

Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine

V. Yusibov^a, D.C. Hooper^a, S.V. Spitsin^a, N. Fleysh^a, R.B. Kean^a, T. Mikheeva^a, D. Deka^a,
A. Karasev^a, S. Cox^a, J. Randall^b, H. Koprowski^{a,*}

^a Biotechnology Foundation Laboratories at Thomas Jefferson University, 1020 Locust Street, Room 346 JAH, Philadelphia, PA 19107, USA

^b Department of Family Medicine, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA 19107, USA

Received 18 May 2001; received in revised form 12 March 2002; accepted 13 May 2002

Abstract

A new approach to the production and delivery of vaccine antigens is the use of engineered amino virus-based vectors. A chimeric peptide containing antigenic determinants from rabies virus glycoprotein (G protein) (amino acids 253–275) and nucleoprotein (N protein) (amino acids 404–418) was PCR-amplified and cloned as a translational fusion product with the alfalfa mosaic virus (AIMV) coat protein (CP). This recombinant CP was expressed in two plant virus-based expression systems. The first one utilized transgenic *Nicotiana tabacum* cv. Samsun NN plants providing replicative functions in trans for full-length infectious RNA3 of AIMV (NF1-g24). The second one utilized *Nicotiana benthamiana* and spinach (*Spinacia oleracea*) plants using autonomously replicating tobacco mosaic virus (TMV) lacking native CP (Av/A4-g24). Recombinant virus containing the chimeric rabies virus epitope was isolated from infected transgenic *N. tabacum* cv. Samsun NN plants and used for parenteral immunization of mice. Mice immunized with recombinant virus were protected against challenge infection. Based on the previously demonstrated efficacy of this plant virus-based experimental rabies vaccine when orally administered to mice in virus-infected unprocessed raw spinach leaves, we assessed its efficacy in human volunteers. Three of five volunteers who had previously been immunized against rabies virus with a conventional vaccine specifically responded against the peptide antigen after ingesting spinach leaves infected with the recombinant virus. When rabies virus non-immune individuals were fed the same material, 5/9 demonstrated significant antibody responses to either rabies virus or AIMV. Following a single dose of conventional rabies virus vaccine, three of these individuals showed detectable levels of rabies virus-neutralizing antibodies, whereas none of five controls revealed these antibodies. These findings provide clear indication of the potential of the plant virus-based expression systems as supplementary oral booster for rabies vaccinations. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Alfalfa mosaic virus; Plant virus-based vaccines; Immunogenicity

1. Introduction

Rabies virus, a rhabdovirus of the genus *Lyssavirus*, remains a significant threat to human and animal health throughout much of the world [1]. In the US alone, 25,000–40,000 persons annually undergo rabies prophylaxis for possible exposure to the virus [2]. According to World Health Organization estimates, there are approximately 50,000 cases of fatal rabies worldwide each year [3]. Free-ranging wildlife, including bats, raccoons, skunks, and foxes are the major reservoirs of rabies virus in the US. In Asia, Africa, and Latin America dogs are important vectors of the virus posing a serious threat to humans. In the last two decades, the extent of wildlife rabies has increased considerably. For example, in the US more than 50,000

cases of rabies among raccoons have been reported to the Centers for Disease Control and Prevention since 1980 [4]. The increase in cases associated with bat rabies is particularly troubling, since from 1980 to 1996, 21/36 human rabies cases were attributed to bats in the US alone [3].

Currently, efficacious rabies vaccines are used in humans for pre- and post-exposure prophylaxis, yet in countries where rabies is endemic, routine vaccination of the general population might be an alternative approach if rabies cannot be contained. The least expensive rabies vaccines, which are still used extensively, are prepared from animal brain and thus, potentially hazardous. Affordable and safe rabies vaccines for humans and animals are needed, preferably utilizing the existing local infrastructure. This is important not only for rabies but also for other infectious diseases prevalent in developing countries.

In the design of a rabies vaccine, representation of both the rabies virus glycoprotein (G protein) and nucleoprotein

* Corresponding author. Tel.: +1-215-503-4761; fax: +1-215-923-6795.
E-mail address: h.koprowski@hendrix.jci.tju.edu (H. Koprowski).

(N protein) antigens is desirable. The G protein is the major antigen responsible for the induction of protective immunity [5], while the N protein triggers rabies virus-specific T cells, facilitating the production of neutralizing antibodies and other immune mechanisms [6]. The only widely used recombinant vector-based vaccine at present consists of vaccinia virus expressing the rabies virus G protein [7]. However, the release of genetically modified live viruses, capable of infecting a variety of species including humans, may pose a safety threat. Instead, the safe and cost-effective management of rabies may depend on the introduction of non-infectious, recombinant vaccines that are effective after oral administration. In this context, oral vaccination of raccoons with baculovirus-expressed G protein or with inactivated rabies virus has been shown to induce production of rabies virus-neutralizing antibodies and protection against a lethal challenge with rabies virus [8,9]. Moreover, oral administration of rabies virus ribonucleoprotein complexes significantly enhanced production of virus-neutralizing antibodies upon subsequent parenteral booster vaccination of mice with inactivated rabies virus [10]. Those studies demonstrated that rabies G and N proteins contain determinants that can be used for oral immunization. Indeed, antigenic determinants of rabies virus G and N proteins have been mapped, and a chimeric peptide (G5–24-N31D) containing a linear epitope of the G protein and an epitope of the N protein were synthesized and found to be immunogenic in mice [11].

Several studies have explored the feasibility of using plant viruses as expression vectors for vaccine antigens [12–21]. Our studies have focused on the use of alfalfa mosaic virus (AIMV) [21] and the tobacco mosaic virus (TMV)-based hybrid vector Av/A4 [22] to express the chimeric peptide G5–24-N31D in virus-infected plants as an in-frame fusion with the coat protein (CP) of AIMV and assembled into virions. Previously, we demonstrated synthesis of mucosal IgA in mice fed on spinach leaves infected with recombinant virus bearing G5–24-N31D [19]. In the present study, we show that mice immunized with recombinant virions recovered from infected tobacco plants are protected against a lethal challenge infection with rabies virus, and that human volunteers fed raw spinach leaves containing experimental plant virus-based rabies vaccine develop a rabies virus-specific immune response.

