Feeding of mice with Arabidopsis thaliana expressing the HIV-1 subtype C p24 antigen gives rise to systemic immune responses

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Arabidopsis thaliana expressing the HIV-1 subtype C p24 antigen gives rise to immune responses in mice after feeding

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Abstract

Development of transgenic edible plants, to be used as production, storage and delivery systems for recombinant vaccine antigens, is a promising strategy to obtain cost effective vaccines against infectious diseases, not the least for use in developing countries. Therefore, we used *Agrobacterium tumefaciens*-mediated gene transfer to introduce the *gag* gene region encoding p24 from HIV-1 subtype C into the *Arabidopsis thaliana* plant genome. Eighteen plant lines were confirmed positive by PCR, four of these lines showed an apparent homozygous phenotype when grown on selective medium and these lines also showed transcription of the *p24* gene into its corresponding mRNA. The mRNA in all four cases generated the p24 protein in planta, as verified by western blot analysis. The plants were shown to contain between 0.2 µg and 0.5 µg p24 protein per g of fresh tissue. Analysis of the localisation of the p24 protein showed that stem tissue contained the largest amount of protein, more than twice as much as leaf tissue, whereas no p24 protein was obtained in roots. By using Southern blotting, we found that 4, 2-3, 2 and 1 T-DNA insertion events took place in the four lines 1, 2, 7, and 10, respectively. The genetic insertions of line 1 were stable from the T_1 to the T_4 generation giving rise to the p24 protein in all cases, as verified by western blotting. In mice fed with fresh transgenic *A. thaliana* (line 10) anti-gag IgG was obtained in serum after a booster injection with recombinant p37Gag. No immune response was observed in mice fed with *A. thaliana* WT or no plants at all after equal booster injection.

1. Introduction

The extensive spread of the Human Immunodeficiency Virus type 1 (HIV-1) is at present an acute worldwide problem. By the end of 2007, the total number of people living with HIV-1 was 33.2 million, out of which 68% were living in sub-Saharan Africa (1). The high mutation rate in HIV-1, approximately one mutation per cycle of replication, has resulted in a substantial genetic variability with several different subtypes of the virus (2-4). Today, antiretroviral therapy is the only way to treat HIV-1 infections, which decreases the risk of developing AIDS, but far from everyone needing this treatment can receive it (5-6). Developing countries have had limited access to these drugs and therefore there is an urgent need for other more cost effective interventions. The most desirable alternative would be an effective and inexpensive vaccine, which could prevent new infections from occurring. The rate of spreading of HIV-1 in sub-Saharan Africa is extremely high and HIV-1 subtype C is one of the dominant virus strains in this area (1,7). Hence, an effective vaccine covering HIV-1 subtype C is a top priority. For safety reasons, i.e. absence of risk for vaccine-induced infections, a subunit vaccine would be preferred in HIV-1 vaccine development.

Plants are ideal as bioreactors and delivery systems for pharmaceuticals and the rigid cell walls protect the antigenic proteins from the stomach acids (8) and enables intact antigen to reach the gut-associated lymphoid tissue (GALT; Ref 9). Plants that can be eaten raw are of particular interest for the production of recombinant vaccines. This reduces the risk of denaturation of the vaccine antigens in a cooking procedure. For instance, boiled transgenic potatoes did not have the same capacity to stimulate the immune system as the raw potatoes had (8). Purification processes also often lead to great loss of recombinant proteins and by eliminating these steps the yield of recombinant protein would increase.

A few models for vaccine antigen expressions in transgenic plants have been described. For instance, hepatitis B virus surface antigen (10), the B subunit of the *E. coli* heat

labile enterotoxin (11), Norwalk virus (12), and measles antigens (13) have been produced. Oral immunization (i.e. feeding) of small animals with some of these edible antigens has provided significant immune responses (8,10-12,14-17). Moreover, pilot experiments in humans resulted in immune responses measurable in serum after oral ingestion of plants expressing some of these antigens (14,18-21).

