Virus Genes 22:1, 73–83, 2001

Expression of Immunogenic Puumala Virus Nucleocapsid Protein in Transgenic Tobacco and Potato Plants

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Received May 29, 2000; Accepted June 5, 2000

Abstract. Transgenic plants, expressing recombinant proteins, are suitable alternatives for the production of relevant immunogens. In the present study, the expression of Puumala virus nucleocapsid protein in tobacco and potato plants (*Nicotiana tabacum* and *Solanum tuberosum*) and its immunogenicity was investigated. After infection of leaf discs of SR1 tobacco and tuber discs of potato cv. ''Desiree'' with the *Agrobacterium* strain LBA4404 (pAL4404, pBinAR-PUU-S) containing the 1302 bp cDNA sequence of S-RNA segment of a Puumala virus, transgenic tobacco and potato plants expressed the Puumala virus nucleocapsid protein under control of the cauliflower 35S promoter. The recombinant proteins were found to be identical to the authentic Puumala virus nucleocapsid protein as analyzed by immunoblotting. Expression of the nucleocapsid protein was investigated over four plant generations (P to F4) and found to be stable (1 ng/3 µg dried leaf tissue). Transgenic tobacco plants were smaller compared to controls. The transformed potato plants were morphologically similar to control plants and produced tubers as the control potatoes. The S-antigen was expressed at a level of 1 ng protein/5 µg and 1 ng protein/4 µg dried leaf and root tissues, respectively, and remained stable in the first generation of vegetatively propagated potato plants. The immunogenicity of the Puumala virus nucleocapsid protein expressed in *Nicotiana tabacum* and *Solanum tuberosum* was investigated in New Zealand white rabbits. They were immunized with leaf extracts from transgenic tobacco and potato plants, and the serum recognized Puumala virus nucleocapsid protein. Transgenic plants expressing hantaviral proteins can thus be used for the development of cost-effective diagnostic systems and for alternative vaccination strategies.

Key words: hemorrhagic fever with renal syndrome, Bunyaviridae, hantavirus, Puumala virus, nucleocapsid protein, recombinant protein, binary vectors, transgenic tobacco plants, transgenic potato plants

Introduction

Hantaviruses are negative-sense, single-stranded RNA-viruses (1) and belong to the *Bunyaviridae* family. Different serotypes are etiologic agents of a number of diseases with renal and/or pulmonal involvement ranging from the hemorrhagic fever with renal syndrome (HFRS; lethality 3–10%) to the more benign nephropathia epidemica (NE, lethality 0.1–1%) and the Hantavirus pulmonary syndrome (lethality $> 50\%$), that was first described in 1993 in the USA (2). The individual hantavirus genotypes are carried by specific rodent hosts. Infection of humans occurs by aerosol-inhalation of contaminated rodent excretions e.g. urine, feces and saliva.

The three segmented genome of hantaviruses codes for the viral RNA-dependent RNA-Polymerase (L-segment), the glycoproteins G1 and G2 (M-segment) and the viral nucleocapsid protein (S-segment). The viral nucleocapsid protein is an important structural protein and is essential for

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packaging of the RNA-segments and encapsidation (3). Major antigenic domains of hantaviruses are also located on the nucleocapsid protein (4). The early immune response is mainly directed against this structural protein, and sera of reconvalescent patients also predominantly contain antibodies against the nucleocapsid protein (5–8). Furthermore, the viral nucleocapsid proteins are, together with the glycoproteins G1 and G2, promising candidates for the development of new vaccination strategies.

In the past years, recombinant nucleocapsid proteins have been expressed in *E. coli*, in Baculo- and Vaccinia Virus expression systems, in silkworms and in mammalian cells (9–15). Recombinant nucleocapsid proteins expressed in insect cells, in bacteria or as chimaeric HBV core particles can evoke protective immunity in animal-models (16–18).

Recent studies have shown that proteins, expressed in plants are effective tools for production of antigen. Bacterial toxins (19) and various viral proteins (20–24) have been successfully expressed in plants and were immunogenic. These proteins were able to elicit specific humoral and mucosal immune responses when administered intraperitoneally or orally and protect the animals against the corresponding bacterial or viral infections (20–24). The expression of the viral proteins in plants have following advantages compared to other expression systems: Firstly, the posttranslational protein modification takes place in eukaryotic cells, secondly, there is no risk of contamination with mammalian viruses or other pathogens, and finally, the production of the antigens based on expression of the proteins is cheap and therefore of economical interest. An example of the potential usefulness of transgenic plants for production and delivery of edible vaccines had been demonstrated for Norwalk virus (20). This investigator succeeded in expression of Norwalk virus capsid protein in transgenic tobacco and potato and showed its immunogenicity after oral application in mice.

