UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

Maestría y Doctorado en Ciencias Bioquímicas

EXPRESIÓN EN SISTEMAS VEGETALES DE LAS PROTEÍNAS DE LA CÁPSIDE DEL POLIOVIRUS SABIN TIPO 1, PARA EL DESARROLLO DE UNA VACUNA ORAL RECOMBINANTE CONTRA LA POLIOMIELITIS

TESIS

QUE PARA OPTAR POR EL GRADO DE:

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Presente

Los miembros del Subcomité Académico en reunión ordinaria del día 24 de agosto del presente año, conocieron su solicitud de asignación de **JURADO DE EXAMEN** para optar por el grado de **DOCTORA EN CIENCIAS**, con la réplica de la tesis **"Expresión en sistemas vegetales de las proteínas de la cápside del poliovirus Sabin tipo 1, para el desarrollo de una vacuna oral recombinante contra la poliomielitis"**, dirigida por el/la Dr(a). **Edda Sciutto Conde**.

De su análisis se acordó nombrar el siguiente jurado integrado por los doctores:

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Sin otro particular por el momento, aprovecho la ocasión para enviarle un cordial saludo.

A t e n t a m e n t e
"POR MI RAZA, HABLARÁ EL ESPÍRITU"
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Dra. Ana Brígida Clorinda Arias Álvarez
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1. RESUMEN

Uno de los hitos más importantes de la vacunología es la reducción del impacto global de la poliomielitis. Las vacunas actuales para combatir la enfermedad comprenden las formulaciones convencionales basadas en virus inactivados o virus atenuados, las cuales fueron desarrolladas por Jonas Salk y Albert Sabin respectivamente. La principal limitación de la primera es su alto costo y la inducción de una baja protección a nivel intestinal, el sitio de replicación del virus; mientras que en la segunda el uso de virus atenuado puede revertir en formas neurovirulentas o nuevas cepas patogénicas.

Las plantas modificadas genéticamente hacen frente a tales limitaciones. Además, ofrecen un bajo costo de producción, almacenaje y entrega de la vacuna. Esta tecnología se ha aplicado recientemente en el desarrollo de prototipos vacunales contra la polio.

En el presente trabajo, exploramos la capacidad de las células de tabaco para expresar las proteínas VP1, VP2, VP3 y VP4 que conforman la cápside viral, las cuales son relevantes para el desarrollo de una vacuna. Evidencias experimentales en ratones demostraron su capacidad para producir respuestas humorales sistémicas y locales cuando se administraron por vía subcutánea y oral. Las VPs producidas en plantas, podrían ser útiles en el desarrollo de una vacuna con una formulación de bajo costo capaz de inducir una inmunidad en mucosas efectiva sin el riesgo asociado al uso de virus atenuados. Por lo tanto, ésta tecnología podría contribuir a la erradicación de la polimielitis.

Palabras clave: vacuna oral, molecular farming, Poliomielitis, vacuna subunitaria, inmunidad en mucosas, plant-made vaccine.

1.1 ABSTRACT

One of the milestones of vaccinology is the depletion of the global impact of Poliomyelitis. Current vaccines to deal with Polio comprise the Sabin and Salk formulations. The main limitation of the former is the use of attenuated viruses that can revert into pathogenic forms, whereas the latter is more expensive and induce no protection in the intestinal tract, the site of virus replication. Genetically engineered plants cope with such limitations. In addition, offer a low-cost alternative for production, storage and delivery of vaccines. This technology has narrowly applied in the development of Polio vaccines.

Herein, we explored the ability of the tobacco cells to express the immunogenic VP1, VP2, VP3 and VP4 Polio antigens, which are relevant for vaccine development. Evidence on the expression of the plant-made Polio VPs is presented and immunogenicity assessment proved their capacity to induce local and systemic humoral responses when administered by subcutaneous and oral routes. The plant-made VPs will be useful in the development of low-cost vaccine formulations able to induce effective mucosal immunity without the risks associated to the use of attenuated viruses; thus there is a potential for this technology to contribute to polio eradication.

Key words: oral vaccine; molecular farming; Poliomyelitis; subunit vaccine; mucosal immunity; plant-made vaccine.

2. INTRODUCCIÓN

2.1 Poliomielitis

La poliomielitis (polio) es una enfermedad viral altamente infecciosa, ocasionada por un enterovirus de ácido ribonucleico (ARN) que puede invadir el sistema nervioso central (SNC) y generar una parálisis irreversible en una de cada 200 personas infectadas (WHO, 2020).

El único reservorio natural del virus es el ser humano, en cual el virus entra por la boca a través de microgotas o secreciones mucosas expulsadas por una persona infectada, mediante el contacto con objetos que contienen al virus o por la ingesta de alimentos contaminados. Las infecciones se propagan rápidamente en lugares con condiciones de saneamiento e higiene deficientes (Nathanson, 2008).

Después de la ingesta, el virus se replica en la mucosa tractoalimentaria particularmente en las amígdalas y las placas de Peyer, que son invadidas como parte de la etapa temprana en la infección. Posteriormente, el virus es transportado por macrófagos y/o células dendríticas infectadas hacia los ganglios linfáticos cervicales profundos y ganglios linfáticos mesentéricos y se replica en ambos sitios eficientemente. Los virus son liberados hacia la linfa y transportados a través de los vasos linfáticos eferentes hacia el torrente sanguíneo provocando una viremia primaria; esto da como resultado la propagación viral hacia otros tejidos. Finalmente, aproximadamente en el 1% de las infecciones, los virus circulantes invaden el SNC; para este proceso se han descrito dos rutas de penetración viral: i) a través de la barrera hematoencefálica y ii) mediante la via neural o transporte axonal retrógrado (Fig. 1) (Blondel et al. 2005).

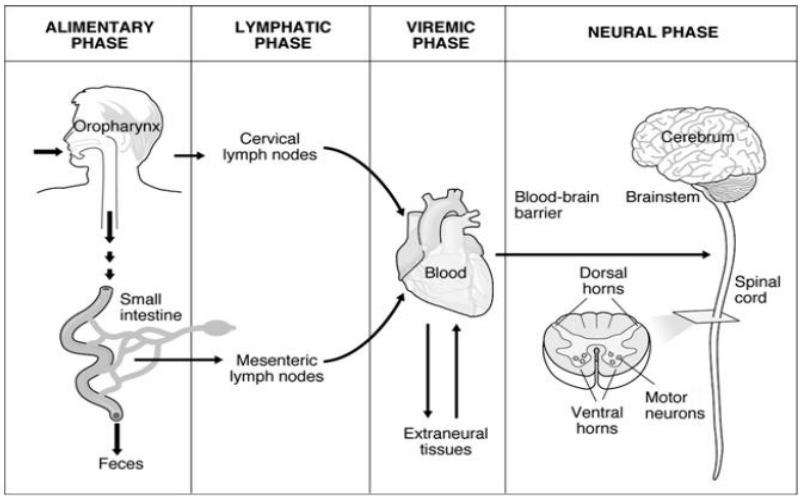

Figura 1. Patogenia del poliovirus. Eventos secuenciales durante la infección de poliovirus en humanos. Las cuatro fases de la infección comprenden: i) fase alimentaria, ii) fase linfática, iii) fase virémica y iv) fase neural.

2.2 Poliovirus, organización genómica y serotipos

El agente etiológico de la polio son los poliovirus (PV) (enterovirus C: Picornaviridae); estos virus se descubrieron en el año 1908 por el inmunólogo Vienés y premio Nobel Karl Landsteiner (1868-1943) y el médico Erwin Popper (1879-1955). Los PV, son virus compuestos por una cadena sencilla de ARN con polaridad positiva (+), no segmentado y con aproximadamente 7,500 pares de bases (pb) de longitud. Las partículas tienen un diámetro de 25-30 nanómetros (nm) y poseen una morfología icosaédrica compuesta por 60 copias de cada una de las 4 proteínas que conforman la cápside (Viral Proteins) i.e. VP1, VP2, VP3 y VP4 (Jiang et al., 2014). La estructura tridimensional fue resulta por rayos-x y se publicó en el año 1985 por Hogle y colaboradores.

La estructura genómica de los PV ha sido ampliamente descrita y es bien conocida. En el extremo 5´ contiene a la proteína viral VPg (22-25 aminoácidos (aa)) unida al ARN mediante un enlace fosfodiéster (Tyr-p-U), seguido a esto; se encuentra la región 5´UTR (untraslated region) que alberga estructuras secundarias en forma de tallo-asa necesarias para la replicación y traducción del

genoma viral. Posteriormente, posee un único ORF (open reading frame) codificante de una poliproteína que contiene proteínas estructurales (VP1, VP2, VP3 y VP4) en la región P1, y proteínas no estructurales (2A-2C y 3A-3D) en las regiones P2 y P3 respectivamente, además una estructura tallo-asa de 61 nucleótidos (nt) requerida para la replicación del material genético se encuentra en la región de la proteína 2C, la cual es llamada cis-replication element (*cre*). Finalmente, en la región 3ÚTR incluye una cola de adeninas (poli-A) (Wimmer et al., 1993).

La formación de viriones infecciosos es iniciada a partir de la poliproteina P1, la cual es modificada con la adición de un grupo miristoilo (miristioilación) y liberada proteolíticamente por la proteasa viral 2Apro. Posteriormente, la P1 adopta una conformación “competente para el procesamiento” mediante la asociación de la chaperona Hsp90 (P1*); consecutivamente P1* es cortada por la proteasa viral 3CDpro para generar las proteínas VP0, VP3 y VP1. Después del corte proteolítico, la chaperona se disocia de las proteínas de la cápside, las cuales, en lugar de dispersarse en el citoplasma, inmediatamente se autoensamblan en protómeros (5S). Los protómeros se condensan entre sí y forman pentámeros 14S que son la base estructural para la formación de la procápside o “cápside vacía” 75S, la cual; en conjunto con ARN viral integran al provirión 150S o virión inmaduro. Finalmente, la maduración del provirión se realiza mediante un corte autocatalítico de la proteína VP0 para generar a las proteínas VP2 y VP4 (Fig. 2) (van der Linder et al., 2015).

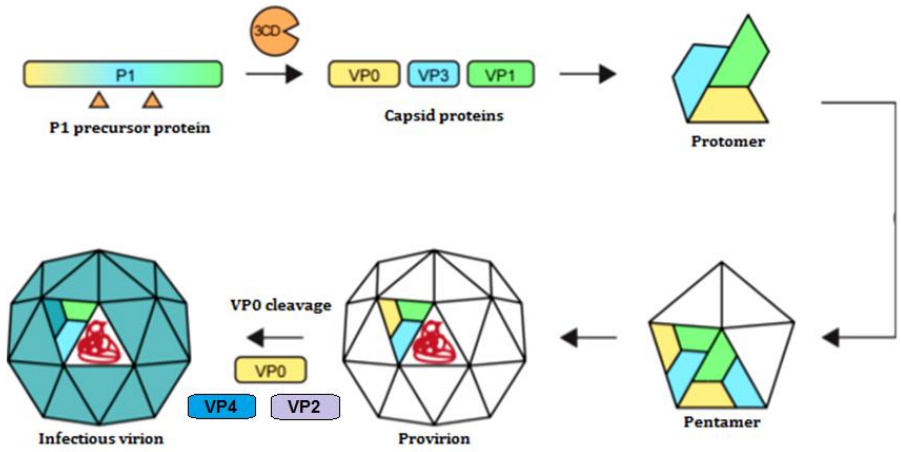

Figura 2. Morfogénesis de Poliovirus. El plegamiento adecuado de la poliproteina P1 es asegurado por la chaperona Hsp90, lo cual permite el corte mediado por la proteasa 3CD para producir las proteínas VP0, VP3 y VP1 que posteriormente forman protómeros, cinco protómeros contruyen un pentámero, los cuales en conjunto con el ARN viral integran al provirión, el paso final es la maduración y generación de los viriones infecciosos mediante el corte de la proteína VP0 en VP4 y VP2.

Las proteínas VP1, VP2 y VP3 están expuestas hacia el exterior de la estructura y contienen los principales sitios antigénicos de neutralización, los cuales son definidos como estructuras lineales o no lineales en donde los anticuerpos se pueden unir y neutralizar al virus (i.e. N-Ag I, N-Ag II, N-Ag IIIA y N-Ag IIIB); mientras que la proteína VP4 reside en el interior de la partícula (Fry and Stuart, 2010). En total, los PV expresan 3 sets únicos en secuencia y/o estructura de los sitios antigénicos de neutralización, por lo cual se han clasificado tres serotipos virales o cepas silvestres (WT), oficialmente descritos en 1953; el serotipo 1 (Mahoney), serotipo 2 (Lansing) y serotipo 3 (León) (Bodian et al., 1949; Salk, 1951). Para el serotipo 1, las secuencias de aa que componen los sitios antigénicos se presentan a continuación: el N-Ag I, contiene los aa 95 al 105 de la proteína VP1, el N-Ag II, se extiende desde los aa 221 al 226 de la proteina VP1 y los aa 164 al 172 de VP2, el N-Ag III presenta dos sitios independientes i) N-Ag III A que posee los aa 58 al 60 y 71 al 73 de la proteína VP3, mientras que el sitio N-

Ag III B alberga el aa 72 de VP2 y 76 al 79 de la proteína VP3 (Hogle et al., 1985). Para el serotipo 3, el sitio N-Ag I se localiza en los aa 89-100 de la proteína VP1, el N-Ag II contiene los aa 162-172 de la proteína VP2, el N-Ag III A alberga los aa 286-290 de la proteína VP1 y finalmente el N-Ag III B está compuesto por los aa 58-60, 70, 71, 77 y 79 de la proteína VP3. La estructura antigénica del serotipo 2 no ha sido descrita tan ampliamente, sin embargo en general es similar a la del serotipo 1 y 3 destacando el sitio N-Ag I (aa 89-100 de la proteína VP1) (Shaw et al., 2018).

Por otra parte, el Dr. Albert Sabin, generó versiones atenuadas de los PV silvestres Mahoney (tipo 1), P712 (tipo 2) y León (tipo 3) mediante pases repetidos de las cepas virales en cultivos celulares de testículo y riñón de mono rhesus y mono cynomolgus. Las cepas producidas pueden multiplicarse en el intestino, pero no poseen la habilidad de replicarse en el SNC; por lo cual forman parte de la formulación de la vacuna oral contra la polio y son denominadas cepas Sabin 1, Sabin 2 y Sabin 3 (Sabin and Boulger, 1973).

Una vez entendidas las bases moleculares de la atenuación de los PV, se determinó que el ARN de las cepas Sabin contiene una serie de mutaciones a lo largo del genoma que producen la baja capacidad de los virus para replicarse en el SNC, los llamados “determinantes de atenuación”. La cepa Sabin tipo 1 difiere en 55 nt que codifican para el cambio en 21 aa de la cepa patogénica parental (Mahoney), y los determinantes de atenuación están localizados en las posiciones 480 de la región 5´UTR, 935 de VP4, 2438 de VP3 y 2795 y 2879 de VP1 (Bouchard et al., 1995). Por otra parte, la cepa Sabin tipo 2 alberga 22 cambios de nt con respecto a la cepa madre P/712 y los determinantes de la atenuación se encuentran en la posición 481 (5´UTR) y el nt 143 de la secuencia perteneciente a VP1 (Moss et al., 1989; Macadam et al., 1993). El genoma del virus Sabin tipo 3 posee 12 cambios de nt que conducen a la diferencia en 3 residuos de aa con la cepa silvestre y los determinantes de atenuación se han mapeado en las posiciones 472 (5´UTR), 3333 (VP1) y 2034 (VP3) (Cann et al., 1984).

2.3 Epidemiología de la enfermedad

Durante el siglo XX, la polio fue una de las enfermedades más temidas alrededor del mundo con un registro de más de 350 000 casos de la enfermedad reportados en 125 países. En el año 1988, la Asamblea Mundial de la Salud aprobó una resolución para erradicar la polio, fue entonces cuando se creó la Iniciativa Global para la Erradicación de la Polio (GPEI), que en conjunto con Rotary International, el Centro para la prevención y control de enfermedades (CDC), el fondo de las naciones unidas para la infancia (UNICEF), la alianza de vacunas (Gavi) y la fundación de Bill y Melinda Gates han logrado la reducción de los casos de polio en un 99% y la contención de los casos generados por las cepas silvestres en sólo dos paises (Afganistán y Pakistán) (Fig. 3) (GPEI, “Polio today, 2020).

En el mundo, se ha certificado la inexistencia de casos de polio en cuatro regiones clasificadas por la OMS. En la región de las Américas el último caso se registró en un niño de Perú y la región se certificó como libre de la enfermedad en el año 1994; la región del Pacífico Occidental se certificó en el año 2000, la región de Europa en junio del año 2002 y el 27 de marzo del 2014 se certificó que la región de Asia Sudoriental estaba exenta de polio (11 países que abarcan desde Indonesia hasta India). Estos logros reafirman el gran avance en la erradicación global de la enfermedad, dado que el 80% de la población mundial actualmente vive en las regiones certificadas como libres de polio (Roberts, 2020).

En México, el último caso se reportó en el año 1990 y ha sido a través de las campañas vacunación que se ha logrado mantener al país como libre de la enfermedad (Esteves-Jaramillo et al., 2012). Sin embargo, en los resultados de la Encuesta Nacional de Nutrición y Salud de Medio Camino 2016 se reportó una cobertura del 51.7% para el esquema completo de la vacuna pentavalente (que incluye a la vacuna inactivada de la polio); además la proporción de dosis adicionales de la vacuna oral fue menor al 80%, lo que podría indicar la existencia de un sector de la población infantil desprotegido (ENSANUT, 2016).

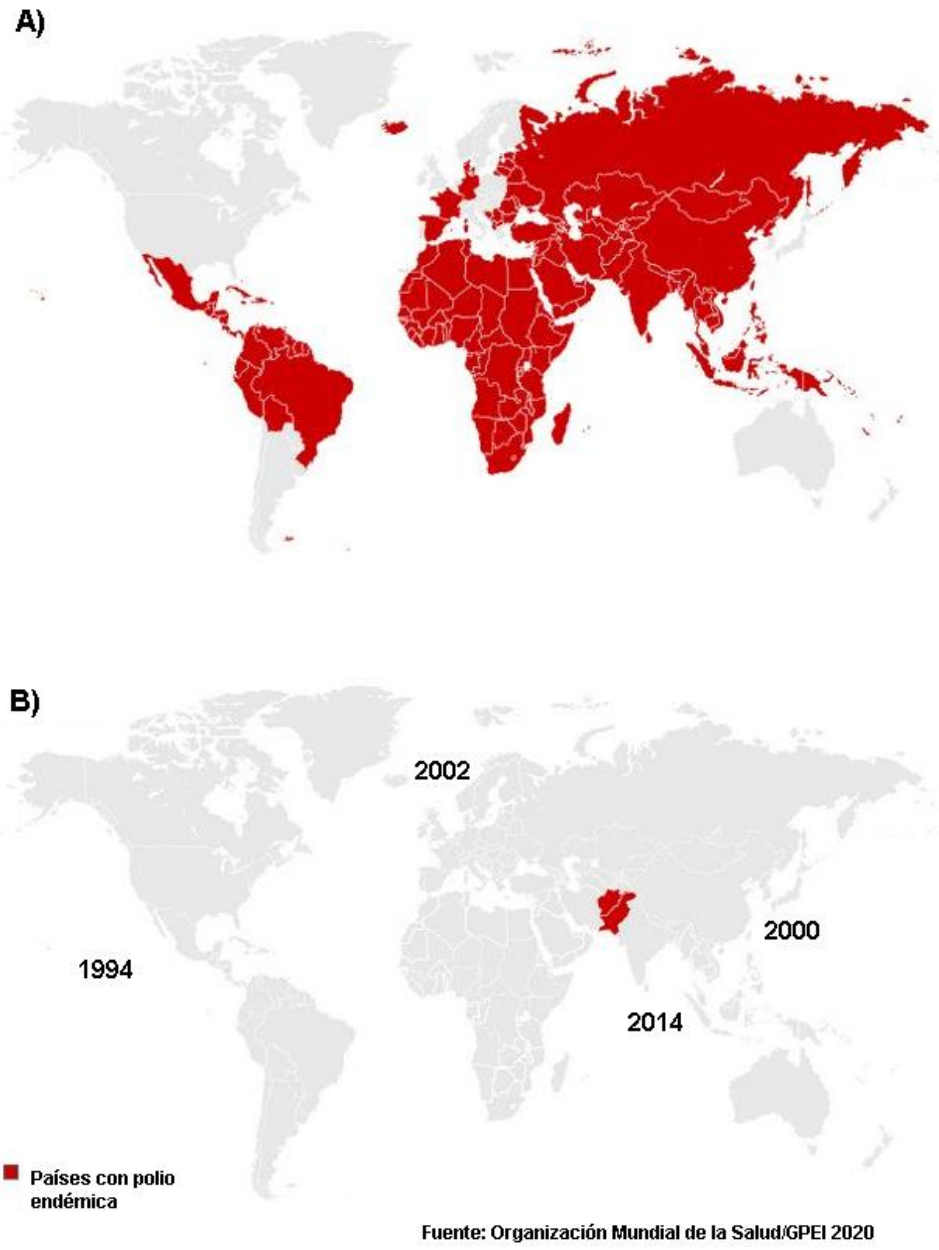

Figura 3. Mapa global de la evolución de los casos de poliomielitis generados por las cepas silvestres. A) En el año 1988, la polio era endémica en 125 países alrededor del mundo con un total de 350 000 casos registrados. B) De acuerdo a la GPEI, la enfermedad es endémica en dos países: Pakistán y Afganistán (fecha de consulta: 17 de septiembre del 2020) con un registro global de 102 casos. Fuente: OMS/GPEI 2020

En cuanto a los serotipos silvestres que generan la enfermedad, en el año 1999, se registró el último caso de polio provocado por el serotipo 2 en Aligarh, Uttar Pradesh (India), por lo que la OMS lo declaró erradicado en septiembre del año 2015; en tanto, en Nigeria en el año 2012 se identificó por última vez un caso relacionado al serotipo 3 y su erradicación se declaró el 24 de octubre del año 2019. De tal forma, que la cepa silvestre del serotipo 1 es la que actualmente se encuentra en circulación produciendo casos de polio en países donde la enfermedad es endémica (GPEI, 2020).

2.4 Respuesta Inmune asociada a la enfermedad

2.4.1 Respuesta inmune innata

El sistema inmune innato está diseñado para desarrollar reacciones celulares rápidas contra microorganismos invasores; para ello los reconoce a través de patrones moleculares asociados a patógenos (PAMPs). Después de esto, se induce la síntesis de proteínas, citocinas, enzimas y se activan vías como la apoptosis que pueden interferir con la replicación del agente infeccioso. El PAMP de los PV es el ARN de doble cadena o bicatenario (ARNbc) producido como intermediario durante la replicación viral, el cual es detectado cuando se encuentra en el citoplasma de la célula infectada.

La respuesta predominante contra una infección viral es la producción y secreción de interferón tipo 1 (INF-1), lo que da como resultado el establecimiento de un estado antiviral en las células contiguas a través de la expresión de proteínas con actividad antiviral. Esta respuesta es iniciada cuando los receptores de reconocimiento de PAMPs RIG-1 (Retinoic acid-Inducible Gene I), MDA-5 (Melanoma Differentiation-Associated protein 5) y TLR3 (Toll like-receptor 3) sensan el ARNbc viral. Estos receptores activan a las cinasas IKKε/TBK (inhibitor of nuclear factor (NF)-κB kinase ε/TANK-binding kinase 1) vía la proteína adaptadora MAVS (mitochondrial antiviral signaling protein; RIG-I and MDA-5) y TRIF (TIR domain-containing adaptor inducing IFN-β; TLR3). Posteriormente, las cinasas IKKε/TBK fosforilan al factor 3 regulador del Interferon (IRF-3), lo que

resulta en su dimerización y translocación al núcleo, donde se induce la transcripción de INF- β (Alexopoulou et al., 2001; Pichlmair et al., 2006). Después de la secreción, el INF- β se une al receptor de interferón tipo 1 (IFNAR1/2) de las células vecinas, resultando en la expresión de una variedad de genes estimulados por el interferón (ISGs).

Al igual que una gran cantidad de virus, los PV son parcialmente resistentes al interferón; debido en parte a la acción de la proteasa viral 2A, la cual puede inhibir la actividad de algunas proteínas ISG inducidas por INF-1, tales como la proteína cinasa K (PKR). Además, los mecanismos de transcripción, traducción y secreción del INF también son afectados por la participación de las proteasas virales 2A y 3C así como por la proteína 3A, la cual es capaz de interactuar con las membranas intracelulares; reprimiendo así la liberación de INF- β por parte de las células infectadas con PV. Por otra parte, MDA-5 puede ser cortada por las proteasas 2A o 3C y enviadas a degradación hacia el proteosoma derivando en la inducción de la apoptosis (Morrison and Racaniello, 2009; Barral et al., 2007; Goldstaub et al., 2000).

2.4.2 Respuesta inmune adaptativa

Después de un tiempo de incubación de 7 días, los PV son excretados en las heces hasta por un periodo de 7 semanas; la fase virémica aparece entonces entre los 3 y 7 días después de la infección. La respuesta inmune adaptativa se genera a partir del tercer día post infección (pi) con la presencia de anticuerpos IgM anti-PV, los cuales alcanzan su título máximo después de los 9 días pi y desaparecen en el transcurso de 4 semanas. La respuesta de anticuerpos IgG aparece 3-4 días después de la exposición al virus, estos anticuerpos alcanzan su título máximo de 3-4 semanas después de la infección y pueden persistir por años con ayuda de la vacunación. Los anticuerpos IgG parecen ser responsables de controlar la viremia, ya que su intensidad máxima correlaciona con la disminución en la concentración de los virus en la sangre (Ogra et al., 1968; Paul et al., 1951; Paffenbarger and Bodian, 1961).

Los anticuerpos IgA secretores (S-IgA) producidos en mucosas son detectables en faringe y muestras de heces una semana después de la infección, mientras que los anticuerpos IgA en sangre (s-IgA) aparecen tres semanas post-exposición. Ambas respuestas alcanzan sus niveles máximos cuatro semanas después de la infección, sin embargo los S-IgA tiene una magnitud mayor en comparación con los s-IgA. De forma importante, la inmunidad en mucosas proporciona resistencia sustancial contra las infecciones secundarias y parece desempeñar un papel importante en la prevención de la propagación del virus a través de las heces (Fig. 4) (Henry et al., 1966).

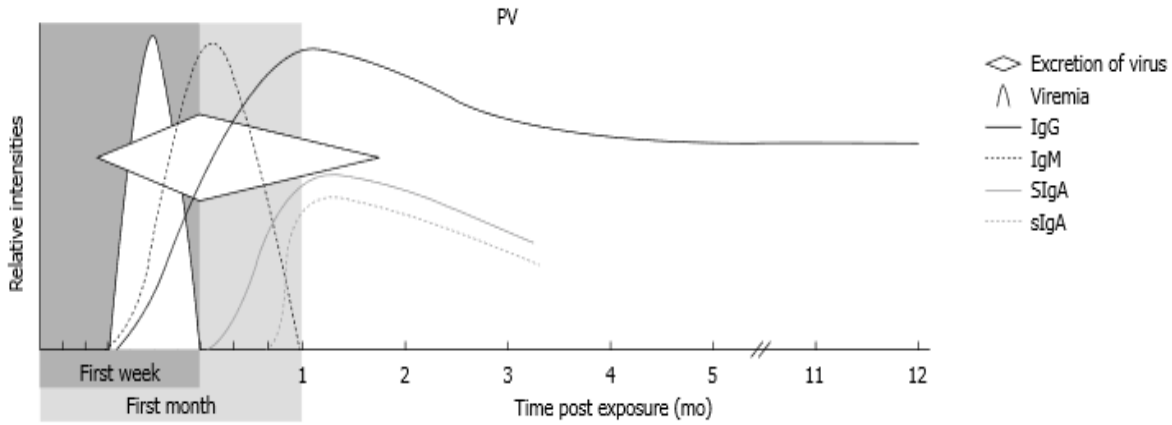

Figura 4. Evolución temporal de la viremia, excreción viral y la respuesta de anticuerpos después de la infección con poliovirus. En la figura se muestral esquemáticamente la duración media y la intensidad relativa de la propagación viral y la respuesta de anticuerpos contra el virus.

2.5 Vacunación contra la poliomielitis

El descenso en la generación de casos se ha debido a los intensivos programas de vacunación coordinados a nivel mundial por la GPEI. Las campañas de vacunación están basadas en la administración de dos tipos de vacunas: i) la vacuna inactivada-inyectable (IPV) y ii) la vacuna atenuada-oral (OPV). Ambas vacunas son producidas bajo métodos convencionales que implican el manejo de las formas infectivas y atenuadas de los poliovirus además, su manejo involucra altos estándares de bioseguridad.

Para la vacuna IPV, las tres cepas WT son proliferadas en células VERO; el cultivo es clarificado, filtrado y concentrado mediante columnas de intercambio aniónico y columnas de filtración en gel, posteriormente las partículas virales son purificadas e inactivadas con formaldehído (1:4000/37°C/12 días). Ésta vacuna induce inmunidad a nivel sistémico, por lo que en las personas inmunizadas con éste biológico, los virus son neutralizados antes de entrar al SNC. Sin embargo, induce baja inmunidad en la mucosa intestinal; el sitio primario de infección, por lo que los virus pueden ser replicados y diseminados a través de las heces. La IPV es significativamente más costosa en comparación a la OPV, por lo tanto es administrada principalmente en países desarrollados (Hawken and Troy, 2012).

Por otra parte, la OPV contiene una, dos o las tres cepas Sabin en una forma atenuada, es administrada oralmente mediante gotas y puede ser fácilmente administrada por personal voluntario, debido a esto se ha convertido en la opción principal en países en desarrollo. No obstante, las principales limitaciones de ésta vacuna son los casos de poliomielitis paralítica asociada a la vacuna (VAPP) que se pueden generar debido a la reversión de la atenuación viral en el hospedero y la generación de nuevas cepas patogénicas denominadas poliovirus derivados de la vacuna (VDPV) produciendo casos de polio de origen vacunal (Minor, 2009). En el año 2019, los casos de polio generados por VDPVs sobrepasaron los casos generados por las cepas WT registrando 368 versus 176. Actualmente, estas cualidades han obstaculizado la etapa final hacia la erradicación de la poliomielitis,

objetivo que exige la eliminación no solo de los casos provocados por las cepas WT, sino también los generados por las cepas vacunales.

En México, como parte del esquema de vacunación nacional, los niños reciben la vacuna hexavalente (toxoide diftérico, toxoide tetánico, toxoide pertússico, antígeno de superficie de la bacteria *Haemophilus influenzae* tipo b, hepatitis B y la IPV) a los 2, 4, 6 y 18 meses de edad, adicionalmente, los niños entre 6 meses y 5 años recién una dosis de la OPV durante la semana nacional de vacunación (dos semanas de vacunación al año).