2. Materials and methods

2.1. DNA constructs

All cloning and cell transformations were performed according to Sambrook et al., [23]. *Escherichia coli* JM109 competent cells (Promega, Madison, WI) were used for transformation. Two recombinant plasmids NF1-g24 and Av/A4-g24 (Fig. 1), were constructed through the in-frame fusion of chimeric peptide representing rabies G (amino

acids 253–275) and N (amino acids 404–418) proteins [24] to the N-terminal coding sequences of the AIMV CP. Sequences encoding chimeric peptide from rabies virus was PCR-amplified and cloned into pSPΔAUG [25], using 5'CGGCTCGAGATGTCCGCAGTATATACCCGATTATGATGAACGGAGGACGACTCAAACGACCCCCAGACCAACTTGTGAACCTC3' as 5'- and 5'GCTGTGACCTCTCTTCCACTACCAGGTGTTTCGATCTCATCCGATCGGAAGTCATGGAGGTTCAAGTTGGTCTGGGGTTCGTTT3' as 3'-primers. These primers contain complementary regions and serve as a template for each other. The resulting PCR product contains introduced *Xho*I and *Sal*I restriction sites (5' and 3', respectively) for ligation into the unique *Xho*I site in pSPΔAUG. Translation of the recombinant CP is initiated from the AUG codon introduced at the 5' end of the recombinant gene, which is upstream of nucleotide sequences encoding the chimeric rabies virus peptide. After sequence confirmation, the recombinant CP containing sequences for the rabies virus G and N proteins was subcloned into full-length RNA3 of AIMV to create NF1-g24 (Fig. 1) or into Av/A4 [22] to obtain Av/A4-g24 (Fig. 1). Expression of the recombinant CP from NF1-g24 and Av/A4-g24 during virus infection of plants is controlled by subgenomic mRNA promoters specific for AIMV and TMV CP, respectively.

2.2. In vitro transcription

In vitro transcripts of recombinant molecules were synthesized using T7 RNA polymerase (Promega, Madison, WI) and plasmid DNA, according to the manufacturer's guidelines. Transcripts were capped using the RNA cap structure analog m7G(5)ppp(5)G (New England Biolabs, Beverly, MA).

2.3. Plant inoculation and virus purification

All plants used in these experiments were grown and maintained in a controlled BL2P greenhouse. For animal experiments, recombinant viral construct NF1-g24 was produced in transgenic *Nicotiana tabacum* cv. Samsun NN plants expressing the AIMV P1 and P2 (P12) replicase genes [26]. Three upper leaves of each plant were inoculated with a mixture (RNA4:RNA3, 1:1000) of in vitro transcription products of recombinant constructs in 2 volumes (v/v) of FES buffer [sodium-pyrophosphate 1% (w/v), macaloid 1% (w/v), celite 1% (w/v), glycine 0.5 M, K₂HPO₄ 0.3 M, pH 8.5, with phosphoric acid]. Inoculum was applied by gentle rubbing on leaves after abrading the leaf surface with carborundum (320 grit; Fisher, Pittsburgh, PA). Recombinant virus was isolated 12–14 days after the inoculum was applied as described [18]. Briefly, leaf tissue was homogenized and the sap separated from cell debris by centrifugation. Virus particles were selectively precipitated using 5% polyethylene glycol. To study the immunogenicity of the experimental rabies vaccine administered orally, three lots of

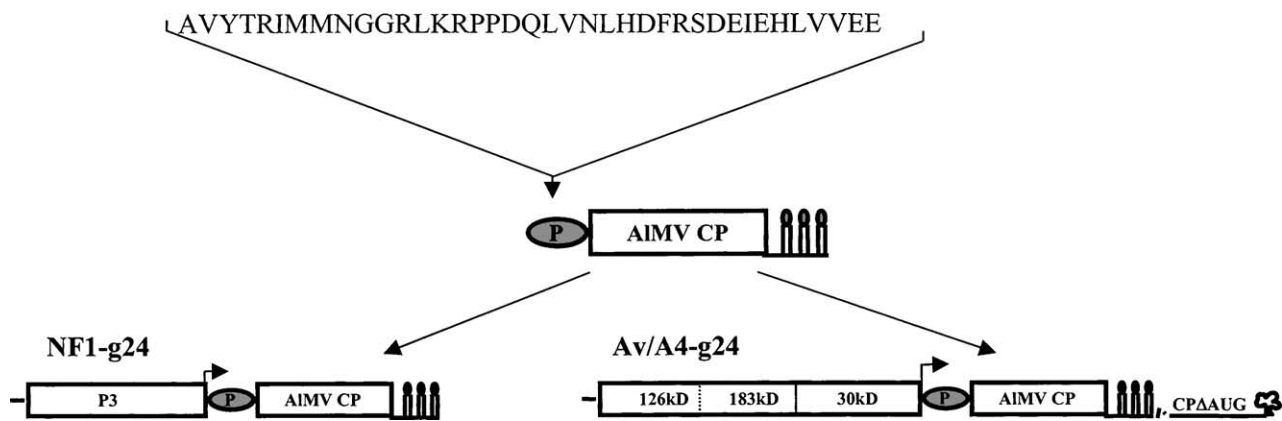


Fig. 1. Schematic representation of NF1-g24 and Av/CP-g24. NF1-g24 was engineered using AIMV RNA3 and sequences of chimeric peptide representing rabies virus G and N proteins. P3, cell-to-cell movement protein; CP, coat protein; P, foreign peptide. Av/CP-g24 is based on the genome of TMV-derived hybrid Av/A4, with systemic movement supported by the introduced AIMV CP. The 126 and 183 kDa proteins are required for replication, the 30 kDa is the viral movement protein, and CPΔAUG indicates the open reading frame of the translation deficient TMV CP gene. T-RNA like structure at the 3' end of Av/A4-g24 represents a ribozyme for self-cleavage of in vitro-synthesized transcripts. Arrowheads within each construct indicate the subgenomic promoters for AIMV and TMV CP, respectively. Amino acid sequences fused at the N-terminus are indicated.

spinach plants (3000 plants per lot) were inoculated with in vitro transcription products of Av/A4-g24, harvested 12–14 days later, washed, analyzed for the presence of rabies virus antigen, packaged in 150 g doses and fed to human volunteers (see Section 2.4).

