *Serrana INTA* and *Spunta* protoplasts (not shown). Immunofluorescence analysis of fixed protoplasts also indicated that although most *Serrana INTA* protoplasts became infected, protoplast-associated fluorescence after 30 h was always clearly less intense than the one observed in infected *Spunta* protoplasts.

In order to check whether general genetic background has any influence on the behaviour of X^1 gene, seedlings belonging to a different *Solanum* species and likely carrying the same gene were used as a source of protoplast for infection with PVX. Figures 3D and 4B (ELISA and nucleic acid hybridization analysis) show that PVX replicative kinetics in protoplasts from immune *S. acaule* PI 320277 genetic background was inhibited essentially to the same extent as in *S. turberosum* cultivar *Serrana INTA*.

Despite this clearcut resistance behaviour, when multiplicity of infection was raised up to highly saturating levels (1 ng PVX/viable protoplast), PVX multiplied in both *Serrana INTA* protoplasts and *S. acaule* PI 320277 to the same extent as in susceptible protoplasts, and no inhibitory effect could be detected (Fig. 3).

Discussion

Previous reports showed that protoplasts isolated from some resistant genotypes of tomato, cucumber, cowpea and potato behaved, at least partially, resistant to viral infections (Motoyoshi and Oshima 1977, 1979, Coutts and Wood 1977, Maule et al. 1980, Kiefer et al. 1984, Sanderson et al. 1985). Some of these reports were successful in establishing the biochemical basis of disease reaction responses. However in most virus-protoplast interactions, resistance does not seem to work at this level (see Furusawa and Okuno 1978 or Adams et al. 1985 for two of several examples).

In the case of potato, there are conflicting results dealing with the expression of resistance genes at the single cell level (Foxe and Prakash 1986, Adams et al. 1985, 1986). In this report, potato protoplasts derived from susceptible and immune genotypes were infected in vitro with a purified PVX virion preparation for comparative analysis of multiplication. In our system we clearly demonstrated that X^1 gene has a distinct effect on PVX replication that

cannot be attributed to general genetic background differences. There is a clearcut reduction on multiplication rate that seems to be inversely related to multiplicity levels of infection. When inoculum densities of up to 0.1 ng PVX/protoplast were used, results essentially reproduced those obtained by Adams et al. (1986) for gene Rx. The lesser accumulation of viral progeny observed in the resistant protoplast system was not due to a delay of a potentially compatible response to the virus, as long term incubations still showed large virus concentration differences with respect to those observed in susceptible protoplast systems. Adams et al. (1986) reported that Rx mediated inhibition of PVX multiplication in protoplasts of cultivar *Cara* did not affect the uncoating of infecting virions, leading to two main possible levels of inhibition: viral genome transcription and/or replication, or expression of late viral functions (i.e. capsid protein synthesis). Nucleic acid hybridization experiments (at both plant and single cell level) presented here obviously favour the first mechanism. In this context, it is likely that resistance gene products restrict somehow transcription or translation of viral genome as it has been suggested for the case of cucumber mosaic virus resistance (Maule et al. 1980). However, at very high density levels (1 ng PVX/protoplast) resistance was not expressed, probably due to oversaturation and bypassing of a putative cellular factor by the high input of viral particles. This kind of threshold level for resistance-susceptibility was shown to occur in other systems when the resistance factor is expressed in limited amounts (for example antisense RNA in Hemenway et al. 1988). We cannot decide whether this threshold level behaviour could explain some of the controversial reports on the expression of resistance at cell level as pointed out above.

Finally, comparison of X^1 gene expression in plants and protoplasts indicates that immunity gene does not completely function as it should at the cell level, since some degree of replication could be detected even at low multiplicity densities. These results contrast to those obtained by artificially inoculating whole plantlets, where even with highly sensitivity methods we failed to detect virus accumulation. Results at the plant level are in agreement with those of Cockerham et al. (1963) and Ross (1952), using plants carrying the same gene. These observations suggest that incompatibility reaction factor needs the tissue structure for full expression. Differential expression of host proteins after infection in the genetically related system of *Huinkul MAG* and *Serrana INTA* at both plant and cell level are under course to further elucidate some of these points.

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We want to greatly acknowledge Ing. Agr. S. F. Nome the supply of PVX_{cp} infected *Nicotiana* leaves for virus purification. We also thank Ing. Agr. Muñoz (Facultad de Ciencias Agrarias, Universidad Nacional de Córdoba) for sending *Nicotiana* leaves infected with a local isolate of PVX. We are grateful to Ing. Agr. Osvaldo Lucarini for generous supply of virus free plant material for micropropagation, prepared at the Balcarce Experimental Station of INTA, and to Lic. B. Orman for supplying PVX cDNA clone. We would like to thank Dr. Jean Masson (Laboratoire de Biologie Cellulaire INRA Versailles, France) for teaching (with good advice and patience) methods of protoplast preparation and culture and to Dr. Cristophe Robaglia for advice, who came to Castelar as a part of a French-Argentine scientific exchange program. This work is also part of a Swedish-Argentine agreement supported by SAREC.

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References

Adams, S. E., Jones, R. A. C. and Coutts, R. H. A. (1985) Infection of protoplasts derived from potato shoot cultures with potato virus X. *J. Gen. Virol.* 66: 1341–1346.

Adams, S. E., Jones, R. A. C. and Coutts, R. H. A. (1986) Expression of potato virus X resistance gene Rx in potato leaf protoplasts. *J. Gen. Virol.* 67: 2341–2345.

Altman, E., Altman, R. K., Ganet, J. M., Grimaila, R. J. and Young, R. (1983) S gene product: identification and membrane localization of a lysis control protein. *J. Bacteriol.* 155: 1130–1137.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Cardman, G. H. (1942) Autotetraploid inheritance in the potato: some new evidence. *J. Genet.* 44: 33–62.

Ceriani, M. F., Saladrigas, M. V., Tozzini, A. T. and Hopp, H. E. (1990) Resistance to replication of PVX in protoplasts of a *Solanum acaule* clone. *Rev. Investig. Agropec.* (in press).

Cockerham, G. (1943) The reactions of potato varieties to viruses X, A, B and C. *Ann. Appl. Biol.* 30: 338–344.

Cockerham, G. (1955) Strains of potato virus X. *In* Proceedings of the Second Conference on Potato Viruses, Lisse-Wageningen, 1954, pp. 89–92.

Cockerham, G. (1958) Experimental breeding in relation to virus resistance. *In* Proceedings of the Third Conference on Potato Virus Diseases, Lisse-Wageningen, pp. 199–203.

Cockerham, G. (1970) General studies on resistance to potato viruses X and Y. *Heredity* 25: 309–348.

Cockerham, G., Davidson, T. M. W. and Mac Arthur, A. W. (1963) Report on the virus content and the reactions to viruses X, S and Y of tuber-bearing *Solanum* collected by the Birmingham University expeditions to Mexico and Central America in 1958. *In* Scottish Plant Station Record, pp. 30–34.

Coutts, R. H. A. and Wood, K. R. (1977) Inoculation of leaf mesophyll protoplasts from a resistant and susceptible cucumber cultivar with cucumber mosaic virus. *FEMS Microbiol. Lett.* 1: 121–124.

Foxe, M. J. and Prakash, J. (1986) Expression of resistance to potato virus X in isolated potato protoplasts. *Arch. Virol.* 88: 167–174.