En el año 2018, Díaz-Quiñónez et al., reportaron la detección de anticuerpos neutralizantes contra el virus de la poliomielitis tipo 1 entre niños de 1 a 4 años de edad en México. La seroprevalencia global fue del 98.39%. Existieron diferencias de acuerdo a la edad, siendo los niños de 3 y 4 años los que tienen el porcentaje más elevado (99.82% y 100% respectivamente) y de acuerdo al número de dosis de IPV (vacuna inactivada contra la poliomielitis) administrada siendo niños con 2 y 4 dosis los que presentaron una prevalencia mayor (100% y 99.92%).

Por otra parte, Altamirado et al. 2018 publicaron un estudio en donde determinaron los patrones de excreción después de administrar la OPV. Las muestras analizadas fueron heces de 2 poblaciones de infantes, 43 niños estadounidenses vacunados con OPV (505 muestras) y de 148 niños mexicanos vacunados con la OPV (1373 muestras). En total, el 84% de los niños estadounidenses y el 78% de los niños mexicanos excretaron cepas de virus vacunales, con una duración media de eliminación de 17,4 días para los niños estadounidenses y 9.3 días para los niños mexicanos. Aunque en México, no se han tenido reportes de casos de polio generados por las cepas virales de origen vacunal, este reporte supone la circulación entre la población de estas cepas patogénicas.

En este sentido, las características ideales que debería poseer la vacuna contra la polio son: evitar la generación de nuevas cepas patogénicas, prescindir del uso del poliovirus infectivos para su fabricación, ser producida a bajo costo e inducir inmunidad en mucosas y a nivel sistémico. En este respecto, un nuevo y asequible

enfoque basado en el diseño racional de una vacuna podría ser la mejor alternativa.

2.6 Vacunas de subunidades

Las vacunas de subunidades son preparaciones biológicas que contienen componentes antigénicos específicos de un patógeno, pero sin incluir cualquier fragmento que lo vuelva infeccioso; eliminando así las preocupaciones derivadas de la recuperación de la virulencia o la inactivación incompleta del agente infeccioso. Este tipo de vacunas son muy estables y seguras de administrar en población vulnerable tales como personas embarazadas, ancianos o con el sistema inmune comprometido. Mediante este enfoque, pueden ser obtenidas vacunas contra patógenos que no pueden ser crecidos en el laboratorio, tal es el caso de los PV cuyas cepas WT están a un paso de ser sacadas de circulación y su manipulación está restringida en los laboratorios de investigación (Temmerman et al. 2011; Storni et al. 2005). No obstante, se ha descrito que las vacunas de subunidades generan una baja inmunogenicidad, un tópico muy importante especialmente en el desarrollo de una vacuna oral. En este respecto, el uso de moléculas acarreadoras tales como la subunidad B de la toxina del cólera (CTB) o la subunidad B de la enterotoxina de *E. coli* (LTB) debe ser abordado durante el diseño de la vacuna, además de la utilización de adyuvantes como saponina, escualeno o quitosano mezclados con el antígeno es ampliamente recomendado para incrementar la inmunogenicidad de la formulación vacunal (Apostólico J de S et al. 2016; Rosales-Mendoza and Salazar-González, 2014).

Actualmente, diversas vacunas de subunidades han sido aprobadas y son administradas ampliamente en los esquemas de inmunización alrededor del mundo; algunas de ellas son: la vacuna contra el virus del papiloma humano (VPH) basada en VLPs (Gardasil ® y Cervarix ®), la vacuna contra el virus de la hepatitis B cuyo principal componente es el antígeno de superficie viral (Engerix-B ® y Recombivax HB®) y las vacunas contra la tos ferina, tétanos y difteria formuladas con toxoides provenientes de las bacterias *Bordetella pertussis, Clostridium tetani y Corynebacterium diphteriae* respectivamente.

2.7 Células vegetales como sistema de expresión de antígenos

Los componentes antigénicos (proteínas, péptidos o polisacáridos) que forman parte de las vacunas de subunidades, pueden ser purificados directamente de los patógenos o pueden ser producidos de forma recombinante en hospederos heterólogos. Una de las plataformas que se ha venido explorando intensivamente durante los últimos 30 años son los sistemas vegetales.

La producción de proteínas en plantas es un enfoque prometedor que ofrece diversas ventajas incluidas la facilidad de escalado, la bioseguridad y el bajo costo. Las plantas al ser un organismo eucariótico, tienen la capacidad de producir proteínas con un ensamble y plegamiento adecuado; además de realizar con éxito la mayoría de las modificaciones pos-traduccionales necesarias para la actividad de proteínas complejas (Merlin et al., 2014).

La inmunogenicidad de las diversas proteínas expresadas en plantas ya ha sido demostrada con éxito, ésta característica podría ser atribuida a la presencia de compuestos adyuvantes en la biomasa vegetal, ya que las plantas producen diversos metabolitos secundarios entre las que se encuentran: saponinas, lectinas, terpenoides, motivos CpGs no metilados y carotenoides (Licciardi and Underwood, 2011; Wang, et al., 2002).

Otra cualidad importante que poseen las células vegetales, es el efecto de bioencapsulación; dicho efecto podría ejercer su potencial mediante la protección del antígeno a través de su paso por el tracto gastroalimentario. Esta capacidad se vuelve relevante en el desarrollo de una vacuna oral, ya que la biodisponibilidad antigénica adecuada es necesaria para inducir una respuesta inmune robusta. Se ha hipotetizado que la degradación de la pared celular es un evento que tiene lugar en el intestino grueso mediada por la acción de microorganismos anaerobios simbióticos que poseen enzimas necesarias para este proceso (Renukuntla et al., 2013; Kong et al., 2001; Flint et al., 2008).

2.7.1 Enfoques para la transformación de células vegetales

Los enfoques para la expresión de proteínas en plantas se clasifican en dos categorías: estable y transitoria. La transformación transitoria se basa en la introducción del ADN foráneo en el genoma nuclear de la planta independientemente del evento de integración. Bajo este enfoque, los antígenos son expresados en un corto plazo en tejidos vegetales de plantas adultas con la cosecha subsecuente del material que es usado típicamente para purificar el antígeno (Márquez-Escobar et al., 2018).

Por otra parte, en la transformación estable, el ADN blanco se puede dirigir e insertar en el genoma del núcleo (estable-nuclear) o del cloroplasto (estable-transplantómico), generando un rasgo hereditario. La metodología para la transformación estable comprende los siguientes 3 pasos generales: 1) entrega del ADN foráneo al tejido vegetal, 2) selección de los tejidos transformados de acuerdo al gen de resistencia utilizado y 3) establecimiento *in vitro* de las líneas (células indiferenciadas, tejidos o plantas completas) (Tzfira et al., 2004). Entre las características importantes de éste enfoque destaca la posibilidad de expresar antígenos en cultivos comestibles para el desarrollo de formulaciones orales, las cuales, son ideales para combatir principalmente patógenos que tienen como vía de entrada la mucosa oral y/o sitio de infección primaria la mucosa gastrointestinal.

La transformación estable se puede desarrollar mediante las siguientes metodologías: i) biobalística, ii) polietilenglicol o iii) *Agrobacterium tumefacines*.

2.7.2 Transformación mediada por Agrobacterium tumefaciens

El método más simple y común para generar la transformación nuclear-estable se basa en el uso de la bacteria *A. tumefaciens,* la cual, es capaz de introducir en las células vegetales grandes segmentos de ADN con alta eficiencia, reordenamientos mínimos y un bajo número de inserciones. *A. tumefaciens* es una proteo bacteria gram negativa (-), causa tumores en las plantas dicotiledóneas conocidos como agallas o tumores de cuello, los cuales crecen principalmente en la zona donde se une la raíz y el tallo.

En biotecnología, la capacidad de transferencia del ADN ha sido extensamente explorada para insertar genes foráneos en el genoma de las plantas. El proceso es liderado por las proteínas vir, la mayoría de ellas codificadas por el plásmido inductor de tumores (pTi) contenido en la bacteria (Fig. 5).

Tras la interacción de *A. tumefacines* con las células dañadas de la planta, la bacteria detecta mediante el receptor VirA los compuestos químicos secretados, entre estos compuestos se encuentra la acetociringona, posteriormente se llevan a cabo una cascada de señalizaciones que culmina en la fosforilación de la proteína VirG, el cual actúa como factor de transcripción con la subsecuente activación del operón vir (Vir B, C, D y E).

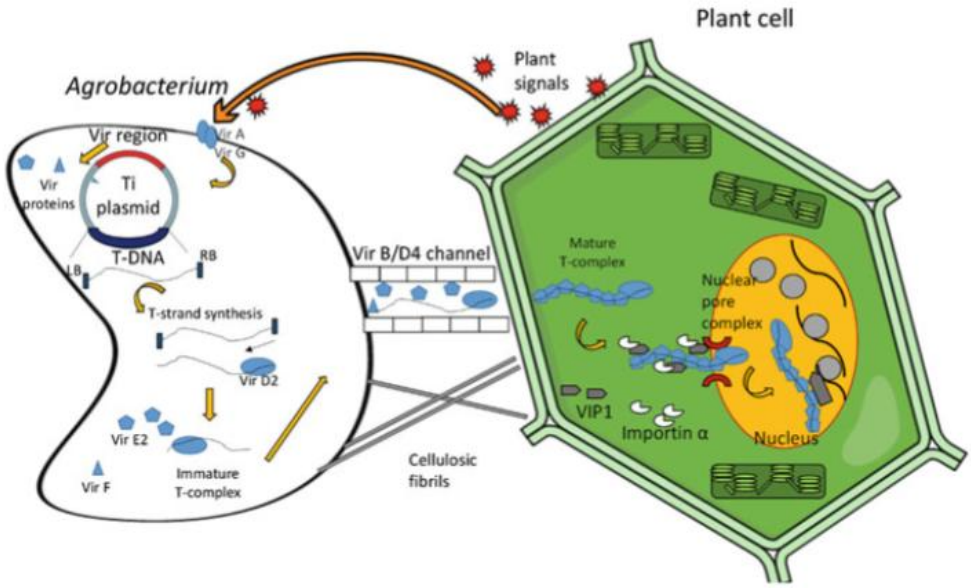

Figura 5. Mecanismo de transferencia del ADN mediado por *Agrobacterium tumefaciens* hacia el genoma de la célula vegetal. Los compuestos fenólicos liberados por las células heridas son reconocidos por VirA y conducen la activación de VirG; posteriormente los genes vir encontrados en el plásmido Ti son expresados. La región del T-DNA es separada del pTi por el complejo VirD1/VirD2 para producir un DNA monocatenario. El canal por el que son transportadas las proteínas vir y el T-DNA inmaduro es generado por las proteínas VirB/VirD4. Una vez en el citosol, las proteínas VirE2 y VirF se adhiere al T-DNA induciendo su maduración con la subsecuente translocación nuclear mediada por VIP1 y por alfa

importinas. Finalmente, el T-DNA llega a la cromatina seguido de la integración del DNA en el genoma nuclear.

El pTi también alberga el ADN de transferencia (T-DNA), dicha secuencia es transportada desde la bacteria al genoma de la planta y contiene diversos genes responsables de la producción de fitohormonas como auxinas y citocininas; además contiene genes encargados de la biosíntesis de opinas que actúan como fuente de carbono para la bacteria. El T-DNA está flanqueado por dos secuencias de 25-28 pb llamados borde izquierdo (LB) y borde derecho (RB) que durante el proceso de escisión del T-DNA son el sitio de corte por parte de las proteínas VirD1/D2. El producto generado es una cadena sencilla en donde la proteína VirD2 queda adherida en el extremo 5´ y VirE2 recubre la hebra formando el complejo de transporte del T-DNA, el cual es exportado al citoplasma de la célula vegetal a través de un canal formado por las proteínas VirD4 y VirB.

Una vez en el citoplasma, las proteínas VirE2 y VirD2 son reconocidas por alfa importinas mediante señales de localización nuclear encontradas en estas proteínas. Las alfa importinas a su vez interactúan con beta importinas y el complejo del poro nuclear permitiendo la transferencia del T-DNA hacia el interior del núcleo. Finalmente, la integración tiene lugar cuando la proteína VIP2 dirige al T-DNA hacia la cromatina que está transcripcionalmente activa y la proteína VirC selecciona y corta el genoma de la planta (Lacroix and Citovsky, 2013; Gelvin 2010; Mangano et al., 2014).

Una vez entendido el mecanismo molecular por el cual *A. tumefaciens* traslada su T-DNA hacia la célula hospedera; se logró desarrollar un mecanismo reemplazando los genes del T-DNA con cassettes de expresión apropiados. Las secuencias del pTi se han dividido en dos vectores creando un sistema binario, el vector shuttle contiene una secuencia que lleva el gen heterólogo flanqueado por los LB y RB, mientras que el plásmido helper codifica los genes vir requeridos para la transformación vegetal (Hoekema et al., 1983). Entre los vectores binarios más destacados y ampliamente utilizados en la transformación de células vegetales se

encuentra el pBI121, en donde; la región del T-DNA contiene el RB, un casete de expresión para el marcador de selección neomycin phosphotransferase II (NPTII) y el gen reportero a-glucoronidase (GUS), y el LB (Chen et al., 2003).

Mediante la transformación nuclear-estable se han logrado expresar una gran variedad de proteínas de interés farmacéutico en distintos cultivos vegetales entre los que destacan: lechuga (*Lactuca sativa*), zanahoria (*Daucus carota*), papa (*Solanum tuberosum*), tomate (*Solanum tuberosum*) y tabaco (*Nicotiana tabacum*).

En este sentido, tabaco se ha convertido en la planta modelo para iniciar con la expresión de antígenos debido a que posee diversas características entre las que destacan: la facilidad de transformación, la elevada cantidad de biomasa generada (hojas), la facilidad de cosecha y el fácil mantenimiento en invernadero.

2.8 Candidatos vacunales contra la polio expresados en plantas

Diversos grupos alrededor del mundo han expresado proteínas en plantas explorando el desarrollo de candidatos vacunales contra la polio. En el año 2006, Fujiyama et al. fusionaron un fragmento de 15 aa derivados de las proteínas VP3 y VP1 (Sabin tipo 1) a la proteína de la cápside del virus del mosaico del tabaco (TMV). La proteína fusión se expresó de forma transitoria en plantas de tabaco obteniendo una producción de 0.2 mg/g de hojas. Ensayos de inmunogenicidad en ratones inyectados una o dos veces de forma intraperitoneal (i.p.) con 200 µg de partículas recombinantes purificadas emulsificadas en monofosforil lipid A como adyuvantes demostraron la producción de anticuerpos en suero específicos para la partícula quimérica.

La tecnología transplantómica para producir la proteína VP1 del serotipo Sabin tipo 1 fue adoptada por Chan et al., 2016. Plantas de tabaco se usaron para generar a la proteína VP1 fusionada al acarreador transmucosal CTB. La inmunogenicidad fue probada en ratones en un esquema que inició con la inmunización subcutánea (s.c.) de la IPV seguida por boosters orales con material vegetal liofilizado emulsificado con escualeno, saponina o ambos. Los títulos de anticuerpos IgG e IgA aumentaron significativamente en suero de ratones alimentados con el tejido vegetal comparados con bajos o nulos títulos cuando no

se administraron boosters orales. Adicionalmente, la actividad neutralizante de los anticuerpos y seropositividad del 70-90% contra los tres serotipos Sabin se observaron cuando se administraron dos dosis de IPV y boosters orales de la proteína VP1 generada en plantas.

En una investigación posterior, un estudio inmunológico a largo plazo involucrando este candidato vacunal fue reportado por Xiao and Daniel (2017), en donde 1 o 25 µg de la proteína VP1 fueron administradas oralmente a ratones previamente inmunizados con la vacuna IPV; tres dosis (una por mes) o un solo booster oral fueron aplicados. Altos niveles de anticuerpos IgG1 e IgA fueron detectados, además la respuesta inmune se mantuvo hasta 400 días protegiendo contra los tres serotipos virales durante ese lapso de tiempo.

Por otra parte, la generación de virus like-particles (VLPs) conteniendo las proteínas de la cápside de poliovirus también ha sido explorada. En el año 2017, Marsian y colaboradores expresaron de forma transitoria en plantas de *Nicotiana benthamiana* la poliproteina P1 de la mutante SktSC8 Sabin tipo 3 produciendo VLPs que mantuvieron la conformación antigénica D nativa. Ratones transgénicos se inmunizaron i.p. dos veces con 0.5 de la dosis humana de las VLPs parcialmente purificas y producidas en plantas logrando su protección después del reto con el poliovirus tipo 3 WT.

Posteriormente, Daniell et al., (2019), empleó lechuga como plataforma de expresión desarrollando diversas líneas transplantómicas. De forma interesante, con la producción de la proteína VP1 se lograron generar VLPs de un tamaño de 22.3 nm aproximadamente. Se observó un aumento en los niveles de anticuerpos IgG1 e IgA específicos y su actividad neutralizante se determinó en suero de ratones inmunizados con la IPV seguida de tres boosters orales con 20 mg de material vegetal liofilizado mezclado con escualeno, saponina o ambos más los péptidos antimicrobianos (LL37 y PG1).

Sin embargo, aunque ya se ha explorado el enfoque transitorio y estable-transplantómico para producir a la proteína VP1, aún falta investigar la capacidad

de las células vegetales para expresar de forma estable-nuclear las proteínas VP1, VP2, VP3 y VP4, así como determinar su potencial antigénico e inmunogénico en vías de contribuir al desarrollo de una vacuna oral segura y de bajo costo.

3. JUSTIFICACIÓN

Actualmente, las limitantes que interfieren con la fase final en la erradicación de la polio se derivan de la manipulación de PV infectivos para la fabricación de vacunas y de la generación de nuevas cepas virulentas asociadas a la administración de la vacuna atenuada.

Este panorama, señala la importancia de disponer de una nueva herramienta que maximice el impacto de los esfuerzos para la erradicación. Por lo tanto, una de las estrategias más novedosas es el desarrollo de una vacuna de subunidades. En los últimos años ha venido cobrando interés la posibilidad de fabricar vacunas recombinantes utilizando células vegetales como plataformas de producción, y para su administración en forma oral, estos sistemas constituyen una plataforma viable para la producción y administración de vacunas a bajo costo; lo cual los posiciona como un sistema idóneo para la generación de una vacuna oral efectiva y económica contra la polio.

4. OBJETIVOS

4.1 OBJETIVO GENERAL

Expresar las cuatro proteínas VPs del poliovirus Sabin tipo 1 en células de tabaco y evaluar su antigenicidad e inmunogenicidad

4.2 OBJETIVOS ESPECÍFICOS

1. Diseñar los genes sintéticos que codifiquen para las proteínas VPs del poliovirus Sabin tipo 1

2. Subclonar en el vector de expresión pBI121 los 4 genes sintéticos

3. Generar y caracterizar a nivel molecular las plantas de tabaco transgénicas

4. Evaluar en ratones la inmunogenicidad de las proteínas expresadas

5. METODOLOGÍA

5.1 Diseño de genes sintéticos

Las secuencias de nucleótidos que codifican para las proteínas VP1, VP2, VP3 y VP4 de la cepa Sabin serotipo 1(GenBank No. Acceso V01150.1) se buscaron en la base de datos del National Center for Biotechnology Information (NCBI). Una vez obtenidas, se adicionaron los sitios de restricción *Sma*I y *Sac*I en los extremos 5´ y 3´ de cada secuencia nucleotídica respectivamente. Las secuencias se mandaron sintetizar con la optimización de codones para su uso en plantas (Anexo 1). La síntesis y optimización se realizó en la empresa GenScript® (Piscataway, NJ, USA); la cual, las clonó en el vector de clonación pUC57 (un vector por cada secuencia).

5.2 Subclonación en el vector de expresión pBI121

Los vectores de clonación pUC57, se resuspendieron en 20 µl de agua estéril. Para liberar el inserto, se tomaron 5 µl y se realizó una digestión con las enzimas *Sma*I/*Sac*I, el producto de la digestión se corrió en un gel de agarosa al 1% y se verificaron los tamaños de los insertos que corresponden a: VP1: 942 pb, VP2: 852 pb, VP3: 750 pb y VP4: 240 pb. Después de corroborar el tamaño correcto de las secuencias, las bandas se purificaron con el kit Wizard® SV Gel and PCR Clean-Up System (promega). Los insertos purificados se subclonaron en el vector binario pBI121 previamente digeridos con las enzimas *Sma*I/*Sac*I, las reacciones de ligación se realizaron con la enzima T4 ligase (NEB cutter) y con los productos se transformó a la bacteria *Escherichia coli* TOP 10. La detección de clonas recombinantes se realizó en agar LB suplementado con kanamicina (Kan) (100 mg/L), en el que las colonias bacterianas que contienen el vector pBI121 crecerán en el agar. Las colonias seleccionadas, se inocularon en medio LB suplementado con kan (100 mg/L) y se incubaron a 37°C/16hrs. Posteriormente, se realizó la extracción de ADN plasmídico por el método de Birnboim (Sambrook and Russell, 2001). Una vez obtenido el ADN, se llevaron a cabo digestiones con las enzimas *Sma*I/*Sac*I y se verificaron los perfiles de restricción mediante desplazamiento

electroforético en geles de agarosa al 1%, además para confirmar la integridad de las secuencias, el ADN de las clonas con los perfiles deseados se mandó secuenciar al Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental (LANBAMA). Posteriormente, las construcciones del vector pBI121-VP se movilizaron por electroporación a la bacteria *A. tumefaciens* GV3101. Finalmente, se seleccionó una clona recombinante de cada construcción para la transformación de tabaco. Total de construcciones: 4 (pBI-VP1, pBI-VP2, pBI-VP3 y pBI-VP4).

5.3 Obtención de las líneas transgénicas de tabaco

La transformación nuclear-estable de tabaco comprendió el siguiente proceso: germinación *in vitro* de las semillas de *Nicotiana tabacum* cv. Petite Havana SR1 en medio MS (Murashige and Skoog, 1962) para generar plantas que sirvieron como fuente de tejido de hoja el cual se utilizó para generar explantes que posteriormente fueron inoculados con los cultivos de *A. tumefacines* que contenían los vectores pBI-VP1, pBI-VP2, pBI-VP3 o pBI-VP4. Subsecuentemente, los explantes infectados se co-cultivaron por un periodo de 48 hrs en la oscuridad usando el medio RMOP [Sales MS y vitaminas, 0.1 mg/L 6-benziladenina (BA) y 0.1 mg/L de ácido naftalenacetico (NAA)].

Posteriormente, los explantes se transfirieron a medio de selección [Medio RMOP + Kan 100 mg/L y Cefotaxima (Cefo) 500 mg/L] y se mantuvieron en una cámara con ambiente controlado (25°C, fotoperiodo: 16 hrs luz/8 hrs oscuridad). Los explantes se cambiaron a medio fresco en intervalos de 15 días. Finalmente, los brotes resistentes a kan rescatados se transfirieron a medio libre de hormonas; las plántulas que generaron raíz exitosamente se trasladaron a suelo y se cultivaron en condiciones de invernadero. Para los ensayos de inmunogenicidad, el tejido de hoja se colectó y se liofilizó en el equipo LABCONO (condiciones: -75 °C/0.133 mbar/48 hrs), la biomasa liofilizada se pulverizó y se almacenó a temperatura ambiente hasta su uso.

5.4 Detección de los transgenes por PCR

Para confirmar la presencia del transgen en las líneas seleccionadas se realizaron ensayos de PCR (polymerase chain reaction). Para ello, se extrajo el ADN total de las líneas putativamente transformadas de acuerdo a Dellaporta et al., 1983. La reacción se llevó a cabo utilizando primers específicos para amplificar cada una de las secuencias VPs (Tabla 1) y con los siguientes componentes en un volumen total de 25 µl: $MgCl_2$ (1.5 mM), dNTPs (1 mM), TaqPol (2.5 U), ADN (50-100 ng). La amplificación se realizó en un termociclador con las etapas: desnaturalización inicial: 94°C/2 min, 35 ciclos a 95°C/30 s (desnaturalización), 55°C/60 s (alineamiento), 72°C/60s (extensión), 72°C/5 min (extensión final). Los productos de PCR se analizaron en un gel de agarosa 1%. Como control positivo se utilizó cada una de las construcciones de los plásmidos (pBI-VP1, pBI-VP2, pBI-VP3 o pBI-VP4).

Tabla 1. Secuencia de los primers usados para la detección de los transgenes mediante PCR

Transgene	Secuencias	Tamaño del amplicon (pb)
***VP*1**	For-5´CGCACAATCCCACTATCCTTCGC3´ Rev-5´TTCCTTCTAAGTTGAACTGTATCCT3´	441
***VP*2**	For-5´CGCACAATCCCACTATCCTTCGC3´ Rev-5´CGAACAATCCCATATCCCTAAGAG3´	352
***VP*3**	For-5´CGCACAATCCCACTATCCTTCGC3´ Rev-5´CAGCCCAATGAGTGTAGTAGTT3´	409
***VP*4**	For-5´GGGTGCTCAAGTTTCTTCTCAAA3 Rev-5´CAAGACCGGCAACAGGATTCAATC 3´	305

pb: pares de bases

5.5 Generación de sueros hiper-inmunes específicos para las proteínas VPs

Para las proteínas VP2, VP3 y VP4, se realizó una búsqueda de epítopes centrada en linfocitos B utilizando el sitio web de la herramienta BCPRED (http://ailab.ist.psu.edu/bcpred/predict.html), los epitopes candidatos (20 aa) fueron seleccionados por su SCORE más alto. Para la proteína VP1 se seleccionó el péptido inmunogénico previamente reportados por Wychowskil et al. 1983 y

Adeyemi et al. 2016. Las secuencias se mandaron sintetizar a la empresa SynPeptide Co. (Beicai pudong new area, Shanghai, China) (Tabla 2). Ratones BALB/cAnN (n=12), hembras y de 8-12 semanas de edad se inmunizaron siguiendo el presente esquema: Semana 1, se inyectó subcutáneamente en el lomo 10 µg de antígeno emulsionados en adyuvante completo de Freud (CFA), en un volumen total de 100 µl. Semana 2, 3 y 4, por vía subcutánea se inyectaron 50 µg de péptido emulsionados con adyuvante incompleto de Freud (IFA). Semana 5, se colectaron muestras de sangre para determinar los niveles de anticuerpos generados. Una vez obtenidos altos niveles, los animales se sacrificaron por dislocación cervical siguiendo las indicaciones de la Norma Oficial Mexicana NOM-062-ZOO- 1999 a fin de obtener una mayor cantidad mayor de antisuero. Los niveles de anticuerpos se evaluaron por ELISA fijando durante toda la noche a los pozos de la placa 1 µg de los péptidos sintéticos VPs; los lavados entre cada uno de los pasos del análisis, se realizaron con PBS-T (Phosphate Buffered Saline 1X+ Tween 0.05%). El bloqueo se realizó con leche libre de grasa al 5%, durante 2hrs a 25°C. Posteriormente, se añadió el suero recolectado en diluciones seriadas de 1:40 hasta 1:320 y se incubaron toda la noche a 4°C; seguido de esto se realizaron 3 lavados con PBS-T y se adicionó el anticuerpo secundario anti-IgG conjugado con peroxidasa durante 2 hrs a 25°C (dilución 1:2000). Después del lavado, se efectuó la detección colorimétrica de peroxidasa mediante la adición de ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) + H_2O_2 1 mM. Después de 30 min de incubación, se determinó la densidad óptica (DO) a 405 nm. Todos los experimentos siguieron los lineamientos del National Institute of Health Guide for Care and Use of Laboratory Animals, los protocolos experimentales fueron revisados y aprobados por el comité de ética para el uso y cuidado de animales de laboratorio del Instituto de Investigaciones Biomédicas (IIB) de la Universidad Nacional Autónoma de México (UNAM) (Número de protocolo 233).

Tabla 2. Secuencia en aminoácidos de los péptidos sintéticos utilizados para la generación de sueros hiperinmunes

Proteína	Secuencia del péptido	Nombre del péptido	Tamaño (aa)
VP1	DNSASTKNKDKL	$VP1_{93-104}$	12
VP2	AGDSNTTTMHTSYQNANPGE	$VP2_{133-154}$	20
VP3	LVSYAPPGADPPKKRKEAML	$VP3_{131-151}$	20
VP4	TINYTTINYYRDSASNAASK	$VP4_{24-44}$	20

aa: aminoácidos

5.6 Inmuno-detección de las proteínas VPs

La presencia e integridad de las proteínas VP se realizó mediante ensayos de dot blot y western blot. Se obtuvieron los extractos solubles a partir de 100 mg tejido fresco de hojas de plantas transgénicas o sin transformar (WT) en 300 µL de buffer de extracción (NaH_2PO_4 25 mM (pH 6.6), NaCl 100 mM, Triton X-100 v/v 0.5%, mM βmercaptoethanol 100 mM y PMSF 1mM). Las suspensiones se sonicaron (5 pulsos/5 s/25% amplitud) y los extractos se clarificaron por centrifugación a 9,660 *x g* a 4°C por 10 mins.

Para los análisis por dot blot, 2 µL del extracto total se aplicaron en una membrana de nitrocelulosa (Bio-Rad, Germany) y se dejaron secar, subsecuentemente, el bloqueo se realizó con leche libre de grasa al 5% por 2 hrs. Para la proteina VP1, se utilizó el Anti-Poliomyelitis Virus 1 (LSc, 2ab strain) antiserum neutralizing (Alpha Diagnostic, Texas, USA) como anticuerpo primario y como control positivo se utilizó la proteina VP1 producida de forma recombinante en *E. coli* (Sabin; POLV1 VP1) con el fin de comparar la producción de señal producida por la única proteína de la cápside de poliovirus disponible de forma comercial (Alpha Diagnostic, TX, USA).

En ausencia de la estructura completa de las proteínas VP2, VP3 y VP4, los péptidos sintéticos $VP2_{133-154}$, $VP3_{131-151,}$ $VP4_{24-44}$ se usaron como controles positivos y sus respectivo anti-suero hiperinmune se añadió para proceder con una

incubación toda la noche a 4°C. Después, se añadió el anticuerpo secundario anti-IgG (dilución 1:2000) por un periodo de 2 hrs a 25°C.

La integridad de las proteínas producidas de forma recombinante en plantas se determinó mediante el ensayo de western blot, los extractos se desnaturalizaron por ebullición durante 5 min a 95°C y las proteínas se separaron por electroforesis en gel de poliacrilamida al 4-12% bajo condiciones desnaturalizantes. Después, se transfirieron a una membrana de nitrocelulosa, la cual se bloqueó con leche al 5% y se incubó con el Anti-Poliomyelitis Virus 1 (LSc, 2ab strain) antiserum neutralizing (Alpha Diagnostic, Texas, USA) o el suero hyperimmune anti-VP4 durante toda la noche. Posteriormente, se incubó con un anticuerpo secundario conjugado con peroxidasa (dilución 1:2000) por un periodo de 2 hrs a temperatura ambiente.