The investigation of the expression of hantaviral proteins in plants is of particular scientific interest, since some members of the family Bunyaviridae, e.g. the genus Tospovirus (25), infect plants. The expression of the nucleocapsid protein of Puumala virus, the causative agent of nephropathia epidemica, in *Nicotiana tabacum* and *Solanum tuberosum* is the goal of the present study.

Material and Methods

Cell Cultures and Viruses

Vero E6 (ATCC C1008) cell culture was obtained from the American Type Culture Collection and propagated in Basal Medium Eagle (BME) supplemented with 10% fetal calf serum, 100 IE/ml penicillin G, and 100 IE/ml streptomycin. Medium and serum were purchased from GibcoBRL (Eggenstein, Germany). The source and propagation of Hantavirus prototype Puumala virus strain CG-1820 used in the study was described elsewhere (15).

Recombinant Plasmids and Bacterial Strains

The properties of recombinant plasmid pCR3.1-PUU-S (6.31 kbp, 15) harboring specific cDNA of the S-RNA segment of Puumala virus strain CG-1820 and the binary vector pBinAR with an insertion of the CaMV 35S-promoter in pBin19 (26) used in this study were described previously (27–29). *E. coli* strain DH5α is $\Delta(lacZYA - argF)$ *endA1 hsdR17* $(r_k m_k^+)$ *supE44 thi-1 recA1 gyrA96 relA1* ϕ80Δ*lacZ* DM15 (BRL), *Agrobacterium tumefaciens* LBA4404 (pAL4404) with a chromosomal background of Ach5 and a deletion in the T-DNA of the Ti-plasmid (30) and *E. coli* K12-strain C600 with the helper plasmid pRK2013::Tn7 (colE1-ori, RKII *tra*-functions) were used for cloning of pBinAR-PUU-S (into DH5α) and transfer experiments.

Construction of Expression Vectors for Plant Cell Transformation

The plasmid vector pCR3.1-PUU-S and the binary vector pBinAR were cleaved with the restriction endonucleases *Acc*65I (isoschizomer to *Asp*718; AGS, Heidelberg) and *Xba*I. The resulting 1.4 kbp DNA fragment was eluted with the QiaexII kit (Quiagen). Subsequently, the vector and the insert were ligated at 4°C. The ligation products were transformed into *E. coli* DH5α by the calcium chloride method. Colonies were selected on kanamycin plates and further investigated for the correct insertion in plasmid pBinAR-PUU-S by cleavage with restriction enzymes *Acc*65I and *Xba*I. DH5α(pBinAR-PUU-S) was conjugated by triparental mating with the agrobacterium strain LBA4404 (pAL4404) and *E. coli* C600 (pRK2013:Tn7) and

transconjugants were screened for resistance to Rif200 and Km50. Strain LBA4404(pAL4404, pBinAR-PUU-S) was confirmed as *A. tumefaciens* with the 3-ketolactose test (Benedict's reagent) forming yellow-orange halos on LB-agar with 1% lactose.

Tobacco Transformation

The pBinAR-PUU-S construct was transferred to the plant genome by cocultivation of agrobacteria with leaf pieces from *Nicotiana tabacum* cv. SR1 plants with an established procedure (31). Leaf pieces were immersed in MS liquid medium (32), containing bacterial cells, blotted dry on sterile filter paper and incubated on the surface of solid MS medium containing 2 µg/ml benzyladenine (BA). After 48 hours cocultivation, leaf pieces were washed with media containing cefotaxime (250 µg/ml) and plated on selective agar media (MS) which contained kanamycin (100 µg/ml), cefotaxime (500 µg/ml), benzyladenine (BA) 2 µg/ml and naphtaleneacetic acid (NAA) 0.1 µg/ml. The putative transformant shoots appearing on the leaf discs were tested for the ability to grow on media containing kanamycin (100 µg/ml) and analyzed by PCR for the presence of the neomycin phosphotransferase (NPTII) gene (33).

Potato Transformation

Virus-free potato (*Solanum tuberosum*) plants of the cultivar Desirée were multiplied *in vitro* on MS salts, vitamins and sucrose (32). Microtubers were produced from shoot cultures on medium as described above except sucrose was increased to 80 g/l and 2 µg/ml kinetin was added. Cultures were kept at 20°C in the dark. Potato microtubers were inoculated according to Snyder and Belknap (34) with LBA4404(pAL4404, pBinAR-PUU-S) as used above for tobacco transformation. Microtubers were cut into 1 to 2 mm discs and submerged 10 min in a liquid MS medium containing *Agrobacterium* cells. The discs were blotted dry on sterile filter paper, and cultured on MS medium containing 2% sucrose. After 2–3 days cocultivation, the discs were washed five times in liquid MS medium containing 2% sucrose, and 500 µg/ml cefotaxime. After blotting dry the discs were transferred onto MS medium containing kanamycin 50 µg/ml, cefotaxime 300 µg/ml, GA_3 0.02 µg/ml, and zeatin 2 µg/ml. Shoots were regenerated from the cell colonies emerging on the discs and plants subsequently multiplied on MS medium containing kanamycin (100 µg/ml). The putative transgenic plants were analyzed by PCR for the presence of NPTII gene.