Fribourg, C. E. (1975) Studies on potato virus strains isolated from Peruvian potatoes. *Potato Res.* 18: 216–226.

Furusawa, I. and Okuno, T. (1978) Infection with BMV of mesophyll protoplasts from five plant species. *J. Gen. Virol.* 40: 489–491.

Gugerli, P. (1978) The detection of two potato viruses by enzyme-linked immunosorbent assay. *Phytopathol. Z.* 92: 51–56.

Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, New York, U.S.A.

Hemenway, C., Fang, R. X., Kaniewski, W. K., Chua, N. H. and Tumer, N. E. (1988) Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO J.* 7: 1273–1280.

Hopp, H. E., Giavedoni, L., Mandel, A., Arese, A., Orman, B., Bravo Almonacid, F., Torres, H. N. and Mentaberry, A. N. (1988) Biotinylated nucleic acid hybridization probes for potato virus detection. *Arch. Virol.* 103: 231–241.

Huarte, M. A., Mendiburu, A. O., Monti, M. C. and Butzonich, I. P. (1986) *Serrana INTA*: a widely adapted virus resistant potato cultivar from Argentina. *Amer. Pot. J.* 63: 695–699.

Jones, R. A. C. (1986) Further studies on resistance-breaking strains of potato virus X. *Plant Pathol.* 34: 182–189.

Khandjian, E. W. (1987) Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. *Biotechnology* 5: 165–167.

Kiefer, M. C., Bruening, G. and Russell, M. L. (1984) RNA and capsid accumulation in cowpea protoplasts that are resistant to cowpea mosaic virus strain SB. *Virol.* 137: 371–381.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory, New York, U.S.A.

Masson, J., Lecerf, M., Tousselle, P., Perennec, P. and Pelletier, G. (1987) Plant regeneration from protoplasts of diploid potato derived from crosses of *Solanum tuberosum* with wild *Solanum* species. *Plant Sci.* 53: 167–176.

Maule, A. J., Boulton, M. I. and Wood, K. R. (1980) Resistance of cucumber protoplasts to cucumber mosaic virus: a comparative study. *J. Gen. Virol.* 51: 271–279.

Maule, A. J. (1983) Infection of protoplasts from several *Brassica* species with cawliflower mosaic virus following inoculation using polyethylene glycol. *J. Gen. Virol.* 64: 2655–2660.

Moreira, A., Jones, R. A. C. and Fribourg, C. E. (1980) Properties of a resistance breaking strain of potato virus X. *Ann. Appl. Biol.* 95: 93–103.

Motoyoshi, F. and Oshima, N. (1977) Expression of genetically controlled resistance to tobacco mosaic virus infection in isolated tomato leaf mesophyll protoplasts. *J. Gen. Virol.* 34: 499–506.

Motoyoshi, F. and Oshima, N. (1979) Standardization in inoculation procedure and effect of a resistance gene on infection of tomato protoplasts with tobacco mosaic virus RNA. *J. Gen. Virol.* 44: 801–806.

Orman, B., Celnik, R. M., Mandel, A. M., Torres, H. N. and Mentaberry, A. N. (1990) Complete cDNA sequence of a South American isolate of potato virus X. *Virus Res.* (in press).

Otsuki, Y., Takabe, I., Honda, Y., Kajita, S. and Matsui, C. (1974) Infection of tobacco mesophyll protoplasts by potato virus X. *J. Gen. Virol.* 22: 375–385.

Prakash, J. and Foxe, M. J. (1985) Optimisation of conditions for infection of isolated potato protoplasts with potato virus X. *Arch. Virol.* 85: 269–279.

Ross, H. (1952) Studies on mosaic resistance in the potato. *In* Proceedings of the Conference on Potato Virus Diseases, Lisse-Wageningen, pp. 40–47.

Ross, H. (1954a) Über die extreme Resistenz von *Solanum acaule* gegen des X-virus. *Mitteilungen der Biologischen BundAnstalt* Berlin Dahlen 80: 144–145.

Ross, H. (1954b) Die Vererbund der Immunität gegen das X-virus in tetraploiden *Solanum acaule*. *Caryologia* 6 (suppl.): 1128–1132.

Sanderson, J. L., Bruening, G. and Russell, M. L. (1985) Possible molecular basis of immunity of cowpeas to cowpea mosaic virus. *In* Cellular and Molecular Biology of Plant Stress. Edited by Kosuge, T. and Ray, J. L. pp. 401–412. New York, U.S.A.

Shalla, T. A. and Petersen, L. J. (1973) Infection of isolated protoplasts with Potato Virus X. *Phytopathology* 63: 1125–1130.

Solomon, R. M. (1985) The relationship of genes X' and Nb for resistance to potato virus X. *Heredity* 55: 135–138.

Torrance, L., Larkins, A. P. and Butcher, G. W. (1986) Characterization of monoclonal antibodies against potato virus X and comparison of serotypes with resistance groups. *J. Gen. Virol.* 67: 57–67.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350–4354.

Widholm, J. M. (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technol.* 47: 189–194.

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RESISTANCE TO REPLICATION OF PVX IN PROTOPLASTS OF A *SOLANUM ACAULE* CLONE

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UMMARY

A germplasm collection was screened for the presence of resistance genes to potato virus . Among selected genotypes, *Solanum acaule* clone PI 320277 of Argentine origin was used for eparation of protoplasts.

Simultaneously, protoplasts were obtained from potato (*Solanum tuberosum tuberosum*) cul- ars Serrana INTA and Huinkul MAG, used as resistant and susceptible controls, respectively. errana INTA is resistant to potato virus X (PVX) infection because it carries the major reaction ne X^1 (believed to be the same as Rx_{acl}) while Huinkul MAG is susceptible. Protoplasts were oculated with a purified preparation of PVX_{cp} (a Peruvian isolate) and then assayed for PVX ncentration at different postinfection times by enzyme-linked immunosorbent assay (ELISA), mmunofluorescence and nucleic acid hybridization. PVX multiplication rates in *S. acaule* were out ninety percent lower than in susceptible genotypes when cell-bound virus levels just after ection were about 0.01 ng PVX per viable protoplast. The comparison of PVX replication netics with Serrana INTA suggests that resistance is likely due to the action of similar genes, at ast in what is concerned to mechanism of reaction.

ESUMEN

Se investigó la presencia de genes de resistencia para el Virus X de la Papa (PVX) en una lección de germoplasma. Entre los genotipos seleccionados, el clon PI 320277 de origen ar- ntino de *Solanum acaule*, fue utilizado en la preparación de protoplastos. Simultáneamente, otoplastos obtenidos de los cultivares de papa (*Solanum tuberosum tuberosum*) Serrana INTA y uinkul MAG fueron utilizados como controles resistentes y susceptibles, respectivamente. Ser- na INTA es resistente a la infección por el Virus X de la Papa porque es portador del gen X^1 ue se cree es el mismo que el Rx_{acl}) mientras que Huinkul MAG es susceptible. Los otoplastos fueron inoculados con una preparación purificada de PVX_{cp} (un aislamiento ruano) y luego se determinó la concentración de PVX a diversos tiempos a partir de la fección por ELISA, inmunofluorescencia e hibridización de ácidos nucléicos. Las tasas de ultiplicación del PVX en *Solanum acaule* fueron un 90% más bajas que en los genotipos sus- ptibles cuando los niveles de virus asociados a las células inmediatamente después de la fección fueron 0.01 ng de PVX por protoplastos viable. La comparación de la cinética de plicación del PVX en Serrana INTA sugiere que la resistencia se debe probablemente a la ción de genes similares, por lo menos en lo que respecta a su mecanismo de acción.

eywords: Potexviruses, Potato virus, *Solanaceae*, *Solanum acaule*, *Solanum tuberosum tuberosum*, Virus-host relationship, virus resistance gene expression, Germplasm.