Para ambos análisis, la unión de anticuerpos se detectó por incubación con Pierce® ECL Western Blotting Substrate solutions (Thermo Scientific, IL, USA) siguiendo las instrucciones del fabricante. La detección de señal se realizó por medio de una película fotográfica y la estimación de la masa molecular se estimó utilizando el Molecular weight marker “Precision Plus Protein™ Dual Color Standars” (BioRad, CAL, USA).

5.7 Cuantificación de las proteínas recombinantes mediante ELISA

Alrededor de 100 mg de tejido fresco vegetal se congelaron y se molieron en mortero frío con 300 µl de buffer de extracción (NaH_2PO_4 25 mM (pH 6.6), NaCl 100 mM, Triton X-100 v/v 0.5% y PMSF 1mM), seguido por la centrifugación a 9,660 *x g* a 4°C por 10 mins. Después se recubrieron placas de poliestireno con el extracto obtenido de las de plantas tabaco transgénicos y sin transformar. Las placas se incubaron toda la noche a 4°C, después se lavaron con PBS-T, se bloquearon con leche libre de grasa al 5% por un periodo de 2 hrs a 25°C, se añadieron anticuerpos primarios dirigidos contra las proteínas de interés y se incubaron toda la noche a 4°C. Posteriormente, se añadió el anticuerpo secundario anti-IgG conjugado con peroxidasa por un periodo de 2 hrs a 25°C

(dilución 1:2000). Finalmente, se determinó la unión de los anticuerpos con ABTS + 1mM H_2O_2 y se determinó la DO a 405 nm. Las lecturas se compararon con las lecturas de una cuerva estándar construida con distintas concentraciones de los péptidos sintéticos.

5.8 Ensayos de inmunogenicidad en ratones

La inmunogenicidad de las proteínas VPs expresadas en las plantas de tabaco se determinó en ratones BALB/c de 7-8 semanas de edad (25 g de peso). Para ello, se emplearon seis grupos experimentales (*n*=5), los cuales se nombraron como grupos 1-6. El esquema de inmunización comprendió la administración de 4 dosis subcutáneas (s.c.), una por semana, seguidas por 4 boosters orales administrados cada dos semanas. Los grupos 1-4 se inmunizaron con los extractos de las plantas transgénicas que expresan a las proteínas VP1, VP2, VP3 y VP4, respectivamente. Los ratones del grupo 5 recibieron extractos de plantas de tabaco sin transformar, mientras que el grupo 6 fue tratado con 200 µl de PBS 1X. Las inmunizaciones s.c. consistieron en la administración de los extractos solubles totales obtenidos al resuspender 10 mg de tejido liofilizado de las líneas transgénicas o WT; los extractos se clarificaron mediante centrifugación a 16,000 x *g* por 15 min. Las dosis s.c. aplicadas de acuerdo a la cuantificación de cada proteína realizada por ELISA fueron 0.03 µg de VP1, 1.40 µg de VP2, 0.43 µg de VP3 y 0.60 µg de VP4. Los boosters orales estuvieron conformados por 10 mg de tejido liofilizado resuspendido en 300 µl de PBS 1X, los cuales se administraron intragastricamente. Todas las inmunizaciones se aplicaron sin la adición de adyuvantes exógenos. Los ratones se sangraron a los días 1, 28 y 105 mediante punción en el seno venoso submandibular y las muestras de sangre se procesaron para obtener suero, el cual se almacenó a -40°C hasta su uso.

El protocolo experimental fue revisado y aprobado por el comité de investigación y docencia de la Facultad de Química de la Universidad Autónoma de San Luis Potosí (Número de registro: CEID201901R1).

5.9 Detección de anticuerpos en suero y heces

Los niveles de IgG y S-IgA se midieron en suero o heces de los ratones inmunizados. Placas de ELISA de 96 pozos se sensibilizaron con 1 ug/pozo de péptidos sintéticos $VP1_{93-104,}$ $VP2_{133-154}$, $VP3_{131-151,}$ $VP4_{24-44}$ y se incubaron toda la noche a 4°C. El bloqueo se realizó durante 2 hrs/25°C con leche libre de grasa al 5% disuelta en PBS 1X, después las placas se incubaron toda la noche a 4°C con cada muestra de suero (diluciones 1:40) o extractos de heces (sin diluir). Posteriormente, las placas se incubaron con anticuerpos anti-IgG o anti-IgA acoplados a peroxidasa por un periodo de 2 h/25°C. Finalmente, los niveles de anticuerpos se midieron después de 30 mins de incubación con ABTS+ H_2O_2 1mM. Los valores de DO a 405 nm se realizaron en el Thermo Scientific Multiskan FC microplate photometer (Thermo Scientific, Waltham, MA).

5.10 Análisis de datos

Para determinar la respuesta de anticuerpos IgG e S-IgA se restaron los valores en DO de los sueros y heces colectados al inicio del experimento (día 1) a los valores obtenidos en los días 28 y 105. Posteriormente, los datos se analizaron por ANOVA usando el software GraphPad Instat 3.1. Los valores de corte se determinaron con los promedios + 2 desviaciones estándar (SD) del grupo de ratones inmunizados con la biomasa WT. Las diferencias se consideraron significativas cuando los valores de *P* fueron <0.05 (*P* <0.05).

6. RESULTADOS Y DISCUSIÓN

En el presente trabajo de tesis se reporta la expresión nuclear-estable de las proteínas VP1, VP2, VP3 y VP4 de la cápside del PV Sabin tipo 1 en plantas de tabaco y su potencial inmunogénico en ratones. La aplicación de esta tecnología se realizó como un esfuerzo para desarrollar una formulación oral capaz de inducir inmunidad intestinal contra el virus a fin de evitar el uso de la cepa infectiva en su forma atenuada o sistemas de producción de vacunas de subunidades costosos.

6.1 Optimización de los genes sintéticos *VP*1, *VP*2, *VP*3 y *VP*4

De manera inicial, las secuencia nucleotídica completa de los cuatro genes VPs se optimizó a través del algoritmo OptimumGene TM, en el cual se ajustó el sesgo de uso de codones a plantas de *N. tabacum*. Además, con esta herramienta se optimizaron otros factores críticos relacionados a la eficiencia de la expresión, estos incluyeron: i) el contenido de Guanina y Citocina (GC) se ajustó en un rango de 30-70%, ii) se evitó la presencia de estructuras tallo-asa y secuencias negativas en cis, iii) se incluyeron los sitios de digestión *Sma*I y *Sac*I en los extremos 5´y 3´ de cada gen respectivamente. La optimización, síntesis y clonación de los genes en vectores pUC57 se realizaron por la empresa GenScript™.

6.2 Generación de líneas transgénicas

Una vez obtenidos los vectores de clonación, se construyeron los vectores de expresión basados en el vector binario pBI121 para expresar de forma nuclear-estable cada una de las proteínas VPs (VP1, VP2, VP3 y VP4) en plantas de tabaco. El mapa de los vectores pBI-VP1, pBI-VP2, pBI-VP3 y pBI-VP4 generados se presentan en la Fig. 6. Los transgenes se introdujeron a las células de tabaco vía transformación mediada por *A. tumefaciens*, en donde los explantes infectados se mantuvieron en medio suplementado con antibiótico y se cambiaron a medio fresco cada 15 días. A través de este procedimiento se generaron exitosamente líneas de tabaco antibiótico-resistentes, las cuales se transfirieron y mantuvieron

en invernadero hasta obtener la biomasa suficiente para conducir los análisis moleculares y de inmunogenicidad (Fig. 7).

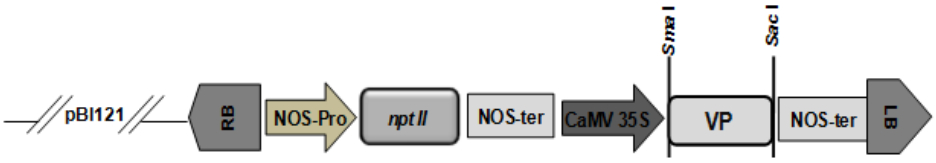

Figura 6. Descripción de los vectores de expresión pBI-VPs. Los vectores pBI-VPs se derivaron del vector binario pBI121. *RB* right border (borde derecho), *LB* left border (borde izquierdo), *nptII* gen de resistencia a kanamicina bajo el control del promotor nopalina sintasa NOS-Pro, NOS-ter terminador nopalina sintasa, VP: genes *VP*1, *VP*2, *VP*3 o *VP*4 expresados bajo el control del promotor 35S del virus del mosaico de la coliflor (CaMV35S). Sitios de digestión *Sma*I y *Sac*I añadidos en los extremos 5´y 3´de los genes respectivamente.

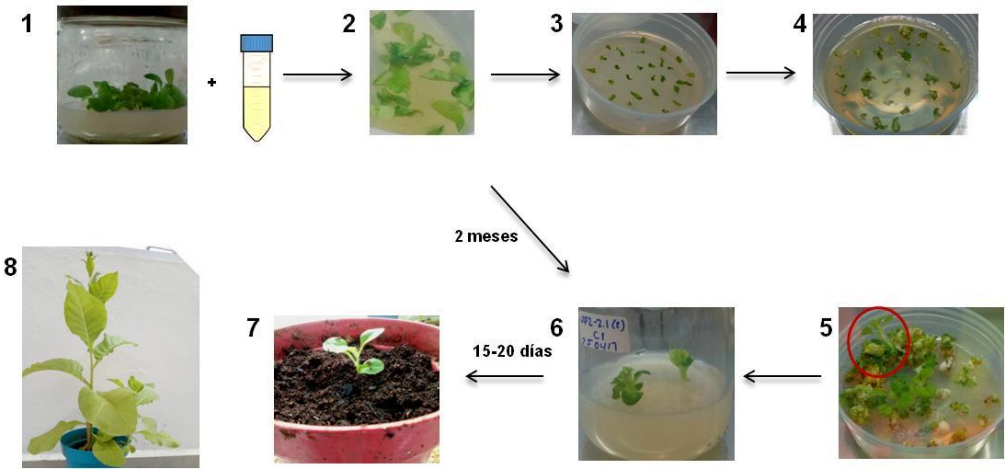

Figura 7. Generación de plantas de tabaco que expresan de forma recombinante las proteínas VPs. 1) germinación y obtención de plántulas que sirvieron como fuente de explantes, **2)** infección de lo explantes con los cultivos bacterianos que contenían los vectores de expresión pBI-VPs, **3)** co-cultivo en medio RMOP sin antibióticos, **4)** transferencia de los explantes a medio RMOP suplementado con kanamicina (100 mg/L) y cefotaxima (500 mg/L), **5)** obtención

de brotes antibiótico-resistentes, **6)** transferencia de brotes a medio para enraizar, **7)** cambio de plántulas a suelo y **8)** cultivo de plantas en invernadero.

6.3 Presencia de los genes VPs en las plantas de tabaco

La detección de los transgenes se evaluó mediante PCR. En la Fig. 8 se observan los amplicones esperados para VP1, VP2, VP3 y VP4 con tamaños de 441 pb, 452 pb, 409 pb y 305 pb respectivamente; estos tamaños coinciden con los obtenidos con las reacciones realizadas con los vectores pBI-VPs utilizados como controles positivos.

El número total de líneas confirmadas mediante este análisis fueron: 1 (P2) para VP1, 6 (P1, P2, P3, P4, P5 y P6) para VP2, 7 (P1, P2, P3, P4, P5, P6 y P7) para VP3 y 4 (P1, P2, P3 y P4) para VP4. No se observaron bandas positivas en las muestras que contenían ADN de tabaco WT o la mezcla de reacción que no contenía ADN.

Desafortunadamente, solo una línea transgénica resultó positiva para el gen VP1, de acuerdo al análisis mencionado anteriormente, en este caso solo dos líneas se analizaron debido al escaso número de líneas rescatas para este transgen. Este hallazgo nos sugiere que la expresión de VP1 ejerce cierta toxicidad en la planta ya que para los demás transgenes se logró el rescate exitoso de una cantidad mayor de líneas.

6.4 Expresión exitosa de las proteínas VPs

La presencia e integridad de las cuatro proteínas VPs expresadas de forma nuclear-estable en plantas de tabaco se realizó mediante las técnicas de dot blot y western blot. Para el análisis por dot blot, se obtuvieron los extractos totales de proteínas de todas las líneas candidatas. Para la inmuno-detección se utilizaron los antisueros Anti-Poliomyelitis Virus 1 antiserum, anti-VP2, anti-VP3 o anti-VP4, con los cuales se produjo una señal positiva en todas las líneas candidatas; mientras una nula señal se obtuvo en los extractos de proteínas de tabacos no transformados (Fig. 9).

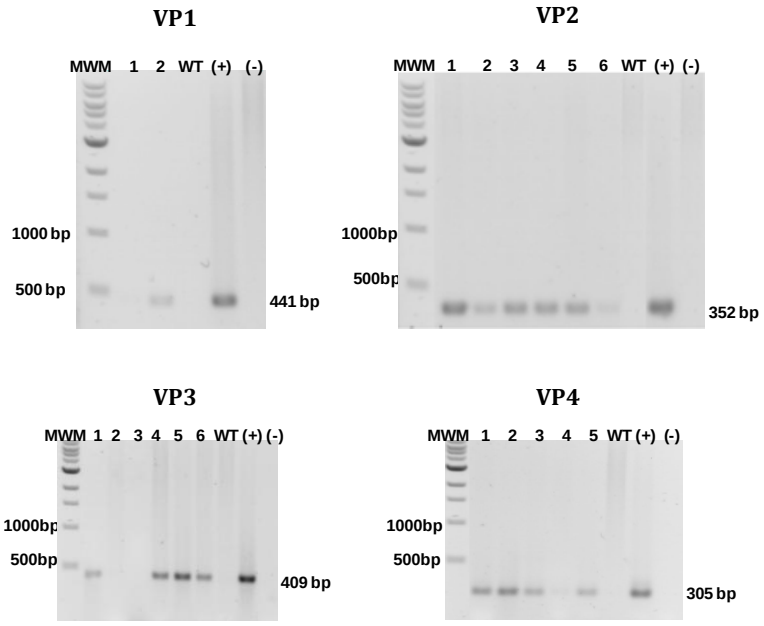

Figura 8. Detección de los transgénes por PCR. MWM, marcador de peso molecular 1Kb, 1-6 líneas candidatas, WT wild type, (+) control positivo pBI-VP1, pBI-VP2, pBI-VP3 o pBI-VP4, (-) control negativo de la reacción (mezcla sin ADN), tamapo de los amplicones esperados a) VP1: 441 pb, b) VP2 352 pb, c) VP3 409 pb y d) VP4 305 pb.

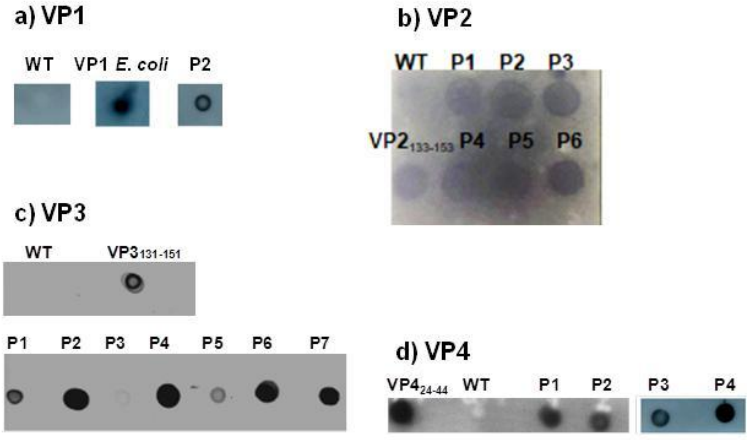

Figura 9. Inmunodetección de las proteínas VPs expresadas en plantas. a) Señal positiva detectada en la línea transgénica P2, VP1 *E. coli:* control positivo, WT extracto de tabaco sin transformar, **b)** P1-P6 líneas transgénicas que expresan a la proteína VP2, VP2 $_{133\text{-}154}$ péptido sintético utilizado como control positivo, WT

extracto de tabaco sin transformar, **c)** P1-P7 líneas transgénicas que expresan a la proteína VP3, $VP3_{131-151}$ péptido sintético utilizado como control positivo, WT extracto de tabaco wildtype, **d)** P1-P4 líneas transgénicas que expresan a la proteína VP4, $VP4_{24-44}$ péptido sintético utilizado como control positivo, WT extracto de tabaco wildtype.

Los análisis por western blot marcados con el antisuero Anti-Poliomyelitis Virus 1 o con el suero hiperinmune anti-VP4 revelaron la presencia de bandas de 34, 30, 27 y 7.5 KDa para las proteínas VP1, VP2, VP3 y VP4 respectivamente; estos pesos moleculares corresponden a los pesos estimados para cada una de las proteínas, lo cual confirma la expresión de las proteínas antigénicas VPs en células vegetales (Fig. 10).

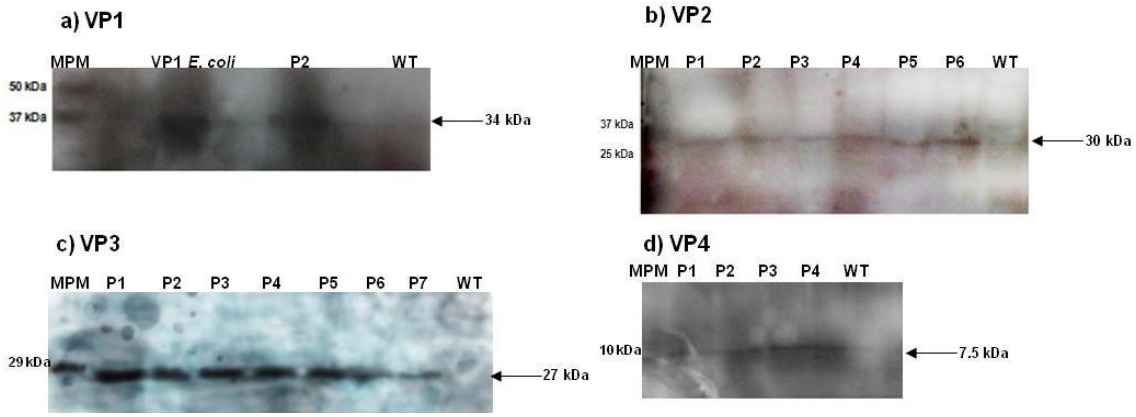

Figura 10. Western blot de las líneas transgénicas de tabaco. Extractos proteicos de cada una de las líneas se corrieron mediante SDS-PAGE y posteriormente se transfirieron a membranas de nitrocelulosa. La detección de las proteínas VP1, VP2 y VP3 se realizó con el antisuero Anti-Poliomyelitis Virus 1, mientras que la proteína VP4 se determinó con el suero hiper-inmune anti-VP4. Las bandas detectadas se marcan con flechas. **a)** VP1, tamaño esperado: 34 KDa, **b)** VP2, tamaño esperado: 30 KDa, **c)** VP3, tamaño esperado: 27 KDa y **d)** VP4, tamaño esperado 7.5 KDa.

Los niveles de expresión de las 4 proteínas VPs se cuantificaron mediante ELISA utilizando un set de curvas estándar realizadas con distintas concentraciones de los péptidos sintéticos. Los niveles de acumulación estimados fueron: 0.3 µg VP1/g, 6.16-16.85 µg VP2/g, 2.74-4.33 µg VP3/g y 0.96-6 µg VP4/g de tejido fresco de hoja (Fig. 11).

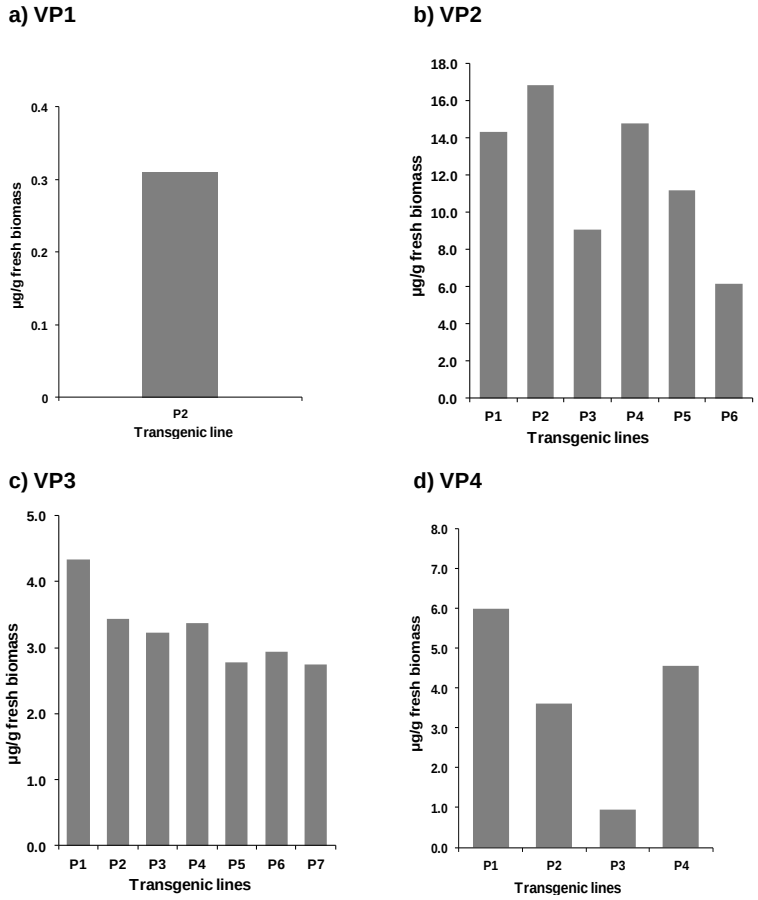

Figura 11. Estimación de los niveles de expresión de las proteínas VPs en plantas. Las curvas estándar para estimar los niveles de acumulación de las proteínas recombinantes se realizaron con los péptidos sintéticos $VP1_{93-104}$, $VP2_{133-154}$, $VP3_{131-151}$ y $VP4_{24-44}$. **a)** Línea transgénica P2 que expresa a la proteína VP1, b) Líneas transgénicas P1-P2 que expresan a la proteína VP2, **c)** Líneas P1-P7 que contienen a la proteína VP3 y **d)** Líneas P1-P4 que expresan a la proteína VP4.

Las cantidades estimadas reportadas en nuestro estudio fueron similares a los niveles alcanzados cuando proteínas de otros virus se expresaron en plantas de tabaco. Tales proteínas incluyen a la proteína VP40 del virus del Ébola en donde su expresión alcanzó hasta 2.6 µg/g de tejido fresco de hoja según lo reportado por Monreal-Escalante E, et al., 2017; el M/L-HBsAg cuya expresión osciló entre 2-10 µg/g (Pniewski et al., 2012), el péptido viral CTB- $MPR_{649-684}$ –HIV-1 con una producción de 18± 2.3 mg/kg de tejido vegetal fresco (Matoba et al., 2009) y el péptido multi-epitópico del virus del Ébola denominado Zerola con una expresión que osciló entre 1-2 µg/g de tejido fresco de hoja (Nieto-Gómez et al., 2019).

Durante los últimos cinco años, la expresión de proteínas inmunogénicas en plantas para la producción de vacunas novedosas contra la polio ha cobrado un auge mayor. Hasta el momento, los esfuerzos solo se han dirigido hacia la proteína VP1, la cual se ha expresado como proteína fusionada a CTB en cloroplastos de tabaco y lechuga. En tabaco, los niveles de expresión alcanzaron hasta 2600 µg/g de peso seco cuando se utilizó la secuencia optimizada, mientras que la proteína VP1 nativa produjo un rendimiento de hasta 54 µg/g de peso seco. En el caso de lechuga, la expresión de la secuencia optimizada generó rendimientos de 9 a 15 veces mayores en comparación a la expresión de la proteína nativa (Chan et al., 2016; Daniell, 2019). En ambos trabajos, los autores afirman que los altos rendimientos en la producción, son consecuencia del elevado número de copias del plastoma y cloroplastos por célula vegetal, así como la ausencia de eventos de silenciamiento de genes. Sin embargo, la expresión de proteínas en cloroplastos tiene ciertas limitaciones que deben ser contempladas, una de ellas es la carente habilidad de realizar modificaciones postraduccionales complejas así como para secretar la proteína (Rosales-Mendoza, 2016). Particularmente, la proteína VP4 requiere una modificación en el segmento amino-terminal, la cual consiste en la adición de ácido mirístico (N-myristoylation N-MYR); este ácido graso está involucrado en procesos moleculares críticos tales como el ensamble de la cápside y el proceso de entrada del virus hacia la célula blanco (Chow et al., 1987). Aunado a estas características, la presencia de esta molécula podría estabilizar la conformación proteica y preservar el mantenimiento

estructural de los epítopes contenidos en la proteína VP4 al igual que en el caso del dominio preS encontrado en el virus de la hepatitis B (Alberti et al., 1990). En plantas, la N-MYR de las proteínas es un proceso que está estrechamente relacionado con la regulación del estrés y la señalización celular (Traverso et al., 2008), por lo tanto, es esperado que esta modificación en la proteína VP4 se lleve a cabo en las células de tabaco modificadas genéticamente.

6.5 Las proteínas VPs producidas en plantas son inmunogénicas en ratones

Basado en los niveles más altos de expresión de las proteínas VP3 y VP4, se seleccionaron las líneas P1 para evaluar su potencial inmunogénico en ratones BALB/c. Por otra parte, para los ensayos de la proteína VP1, se utilizó la única línea transgénica caracterizada, mientras que en el caso de la proteína VP2, la línea P1 se prefirió sobre la línea P2 debido a la cantidad insuficiente de material vegetal para realizar la prueba. Grupos de 5 ratones se inmunizaron s.c. con los extractos de proteínas totales obtenidos de las plantas seguido por boosters orales con suspensiones de material vegetal liofilizado que contenía las proteínas VP1, VP2, VP3 y VP4 recombinantes; las administraciones se efectuaron siguiendo el esquema de inmunización presentado en la Fig. 12.

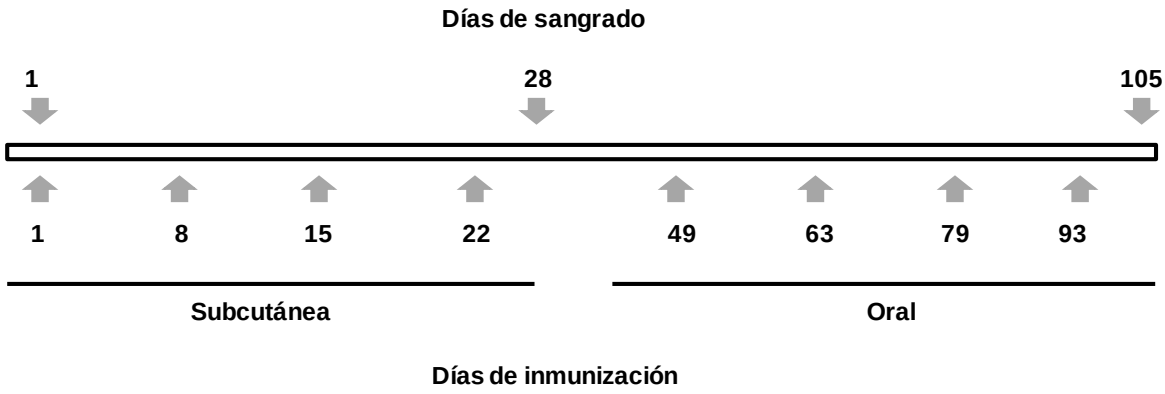

Figura 12. Esquema de inmunización. Grupos de 5 ratones BALB/c se inmunizaron con extractos (s.c.) o suspensiones (oralmente) preparados a partir de tejido vegetal liofilizado de las líneas transgénicas de tabaco, los cuales

incluyeron aproximadamente 0.03 µg de VP1, 1.40 de VP2, 0.43 µg de VP3, y 0.60 µg de VP4 en cada dosis.

Los datos obtenidos mediante ELISA revelaron la inducción de anticuerpos específicos IgG anti-VP3 y anti-VP4 en suero de los ratones inmunizados s.c. cuatro veces (día 28) con las proteínas correspondientes; para ambos grupos los niveles de anticuerpos IgG incrementaron después de las cuatro administraciones orales (día 105) con el tejido vegetal liofilizado, sin embargo, las diferencias entre el día 28 y el día 105 no fueron significativas (Fig. 13c, Fig. 13d). La demostración de las propiedades inmunogénicas de VP3 y VP4 nos sugieren que ambas proteínas se ensamblaron, modificaron y se produjeron de manera funcional. Además, al igual que las proteínas VP1 y VP2 conservaron sus propiedades antigénicas tal como se evidenció a través de los inmuno-análisis de dot blot y westernblot utilizando antisueros específicos para cada una de las proteínas de interés.

En los ratones inmunizados con la proteína VP1, no se detectaron niveles importantes de anticuerpos específicos después de las cuatro inyecciones s.c. (día 28); interesantemente la respuesta de IgG en suero se produjo después del primer refuerzo oral y la producción de anticuerpos aumentó en 2 de 5 ratones significativamente con los tres subsecuentes boosters orales (día 105) (Fig. 13a). Entre las diferentes modalidades de esquemas de vacunación, la estrategia prime-boost puede mejorar la inmunidad celular y humoral en distintos modelos animales. Este enfoque ha sido aplicado en el desarrollo de vacuna contra diversas enfermedades infecciosas provocadas por virus, lo cual indica resultados prometedores incluso en ensayos clínicos. Sin embargo, diversos factores incluyendo la selección del antígeno, tipo de vacuna, ruta de entrega, dosis, adyuvantes, régimen del boost y el orden de administración pueden influir en el resultado del enfoque de inmunización prime-boost (Kardani et al., 2016). En cuanto a las vacunas de subunidades las cuales están basadas en proteínas, péptidos o polisacáridos que contienen a los epítopes protectores, se sabe que

son poco inmunogénicas y requieren algunos componentes adicionales para aumentar su potencia además de encontrar la combinación de dosis/frecuencia/cantidad de inmunizaciones para lograr una respuesta inmune protectora (Hansson et al., 2000). En el caso de la proteína VP1 producida en plantas, la detección de niveles significativos de anticuerpos IgG específicos después de seguir el esquema de inmunización que combinó cuatro inmunizaciones s.c. y cuatro boost orales y una dosis baja del antígeno en nuestro análisis nos condujo al enfoque correcto para lograr una respuesta inmune humoral.

La presencia de altos niveles de anticuerpos IgG específicos para la proteína VP2 en los ratones inmunizados con el tejido vegetal correspondiente no fue detectada, sin embargo, la cantidad de anticuerpos aumentó ligeramente cuando todos los boosters orales fueron administrados y altos niveles de IgG se produjeron solo en un ratón al final de todas las dosis s.c. y orales administradas (Fig. 13b).

