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Arabidopsis thaliana plants expressing Rift Valley fever virus antigens: Mice exhibit systemic immune responses as the result of oral administration of the transgenic plants

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ABSTRACT

The zoonotic Rift Valley fever virus affects livestock and humans in Africa and on the Arabian Peninsula. The economic impact of this pathogen due to livestock losses, as well as its relevance to public health, underscores the importance of developing effective and easily distributed vaccines. Vaccines that can be delivered orally are of particular interest.

Here, we report the expression in transformed plants (*Arabidopsis thaliana*) of Rift Valley fever virus antigens. The antigens used in this study were the N protein and a deletion mutant of the Gn glycoprotein. Transformed lines were analysed for specific mRNA and protein content by RT-PCR and Western blotting, respectively. Furthermore, the plant-expressed antigens were evaluated for their immunogenicity in mice fed the transgenic plants. After oral intake of fresh transgenic plant material, a proportion of the mice elicited specific IgG antibody responses, as compared to the control animals that were fed wild-type plants and of which none sero-converted.

Thus, we show that transgenic plants can be readily used to express and produce Rift Valley Fever virus proteins, and that the plants are immunogenic when given orally to mice. These are promising findings and provide a basis for further studies on edible plant vaccines against the Rift Valley fever virus.

1. Introduction

Rift Valley Fever (RVF) is an emerging viral zoonosis of great concern for veterinary and public health. Human morbidity and mortality and massive losses of meat- and milk-producing domesticated ruminants are typically a consequence of Rift Valley Fever virus (RVFV) outbreaks [1,2]. Vaccination is a relevant strategy to counteract the devastating impacts on health and economy by RVFV [3]. As yet, there is no commercial vaccine available to prevent infections in humans, but RVFV is well suited for a one-health approach, preventing both animal and human disease through livestock vaccination [3]. Vaccinations of livestock with live-attenuated virus preparation or formalin-inactivated RVFV vaccines are currently the main measures used to control RVFV in endemic areas [4]. However, these vaccines have some severe drawbacks including adverse effects in pregnant animals and low immunogenicity [5,6].

RVFV is found in Africa and in the Arabian Peninsula [7] and often in areas where it may be difficult to maintain sterility of hypodermic medical equipment. The reuse of needles during an outbreak in northeast Africa in the year of 2000 resulted in

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unnecessary spread of RVFV among animals when vaccine was administered to livestock at times when virulent RVFV was circulating [8]. Also, the variability in temperature in outback Africa negatively impacts on the stability of candidate vaccines against RVFV [9]. Oral delivery could overcome both safety concerns and costly logistics and is hence one of the most attractive vaccine administration routes for the future [10].

Development of transgenic plants that could be used for oral delivery of recombinant vaccine antigens is an interesting strategy for obtaining cost-effective and logistically advantageous vaccines [11]. Various viral proteins have successfully been expressed in plants and have shown antigenic properties in animal experiments [12–17]. Whereas some of these proteins were delivered invasively [12,16], others, such as antigens for Crimean-Congo hemorrhagic fever virus, human immunodeficiency virus, and Norwalk virus, were able to elicit systemic immune responses when administered orally, either as pellets, plant extracts or fresh plant material [13–15]. Plants are ideal as oral delivery systems since the natural bio-encapsulation of vaccine antigens allows for safe passage through the acidic environment in the stomach of non-ruminants, such as humans or laboratory animals, into the duodenum and the small intestine [18]. Thus, this enables intact antigen to reach the gut-associated lymphoid tissue, where the antigen is recognized by specialized immune cells (i.e. M-cells) [19]. Plants that can be eaten raw are of particular interest, since it has been shown that raw consumption reduces the risk of denaturation of the vaccine antigen [18].