INTRODUCTION

The incorporation of pathogen resistance genes into agronomic important crops from wild relatives of is a general practice in plant breeding. In the case of potato virus X, two groups of characterized reaction genes have been used. The first group includes the hypersensitive reaction genes Nx and Nb (Cockerham, 1955, 1970) which are present in many commercial cultivars (Cadman, 1942; Cockerham, 1958). The second group of genes confers extreme resistance to PVX (Cockerham, 1958; Ross, 1954a, 1954b; Solomon, 1985) and is present in many wild potato genotypes and in some few *S. tuberosum* clones. This group includes the gene X^1 (identical to Rx_{acl}) (Cockerham, 1970) present in cultivar Serrana INTA (Huarte *et al.*, 1986) and the gene Rx_{adg} present in cultivar Cara (Adams *et al.*, 1986).

The use of artificially inoculated protoplasts provides a controlled system for the study of the action of resistance genes on virus replication at the single cell level, enabling the characterization of the genes and eventually their molecular cloning. It was shown that resistance to PVX could be analyzed under these conditions. While results for gene Nx were rather conflicting (Adams *et al.*, 1985, 1986; Foxe and Prakash, 1986), protoplasts obtained from potato cultivars Cara and Serrana INTA (carrying immunity genes Rx and X^1 respectively), clearly resisted the multiplication of PVX (Adams *et al.*, 1986; Saladrigas *et al.*, 1990).

In this paper we arrive to similar conclusions analyzing the expression o resistance in protoplasts of a *Solanum acaule* clone selected by germplasm screening and we compare the gene action mechanisms with those of the Rx gene group.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; PVX, potato virus X; PI, plant introduction number from the Potato Introduction Station, Sturgeon Bay, USA.

MATERIALS AND METHODS

Solanum acaule clones PI 320277 (conferring resistance to PVX) and PI 175396 susceptible segregants (used as homologous species susceptible control) of Argentine origin were kept and multiplied by the Potato Introduction Station, Sturgeon Bay, USA. This Station generously sent us sib seeds of the mentioned clones. Control *Solanum tuberosum* L. spp *tuberosum* cultivars were Huinkul MAG and Serrana INTA (Huarte *et al.*, 1986; Saladrigas *et al.*, 1990). PVX virions from the Peruvian isolate cp (Fribourg, 1975; Orman *et al.*, 1990) were purified as described (Orman *et al.*, 1990). PVXcp cDNA clone pX1 (Hopp *et al.*, 1988; Orman *et al.*, 1990) was labelled by oligonucleotide primed incorporation of α-^{32}P-dCTP with a specific activity of 10^9 dpm/μg DNA and used as hybridization probe (Hopp *et al.*, 1988).

Virus-free seedlings were handled in sterile conditions an

micropropagated as described by Masson *et al.* (1987). Leaf protoplasts from shoot cultures were obtained as reported (Masson *et al.*, 1987). *In vitro* infection was carried out at infection densities (multiplicities) of about 0.01 ng of cell-bound PVX virions per viable protoplast per μl. Protoplast suspensions were incubated one minute and a half at a final polyethylene glycol 8,000 concentration of 25% (w/v) (Saladrigas *et al.*, 1990). Mock inoculations were performed in the same way in absence of virus. Protoplasts were incubated at different times in an excess of medium containing nutrients, hormones, etc. (medium A) as stated in Masson *et al.* (1987) at 21°C under continuous fluorescent light for up to 50 h. At each post-infection time, protoplast samples were concentrated by centrifugation and resuspended in specific buffers either for viability testing, estimation of PVX concentration by ELISA or slot-blot hybridization (Saladrigas *et al.*, 1990).

For estimation of virus content by ELISA, aliquots of protoplasts were resuspended in 200 μl of buffer, added to wells precoated with polyclonal anti-PVX immunoglobulin G and incubated overnight. After reaction with specific biotinylated immunoglobulin G, wells were incubated with a streptavidine-alkaline phosphatase complex as reported (Hopp *et al.*, 1988) p-nitrophenyl phosphate was added and the absorbance at 405 nm was recorded and compared to a plot of values obtained from wells containing known amounts of purified PVX virions that were assayed simultaneously in the same ELISA plate.

For slot-blot assays, equal number of protoplasts belonging to cultivars Huinkul MAG, Serrana INTA or *S. acaule* (PI 320277 and PI 175396) were resuspended and sampled into pre-wetted nylon membranes with the aid of a slot-blot manifold (Saladrigas *et al.*, 1990). RNA was fixed to the membrane and hybridization, washing and autoradiography were performed as in (Hopp *et al.*, 1988).

RESULTS AND DISCUSSION

Wild potato plants (*Solanum acaule* P.I. 320277) from Argentine origin, as well as resistant and susceptible controls (*S. tuberosum tuberosum* cv. Serrana INTA, carrying immunity gene X^1 and cv. Huinkul MAG, respectively) were used for establishing *in vitro* lines that were multiplied by micro-propagation of internode sections. These virus-free *in vitro* micropropagated plants were mechanically inoculated with a purified preparation of the South American strain PVXcp, to analyze resistance gene action over the viral multiplication. Plantlets were assayed one month later by ELISA in the presence of standards with known amounts of purified virions (see table I). Resulting levels of PVX were quantified by spectrophotometry and percentage of inhibition of virus replication was calculated. Table 1 clearly indicates that only basal levels of virus particles could be detected in *S. acaule* plantlets confirming extreme resistance to PVX. That inhibition was not due to some non-specific clone (a susceptible segregant of PI 175396). Quantification of virus accumulation showed that this control was as susceptible to PVX as the *tuberosum* cultivar Huinkul MAG (Table 1).

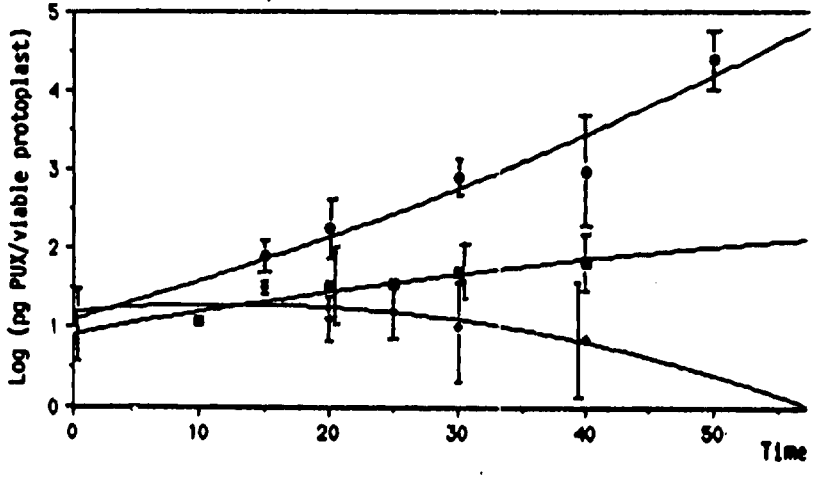
Viral multiplication at the cellular level was monitored by ELISA (Fig. and slot-blot hybridization (Fig. 2).