La detección de anticuerpos en heces de ratones después de los cuatro boosters orales reveló la presencia de niveles significativos de S-IgA intestinales en 3 de 5 animales de los grupos de ratones alimentados con el material vegetal que contenían a las proteínas VP1, VP2, VP3 o VP4 comparados con el mismo grupo después de las cuatro inyecciones s.c.. Sin embargo, para el caso de los anticuerpos S-IgA específicos para la proteína VP4, los niveles solo sobrepasaron el valor de corte establecido en un ratón inmunizado (Fig. 14).

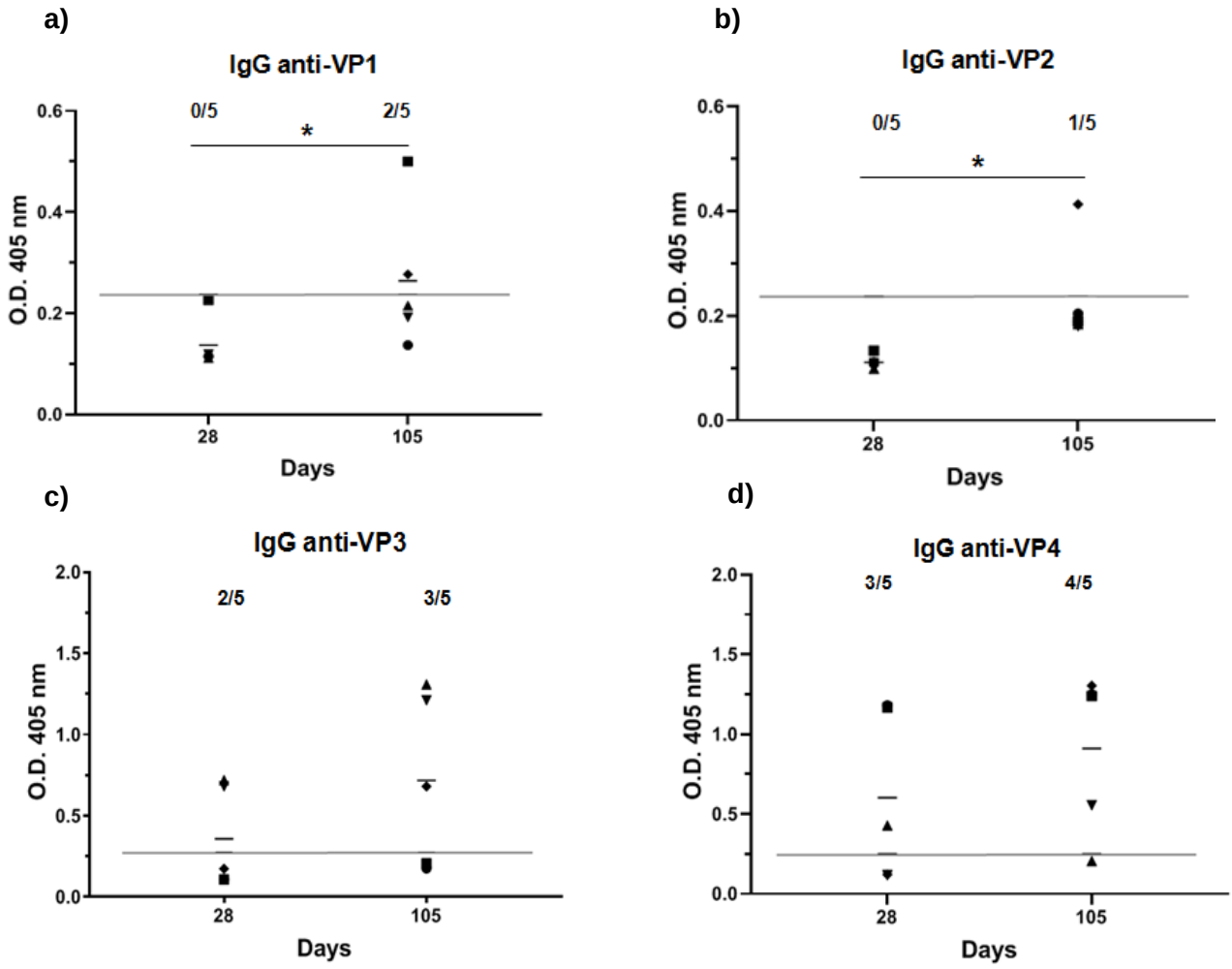

Figura 13. Niveles de anticuerpos IgG generados en sueros de ratones BALB/c inmunizados con las proteínas VPs expresadas en plantas. a) anticuerpos IgG anti-VP1, **b)** anticuerpos IgG anti-VP2, **c)** anticuerpos IgG anti-VP3 y **d)** anticuerpos IgG anti-VP4. La línea gris representa el valor de corte (promedio WT + 2SD). Los asteriscos denotan las diferencias significativas entre medias (marcadas por líneas cortas) de los días 28 y 105 (*$P < 0.05$).

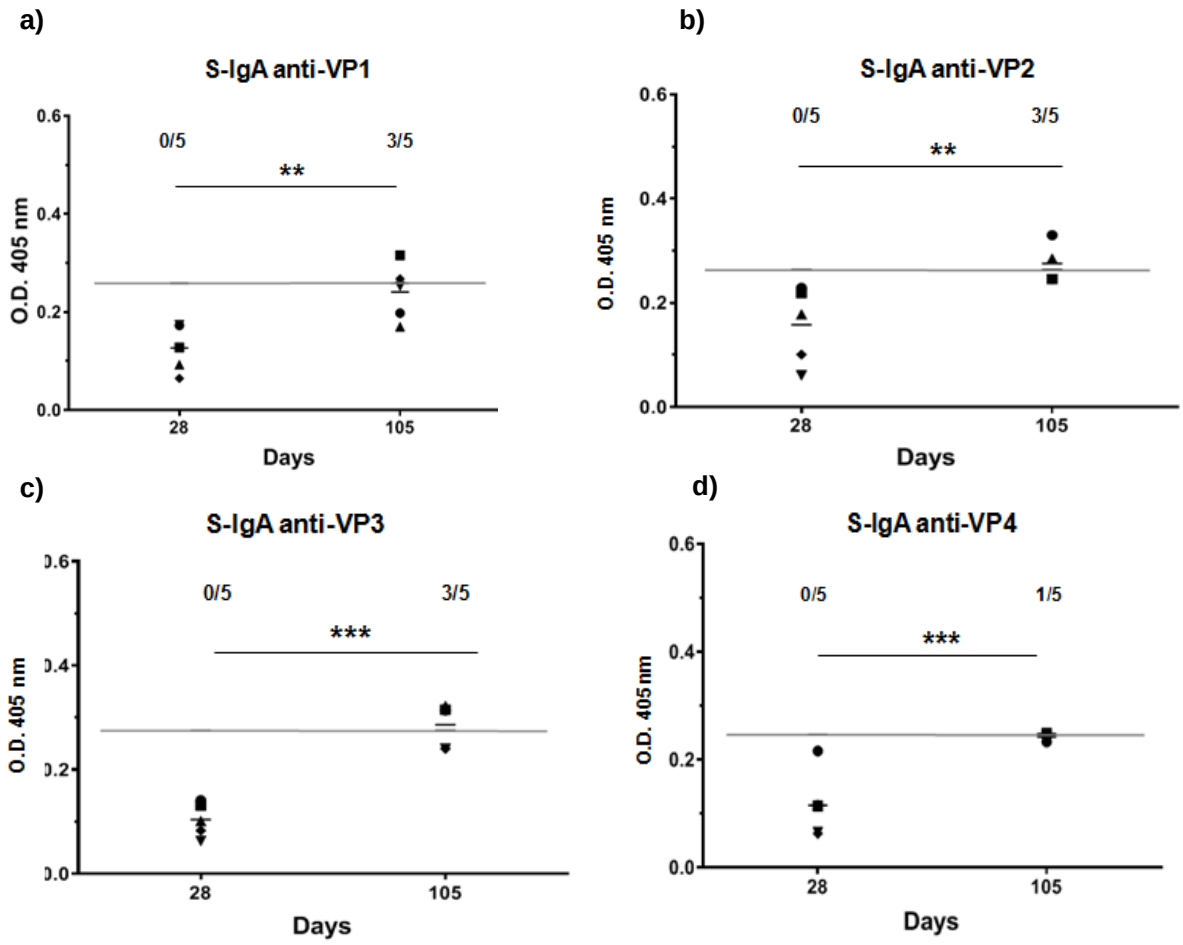

Figura 14. Niveles de anticuerpos S-IgA generados en las heces de ratones BALB/c inmunizados con las proteínas VPs expresadas en plantas. a) anticuerpos S-IgA anti-VP1, **b)** anticuerpos S-IgA anti-VP2, **c)** anticuerpos S-IgA anti-VP3 y **d)** anticuerpos S-IgA anti-VP4. La línea gris representa el valor de corte (promedio WT + 2SD). Los asteriscos denotan las diferencias significativas entre medias (marcadas por líneas cortas) de los días 28 y 105 (**$P < 0.01$, ***$P < 0.001$).

De acuerdo a los datos epidemiológicos y de inmunogenicidad, el uso combinado de la vacuna IPV y OPV conducen a la prevención de la polio en los niños vacunados, de igual manera es posible interrumpir la transmisión viral. Este último

hecho es conducido principalmente por la administración de la OPV que genera una respuesta inmune en mucosas, lo cual tiene un papel clave para limitar la infección entérica o faríngea y por tanto es crucial para disminuir la eficiencia en la transmisión viral. Esta eficacia se debe en parte a la naturaleza de ambas vacunas, debido a que están formuladas por los virus completos de forma inactivada o atenuada y en consecuencia son altamente inmunogénicas (Wright et al., 2016).

En contraste, las vacunas de subunidades producen una baja inmunogenicidad y es necesario el uso de compuestos adyuvantes mezclados en la formulación biológica. De forma notable, los prototipos vacunales desarrollados en el presente trabajo generaron exitosamente una respuesta inmune sistémica y en mucosas sin la co-administración de adyuvantes exógenos cuando se administraron de forma oral y s.c.

El desarrollo de ambas respuestas inmunes con dos formulaciones distintas partiendo de la misma biomasa vegetal que contiene a las proteínas VPs supone una importancia mayor. Ya que, además de no utilizar a los virus infectivos completos, mediante esta estrategia se podría generar un biológico a un bajo costo de producción y mantenimiento en comparación con las vacunas utilizadas actualmente.

Estos resultados apoyan también la teoría de que la inmunogenicidad de las proteínas expresadas en células vegetales se ve favorecida por la presencia de diversos metabolitos encontrados en la biomasa vegetal. De igual forma, la respuesta inmune generada en la mucosa intestinal apoya la conjetura de que las células vegetales tienen la capacidad de bioencapsular y proteger al antígeno durante su trayecto por el tracto gastroalimentario para su posterior captación por las células del sistema inmune encontradas en el intestino.

En concordancia con nuestros hallazgos, Chen et al., (2006) reportaron la inmunogenicidad oral de la proteína VP1 del enterovirus 71 fusionada a una señal de retención en el retículo endoplasmático, dicha fusión se expresó en frutos de

tomate. Por otra parte, Pniewski y cols (2011) inmunizaron ratones por vía oral con una dosis baja (100 ng) que contenía VLPs del antígeno de superficie del virus de la hepatitis B (HBsAg-VLPs) expresadas en lechuga, estas dosis se administraron sin adyuvantes exógenos; los refuerzos se administraron con un intervalo de 60 días y los resultados mostraron la producción de anticuerpos S-IgA en heces así como IgG en suero a niveles protectores. Resultados similares se obtuvieron cuando se administraron oralmente en ratones dosis bajas del mismo antígeno (HBsAg) expresado en tabaco; mientras que dosis elevadas condujeron a la activación de células T reguladoras asociadas a tolerancia oral con la subsecuente supresión de la respuesta inmune específica al antígeno (Kostrzak et al., 2009). Probablemente, en nuestro estudio este fenómeno podría haber ocurrido en los ratones inmunizados con la proteína VP2, ya que de todas las dosis administradas, las dosis de la proteína VP2 fueron las más altas en comparación a las demás proteínas y solo en un ratón se generó una respuesta inmune significativa.

Por otra parte, Pniewski et al., 2018 reportaron la evaluación de los factores que afectan la inmunización con boosters orales, particularmente la dosis de antígeno y la composición vacunal. Para ello, administraron oralmente en ratones BALB/c 2 dosis del antígeno S-HBsAg contenido en tejido de lechuga liofilizado, las cantidades utilizadas oscilaron desde 5 ng hasta 500 ng en diversas combinaciones precedidas por 1 priming intramuscular (i.m.) con 500 ng de la vacuna comercial Engerix B (S-HBsAg). La respuesta significativamente más alta se obtuvo utilizando oralmente 200 ng del antígeno seguido por las inmunizaciones con 5 ng, ambas dosis fueron efectivamente comparables o incluso excedieron al final la respuesta inducida por 3 inyecciones i.m. con Engerix B. En, el presente trabajo, las concentraciones de proteínas recombinantes administradas oscilaron desde 30 ng para VP1 hasta 1400 ng para VP2, obteniendo en general una respuesta inmune humoral significativa en mucosas para las 4 proteínas VPs y sistémica para VP1 y VP2.

La respuesta en mucosas determinada por los niveles de S-IgA anti-HBs no excedió los valores de fondo; por lo tanto administrar el extracto del tejido no es una forma adecuada de vacunación oral contra el virus del hepatitis B; en contraste, en los resultados obtenidos en este trabajo, se demostró que las proteínas VPs expresadas en plantas de tabaco transgénicas y administradas directamente como el tejido liofilizado resuspendido generaron una respuesta inmune significativa en la mucosa intestinal, lo cual apoya la teoría de que las células vegetales provee estabilidad y protección al antígeno de la degradación.

Además, en este mismo estudio los autores demostraron que los adyuvantes CTB, saponinas o alhydrogel no tenían un impacto favorecedor en la respuesta inmune sistémica. De forma interesante, para el S-HBsAg el uso de CTB disminuyó la respuesta inmune específica para el antígeno y aumentó la reacción específica a los componentes de la planta, ya que la respuesta inmune generada hacia liofilizado utilizado como control negativo excedió a la preparación vegetal que contenía el S-HBsAg.

Cabe señalar que en el presente proyecto las proteínas VPs expresadas en tabaco se inmunizaron de forma subcutánea seguida por refuerzos orales sin la adición de adyuvantes externos, y mediante este esquema se logró generar una respuesta inmune humoral significativa en ratones BALB/c. Aunque no todos los ratones produjeron anticuerpos IgG, los promedios de VP1, VP3 y VP4 excedieron el límite de corte establecido; incluso después de la primera inmunización parenteral (para VP4).

La respuesta inmune sistémica se caracterizó por el aumento de los anticuerpos IgG en los cuatro grupos después de los boosters orales. El mecanismo molecular por el que sucede este fenómeno no se conoce con precisión, sin embargo, es posible proponer una probable vía. Primero, el antígeno administrado por la vía parenteral estimula de forma eficaz a las células T helper (Th) que posteriormente circulan, después de la administración oral, las células M del epitelio intestinal captan al antígeno o las célula dendríticas y los macrófagos desde la lámina propia (Wang et al., 2014; Vela et al., 2017). Estas células presentadoras de

antígeno se mueven a los ganglios linfáticos mesentéricos en donde entran en contacto con las células Th específicas previamente sensibilizadas que migran desde los vasos sanguíneos sub-intestinales. De esta forma, la respuesta inmune sistémica podría acelerarse significativamente.

En los trabajos previos publicados con respecto a la proteína VP1 producida en cloroplastos de tabaco y lechuga, las inmunizaciones requirieron el uso de adyuvantes externos tales como saponina, escualeno y los péptidos antimicrobianos inmunomoduladores protegrina-1 y LL37 para inducir respuestas inmunes significativas además de un priming con la IPV (Daniell, 2019; Xiao and Daniell, 2017; Chan et al. 2016). Aunque en nuestros resultados se logró obtener una respuesta inmune significativa de anticuerpos IgG específicos para la proteína VP1, los niveles fueron bajos en comparación los reportados previamente o comparándolos con los producidos por las proteínas VP3 o VP4, este hallazgo podría suponer que la proteína VP1 expresada en sistemas vegetales requiere el uso de adyuvantes mezclados con las preparaciones vegetales para aumentar su inmunogenicidad cuando son administradas en ratones. Por lo tanto, para probar este supuesto, es necesario realizar experimentos adicionales utilizando adyuvantes como saponina, escualeno, CTB o LTB.

Por otra parte, las proteínas VPs expresadas en plantas también han sido reportadas por otros grupos de investigación. En el año 2017, Marsian J y cols, utilizaron ratones transgénicos que expresan el receptor humano CD155 (TgPVR) para evaluar la respuesta inmune después de administrar una o dos inyecciones intraperitoneales conteniendo 0.5 (dosis humana) de VLPs del virus de la polio serotipo 3 purificadas. Estas VLPs se generaron de manera transitoria en plantas de *N. benthamiana*, y los autores reportaron la generación de altos títulos de anticuerpos neutralizantes y la protección de los ratones después del reto viral. Sin embargo, la respuesta inmune en mucosas no fue caracterizada, lo que es de gran relevancia dada la naturaleza del poliovirus, lo que exige la generación de protección intestinal para evitar la propagación del virus a través de las heces.

Para mejorar la inmunogenicidad de las proteínas VPs producidas en tabaco y presentadas en este trabajo, se llevarán a cabo experimentos adicionales en donde se pruebe el efecto en la adición de adyuvantes externos. Otro tema a abordar, es el ajuste de dosis. Por ejemplo, las dosis de las cuatro proteínas debe ser igualada para determinar así si las diferencias en el potencial inmunogénico podrían atribuirse a la cantidad de proteína administrada o a la naturaleza del antígeno per se. Este ajuste quizá podría mejorar la inmunogenicidad de todas las proteínas, especialmente para el caso de VP2 ya que solo un ratón resultó ser respondedor; al igual que para los casos de VP3 y VP4 debido a que no mostraron aumento significativo en la respuesta sistémica humoral tras los refuerzos orales.

El éxito de la inmunización oral para generar una respuesta inmune adecuada depende de diversos factores, entre los que se encuentran: la dosis administrada, el sistema de entrega del antígeno, el esquema de inmunización y la composición de la vacuna; por otra parte, el éxito también depende de las propiedades biológicas del antígeno entre los que destacan: el tamaño de la molécula y su capacidad de interactuar con las moléculas de agua (hidrofóbicidad o hidrofílicidad). Estas características pueden influir directamente en la producción de la respuesta inmune humoral deseada.

Las macromoléculas de mayor tamaño son reconocidas de forma más eficiente por los receptores de células B (BCR) encontrados en la superficie de los linfocitos B, estas macromoléculas se absorben a través de endocitosis, se degradan y se presentan a las células T helper como pequeñas piezas peptídicas en el complejo principal de histocompatibilidad clase II (MHC-II-péptido) encontrado en la membrana celular. Las células T helper reconocen y se unen a este complejo a través de su receptor de células T (TCR), este receptor reconoce principalmente antígenos asociados a las células y antígenos no solubles. Después de esta unión, las células T expresan en la superficie diversas proteínas con función co-estimuladora, además de secretar diversas citocinas, con el fin de activar al linfocito B y promover su proliferación y posterior producción de anticuerpos (Abbas et al., 2015)

Aplicando estos principios a nuestros resultados, aunque se observó una diferencia significativa en la producción de S-IgA anti-VP4 en el día 105, sólo un ratón superó el valor de corte establecido. Este fenómeno podría atribuirse a las características biológicas de la molécula debido a que es una proteina muy pequeña, con bajo peso molecular (7.5 KDa) y alta solubilidad (Hebditch et al., 2017), lo que podría favorecer el desarrollo de una respuesta inmune insuficiente. En comparación con las proteínas VP1, VP2 y VP3, la proteína VP4 es la que posee el peso molecular más bajo y mediante el uso del servidor Protein-sol (https://protein-sol.manchester.ac.uk/) para la predicción de la solubilidad de las proteínas, se determinó que la proteína VP4 tiene una solubilidad mayor en comparación con las 3 proteínas restantes. Por lo tanto, el ajuste de las dosis o el uso de adyuvantes también podrían aportar una solución al efecto en la respuesta inmune limitada en mucosas.

La presencia de anticuerpos neutralizantes en suero es considerado como el estándar de oro para determinar la protección contra el virus de la polio. El método de microneutralización utilizando un cultivo celular HEP-2 y las cepas de poliovirus silvestre serotipos 1, 2 y 3 es recomendado como método de rutina de acuerdo a la OMS, WHO, 1993. Sin embargo, la capacidad neutralizante de los anticuerpos generados durante nuestro estudio no pudo ser determinada debido a que la manipulación de las cepas poliovirales en México está restringida y limitada sólo a algunos laboratorios en el mundo para su uso en el área diagnóstica. Esta manipulación conlleva el riesgo biológico y potencial escape de las cepas virales, por lo que este compromiso implica que cada país prohíba el manejo, retención o subsecuente adquisición de materiales de poliovirus en todas las instalaciones no esenciales incluidos los laboratorios de investigación. Cabe señalar que sería de gran relevancia determinar esta característica ya que la producción de anticuerpos neutralizantes es el objetivo primordial de una vacuna contra la poliomielitis (SSA, 2018).

Finalmente, el desarrollo de una vacuna oral compuesta por las proteínas VPs de la cápside del poliovirus Sabin tipo 1 y usando a las plantas como sistema de

expresión, posee diversas ventajas entre las que destacan: i) la eliminación del uso y manipulación de poliovirus infectivos para su producción, ii) la utilización de cultivos vegetales para la generación de la vacuna reduce el costo, el riesgo biológico y mantenimiento del producto, iii) mediante este enfoque se lograron fabricar dos formulaciones distintas partiendo de la misma biomasa vegetal, iv) siguiendo el esquema de inmunización oral precedida de la inmunización parenteral se logró generar y aumentar una respuesta inmune humoral sistémica y en la mucosa intestinal.

7. CONCLUSIÓN

Las cuatro proteínas de la cápside (VPs) del poliovirus Sabin tipo 1 se expresaron de manera nuclear-estable en células de tabaco, las cuales preservaron su antigenicidad e inmunogenicidad. Las proteínas generaron una respuesta inmune humoral sistémica y en mucosas discreta, resultando VP1, VP3 y VP4 las más inmunogénicas siguiendo un esquema que combinó la administración parenteral y oral. Los resultados obtenidos permiten sostener que las proteínas VP expresadas en plantas tienen capacidad inmunogénica resultados que señalan su potencial interés para utilizarse como inmunógenos así como antígenos para evaluar la seroprevalencia en la población. Estos resultados señalan el interés de optimizar la producción de VP de polio en plantas para ser utilizadas como vacuna segura de bajo costo y fácil distribución.

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9. ANEXOS

Anexo 1. Secuencias optimizadas de los genes VP1, VP2, VP3 y VP4

VP1

GGATCCCGGGATGGGACTTGGTCAAATGTTGGAATCAATGATCGATAACACTGTTAGAGAGACAGTTGGAGCT
GCTACTTCTAGGGATGCTCTTCCAAATACAGAAGCTTCTGGTCCTGCTCATTCAAAAGAAATTCCAGCTCTTA
CTGCTGTTGAGACTGGAGCTACAAATCCATTGGTTCCTTCAGATACTGTTCAAACAAGACATGTTGTTCAACA
TAGATCTAGATCAGAATCTTCAATTGAGTCTTTCTTTGCTAGGGGTGCTTGTGTTGCTATTATTACTGTTGAT
AACTCTGCTTCAACAAAGAATAAGGATAAGCTTTTTACTGTTTGGAAGATCACTTATAAGGATACAGTTCAAC
TTAGAAGGAAATTGGAATTTTTCACATACTCAAGATTCGATATGGAGTTTACTTTCGTTGTTACAGCTAACTT
CACTGAAACAAATAACGGACATGCTTTGAACCAAGTTTACCAAATTATGTATGTTCCACCAGGTGCTCCAGTT
CCTGAGAAGTGGGATGATTATACTTGGCAAACATCTTCAAACCCTTCAATTTTCTATACTTACGGAACAGCTC
CAGCTAGAATTTCTGTTCCTTACGTTGGAATCTCTAACGCTTACTCACATTTCTACGATGGTTTTTCTAAGGT
TCCTTTGAAGGATCAATCTGCTGCTCTTGGAGATTCATTGTATGGTGCTGCTTCTCTTAATGATTTTGGTATT
TTGGCTGTTAGGGTTGTTAACGATCATAACCCAACTAAGGTTACATCAAAGATTAGAGTTTATCTTAAGCCTA
AGCATATCAGGGTTTGGTGCCCAAGACCACCTAGGGCTGTTGCTTATTACGGACCTGGTGTTGATTATAAGGA
TGGAACTCTTACACCATTGTCTACTAAGGATTTGACTACATACTAAGAGCTCGGCGCGCCCTGCAG

VP2

GGATCCCGGGATGTCTCCAAATATTGAAGCTTGTGGATATTCAGATAGAGTTCTTCAATTGACACTTGGTAAC
TCTACTATCACTACACAAGAGGCTGCTAATTCAGTTGTTGCTTATGGAAGATGGCCAGAATACTTGAGGGATT
CTGAGGCTAATCCTGTTGATCAACCAACAGAACCTGATGTTGCTGCTTGCAGATTTTACACACTTGATACTGT
TTCTTGGACTAAGGAGTCAAGAGGTTGGTGGTGGAAATTGCCAGATGCTCTTAGGGATATGGGATTGTTCGGT
CAAAACATGTACTACCATTATCTTGGAAGGTCTGGTTACACAGTTCATGTTCAATGTAATGCTTCAAAGTTTC
ATCAAGGAGCTTTGGGTGTTTTTGCTGTTCCTGAAATGTGCCTTGCTGGAGATTCTAACACTACAACTATGCA
TACTTCATACCAAAACGCTAATCCAGGAGAGAAGGGAGGAACTTTTACTGGAACTTTTACTCCTGATGATAAT
CAAACATCACCAGCTAGAAGGTTCTGTCCTGTTGATTATTTGTTCGGAAATGGTACTCTTTTGGGTAATGCTT
TCGTTTTCCCACATCAAATTATTAATTTGAGGACAAATAACTGCGCTACTTTGGTTCTTCCTTACGTTAACTC
TCTTTCAATCGATTCTATGGTTAAGCATAATAACTGGGGAATTGCTATTTTGCCACTTGCTCCTCTTAATTTT
GCTTCAGAATCTTCACCAGAGATTCCTATTACATTGACTATTGCTCCAATGTGTTGCGAGTTTAATGGTCTTA
GAAACATCACTTTGCCTAGGCTTCAATAAGAGCTCGGCGCGCCCTGCAG

VP3

GGATCCCGGGATGGGATTGCCAGTTATGAATACTCCTGGTTCAAACCAATATTTGACAGCTGATAACTTTCAA
TCTCCATGTGCTCTTCCTGAATTTGATGTTACTCCACCTATCGATATTCCAGGAGAGGTTAAAAACATGATGG
AATTGGCTGAGATCGATACTATGATCCCTTTCGATCTTTCAGCTAAGAAAAAGAATACTATGGAAATGTACAG
AGTTAGGTTGTCTGATAAGCCACATACTGATGATCCTATTCTTTGTTTGTCTCTTTCACCAGCTTCTGATCCT
AGACTTTCACATACAATGTTGGGAGAGATCCTTAACTACTACACTCATTGGGCTGGTTCTTTGAAGTTTACAT
TTTTGTTTTGTGGATCAATGATGGCTACAGGAAAGCTTTTGGTTTCTTATGCTCCACCTGGAGCTGATCCACC
TAAAAAGAGAAAAGAAGCTATGTTGGGAACTCATGTTATTTGGGATATTGGTCTTCAATCTTCATGTACAATG

GTTGTTCCATGGATCTCAAACACTACATACAGGCAAACTATCGATGATTCTTTTACAGAAGGAGGTTACATCT
CAGTTTTCTATCAAACTAGAATCGTTGTTCCATTGTCTACACCTAGGGAGATGGATATTCTTGGTTTTGTTTC
TGCTTGCAATGATTTTTCAGTTAGATTGATGAGGGATACTACACATATTGAGCAAAAGGCTCTTGCTCAATAA
GAGCTCGGCGCGCCCTGCAG

VP4

GGATCCCGGGATGGGTGCTCAAGTTTCTTCTCAAAAGGTTGGTGCTCATGAAAATTCTAATAGAGCTTATGGA
GGTTCAACTATTAATTACACTACAATTAATTACTACAGGGATTCTGCTTCAAATGCTGCTTCTAAGCAAGATT
TCTCTCAAGATCCATCTAAGTTTACTGAGCCTATTAAGGATGTTCTTATTAAAACATCACCAATGTTGAATTA
AGAGCTCGGCGCGCCCTGCAG

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Expression of immunogenic poliovirus Sabin type 1 VP proteins in transgenic tobacco

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ABSTRACT

One of the milestones of vaccinology is the depletion of the global impact of Poliomyelitis. The current vaccines to deal with Polio comprise the Sabin and Salk formulations. The main limitation of the former is the use of attenuated viruses that can revert into pathogenic forms, whereas the latter is more expensive and induces no protection in the intestinal tract; the site of virus replication. Genetically engineered plants cope with such limitations. In addition, they offer a low-cost alternative for production, storage and delivery of vaccines. This technology has been narrowly applied in the development of Polio vaccines.

Herein, we explored the ability of tobacco cells to express the immunogenic VP1, VP2, VP3, and VP4 Polio antigens, which are relevant for vaccine development. Evidence on the expression of the plant-made Polio VPs is presented and an immunogenicity assessment proved their capacity to induce local and systemic humoral responses when administered by subcutaneous and oral routes. The plant-made VPs will be useful in the development of low-cost vaccine formulations able to induce effective mucosal immunity without the risks associated to the use of attenuated viruses; therefore there is a potential for this technology to contribute toward Polio eradication.

1. Introduction

Polioviruses possess an RNA positive sense genome (of ~7500 pb in length) and show an icosahedral morphology composed of 60 copies of each of the four proteins that form their capsid (VP1, VP2, VP3, and VP4). There are three serotypes of poliovirus with the wild serotype 1 currently in circulation generating poliomyelitis cases in endemic countries; therefore it is the most important Poliovirus from an epidemiological scope (WHO, 2019).