An important HIV-1 subunit vaccine antigen candidate is the p24 protein. This structural capsid protein is relatively conserved between subtypes and is capable of eliciting a strong immune response covering both B- and T-cell epitopes (4,22-26). Furthermore, an antibody response to the p24 protein can be detected early in infected individuals and the maintenance of a high anti-p24 response has been shown to correlate to non- or slow progression to AIDS, thus indicating the important role of the p24 protein for induction of a protective immune response (26-28). These characteristics make the p24 antigen an interesting subunit vaccine antigen candidate. In plants, the intact HIV-1 p24 protein has previously only been expressed in transgenic tobacco (29). A genetically engineered construct consisting of sequences encoding antigenic determinants of HIV-1 *env* (gp41) and *gag* (p24) proteins together with HBsAg expressed in tomato has also been described (30).

In this study, we have transformed the model plant *Arabidopsis thaliana* to express the near full-length p24 protein from HIV-1 subtype C and identified the specific p24 antigens in the transgenic plants. The transgenic *A. thaliana* plants are primarily to be used as an edible vaccine candidate for laboratory animal studies. However, *A. thaliana* WT plants have a close relationship with e.g. *Brassica* species, vegetables that are eaten raw by humans, and therefore of interesting relevance for further studies. In a preliminary feeding experiment with mice, our construct induced a systemic immune response.

2. Materials and methods

2.1. Construction of the vector

HIV-1 subtype C RNA was isolated from a virus suspension (Karolinska Institute, Stockholm, Sweden), emanating from an HIV-1 subtype C infected patient, using QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For the complementary DNA (cDNA) synthesis, 1 μg of total RNA and 200 units reverse transcriptase from Moloney Murine Leukemia Virus (Invitrogen, Carlsbad, CA, USA) were used in a 20 μl reaction mixture. The *gag* gene region encoding the p24 protein was amplified by RT-PCR using an 5 μl aliquot of the cDNA reaction, 1 μM of gene-specific primers (forward primer G60 [5'-TAG AAC GGA TCC TAT GAG CCA AAA TTA CCC TAT AGT GCA-3'], reverse primer G35 [5'-CAA GAT GGA TCC GTT AAC ATG CTG TCA TTT CTT CTA-3'] (Scandinavian Gene Synthesis AB, Köping, Sweden), 0.8 mM dNTPs, and 1 unit AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) in a 50 μl solution. The RT-PCR was performed as follows: preheating at 94ºC for 10 min, then 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC and extension at 72ºC for 1 min, followed by a final extension at 72ºC for 10 min. Both primers contained a *Bam*HI site and the reverse primer also had a stop codon at the 5' end. The amplified PCR product was digested with *Bam*HI (Fermentas, Vilnius, Lithuania) and the 683-bp fragment was cloned into the 35S-CaMV cassette, which was subcloned into the plant vector pGreen0229 using the *Eco*RV cloning site (Ref. 31; www.pgreen.ac.uk). The cauliflower mosaic virus (CaMV) 35S promoter was in control of the transcription of the recombinant *p24* gene. The pGreen0229 vector contained two selectable marker sequences: a herbicide resistance gene (BASTA) in the T-DNA region for plant selection and a kanamycin resistance gene for bacterial selection. The cassette and the two vectors were kindly provided by Drs. P. Mullineaux and R. Hellens, John Innes Centre and the Biotechnology and Biological Sciences Research Council, Norwich

Research Park, UK. The binary vector was introduced into *E. coli* TOP10 cells (Invitrogen) by heat-shock standard transformation techniques. Finally, the pGreen0229/p24 vector was purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) and confirmed by DNA sequencing (ABI PRISM 310 GeneticAnalyser, Applied Biosystems).

2.2. Transformation of Agrobacterium tumefaciens *and* Arabidopsis thaliana

The pGreen0229/p24 vector was introduced into *A. tumefaciens* strain EHA105, kindly provided by E.E. Hood (Department of Biology, Utah State University), by electroporation (www.pgreen.ac.uk). Positive transformants were grown on LB medium supplemented with kanamycin (50 µg/ml) and the presence of the *p24* gene was verified by PCR using AmpliTaq polymerase (Applied Biosystems) and the G60/G35 primer pair previously described.