PCR Analysis

The PCR-approach to show the transformation of tobacco and potato plants, was based on the amplification of a 700 bp fragment of the NPTII gene integrated in the plant genome. Single leaves or tubers were ground in CTAB buffer according to Doyle and Doyle (35). Each PCR mixture (total volume, 25 µl) contained 20 ng of total nucleic acid extracted from plant tissue with 0.6 units of *Taq* DNA polymerase in an optimized buffer (DyNazyme). Amplification was done with 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 5 min in a thermal cycler (PTC-100 MJ Research, Inc.). PCR products were analyzed on a 1% agarose gel, stained with ethidium bromide, and DNA bands visualized on a UV transilluminator.

Sera and Antibodies

Antiserum against recombinant N proteins of Puumala virus strain CG-1820 was induced in New Zealand white rabbits. Recombinant N proteins were generated as described elsewhere (11,36). Rabbits were inoculated subcutaneously with 100 µg of recombinant N proteins (100 µg/ml) as described previously (15). The sensitivity of rabbit antisera was determined by Western blot analysis. The rabbit antiserum (dilution: 1 : 2000) raised against Puumala virus N protein was able to recognize 0.125 ng of recombinant viral N protein.

Immunoblot Analysis

Plant tissues including leaves and roots were harvested, frozen in liquid nitrogen and dried for 72 hours at 50°C. Samples of leaf tissue powder (200 µg) were dissolved in lysis buffer (600 µl with 0.01 M Tris HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% (w/v) bromophenol blue, pH 8), heated for five min at 95°C. The insoluble fraction was removed by centrifugation at 17,000 g for 5 min. The protein

concentration was measured according to Bradford (37). Proteins were separated by SDS-PAGE (15 V/cm) (38). Proteins derived from plant tissues or recombinant N protein were separated by SDS-PAGE and electroblotted onto nitrocellulose filters using semi-dry electroblotting chambers (Renner, Dannstadt, Germany) as described previously (5,11,39,15). Transfer efficiency was monitored by Ponceau staining (Sigma, Munich, Germany). Filters were blocked for 1 hour and incubated with a 1 : 1000 and 1 : 2000 dilution of rabbit antiserum. Alkaline phosphatase conjugated antibodies (anti rabbit Ig-AP, Roche Diagnostics, Germany) were used to detect interaction of the rabbit antiserum with hantaviral protein.

Polymerase Chain Reaction

Oligonucleotide primers were synthesized with an Oligo 1000 M DNA Synthesizer (Beckman Instruments GmbH, Munich, FRG) deduced from the Puumala S-RNA segments (40–42). The properties of primers are summarized in Table 1. PCR was performed with DNA from 1×10^3 cells in 100 µl containing 80 mM Tris HCl, pH 8.9; 20 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 12.5 nmol of each dNTP, 5 pmol of each primer and 2.5 units of *Taq* DNA Polymerase (Applied Biosystems GmbH, Weiterstadt, FRG). Thirty-five cycles were run in an automated temperature cycling reactor (GeneE, Techne, Cambridge, UK) at 96° for 30 sec, 55° for 1 min, and 72° for 2 min per cycle.

Immunogenicity Tests of Puumala Virus Nucleocapsid Protein Expressed in Transgenic Nicotiana tabacum *and in* Solanum tuberosum

Freshly harvested leaves (10 g) of the corresponding transgenic plants were mixed with 10 ml PBS (pH 7.0) and frozen (− 70°C) and thawed three times. The cell debris was removed by centrifugation of the mixtures at 4°C for 10 min by 6000 rpm. The immunogenicity of the hantaviral gene product expressed in *Nicotiana tabacum* and in *Solanum tuberosum* was tested in New Zealand white rabbits. The animals were inoculated with the leaf tissue extracts from NT-PUU-S-clone 3 and ST-PUU-S-clone 1 intramuscularly and intraperitoneally with an aliquot of the leaf extracts (2 ml) that were mixed with 0.5 ml complete ABM-ZK (Linaris, Germany). Inoculation was repeated four times at a two weeks interval.