2.4. Western blot analysis

AIMV CP produced in virus-infected plants was analyzed by Western blotting [18] using antibodies specific for AIMV CP (Agdia, Elkhart, IN) or rabies virus G protein. Proteins from crude plant extracts or from purified virus particles were separated electrophoretically on SDS-polyacrylamide gels and electroblotted onto a nylon membrane overnight at 33 mA. After blocking with milk (Kirkegaard and Perry; Gaithersburg, MD), proteins were allowed to react with appropriate antibodies and detected using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

2.5. Immunization of mice with recombinant plant virus particles and challenge

Eight-week-old C3H mice (10 in each group) were injected intraperitoneally three times at 2-week intervals with purified particles of NF1-g24 (250 µg per injection), in the presence of Freund's complete (first injection) or incomplete (second) adjuvant, while control groups received synthetic rabies virus peptide (35 µg per injection) or AIMV particles without the rabies insert (250 µg per injection). A 250 µg dose of recombinant NF1-g24 contains 35 µg of chimeric rabies peptide. Serum samples were collected on days 0, 28 and 40. At 120 days after the last immunization, mice were challenged intramuscularly with canine street rabies virus 3374L and observed for clinical signs of rabies infection. Animals were euthanized by CO₂ inhalation. Brains were

removed and tested for rabies by the fluorescent antibody test.

2.6. Neutralization assay

Sera from mice, inoculated with recombinant AIMV particles containing chimeric rabies virus peptide, synthetic rabies virus peptide and control virus (AIMV) were heat-inactivated at 56 °C for 30 min and incubated with CVS-11 strain rabies virus. The neutralizing activity of rabies virus-specific serum antibodies was determined using a rapid fluorescent focus inhibition test, as described [27]. Similarly, rabies-specific virus-neutralizing antibody titers were assessed in sera collected from human volunteers.

2.7. Ingestion of raw spinach leaves containing experimental rabies vaccine by human volunteers

2.7.1. Group 1

Five individuals of both genders, immunized against rabies virus, were fed with 20 g (0.6 mg of recombinant virus contains 84 µg of chimeric rabies peptide) of raw spinach leaves containing rabies virus antigen. Each individual received three doses of experimental vaccine at 2-week intervals. The control group (five individuals immunized against rabies virus), received raw spinach leaves only. Blood samples (10 ml) were obtained by venipuncture on days 0, 14, 28 and 42. Sera were analyzed by ELISA to determine if there is an increase in rabies virus-specific antibodies resulting from experimental spinach feeding.

2.7.2. Group 2

Nine volunteers (non-immunized against rabies virus), between ages 21 and 60 (both genders) were fed three times with fresh spinach leaves containing rabies virus antigen at

2-week intervals. Individuals received 150 g of leaf tissue, equivalent to a medium-size salad, in each feeding. This amount of leaf tissue contained approximately 5 mg of virus, which equaled to 700 µg of rabies peptide per dose. The control group (five non-immunized volunteers) was fed spinach leaves without rabies peptide per dose. Seven days after the third feeding (day 35), all participants received a single dose (boost) of commercial rabies vaccine intramuscularly. Blood samples (10 ml) were obtained by venipuncture at days 0, 35 and 42. Sera were analyzed for rabies virus-specific antibodies and for any boosting effect of the commercial vaccine.

2.8. ELISA

Sera were analyzed for the presence of antigen-specific antibody by solid phase ELISA as described [18], using 96-well plates coated overnight at 24 °C with 100 µl per well of inactivated ERA strain rabies virus or synthetic peptide G5–24. Peroxidase-conjugated goat anti-mouse IgG (whole) (Sigma) was used as secondary antibody for mouse sera, and biotin-conjugated goat anti-human IgG (H plus L) (Rockland, Gilbertsville, PA) for human IgG as well as biotin-conjugated mouse monoclonal anti-human IgA1/IgA2 (Pharmingen, San Diego, CA) for human sera.

3. Results

3.1. Plant virus-based expression systems

Two plant virus-based expression systems were used. The first one was based on the full-length infectious cDNA clone of AIMV RNA3. AIMV is a tri-*p*[artite] plant positive-strand ssRNA virus. RNA1 and RNA2 are indispensable for AIMV replication, while RNA3 is necessary for cell-to-cell and long distance movement, and also codes for the viral CP. The CP is expressed from a small subgenomic RNA4. Besides the assembly function, the CP is also involved in the AIMV genome activation, and thus either CP or its messenger, RNA4, has to be present in the inoculum to initiate normal AIMV genome replication. Therefore, the RNA3 transcripts used for inoculations were always supplemented with the RNA4 transcript to provide sufficient amount of the CP to initiate infection [25]. RNA1 and RNA2 code for replication-associated proteins P1 and P2, and in this particular system are provided in trans in P12 transgenic tobacco plants [26].

The second system used is based on an autonomously replicating TMV-based Vector Av/A4 which lacks the TMV CP. In this particular system it was replaced by a fusion between the rabies peptide, g24, and the AIMV CP. The recombinant virus, a TMV-derivative carrying the AIMV gene was shown previously to be viable and even able to utilize AIMV for the long distance movement through the plant [22].

3.2. Expression of chimeric rabies peptide in plants inoculated with NF1-g24 of Av/A4-g24

3.2.1. NF1-g24

To express chimeric rabies virus peptide fused to AIMV CP at the N-terminus, greenhouse-grown transgenic P12 plants at the six-leaf stage were mechanically inoculated with a mixture of in vitro synthesized transcripts of NF1-g24 (AIMV genomic RNA3) and AIMV RNA4. Within 10–12 days of inoculation, accumulation of recombinant AIMV CP containing rabies virus antigen was detected in the upper uninoculated leaves. Amplification of recombinant NF1-g24 in locally inoculated leaves resulted in occasional necrotic lesions surrounded with concentric rings. Movement of recombinant virus into the upper uninoculated leaves and systemic spread of infection resulted in mild mosaic symptoms, vein clearing, slight curling of the uppermost leaves, and stunting of plant growth. As the leaves grew larger, signs of virus infection began to regress and leaf curling was reduced, although rare localized areas of vein necrosis, appearing on systemically infected leaves, as well as necrotic lesions on locally inoculated leaves remained present throughout the infection. Over a 2-week period, significant quantities of recombinant CP accumulated in systemically infected leaves, with average levels reaching 0.4 ± 0.07 mg/g of fresh leaf tissue. Up to 70% of expressed recombinant protein (0.3 ± 0.03 mg/g of fresh tissue on average) was recovered from infected leaf tissue as virus particles. Western analysis of recombinant virions isolated from locally inoculated and systemically infected leaves revealed a protein of the expected size (29.3 kDa), which reacted with antibodies specific for both AIMV CP (carrier molecule) and rabies virus G protein (Fig. 2). Native AIMV CP reacted only with AIMV CP-specific antibodies (Fig. 2A) and did not bind antibodies specific for rabies virus G protein (Fig. 2B). Together, these data demonstrate that when expressed in plants, recombinant AIMV CP carrying the rabies virus peptide is assembled into virions that can be recovered efficiently and at high homogeneity by standard virus purification procedures.