A. thaliana wild type (WT) plants of the Columbia-0 ecotype (Col-0) (The European Arabidopsis Stock Centre, Loughborough, UK) were grown to flowering (6 weeks old) in growth chambers (22°C, 16 h light, 8 h darkness) and, before transformation, prepared by cutting off flowers and siliques that were already visible. Plants were transformed via the floral dip method of *A. tumefaciens*-mediated gene transformation (32). Seeds were harvested in bulk from the plants that had undergone the floral dip, spread onto 0.8 % MS agar plates containing BASTA (10 µg/ml) and grown under normal conditions. After 2-3 weeks, BASTA resistant plants (T_0) were transferred to soil and grown under normal conditions. T_1 seeds were collected separately from each T_0 plant and sown on BASTA medium. After germination the segregation pattern was checked. To verify the presence of the *p24* gene in *A. thaliana*, DNA from the plants was isolated (DNeasy® Plant Mini Kit; Qiagen), PCR was performed using AmpliTaq polymerase (Applied Biosystems) and the G60/G35 primer pair

previously described. The positive PCR products were sequenced using an ABI PRISM™ 310 Gene Analyzer (Applied Biosystems).

2.3. RNA isolation and northern blot analysis

Isolation of RNA was performed according to the protocol previously described by Carpenter and Simon (33). The samples containing 10 µg of total RNA were electrophoretically separated on 1.2% agarose gel and transferred to a Hybond-N membrane (GE Healthcare, Uppsala, Sweden). The *p24* probe was labelled with ^{32}P-dCTP using the random primers DNA labelling system (Invitrogen). Blotting and hybridization was performed according to Kalbina and Strid (34) with minor modifications. Loading of RNA and northern transfer were controlled by hybridization with a radiolabelled *Pisum sativum 18S* ribosomal RNA cDNA probe (35) to the same membrane after stripping. Membranes were exposed to X-ray film (Kodak, Rochester, NY, USA) overnight, using an amplifying screen at -80ºC. The RNA isolations and northern blotting were repeated twice.

2.4. SDS-PAGE and western blot analysis

Leaves, stems or roots of mature plants (50 mg) were homogenized in 200 µl of 50 mM Tris-HCl, pH 7.0, and centrifuged at 4ºC for 10 minutes (16 100×g). 15 µl of plant extracts or 10 ng of recombinant HIV-1 p24 protein (Aalto Bio Reagents Ltd., Dublin, Ireland), respectively, were boiled together with loading buffer (containing 10% dithiotreitol) for 5 minutes and proteins were separated by 12% SDS-PAGE.

For western blot analysis, proteins were transferred onto a nitrocellulose membrane, Hybond-C (GE Healthcare), and blocked in 5% dry milk in TBS-T (0.02 M Tris, 0.15 M NaCl, 0.05% Tween, [pH 7.5]) at 4ºC overnight. The membrane was incubated with monoclonal mouse IgG-anti-HIV-1-p24-antibodies (Aalto Bio Reagents Ltd.), diluted 1:10

000 and subsequently incubated with a 1:7 500 dilution of AP-conjugated secondary goat anti-mouse-IgG-antibodies (Promega). Proteins were visualized with 0.66% 4-nitroblue tetrazolium chloride and 0.33% 5-bromo-4-chloro-3-indolyl-phosphate in 100 mM Tris, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$.

2.5. DNA isolation and Southern blot analysis

Plant genomic DNA was isolated according to Doyle and Doyle (36) and 15 µg DNA was digested with the restriction enzymes *Hind*III, *Eco*RI, *Xho*I, and *Kpn*I (Fermentas), respectively. These enzymes lack recognition sites in the *p24* coding sequence. The DNA fragments were separated on a 1% agarose gel and subsequently transferred to a nylon membrane (Hybond-N, GE Healthcare) using 75 mmHg pressure for 1 h. The membrane was probed with *p24* cDNA labelled with ^{32}P-dCTP and treated according to the same protocol as was previously described for northern blotting. The number of bands observed on the X-ray film corresponded to the number of T-DNA insertions in the plant genomes.