Results

Construction of an Expression Vector with the cDNA of the Puumala Virus S-RNA Segment

In order to transform plant cells the cDNA sequence of the S-RNA segment of the Puumala virus was inserted into pBinAR from nucleotide positions 43 (ATG start codon) to 1344 (TAA stop codon) corresponding to the translation unit of the Puumala virus N protein (Fig. 1). The resulting plasmid

Table 1. Oligonucleotide primers for analysis of the cDNA of Puumala virus nucleocapsid protein and neomycin phosphotransferase (NPTII)

Properties of Oligonucleotide Primers	cDNA Nucleotide Position	Use/Size of Product
P1-PUU-S (23-mer, Ta = 63°C, forward primer) 5′-ACGTGGAAGACAGACTGTAAAGG-3′	Puumala virus 471–493	PCR
P2-PUU-S (22-mer, Ta = 63°C, reverse primer) 5′-GCACCTAATTCAGCCATCCCAG-3′	Puumala virus 1022–1043	PCR
P1-PUU-S + P2-PUU-S		Signal at 573 bp
NPTIIf (21-mer, Tm = **72**°C, forward primer) 5′-GAGGCGAGGCGGCTATGACTG-3′	Neomycin phosphotransferase 201–222	PCR
NPTIIr (21-mer, Tm = **73**°C, reverse primer) 5′-ATCGGGAGCGGCGATACCGTA-3′	Neomycin phosphotransferase 900-879	PCR
NPTIIf + NPTIIr		Signal at 700 bp

Fig. 1. Map for the insertion of the cDNA of the Puumala virus S-RNA segment in vector pBinAR a derivative of Bin 19 vector (11,777 bp; AC#: U09365 (49)). LB, RB: left and right borders of T-DNA; P_{CaMV}: CaMV 35S promoter; E, *Eco*RI; A, *Acc*65I (*Asp*718); H, *Hind*III; X, *Xba*I.

pBinAR-PUU-S was transferred into the *Agrobacterium* strain LBA4404 (pAL4404).

Plant Transformations

Leaf discs of SR1 tobacco and tuber discs of potato cv. Desirée were inoculated with *A. tumefaciens* LBA4404 (pAL4404, pBinAR-PUU-S). Transgenic tobacco plants, which were grown for several months, were smaller compared to the controls. The flowers of transformed plants were not significantly reduced in size. PCR analysis showed the expected 700 bp amplification product of the NPTII gene (Fig. 2).

The transformed potato plants were morphologically as the control plants. Replicates of transgenic plants obtained by clonal propagation were transferred to soil and grown in the greenhouse. No difference in growth behavior, final height and tuber production could be seen between untransformed and transformed potatoes. PCR analysis of DNA from potato leaves and tubers showed the expected 700 bp product (Fig. 2).

Detection of Puumala Virus Nucleocapsid Protein in Nicotiana tabacum

In order to detect the expression of the Puumala virus N protein in *Nicotiana tabacum*, NT-PUU-S plants were screened by immunoblot analysis as described in Material and Methods. Six out of nine transformed tobacco plants were found to express Puumala virus nucleocapsid protein (Table 2). An example of the results of this analysis for six transformed tobacco plants (NT-PUU-S-clone 0–5) is shown in Fig. 3 lanes 10 to 15. Those tobacco plants were used for further propagation. The NT-PUU-S-clone 3 that expressed hantaviral nucleocapsid protein at a high level (Fig. 4, lanes 1 to 3) was selected for production of seeds and establishing a transgenic *Nicotiana tabacum* line. The level of viral nucleocapsid protein was found to be 1 ng/3 µg of dried leaf tissue.

Expression of viral nucleocapsid protein in NT-PUU-S-clone 3 was stable when propagated via seeds from F1 to F4 (Table 2, Figs. 5 and 6). All 11 plants from the F4 generation tested, expressed high levels of Puumala virus nucleocapsid protein. This indicates that the NT-PUU-S-3 can be considered as a stable transgenic *Nicotiana tabacum* line.

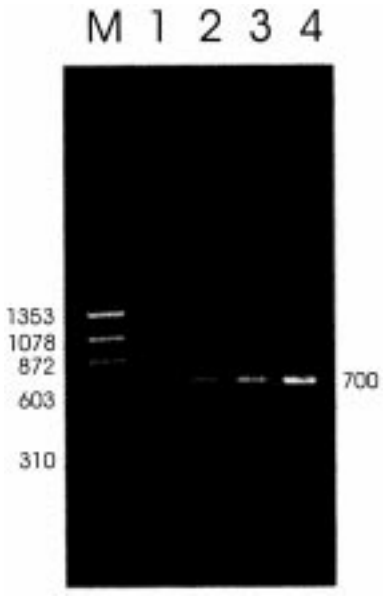

Fig. 2. Polymerase chain reactions to show the integration of cDNA of Puumala virus S-RNA segment encoding the viral nucleocapsid protein in the plant genome. DNA was extracted from plant tissue, amplified with primers NPTIIf and NPTIIr and the PCR sample loaded on a 1% agarose gel. Lane M: marker; lanes 1 and 2: from leaves of tobacco clones; lane 3: from leaf of clone 1 of transformed potato; lane 4: from tuber of clone 1 of transformed potato.