The model plant *Arabidopsis thaliana (A. thaliana)* has little agricultural use but is closely related to vegetables that can be consumed raw (for instance the *Brassicaceae* including cabbage, broccoli, and cauliflower). Combined with other advantageous characteristics such as its small size, rapid generation time, high fecundity, and the fact that functional transformation methods [20] have been developed for this plant, makes it a useful system for proof-of-principle studies of protein production and antigenicity [21].

Passive immunization has previously been shown to protect against RVFV infection in animal models, suggesting that humoral immune responses play an important role for protective immunity [22,23]. The nucleocapsid (N) protein of RVFV encapsidates the three genome segments of the virus (the L, M, and S segments). It is considered a major immunogen and immunizations using the N antigen have been shown to evoke partial protective immunity in animal models [24–26]. The Gn and Gc glycoproteins protrudes from the surface of the virions and mediate attachment to the cell receptor [27]. Therefore, these glycoproteins are the main targets for virus neutralizing antibodies and have frequently been used as subunit vaccines in early animal trials [28].

Here, we report the expression of RVFV N protein in *A. thaliana* and detection of specific mRNA in plants transformed with a deletion mutant of the RVFV Gn glycoprotein. Further, we show that fresh transgenic plant material can induce systemic immune responses in orally immunized mice. This study provides proof that RVFV antigens can readily be produced in transgenic plants and that such antigens are orally immunogenic.

2. Materials and methods

2.1. Construction of plant expression vectors

DNA encoding the full length N protein and a deletion mutant of the Gn protein (denoted ΔGn) was amplified from S-segment (GenBank ID DQ380151) and M-segment (GenBank ID DQ380206) RVFV cDNA, respectively, using gene specific primer pairs (Table 1). The Sseg primers contained XbaI sites and the Mseg1 primers BamH1 sites in addition to stop codons in the reverse primer. All primers were purchased from TAG Copenhagen A/S. DNA amplification was performed using Platinum Taq DNA polymerase (Invitrogen) and the amplified PCR products were digested with XbaI or BamHI (New England Biolabs) followed by ligation into the 35S-CaMV cassette of the pGreen0229 plant vector [29]. The correctness of the binary vectors pGreen0229/N and pGreen0229/ΔGn was verified by sequencing (GATC Biotech).

2.2. Plant transformation

The pGreen0229/N and pGreen0229/ΔGn plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation as described before [14]. Transformed bacteria were selected for by using kanamycin (50 μg/ml) and the presence of the

Table 1
Primer characteristics.

Primer[a]	Primer sequence[b] (5' to 3')	Amplicon	
		Primer set	Size (bp)
Sseg fwd	AGATTCTAGAGATGGACAACTATCAAGAGCTTGCG	S/1f - S/1r	760
Sseg rev	AGATTCTAGAGTTAGGCTGCTGTCTTGTAAGCCTG		
Mseg1 fwd	TAGAACGGATCCTATGAAGACACACTGTCCAAATGAC	M/1f - M/1r	905
Mseg1 rev	CAAGATGGATCCGTTATATGTCCCCCCCAGAAGACTG		
Mseg2 fwd	AAATGAAAGGGGTCTGCG	M/3f - M/3r	189
Mseg2 rev	GTCCCTTATGCTCGAAAC		
35S fwd	GAGCATCGTGGAAAAAGAAGA	35Sf - 35Sr[c]	1038 (N) 1059 (ΔGn)
35S rev	CTTATCGGGAAACTACTCACACAT		

[a]Sseg and Mseg corresponds to the RVFV S (GenBank ID DQ380151) and M (GenBank ID DQ380206) genome segment specificity, respectively; [b]Non-gene-specific nucleotides are boxed; [c]Expected size of the amplicon with 35S primers, when the N or ΔGn constructs are inserted into the 35S cassette.

transgenes were thereafter verified by PCR and primer pairs Sseg, Mseg1 and 35S (Table 1). *A. thaliana* wild-type (WT) plants of the Columbia-0 ecotype (The European Arabidopsis Stock Centre, NASC) were grown as previously described [14] and transformed using the floral dip method of *Agrobacterium*-mediated gene transfer [20]. To verify the presence of the N and ΔGn inserts in transformed *A. thaliana*, genomic DNA was extracted from leaf tissue using REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich) and amplified using the 35S primers. The reaction mixtures were cycled at 94 °C 10 min, followed by 40 cycles of 94 °C 60 s, 58 °C 60 s and 72 °C 60 s and a final extension for 10 min at 72 °C. The resulting PCR products were analysed by agarose electrophoresis and were thereafter sequenced (GATC Biotech).