Table 1. Quantification of resistance to viral replication in *in vi* plantlets.

Plant material	PVX Concentration	% Inhibition
S. acaule PI 320277	1300	90
S. acaule PI 175396	16154	4
Resistant control	1860	89
Susceptible cont.	16740	-

Plants of *Solanum acaule* clones PI 320277 and susceptible segregants of PI 175396 well as resistant and susceptible controls (Serrana INTA and Huinkul MAG, respectively) w used for establishing *in vitro* lines that were multiplied by micro-propagation of internode s tions. These virus-free *in vitro* micropropagated plants were mechanically inoculated wit preparation of PVXcp. Plantlets were assayed one month later by ELISA in the presenc standards with known amounts of virion RNA in the same plate. Resulting levels of PVX w quantified by spectrophotometry and represented in the first column as ng PVX/g leaf wei Second column indicates percentage of inhibition of virus replication respect to susceptible c trol.

Fig. 1. Time course accumulation of PVX in susceptible and resist protoplasts.



Potato leaf protoplasts prepared from cultivars Huinkul MAG (●—●) Serrana I

imes except in those cases where only one measurement was available.

Absolute PVX accumulation in inoculated protoplasts showed a great deal of variation from experiment to experiment. Best relative accumulations with good reproducibility were obtained with a multiplicity of 0.01 ng PVX/viable infected protoplast. Fig. 1 shows typical time course accumulation plots of PVX after *in vitro* infection of protoplasts obtained from the Argentine susceptible cultivar Huinkul MAG. After a period of 15-20 h post infection there was a logarithmic increase of capsid protein synthesis reaching maximum levels at 40-50 h post-infection. Protoplasts were found to support PVX virion increases after 40 h of incubation that reach 94 times the concentration of cell-bound virion levels immediately after infection. Similar shapes were observed when virus replication was scored at the genomic level by slot blot hybridization densitometry in the same experiment.

Essentially similar results were obtained with protoplasts belonging to the susceptible *S. acaule* clone PI 175396, resembling the behaviour at the plant level shown above. These results suggest that, under the conditions used, the kinetics of PVX replication in *Solanum acaule* protoplasts is not largely affected by differences in the general genetic background with *S. tuberosum*.

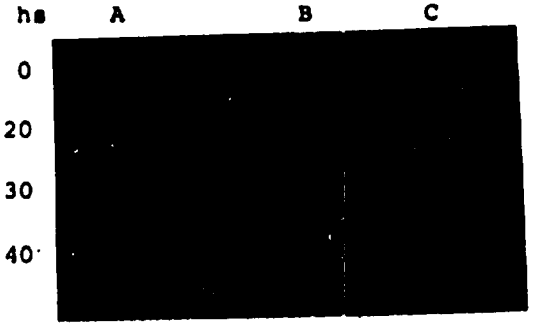
Previous reports showed that *in vitro* infection of protoplasts allowed the analysis of resistance gene action at the cellular level (Ponz and Bruening, 1986). In most cases, protoplasts derived from plants carrying resistance genes showed to support pathogen replication at a similar extent than the roplication observed in protoplasts derived from susceptible plants (Adams *et al.*, 1985; Furosawa and Okuno, 1978; Ponz and Bruening, 1986) for some of several examples). However, resistance at the cellular level could be demonstrated in a number of cases that showed, at least partially, resistance to viral infections (Adams *et al.*, 1985, 1986; Foxe and Prakash, 1986; Coutts and Wood, 1977; Kiefer *et al.*, 1986; Maule *et al.* 1980; Motiyoshi and Oshima, 1977; Ponz and Bruening, 1986; Sanderson *et al.*, 1985).

In this work, potato protoplasts derived from susceptible and immune genotypes were infected *in vitro* with a purified PVX virion preparation for comparative analysis of multiplication. Figure 1 shows a compilation of representative experiments where accumulation rates of PVX in protoplasts derived from the immune *S. acaule* PI 320277 showed significant difference to susceptible controls at multiplicity levels of 0.01 ng PVX/viable infected protoplast. The reproducibility of this behaviour was confirmed after repeating these experiments several times using Huinkul MAG as susceptible control. The increase of protoplast-associated virions in the immune *S. acaule* clone was not only slower but also smaller since even after long-term incubations, virus concentration remained low compared to susceptible controls.

In order to assess whether viral RNA production was correlatively af-

tant plants were assayed by slot-blot hybridization (Fig. 2).

Fig. 2. Slot blot hybridization analysis of susceptible and resistant PV inoculated protoplasts.



Slot blot hybridizations of total RNA from equal amounts of PVX-inoculated or mock-ino lated protoplasts of genotypes Huinkul MAG (A), *S. acaule* PI 320277 (B), and Serrana INTA with ^{32}P-labelled pX1 cDNA. At each post-infection time, cells were centrifuged, processed indicated in Materials and Methods for total RNA isolation and sampled onto nylon membran Lanes 0 to 40: Viral RNA production at 0, 20, 30 and 40 h after infection, respectively.

Densitometry analysis clearly indicated that, although absolute increa of total viral RNA concentration was smaller than capsid protein, there wa ninety percent reduction of virus accumulation rates following a sharp pa lelism to the behaviour of resistant Serrana INTA control and overall relat inhibition measured by ELISA in the same experiment. Both, ELISA molecular hybridization experiments indicated that consistently sim amounts of cell-bound viral antigen or genome were detected in the th types of protoplasts at 0 time after infection. These results suggest lesser production of PVX RNA or capsid antigen in *S. acaule* clone is n function of the infection methodology or a differential binding of virions. demonstrates that *S. acaule* protoplasts express a distinct effect on replication that cannot be attributed to general genetic background diffe ces following a sharp parallel to the behaviour of Serrana INTA.

Results essentially reproduced those obtained by us (Saladrigas *et* 1990) and by Adams *et al.*(1986) for Rx gene group. Furthermore, as for other two systems, the lesser accumulation of viral progeny observed in resistant protoplast system was not due to a delay of a potentially compa response to the virus. As mentioned above, long term incubations showed large virus concentration differences with respect to those obse in susceptible protoplast systems. In this context, it is likely that resist gene products restrict somehow transcription or early translation of

the entire resistance gene group Rx. However, we do not set apart the possibility of an inhibitory step at the viral uncoating level. PVX RNA transfection experiments are on course to clarify this hypothesis.

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REFERENCES

ADAMS, S.E., JONES, R.A.C. and R.H.A. COUTTS. 1985. Infection of protoplasts derived from potato shoot cultures with potato virus X. J. Gen. Virol. 66: 1341-1346.

ADAMS, S.E., JONES, R.A.C. and R.H.A. COUTTS. 1986. Expression of potato virus X resistance gene Rx in potato leaf protoplasts. J. Gen. Virol. 67: 2341-2345.

CADMAN, G.H. 1942. Autotetraploid inheritance in the potato: some new evidence. J. Genet. 44: 33-62.

COCKERHAM, G. 1955. Strains of potato virus X. Proc. Second Conf. Pot. Virus Lisse-Wageningen, 1954, pp. 89-92.

COCKERHAM, G. 1958. Experimental breeding in relation to virus resistance. Proc. Third Conf. Pot. Virus Dis. Lisse-Wageningen, pp. 199-203.