Table 2. Transgenic plants expressing the Puumala virus N protein

Plant Line	Generation	Positive Plants/Plants Tested	% of Plants Expressing NP
NT-PUU-S-T0-8	P	6/9	66
NT-PUU-S-C3	F1[a]	15/24	62
NT-PUU-S-C3	F2[a]	7/9	78
NT-PUU-S-C3	F3[a]	9/11	82
NT-PUU-S-C3	F4[a]	11/11	100
TS-PUU-S-P1-7	P	4/7	57
TS-PUU-S	F1[b]	3/3	100

[a]From seeds of NT-PUU-S-C3.
[b]Vegetative propagation from tubers of P generation.

Detection of Puumala Virus Nucleocapsid Protein in Solanum tuberosum

The expression of the Puumala virus nucleocapsid protein in *Solanum tuberosum* plants was also determined by immunoblot analysis. Four out of seven transformed potato plants (ST-PUU-S-clone 1–7) expressed Puumala virus nucleocapsid protein (Table 2, Fig. 3 lanes 2 to 8). The positive lines ST-PUU-S-clone 1–3, and 6 were further grown, and ST-PUU-S-clone 1 with the highest expression was vegetatively propagated. The level of NP expression in transformed ST-PUU-S-clone 1 was found to be 1 ng/5 µg, 1 ng/4 µg, and 1 ng/10 mg in leaf (dried tissue), root (dried tissue), and in tubers (fresh tissue), respectively (Fig. 7).

The potato plant can be considered to be a stable transgenic *Solanum tuberosum* line expressing Puumala virus nucleocapsid protein, although propagation of the plants through the seeds has not been done.

Immunogenicity of Puumala Virus Nucleocapsid Protein Expressed in Transgenic Nicotiana tabacum *and in* Solanum tuberosum

In order to detect the immunogenicity of the hantaviral gene product expressed in transgenic

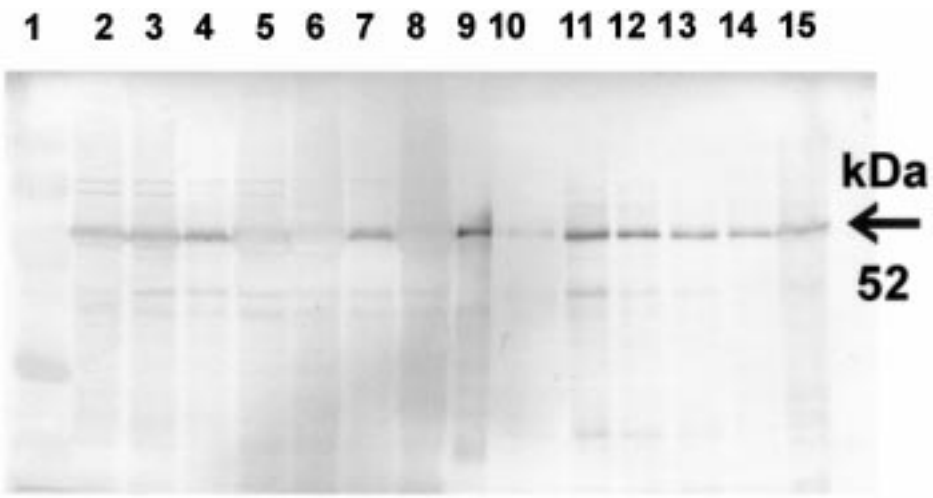

Fig. 3. Expression of Puumala virus nucleocapsid protein in tobacco (NT-PUU-S) and potato (ST-PUU-S) plants, screened by immunoblotting. Rabbit antisera raised against recombinant N protein of Puumala virus (1 : 1000 dilution) and alkaline phosphatase conjugated antibodies (anti rabbit Ig-AP, 1 : 2000 dilution) were applied as described in Material and Methods. Lane 1: molecular weight markers (not stained); lanes 2 to 8: protein from tissue of potato plants (ST-PUU-S-clone 1–7); lane 9: recombinant N protein of Puumala virus (10 ng); lanes 10 to 15: protein from tobacco plants (NT-PUU-S-clone 0–5). Arrow: position of Puumala virus nucleocapsid protein.