2.3. Protein detection in transgenic plants

The presence of RVFV antigens in the transgenic plants was analysed by Western blotting. Different plant tissues from N transformant lines were ground in liquid nitrogen and mixed with 0.1 M Tris-buffer (pH 8.0) supplemented with cOmplete™ protease inhibitor cocktail (Roche Diagnostics). The extracts were loaded onto 12% SDS PAGE electrophoresis gels (Life Technologies) and transferred to PVDF membranes (Millipore). After blocking with 10% fat-free milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) the membranes were incubated for 1 h at 22 °C with polyclonal mouse anti-N antibody (produced in-house in DNA vaccinated mice; Ref. [26]) diluted 1:100 in 5% fat-free milk in TBS-T. After washing 3 × 10 min in TBS-T, peroxidase conjugated anti-mouse IgG antibody (Jacksson ImmunoResearch) diluted 1:5000 in 5% milk in TBS-T was added followed by incubation for 1 h at 22 °C. After another three washings as described above, bound antibody was detected using the Amersham ECL reagent (GE Healthcare).

2.4. RNA detection in transgenic plants

Fresh plant tissue, 0.10–0.15 g of each ΔGn transformant line, was homogenized by using steel beads in a TissueLyser (Qiagen) before total plant RNA was extracted using Trizol LS reagent (Life Technologies), all according to the manufacturers' instructions. DNA contamination was removed using Ambion TURBO DNase (Life Technologies). Using primer pair Mseg2 fwd and Mseg2 rev (Table 1), the presence of RNA and DNA was analysed using Superscript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) and Platinum *Taq* DNA polymerase (Invitrogen), respectively. The cycling profile was 50 °C 30 min, 95 °C 2 min, and 45 cycles of 95 °C 20 s, 55 °C 30 s, and 63 °C 30 s followed by 72 °C for 10 min. The resulting PCR products were analysed by electrophoresis.

2.5. Animal immunization and challenge

This study was carried out in strict accordance with the provisions and general guidelines of the Swedish Animal Welfare Agency. The protocol was approved by the Committee on the Ethics of Animal Experiments at the Karolinska Institutet, Stockholm (Permit Number: N105/12). C57/Bl female mice, 6–8 weeks old, were immunized according to the scheme shown in Table 2. The mice were kept four and four in cages with free access to food and water during the whole immunization routine, except during the days of immunization, when fresh plant material replaced their normal food for 24 h. In the first experiment, animals were provided 21 g fresh weight of intact plant material per four animals and blood samples were collected from the tail vein of the mice two weeks after the primary immunization (Table 2). In the second experiment, animals were provided 26 g of fresh leaves during each of the three immunization rounds and samples were collected at weeks 0, 2, 4, 5, and 6 (Table 2). The sera were stored at −20 °C pending analysis.

Table 2
Immunization schedule.

Vaccine groups	No. of animals	Plant line	Immunization at week[a]		
			0	2	4
N(a)	4	N2	21 g plant		
ΔGn(a)	4	ΔGn2	21 g plant		
WT(a)	8	WT	21 g plant		
N(b)	4	N2	26 g plant	26 g	26 g
ΔGn(b)	4	ΔGn2	26 g plant	26 g	26 g
WT(b)	4	WT	26 g plant	26 g	26 g

[a] Mice in groups of four were orally administered transgenic *Arabidopsis thaliana* (21 g or 26 g fresh weight) during 24 h. WT; wild-type.