Poliomyelitis (Polio) is a highly infectious viral disease, caused by a poliovirus upon infection of the central nervous system; leading to irreversible paralysis in one out of 200 infected people. To date, Polio is endemic in three countries: Afghanistan, Pakistan, and Nigeria. Since the creation of the Global Polio Eradication Initiative (GPEI) the incidence of the disease has been reduced by 99 %. To achieve this goal, two vaccines have been administered around the world for over 50 years: 1) the Inactivated Polio Vaccine (IPV) and the Oral Polio Vaccine (OPV). Manufacturing these vaccines involves handling the infective form of the pathogen; requiring high biosafety facilities. Limitations of OPV are the vaccine-associated paralytic polio cases (VAPP) and the generation of new pathogenic strains leading to outbreaks of vaccine origin. These limitations have hampered the advances towards Polio eradication, a goal that demands the elimination not only of the wild circulating viruses but also the ones derived from OPV (GPEI, 2019).

The recombinant expression of the virus capsid proteins with the formation of virus-like particles (VLPs) is a novel strategy to be used in vaccine manufacturing. In this sense, the approach of using plant cells to express recombinant proteins is a great alternative to generate a new vaccine without the use of inactivated or attenuated viruses, produced at low cost and avoiding the risk of biological contaminants (Rosales-

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Mendoza and Nieto-Gómez, 2018).

In the last two decades, the use of plant-based vaccines has become of great importance due to their numerous advantages over conventional vaccines, which include simple production, cost-effectiveness, lower or null toxicity, high stability of freeze-dried formulations, and simple delivery (oral vaccination) (Kumar et al., 2018). Some biopharmaceutical companies are currently focused on plant-based platforms for the production of biopharmaceuticals. For instance, the production of insulin in transgenic safflower was implemented by SemBioSys in Canada and the Flt3 growth factor was produced in transgenic barley by ORF Genetics in Iceland. Outstandingly, Protalix BioTherapeutics produced taliglucerase alfa in carrot cell lines; this became the first plant-made biopharmaceutical commercialized for human use. In the case of vaccines, Medicago in Canada have developed influenza vaccines that are currently under phase II clinical trials. Another key example is a monoclonal antibodies cocktail used to treat patients infected with the Ebola virus, developed by Mapp Biopharmaceutical in USA (Faye and Gomord, 2010; McCarthy, 2014; Laere et al., 2016).

Recently, in the plant-made Polio vaccine realm some important works have been reported. Fujiyama et al. (2006) fused 15 amino acids from VP1 and VP3 of the Sabin 1 strain to the coat protein (CP) of the *Tobacco Mosaic Virus* (TMV) creating a viral vector used to inoculate *Nicotiana tabacum* plants; mice immunized intraperitoneally (i.p.) generated antibodies against the fused fragment. The transplastomic approach expressing VP1 in *N. tabacum* was applied by Chan et al. (2016), the codon optimized (CO) protein was fused to the transmucosal carrier CTB. Immunogenicity of the heterologous protein was assessed in mice previously immunized with a single subcutaneous (s.c.) dose of IPV and subsequent oral boosts with freeze-dried plant material. The results showed an increment of the specific IgG and IgA titers in mice orally boosted when compared to mice with no boosts. Moreover, seropositivity (70–90 %) and neutralizing antibodies against all three poliovirus Sabin strains were induced, whereas a single dose of IPV resulted in poor neutralization. In a subsequent study, the same fusion protein was produced in transplastomic lettuce lines; astonishingly, VP1-VLPs of 22.3 nm in size were observed (Daniell et al., 2019). On the other hand, VLPs that retained the native D antigenicity were produced by Marsian et al. (2017); the particles were generated transiently in *Nicotiana benthamiana* plants expressing the P1 polyprotein from the mutant (SktSC8) of the Sabin type 3 strain, transgenic mice TgPVR immunized i.p. with partially purified VLPs accomplished protection upon a challenge with infectious poliovirus (Saukett strain).

According to the above panorama, in the present study the ability of tobacco cells to produce the immunogenic VP1, VP2, VP3, and VP4 Polio antigens was explored in order to investigate the potential of this platform for the development of innovative vaccines; moreover, the immunogenic potential upon subcutaneous and oral administration was determined. The use of plant-made Polio antigens administered by those routes could lead to effective approaches to achieve immunity at the gastrointestinal tract, which is critical to fight against poliomyelitis.

2. Materials and methods

2.1. Gene desing and cloning

Synthetic genes coding for the full length VP1, VP2, VP3 and VP4 proteins from the Sabin strain serotype 1 (GenBank accession no. CAA24465.1) were designed following codon optimization. In these genes the 5´ *Sma*I and 3´*Sac*I restriction sites were added to facilitate cloning into the expression vector. The optimized sequences are shown in Fig. S1. The genes were synthesized by GenScript® (Piscataway, NJ, USA) and subcloned into the pBI121 vector following standard procedures to yield the pBI-VP1, pBI-VP2, pBI-VP3, and pBI-VP4 vectors (Jefferson, 1987). *Escherichia coli* carrying positive clones were detected by plasmid restriction profile and conventional sequencing, a positive clone for each construct was electroporated into the *Agrobacterium tumefaciens* GV3101 strain according to the method described by Cangelosi et al. (1991). Recombinant *A. tumefaciens* clones were identified by antibiotic resistance and PCR to confirm the presence of the recombinant plasmid (Sambrook and Russell, 2001).

2.2. Plant transformation

Tobacco genetic transformation comprised the in vitro germination of seeds of *Nicotiana tabacum* cv. Petite Havana SR1 in MS medium (Murashige and Skoog, 1962) to generate plants that served as the source of leaf tissues used as explants, which were inoculated with an overnight-grown *A. tumefaciens* culture carrying the pBI-VP1, pBI-VP2, pBI-VP3, or pBI-VP4 vectors. Infected explants were subsequently co-cultivated for a period of 48 h in darkness using the RMOP medium [MS salts and vitamins, 0.1 mg/L 6-benzyladenine (BA), and 0.1 mg/L naphthaleneacetic acid (NAA)]. Explants were afterwards transferred to selective medium [RMOP medium plus 100 mg/L kanamycin (Kan) and 500 mg/L cefotaxime (CEFO)] and maintained in a controlled environment chamber (25 °C) under a 16 h-light/8 h-dark photoperiod. Explants were sub-cultured at 2-week intervals in a fresh selective medium. The rescued kanamycin-resistant shoots were rooted in hormone-free medium; the plantlets successfully rooted were finally transferred to soil, acclimatized, and cultivated under greenhouse conditions. For the immunogenicity assays the leaf tissues were collected and freeze-dried in a LABCONCO equipment (conditions: −75 °C and 0.133 mbar for 48 h), the lyophilized biomass was milled and stored at room temperature for further characterization.

2.3. PCR detection of heterologous DNA

The presence of the transgenes in the selected lines was tested by PCR. Total DNA was isolated from fresh leaves of transformed and wild-type plants according to Dellaporta et al. (1983). PCR reactions (total volume of 25 µL) were prepared with 1x PCR buffer, $MgCl_2$ (1.5 mM), dNTPs (1 mM), TaqPol (2.5 U), DNA (50 − 100 ng), and 1 µM of specific primers to amplify the VP transgenes (Table 1). The cycling conditions comprised: initial denaturation at 94 °C/2 min, 35 cycles at 95 °C for 30 s (denaturation), 55 °C/60 s (annealing), 72 °C/1 min (extension), and 72 °C/5 min (final extension). The PCR products were analyzed by electrophoresis in 1.0 % agarose gels. The pBI-VP1, pBI-VP2, pBI-VP3, and pBI-VP4 constructs were used as positive controls (10 ng).

2.4. Hyper-immune Sera production

Anti-sera against the VPs proteins were obtained with theoretically predicted antigenic peptides from VP2, VP3, and VP4 proteins using the BCPRED tool website (http://ailab.ist.psu.edu/bcpred/predict.html) and the antigenic peptide from VP1 previously reported by Wychowski et al. (1983). Ten-week-old BALB/c female mice ($n = 3$) were subcutaneously immunized at day 1 with 10 µg of $VP1_{93\text{-}104}$ (DNSASTKNKDKL), $VP2_{133\text{-}154}$ (AGDSNTTTMHTSYQNANPGE), $VP3_{131\text{-}151}$ (LVSYAPPGADPPKKRKEAML), or $VP4_{24\text{-}44}$ (TINYTTINYYRDSASNAASK) synthetic peptides (GenScript®) emulsified in 20 µL of complete Freud´s adjuvant (CFA). The subsequent doses were administered by the same route on days 8, 16, 24, and 31 using 50 µg of the respective peptide emulsified in one volume of incomplete Freud´s adjuvant (IFA). Mice were bled at day 43 to measure antibody titers by ELISA. The animals were subsequently sacrificed to collect sera. All experiments followed the guidelines from the National Institutes of Health Guide for Care and Use of Laboratory Animals, the experimental protocols were reviewed and approved by the Ethical Committee for the Care and Use of Laboratory Animals (Protocol Number 233) at the Biomedical Research Institute from the Universidad Nacional Autónoma de México (UNAM).

2.5. Protein analysis

The presence and integrity of the VPs proteins were assessed by dot-blot and western-blot analyses. Soluble protein extracts were obtained by resuspending 100 mg of fresh leaves tissue from WT (wild type) or transgenic plants in 300 µL of extraction buffer (25 mM sodium phosphate at pH 6.6, 100 mM NaCl, 0.5 % Triton X-100 v/v, 100 mM β-mercaptoethanol, and 1 mM PMSF). Suspensions were sonicated (5 pulses of 5 s at 25 % amplitude) and extracts were clarified by centrifugation at 9660*g* for 10 min at 4 °C.

For dot blot analysis, 2 µL of total soluble protein extracts were applied onto a nitrocellulose membrane; after drying, the membrane was subsequently blocked using 5 % fat-free milk for 2 h. For the VP1 protein, the Rabbit Anti-Poliomyelitis Virus 1 (LSc, 2ab strain) antiserum (Alpha Diagnostic, TX, USA) was used as primary antibody and pure VP1 (Sabin; POLV1-VP1, Alpha Diagnostic, TX, USA) was used as positive control. In the absence of the pure VP2, the VP3 and VP4 proteins; the synthetic peptides $VP2_{133-154}$, $VP3_{131-151,}$ $VP4_{24-44}$ were used as positive controls; the respective specific mouse anti-sera allowed labeling for the corresponding antigen by an overnight incubation at 4 °C. Afterwards, a secondary anti-IgG antibody (1:2000) was added for a period of 2 h at 25 °C. Finally, the reaction was developed by adding the Pierce® ECL Western Blotting Substrate solutions (Thermo Scientific, IL, USA) according to instructions from the manufacturer; followed by exposure of an X-ray film that was finally treated with standard developer and fixer solutions.

The integrity of the plant-made VPs proteins was assessed by Western blot analysis. Protein extracts were denatured by boiling the samples in the presence of 1× reducing buffer for 5 min at 95 °C. Proteins were resolved by electrophoresis in 4–12 % or 20 % polyacrylamide gels under denaturing conditions that were blotted onto nitrocellulose membranes (Bio-Rad, Germany). Blots were blocked for 2 h at 25 °C with 5 % fat-free milk dissolved in phosphate saline buffer (PBS 1×) and subsequently incubated overnight with Rabbit Anti-Poliomyelitis Virus 1 (LSc, 2ab strain) antiserum (Alpha Diagnostic, TX, USA) (1:2000 dilution) or hyperimmune anti-$VP4_{24-44}$ serum (1:500 dilution). The antigen used to generate the commercial Anti-Poliomyelitis Virus 1 anti-serum was the purified Poliomyelitis Virus 1 (LSc, 2ab strain); therefore the proteins mainly detected were VP1, VP2 and VP3; which are exposed outside of the capsid surface. Since VP4 is located inside of the poliovirus structure, its null detection could be considered as a false negative result; consequently, the hyperimmune anti-$VP4_{24-44}$ serum was used to detect VP4. Recombinant VP1 expressed in *E. coli* (Sabin; POLV1-VP1) was used as positive control. Horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) was added and incubated for 2 h at 25 °C. Antibody binding was detected by incubation with Pierce® ECL Western Blotting Substrate solutions (Thermo Scientific, IL, USA) following the instructions from the manufacturer. Molecular weight marker "Precision Plus Protein™ Dual Color Standars" (BioRad, CAL, USA) was used to determine the size of the immunoreactive proteins.

2.6. Quantitative ELISA

Total soluble protein extracts were obtained as described above, but omitting mercaptoethanol in the extraction buffer. ELISA plates were coated overnight at 4 °C with 1:32 dilutions of protein extracts using carbonate buffer (pH 9.6). Blocking with 5 % non-fat milk for 2 h at room temperature was performed and plates were afterwards washed thrice with PBS + 0.05 % Tween (PBS-T). Hyperimmune sera against specific antigens (VP1, VP2, VP3, or VP4) were subsequently added (1:500 dilution) and plates incubated overnight at 4 °C. After three washes with PBS-T, a rabbit horseradish peroxidase-conjugated anti-mouse IgG (Sigma, St. Louis, MO) secondary antibody diluted 1:2000 in PBS was added and plates were incubated at room temperature for 2 h. Finally, plates were washed with PBS-T and antibody binding was revealed with an ABTS substrate [2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), 0.1 M citric acid, pH 4.35 plus 1 mM H_2O_2 (Sigma, St. Louis, MO)]. After 30 min of incubation, the optical density (OD) values at 405 nm were measured in a Multiskan FC Microplate Photometer (Thermo, IL, USA). A standard curve was set using the synthetic peptides $VP1_{93-104}$, $VP2_{133-154}$, $VP3_{131-151}$, or $VP4_{24-44}$ (Genescript ®) to estimate recombinant protein levels.

2.7. Immunogenicity assessment

The immunogenicity of the VPs proteins expressed in tobacco plants was assessed in 7 − 8-week old BALB/c male mice (25 g of body weight). Six experimental groups ($n = 5$) were randomly established and named as groups 1 − 6. The immunization schedule comprised four doses administered once a week subcutaneously (s.c.); followed by four oral boosters administered at two-week intervals. Groups 1–4 were immunized with clarified extracts from lyophilized transgenic leaf tissues (s.c. immunization) or lyophilized transgenic leaf tissues resuspended in PBS (for oral immunization), while mice from group 5 received respective preparations from a wild type tobacco plant (untransformed) and group 6 was treated with PBS.

Subcutaneous treatments consisted of the administration of total soluble protein extracts (200 µL) obtained from 10 mg of lyophilized plant powder from the corresponding VP-expressing plant line; the extracts were prepared by adding PBS to the plant powder, sonicating for 10 s and then centrifuging at 16,000*g* for 15 min with the subsequent recovery of the supernatant. Oral boosting consisted of 10 mg of lyophilized plant powder resuspended in 300 µL of PBS, which were administered intragastrically.

The s.c. and oral doses administered were identical, containing approximately 0.8 µg of VP1, 1.40 µg of VP2, 0.43 µg of VP3, and 0.60 µg of VP4 as determined by ELISA. All immunizations were given without exogenous adjuvants. Test mice were bled on days 1, 28, and 105 by punction in the submandibular venous sinus. Blood samples were processed to obtain sera, which were stored at − 40 °C until further use (Fig. 7). The experimental protocols were reviewed and approved by the Research and Teaching Committee of the Chemical Faculty at the University of San Luis Potosi, México (Register Number: CEID201901R1).

2.8. Antibody levels in sera and feces

IgG and S-IgA levels were measured in sera and fecal extracts of immunized mice by ELISA as follows. Ninety-six-well plates were coated overnight at 4 °C with 1 µg/well of one of the following peptides: $VP1_{93-104}$, $VP2_{133-154}$, $VP3_{131-151}$, or $VP4_{24-44}$ diluted in bicarbonate buffer (pH 9.6) and subsequently blocked with 5 % fat-free dry milk for 2 h at 25 °C. Plates were washed 3 times with PBS-T between each incubation step. For IgG determination, plates were incubated overnight at 4 °C with serial dilutions of sera samples prepared in PBS 1× (1:20 to 1:40 dilutions). For S-IgA measurements, samples were resuspended in PBS 1× (100 mg/0.5 mL) with 5 % non-fat milk supplemented with 1 mM PMSF, after clarifying at 6700*g* for 15 min at 4 °C; 50 µL samples were loaded into the wells. Afterwards, plates were incubated with a secondary anti-IgG or anti-IgA HRP antibody for 2 h at 25 °C. Finally, immunodetection was revealed by adding an ABTS substrate solution [0.6 mM 2, 20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), 0.1 M citric acid, 1 mM of H_2O_2, pH 4.35 (Sigma, St. Louis, MO)] and incubating the plates at 25 °C for 30 min. OD values at 405 nm were recorded in a Multiskan FC microplate photometer (Thermo Scientific, Waltham, MA).

2.9. Statistical analysis

The ELISA data were analyzed by one-way ANOVA using the GraphPad Instat 3.1 software. The cut-off value was determined with

the means + 2 S.D. (standard deviation) of the group of mice immunized with WT biomass. Differences were considered significant at $P < 0.05$.

3. Results

3.1. The VP1, VP2, VP3 and VP4 genes were optimized for the efficient expression in plants

The expression of a foreign gene in a phylogenetically distant host requires coping with low translation rate due to the differential codon usage of such organisms. This is the case of the expression of viral *VP* genes and the plant cell as host. Hence, the full length *VP* genes were subjected to optimization through the OptimumGene™ algorithm, which adjusts codon usage bias in *N. tabacum* plants. Moreover, this tool optimizes other critical factors related to expression efficiency, including the GC content in a range between 30–70 % to prolong the half-life of the RNAs; while stem-loops structures, which impact ribosomal binding and stability of mRNA, and negative cis-acting sequences are avoided. The gene synthesis also allowed the straightforward inclusion of the *Sma*I and *Sac*I restriction sites at the 5´ and 3´ ends, respectively; to facilitate the construction of expression vectors. The *VP*s genes were synthesized by GenScript and supplied in the pUC57 cloning vector.

3.2. Synthetic VPs genes are present in the transgenic lines of tobacco

Expression vectors based on the pBI121 backbone were constructed to mediate the production of the VP proteins in plants (Fig. 1). Foreign genes were introduced in tobacco cells via *Agrobacterium*-mediated transformation. Infected explants were maintained in antibiotic-supplemented medium; changing to fresh medium every 15 days. Kanamycin-resistant tobacco lines were successfully generated and transferred to a greenhouse to obtain the sufficient biomass to conduct molecular and immunogenicity analyses (Fig. 2). The presence of the transgenes was assessed by PCR.

Figs. 3**a**, 4 **a**, 5 **a**, and 6 **a** show the presence of the expected amplicons for the VP1 (P2), VP2 (P1, P2, P3, P4, P5, and P6), VP3 (P1, P2, P3, and P4), and VP4 genes (P1, P2, P3, and P4) (441, 352, 409, and 305 bp in size; respectively); matching with those obtained with the plasmids used as positive controls. The following numbers of positive lines were confirmed by the analysis: one for VP1, six for VP2, seven for VP3, and four for VP4. No positive signals were observed for either the WT DNA sample or the reaction mixture lacking DNA.

3.3. Tobacco plant succesfully express the capsid proteins

The presence and integrity of the four VP proteins expressed in plant cells were performed through dot blot and western blot analyses. Total soluble protein extracts were obtained from all candidate lines, the dot blot analysis was performed under native condition and labeling with Anti-Poliomyelitis Virus 1 antiserum or anti-VP2, anti-VP3, or anti-VP4 hyperimmune sera revealed a positive signal in all transgenic lines, whereas no signal was observed in the extracts obtained from the untransformed tobacco plant (WT) Figs. 3**b**, 4 **b**, 5 **b**, and 6 **b**).

As shown in Figs. 3**c**, 4 **c**, 5 **c**, and 6 **c**; the western blot analysis labeled with the Anti-Poliomyelitis Virus 1 antiserum or anti-VP4 hyperimmune sera revealed positive reactivity with bands of 34, 30, 27, and 7.5 kDa for VP1, VP2, VP3, and VP4; respectively, which confirms the expression of the antigenic polio VP proteins in plant cells.

The expression levels of the recombinant plant-made VPs proteins were quantified by ELISA using standard curves constructed with synthetic peptides. The determined accumulation levels were: 0.3 µg VP1/g of fresh biomass, 6.16–16.85 µg VP2/g, 2.74–4.33 µg VP3/g of fresh biomass, and 0.96 – 6 µg VP4/g of fresh leaves tissue (Figs. 3**d**, 4 **d**, 5 **d**, and 6 **d**).

3.4. The Plant-Made VPs proteins are immunogenic in mice

Based on the highest expression levels of the VP3 and VP4 plant-made proteins, the lines P1 were selected to assess their immunogenic potential in BALB/c mice. For VP1 the only characterized transgenic line (P2) was used and in the case of the VP2 tobacco-made protein; the transgenic line P1 was preferred upon P2 due to insufficient plant material. Groups of 5 mice were primed subcutaneously with total soluble protein extracts and orally boosted with suspensions of lyophilized plant powder containing the VP1, VP2, VP3, or VP4 tobacco-made proteins; following the immunization schedule presented in Fig. 7. ELISA revealed the induction of anti-VP3 and anti-VP4 IgG antibodies in serum from mice immunized four times s.c. with the corresponding plant-made antigens (day 28); for both groups the levels of specific IgG antibodies increased after the four oral boosts with suspensions of lyophilized plant powder, nonetheless the differences between day 28 and day 105 were non-significant. For mice immunized with plant-derived VP1, important levels of IgG antibodies were not detected in serum after all s.c. injections, interestingly; specific IgG response was elicited following the first oral immunization (data not shown) and the antibody production was enhanced significantly with the subsequent three orally delivered doses (day 105). The presence of high levels of IgG antibodies in sera from mice treated s.c. with plant-made VP2 protein was not detected, however the amount of antibodies slightly increased with all doses of protein orally administered and high levels of IgG were elicited in only one mouse at the end of the all s.c. and oral doses (Fig. 8**a**, **c**, **e**, and **g**).

Antibody detection in feces from mice after being subjected to four oral boosters revealed the presence of significant intestinal S-IgA levels in mice groups fed with tobacco-made VP1, VP2, VP3, or VP4 proteins compared to the same group after all subcutaneous immunizations. Nevertheless, for the case of S-IgA antibodies for VP4; only a single mouse increased the levels above the established cut-off (Fig. 8**b**, **d**, **f**, and h). The Fig. 9 showed the overall systemic and mucosal antibody responses against each plant-made VP protein at the end of the immunization schedule (day 105).

4. Discussion

In the present study, the nuclear-based expression in *N. tabacum* of the VP1, VP2, VP3, and VP4 capsid proteins from poliovirus Sabin type 1 and their immunogenic properties assessment are reported for the first time as an effort to begin the development of oral immunization approaches able to induce intestinal immunity against the virus; seeking to avoid the use of attenuated strains or costly subunit vaccine production systems.

Tobacco plants carrying the synthetic genes coding the target VPs

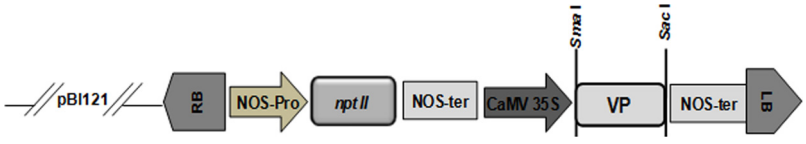

Fig. 1. Description of the pBI-VPs expression vector. The vector is derived from the pBI121 binary vector. *RB* right border, *LB* left border, *nptII* kanamycin resistance gene under control of the nopaline synthase promoter NOS-Pro, NOS-ter nopaline synthase terminator, VP: *VP1, VP2, VP3* or *VP4* genes are expressed under cauliflower mosaic virus 35S constitutive promoter (CaMV35S). *Sma*I and *Sac*I digestion sites were added flanking the 5´ and 3´ ends of the genes.

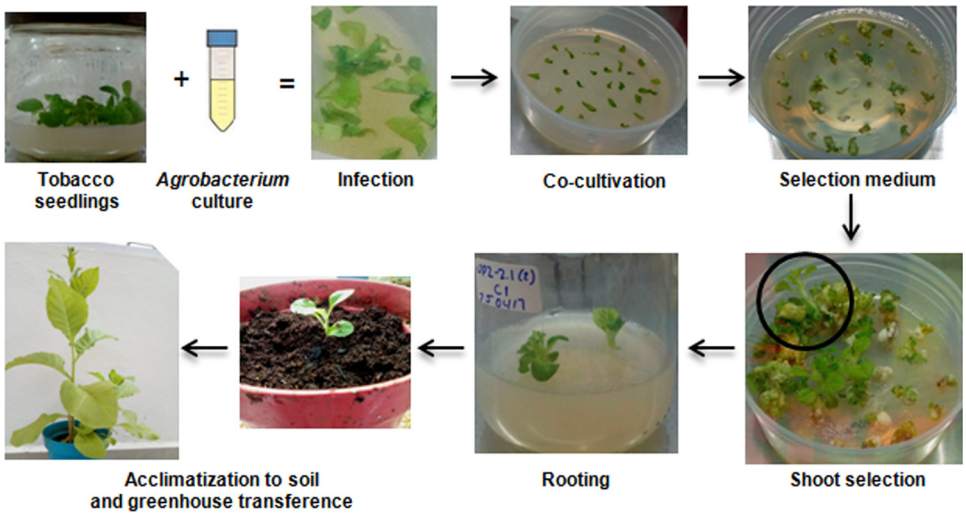

Fig. 2. Generation of the VPs plant-made proteins. Foreign genes were introduced in tobacco cells via the *A. tumefaciens* method, explants infected were maintained in antibiotic-supplemented medium and changed to fresh medium every 15 days; after rooting the lines were transferred to a greenhouse to increase biomass. Fresh material was collected to confirm stable integration of the genes and molecular characterization of the proteins. Harvested leaves were lyophilized, powdered, and stored at room temperature.

were successfully engineered as confirmed by PCR. Unfortunately, only one line was positive for VP1 according to the conducted PCR analysis, in which only two lines were analyzed due to the reduced number of lines rescued for this transgene. This finding suggests that VP1 expression exerts some toxicity in the plant since for the other genes the rescue of several lines was achieved. Dot blot analysis allowed detecting each VP protein and the expected molecular weight of VP1 (34 kDa), VP2 (30 kDa), VP3 (27 kDa), and VP4 (7.5 kDa) was confirmed by Western blot analysis. The expression levels obtained herein are similar to those attained for other viral proteins expressed in tobacco plants such as the VP40 protein from Ebola Virus that reached up to 2.6 µg/g fresh leaf tissue (Monreal-Escalante et al., 2017), the M/L-HBsAg whose expression ranged 2 – 10 µg/g (Pniewski et al., 2012), the immunogenic viral peptides CTB-MPR$_{649-684}$ –HIV-1 with yields of 18.1 ± 2.3 mg/kg of fresh leaf material (Matoba et al., 2009), and the multiepitopic peptides of the Ebola virus termed Zerola with expression ranging 1 – 2 µg/g fresh weight leaf tissues (Nieto-Gómez et al., 2019). The difference in protein yields among the modified tobacco lines may be associated to diverse factors such as differential transgene copy number and insertion sites along the plant genome; owing to the non-specific and no-homologous random recombination mediated by *A. tumefaciens* (Kim et al., 2007). No phenotypic changes were observed in the generated tobacco plants; including those with the highest expression levels, which guarantees the scale-up of the production of the target antigens for further applications. Although the plant expression systems with the highest protein yields are based on transient transformation using vectors with viral elements, it should be considered that those systems require the purification of the protein since agrobacteria residues are present in the plant tissue. In contrast, our approach based on nuclear stable transformation allow avoiding purification process since

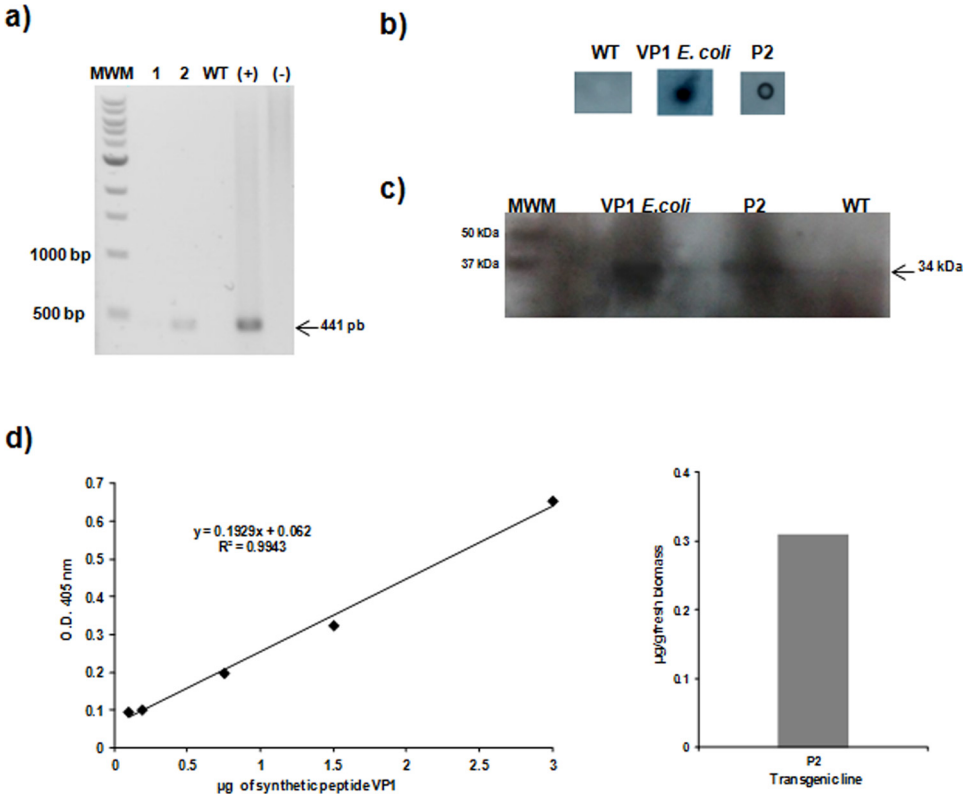

Fig. 3. Characterization of tobacco lines expressing codon-optimized VP1 protein. a) Transgene detection in PCR analysis. MWM: Molecular Weight Marker 1 kb, 1-2: candidate lines, WT: Wild type, (+): positive control pBI-VP1 expression vector, (-): negative reaction control (water). The VP1 amplicon, which is 441 bp in length, is indicated by the arrow. **b)** Immunodetection of VP1 protein. A positive dot blot signal was detected in the transgenic line P2 exposed to the Rabbit Anti-Poliomyelitis Virus 1 (1:2000 dilution) antibody. **c)** Western blot of recombinant VP1 expressed in *E. coli* (Sabin; POLV1-VP1) (350 ng/well), VP1 expressed in the P2 transgenic line and untransformed plant extracts probed with Rabbit Anti-Poliomyelitis Virus 1 (1:2000 dilution). The 34 kDa band corresponding to the VP1 protein is indicated by the arrow. **d)** Quantification of protein expression. The standard curve made with the VP1$_{93-104}$ synthetic peptide was used to estimate the accumulation levels of the recombinant protein.

