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Neutralizing immunogenicity of transgenic carrot (*Daucus carota* L.)-derived measles virus hemagglutinin

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Abstract

Although edible vaccines seem to be feasible, antigens of human pathogens have mostly been expressed in plants that are not attractive for human consumption (such as potatoes) unless they are cooked. Boiling may reduce the immunogenicity of many antigens. More recently, the technology to transform fruit and vegetable plants have become perfected. We transformed carrot plants with *Agrobacterium tumefaciens* to generate plants (which can be eaten raw) transgenic for an immunodominant antigen of the measles virus, a major pathogen in man. The hemagglutinin (H) glycoprotein is the principle target of neutralizing and protective antibodies against measles. Copy numbers of the H transgene were verified by Southern blot and specific transcription was confirmed by RT-PCR. The H protein was detected by western blot in the membrane fraction of transformed carrot plants. The recombinant protein seemed to have a 8% lower molecular weight than the viral protein. Although this suggests a different glycosylation pattern, proper folding of the transgenic protein was confirmed by conformational-dependent monoclonal antibodies. Immunization of mice with leaf or root extracts induced high titres of IgG1 and IgG2a antibodies that cross-reacted strongly with the measles virus and neutralized the virus *in vitro*. These results demonstrate that transgenic carrot plants can be used as an efficient expression system to produce highly immunogenic viral antigens. Our study may pave the way towards an edible vaccine against measles which could be complementary to the current live-attenuated vaccine.

Abbreviations: CaMV, cauliflower mosaic virus; H, hemagglutinin protein; IP, intraperitoneal; MABs, monoclonal antibodies; MV, measles virus; OD, optical density; RT, reverse transcription; TEV, tobacco etch virus; wt, wild-type

Introduction

The development of genetic transformation technology has allowed the expression of foreign genes in an increasing number of plant species. The use of plants for the production of foreign antigen proteins that could serve as experimental immunogens was first reported in the early 1990s (Cardineau and Curtis, 1990; Mason *et al.*, 1992). Since then, a number of viral and bacterial antigens have been expressed in a variety of plant species (McGarvey *et al.*, 1995; Thanavala *et al.*, 1995; Carrillo *et al.*, 1998; Gomez *et al.*, 1998; Modelska *et al.*, 1998; Tacket *et al.*, 1998; Wigdorovitz *et al.*, 1999). Despite differences in post-translational processing viral and bacterial antigens preserved their immunogenic properties when produced in plants and induced cross-reactive and sometimes neutralizing and protective antibodies. Plants could therefore be an inexpensive source of antigens that could be easily purified for parenteral inoculation (Thanavala *et al.*, 1995; Gomez *et al.*, 1998). Moreover, oral ingestion of plants expressing high levels of antigens bear

the potential of edible vaccines (Kong *et al.*, 2001). Strategies based on potent mucosal adjuvants such as cholera toxin and heat-labile enterotoxin of *Escherichia coli* (Haq *et al.*, 1995; Arakawa *et al.*, 1998a, b) may pave the road for oral immunization.

Research on edible plant vaccines has been carried out mostly in plant species largely inappropriate for human consumption (e.g. tobacco, *Nicotiana tabacum*, Thanavala *et al.*, 1995; Mason *et al.*, 1996; Huang *et al.*, 2001; *Nicotiana benthamiana*, Modelska *et al.*, 1998; *Arabidopsis thaliana*, Carrillo *et al.*, 1998; Gomez *et al.*, 1998). For most proteins of human pathogens expressed in edible plants, potatoes were used, which are normally boiled before consumption (Mason *et al.*, 1996; Arakawa *et al.*, 1998a; Richter *et al.*, 2000; Kong *et al.*, 2001). It can be anticipated that many antigens would not resist cooking without being denatured and that cooked plant material is less immunogenic than raw plants (Kong *et al.*, 2001). Therefore, there is a need to develop other and more appropriate transgenic plant species that can serve as edible vaccines. The technology for creating other transgenic edible plants, including fruits and vegetables has been further perfected (Schenk *et al.*, 2001; Brodzik *et al.* 2000). We chose to transform transgenic carrots, which can be grown in most parts of the world, which can be eaten both raw and cooked, and are part of the early diet of infants.

Current life-attenuated measles vaccines are given routinely at 9 to 15 months of age. After a single injection, seroconversion rates are high, complications are rare and protection is long-lasting. These advantages are difficult to match by other experimental measles vaccine. However, after 25 years 50% of vaccinees are thought to have lost protective levels of antibodies (Mossong *et al.*, 1999). Revaccination with an oral vaccine which would boost the residual immunity would be a preferred strategy since it can be self-administered, requires less training of health workers and avoids the risks associated with needle injections. The potential of a parenteral/oral prime-booster schedule has been demonstrated for a number of pathogens (Kong *et al.*, 2001; Mantis *et al.*, 2001). Such a schedule is probably less liable to problems of weak and variable responses after oral vaccination. A strategy based on oral vaccination could be a particularly useful for large-scale booster immunization in developing countries where the need to deliver parenteral vaccines may hamper eradication efforts.