3.2.2. Av/A4-g24

Leaves of 6-week-old spinach plants were mechanically inoculated with in vitro synthesized transcripts of Av/A4-g24. Within 10–12 days of inoculation (the time established as optimal for maximum accumulation of Av/A4-g24 while plants are still suitable for ingestion), samples of leaf tissue were randomly collected and analyzed by Western blotting. Accumulation of recombinant CP was detected in both inoculated and uninoculated leaves (Fig. 3), indicating systemic movement of the recombinant virus in the plants. Protein of the expected size (19.3 kDa) was detected with antibodies specific for both AIMV CP and rabies virus G protein (Fig. 3). However, the virus was not detected in all leaves analyzed. Approximately 70% of the leaves, as determined by Western analysis (data

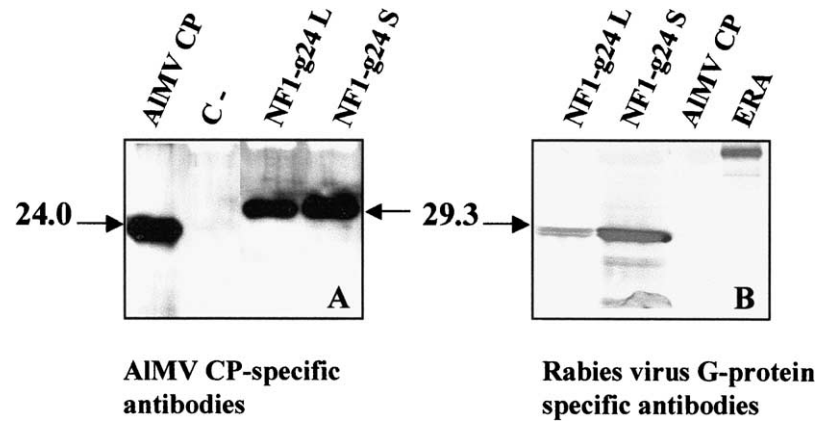


Fig. 2. Western blot analysis of recombinant CP in virus particles purified from leaves (locally inoculated (L) and systemically infected (S)) of P12 transgenic plants infected with wild-type AIMV or NF1-g24. Proteins were separated electrophoretically on a 12% SDS-polyacrylamide gel, transferred to a membrane, and detected with different antibodies. Monoclonal antibodies specific for AIMV CP (panel A) recognized 24.0 kDa (AIMV CP) and 29.3 kDa (NF1-g24) proteins, whereas antibodies specific for rabies virus G protein recognized only NF1-g24 and G protein from rabies virus (ERA strain, panel B). Wild-type-AIMV did not react with antibodies specific for rabies virus. Extracts from uninoculated (C-) plants showed no reactivity for AIMV CP-specific antibodies.

not shown), were infected, containing sufficient levels of recombinant CP to recover up to $50 \pm 12 \mu\text{g}$ of recombinant virus from 1 g of fresh tissue. Amounts of recombinant Av/A4-g24 recovered from spinach plants were similar to those of Av/A4 ($60 \pm 9 \mu\text{g/g}$ fresh tissue). Infection of only 70% of leaves might reflect the absence of a stem structure in spinach plants at 6 weeks of age, which might have impeded the uniform movement of virus into all uninoculated leaves. The Av/A4-g24-infected spinach plants showed no evidence of symptoms or any significant reduction in leaf growth as compared to that of controls.

Western blot analysis of unprocessed spinach leaves stored at 4°C revealed the presence of recombinant AIMV

CP containing rabies peptide, which was detectable in leaves up to 35 days of storage (data not shown). Thereafter, tissue degradation became visible and the recombinant protein was no longer detectable.

3.3. Immunization of mice with recombinant AIMV particles containing chimeric rabies peptide

C3H mice (10 in each group) were given three doses of recombinant virus particles intraperitoneally at 2-week intervals. Each mouse received $250 \mu\text{g}$ of virus per dose equivalent to $35 \mu\text{g}$ of rabies peptide. Mice in control groups received either $35 \mu\text{g}$ of synthetic peptide or $250 \mu\text{g}$

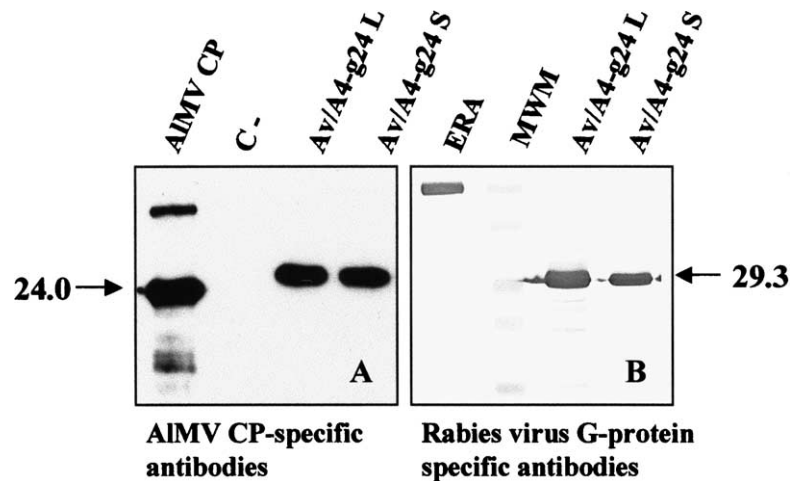


Fig. 3. Western blot analysis of recombinant CP accumulation in spinach leaves (locally inoculated (L) and systemically infected (S)) infected with Av/A4-g24. Proteins were separated electrophoretically on a 12% SDS-polyacrylamide gel, transferred to a membrane, and detected with different antibodies. Monoclonal antibodies specific for AIMV CP recognized 24.0 kDa (AIMV CP) and 29.3 kDa (Av/A4-g24) proteins (panel A), whereas antibodies specific for rabies G protein recognized Av/A4-g24 and G protein from rabies virus (ERA strain) (panel B). Extracts from uninoculated (C-) plants were not recognized by AIMV CP-specific antibodies. MWM is molecular weight marker lane.