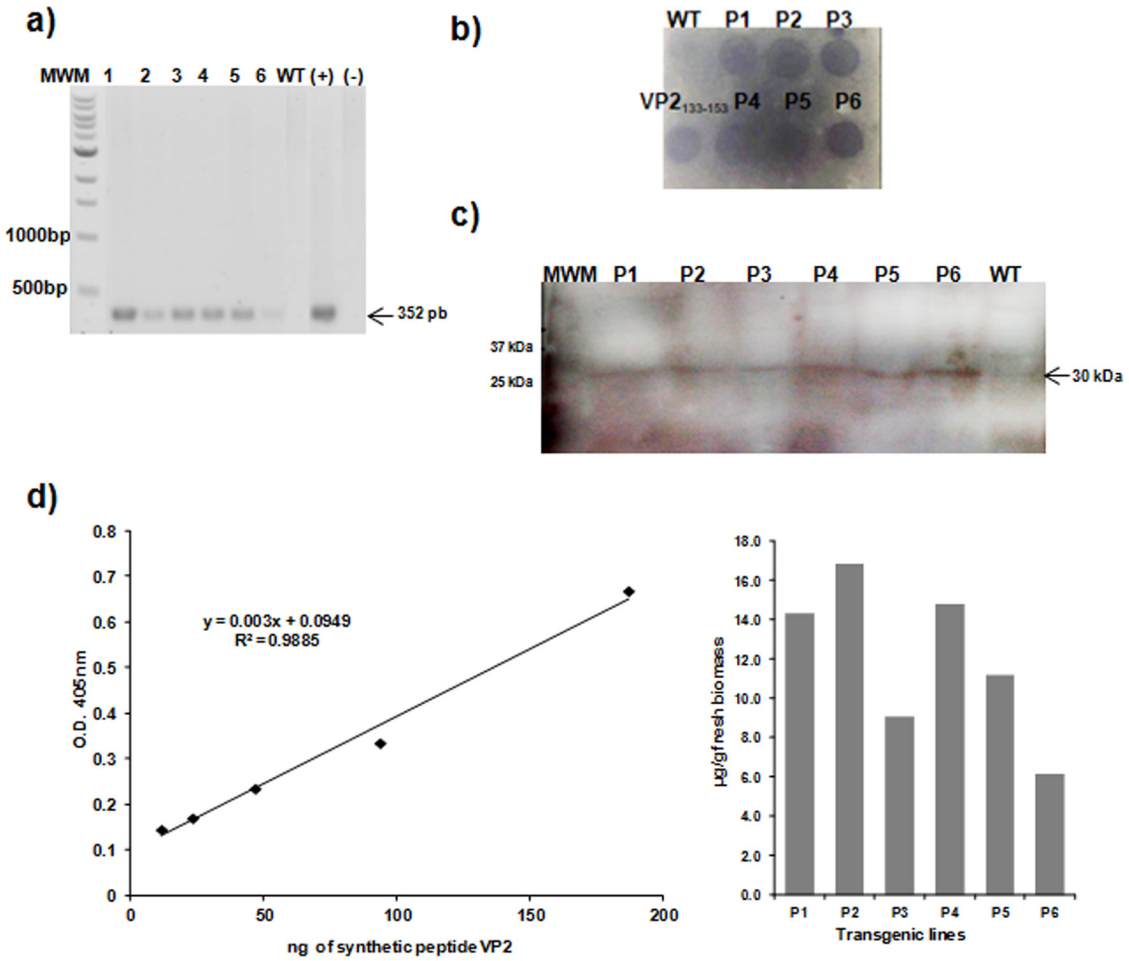

Fig. 4. Characterization of tobacco lines expressing codon-optimized VP2 protein. a) Transgene detection in PCR analysis. MWM: Molecular Weight Marker 1 kb, 1-6: candidate lines, WT: Wild type, (+): positive control pBI-VP2 expression vector, (-): negative reaction control (water). The VP2 amplicon, which is 352 bp in length, is indicated by the arrow. **b)** Immunodetection of VP2 protein. A positive dot blot signal was detected in transgenic lines exposed with the VP2 hyperimmune sera (1:300 dilution). **c)** Western blot of VP2 expressed in the P1, P2, P3, P4, P5, and P6 transgenic lines and untransformed plant extracts probed with the Rabbit Anti-Poliomyelitis Virus 1 antibody (1:2000 dilution). The 30 kDa band corresponding to the VP2 protein is indicated by the arrow. **d)** Quantification of protein expression. The standard curve made with the $VP2_{133-154}$ synthetic peptide was used to estimate the accumulation levels of the recombinant protein.

once providing a proof-of-concept in tobacco; we are working in the development of carrot stably-transformed lines that will be used for oral immunization with no need of purification processes. In this way oral boosting can be performed at very low cost with simple delivery. Other factors are the bioencapsulation and adjuvant effects that the plant cells exert upon oral administration, which optimize the effect of the administered dose (Rosales Mendoza and Salazar-González, 2014).

The antigenic and immunogenic properties of the monomeric VPs have already been investigated through in vitro and in vivo assays, which evidenced the induction of high levels of neutralizing IgG antibodies and S-IgA (van Wezel et al., 1983; van der Marel et al., 1983; Zhaori et al., 1988; Emini et al., 1983).

The application of molecular farming for the production of innovative Polio vaccines has been recently initiated. Thus far only the VP1 protein has been targeted, which was expressed as fusion protein along with the cholera non-toxin B subunit (CTB) in tobacco and lettuce chloroplasts. In tobacco, the expression levels reached up to 2600 µg/g dry weight when a codon-optimized VP1 (CTB-VP1co) sequence was used, whereas the native VP1 (CTB-VP1n) gene led to a yield up to 54 µg/g DW. In the case of lettuce, the expression of CTB-VP1co led to 9−15-fold higher yields than CTB-VP1n (Chan et al., 2016; Daniell et al., 2019). The authors claimed that these high yields are consequence of the high copy number of both the plastome and chloroplast per cell; as well as the absence of gene silencing events (Koop et al., 1996). However, the expression in chloroplast-based systems has some limitations that should be contemplated such as the lack of ability to achieve complex post-translational modifications and to secrete the protein (Rosales-Mendoza, 2016). In particular, VP4 is a protein that requires modification at the N-terminal segment that consists of the attachment of myristic acid (N-myristoylation N-MYR), which is involved in critical processes such as capsid assembly and viral entry (Chow et al., 1987). The presence of this fatty acid could stabilize the structural conformation and preserve the maintenance of the epitopes contained in the VP4 protein as in the preS domain of Hepatitis B virus (Alberti et al., 1990). In plants, the modification of nuclear-encoded proteins with N-MYR is a proven process that is related to regulation of stress and energy signaling (Traverso et al., 2008), therefore the N-MYR modification is expected for the VP4 protein expressed in nuclear-transformed plant cells.

Our findings revealed high levels of specific IgG systemic antibodies induced by the plant-made VP4 protein after the fourth s.c. immunization. Interestingly, this response was sustained by administering four oral boosters with suspensions of lyophilized plant powder from the same transgenic lines. The immunogenic properties of the plant-made VP4 suggest that it was properly assembled, modified, and functionally produced. Similarly, the plant-made VP1, VP2, and VP3 retained their antigen determinants as evidenced by dot blot and Western blot analyses using a specific anti-serum against the target

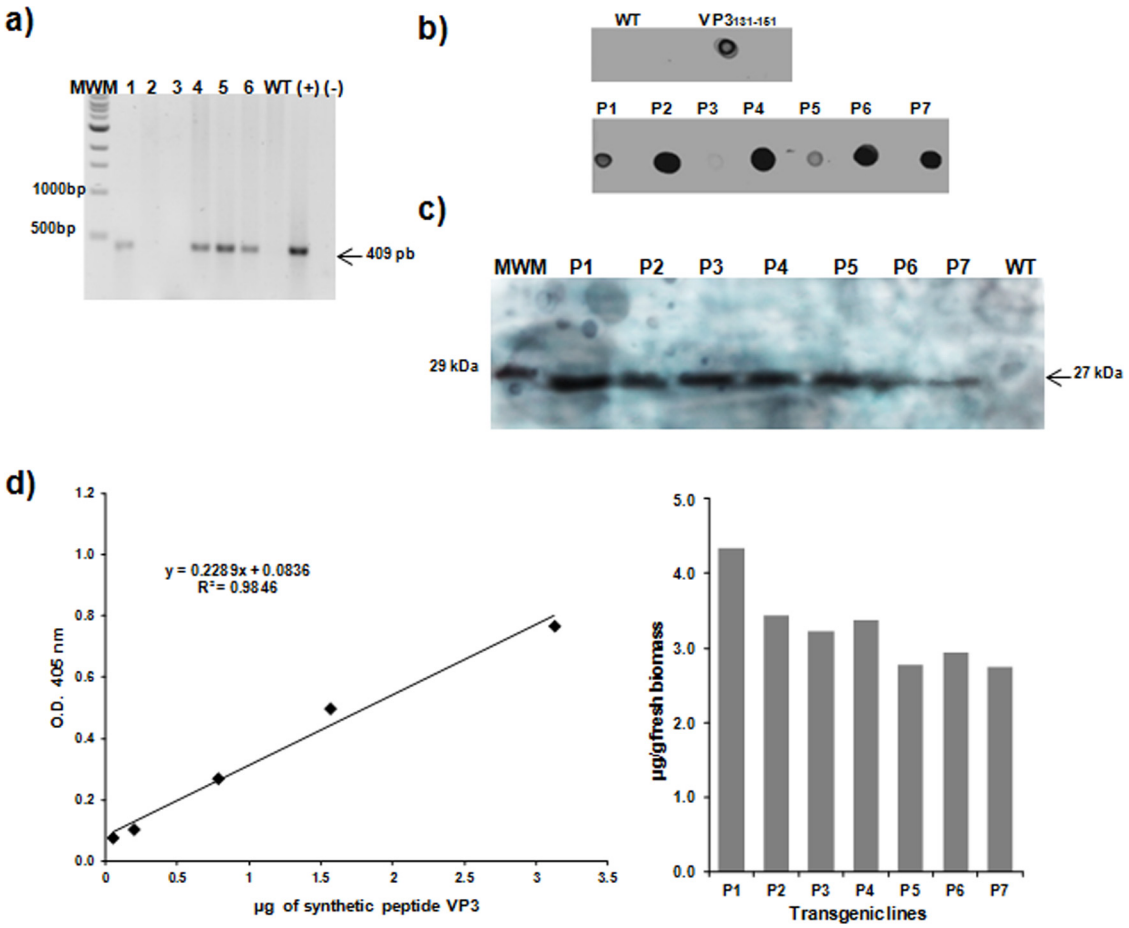

Fig. 5. Characterization of tobacco lines expressing codon-optimized VP3 protein. a) Transgene detection in PCR analysis. MWM: Molecular Weight Marker 1 kb, 1-6: candidate lines, WT: Wild type, (+): positive control pBI-VP3 expression vector, (-): negative reaction control (water). The VP3 amplicon, which is 409 bp in length, is indicated by the arrow. **b)** Immunodetection of VP3 protein. A positive dot blot signal was detected in transgenic lines exposed with the VP3 hyper-immune sera (1:350 dilution). **c)** Western blot of VP3 expressed in the P1, P2, P3, P4, P5, P6, and P7 transgenic lines and untransformed plant extracts probed with Rabbit Anti-Poliomyelitis Virus 1 antibody (1:2000 dilution). The 27 kDa band corresponding to the VP3 protein is indicated by the arrow. **d)** Quantification of protein expression. The standard curve made with the $VP3_{131-151}$ synthetic peptide was used to estimate the accumulation levels of the recombinant protein.

proteins. In terms of immunogenicity, the ELISA data revealed that the VP1 protein induced specific antibodies upon a single oral dose and the response gradually increased upon boosting. The presence of IgG VP3 antibodies was detected after the third s.c. immunization and the response increased with the subsequent oral boosts. The anti-VP2 IgG response was triggered only in a single mouse at the end of all immunizations. Interestingly, humoral and mucosal responses were successfully achieved without the presence of co-administered adjuvants in both subcutaneous and oral immunization schemes.

In line with our findings, Chen et al. (2006) reported the oral immunogenicity of the VP1 protein from Enterovirus 71 fused to an ER retention signal expressed in tomato fruit. Pniewski et al. (2011) orally immunized mice with a low dose (100 ng) of HBsAg-VLPs lettuce-made without adjuvants with an interval of up to 60 days between priming and boosting; resulting in the induction of both secretory IgA in feces and IgG in serum at protective levels. Comparable results were obtained in oral immunization experiments with low doses of lyophilized tobacco-derived HBsAg, while high doses led the activation of regulatory T cells associated to oral tolerance with a consequent suppression of the antigen-specific immune response (Kostrzak et al., 2009). It is likely that this phenomenon occurred in mice immunized with the VP2 protein in the present study since it was administered at the highest dose compared to the VP1, VP3, or VP4 tobacco-made proteins; only one mouse elicited significant humoral responses. Further research will be focused on evaluating different doses of the plant-made VPs; this would be especially interesting in the case of VP2 in which lower doses could lead to better humoral response.

Interestingly, Pniewski et al. (2018) reported the efficiency of the immune response with a combining i.m. priming and oral boosters series using plant lyophilized tissue containing the S-HBsAg antigen. Additionally, the authors showed that mucosal adjuvants (CTB, Quillaja bark saponins or alhydrogel) had no impact on the immune response indicating that a low-dose as an oral dose may not require exogenous adjuvants.

It should be noted that our tobacco-made VPs proteins delivered through parenteral immunizations followed by oral boosters and without external adjuvants were capable of eliciting significant humoral responses in mice. Although not all mice generated antibodies; the means of VP1, VP3, and VP4 exceeded the cutoff established with the serum from mice treated with wildtype lyophilized tobacco tissue; even with the first parenteral immunization (VP4) (data not shown). However, in previous reports focused on the VP1 protein from poliovirus Sabin 1 expressed in plant cells; accessory adjuvants such as saponin, squalene, and the immune-modulating antimicrobial peptides protegrin-1 and LL37 were required to induce significant responses; or priming with the IPV (Daniell et al., 2019; Xiao and Daniell, 2017; Chan et al., 2016). Highly immunogenic plant-made proteins have been reported by other groups. For instance, Marsian et al. (2017) used transgenic mice expressing the human PV receptor (TgPVR) to evaluate the response upon a one or two intraperitoneal injections of 0.5 human

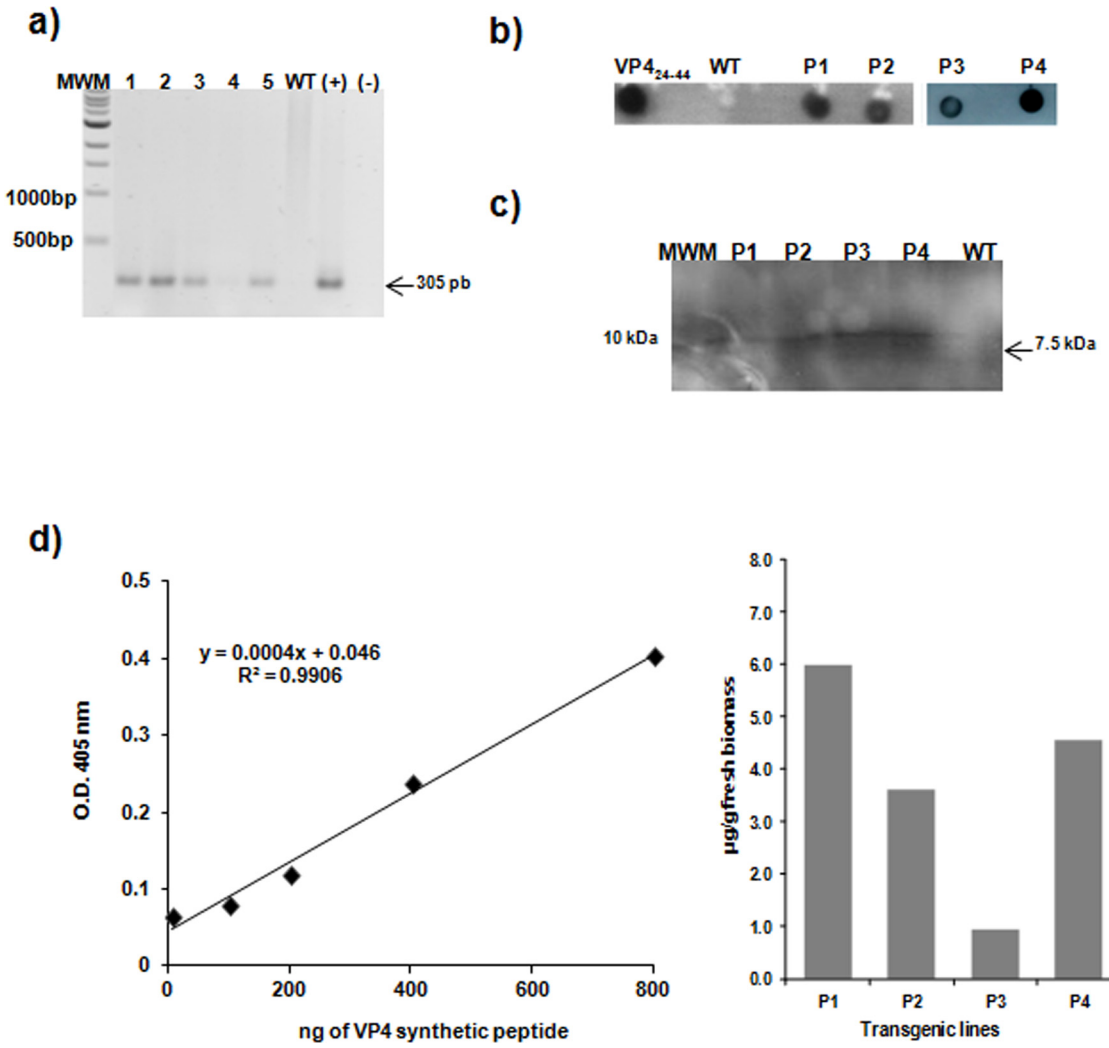

Fig. 6. Characterization of tobacco lines expressing codon-optimized VP4 protein. a) Transgene detection in PCR analysis. MWM: Molecular Weight Marker 1 kb, 1-5: candidate lines, WT: Wild type, (+): positive control pBI-VP4 expression vector, (-): negative reaction control (water). The VP4 amplicon, which is 305 bp in length, is indicated by the arrow. **b)** Immunodetection of VP4 protein. A positive dot blot signal was detected in transgenic lines exposed with the VP4 hyperimmune sera (1:500 dilution). **c)** Western blot of VP4 expressed in the P1, P2, P3, and P4 transgenic lines and untransformed plant extracts probed with the VP4 hyperimmune sera (1:500 dilution). The 7.5 kDa band corresponding to the VP4 protein is indicated by the arrow. **d)** Quantification of protein expression. The standard curve made with $VP4_{24-44}$ synthetic peptide was used to estimate the accumulation levels of the recombinant protein.

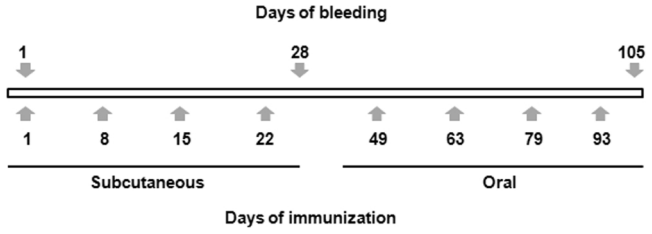

Fig. 7. Immunization schedule. Groups of five BALB/c mice were immunized with soluble protein extracts (s.c.) or suspensions (orally) prepared with lyophilized plant tissues of transgenic tobacco, containing a dose of approximately 0.8 µg of VP1, 1.40 µg of VP2, 0.43 µg of VP3, and 0.60 µg of VP4. The doses were defined considering as reference the study by Pniewski et al. (2018). Untransformed tobacco (WT) was used in control group.

doses of purified polio type 3 stabilized VLPs transiently expressed in *N. benthamiana* plants; observing that mice developed high neutralizing antibodies titers and protection against a viral challenge. Nevertheless, the mucosal immune response was not characterized, which is a highly relevant aspect given the nature of this pathogen; demanding intestinal protection to avoid the spread of poliovirus through feces.

Further studies employing natural plant-derived or synthetic adjuvants to immunize mice with the plant-made VPs proteins will be conducted to improve their immunogenicity. Another topic to address in future investigations is dose adjustment. For instance, the four plant-made antigen concentrations should be equalized to determine if the differences in immunogenicity potential are attributed to the amount of protein delivered or to the nature of antigen per se. The adjustment perhaps could improve the immunogenicity of all proteins, especially for the case of VP2 since only one mouse responded; moreover for the cases of VP3 and VP4, which showed no significant increase in the humoral response upon oral boostings.

On the other hand, although there was a significant difference in S-IgA anti-VP4 production at day 105; only one mouse overcomes the cut-off established value. This phenomenon could be attributed to the biologic characteristics of the molecule due to its low molecular weight (7.5 kDa) and high solubility (Hebditch et al., 2017), which could drive oral tolerance development; therefore, adjustment of the oral doses could provide a solution to this limited response.

Finally, a comparison of the humoral response observed at the end of the assessed immunization scheme clearly exhibits a differential immunogenicity for each protein. In case of the IgG systemic response,

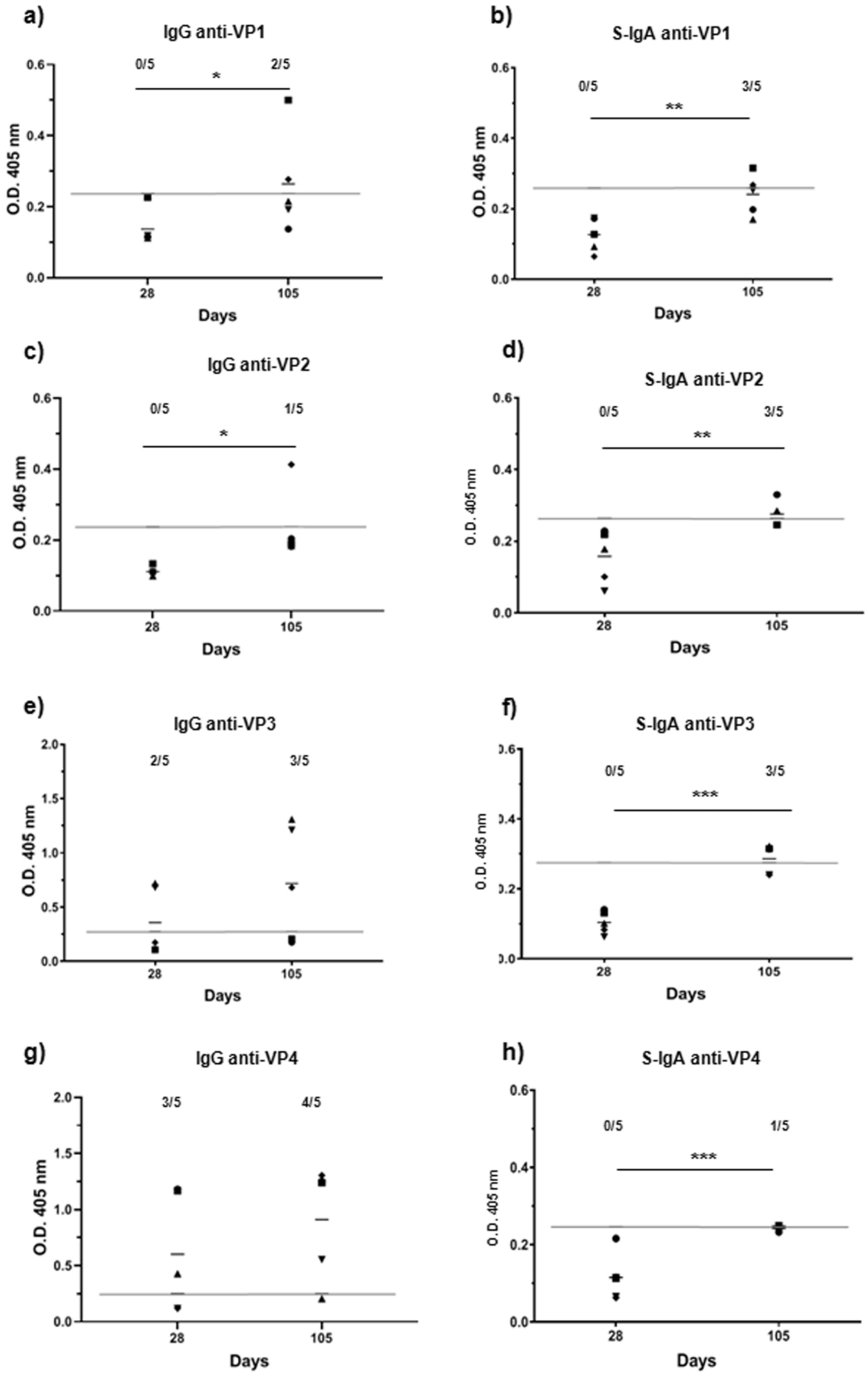

Fig. 8. Assessment of the immunological potential of VP plant-made antigens in BALB/c mice. Determination of IgG and S-IgA antibodies upon four subcutaneous injections and four oral immunizations with transgenic lines expressing VP antigens. The panels **a**, **c**, **e**, and **g** show sera IgG production after administrations of the VP1, VP2, VP3 and VP4 plant-made proteins; respectively. The panels **b**, **d**, **f**, and **h** represent the S-IgA elicited prior and after orally deliver of the VP1, VP2, VP3, and VP4 antigens; respectively. Both serum and feces were collected on days 28 and 105 and the levels of IgG and S-IgA were assayed by ELISA in 96-well plates coated with the $VP1_{93\text{-}104}$, $VP2_{133\text{-}154}$, $VP3_{131\text{-}151}$, or $VP4_{24\text{-}44}$ synthetic peptides. The line represents the cut-off value (mean + 2 S.D.). The asterisk denotes significant differences between means (marked by short line segments) for days 28 and 105 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

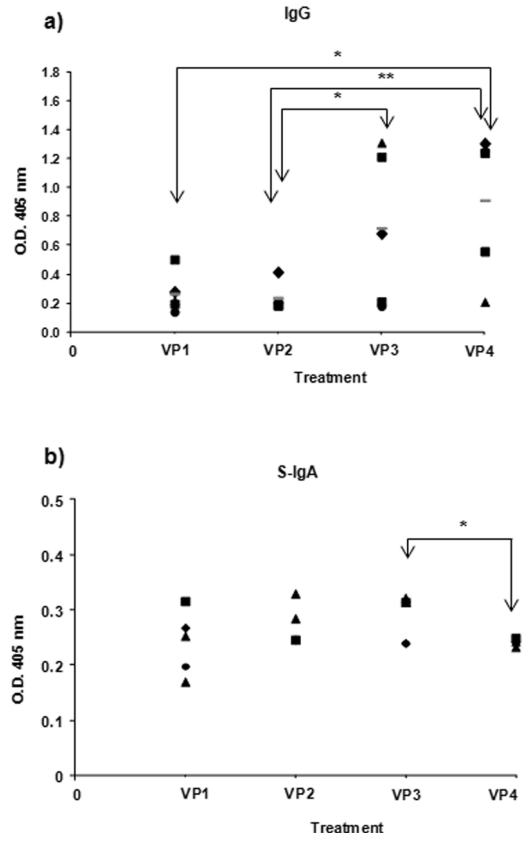

Fig. 9. Evaluation of the VP plant-made immunogenicity upon subcutaneous and oral administrations in BALB/c mice. a) Comparison of VP IgG specific antibodies elicited in sera collected on day 105. **b)** Comparison of mucosal response of mice subjected to immunizations with the VP1, VP2, VP3, or VP4 plant-made antigens. The asterisk denotes significant differences between means (marked by short line segments) for day 105 ($*P < 0.05$).

VP3 and VP4 proteins induced a higher response, even at the priming phase comprising s.c. immunizations; indicating that these proteins are highly immunogenic considering that no accessory adjuvants were used. However, a mucosal IgA response was only triggered upon the oral boosters with the corresponding VPs. Interestingly, such mucosal immune response was comparable for the tested VPs as non-significant differences was observed, except for the case of VP4. These comparisons strengthen the need to readjust the doses in future works and highlights the potential of the plant-made VPs to serve as effective boosting agents to promote an intestinal humoral response, which is critical to protect against Poliovirus.

5. Conclusions

The capsid proteins VPs can be functionally produced in plant cells; preserving their antigenicity and ability to induce humoral responses in mice. The plant-made proteins elicited both humoral systemic and mucosal antibody responses with VP1, VP3, and VP4 proteins as the most immunogenic upon a schedule combining parenteral and oral vaccine administration. Therefore, the plant-made VPs reported herein are promising immunogens that will have implications in the fight against poliomyelitis by supporting the generation of subunit vaccines having low cost and enhanced safety as main attributes. Importantly, these plant-made VPs having the capacity to induce immunity at the gastrointestinal tract, which is the port of virus entry and the initial site for its replication and transmission.

Author's contribution

All authors discuss the results, read and approved the final version of the manuscript.

Role of the funding source

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CRediT authorship contribution statement

Omayra C. Bolaños-Martínez: Investigation, Methodology, Formal analysis, Writing - original draft. **Dania O. Govea-Alonso:** Resources, Methodology. **Jacquelynne Cervantes-Torres:** Resources, Methodology. **Marisela Hernández:** Resources, Methodology. **Gladis Fragoso:** Writing - review & editing. **Edda Sciutto-Conde:** Writing - review & editing, Conceptualization, Supervision, Funding acquisition. **Sergio Rosales-Mendoza:** Writing - original draft, Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2020.07.007.

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The potential of plant-made vaccines to fight picornavirus

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REVIEW

The potential of plant-made vaccines to fight picornavirus

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ABSTRACT

Introduction: Several *Picornaviruses* are pathogens that generate serious problems for human and animal health worldwide. Vaccination is an attractive approach to fight against picornaviruses. In this regard, the development of low-cost vaccines is a priority to ensure coverage; especially in developing and low-income countries. In this context, plant-made vaccines are a convenient technology since plant cells are low-cost bioreactors capable of producing complex antigens that preserve their antigenic determinants; moreover, they can serve as biocapsules to achieve oral delivery.

Areas covered: In the present review the advances in the development of plant-made vaccines against picornaviruses are summarized and placed in perspective. The main diseases that have been targeted using this approach include Poliovirus, Food and mouth disease virus, Hepatitis A virus, and Enterovirus 71.

Expert opinion: Several vaccine candidates against picornavirus have been characterized at the preclinical level; with many of them capable of inducing humoral and cellular responses that led to neutralization of pathogens when evaluated in vitro and test animal challenge assays. Plant-made vaccines are a promise to fight picornaviruses; especially in the developing world where limited resources hamper vaccination coverage. A critical analysis of the road ahead for this technology is provided.