2.6. Preparation of viral antigens for ELISA

RVFV (strain ZH548) grown in Vero cells (ATCC no. CCL-81) was centrifuged at 2500 × g at 4 °C for 10 min. Purification of viral particles was performed by centrifugation through a 20% sucrose cushion (w/w) in 0.05 M Tris, 1 mM EDTA (pH7.5) using Beckman ultraclear ultracentrifuge tubes in a SW41 rotor at 36,000 rpm at 4 °C for 2 h in a Beckman L-80 ultracentrifuge. The pellet was resuspended in 0.05 M Tris, 1 mM EDTA (pH7.5) and was stored at −70 °C until used. All work involving viable RVFV was performed in biosafety level 3 containment laboratories. Recombinant N (recN) protein with a 6×His tag was expressed from the prokaryotic expression vector pET14b (Novagen). Protein expression from transformed BL21 *Escherichia coli* (Invitrogen) was induced for 4 h using 0.5 mM isopropyl-β-D-thiogalactopyranoside (Calbiochem) and protein was purified under native conditions by gravity-flow chromatography using Ni-NTA Agarose (Qiagen) according to the manufacturer's instructions. Thereafter, recN protein was dialysed twice against sterile phosphate-buffered saline (PBS) using SLIDE-a-Lyzer Dialysis Cassette with 10 K molecular-weight cut-off (Pierce) before the concentration was determined using an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies).

2.7. Detection of immunization-induced antibody responses

ELISA was performed using plates (Nunc MaxiSorp) coated with 50μL/well of either 3 μg/mL recN diluted in 0.05 M Na_2CO_3 (pH 9.6) or sucrose-extracted RVFV diluted 1:200 in PBS. ELISA based on recN and purified RVFV was performed according to protocols described previously [30,31]. Serum was two-fold serially diluted (1:50–1:51200) and reactive antibodies were detected by HRP-conjugated goat anti-mouse IgG (Jacksson ImmunoResearch), goat anti-mouse IgG1 (Dako) or goat anti-mouse IgG2c (Thermo Scientific) antibodies diluted 1:5000, 1:4000 or 1:3000, respectively. Detection of bound antibody was visualized by adding TMB substrate (Sigma-Aldrich) and the reaction was stopped with 0.5 mM H_2SO_4 after which the absorbance was measured at 450 nm. Negative and positive control samples were present in duplicate on each plate. The cut-off was calculated as the mean absorbance value plus three times the standard deviation obtained from dilutions of pre-immunization sera. The ELISA titer was determined as the reciprocal of the highest serum dilution that resulted in a reading above the cut-off in duplicate samples.

3. Results

3.1. Expression vectors and selection of transformed plants

For expression in *A. thaliana* plants, genes encoding RVFV antigens were subcloned into the 35S cassette of the pGreen0229 binary vector, resulting in pGreen0229/N and pGreen0229/ΔGn. The pGreen0229/N construct contained the gene encoding the complete RVFV N protein (25 kDa, 760bp) and the ΔGn insert (905bp) encoding amino acids 91-382 of the mature Gn protein. The transfer, insertion, and stable integration of DNA encoding N and ΔGn protein at random sites in the plant nuclear chromosomal DNA was mediated by *Agrobacterium tumefaciens* [21]. Transgenic expression was under the control of the cauliflower mosaic virus 35S promoter, which is known to provide a strong and constitutive expression in *A. thaliana* [32]. The integration of the N or the ΔGn gene into the plant genome was confirmed by PCR (Fig. 1). Genomic DNA was extracted and used for PCR analysis using primers targeting the 35S cassette in the pGreen vector. PCR analysis showed the presence of correctly sized products of 1038 bp for the N insert (Fig. 1A) and 1059 bp for the ΔGn insert (Fig. 1B). Efficient transformation was observed in 70–90% of the regenerated plants (data not shown).