Table 1

Antibody response and survival of C3H mice immunized with AIMV containing chimeric peptide from rabies virus

Group of mice	Codes	Dose of antigen (μg per dose)	Rabies neutralizing antibodies	Survival after challenge with rabies virus
NF1-g24	A	250	4/5	5/5
	B		5/5	5/5
AIMV	C	250	0/5	2/5
	D		0/5	0/5
G5–24–N31D	E	25	1/5 ^a	1/3 ^a
	F		4/5	2/4 ^b

Mice received three doses of antigen intraperitoneally on days 1, 14 and 28. Serum samples for rabies virus-neutralizing antibody analysis were collected 12 days after the last immunization. At 120 days after the last immunization, mice were challenged intramuscularly with a lethal dose of rabies virus.

^a Two mice died before challenge.

^b One mouse died before challenge.

of wild-type AIMV particles per dose. At 12 days after the third immunization, serum samples from 9/10 mice immunized with recombinant NF1-g24 particles showed the presence of rabies neutralizing antibodies (Table 1). At 120 days after the last immunization, mice were challenged intramuscularly with 50 μl of canine street rabies virus 3374L ($10^{7.7}$ MICLD₅₀) and observed for clinical signs of infection. All 10 mice immunized with NF1-g24 particles survived the challenge, while 3/7 mice that received the synthetic rabies peptide and 2/10 that received the wild-type AIMV survived (Table 1). The remaining mice developed clinical signs of rabies and succumbed to infection 10–20 days after rabies virus challenge. No rabies virus was isolated from the tissue of mice surviving challenge 5 months after challenge.

3.4. Immunogenicity of experimental plant virus-based rabies vaccine consumed in food

As an initial assessment of the immunogenicity of Av/A4-g24 in humans, five volunteers who had previously received a full course of conventional rabies virus vaccine (four intramuscular injections) were fed three doses of raw spinach leaves containing rabies antigen (each 20 g containing 0.6 mg of recombinant virus equivalent to 84 μg chimeric rabies peptide) at 2-week intervals. Five control individuals received spinach inoculated with the recombinant Av/A4 vector alone. ELISA analysis of sera collected before feeding (pre-F) and after the third feeding (post-F) revealed a significant increase in the already high levels of rabies-specific antibodies in individuals 4, 7 and 9 (Fig. 4A;

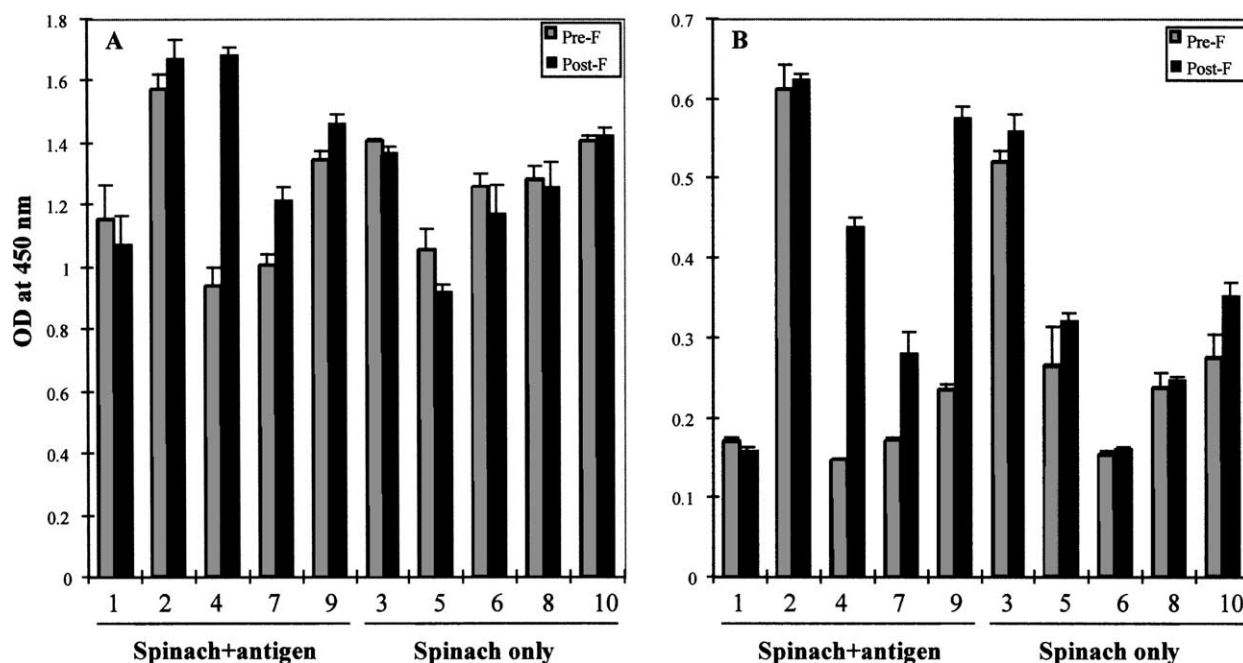


Fig. 4. Rabies virus-specific serum antibody (IgG) response generated in human volunteers after injection of spinach infected with Av/A4-g24. Rabies virus-specific serum antibody responses were measured by ELISA on plates coated with either inactivated rabies strain ERA (panel A) or synthetic peptide resembling the linear epitope G5–24 of rabies virus G protein. Data are mean values of six replicates obtained using sera collected on day 0 of trial (pre-F) and after the third (post-F) feeding. Numbers on the axis indicate volunteer designations.