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KEYWORDS
Enterovirus; hepatitis A virus; poliovirus; bioencapsulation; molecular farming; oral vaccine; recombinant antigen

1. Introduction

Picornaviridae is one of the largest viral families, which according to the International Committee on Taxonomy of Viruses (ICTV) comprise 35 genera enclosing 80 viral species; many other are on the list to be classified. All members are ~30-32 nm in diameter with an icosahedral structure composed of 60 identical units (protomers) [1]. The members of this family have a genome composed of a single-stranded, positive-sense, and non-segmented RNA; with a length ranging 6.7–10.1 kb. The ORF is flanked by two untranslated regions (UTR); with the 5´end containing diverse RNA secondary structures implicated in replication and associated with the VPg protein that plays an important role in translation. The 3´UTR contains a poly (A) tail that mimics mRNA from the host providing genome stability (Figure 1). Picornaviruses possess four capsid proteins having b-barrel folding and code for a polyprotein that is processed by virus-encoded cysteine proteinases; their replication is performed by an RNA-dependent RNA polymerase containing the YGDD sequence motif. Picornaviruses are transmitted through the oral-fecal or aerial routes and many of them affect humans and animals; causing subclinical infections, mild febrile illness, and mild diseases in the gastrointestinal or respiratory tracts; as well as severe heart, liver, and central nervous system diseases. Picornaviruses of the genera *Cardiovirus, Cosavirus, Enterovirus, Hepatovirus, Kobuvirus, Parechovirus*, and *Salivirus* infect humans [2].

Plant-based vaccines are antigenic formulations obtained from plant tissues or cells, which are genetically engineered to produce antigens intended to induce immunity against a specific disease. Plant-based vaccines are a promise in the fight against several diseases (infectious and non-communicable); having singular advantages that include low cost and high safety associated with the absence of replication of human pathogens, which contrasts with the conventional hosts (i.e. *E. coli* and insect and mammalian cells). Plant-based vaccines can be developed by delivering a transgene for nuclear (transiently or stably) or chloroplast (stably) expression into the plant cell. Plant tissues or cells are subsequently used to extract the antigen when transient expression is performed or, in the case of stable transformation, they are subjected to a selection process to generate cell lines, tissues, or whole plants expressing the antigen. The use of edible plant species adds a great advantage since their biomass can be simply processed by lyophilization and milling to produce oral vaccines dosed as capsules or tablets; dramatically reducing the production cost [3]. However, this objective requires a rational antigen design to ensure its efficient uptake at the gastrointestinal tract and proper processing by immune cells in the gut-associated lymphoid tissues (GALT) such that the tolerogenic nature of the system is overcome. Interestingly, the plant cell can act as a biocapsule that protects the antigen from degradation in the gastrointestinal tract and may provide

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Article Highlights

- *Picornaviruses* are pathogens that affect humans and animals
- Plant-made vaccines (PMV) are attractive tools to fight infections by *Picornaviruses*
- Several immunoprotective PMV against *Picornaviruses* have been generated
- The most explored targets comprise Poliovirus and Food and mouth disease virus
- PMV against Poliovirus are promising oral boosting agents

immunostimulatory compounds to enhance the vaccine immunogenicity [4].

Another approach consists in performing the purification of the plant-made antigen to formulate conventional, parenteral vaccines. Under this approach, plant-made influenza vaccines are currently produced and under clinical evaluations with promising results; they have the potential to be commercialized [5]. Plant-made vaccines are acquiring relevance, but they depend on their adoption by the industry; some governments and companies have invested to generate the first plant-made biopharmaceuticals and vaccines in the market [6,7]. In particular, plant-based vaccines have been proposed as effective tools to promote the One Health initiative, which implies promoting the use of diagnostic methods and vaccines for both humans and animals [8].

The potential of plant-based platforms to generate new products in the clinic is real and evidenced by the approval of Elelyso® (Protalix Bio Therapeutics, Karmiel, Israel, which is a plant-made version of the glucocerebrosidase used as replacement therapy for Gaucher's disease. This target is expressed in carrot cells using bioreactors and purified to be administered parenterally to patients. Besides attractive costs, this plant-based system confers an appropriate glycans pattern to the biopharmaceutical; since the enzyme expressed in mammalian cells possess terminal sialic acid, galactose, and N-acetylglucosamine residues on the glycans chains that inhibit the mannose receptors-mediated endocytosis by macrophages. In contrast, the carrot-made enzyme lacks sialic acid and targeting it to the vacuole prevents the extension of terminal mannose residues, which results in an enzyme that is efficiently captured by macrophages [9]. Another remarkable example on the potential of plant-made biopharmaceuticals is ZMapp™ (developed by Leaf Biopharmaceutical, San Diego, CA, USA), which consists of a cocktail of three monoclonal antibodies to treat Ebola. This product is obtained in transiently transformed *N. benthamiana* plants and it has been approved for its use before completion of clinical testing during an outbreak of the viral disease in West Africa. Seven patients were treated with the available ZMapp stock and five of them ultimately recovered. At present, ZMapp is under clinical evaluations [10]. A final example are the influenza vaccines developed by Medicago (Québec, Canada/Durham, NC, USA), which are based on agroinfiltration of the *N. benthamiana* plants for the production of virus-like particles (VLPs) as vaccine candidates. Their products include the human quadrivalent seasonal influenza that has already completed phase II and phase III clinical trials, the pandemic influenza with the phase II clinical trial finished, the rotavirus vaccine with the phase I clinical trial complete, and norovirus with preclinical studies accomplished [11].

On the other hand, in 2006 the US department of Agriculture (USDA) approved the first molecular farming product generated in tobacco NT-1 cells as veterinary vaccine against a viral disease (Dow AgroSciences, Zionsville, IN, USA) [12]. The vaccine consists of the hemagglutinin-neuraminidase glycoprotein (HN) of the Newcastle disease virus and it is used to induce a protective immune response in poultry and other avian species.

Since picornaviruses are pathogens for human and animals, the exploitation of the plant-made vaccine technology has deserved the attention of several research groups around the world and this review transmits the state of the art in this field (Table 1).

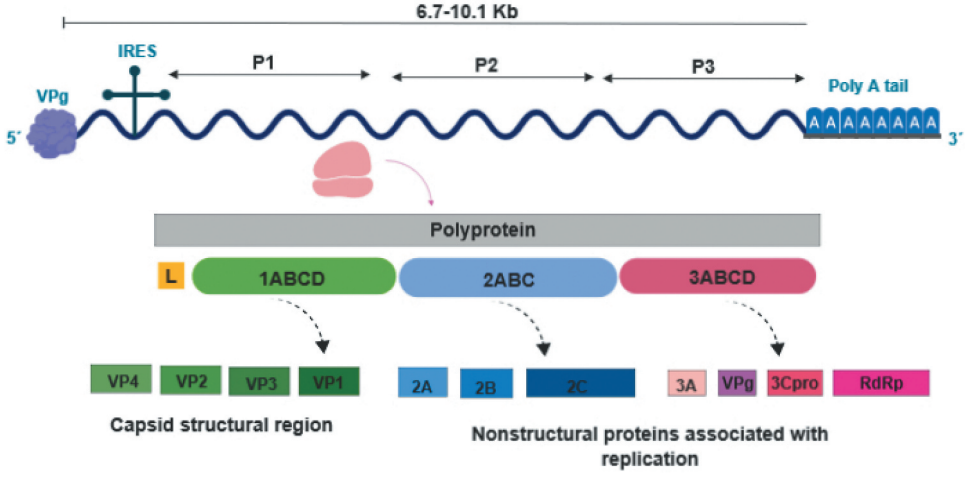

Figure 1. Genome structure of picornavirus. The single-strand genomic RNA ranges from 6.7–10.1 kb in length and the 5´end is covalently linked to the viral protein g (VPg), while the 3´end has poly (A). The 5´ NTR contains an internal ribosome entry site (IRES) that directs polyprotein translation. The single open reading frame is organized as 1ABCD-2ABC-3ABC. The P1 region encodes the capsid structural polypeptides, while P2 and P3 regions encode for the nonstructural proteins associated with replication.

Table 1. Compilation of plant-made vaccine prototypes against picornaviruses.

Genera/Species	Plant host (expression strategy)	Protein expressed [concentration obtained]	Immunization route	Immunological findings	References
Enterovirus/Enterovirus 71 (EV71)	Tomato (stable)	VP1 fused to ER retention signal [27 µg/g FLT]	Oral	VP1-specific IgA and serum IgG developed in BALB/c mice. Neutralization of EV71 infection to RD cells using serum from immunized mice. Humoral and cellular response observed.	Chen et al. (2006)
Aphthovirus/Foot-and-Mouth Disease Virus (FMDV)	Alfalfa (stable)	Polyprotein P1 and protease 3 C	i.p. + IFA	Strong neutralizing antibody response and mice protected against the challenge test with the virulent FMDV virus	Dus Santos et al. (2005)
	Nicotiana benthamiana (transient)	VP1 (TMV.VP1) [50–150 µg/g FLT]	i.p.+ IFA	Immunized mice developed a protective immune response against the challenge test with virulent FMDV. Specific IgG antibody response against VP1 in BALB/c mice	Wigdorovitz et al. (1999)
	Tomato (stable)	Polyprotein P1-2A and protease 3 C	i.m. + IFA	Immunized guinea pigs developed a virus-specific IgG antibody response against FMDV and were fully protected against a challenge test infection.	Pan et al. (2008)
	Rice (stable)	Polyprotein P1 [0.6–1.3 µg/mg of TSP]	i.p. + IFA and oral	FMDV-specific neutralizing antibodies detected in sera. FMDV-specific IgA and IgG were detected in intestinal wash and in vitro tissue; both were significantly higher than the mice fed with the *E. coli*-derived P1 protein.	Wang et al. (2012)
	Nicotiana benthamiana (transient)	Polyprotein P1-2A [~0.03 µg/g FLT]	s.c. + Montanide ISA 50 V 2 (Seppic) Adjuvant	Assembly of proteins into VLPs. Immunological assays demonstrated the production of FMDV-specific IgG antibodies in mice.	Veerapen et al. (2018)
	Nicotiana benthamiana (transient)	Polyprotein P1-2A and protease 3 C [3–4 mg/kg FLT]	i.p. + Montanide ISA 50 (Seppic)	Production of FMD VLPs. Mice immunized with purified VLPs or crude leaf extract elicited a specific humoral response.	Ruiz et al. (2018)
	Crotalaria juncea (stable)	Tandem-linked VP1-serotype A and O	i.m.+ IFA and oral	Humoral- and cell-mediated immune response in guinea pigs immunized orally or parenterally. Serum IgG antibodies and reactive lymphocytes. Secretory IgA antibody response in orally immunized pigs. Animals protected against the challenge test with the virus serotype A and O.	Rao et al. (2012)
	Nicotiana benthamiana (transient)	Polyepitope protein (B cell epitopes from VP1 and VP4/T cell epitopes from 2 C, 3D proteins) designated as H-PE proteins [67 mg/kg plant tissue]	i.m.	Guinea pigs immunized with purified H-PE induced an efficient immune response against FMDV and protection against the disease	Andrianova et al. (2010)
	Chenopodium quinoa (transient)	Amino acids 128–164 of VP1 inserted in pBS-d35CP viral vector [14.3% of the total viral coat]	i.m. + Montanide ISA 206	High levels of anti-VP1 IgG antibodies elicited. Neutralizing antibodies detected in sera of the swine group vaccinated with the BVP1 recombined virus. Immunoprotection in swine after the FMDV challenge test.	Yang et al. (2007)
	Nicotiana tabacum (stable)	VP1 epitope 140–160 aa into HBcAg [0.05% TSP]	i.p. + IFA and CFA	Specific antibody responses to both HBcAg and FMDV VP. Mice highly protected against virulent FMDV.	Huang et al. (2005)
	Arabidopsis thaliana (stable)	VP1	i.p. + IFA	Specific antibody response and protection against the challenge with the virulent FMDV	Carrillo et al. (1998)
	Potato (stable)	VP1 [0.005–0.01% TSP]	i.p. + IFA	Specific IgG antibodies against FMDV and protection against the experimental challenge	Carrillo et al. (2001)
	Potato (stable)	VP1-CTB [0.1–0.13% TSP]	NA	NA	He et al. (2007)
	Alfalfa (stable)	VP1 135–160 fused to *gus* A [0.5–1 mg/g TSP]	i.p. + IFA	Mice developed strong anti-FMDV antibody response and were completely protected against the experimental challenge with the virulent virus.	Dus Santos et al. (2002)
Hepatitis A Virus (HAV)	*Nicotiana benthamiana* (transient)	VP1-Fc inserted in BCTV based vector [0.6% TSP]	i.p. + CFA and IFA	Production of IgG antibodies in serum of immunized mice. Expressions of IFN-γ and IL-4 increased in splenocytes at the time of sacrifice.	Chung et al. (2011)
Polio Virus (PV)	*Nicotiana tabacum* (transient)	Amino acids of VP3 and VP1 fused to TMV-based vector	i.p. + MPL+ TDM	The TMVCP-PVP virus particle induced antibodies against poliovirus peptide; as well as TMVCP in mice.	Fujiyama et al. (2006)

(*Continued*)

Table 1. (Continued).

Genera/Species	Plant host (expression strategy)	Protein expressed [concentration obtained]	Immunization route	Immunological findings	References
	Nicotiana tabacum (chloroplast)	VP1 and CTB-VP1 [54 µg/g DW (0.1%) and 2.6 mg/g DW (4–5%) TSP]	s.c. and oral boosters + squalene or saponin or both	VP1-IgG1 and VP1-IgA titers increased significantly. Neutralizing antibody titers (~3.17–10.17 log2 titer) and seropositivity (70–90%) against all three poliovirus Sabin serotypes	Chan et al. (2016)
	Nicotiana benthamiana (transient)	P1 polyprotein [0.04%-0.06%]	i.p.	The VLPs developed were predominantly in the native D antigenic conformation. Immunized mice developed neutralizing antibodies and were protected from wild type poliovirus infection.	Marsian et al. (2017)
	Lettuce (chloroplast)	CTB-VP1 n and CTB-VP1co	s.c. and oral boosters + squalene or saponin or both, plus PG1 or LL37 or both	VP1-VLPs of 22.3 nm size were developed. VP1-specific IgG1 and VP1-IgA enhanced titers with oral plant-made VP1 boosters. 100% seropositivity of Sabin −1, 2, and 3.	Daniell et al. (2019)

FTF: Fresh tomato fruit, **RD cells**: Rhabdomyosarcoma cells, **ER**: Endoplasmic reticulum, **i.p.** Intraperitoneally, **IFA** Incomplete Freund´s Adjuvant, **TMV**: Tomato mosaic virus vector, **FLT**: Fresh Leaf Tissue, **i.m.** Intramuscularly, **TSP**: Total soluble protein, **s.c.** subcutaneously, **NAs**: Neutralizing antibodies, **HBcAg**: Hepatitis B core antigen gene, **CFA**: Complete Freund´s Adjuvant, **MPL**: Monophosphoril lipid A, **TLP**: Total leaf protein, **FW**: Fresh weight.

2. Plant-made vaccine candidates

2.1. Enterovirus 71 (EV71)

Enterovirus 71 is a member of the enterovirus species A that causes infections in humans and has become a serious threat to global public health. Infants and young children are the main affected population; developing hand, foot and mouth disease (HFMD), aseptic meningitis, poliomyelitis-like acute flaccid paralysis, brainstem encephalitis, and other severe systemic disorders such as pulmonary edema, cardiorespiratory collapse, or even death [13]. Since the first EV71 isolation occurred 50 years ago, several outbreaks have been reported mainly in the Asia-Pacific region. One of the most important outbreak originated in Taiwan in 1988 that resulted in more than 100,000 cases with 78 deaths [14]. During 2008 and 2009, infections by EV71 caused the death of 479 infants in several provinces and cities of mainland China [15]. In Vietnam, EV71 was first isolated in 2003 and a large outbreak of HFMD occurred in 2005 that included 51 cases with neurological complications causing three deaths in southern Vietnam [16]. The first vaccine against EV71 was licensed by the Chinese Food and Drug Administration (CFDA) in 2015 and manufactured by the Institute of Medical Biology (Chinese Academy of Medical Science). The vaccine contains the whole inactivated virus and it is administered by injection; requiring two immunizations to provide full protection [17].

Looking to develop a plant-based subunit vaccine against EV, the VP1 protein was expressed in tomato plants [18]. The *VP1* gene was fused to a signal peptide (SP) and its accumulation in the endoplasmic reticulum (ER) of the plant cells was favored. Tomato plants were obtained by *Agrobacterium*-mediated transformation and the presence of the transgene resulted in the production of the heterologous protein.

The immunogenicity of the plant-made VP1 antigen was assessed by oral administration of transgenic fruits to BALB/c mice (60–80 µg of VP1 contained in 3–5 g of plant biomass). Doses were administered on days 0, 7, 14, and 51; significant IgG (serum) and IgA (feces) levels were observed in mice immunized with plant fresh biomass. Moreover, serum from immunized mice was able to neutralize EV71 in vitro. In addition, splenocyte proliferation assays revealed that the plant-made VP1 protein triggered cellular responses. Furthermore, significant secretion of INF-γ, IL-5, and TGF-β was detected in the supernatants of stimulated spleen cells. Interestingly, the levels of INF-γ in mice fed with transgenic tomato and bacterial VP1 showed a two to fivefold increase; suggesting a Th1 response. This work reveals that the tomato-made VP1 vaccine is functional and highly attractive; since it is produced in an edible crop, which adds the significant advantage of not requiring purification. Determining the protective potential of the vaccine in challenge studies is the next step for this vaccine.

2.2. Foot and Mouth Disease Virus (FMDV)

The Foot and Mouth Disease Virus (FMDV) belongs to the *Aphtovirus* genus that comprises seven serotypes (A, O, C,

SAT-1, SAT-2, SAT-3, and ASIA-1). The A, O, and C serotypes prevail mainly in America and Europa, whereas SAT-type viruses have been described in Africa and the Middle East. The ASIA-A serotype is found predominantly in Asia. No cross-immunity is developed among the seven serotypes, which can be highly infectious and transmissible in cattle, sheep, pigs, and goats [19].

Several FMDV outbreaks have been reported worldwide. In 2017, two important notifications were emitted in Colombia with 1341 cases and 5 deaths associated with serotype O [20]. Over the last 65 years, the disease has become endemic in Egypt with A, O, and SAT-2 as the circulating serotypes. Serotype A was detected in 34 outbreaks in 9 Egyptian governorates affecting >7,500 animals in 2006 [21]. From 2010 to 2015, 1333 livestock holdings reported FMDV outbreaks in Nepal with a mean of 71.2 animals affected in each outbreak and a 3.6% fatality rate [22].

Vaccines for FMDV were developed 70 years ago; most of them are produced by propagating the virulent FMDV in baby hamster kidney-21 cells. Upon subsequent inactivation of the virus with binary ethyleneimine (BEI), the vaccine is partially purified and formulated with an appropriate adjuvant such as aluminum hydroxide/saponin [23]. However, FMD remains endemic in diverse regions worldwide, which is favored by the rapidly changing environment, increased trade, population growth, international travel, and migration. These conditions constitute a challenge for the capabilities of any available vaccine, which should be improved in antigenic coverage [24]. Currently, new strategies are being developed in order to improve the control and eradication of the disease. In plants, the antigens VP1, 2A, 2 C, 3 C, 3D, and P1; and epitopes from VP4 and VP1 have been explored for their potential as plant-based vaccines.

In 1998, Carrillo et al. reported the expression of the VP1 protein in transgenic *Arabidopsis thaliana* plants. The gene was introduced into plants by vacuum infiltration using an *Agrobacterium tumefaciens* culture; transgenic T1 seeds were germinated and antibiotic-resistant plants analyzed [25]. BALB/c mice were immunized intraperitoneally (i.p.) with 0.5 mL of the plant extract emulsified in Incomplete Freund´s Adjuvant (IFA). Serum from immunized animals showed a strong reaction against both a synthetic VP1 peptide (aa 135–160) and intact FMDV particles. Finally, the protective effect was assessed by challenging the immunized mice; observing full protection against i.p. lethal doses of FMDV O1 C. This was the first report showing the protection against this virus by using a plant-made antigen.

Wigdorovitz et al. (1999) produced a chimeric version of the tobacco mosaic virus (TMV) expressing the full-length VP1 protein from the serotype O1 Campos (O1 C) in *Nicotiana benthamiana* plants [26]. VP1 protein levels ranged from 50 to 150 µg/g of freshly harvested leaves. Significant titers of IgG antibodies in sera directed against the p135-160 epitope of VP1, as well as to FMDV particles, were elicited in BALB/c mice injected i.p. with 0.5–1 µg of recombinant VP1 protein. Remarkably, all immunized mice developed a protected immune response against an experimental challenge with virulent FMDV O1 C.

The VP1 protein has also been expressed in transgenic potato plants [27]. BALB/c mice were immunized i.p. four times with 150 µL of leaf extracts from plants expressing VP1 or the unrelated foreign gene, which contained 15 to 20 mg of fresh tissue emulsified in IFA. Sera from immunized mice revealed positive humoral responses against FMDV and 91% protection against the experimental challenge with the virus.

Another interesting work was reported by Dus Santos (2002) in which a new immunogen was produced in alfalfa plants; consisting of a highly immunogenic epitope from FMDV O1 C (i.e. VP 135–160) fused to the glucuronidase reporter gene (*gus* A) [28]. The level of antigen accumulation was particularly high and ranged from 0.5 to 1 mg/g of total soluble protein (TSP). BALB/c mice were immunized i.p. twice with 200 µL of plant tissue extracts containing approximately 100 mg of fresh leaf tissue emulsified in IFA. An ELISA showed strong anti-FMDV antibody response after administration of the plant-made VP-GUS. Additionally, these mice were completely protected against an experimental challenge with the virulent virus. This was the first report of an epitope-based FMDV vaccine produced in transgenic plants inducing a protective immune response against FMDV in an experimental host.

He et al. (2007) used potato plants for the expression of a chimeric protein comprising the VP1 antigen and the cholera toxin B subunit (CTB) as an immunogenic carrier, a flexible linker tetrapeptide (GPGP) in between, and the ER targeting signal (SEKDEL) in the C-terminal [29]. The levels of the chimeric protein ranged from 0.1% to 0.3% TSP. Although this is an interesting candidate due to the mucosal adjuvanticity of CTB that could account for the potential of an oral vaccine, immunogenicity and immunoprotection analyses were not reported.

Sunn hemp plants were engineered to generate a prototype of a bivalent vaccine targeting tandem-linked VP1 proteins from serotypes A and O [30]. The presence of the VP1 O-A construct and the synthesis of the chimeric protein in plants were confirmed. The accumulation levels ranged from 1 to 12 µg/g of TSP. Guinea pigs were immunized orally or parenterally with 1 g of leaf material from transgenic plants or wild type plants as negative control. Both, the parenteral and oral approaches were able to induce IgG antibodies in sera and the stimulation index (SI, lymphocyte stimulation assay) were three fold higher than the control group. In a challenge experiment, 66% of the animals were protected against both virus serotypes. This study is relevant as it constitutes the first report on the development of a bivalent FMD vaccine using a forage crop.

Huang et al. (2005) reported the expression of relevant epitopes from the VP1 and VP4 proteins [31]. The authors fused a VP21 epitope (aa 140–160 at the G-H loop from the VP1 protein) into the internal region of the hepatitis B virus core antigen (HBcAg). Transgenic tobacco plants were obtained via *Agrobacterium*-mediated transformation. The highest concentration of recombinant HBCVP protein was 0.05% of TSP. Interestingly, this fusion protein formed a VLP structure. BALB/c mice were i.p. immunized four times with 0.25 mL of transgenic tobacco leaf extracts (containing 1.25 µg of recombinant HBCVP protein). Sera analyses showed specific

IgG antibody responses to both HBcAg and FMDV VP1, moreover, a virus challenge experiment demonstrated that the immunized mice were protected against the virulent FMDV serotype O.

Later, Yang et al. (2007) developed a chimeric virus based on the *Bamboo Mosaic Virus* (BaMV) and the epitopes T128-N164 from FMDV VP1 serotype O/Taiwan/97 [32]. *Chenopodium quinoa* plants were infected, leaves were harvested and the recombined virions (BVP1) purified. The yield of the virus was estimated at 0.2–0.5 mg/g of fresh leaf tissue. Two groups of three pigs were immunized intramuscularly (i.m.) with 10 or 5 mg of BVP1 virions emulsified with Montanide ISA 206, a booster dose was administered 6 weeks later. Both groups of swine showed high levels of IgG anti-VP1 antibodies and neutralizing antibodies were detected in sera. Additionally, IFN-γ mRNA was detected in peripheral blood mononuclear cells (PBMC) upon VP1 antigen stimulation. Interestingly, swine immunized with BVP1 and challenged with a virulent FMDV showed no FMD symptoms during 14 days.

Another interesting work was reported by Adrianova et al. (2010) based on the production of an immunogen consisting of B cell epitopes from VP1 (aa 135–160 and 200–213) and VP4 (aa 21–40) and T cell epitopes from 2 C (aa 68–70) and 3D (aa 1–115 and 421–460) from the serotype O/Taiwan/99 fused to a potato X virus vector [33]. *N. benthamiana* plants were infected with *A. tumefaciens* harboring the viral vector. Epitopes detection was successfully performed by Western blot; having yields up to 67 mg/kg plant tissue. Three groups of Guinea pigs were immunized i.m. with different doses of the protein (350, 120, and 40 µg) in oil adjuvant Montanide ISA 70. Interestingly, a single dose of 350 or 120 µg triggered humoral responses against a purified FMDV antigen; displaying neutralizing activity against the FMD virus type O/Taiwan/99.

Dus Santos et al. (2005) generated alfalfa plants expressing P1, the 2A sequence, the first 61 amino acid residues of the N terminus of 2B, the complete sequence of 3B1, 3B2, 3B3, and 3 C; and the first 16 amino acid residues of the 3D N terminus of FMDV O1 C [34]. Transgenic plants were developed by *A. tumefaciens*-mediated transformation; generating empty capsids of FMDV. Mice were immunized i.p. four times with 150 µL of leaf extract obtained from 15 to 20 mg of fresh tissue; leading to the induction of humoral responses against the structural protein VP1 and purified FMDV particles. Mice were subsequently challenged with FMDV observing full protection against infection. This interesting work demonstrated the capability of the plant-made FMDV-P1 to induce a protective antibody response.

The polyprotein P1-2A and the protease 3 C were also expressed in tomato plants [35]. Guinea pigs were i.m. immunized three times with plant extracts, first with 400 µL of foliar extract containing 50 mg of fresh leaf tissue emulsified in CFA; and two boosts consisting of 800 µL of foliar extracts containing 100 mg emulsified in IFA were subsequently administered. The animals developed a virus-specific antibody response against FMDV; resulting in complete protection against a challenge infection. In this work, the concentration of the recombinant protein was not reported, neither the formation of VLPs demonstrated.

In another report, Wang et al. (2012) generated transgenic rice expressing the polypeptide P1 [36]. The protein was expressed in a range of 0.6–1.3 µg/mg of TSP. Mice were i.p. immunized with 10 µg of P1 protein and 3 subsequent boosts were applied; observing the elicitation of FMDV-specific neutralizing antibodies with the capacity to clear virus from their sera after FMDV challenge. Additionally, oral immunogenicity was assessed; observing the induction of FMDV-specific IgA responses in intestinal washes and partial clearance after FMDV challenge. This study shows the potential of using transgenic rice-based expression systems as alternative bioreactors to produce FMDV subunit vaccines.

Recently, Veerapen et al. (2018) transiently expressed the P1-2A and 3 C protease proteins in *N. benthamiana* plants [37]. These proteins were assembled into VLPs and the estimated yield was ~0.03 µg VLPs per gram of fresh leaf tissue. The immunogenicity of the partially purified VLPs was tested in mice injected subcutaneously (s.c.) four times with 5 µg of P1-2A protein. Mice immunized with adjuvanted P1-2A showed four-fold increase in ELISA absorbance readings when compared to the control group, whereas those vaccinated with P1-2A only showed two-fold increase. This study revealed for first time that the capsid precursor of FMDV can be proteolytically cleaved into the capsid proteins (VP0, VP1, and VP3) in the absence of the 3 C protease, which constitutes an advantage over other expression platforms. The protective capacity of this vaccine remains to be determined.

In another study, VLPs from FMDV were also produced by transient expression in *N. benthamiana* plants. The polyprotein P1-2A and 3 C protease were expressed and the recombinant protein yield reached 3–4 mg/kg of fresh tissue. The immunogenicity of the generated particles was evaluated in mice under two different conditions: i) i.p. administration of purified VLPs + Montanide ISA 50 as adjuvant and ii) i.p. administration of the crude leaf extract + Montanide ISA 50. Both groups showed specific humoral responses at similar magnitude. This research showed that the use of minimally processed plant material, containing VLPs as an immunogen, could be a scalable and cost-effective technology for the production of a recombinant vaccine against FMDV [38].

2.3. Hepatitis A Virus (HAV)

The Hepatitis A virus (HAV) is an atypical member of the *Picornaviridae* family classified taxonomically within a unique picornaviral genus: *Hepatovirus*, which comprises a single serotype of human HAV and other closely related mammalian viruses [39]. Infection by HAV is typically acquired by ingestion and the virus replicates within hepatocytes, the epithelial cells lining the crypts of the small intestine have been suggested as primary replication sites. Approximately 1.5 million people are infected annually with HAV and the incidence is related to socio-economic conditions such as density of housing, sanitation, and quality of water. The disease is characterized by jaundice and leads to acute liver failure. Prevention of Hepatitis A can be achieved by vaccination and adequate

sanitation. Nowadays two types of HAV vaccines are used, which are based on formaldehyde-killed or attenuated HAV. Most countries have opted for the use of killed vaccines for pre- and post-exposure prophylaxis; considering their superior immunogenicity and the low risk of reversion to virulence [40,41].

Plant-based vaccines are being adopted as an alternative to generate new HAV vaccines. In 2011, Chung et al. reported the expression and immunogenicity of a recombinant chimeric protein (HAV VP1-Fc) containing the VP1 viral protein and an Fc antibody fragment. The chimeric protein was detected and subsequently purified. The expression levels reached up to 0.6% TSP. The immunogenicity was assessed in mice after i.p. immunization (4 times at 2-week intervals with 30 µg of VP1). Strong IgG response to VP1-Fc in sera was obtained from the group immunized with the plant-made antigen. Furthermore, splenocytes from immunized mice showed increased INF-γ and IL-4 production upon antigenic stimuli. These results indicated the induction of Th1 and Th2-type cytokines [42]. The assessment of the protective capacity of this vaccine in appropriate animal models will provide solid evidence of its true potential.

2.4. Polio Virus (PV)

One of the most important members of the *Picornaviridae* family is Poliovirus (PV), which belongs to the enterovirus genus and causes poliomyelitis. There are three wild PV serotypes, from which type 2 was officially declared as eradicated in 2015 and type 3 in 2019. Therefore, type 1 is the only wild PV that remains in circulation worldwide [43].