2.6. Concentration levels of p24 protein in transgenic **Arabidopsis thaliana**

The concentration levels of the p24 protein in different parts of the plant were determined by enzyme-linked immunosorbent assay (ELISA), using the INNOTEST® HIV Antigen mAb (Innogenetics, Gent, Belgium) according to the manufacturer's protocol. Stems or leaves were ground in liquid nitrogen, mixed phosphate buffer (50 mM, pH 7.0) and phenylmethylsulphonyl fluoride (1 mM) (3 ml/g plant tissue) and centrifuged at 4ºC for 15 minutes (16 100×g). Centrifugation was repeated and the supernatant was diluted and used for ELISA analysis. In parallel, confirmation of HIV-1 p24 antigen expression in the plants was also performed using the Abbott Architect HIV Ag/Ab Combo Assay (Abbott, Abbott Park,

IL, USA). This method is a combination enzyme immunoassay for simultaneous detection of HIV-1 p24 antigen and antibodies toward HIV.

2.7. Immunization experiments

Two pilot experiments were conducted. First, five HLA-A2 transgenic C57BL/6 (37) mice were given fresh transgenic *A. thaliana* (line 1) day 0 (d0) and d14, between 10-12 g fresh weight (FW) per feeding event. The mice were allowed to eat free quantities of the plants for 48 hours in parallel to their ordinary food (a surplus of fresh plant material was provided), the plant material still left after 48 hours was removed. The mice were bled d0 and d28 and analysed for serum anti-gag IgG by ELISA, as described (38). Briefly, ELISA plates (Nunc Maxisorp, Odense, Denmark) were coated with recombinant subtype B p37Gag (1 µg/ml) (Prospec-Tany TechnoGene Ltd., Rehovot, Israel). Plates were blocked with 5% fat-free milk in PBS and serum was diluted and added to wells. Reactive antibodies were detected with goat anti-mouse IgG antibodies conjugated to HRP (DAKO PO449) diluted 1:3 500. Plates were then developed for 15 min by adding O-phenylene diamine buffer (Sigma-Aldrich, St. Louis, MO, USA). The color reaction was stopped with 2.5 M H_2SO_4 and the optical density (OD) was read at 490 nm. Samples were considered positive if OD exceeded the mean value for negative samples (untreated animals and preimmunization bleedings) plus 3 standard deviations.

Second, three groups of mice (HLA-A2 transgenic C57BL/6), six per group, were given i) fresh transgenic *A. thaliana* (line 10) (10-12 g FW/feeding); ii) freeze-dried transgenic *A. thaliana* (line 1) (10-12 g of FW was freeze-dried/feeding resulting in 0.8-1 g DW/feeding); iii) freeze-dried *A. thaliana* WT (negative control 1) (10-12 g of FW was freeze-dried/feeding resulting in 0.8-1 g DW/feeding). A fourth group did not receive any *A. thaliana* plant material (negative control 2). Feeding of plant material was done d0, d14, d35

and d70. Again, the plants were provided in parallel with ordinary food for 48 hours and the mice were allowed to eat free quantities. The freeze dried plant material was mixed with 1 ml water to form a paste. After 48 hours, plant material still left was removed. On d175 (25 weeks) each group was divided into two subgroups with three mice in each (one mouse/subgroup in group 4). One subgroup per group was given intramuscular injection of 100 µg plasmid DNA encoding p37Gag (38). The other subgroup received an intramuscular injection of 10 µg recombinant p37Gag (ProSpec-Tany TechnoGene Ltd). All mice were bled on d28, d49, d84 and d189 and analysed for serum anti-gag IgG by ELISA, as described above.