COCKERHAM, G. 1970. General studies on resistance to potato viruses X and Y. Heredity 25: 309-348.

COUTTS, R.H.A. and K.R. WOOD. 1977. Inoculation of leaf mesophyll protoplasts from a resistant and susceptible cucumber cultivar with cucumber mosaic virus. FEMS Microbiology Letters 1: 121-124.

FOXE, M.J. and J. PRAKASH. 1986. Expression of resistance to potato virus X in isolated

FRIBOURG, C.E. 1975. Studies on potato virus strains isolated from Peruvian potatoes. Res. 18: 216-226.

FUROSAWA, I. and T. OKUNO. 1978. Infection with BMV of mesophyll protoplasts fr plant species. J. Gen. Virol. 40: 4[illegible]-491.

HOPP, H.E., GIAVEDONI, L., MANDEL, A., ARESE, A., ORMAN, B., BRAVO ALMONA TORRES, H.N. and A.N. MENTABERRY. 1988. Biotinylated nucleic acid hybri probes for potato virus detection. Arch. Virol. 103: 231-241.

HUARTE, M.A., MENDIBURU, A.O., MONTI, M.C. and I.P. BUTZONICH. 1986. Serran a widely adapted virus resistant potato cultivar from Argentina. Am. Pot. J. 63: 695-

KIEFER, M.C., BRUENING, G., and M.L. RUSSELL. 1984. RNA and capsid accumul cowpea protoplasts that are resistant to cowpea mosaic virus strain SB. 137: 371-3

MASSON, J., LECERF, M., TOUSSELLE, P., PERENNEC, P. and G. PELLETIER. 198 regeneration from protoplasts of diploid potato derived from crosses of Solanum tu with wild Solanum species. Plant Science, 53: 167-176.

MAULE, A.J., BOULTON, M.I. and K.R. WOOD. 1980. Resistance of cucumber protop cucumber mosaic virus: a comparative study. J. Gen. Virol. 51: 271-279.

MOTOYOSHI, F. and N. OSHIMA. 1977. Expression of genetically controlled resista tobacco mosaic virus infection in isolated tomato leaf mesophyll protoplasts. J. Ge 34: 499-506.

MOTOYOSHI, F. and N. OSHIMA. 1979. Standardization in inoculation procedure and e a resistance gene on infection of tomato protoplasts with tobacco mosaic virus R Gen. Virol. 44: 801-806.

ORMAN, B., CELNIK, R.M., MANDEL, A.M., TORRES, H.N. and A.N. MENTABERRY. Complete cDNA sequence of a South American isolate of potato virus X. Virus R press).

PONZ, F. and G. BRUENING. 1986. Mechanisms of resistance to plant viruses. Ann Phytopathol. 24: 355-381.

ROSS, H. 1954a. Uber die extreme Resistenz von Solanum acaule gegen das X-virus. Mi Bund. Berlin Dahlen, pp. 80.

ROSS, H. 1954b. Die Vererbund der Immunitat gegen das X-virus in tetraploiden S acaule. Caryologia 6 (suppl.): 1128-1132.

SALADRIGAS, M.V., CERIANI, M.F., TOZZINI, A.C., ARESE, A.I. and H.E. HOPP. 1990. Cell Physiol. (submitted for publication).

SANDERSON, J.L., BRUENING, G. and M.L. RUSSELL. 1985. Possible molecular basis munity of cowpeas to cowpea mosaic virus. In UCLA: Cellular and Molecular Biol Plant Stress. Edited by Kosuge, T. and Ray, J.L. New York, USA. pp. 401-412.

Extreme resistance to infection by potato virus X in genotypes of wild tuber-bearing *Solanum* species

Running title: Resistance to PVX in *Solanum*

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Summary

Clones derived from thirty one different accessions (nineteen of Argentine origin) belonging to eight *Solanum* species were screened for resistance to infection by potato virus X strain cp (PVXcp) by mechanical inoculation of plantlets that had been micropropagated *in vitro*. Estimates of PVX multiplication obtained by enzyme linked immunosorbent assay and slot blot nucleic acid hybridization allowed the identification of resistant clones derived from five accessions belonging to *S. commersonii*, *S. oplocense*, *S. sparsipilum* and *S. tuberosum andigena*. Resistant genotypes supported PVX concentrations 5 to 15 times smaller than did the susceptible control cultivar Spunta. Graft inoculation test confirmed the presence of extreme resistance similar to that conferred by the "immunity" gene X^1 (also called Rx_{acl}).

Introduction

The genetic pool in wild tuber-bearing species of *Solanum* has been, and will continue to be used as a source of resistance to viruses in potato breeding, because the range of genes available allows the plant breeder to combat newly occurring virulent strains. Resistance genes can be incorporated into cultivars by, for example, sexual crossing (near or wide), protoplast fusion or genetic transformation after cloning those genes. Although some germplasm selections has been evaluated (Hanneman & Bamberg, 1986; Webb & Schultz, 1961), there are thousands of accessions of wild *Solanum* species that have not yet been surveyed for resistance to viruses.

This paper reports the screening of 31 accessions (19 of Argentine origin) belonging to eight Solanum species. Three of these species, *S. gourlayi* Hawkes, *S. sparsipilum* (Bitter) Juz. et Buk. and *S. tuberosum* subsp. andigena (Juz. et Buk.) Hawkes, had been tested previously, but the

other five, *S. commersonii* Dunal, *S. megistacrolobum* Okada et Clausen, *S. neorossii* Hawkes et Hjerting, *S. oplocense* Hawkes, and *S. vidaurrei* Cardenas, were tested for the first time for resistence to PVX multiplication. Recently *S. vidaurrei* Cardenas was reclassified as *S. gourlayi* subsp. *vidaurrei* (Card.) Hawkes et Hjerting (A. M. Clausen, personal communication). A highly sensitive *in vitro* test using micropropagated plants resulted in five new sources of resistance being identified.

Materials and Methods

Plants of potato cultivars derived from *in vitro* culture have previously been shown to be suitable for quantitative determination of resistance to PVX multiplication (Kürzinger & Schenk, 1988). Kürzinger & Schenk (1988) also demonstrated that estimation of the virus concentration in primary infected plants was in good agreement with established resistance ranking. We modified this method for screening wild *Solanum* species. The concentrations of virus in each genotype were assessed quantitatively by both ELISA (Kürzinger & Neitzel, 1985) and nucleic acid hybridization (Hopp et al., 1991).

Plant material: Most of the accessions used in this study had originally been collected in Argentina (Fig. 2); others originated in Bolivia, Guatemala and Perú (Fig. 2). No specific information was available about their reactions against PVX infection except for the two known resistant clones of *S. acaule* (derived from accessions PI 175395 and PI 230554) that were used as resistant controls. *S. tuberosum* subsp. *andigena* WRF 1758 is a cross between *S. tuberosum* subsp. *andigena* PI 279291 and *S. tuberosum* *andigena* PI 306302 (J.B. Bamberg, personal communication). Two cultivars of *S. tuberosum* subsp. *tuberosum*, cultivars Serrana INTA and Spunta were also included as resistant and susceptible controls respectively. Serrana INTA carries the "immunity" gene Rx^1 (also called Rx_{Ser1}) (Huarte et al., 1986, Saladrigas et al., 1990).