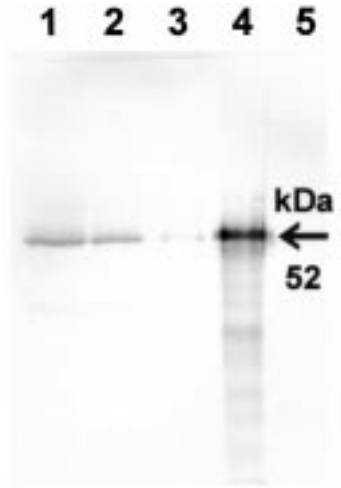

Fig. 4. Analysis of Puumala virus nucleocapsid protein in transformed tobacco (NT-PUU-S-clone 3) plants by an immunoblot assay. Tobacco leaf powder (6 mg) was dissolved in 2 ml protein extraction buffer, separated by SDS-PAGE, electroblotted and developed as described in Fig. 3. Decreasing amounts of protein from the tobacco plant TN-PUU-S-clone 1 were loaded. Lane 1: 20 µl; 2: 10 µl; 3: 1 µl. Lane 4: recombinant N protein of Puumala virus (10 ng); lane 5: molecular weight markers (not stained). Arrow: position of Puumala virus nucleocapsid protein.

Nicotiana tabacum and in *Solanum tuberosum* New Zealand white rabbits were inoculated intramuscularly and intraperitoneally with the leaf tissue extracts of NT-PUU-S-clone 3 and ST-PUU-S-clone 1 as described in Material and Methods. The sensitivity of the rabbit antisera was determined by immunoblot analysis (Fig. 8). Leaf extracts from NT-PUU-S-clone 3 and ST-PUU-S-clone 1 raised antibodies that were able to recognize the authentic Puumala virus nucleocapsid protein in infected Vero E6 cells at the dilution of about 1 : 1000 as shown in Fig. 8 B and C.

Discussion

Gene-transfer into plants possesses the potential for expression of foreign genes. Production of different biological active recombinant proteins including viral immunogenic proteins has been reported (43,44). The use of plants as an economical alternative for expression of hantavirus antigens was investigated in this report. This study shows that the expression of hantaviral proteins in *Nicotiana tabacum* and in *Solanum tuberosum* is possible. The transgenic plants give a novel concept for production of recombinant hantaviral nucleocapsid proteins for the use in basic science, development of new generation of diagnostic systems, and their application for vaccination studies. In addition, the use of plants is of special interest as they can be easily manipulated and grown. Furthermore, they allow production of recombinant proteins in large quantities and of relatively low cost. Plants have similar pathways of protein synthesis, secretion, folding and post-translational modifications as known for animal cells.

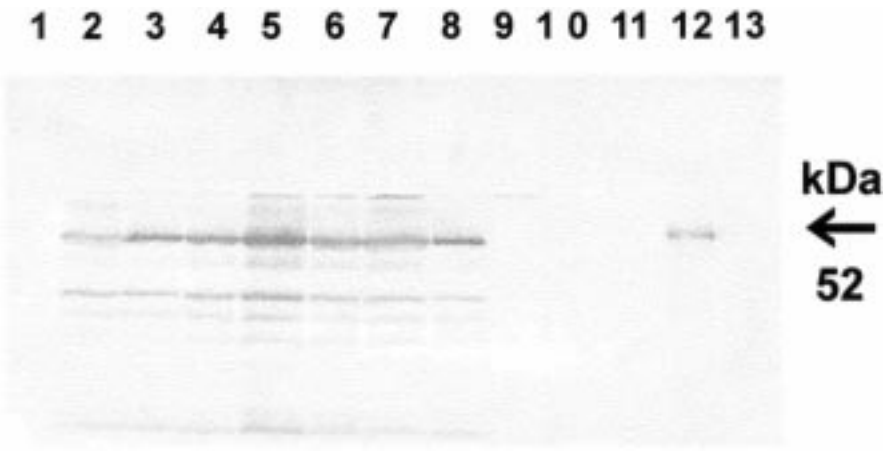

Fig. 5. Screening of tobacco plants germinated from seeds of the NT-PUU-S-clone 3 (second generation = F2) by immunoblotting. The assays were done as described in Figs. 3 and 4. Lane 1: protein from plant NT-PUU-S-3-F3, clone 2; lanes 2–5: clones 4–7; lanes 6–9: clones 9–12; lane 10: loading buffer served as negative control; lane 11: recombinant N protein of Puumala virus (10 ng); lane 12: molecular weight markers (not stained). Arrow: position of Puumala virus nucleocapsid protein.