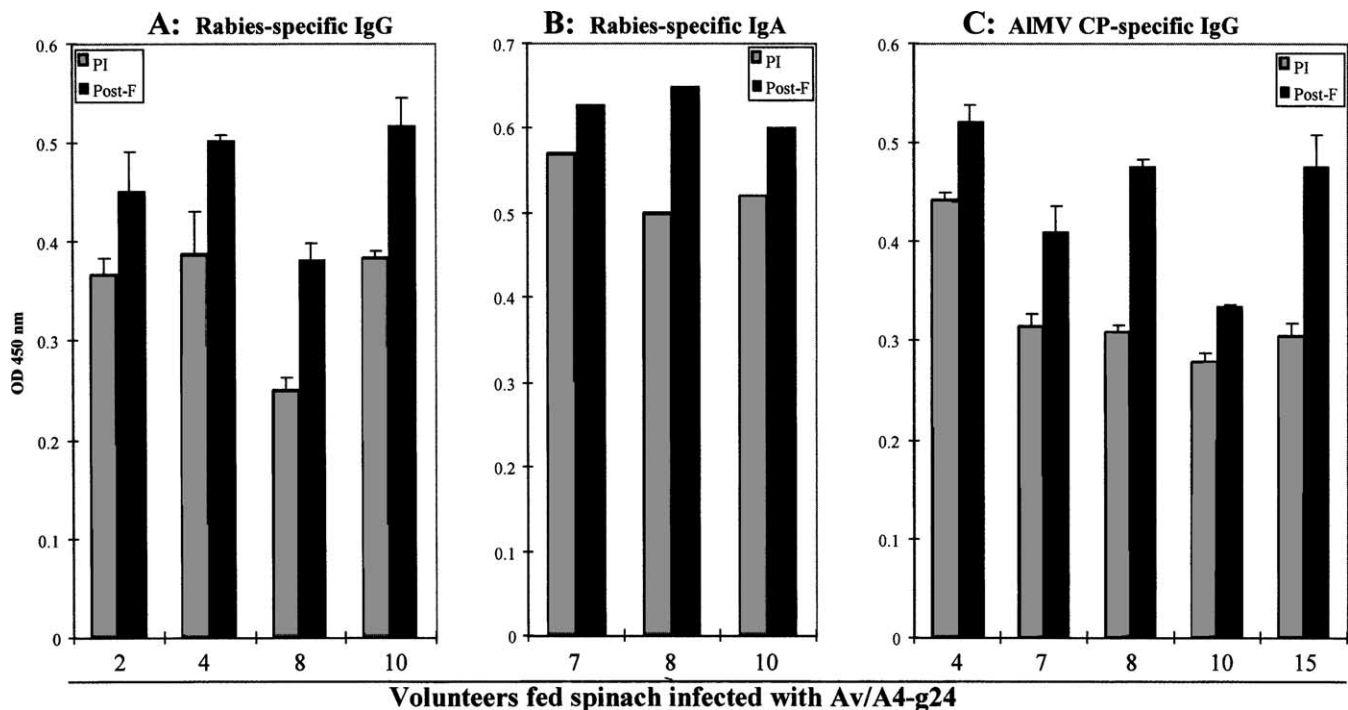


Fig. 5. Rabies- and AIMV CP-specific serum antibody (IgG and IgA) response generated in immune-naïve human volunteers after ingestion of spinach infected with Av/A4-g24. Rabies-specific and AIMV-specific serum antibody responses were measured by ELISA on plates coated with inactivated rabies strain ERA (A and B) and on plates coated with AIMV CP (C), respectively. Data are mean values of six replicates (except in B) obtained using pre-immune and sera after third administration of antigen. Numbers on the axis indicate volunteer designations.

$P = 0.003$, 0.001 and 0.005 , respectively) at a serum dilution of 1:164. Increases in rabies-specific serum IgG antibodies became even more obvious in ELISA performed using G5-24 peptide-coated plants (Fig. 4B; individuals 4, 7 and 9; $P = 0.002$, 0.007 and 0.005 , respectively). Elevation in rabies-specific serum IgG levels in these three volunteers was observed after each dose of spinach infected with the recombinant virus (data not shown). One of the two individuals who did not respond to the peptide had exhibited high levels of peptide-specific antibody prior to the study. Rabies virus-specific antibodies did not become elevated in any of the control individuals. Throughout these studies, there were no instances of nausea, vomiting, mild cramps, fever or diarrhea among volunteers after ingestion of Av/A4-g24-infected spinach or spinach only.

Based on the lack of any evident untoward effect on pre-existing immunity to rabies virus, together with some evidence of enhanced immunity against the rabies virus peptide, the immunogenicity of this recombinant virus was assessed in non-vaccinated individuals who had no rabies antibodies in their blood. Nine volunteers received three doses of raw spinach leaves (150 g per dose) infected with the recombinant virus, while five volunteers received an equal amount of healthy spinach. Seven days after the third feeding, all participants received a single dose of commercial rabies vaccine (HDCV). In addition to pre-immune samples, sera were collected 7 days after the third feeding and 7 days after administration of the commercial vaccine.

Each serum sample collected before administration of the commercial vaccine was assessed for antibodies (IgG and IgA) against rabies virus, AIMV, and for neutralization (data not shown). At serum dilutions of 1:10, no control samples showed significant elevations in antibodies reactive with either AIMV CP or rabies virus. On the other hand, 6/9 volunteers who had ingested spinach infected with the recombinant virus showed significant elevations in serum antibodies specific for either AIMV CP or rabies virus (Fig. 5A–C). Four volunteers produced rabies virus-specific IgG (Fig. 5A) and two of these four and volunteer 7 also produced rabies virus-specific IgA (Fig. 5B). Four individuals who produced rabies virus-specific IgG or IgA (volunteers 4, 7, 8 and 10) as well as volunteer 15 demonstrated significant elevations in AIMV CP-specific IgG (Fig. 5C). While no neutralizing antibody was detected before immunization with commercial vaccine, only recipients (3/9) of Av/A4-g24-infected spinach became positive for rabies virus-neutralizing antibodies following parenteral administration of a single dose of conventional vaccine (Fig. 6).

4. Discussion

The identification of key antigenic determinants responsible for the generation of protective immunity against corresponding viral or bacterial pathogens has enabled the development of subunit vaccines. Non-replicating subunit

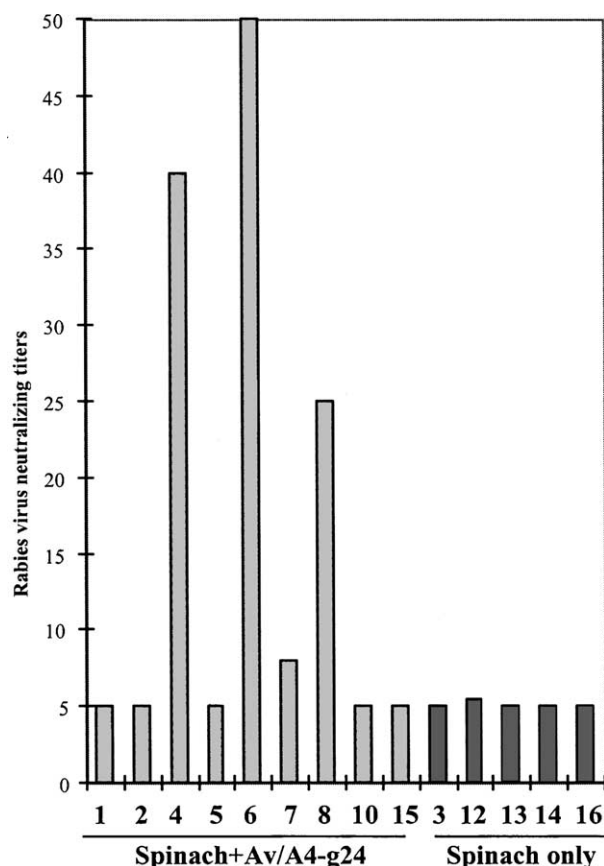


Fig. 6. Rabies virus-neutralizing antibody titers in sera of individuals who received a single dose of commercial rabies vaccine 7 days after the third feeding with spinach. Sera were collected 7 days (on day 42) after the administration of the commercial rabies vaccine. Numbers on the axis indicate volunteer designations.