Virus material: The inoculum source was potato virus X strain cp (PVXcp), a Peruvian isolate (Fribourg, 1975, Jones, 1986, Moreira et al., 1980) recently characterized by nucleotide sequencing (Orman et al., 1990).

and replication analysis in potato protoplasts (Saladrigas et al., 1990). It was multiplied in *Nicotiana tabacum* plants and purified as described by Orman et al. (1990).

Plant Culture. True seeds were sterilized by sodium hypochlorite treatment (Gamborg et al., 1981), germinated aseptically in 2 cm diameter borosilicate glass tubes containing 10 ml of medium F (Masson et al., 1987) and the resulting seedlings micropropagated as described by Masson et al. (1987). Seedling were individually numbered and micropropagated singly. The growth conditions were 16/8 h (day/night) photoperiod at 22 °C with a light intensity of 6000 lux/cm^2 provided by fluorescent lights (Phillips fluorescent tubes, TLD 36W 54DL8).

Mechanical inoculation. Plantlets selected for inoculation were about 10 days old, had two or three nodes, and were just starting to develop roots. Before inoculation, 0.09 ml of a nystatin suspension (2 mg/ml in 50% ethanol) was added to the medium to control fungal contamination derived from virus inoculum which was prepared in non-sterile conditions.

PVX infected tobacco leaves were ground with a pestle in mortar in 10 ml/g viral extraction buffer (8 g/l NaCl, 0.2 g/l KH_2PO_4, 2.9 g/l $Na_2HPO_4.12H_2O$, 0.2 g/l KCl, 0.5 ml/l Tween 20 and 20 g/l polyvinylpirrolidone (PVP) 10,000, pH 7.4). The suspension was briefly centrifuged (5000 rpm for 5 min), and an inoculum preparad by adding 150 mg/ml of carborundum to the supernatant. This inoculum, wich had a PVX concentration of about 1 mg/ml (as estimated by ELISA), was applied to the leaves with a cotton swap. After 12 - 30 days, the leaves were harvested and weighted (0.05 to 0.2 g/plantlet, depending on the genotype).

Between 5 to 12 individual seeds per accession were tested, the larger numbers corresponding to those that were found to be resistant to PVX infection, and at least 3 micropropagated clones derived from an individual seed were inoculated per assay.

Grafting inoculation. Two susceptible potato cultivars (Spunta and Huinkul MAG) were mechanically inoculated and after 12 days, the shoots were grafted onto those of putative resistant genotypes. After ten days, putative resistant plants were excised at the base of the shoot. Maintenance of turgidity was used as an indicator of effective vascular union between the plants. Leaves from both shoots were then processed for

ELISA as described in *Sample analysis* and their PVX concentrations were compared

Sample analysis: ELISA was carried out as described (Hopp et al., 1991) Tissue sample in 7 ml/g viral extraction buffer were in a Potter-Elvehjem. Homogenates were transferred to 1.5 ml Eppendorf tubes and kept at -20 °C until required. They were then thawed, vortexed and clarified by brief centrifugation (5000 rpm for 5 min). Assays were made in microtitre plates (Nunc Immunoplates I, Denmark) using a Titertek Multiscan MC eight-channel automatic spectrophotometer (Flow Laboratory). Double antibody sandwich ELISA was carried out using polyclonal antibodies against capsid proteins as coating antibodies, (provided by Dr. S. Pérez Talavera, Centro de Ingeniería Genética y Biotecnología de La Habana, Cuba). Immunoglobulins were coupled to biotin-N-succinimide ester derivative (Amersham, UK) and used as recommended by Zrein et al. (1986). After reaction with specific biotinylated immunoglobulin G, wells were incubated with streptavidin-alkaline phosphatase complex as recommended by Amersham (UK). p-Nitrophenyl phosphate was added and the absorbance at 405 nm was recorded. To assess PVX levels, an internal calibration curve was constructed by sampling a range of 4 to 600 ng of purified PVX per well in each ELISA plate

Samples for slot-blot hybridization were homogenized in a freshly prepared solution of 10 U/ml human placental ribonuclease inhibitor (RNAsin, Promega Biotec, USA), 6% formaldehyde, 1% Na N-lauroylsarcosine, 2.4 M NaCl and 0.24 M Na citrate pH 7 at 65 °C and then maintained at this temperature for 15 min. After rapid cooling with ice, the homogenate was centrifuged as for ELISA and the corresponding supernatant sampled into pre-wetted Nytran membranes (Schleicher and Schuell, Germany) using a manifold (Schleicher and Schuell, minifold II). After washing twice with 0.1 ml of 3 M NaCl, 0.3 M Na citrate, RNA was fixed by exposing the wet membrane to UV light (Khandjian, 1987) followed by vacuum baking for 15 min at 80 °C in a slab gel dryer (Hoeffer, USA). Hybridization was carried out as described in Hopp et al.(1988). The PVX cDNA clone used was a 3,555 nucleotide insert corresponding to the 3' end of the PVXcp genome (Hopp et al., 1991, Orman et al., 1990), cloned in the Sma I site of pBluescript SK M13(+) phagemid (Stratagene, USA). The probe was labelled with ^{32}P-dCTP by oligonucleotide primed labelling to a specific activity of 10^{12} cpm/mg DNA (Hopp et al., 1988).

Results and Discussion

PVX multiplied rapidly in mechanically inoculated plantlets and reached a plateau in about one to two weeks. The levels fluctuated but little over a period at least one month after inoculation; the plateau levels depending on genotype (Fig. 1). The test discriminated between a resistant cultivar (e.g., Serrana INTA carrying the "immunity" gene X^i) and a susceptible cultivar (e.g., Spunta) that differed in a concentration level of PVX by one order of magnitude (Fig. 2). When levels of individuals seedlings derivatives were compared no evidence of segregation within accessions was found, so the data from each accession were pooled and the pooled resulted used to prepare Figure 2.

Clones derived from several accessions supported higher levels of PVX concentration than did cv Spunta. Unexpectedly, some concentration values were higher than those of tobacco plants inoculated for virus multiplication (Fig. 2).

Five accessions; *S. commersonii* PI 243503, *S. sparsipilum* PI 310972, *S. tuberosum andigena* WRF 1758, *S. oplocense* PI 435079 and PI 435080 supported low levels of PVX multiplication, similar to those of plants carrying gene X^i and "immune" *S. acaule* clones. PVX concentrations were 5 to 15 times lower than in the susceptible control Spunta as assessed by ELISA (Fig. 2). Resistance behaviour in plantlets of these accessions was reproducible and no segregation of susceptible plants was detected after testing at least 10 different seedling genotypes per putative resistant accession family. Such a number seems to exclude the possibility of heterozygosity of the accessions belonging to diploid species. However, it is still possible that alleles for susceptibility could be present in the accessions of tetraploid species. Comparison of the ELISA values of clones derived from the same seed with those derived from different seeds of the same accession did not show significant differences, suggesting that differences in the genetic background between sibs do not affect expression of the character.

Nucleic acid hybridization using a characterized PVX cDNA clone as a probe was used to verify whether viral RNA production was affected and to confirm data obtained by ELISA. Fig. 3 shows that slots corresponding to RNA of genotypes with resistance to PVX multiplication did not hybridize to the probe, while susceptible ones did so (with one exception, slot 19), a result closely paralleling that obtained by ELISA.