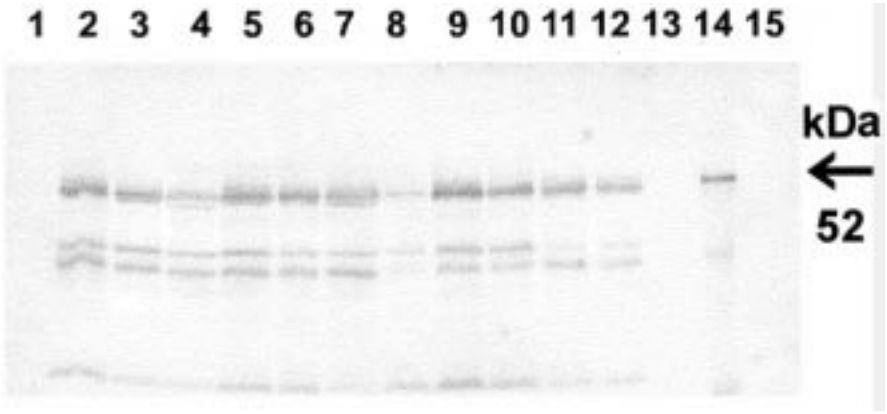

Fig. 6. Screening of tobacco plants germinated from seeds of the NT-PUU-S-clone 3 (forth generation = F4) by immunoblotting. The assays were done as described in Figs. 3 and 4. Lanes 1 and 15: molecular weight markers (not stained); lanes 2 to 12: protein extracts from plant NT-PUU-S-3-F4 clones 1–11; lane 13: loading buffer served as negative control; lane 14: recombinant N protein of Puumala virus (10 ng); lane 15: molecular weight markers (not stained). Arrow: position of Puumala virus nucleocapsid protein.

Heterologous proteins accumulate to high levels in plant cells and for instance antibodies produced in plants are indistinguishable from those produced by hybridoma. In the last decade, the production of foreign proteins in plants has become an attractive alternative to conventional production systems (bacterial and yeast production systems). Recently, chimaeric human-mouse therapeutic antibodies were produced in plants in sufficient quantities for preclinical trials (45). Current applications of plants in biotechnology include the tailor-made plant polymers or low molecular weight compounds, increased resistance towards pathogens and pesticides, improved food quality, the production of polypeptides, and vaccines or antibodies for pharmaceutical and diagnostic use.

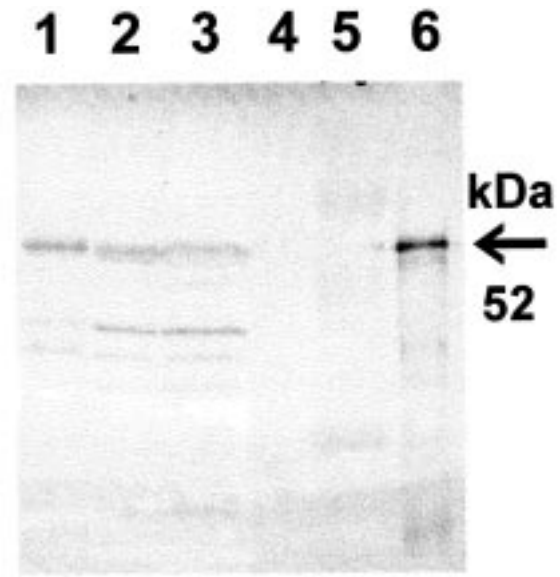

Fig. 7. Screening of vegetatively propagated potato plants (ST-PUU-S) by immunoblotting. The assays were done as described in Figs. 3 and 4. Lane 1: protein from plants ST-PUU-S-C1-F1; lane 2: from ST-PUU-S-CU1-F1; lane 3: from ST-PUU-S-CU2-F1; lane 4: loading buffer served as negative control; lane 5: recombinant N protein of Puumala virus (10 ng); lane 6: molecular weight markers (not stained). Arrow: position of Puumala virus nucleocapsid protein.

In the last decade, a variety of procaryotic and eucaryotic vectors for expression of hantaviral proteins were tested. Although different viral proteins had been successfully expressed in mammalian cells, the establishment of transgenic plants expressing hantaviral proteins is not reported to our knowledge so far. In this study we present data on the stable expression of the Puumala virus nucleocapsid protein in *Nicotiana tabacum* and *Solanum tuberosum* and its immunogenicity in *in vivo*. The cDNA sequence of the translation unit of S-RNA segment of Puumala virus was transferred into the plants chromosomes using a binary vector pBinAR-PUU-S and *Agrobacterium* strain LBA4404 (pAL4404, pBinAR-PUU-S). In this system the gene expression is under the control of the cauliflower 35S promoter. The transformed potato plants were morphologically the same as the untransformed control plants and produced tubers similar to the controls. However,