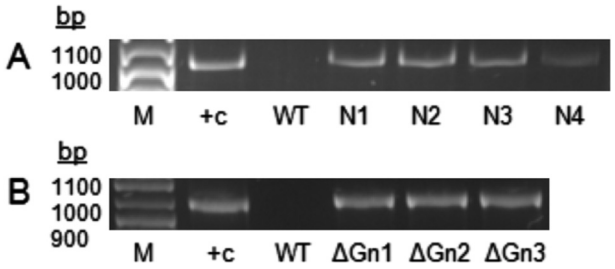

Fig. 1. PCR analysis of transformed plants. The integration of the RVFV N or ΔGn encoding sequence into the plant genome was confirmed by PCR. Total DNA was extracted from plants transformed with pGreen0229/N (A) and pGreen0229/ΔGn (B) and PCR was performed using 35S primers. Total DNA extracted from wild type (WT) *A. thaliana* and pGreen0229/N or pGreen0229/ΔGn plasmids (+c) were included in the PCR reactions. M; size marker.

3.2. Analysis of transformed plants

The presence of heterogeneously expressed N protein in transformed plants was analysed by Western blotting (Fig. 2A and B). The N protein could readily be detected in leaf tissue from all four PCR positive N transformant lines using polyclonal anti-N antibody (Fig. 2A). Since line N2 showed high abundance of N protein (Fig. 2A), this transgenic plant line was selected for further analysis.

Tissue-specific expression and abundance of N protein in soluble plant extracts from leaf, root and stem tissue was investigated using Western blotting (Fig. 2B) along with a standard consisting of serial dilutions of purified bacterially expressed recN with a previously determined concentration (not shown). The highest amount of N protein was found in leaf tissue with approximately 3.8 μg N/g fresh weight (Fig. 2B). Root tissue contained slightly less, about 3.3 μg N/g fresh weight and stem tissue 1.9 μg N/g fresh weight (Fig. 2B). Whether the N protein self-assembled to nucleocapsid-like particles in the plant cells is not known.

Despite several attempts, we could not detect specific protein expression in plant tissue from ΔGn transformant lines by Western blotting using polyclonal bovine, mice, or sheep anti-RVFV sera from infected animals (data not shown). Instead, these lines were analysed for specific mRNA expression by RT-PCR performed on total RNA extracted from ΔGn transgenic plants (Fig. 2C). The ΔGn2 line was found to contain the specific transgenic cDNA of 189 nucleotides (Fig. 2C). To verify that the RNA was not contaminated by DNA, the reverse transcriptase was omitted and replaced with DNA polymerase. No DNA could be detected in samples of the mRNA positive ΔGn2 line (Fig. 2C). The plant lines N2 and ΔGn2 were chosen for the subsequent oral immunization trials with mice.

3.3. Oral immunogenicity of plant derived RVFV antigens in mice

We conducted two experiments in mice to study whether our transgenic plants were immunogenic after oral intake by mice. Initially, the mice were fed fresh plant material once. Two weeks later the presence of anti-N or anti-RVFV antibodies in sera were analysed by ELISA (Table 2 and Fig. 3). After this single administration with fresh plants expressing N protein, two out of four mice in the N(a) vaccine group induced specific IgG antibody responses with titers ranging from 1600 to 6400 (Fig. 3A). The antibody responses were evaluated in more detail by analysing IgG1 and IgG2c subclasses (data not shown). The animal that responded with the highest IgG antibody titer (Fig. 3A) had low, but detectable levels of IgG1 (titer 50), but was negative for IgG2c antibodies. The remaining animals in the N(a) vaccine group did not respond in any of these ELISA assays. Similar results were obtained for animals in the ΔGn(a) vaccine group: two animals sero-converted after a