3. Results

3.1. Cloning and confirmation of transgenic plant lines

The new construct pGreen0229/p24, containing the *gag* gene region encoding the HIV-1 subtype C p24 protein, was obtained. *A. thaliana* WT plants were transformed with the pGreen0229/p24 vector and twenty transgenic plant lines (T_0) were selected after growing seeds on Murashige-Skoog (MS) agar/BASTA medium. Integration of the recombinant *p24* DNA into the plant nuclear chromosomal DNA was confirmed in 18 out of the 20 plants by PCR using the *p24*-specific primers G60/G35 (see Materials and Methods for details). The purified pGreen0229/p24 vector was used as a positive control in the PCR reaction. Both the transformants (18 lines) as well as the positive control generated a PCR product of the expected size, approximately 700 bp (Fig. 1). In addition, the nucleotide sequences of the PCR products were confirmed by DNA sequence analysis, which showed the presence of the *p24* gene in the plant genome (data not shown). Subsequently, BASTA screening of the 18 positive transformants resulted in four independent plant lines (lines 1, 2, 7, and 10) having

growth patterns, in both the T_1 and the T_2 generation, indicating potential homozygousity with respect to the transgene.

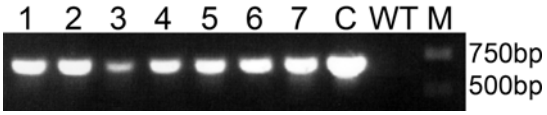

Figure 1. PCR analysis of A. thaliana candidate transformants using the G60/G35 primer pair. Lanes 1-7 show transformed plants with the p24 gene incorporated into their genome (~700 bp). Positive transformants 8-18 are not shown. The positive control (C), the pGreen0229/p24 vector PCR product, has an identical size as the transformants (~700bp). A. thaliana Col-0 wild type (WT) was used as a negative control. DNA size markers (M) are indicated to the right.

3.2. Analysis of transgene insertion and p24 production in planta

Restriction enzymes (*Hind*III, *Eco*RI, *Xho*I, and *Kpn*I) were used to cleave the DNA isolated from the four lines (1, 2, 7, and 10) and the resulting fragments were analyzed by Southern blotting. The analysis showed that 4, 2 and 1 T-DNA inserts had been integrated into genomes of lines 1, 7 and 10, respectively, whereas 2-3 T-DNA inserts were found in line 2, depending on what restriction pattern was studied. As an example, the restriction pattern obtained by using the *XhoI* enzyme is shown in Fig. 2. As expected, no hybridization was detected between the radioactive probe and the DNA from *A. thaliana* WT plants. Since more than one *p24* gene insertion had occurred in each plant line, except for line 10 as demonstrated by Southern blotting (Fig. 2), only line 10 was actually positively shown to be homozygous with respect to the *p24* genotype. However, most likely, considering the growth patterns on BASTA medium, the three other lines are homozygous to one or more of the *p24* genetic inserts. Although different numbers of *p24* gene insertion events occurred in different lines, this did not visually influence the phenotype of the plants. In addition, the transformants had an identical morphological appearance compared with the *A. thaliana* WT plants.

The four plant lines were also analysed by northern blotting. All four lines produced mRNA corresponding to the *p24* gene. Line 1 had the highest abundance of *p24*

mRNA of these transgenic lines (Fig. 3), probably depending on the fact that this line contained the highest number of insertions of the *p24* gene.

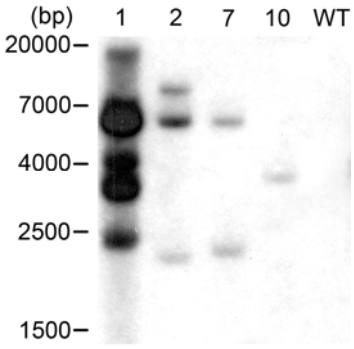

Figure 2. Southern blot analysis using the XhoI restriction enzyme with the four different transformant lines (lines 1, 2, 7, and 10), showing 4, 3, 2 and 1 inserts, respectively. A. thaliana Col-0 wild type was used as a negative control (WT). The sizes of molecular mass standards are indicated as the number of base pairs (bp) to the left.

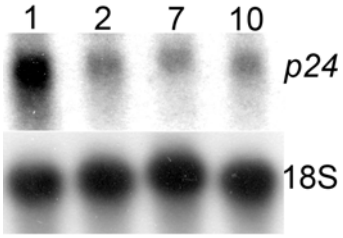

Figure 3. Autoradiographs showing the p24 and 18S rRNA transcript levels in A. thaliana transgenic lines 1, 2, 7, and 10. The northern blots were hybridized with a ^{32}P-labelled p24 cDNA probe. Hybridization with an 18S rRNA cDNA was employed as a control of equal loading and transfer of RNA to the membrane.