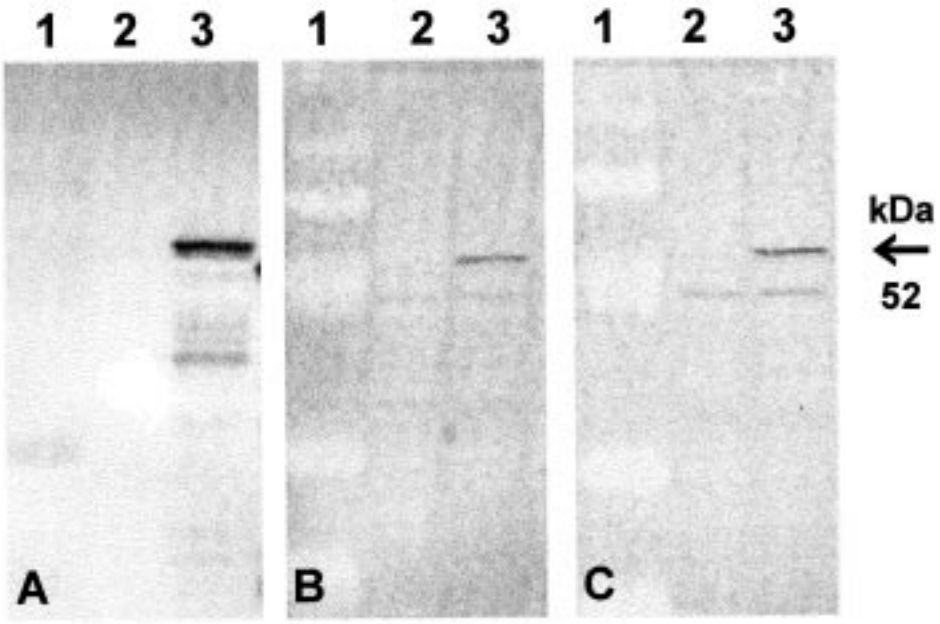

Fig. 8. Identification of the immune reactivity of sera of rabbits immunized with leaf extracts from transgenic tobacco and potato. Panel A: rabbit antisera raised against recombinant N protein of Puumala virus. Panel B: extracts from tobacco; panel C: extracts from potato plants expressing nucleocapsid protein of Puumala virus. Lane 1: molecular weight markers (not stained); lane 2: protein from Vero E6 cells; lane 3: protein from Vero E6 cells, also infected with Puumala virus. The gels were developed as described in Fig. 3. Arrow: position of Puumala virus nucleocapsid protein.

the transgenic tobacco plants were smaller compared to the controls but with a normal shape. The transgenic *Nicotiana tabacum* NT-PUU-S-clone 3 and transgenic *Solanum tuberosum* ST-PUU-S-clone 1 that expressed hantaviral nucleocapsid protein in higher levels was selected for further growing and production of seeds and/or vegetative propagation of plants. The level of viral nucleocapsid protein was found to be 1 ng/3 µg of dried leaf tissue of NT-PUU-S-clone 3. The stability of the expression hantaviral nucleocapsid protein in NT-PUU-S-clone 3 was investigated by propagation of the individual transformed tobacco plants germinated from the seeds of the NT-PUU-S-clone 3 through to the F4 generations. A special advantage of the produced transgenic seeds is that the modified genetic material can be stably stored and required no special maintenance and has a relatively long shelf life. The level of hantaviral NP expression in transformed ST-PUU-S-clone 1 was found to be rather high, 1 ng/5 µg and 1 ng/4 µg of dried leaf and route tissues, respectively. This corresponds to about 20 µg nucleocapsid protein/g fresh tuber tissue representing approximately 0.01% of total protein. Expression of *E. coli* LT-B protein in potato tubers resulted in 7.3–17.3 µg LTB protein/g fresh tuber tissue (46). Vegetatively propagated transformed potato plants also expressed hantaviral nucleocapsid protein at a high level. The immunogenicity of the hantaviral gene product expressed in transgenic *Nicotiana tabacum* and in *Solanum tuberosum* was investigated in New Zealand white rabbits. It was found that the New Zealand white rabbits that were immunized with the leaf extracts from NT-PUU-S-clone 3 and ST-PUU-S-clone 1 raised antibodies that were able to recognize the authentic Puumala virus nucleocapsid protein in infected Vero E6 cells. Other virus antigens that have been successfully produced in transgenic plants include the capsid protein of Norwalk virus (20), and the hepatitis B surface antigen (47). Recently, transgenic potato tubers expressing *E. coli* LT toxin were used to feed human volunteers. The significant mucosal and systemic immune response in the volunteer bodies proved that the plant-produced protein was immunogenic for humans (48). The stable expression of hantaviral proteins in plants is

of economical and medical advantage and provides the possibility to produce the viral antigens in high amounts. Consequently the use of such antigens for development of a diagnostic and prophylactic system is of low production cost and allows its application worldwide.

Acknowledgments

This work is dedicated to Professor Hartmut Hoffmann-Berling on the occasion of his 80th birthday. This work was supported by OTKA Grant T25014 to S.S.

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