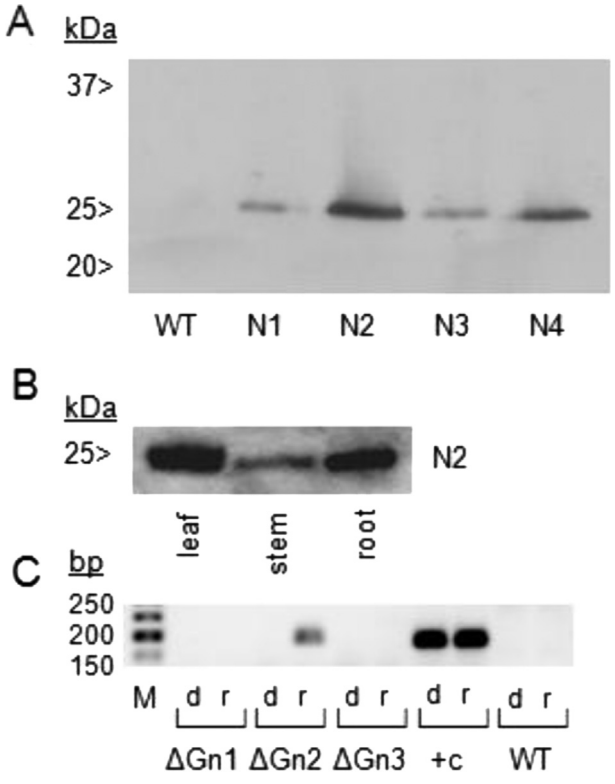

Fig. 2. Analyses of protein and mRNA expression in transformed plants. Protein expression in N transformant lines (N1–N4) was analysed using Western blotting (A). Five to ten leaves of each N transformant line were ground and subjected to SDS-PAGE. Membranes were incubated with polyclonal anti-N antibodies. The amount of N protein in different plant tissues of line N2 was analysed by Western blotting (B). Wells were loaded with 0.02 g plant tissue (leaf, stem or root) and the analysis was preformed as above. The presence of specific mRNA in ΔGn transformant lines was analysed by RT-PCR (C). Total plant RNA was extracted and treated with DNAse followed by PCR using either DNA polymerase (d) or reverse transcriptase (r). RNA extracted from WT plants was present as negative control and pGreen0229/ΔGn vector in (d) or viral RNA in (r) as positive controls (+c). The figures are representative for at least two independent experiments. M; size marker, kDa; kilodalton, bp; base pairs.

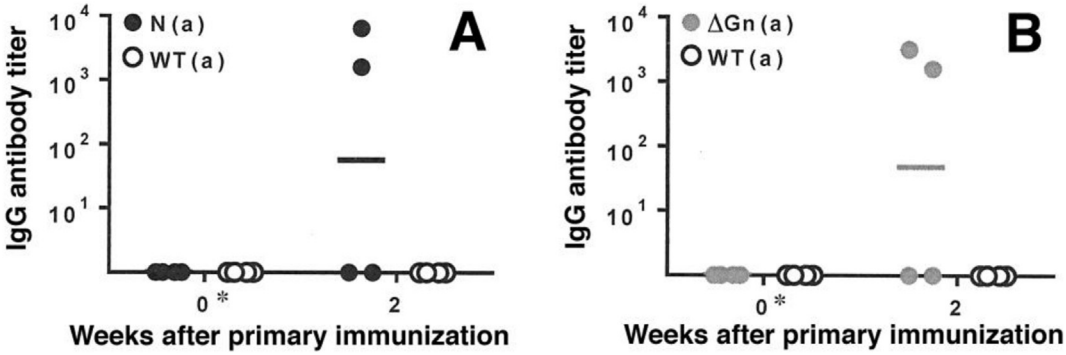

Fig. 3. Antibody responses induced after oral immunization in groups receiving one dose. Vaccine groups N(a) and ΔGn(a) each representing four mice and a control group consisting of eight animals, WT(a), were fed fresh N or ΔGn transgenic plant or wild type plant material, respectively. Collected sera were analysed for the presence of anti-N (A) or anti-RVFV (B) specific IgG antibodies by ELISA two weeks after the primary immunization. Of the eight animals in the WT(a) group, sera from four animals were analysed using ELISA based on N protein (A), and four using ELISA based on intact RVFV particles (B). Each dot represents one animal and horizontal lines show the geometric mean antibody titer in specified vaccine groups. Superscript asterisks along the x-axis indicate immunization.