Using western blotting, specific monoclonal anti-p24 antibodies were used to detect p24 protein in the same four lines. Proteins of the expected molecular weight (24 kDa) were found in each line (1, 2, 7, and 10) as well as in the positive control, whereas no p24 protein was detected in the *A. thaliana* WT plants (Fig. 4).

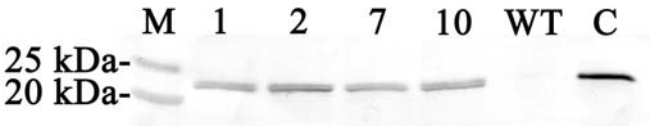

Figure 4. Western blot analysis of leaf extracts from plant lines 1, 2, 7, and 10 using monoclonal anti-p24-antibodies. Bands similar to the expected size were visible in all plant lines as well as in the positive control (C), 10 ng of recombinant p24 protein. Extracts from A. thaliana Col-0 wild type was used as a negative control (WT). The sizes of the molecular weight standards are indicated in kDa.

3.3. Accumulation of p24 protein in stem and leaf but not root

Due to the high abundance of *p24* mRNA in line 1, this transgenic plant line was selected for analysis of tissue localisation of the p24 protein. Soluble plant extracts from stems, leaves and roots were analysed by immunoblotting. No p24 protein was detected in roots of the transgenic plants whereas both stem and leaf contained proteins similar to the positive control and the expected molecular weight (24 kDa). Corresponding tissue samples of the *A. thaliana* WT plants were all negative. The analysis indicated that the stem of plant line 1 contained larger amounts of p24 protein per g fresh tissue than the leaves (not shown).

ELISA analysis of the p24 protein in the transgenic plant line 1 verified the previous results. According to the ELISA, the estimated yield in plant line 1 was approximately 0.5 µg of p24 protein per g of fresh weight in stem and 0.2 µg p24 protein per g of fresh weight in leaves. *A. thaliana* WT plants were all negative in the ELISA (data not shown). Also, a positive reaction in the Architect assay was confirmed by absorption with anti-HIV-1 p24 monoclonal antibodies which abolished the reactivity (data not shown). Again, by this method, stem extracts from line 1 gave higher signals than leaf extracts from the same line, demonstrating that the stem contained more p24 protein than the leaf (line 1). Plant extracts from *A. thaliana* WT plants did not give any signal in the Architect assay and, therefore, proving that the plant extract did not affect the analysis (data not shown). Moreover, approximately twice as much p24 protein was produced in transgenic plant line 1 compared to line 10, which only had one copy of the *p24* gene as confirmed by ELISA (data not shown).

3.4. Stability of the transgenes in subsequent generations

In order to establish the stability of the genetic construct over several plant generations, T_1 transformants of line 1 were grown and leaves from 32 of these individuals (N=32) were sampled for their content of p24 protein using western blotting. Seeds were harvested from these plants and the same procedure was repeated for the T_2 (N=22), T_3 (N=24) and T_4 (N=24) generation plants, respectively. All plants were positive with respect to the content of the p24 protein (data not shown).

3.5. Immune responses induced in mice by the transgenes

In the first pilot experiment, five mice were their own controls, i.e. anti-gag-specific IgG was measured before and after the feedings of the high insertion event *A. thaliana* line 1. Three mice showed a clear increase of anti-gag-specific IgG in serum, above background levels, after feedings, proving that transgenic *A. thaliana* (line 1) is capable of inducing immune response in mice after oral ingestion of the fresh transgenic plant material (data not shown). However, two mice lacked immune response (anti-gag IgG in serum) after the feedings.