PV has humans as the only natural reservoir, it is transmitted through the oral-fecal route and quickly spreads in places with poor sanitation. PV multiplies in the intestine and can invade the central nervous system causing irreversible paralysis in 1 out of 200 infected people. At present, two types of vaccines are used to prevent the disease: 1) The inactivated polio vaccine (IPV) that contains poliovirus types 1, 2, and 3 (inactivated with formaldehyde) and 2) the oral polio vaccine (OPV) that may contain one, two, or the three serotypes in an attenuated form. Although the number of cases of poliomyelitis has decreased by 99%, infections still occur in three endemic countries (Afghanistan, Nigeria, and Pakistan) and seven 'outbreak countries' where the circulation of wild PV has stopped, but they are experiencing re-infection by importation (Democratic Republic of the Congo, Indonesia, Somalia, Kenya, Papua New Guinea, Niger, and Mozambique) [44].

The limitations of these vaccines comprise the requirement of large-scale cultivation of PV for vaccine production and the polio cases caused by reversion of the strains used in OPV, which are factors likely limiting polio eradication. Therefore, the Global Polio Eradication Initiative (GPEI) has set as a priority the development of affordable and safer vaccines. In this context, the approach of recombinant proteins expressed in plant cells is a great alternative to generate a vaccine without the use of the live or attenuated virus; moreover, these vaccines are produced at low costs and avoid the risk of biological contaminants.

Fujiyama et al. (2006) fused 15 amino acids (PVP) from the viral capsid proteins VP1 and VP3 of type 1 poliovirus (Sabin strain) to the *Tobacco Mosaic Virus* (TMV) coat protein (CP). The chimeric particles were obtained at a yield up to 0.2 mg/g leaves of *Nicotiana tabacum* plants. Mice were i.p. immunized once or twice with approximately 200 µg of purified particles emulsified with monophosphoryl lipid A (MPL) + trehalose dimycolate (TDM) as adjuvants. Specific antibodies against PVP and TMVCP were induced after immunization [45]. These data supported the potential of TMV as an antigen-carrying agent in plants.

A novel plant-based approach to produce VP1 was implemented by Chan et al. (2016) by applying transplastomic technologies in *N. tabacum*. A codon-optimized *VP1* gene fused to CTB and the native *VP1* gene were produced. The immunogenicity of the CTB-VP1CO was assessed in mice in a scheme comprising oral boosts with adjuvanted freeze-dried plant material after a single IPV s.c. dose. Boosters with the plant-made CTB-VP1 or CTB-VP1CO significantly increased VP1-IgG1 and VP1-IgA titers when compared to not using boosts. Neutralizing antibodies and seropositivity (70–90%) against all three poliovirus Sabin serotypes were induced with two doses of IPV and plant-cell oral boosters, whereas a single dose of IPV resulted in poor neutralization [46]. In a later study, Yuhong and Daniell (2017) reported a long-term study for this vaccine candidate. CD-1 mice were primed s.c. with IPV and orally boosted with 1 or 25 µg of recombinant adjuvanted protein once a month for 3 months or a single booster 1 year after the first prime. Interestingly, the use of the plant-made CTB-VP1 antigen-induced high levels of IgG1 and IgA; having systemic humoral response neutralizing potential (4.3–6.8 log 2 titers). In terms of the duration of the response, it was sustained from 29 to 400 days and conferred the same level of protection against all three serotypes during this period [47]. The antigen was stable in lyophilized tissue when stored at room temperature. Therefore, this candidate is proposed as a cold chain-free vaccine, which makes it very attractive in terms of cost and distribution.

Considering that tobacco produces toxic compounds; making it improper when pursuing safe oral delivery of antigens in humans, the authors thereafter developed transplastomic lettuce lines that rendered edible biomass acceptable for oral immunization [48]. Integration of the transgene and homoplasmy were confirmed; surprisingly, the assembly of VP1-VLPs of 22.3 nm in size was observed. Mice primed s.c. with IPV and boosted three times with 20 mg of lyophilized tissue enhanced specific IgG1 and IgA levels; as well as the neutralization activity (80–100% seropositivity of Sabin 1, 2, and 3) when compared to a treatment based on an IPV single dose or mice orally boosted with CTB-VP1CO without IPV priming. These are considered very promising results since population worldwide is receiving IPV at a single dose; therefore, a booster cold-chain free vaccine might be used to aid in polio eradication.

Another important work on polio VLP production in plants was reported in 2017 by Marsian et al. *N. benthamiana* plants were transiently transformed by agro-infiltration to express P1 from the Poliovirus type 3 Sabin (PV3) mutant SktSC8; rendering VLPs that retained the native D antigenicity conformation.

Mice carrying the gene for the human PV receptor (TgPVR) were i.p. immunized two times with partially purified VLPs; achieving protection upon a challenge with poliovirus wtPV3 (Saukett) [49].

3. Expert opinion

The race for developing therapeutic and prophylactic approaches against viral diseases comprises several emerging technologies. Among them, plant-based vaccines have been explored by several research groups around the world for this purpose. The literature review performed herein revealed that several plant-made vaccine candidates against picornaviruses are promising since they are capable of providing protection against pathogen challenges or inducing antibodies with an in vitro-tested neutralizing activity. The ambitious goal of using plant biomass to easily obtain purification-free vaccines is moving slowly; nonetheless, there is solid evidence that plant cells containing the target antigen can be used at least as boosting agents. Therefore, these formulations are proposed as cold-chain free vaccines; being low-cost alternatives in terms of production, distribution, and administration. Moreover, they can be highly accepted by patients [3,50].

The developed Polio-made vaccines are good examples of the potential of this technology to generate low-cost boosting vaccines. Given that the current polio vaccination schedule comprises priming with the inactivated vaccine and four or more oral boosts with the oral attenuated vaccine, the plant-made, and purification-free vaccines could replace the conventional oral vaccine [51]. The expression of VP2, VP3, and VP4 also deserves attention since they are relevant to confer immunoprotection [52]. Among picornaviruses, FMDV has been the most explored target with plant-made vaccines using epitopes from VP1 and VP4, the full-length VP1 protein, and the Polyprotein P1; mainly expressed in tobacco or Arabidopsis. Although some groups have focused on edible crops such as potatoes, tomatoes, and quinoa; their potential for oral immunization has been narrowly assessed. One remarkable contribution is the report from Wang et al. (2012) where significantly higher FMDV-specific IgA and IgG were detected in intestinal washes of mice orally immunized with rice expressing the Polyprotein P1.

Nowadays, the production of vaccines based on VLPs has become one of the most promising tools in vaccinology since they are stable structures and structurally and antigenically indistinguishable from the native virus. VLPs do not contain DNA; therefore, they are safer (non-infective) and able to stimulate strong immune responses [53]. Recently, picornavirus VLPs have been produced in plants with relevant results. VLPs of FMDV transiently expressed in *N. benthamiana* plants in absence of the 3 C protease constituted a pioneering study not even explored in conventional systems (i.e. mammalian and insect cells). Poliovirus VLPs have also been generated by expressing the P1 Polyprotein and surprisingly only with the VP1 protein, which is the first report on the production of poliovirus VLPs based on a single capsid protein; in this sense, it will be interesting to assess the formation of VLPs with other viral proteins such as VP2 or VP3.

However, in the case of plant-made vaccines against Picornaviruses the road to derive benefits from this technology in the clinic is still long and the following goals remain pending:

Enhancing and optimizing the development of oral formulations. This goal has been narrowly explored for the picornavirus family. From the reports summarized in this review only five cases focused on oral immunization (the case of VP1 tomato-made from EV71 [18], P1 from FMDV generated in rice [36], VP1 from FMDV produced in *Crotalaria juncea* [30], and CTB-VP1 expressed in tobacco and lettuce used as oral boosters [46, 48). Oral delivery must attract research interest since most of the viruses enters the body through the mucosal portal and this route is the most effective for inducing local immunity. The use of CTB or LTB as adjuvants/transmucosal carriers offers a potential approach that could lead to optimal induction of mucosal immune responses [54]. Other promising mucosal adjuvants include those based on toxin derivatives such as ADP-ribosyl transferase enterotoxins, adenylate cyclase toxins, MPL, CpG ODNs, flagellin (a TLR agonist), and immune-modulators such as NKT cells ligands, mast cells activators, and stimulator of interferon gamma genes (STING) ligands. These adjuvants could be explored in combination with plant extracts containing the picornaviral antigens [55].

Another aspect to implement is to move toward more attractive plant species since tobacco and *N. benthamiana* are not appropriate hosts for the formulation of purification-free oral vaccines due to their toxic alkaloid content. Among the attributes that should be contemplated during plant host election are shelf life of plant tissues (with seeds being a highly advantage case), growth rate, and genetic transformation efficiency [56].

Increasing antigen yields. Low yields constituted one of the main obstacles initially observed for plant-based manufactured biopharmaceuticals. In particular plant cell suspension cultures possess a slower growth rate than other organisms (e.g. bacteria, yeast, and mammalian cells) and a long time framework is required in particular for the generation of stably transformed lines. In the case of cell cultures, the presence of a dominant vacuole increases cell size and volume, which results in lower cellular protein content when compared to bacteria or yeast cultures [12]. Many technologies are currently available to address these drawbacks. The conventional nuclear expression approaches should be substituted by the next generation approaches such as those based on highly efficient vectors carrying optimized viral elements or replicative vectors, as in the case of the vaccine against swine Hepatitis B reported by Zahmanova et al. (2019) [57], and the transplastomic technologies adopted by Daniell et al. (2016) [58]. Moreover, there is a relevant niche to be explored by using seed accumulation strategies [59,60]. Among other approaches that might positively impact antigen yields are the following: using RNA silencing, knockout technology to modulate the glycosylation patterns or protease-encoding genes, introduction of recombinant protease inhibitors, and manipulation of chaperones and intracellular trafficking pathways [61]. Another novel molecular strategy available for the generation of genome engineered plants is the CRISPR system, whose main application in molecular farming is the

modification of the glycosylation pathways by interrupting the expression of certain glycosyl-transferases to avoid the presence of undesired glycans in the recombinant protein; although it should be considered that in some cases plant glycosylations led to the production of antigens with enhanced immunogenicity, which is desirable for vaccine design [62].

Surpassing the valley of death. Since many of the reported vaccine candidates are not continued into the completion of the preclinical validation to enter clinical trials, efforts to ensure these evaluations should be increased. For this purpose, the following steps are critical: performing toxicological and stability analyses (to justify the beginning of clinical trials) and establishing and scaling-up the GMPc production process in such a way that sufficient plant material can be obtained to cover further evaluations [63].

The plant-based vaccines must follow the guidelines defined by the FDA and the US Department of Agriculture that establish the evaluation of: 1) the presence of allergenic or toxic compounds, 2) the method of the plant production and propagation, 3) the characterization of the recombinant DNA, and 4) genetic stability for those cases based on stable transformation events [64]. On the other hand, environmental concerns should be taken into consideration to control the spread of the bioengineered pharmaceutical plants and meet regulations for global commercialization. Moreover, as for any technology, social acceptance is a critical factor and in the case of technologies involving GMOs; robust programs to make the society aware of the benefits and the minimal risks involved with this approach are crucial [65].

Interestingly, these goals have been overcome by Protalix Biotherapeutics; a company that developed a novel, low-cost bioreactor system based on large flexible polyethylene bags (ProCellEx® system) used to propagate plant cell cultures. The system provides high volume, multiuse, and disposable bioreactor; suitable for a variety of cell cultures. This GMPc methodology allowed producing Elelyso®, whose safety and efficacy was proven in preclinical and clinical trials [61]. This technology and experience are under exploitation to develop other biopharmaceuticals.

In conclusion, although many picornaviral vaccine candidates have been reported in plants; most of them not progressed beyond the pre-clinical phase, which indicates the need to reinforce this field by the adoption of new technologies that have the capacity to overcome some of the identified obstacles (i.e. low antigen yields and suboptimal immunogenicity). The adoption of new technologies by some groups is leading to innovative candidates that augurs a path for the generation of new, affordable, and safe vaccines to decrease the negative impact of picornaviruses on global health.

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Author contribution statement

S Rosales-Mendoza generated the outline and supervised information compilation and analysis. O C Bolaños-Martínez conducted the main literature search, writing, and generated the figures and tables.

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Toward the Optimization of a Plant-Based Oral Vaccine Against Cysticercosis

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Abstract It is recognized that an effective, low-cost oral vaccine may effectively contribute to prevent *Taenia solium* cysticercosis; plant-based vaccines, on the other hand, can make this goal feasible. Plants are optimal platforms for the massive production of oral vaccines. In this chapter, advances toward the development of oral plant-based vaccine against cysticercosis are reviewed.

Keywords Oral vaccine · Transgenic plant · Transplastomic plant
Carica papaya · *Daucus carota* · *Nicotiana tabacum* · *Taenia solium*

1 Introduction

1.1 Relevance of Cysticercosis

Taeniasis/cysticercosis is a parasitic infection caused by *Taenia solium.* It is prevalent in areas with low socioeconomic and sanitary standards in developing

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countries. *T. solium* cysticercosis is still endemic in most countries of Asia, Africa, and Latin America despite significant progress in developing effective tools for its prevention, diagnosis, and treatment (Fleury et al. 2012, 2013).

T. solium cysticercosis can be acquired by humans (the definitive host) and pigs (the intermediate host) after ingesting parasite eggs in food or water contaminated with feces from human *T. solium* tapeworm carriers (Sciutto et al. 2000). A single tapeworm carrier may produce thousands of *T. solium* eggs per day, which are shed to the environment, contaminating vegetables, running waters, and soils, upon open-air defecation (De Aluja 2008). Humans also acquire intestinal tapeworms by eating insufficiently cooked meat from cysticercus-infected pigs.

In humans, cysticerci frequently establishes in the central nervous system (CNS), causing neurocysticercosis (NC), the most severe form of the disease. NC is a clinically and radiologically heterogeneous disease, ranging from an asymptomatic infection to a severe and eventually fatal disease. NC severity mainly depends on the location of the parasite. The most severe clinical forms occur when parasites are located in the subarachnoid space at the base of the brain. This form of the disease is also less susceptible to cysticidal drugs and more difficult to diagnose based on neuroradiological studies (Marcin Sierra et al. 2017).

Pig vaccination may curtail *T. solium* transmission by reducing the number of cysticerci, and thence the incidence of adult intestinal tapeworms in humans. Although several vaccines have been developed and successfully field-trial tested, no cysticercosis vaccines for pigs have been approved for commercialization. All of them induced high level of protection, but in all cases they are injectable vaccines (Huerta et al. 2001; Morales et al. 2008; Assana et al. 2010). Injectable vaccines are costly and their administration at a massive scale implies logistic difficulties, since pigs roaming in rural areas must be captured by trained personal for vaccination (Huerta et al. 2001; Morales et al. 2008; Assana et al. 2010). These difficulties limit the use of parenteral vaccines in nationwide programs and may underlie the lack of interest in companies to produce the vaccine commercially. An orally administered vaccine, which could be delivered by pig owners, would elude these difficulties.

1.2 Context of Veterinary Vaccines

Veterinary vaccines are aimed to reduce morbidity in animals for human consumption (chickens, cows, fish, and pigs), pets, and in wildlife species, to prevent the loss or contamination of animal derivatives (improve productivity), and to reduce the risk of disease transmission from animals to humans (zoonosis) (Meeusen et al. 2007).

According to the World Organization for Animal Health (OIE), 116 animal infections are included in the 2017 list of diseases, infections, and infestations. Most of these diseases are caused by bacteria such as *Salmonella* spp., *Listeria monocytogenes,* and *Escherichia coli;* viruses such as avian influenza virus, norovirus,

and rabies virus, as well as parasites such as *Taenia saginata*, *T. solium*, *Toxoplasma gondii,* and *Giardia duodenalis* (www.oie.int/es).

Several conventional commercial pig vaccines are currently being used to prevent infections in pigs caused by pathogenic *E. coli*, *Salmonella*, and *Lawsonia intracellularis* strains (Table 1) (Meeusen et al. 2007).

While most veterinary vaccines have been formulated for parenteral administration, the number of oral vaccines has increased in recent years, considering the advantages of this alternative route. Oral administration of vaccines can be performed by animal owners themselves. In addition, oral administration is less-costly than the parenteral route since neither trained personal for administration nor the use of needles are required. Moreover, the oral route is non-invasive and particularly attractive for the prevention of orally acquired infections like cysticercosis (Wang and Coppel 2008). Oral vaccines efficiently stimulate the mucosal system, improving protection by emulating the entry path of most pathogens. In addition, oral immunization can induce a concomitant systemic immune response (Muir et al. 2000; Mutoloki et al. 2015).

The mentioned advantages are especially relevant when designing an economic vaccine to prevent these neglected tropical diseases (NTDs) (WHO 2010) that affect rural pigs in marginalized populations of poor countries. In this context, an oral recombinant vaccine could overcome these limitations.

Several avirulent and live-attenuated oral vaccines are applied to pigs to prevent diseases like salmonellosis and erysipeloid. As in the case of poultry, a lyophilizate can be mixed with sterile water and administered as a beverage to 3–8 weeks-old pigs.

On the other hand, subunit vaccines based in immunoprotective proteins provide a safer approach for vaccination. An example is the rabies vaccine known as Raboral, in which glycoprotein G is used; it is administered as bait to wild animals in France, Belgium, Germany, and the USA (Bano et al. 2017).

Table 1 Orally-administered, commercial pig vaccines against bacterial pathogens

Pathogen	Disease	Brand name	Distributor	Composition	References
Lawsonia intracellularis	Porcine proliferative enteropathy	Enterisol® Ileitis	Boehringer Ingelheim Vetmedica, Inc.	LAT	Park et al. (2013)
Salmonella choleraesuis and *typhimurium*	Salmonellosis	Enterisol® Salmonella T/C		LAV	Wales and Davies (2017)
Salmonella choleraesuis		Enterisol® SC-54		LAV	
Erysipelothrix rhusiopathiae	Erysipelas	Ingelvac® ERY-ALC		LAV	http://www.bivetmedica.com/
Escherichia coli strain K-88	Enteritis	Entero Vac	Arko labs	LAV	Cox et al. (2014)

LAT live attenuated; *LAV* live avirulent

In addition to enhanced safety, an advantage of subunit vaccines is their ability to address multiple genetic variants of a pathogen in a single chimeric protein. Several pathogens, such as RNA viruses, exhibit a high mutation rate, resulting in a great variability within a short period; in addition, multiple serotypes are reported for several virus strains. Since many existing viral vaccines cannot recognize new viral strains, novel strategies to produce vaccines against these new infectious variants are much needed (Meeusen et al. 2007). In the case of parasitic diseases, an immune response against several antigens is desired to achieve an efficient immunoprotection. Subunit vaccines constitute an alternative to address these challenges, since the development of multivalent vaccines based in mixtures of several antigen variants or even in multiepitopic proteins carrying a set of relevant epitopes can provide broad immune responses.

1.3 Advances in the Development of Plant-Based Oral Vaccines

The expression of recombinant vaccine antigens in plants to elicit and maintain protective immunity is an attractive option that has been explored for the last two decades. Edible vaccines currently under development are based on fruits, leaves, and seeds of transgenic plants. Such vaccines are prepared without expensive antigen purification steps, commonly required for parenterally administered vaccines (Lugade et al. 2010) (Table 2).

Besides protecting against pathogen viruses and bacteria infecting domestic animal species, this strategy is appropriate to delivery parasite-derived antigens to gut-associated lymphoid tissues (e.g. for fasciolosis, schistosomiasis, coccidiosis, cysticercosis, and ascariasis) (Walmsley and Arntzen 2000; Chambers et al. 2016).

1.4 Transgenic Plants as Alternative Platforms to Produce an Anti-cysticercosis Vaccine

Significant progress has been attained in the development of oral plant-based vaccine candidates against porcine cysticercosis (Table 3). An oral vaccine against cysticercosis was developed expressing the three peptides (KETc1, KETc12, and KETc7) that constitute the injectable vaccine against pig cysticercosis named S3Pvac. When parenterally applied, synthetically and recombinantly expressed S3Pvac reduced by 50% the number of infected pigs, and by 80–90% the number of established cysticerci under natural conditions of transmission (Huerta et al. 2001; Morales et al. 2008). To develop the oral version of the vaccine, the three peptides were expressed in three independent papaya embryogenic cell lines, obtained by bioballistics at the nuclear level under the CaMV35S promoter (Hernández et al.

Table 2 Experiences in immune response induced by oral vaccination with transgenic plants

Disease	Antigen	Plant	Dose	No. doses (interval)	Immune response	References
Gastroenteritis	LT-B	Potato tubers	20–50 μg	3 (wk)	Specific IgG and IgA Abs; partial protection	Mason et al. (1998)
Hepatitis	HBsAg	Potato tubers	20 μg	3 (wk)	Specific IgG Abs on day 36–50 after first feeding	Rukavtsova et al. (2015)
Dengue	cEDIII-CTB	Rice calli	100 μg	4 (0, 2 4, 9 wk)	cEDIII specific IgG and IgA Abs; splenic T cell proliferation	Kim et al. (2016)
Fasciolosis	CPFhW-HBcAg	Lettuce leaves	10 μg	2 (0, 4 wk)	65.4% protection; specific IgG1 and IgM Abs; increased blood CD4 + and CD8+	Kesik-Brodacka et al. (2017)
Cysticercosis	KETc1, KETc7, KETc12	Papaya calli	0.1–10 μg	2 (1, 10 days)	55–89% protection; specific IgG Abs; CD4 and CD8 proliferation	Fragoso et al. (2017)
Rheumatoid arthritis	APL-CII	Rice seeds	100–120 μg	14 (daily)	IL-10 production in spleen; reduced joint inflammation	Lizuka et al. (2014)

LT-B: *E. coli* heat-labile enterotoxin B subunit; HBsAg: surface hepatitis B antigen; cEDIII-CTB: envelope protein domain III (cEDIII) fused to cholera toxin B subunit; CPFhW-HBcAg: Cysteine proteinases from *F. hepatica;* APL-CII: Altered peptide ligands fused of type II collagen; wk: weekly; Abs: antibodies

Table 3 Transgenic plants and antigens evaluated to design an anti cysticercosis vaccine

Plant	Antigen	Expression Vector	Transformation	Specie	/Pathogen/ #immunizations/ route	% Protection	References
Carica papaya L.	KETc1, KETc12, KETc7	pUI235–5.1	Bioballistics	Mouse	*T. crassiceps*/ Two/sc		Hernández et al. (2007), Rosales-Mendoza et al. (2012)
				Rabbit	*T. pisiformis* Two/oral		Betancourt et al. (2012)
				Mouse	*T. crassiceps*/ Two/oral	55–66	Fragoso et al. (2017)
				Pig	*T. solium*/Two/ oral	ND	
Nicotiana tabacum	KETc1 KETc12 KETc7, GK1 HP6/TSOL18	pBI-Helios2A polyprotein system	*A. tumefaciens GV3101* strain	Mouse	Three/sc	ND	Monreal-Escalante et al. (2015)
Daucus carota L.	HP6/TSOL18	pBin	*A. tumefaciens* GV3101 strain	Mouse	*T. crassiceps*/ Two/oral	80%	Monreal-Escalante et al. (2016)

sc subcutaneous

2007). The combination of three embryogenic transgenic papaya callus lines was designated as S3Pvac-papaya. The expression of the respective peptide in each clone was confirmed at the transcriptional level by RT-PCR. Soluble extracts from the transgenic papaya clones were found to be immunogenic when subcutaneously administered to mice. Indeed, all three clones expressing the vaccine peptides induced high levels of protection against murine cysticercosis when injected to mice (Hernández et al. 2007).

Furthermore, orally administered S3Pvac-papaya was found to be protective against murine and rabbit cysticercosis caused by *T. crassiceps* and *T. pisiformis,* respectively (Betancourt et al. 2012; Fragoso et al. 2017). The protective properties of the vaccine were maintained when formulated with different excipients that could eventually be attractive for pigs (Fragoso et al. 2017).

The effectiveness of the vaccine against these highly predictive experimental models let us consider its usefulness to be employed for pig cysticercosis prevention. To further examine this possibility, the immunity of pigs orally vaccinated with S3Pvac-papaya was explored. Oral vaccination with S3Pvac-papaya elicited an exacerbated humoral and cellular response in pigs (Fragoso et al. 2017).

Given the promising potential of papaya-made *T. solium* antigens, their expression in plant systems has been expanded to add new advantages to the plant-made vaccine candidates. The simultaneous expression of *T. solium* antigens in a single plant line would facilitate vaccine formulation; thus, innovative approaches have been recently explored to address this objective. An alternative Helios2A polyprotein system was developed, which relies on the use of the 2A sequence (LLNFDLLKLAGDVESNPG-P) of the foot and mouth disease virus that is placed between each of the antigens in a translational fusion arrangement. During the translation process of the polyprotein-encoding mRNA coding for the target antigens, the 2A sequence induces self-cleavage events by modifying the activity of the ribosome to allow hydrolysis of the ester linkage 2A-tRNAgly to be released, while the translation of the downstream product continues (Ryan and Drew 1994). Thus, this approach would allow the production of a multicomponent vaccine through the insertion of a single expression cassette coding for the polyprotein arrangement (Liu et al. 2007; Minskaia et al. 2013; Minskaia and Ryan 2013). Following a 2A-based polyprotein expression approach, a new multicomponent vaccine called Helios-2A, comprising the KETc1, KETc12, and KETc7 peptides from the S3Pvac along with the TSOL18/HP6-TSOL protective antigen was generated. The latter was included to assess whether vaccine efficacy is improved, since it has been reported as a highly protective antigen against porcine cysticercosis (Lightowlers et al. 2016), The Helios-2A system allowed the successful expression of the KETc1, KETc12, KETc7, GK1 (a short protective sequence inserted in the KETc7 peptide), and Tsol18/HP6 individual antigens in tobacco plants transformed with *Agrobacterium tumefaciens* at the nuclear level using the CaMV35S promoter. Interestingly, plant-made Helios-2A antigens were recognized by cerebral spinal fluid of neurocysticercosis patients and induced humoral responses in mice upon subcutaneous immunization (Monreal-Escalante et al. 2015). Although the efficacy of the Helios-2A is still under assessment, it is proposed as a highly convenient

vaccine that could be produced by propagating and characterizing a single transformed line (instead of the three lines required to formulate S3Pvac-papaya), and possibly conferring higher protection than the original S3Pvac vaccine.

Another innovation developed by our group consisted in the use of carrot cells as expression host to produce anti-cysticercosis vaccines. Carrot cell lines constitute a pioneering case in the molecular pharming arena, since the first commercialized plant-made biopharmaceutical was produced in this system. Thus, carrot was adopted to produce a candidate vaccine against cysticercosis based in cell lines expressing the TSOL18/HP6-Tsol antigen. Carrot lines were obtained by *A. tumefaciens* transformation at the nuclear level to express the TSOL18/HP6-Tsol antigen under the control of the CaMV35S promoter. Upon oral immunization with carrot-made TSOL18/HP6-Tsol, mice developed humoral responses and were protected against *T. crassiceps* challenge (Monreal-Escalante et al. 2016). Immunization trials to compare the efficacy of this vaccine with that of S3Pvac are ongoing.

Looking to enhance antigen yields, transplastomic approaches have been implemented to produce the target *T. solium* antigens. The S3Pvac-papaya components were produced along with the TSOL18/HP6-Tsol antigen in tobacco plastids.

Synthetic operons under the control of the Prnn promoter led to the expression of individual target antigens through a single transformation event. Chloroplast-made antigens retained their immunogenic properties, as revealed by immunization experiments in mice. The immunoprotective properties of this transplastomic vaccine are currently being assessed (Rosales-Mendoza et al., unpublished). As an additional advantage, this vaccine offers enhanced biosafety with respect to the nuclear transformed plants, since plastomes are maternally inherited, and thus transgene transmission via pollen is avoided. Thus, the transplastomic approach is likely to yield an optimized anti-cysticercosis vaccine; however, its detailed characterization and the assessment of its protective efficacy are still in progress.

2 Conclusions and Perspectives

Cysticercosis control is theoretically possible, and the disease was declared to be eradicable by the International Task Force for Disease Eradication in 1993. However, *T. solium* cysticercosis persists to date, and new cases are continually reported in non-developed countries, where the parasite life cycle is well established, and also in developed regions due to immigration of infected individuals. Control strategies based on mass-treatment for human taeniasis in identified transmission foci have been proposed by WHO (2010) and the Pan American Health Organization. The inclusion of an effective oral, low-cost vaccine that could be administered directly by pig owners may significantly improve the effectiveness of a control program. The production of anti-cysticercosis vaccines using plants can accomplish this goal. Substantial advances have been achieved over the last

10 years in this area. Both nuclear and transplastomic approaches have been assessed to test the biosynthetic capacity of plants to produce immunoprotective *T. solium* antigens. S3Pvac-papaya vaccine was a pioneering case for a vaccine tested in the field (Hernández et al. 2007); this first experience demonstrated that plants are promising biofactories for anti-cysticercosis vaccines, and justify the projection to generate other vaccine candidates, facilitate vaccine formulation, and maximize antigen productivity. The promising results reported in pigs prompt us to start the scale-up process to produce an oral vaccine in airlift bioreactors and obtain enough material for conducting field trials. On the other hand, a vaccine based in carrot cell lines expressing the TSOL18/HP6-Tsol provided the first evidence on the production of the functional antigen at appropriate levels to immunize mice. Since TSOL18/HP6-Tsol also confers immunoprotection against *T. saginata* and the S3Pvac peptides are highly conserved in this parasite, this vaccine candidate will allow us not only to perform studies on its role as a supplementary antigen for the S3Pvac vaccine, but also to develop a new anti-*T. saginata* vaccine for cows and cattle (Parkhouse et al. 2008).

Vaccines produced in tobacco, either by nuclear or plastid expression, exemplify the potential of synthetic polycistrons and viral sequences to engineer plant cells as efficient biofactories to produce multicomponent vaccines in a single transformed line. This expression modality will greatly facilitate vaccine formulation, since the upstream process will deal with a single seed stock, and during downstream processing a single line will be used for antigen quantification and encapsulation prior to dosage. Preclinical evaluation of these 'single line' vaccine candidates will be completed soon, and will provide the basis for field evaluations in pigs.

In conclusion, plants have proven to be suitable platforms to produce anti-cysticercosis vaccines, and promising prospects are being projected in terms of field evaluations and the development of innovative candidates, based on alternative expression approaches. Such plant-based vaccines will be valuable tools to control cyticercosis especially in poor countries, since formulations based on freeze-dried plant biomass have very low production costs and lower logistic costs, since they are easy to apply and do not require purification, cold-chain, sterile devices nor trained personnel to be applied. Altogether, these features would make a more robust and easier to handle vaccine (Hirlekar and Bhairy 2017).

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