vaccines are safer than pathogen-based vaccines and can be readily formulated to contain multiple antigenic determinants from different pathogens. Consequently, subunit vaccines are preferred for manufacturing purposes. However, traditional procedures for the development, storage and distribution of subunit vaccines remain costly, thus limiting their global accessibility.

In recent years, several groups have begun to investigate the utility of plants as vehicles for the expression of vaccine antigens [28–36]. One approach involves the engineering of plant virus CPs as carrier molecules for fused antigenic peptides. Such carrier proteins can self-assemble and form virus particles displaying the desired antigenic peptide on their surfaces. This may be expected to be more immunogenic than synthetic peptide alone. Here, we used AIMV- and TMV-based plant virus expression vectors to produce a chimeric peptide from rabies virus as an in-frame fusion with the AIMV CP. We previously have demonstrated that immunogenic rabies peptide could be expressed using TMV-based vector 30B [18]. However, the quantities of recombinant protein obtained using that vector were somewhat limited (5–10% of total viral protein purified). In the

case of AIMV-based NF1-g24 or the hybrid Av/A4-g24, every subunit of virus CP contains the rabies virus peptide. Thus, significantly higher quantities of the antigen are produced. It has been demonstrated by a number of groups that plant virus particles containing foreign peptides can be readily purified and used to induce protective immunity in animals [20,21]. Until the present investigation, the longevity of protective immune responses induced by plant virus-expressed antigens remained unclear [18–21]. Here, we demonstrate that mice remain protected against challenge with a lethal dose of rabies virus for at least 120 days following parenteral immunization with three doses of recombinant NF1-g24 particles. This suggests that the reagent establishes conventional immunological memory to the rabies virus epitope. Moreover, immunization of mice with the peptide expressed in the context of the plant virus particle appeared to be more immunogenic than administration of an equivalent amount of the same peptide in adjuvant.

While parenteral immunization with a recombinant plant virus evidently can be effective, the ultimate objective for such reagents is oral immunization which is more suited for widespread use. The successful use of transgenic plants to orally deliver vaccine antigens, such as *E. coli* heat-labile enterotoxin [35], Norwalk virus capsid protein [36], and hepatitis B virus surface antigen [34], to human volunteers has been reported. We demonstrate here that the same goal can be achieved using plant tissue infected with a recombinant virus. Fresh spinach leaves infected with virus expressing rabies antigenic determinants were consumed by two groups of volunteers, one consisting of individuals who had previously been vaccinated against rabies virus, and the second composed of rabies-naïve individuals. Despite the fact that this could only have resulted in exposure to relatively small doses of antigen, this resulted in elevations of antigen-specific serum IgG levels in 3/5 of the previously immunized subjects. Different patterns of reactivity to the rabies and AIMV antigens were seen in the individuals from previously rabies non-immune group. Low but significant elevations in rabies-specific serum IgG levels were seen in 5/9 volunteers after three doses of infected spinach leaves. Evidence of increased serum rabies-specific IgA antibodies was detected in 3/5 individuals while two of these and one other individual also had significantly increased levels of AIMV CP-specific serum IgG. Although none of these volunteers showed rabies virus-neutralizing antibodies following the feeding regimen, 3/9 had significant levels of rabies virus-neutralizing antibody in sera following receipt of a single parenteral dose of commercial rabies vaccine. This contrasts with the fact that one of the five individuals that received control spinach developed rabies virus-neutralizing antibodies following similar administration of the commercial vaccine. Taken together, these findings suggest that engineered plant viruses delivered in edible material may be a viable approach to the development of vaccines for widespread distribution. The induction of tolerance to the peptide antigen expressed in the context of a plant virus

in plant material was evidently not an issue. While only low rabies virus-specific antibody levels were detected in select individuals, this outcome is not unexpected due to exposure to a low amount of peptide which may differ in immunogenicity in different individuals. We speculate that this response may have been facilitated by contact with the antigen in the oropharynx during the chewing process.

In summary, plant virus vectors can be used as an alternative system to express foreign sequences with specific biological activity. When fused to viral CPs, these peptides become part of a virus particle which can be easily recovered from infected plant tissue to a high homogeneity, utilizing simple virus isolation procedures. The two important points which deserve noting are: (i) relatively mild symptoms in inoculated plants allowing high yield of the expressed antigen; and (ii) lack of the adverse reactions in individuals after oral immunization with spinach expressing the rabies protective antigen. More importantly in the context of this investigation, engineered virus particles containing antigenic peptides may be developed to induce specific immune responses upon oral delivery as part of unprocessed food.

Acknowledgements

The authors thank Dr. Sue Loesch-Fries for the infectious cDNA clone of AIMV RNA4, and Mike Bertovich, Genadi Golovin, and Michael Niezgoda for excellent technical assistance. Research at the Biotechnology Foundation Laboratories is supported by grants from the Commonwealth of Pennsylvania, CDC, and the US Department of Agriculture.