In the second feeding experiment we wanted to compare single insertion *A. thaliana* line 10 plants with multiple insertion *A. thaliana* line 1 plants, which was the line used in the first study. In parallel, we also wanted to see if freeze-dried plants *A. thaliana* line 1 (high insertion number) could induce immune responses comparable with that of fresh plants of the same line. No antibody responses in serum were detected after four feedings with transgenic plants (data not shown). However, after subsequent intramuscular injection of recombinant p37Gag all three mice that had eaten fresh transgenic *A. thaliana* line 10 (group 1) had significantly higher anti-gag IgG levels in serum, compared with the mice that had eaten freeze-dried *A. thaliana* WT plants (group 2) or no plants at all (group 4). In the group that had received freeze-dried transgenic *A. thaliana* line 1 (group 3), one of three mice

exhibited a positive result after the booster injection as judged by anti-gag IgG ELISA, but at a lower level than the mice of group 1 (Fig. 5). The mice (three per group/one in group 4) that were injected with plasmid DNA encoding p37Gag were all negative.

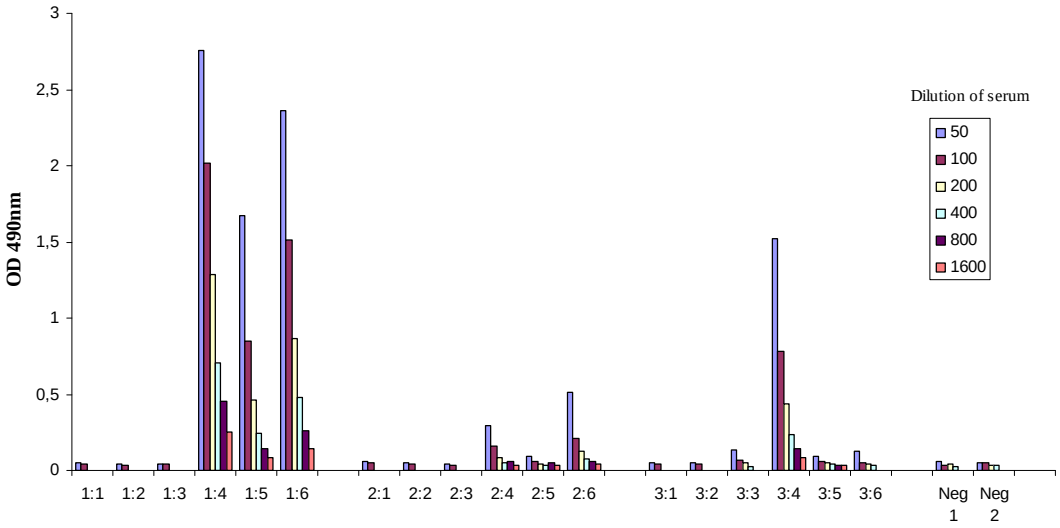

Figure 5. ELISA analysis of serum after a feeding study with mice. The study consisted of three groups with six mice per group and a fourth group with two mice. Group 1 ate fresh transgenic A. thaliana line 10 (1:1-1:6), whereas group 2 and group 3 consumed a paste of freeze dried plant material, A. thaliana WT (2:1-2:6) and A. thaliana line 1 (3:1-3:6), respectively. The fourth group did not receive any plant material (Neg1, Neg2). After four feedings, mice 1-3 in group 1-3 and Neg1 in group 4 received a booster injection of plasmid DNA encoding p37Gag (100 µg i.m.). Mice 4-6 and Neg2 received a booster injection using recombinant p37Gag (10 µg i.m.). The recombinant p37Gag induced a serum IgG response in all mice fed with fresh transgenic plants (line 10) and in one mouse fed with transgenic paste (line 1), whereas no serum IgG was produced in groups 2 and 4, i.e. the negative control groups. No reaction was observed towards the plasmid DNA encoding p37Gag booster treatment in any of the groups.

4. Discussion

In this study, we have shown the feasibility of transforming the model plant *A. thaliana* with the HIV-1 subtype C *p24* gene and the production of the corresponding protein in plant tissue. The recombinant p24 protein appears to maintain its antigenicity in the plants since specific monoclonal p24 antibodies in the western blot analysis, in ELISA and in the Abbott Architect

Ag/Ab Combo assay specifically interact with the antigen. In two pilot immunization experiments we demonstrated that the transgenic plants are able to induce a systemic immune response against the HIV-1 p24 protein in mice after feeding.