S. sparsipilum PI 310972 showed strong chlorosis and the inoculated leaves died suggesting that the resistant gene(s) caused a hypersensitive reaction. The other genotypes reacted to inoculation in a way similar to the reaction of cv. Serrana INTA and the resistant clones of *S. acaule*, that is without characteristic symptoms of hypersensitive reactions such as chlorotic spots or top necrosis. This behaviour suggests that the corresponding reaction genes could be of the Rx type (Cockerham, 1970), as might be expected in clones of *S. tuberosum* subsp. *andigena* in which such a gene has been shown to occur (Cockerham, 1943, 1958).

Previous reports indicate that some genotypes that show resistance to virus inoculation may be susceptible when graft inoculated (Webb & Schultz, 1961). Grafting PVX infected susceptible plantlets with the five resistant genotypes confirmed extreme resistance in these plants (Table 1). The reaction of the resistant genotypes resembled that of the immune controls, with the exception of *S. commersonii* PI 243503 derivatives which showed mild chlorosis in leaf margins.

For each accession showing resistance to PVX, three clones were numbered and sent to the germplasm bank of INTA, Balcarce, for maintainance and distribution to plant breeders. The accessions numbers of the clones at INTA Balcarce germplasm bank are: for *S. commersonii* PI 243503/1,2 and 3: Bal 9113/1,2 and 3 respectively; for *S. oplocense* PI 435079/1,2 and 3: Bal 9114/1,2 and 3 respectively; for *S. oplocense* PI 435080/1,2 and 3: Bal 9115/1,2 and 3 respectively, for *S. sparsipilum* PI 310972/1,2 and 3: Bal 9116/1,2 and 3 respectively; and for *S. tuberosum andigena* WPF 1758/1,2 and 3: Bal 9117/1,2 and 3 respectively.

In summary, this paper reports five new sources of extreme resistance to PVX multiplication in four wild *Solanum* species. This source behave similarly to known extreme resistance reaction genes. Experiments are in hand, using *in vitro* infected protoplasts to assess whether the corresponding genes have a similar action on virus multiplication (Ceriani et al., 1990, Caladrigas et al. 1990). However, genetic cross analysis will be

necessary to establish chromosome location and the allele relationships with other known resistance genes.

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References

Ceriani, M.F., Caladrigas, M.V., Tozzini, A.C., & Hopp, H.E., 1990. Resistance to replication of PVX in protoplasts of a *Solanum acaule* clone. Revista de Investigaciones Agropecuarias INTA (in press)

Cockerham, G., 1943. The reaction of potato varieties to viruses X, A, B and C. Annals of Applied Biology 30: 338-344.

Cockerham, G., 1958. Experimental breeding in relation to virus resistance. Proceedings Third Conference Potato Virus Disease (Lisse-Wageningen): 199-203.

Cockerham, G., 1970. General studies on resistance to potato viruses X and Y. Heredity 25: 309-348.

Fribourg, C.E., 1975. Studies on potato virus strains isolated from Peruvian potatoes. Potato Research 18: 216-226

Gamborg, O.L., Shyluk, J.P., & Shahin, E.A., 1981. Isolation, fusion and culture of protoplasts. Plant tissue culture and its application in agriculture Ed. Thorpe T.A. Academic Press Inc. (New York): 115-153

Hanneman, Jr, R.E., & Bamberg, J.B., 1986. Inventory of Tuber-bearing *Solanum* species. Bulletin of the University of Wisconsin, Madison, Wisconsin.

Hopp, H.E., Giavedoni, L., Mandel, A., Arese, A., Orman, B., Bravo Almonacid, F. Torres, H.N. & Mentaberry, A.N., 1988. Biotinylated nucleic acid hybridization probes for potato virus detection. Archives of Virology 103: 231-241.

Hopp, H.E., Hain, L., Bravo Almonacid, F., Arese, A.I., Tozzini, A.C., Ceriani, M.F., Caladrigas, M.V., Orman, B., Celnik, R., & Mentaberry, A.N., 1991. Development and application of a nonradiactive nucleic acid hybridization system for the simultaneous detection of four potato pathogens. Journal of Virological Methods, 31: 11-30

Huarte, M.A., Mendiburu, A.O., Monti, M.C. & Butzonich, I.P., 1986. *Serrana INTA* a widely adapted virus resistant potato cultivar from Argentina. American Potato Journal 63: 695-699.

Jones, R.A.C., 1986. Further studies on resistance-breaking strains of potato virus X. Plant Pathology 34: 182-189.

Khandjian, E.W., 1987. Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. Biotechnology 5: 165-167.

Kurzinger, B & Neitzel, K., 1985. Studies on the concentration of potato virus X in primarily infected potato plants, using ELISA as a basis for

determining relative virus resistance (Short comunication). Archiv für Zuchtungsforschung 15(6):389-393.

Kurzinger, B & Schenk,G., 1988. Beitrag zur Bestimmung der relativen Resistenz gegen Potato Virus X (PVX) an In-vitro-Pflanzen der Kartoffel. Potato Research 31(1):49-54.

Masson, J., Lecerf, M., Rousselle, P., Perennec, P. & Pelletier, G., 1987. Plant regeneration from protoplasts of diploid potato derived from crosses of *Solanum tuberosum* with wild *Solanum* species. Plant Science 53: 167-176.

Moreira, A., Jones R.A.C. & Fribourg, C.E., 1980. Properties of a resistance breaking strain of potato virus X. Annals of Applied Biology 95: 93-103.

Orman, B., Celnik, R.M., Mandel, A.M., Torres, H.N. & Mentaberry, A.N., 1990. Complete cDNA sequence of a South American isolate of potato virus X. Virus Research 16:293-306.

Saladrigas, M.V., Ceriani, M.F., Tozzini, A.C., Arese, A.I. & Hopp, H.E., 1990. Potato gene X^1 confers inoculum dependent resistance to potato virus X replication in protoplasts. Plant Cell Physiology 31:749-755.

Webb, R.E. & Schultz, 1961. Resistance of solanum species to potato viruses A, X and Y. American Potato Journal 38:137-151.

Zrein, M., Burckard, J. & Van Regenmortel, M.H.V., 1986. Use of the biotin-avidin system for detecting a broad range of serologically related plant viruses by ELISA. Journal of Virological Methods 13, 121-128.

Fig 1. Time course of PVX multiplication in mechanically inoculated plantlets susceptible or resistant to infection. *In vitro* grown plantlets were inoculated and PVX levels quantified by ELISA at different times after infection. —o— *S. tuberosum tuberosum cv. Spunta* (susceptible); —●— *S. acaule* 175395 (resistant).