References

- [1] Meslin F-X, Fishbein DB, Matter HC. Rationale and prospects for rabies elimination in developing countries. In: Rupprecht CE, Dietzschold B, Koprowski H, editors. *Lyssaviruses*. Berlin: Springer, 1994. p. 1–26.
- [2] Rupprecht C, Wiktor T, Johnston D, Hamir A, Dietzschold B, Wunner W, et al. Oral immunization and protection of raccoons (*Procyon lotor*) with a vaccinia–rabies glycoprotein recombinant virus vaccine. *Proc Natl Acad Sci USA* 1986;83:7947–50.
- [3] Plotkin SA. Rabies. *Clin Infect Dis* 2000;30:4–12.
- [4] Child JE, Curns AT, Dey ME, Real LA, Feinstein L, Bjornstad ON, et al. Predicting the local dynamics of epizootic rabies among raccoons in the United States. *Proc Natl Acad Sci USA* 2000;97:13666–71.
- [5] Cox JH, Dietzschold B, Schneider LG. Rabies virus glycoprotein. Part II. Biological and serological characterization. *Infect Immun* 1977;16:754–9.
- [6] Tollis M, Dietzschold B, Viola CB, Koprowski H. Immunization of monkeys with rabies ribonucleoprotein (RNP) confers protective immunity against rabies. *Vaccine* 1991;9:134–6.
- [7] Wiktor TJ, MacFarlan RI, Foggin CM, Koprowski H. Antigenic analysis of rabies and Mokola virus from Zimbabwe using monoclonal antibodies. *Dev Biol Stand* 1984;57:199–211.
- [8] Fu ZF, Rupprecht C, Dietzschold B, Saikumar R, Niu HS, Babka I, et al. Oral vaccination of raccoons (*Procyon lotor*) with baculovirus-expressed rabies virus glycoprotein. *Vaccine* 1993;11:925–8.
- [9] Rupprecht CE, Dietzschold B, Campbell JB, Charlton KM, Koprowski H. Consideration of inactivated rabies vaccines as oral immunogens of wild carnivores. *J Wildlife Dis* 1992;28:629–35.
- [10] Hooper DC, Pierard I, Modelska A, Otvos L, Fu ZF, Koprowski H, et al. Rabies nucleocapsid as an oral immunogen and immunological enhancer. *Proc Natl Acad Sci USA* 1994;91:10908–12.
- [11] Dietzschold B, Gore M, Marchadier D, Niu H-S, Bunschoten HM, Otvos L, et al. Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. *J Virol* 1990;64:3804–9.
- [12] Hamamoto H, Sugiyama Y, Nakagawa N, Hashida E, Matsunaga Y, Takemoto S, et al. A new tobacco mosaic virus vector and its use for the systemic production of angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. *Biotechnology* 1993;11:930–2.
- [13] Usha R, Rohll JB, Spall VE, Shanks M, Maule AJ, Johnson JE, et al. Expression of an animal virus antigenic site on the surface of a plant virus particle. *Virology* 1993;197:366–74.
- [14] Porta C, Spall VE, Loveland J, Johnson JE, Barker PJ, Lomonosoff G. Development of cowpea mosaic virus as a high-yielding system for the presentation of foreign peptides. *Virology* 1994;202:949–55.
- [15] Fitchen J, Beachy RN, Hein MB. Plant virus expressing hybrid coat protein with added murine epitope elicits autoantibody response. *Vaccine* 1995;13:1051–7.
- [16] McLain L, Porta C, Lomonosoff G, Durrani Z, Dimmock NJ. Human immunodeficiency virus type 1-neutralizing antibodies raised to a glycoprotein 41 peptide expressed on the surface of a plant virus. *AIDS Res Hum Retroviruses* 1995;11:327–34.
- [17] Turpen TH, Reini SJ, Charoenvit Y, Hoffman SL, Fallarme V, Grill LK. Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Biotechnology* 1995;13:53–7.
- [18] Yusibov V, Modelska A, Stepewski K, Agadjanyan M, Weiner D, Hooper C, et al. Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1. *Proc Natl Acad Sci USA* 1997;94:5784–8.
- [19] Modelska A, Dietzschold B, Fleish N, Fu ZF, Stepewski K, Hooper C, et al. Immunization against rabies with plant-derived antigen. *Proc Natl Acad Sci USA* 1998;95:2481–5.
- [20] Dalsgaard K, Utenthal A, Jones TD, Xu F, Merryweather A, Hamilton WDO, et al. Plant-derived vaccine protects target animals against viral disease. *Nat Biotechnol* 1997;15:248–52.
- [21] Belanger H, Fleish N, Cox S, Bartman B, Deepali D, Trudel M, et al. Human respiratory syncytial virus vaccine antigen produced in plants. *FASEB J* 2000;78:1213–7.
- [22] Spitsin S, Stepewski K, Flyesh N, Belanger H, Micheeva T, Shivprasad S, et al. The coat protein alfalfa mosaic virus supports the long distance movement of TMV deficient in production of TMV coat protein. *Proc Natl Acad Sci USA* 1999;96:2549–53.
- [23] Sambrook S, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*, 2nd ed. Plainview, NY: Cold Spring Harbor Laboratory Press, 1989.
- [24] Dietzschold B, Ertl HC. New developments in the pre- and post-exposure treatment of rabies. *Crit Rev Immunol* 1991;10:427–39.
- [25] Yusibov V, Loesch-Fries LS. N-terminal basic amino acids of alfalfa mosaic virus coat protein involved in the initiation of infection. *Virology* 1995;208:405–7.
- [26] Taschner PEM, van der Kuyl AC, Neeleman L, Bol JF. Replication of incomplete alfalfa mosaic virus genome in plants transformed with viral replicase genes. *Virology* 1991;181:445–50.
- [27] Smith JS, Yager PA, Baer GM. A rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralizing antibody. In: Meslin F-X, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*. Geneva: World Health Organization, 1995. p. 181–92.
- [28] Mason HS, Lam DM-K, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. *Proc Natl Acad Sci USA* 1992;89:11745–9.

- [29] Haq TA, Mason H, Clements JD, Arntzen CJ. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* 1995;268:714–6.
- [30] McGarvey PB, Hammond J, Dienelt MM, Hooper DC, Fu ZF, Dietzschold B, et al. Expression of the rabies virus glycoprotein in transgenic tomatoes. *Biotechnology* 1995;13:1484–7.
- [31] Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ, et al. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc Natl Acad Sci USA* 1996;93:5335–40.
- [32] Hiatt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants. *Nature* 1989;342:76–8.
- [33] Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, et al. Generation and assembly of secretory antibodies in plants. *Science* 1995;268:716–9.
- [34] Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O, et al. A plant derived edible vaccine against hepatitis B virus. *FASEB J* 1999;13:1796–9.
- [35] Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen CJ. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat Med* 1998;4:607–9.
- [36] Tacket CO, Mason HS, Losonsky G, Estes MK, Levin MM, Arntzen CJ. Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. *J Infect Dis* 2000;182:302–5.