The advantages of using edible transgenic plants for vaccinations include the simple delivery not requiring sterile injections or cold chain, cost-efficient and possibly local production, safe non-infectious products and possibilities of frequently repeated boosters. Previous studies have demonstrated the feasibility of using this approach in animals and humans, confirming that transgenic plants can be used as both an expression system and an oral delivery route for vaccine antigens (8, 10-12, 14-21). The potential of the GALT for induction of protective immune responses has hitherto only marginally been explored, although two successful oral vaccines for humans exist (against poliomyelitis and cholera/ETEC). Taken together this approach for vaccination provides an opportunity for increased global access to new vaccines.

The model plant *A. thaliana* is frequently used by plant scientists and its close relationship with e.g. *Brassica* species is of great interest since *Brassica* vegetables are eaten raw and are accepted as food plants in many countries throughout the world. Our aim is to create a model system for edible subunit vaccines in *A. thaliana* and then transfer the technique to e.g. cabbage. Furthermore, as demonstrated in this study, *A. thaliana* is eaten raw by mice and can therefore function as a model system in laboratory trials.

It is necessary to control the stability of the genetic construct in the offspring since gene silencing sometimes can occur in subsequent plant generations. The demonstration that our constructs were stable over at least five generations is important for the possibilities to scale up transgenic plant production.

With regards to the content of recombinant protein in plants, the actual p24 protein concentration levels may be higher than the result from the ELISA indicates, since the

positive calibration control used in the ELISA assay is a p24 protein derived from HIV-1 subtype B, against which also the monoclonal antibodies were directed, whereas our construct was derived from HIV-1 subtype C. Since there are amino acid composition differences between the two subtypes, the standard curve established using the subtype B p24 protein may have given too low an estimation of the actual concentration of the subtype C p24 levels in the plants.

Our first quite limited immunization experiment showed a low level of antigen-specific antibody production in serum in three of five animals using fresh plants from the line with the highest p24 concentration. This was important as a principle and led us to go on with a second experiment including fresh as well a dried plant material and boost with i.m. injections of DNA plasmids or recombinant HIV-1 p24 proteins. The fact that two mice in the first feeding experiment lacked immune response may be explained by the free eating procedure, which made it difficult to estimate the levels of transgenic plant consumption for each mouse. It is possible that these two mice had a lower intake of the plants, or none at all, compared with the other three. Furthermore, after 48 hours there were still plants left in the cage showing that the mice were not capable to eat the quantities provided.

The results in the second experiment showed that fresh single insertion *A. thaliana* line 10 did not induce immune response after oral feeding as the multiple insertion *A. thaliana* line 1 did in the previous study. Probably, the lower p24 protein concentration level in *A. thaliana* line 10 was not capable of inducing an immune response by oral feeding. However, the booster injection of recombinant p37 gag resulted in immune responses of anti-gag IgG in serum in all three mice (the p37 gag is the precursor gag protein containing the entire p24). Thus, following the systemic protein boost, we could demonstrate that the fresh transgenic *A. thaliana* line 10 was able to prime a gag-specific B cell response in mice. The fact that the freeze-dried *A. thaliana* line 1 gave poor immune response in mice could be due

to a negative effect on the p24 protein caused by the freeze-drying process or that the mice actually preferred to consume fresh plants. New experiments are under way to address these issues. The p37 gag-encoding DNA vaccine was not sufficient to induce a systemic B-cell response in the plant-primed mice. This is not surprising since DNA plasmid vaccines, especially when injected intramuscularly, are known to be weak inducers of antibody responses but good for priming of T-cells (39, 40). Furthermore, the plasmid used in this experiment encodes a non-secreted (cytoplasmic) protein, a feature that generally drives a cellular immune response (38, 41).

To conclude, we showed stable transformation of *A. thaliana* with the HIV-1 subtype C capsid protein *p24* gene. In the transformants, the p24 protein is stably expressed over generations in the plant leaves and stem and constitutes a first step towards the development of an edible plant-based vaccine candidate against HIV. Feeding of laboratory mice with transgenic plants gave rise to formation of anti-gag IgG in serum.

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