single feeding with titers ranging from 1600 to 3200 (Fig. 3B) and one of those animals had an IgG1 antibody titer of 200, whereas no animal had detectable levels of IgG2c antibodies. None of the animals that had consumed WT plants induced any detectable anti-N or anti-RVFV antibody responses (Fig. 3A and B).

The antigen dose has previously been shown to be a factor influencing the inducible immune response after oral administration [13]. Since only half of the animals responded in the first experiment when 21 g of plant material was administered per four animals, the amount of plant material in the second experiment was slightly increased to 26 g per four animals (Table 2). In addition to this, the vaccination period was extended to include a priming dose and two booster doses over a period of four weeks (Table 2). Confirming the findings from the first experiment, half of the animals in the group that were fed plants expressing the N protein had detectable levels of IgG antibodies, while three out of four responded in the group administered ΔGn transgenic plants (Fig. 4). Both animals that sero-converted in the N(b) vaccine group had detectable levels of IgG1 antibodies (titers between 200 and 1600) as well as two out of three responders in the ΔGn(b) vaccine group (titers between 200 and 400). Additionally, two animals in the ΔGn(b) vaccine group had IgG2c antibodies in the lower range

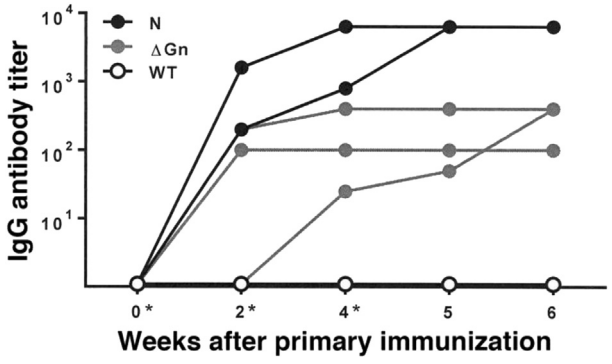

Fig. 4. Immunization-induced antibody responses in vaccine groups receiving three doses. Vaccine groups N(b) and ΔGn(b), and a control group WT(b), each representing four mice, were fed fresh N or ΔGn transgenic or wild type plant material three times, respectively. Collected sera were analysed for the presence of anti-N or anti-RVFV specific IgG antibodies by ELISA two, four, five and six weeks after the primary immunization. Serum from animals in the WT(b) group were analysed using both ELISA based on N protein and ELISA based on intact RVFV particles. Each line corresponds to one animal and represents the mean titer of duplicate samples. Superscript asterisks along the x-axis indicate immunization.

(titers between 50 and 200; data not shown). Similarly to the case in the first experiment, neither of the control mice in the WT(b) vaccine group showed any detectable levels of anti-N or anti-RVFV antibodies (Fig. 4).

4. Discussion

The feasibility of oral delivery of plant-expressed recombinant viral subunit vaccines for human and animal use has been demonstrated in several pre-clinical and clinical studies [11]. In this work, we have developed transgenic production of RVFV antigens in *A. thaliana* Since a non-invasive vaccine delivery route would be of particular interest for RVFV, we evaluated if the plant-expressed antigens were immunogenic in mice after oral intake.

The RVFV antigens chosen for this study were the highly immunogenic N protein and a deletion mutant of the Gn protein, a viral glycosylated surface protein that is the structure responsible for the attachment to the target cell [27] and that is known to induce and interact with virus-neutralizing antibodies [33]. Viral capsid proteins have previously been shown to be easily expressed in plants [12,13,34] and here we show that this is the case also for the RVFV N protein, as is evident from its expression at high concentrations. To the best of our knowledge, this is the first time RVFV antigen expression has been demonstrated in plants.