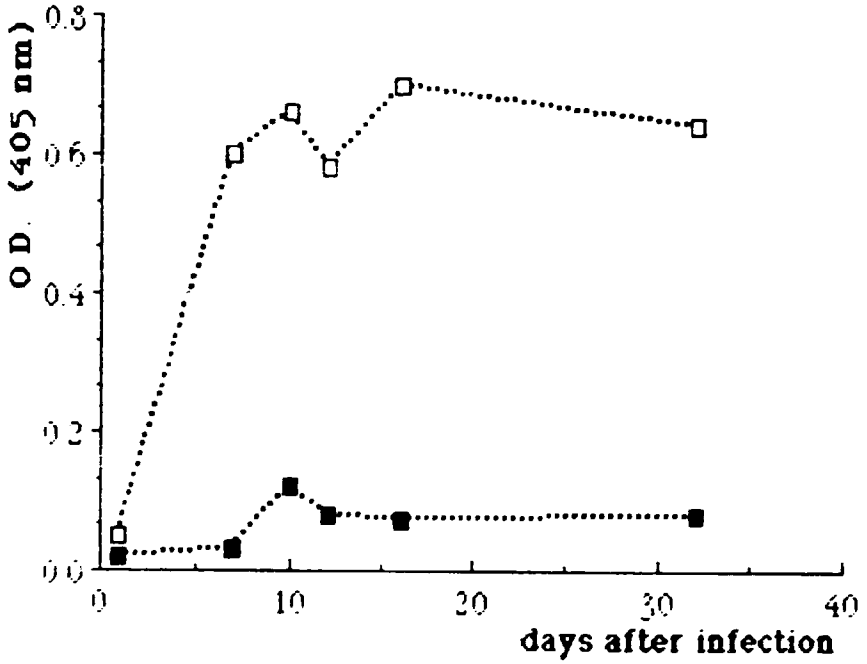

Figure 2: PVX concentration levels; in the left column indicates all the accessions surveyed, positive and negative controls are underlined. Mean multiplication of PVX in each accession family (as assessed by ELISA) is shown in the central column, the bars represent the standard deviation. The concentration level in *Nicotiana* is included as a reference.

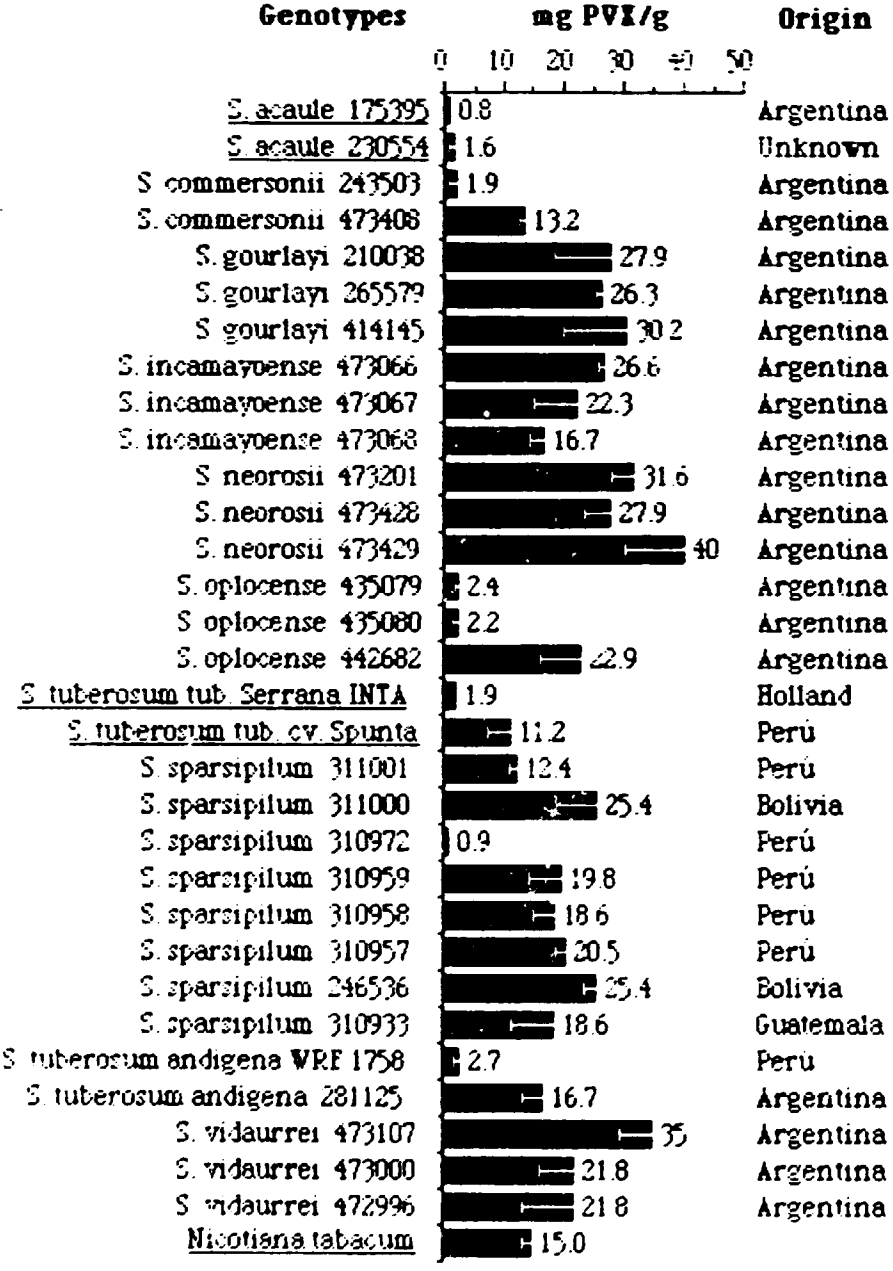

Fig. 3. Slot blot hybridization of mechanically infected plantlets with a PVX cDNA probe. Infected leaves were processed for hybridization with ^{32}P labelled pX1 clone and then exposed overnight to autoradiography. Each slot was sampled with extracts from independent plantlets, except for slots 1 to 6 and 13 to 17 that contain 686, 343, 172, 86, 21, 0, 600, 300, 150, 43 and 10 ng of purified PVX virions, respectively. Slots 7, 8, 9, 11 and 12: *S. sparsipilum* 310972 clones 1,2,2,3 and 5 (resistant). Slots 10, 19 and 20: *S. spegazzinii* 442582 clones 1,2 and 3 (susceptible). Slots 18 and 31: *S. spegazzinii* 435080 clones 1 and 3 (resistant). Slots 21 to 24: *S. tuberosum* subsp *andigena* WRF 1758 clones 3,2,1 and 1 (resistant). Slots 25 to 28: *S. commersonii* 243503 clones 1,1,2 and 3 (resistant). Slots 29 and 30: *S. spegazzinii* 435079 clones 1 and 2 (resistant). Slots 32 and 33: *S. sparsipilum* 311000 clones 1 and 2 (susceptible). Slots 34 to 36: *S. tuberosum* subsp *tuberosum cv* Serrana INTA (resistant).

Table 1. Relative PVX concentration levels in five genotypes inoculated by grafting. PVX infected plantlets of *S. tuberosum* subsp *tuberosum cv.* Spunta or cv. Huinkul MAG were grafted with non infected putative resistant genotypes, *S. sparsipilum* 310972/1/2/3; *S. tuberosum andig.* WRF 1758/1/2/3; *S. oplocense* 435079/1/2/3; *S. oplocense* 435080/1/2/3; *S. commersonii* 243503/1/2/3. After 25 days, PVX concentration levels were assessed by ELISA and these one expressed in the table as ratios between the PVX concentration level in the tested genotype and in its susceptible grafted partner.

Genotypes	**Relative PVX concentration**
Huinkul MAG/Spunta	1.00
S. sparsipilum 310972	0.40
S. tuberosum subsp *andig.* WRF 1758	0.32
S. oplocense 435079	0.14
S. oplocense 435080	0.10
S. commersonii 243503	0.05

Footnotes

[1] PI. plant introduction number of the Sturgeon Bay germplasm accessions (Hanneman & Bamberg, 1986).