Since the glycoprotein Gn contains transmembrane regions, i.e. structural elements that are known to negatively affect transgenic protein expression and solubility in plants, this protein was truncated by removing the transmembrane segments. The resulting construct corresponded in size to a Gn antigen that has previously been shown to induce protective immunity in mice [35]. Although several attempts were made, we were not able to detect ΔGn protein expression in the transgenic lines, neither as a soluble protein nor as inclusion bodies in the insoluble fraction of the plant extracts, indicating that the truncation did not lead to insolubility of the protein. Instead, there may be a number of other reasons for the lack of detectable ΔGn protein. One is that the truncation itself affected the presentation of the protein to the antibodies of the sera from RVFV-infected animals that was used for its detection and that it thereby escaped identification. A more likely reason may be the lack of a signal [36] in the ΔGn polypeptide directing it into the plant cell endoplasmic reticulum. Therefore, the protein may have been formed in a non-glycosylated form in the cytoplasm and to a large degree been degraded. However, independently of why the protein escaped immunoblot detection in our hands, the amounts of ΔGn protein formed in the plants were large enough for oral

induction of a systemic immune response in mice (see below). Instead, to follow ΔGn expression, we assayed the occurrence of Gn specific mRNA in the ΔGn transgenic Arabidopsis lines. Although mRNA is not a proof of the existence of the corresponding protein, we decided to continue working with the mRNA positive ΔGn-transgenic line.

To explore the possibility of using these transgenic plants as vectors for vaccine antigen administration, we conducted two limited mice experiments. In the first experiment, half of the animals in vaccine groups N(a) and ΔGn(a) sero-converted after a single dose of fresh transgenic plants orally administered. Due to the lack of detection of the ΔGn protein, it is of particular interest that animals consuming those plants did indeed sero-convert. To determine if it was possible to increase the response rate, we conducted yet another experiment, this time slightly increasing the amount of plant material shared by the four animals. Two booster doses were also applied for the same reason. Again two out of four animals responded in the N(b) vaccine group. However, three out of four responded in the ΔGn(b) vaccine group, out of which one mouse did so only after two immunizations. Hence, the response rate was similar after one immunization in the two different experiments. The increased dose and the repeated feedings used in the second experiment did not influence the observed IgG titers, which were in the range of 10^3–10^4 in the first and 10^1–10^4 in the second experiment.

The fact that only a proportion of the animals elicited specific antibody responses subsequent to immunization might be explained by the free feeding procedure, which was conducted in groups of 4 animals, making it hard to ensure equal consumption of plant material on an individual basis. It is therefore likely that the non-responders had lower intake, or none at all, compared with the responders. Others have previously shown that plant extracts delivered by gastric intubation [13] or pelleted plant material [15] are feasible alternatives to free feeding. These are delivery modes that would help to ensure that every animal would consume equal amounts of plant material.

To further assess the antibody responses, we analysed IgG subclasses IgG1 and IgG2c as indicators for a Th2 or Th1 cellular response, respectively [37]. Among the IgG responders, IgG1 positive mice were overrepresented compared to animals with detectable IgG2c antibodies. This indicates that Th2 responses are elicited after oral immunization using these antigens.

5. Conclusions

We have shown that the plant *Arabidopsis thaliana* can be transformed to express RVFV N protein and at least the mRNA of a truncated version of the RVFV Gn glycoprotein. On a proof-of-principle basis, oral delivery of either RVFV antigen-transgenic plant lines to mice stimulated the induction of antibody responses. Thus, this study confirms that consumption of recombinant plant material by free feeding allows immunogenic responses and provides an initial step towards a plant-based subunit vaccine against RVFV.

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