Measles is caused by a paramyxovirus (MV) which projects two glycoproteins, the hemagglutinin (H) and the fusion protein, from the outer viral envelope. The H protein is responsible for the attachment of the virus to the host cell (Naniche *et al.*, 1993), whereas the fusion protein is directly involved in the fusion of viral and target cell membranes required for the penetration of the virus (Wild *et al.*, 1991). Virus-neutralizing and protective antibodies are mainly directed against the hemagglutinin and, to a lesser extent, the fusion protein (McFalin *et al.*, 1980; Giraudon and Wild, 1985).

The aim of this study was (1) to explore the potential of carrots as an expression system for antigens that is suitable for human consumption, and (2) to test whether the measles virus hemagglutinin glycoprotein would preserve its neutralizing immunogenicity in this system. Although some work has been done with transgenic carrot callus cells (Brodzik *et al.*, 2000), this is one of the first reports of the expression of a transgenic antigen in mature carrots, showing that high levels of virus-neutralizing antibodies can be induced with a glycoprotein produced in this plant.

Materials and methods

Construction of a plant expression vector

The coding sequence corresponding to the measles virus hemagglutinin (MV-H) protein (Bouche *et al.*, 1998a) was subcloned into the expression cassette of the pRTL2 vector at the *Nco*I-*Bam*HI sites (Restrepo *et al.*, 1990). In this vector, the MV-H sequence was under the control of the constitutively expressed cauliflower mosaic virus (CaMV) 35S promoter fused to the tobacco etch virus (TEV) 5′-untranslated region, a translational enhancer, and the CaMV 35S terminator (Odell *et al.*, 1985; Pietrzak *et al.*, 1986). These control sequences were flanked with *Hin*dIII restriction sites that allowed their transfer, together with the H sequence, into the T-DNA region presents in binary vector pBIN19 (Bevan, 1984), creating the recombinant plasmid pBIN19-MVH. The T-DNA region, delimited by the right and left border sequences, also contains the neomycin phosphotransferase II gene (*nptII*) that provides neomycin and kanamycin resistance to transformed plants (Figure 1). After subcloning of the expression cassette in pBIN19 and transformation of XL1-Blue cells kanamycin-resistant colonies were picked and checked for the presence of the expression cassette by PCR and automated sequencing (373 A model, Perkin Elmer, Netherlands).

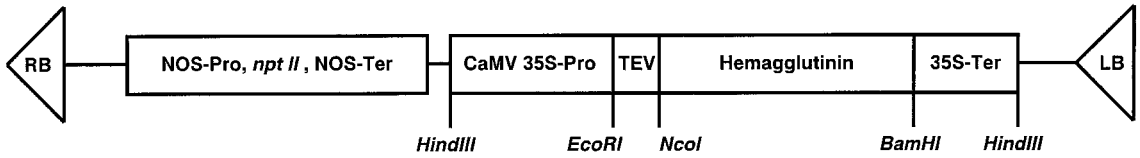

Figure 1. Transferred DNA region of the binary vector pBIN19-MVH used for *Agrobacterium*-mediated transformation of carrot plants. This region contains the *nptII* expression cassette providing kanamycin resistance, the CaMV 35S promotor and terminator, the TEV leader sequence, and the MV-H target sequence. Restriction sites used for the construction of pBin19-HMV and genetic analysis of the transformed plants as well as the right (RB) and left boarders (LB) are shown.

Plasmid DNA was then isolated from a positive clone and introduced in *Agrobacterium tumefaciens* strain LBA4404 by electroporation (25 μF, 2500 V, 400 Ω). Transformed bacteria were selected on YEB-agar solid medium containing 50 μg/ml kanamycin (28 °C, 48 h) and were used for subsequent carrot transformation.

Plant transformation

The protocols of *A. tumefaciens*-mediated transformation of hypocotyls were modified for the production of transgenic carrot plants (Hardegger and Sturm, 1998; Tokuji and Fukuda, 1999; Brodzik *et al.*, 2000). Sterilized carrot seeds (Herrera-Estrella and Simpson, 1988; *Daucus carota* cv. Senkou-Gosun, kindly given by Dr Tokuji, Japan) were sown in 0.8% agar in the dark at 25 °C. After 14 days hypocotyl segments were harvested in Gamborg B5 liquid medium (B5) supplemented with 3% sucrose and with the phytohormone 2,4-dichlorophenoxyacetic acid (1 mg/l) for 48 h at 25 °C. After washing, the segments were placed for 5 days on phytohormone-free B5 solid medium (0.8% agar) containing 3% sucrose. The hypocotyl fragments were then transformed by immersion (2 h) in the bacterial suspension of *A. tumefaciens* containing the recombinant pBIN19-MVH binary plasmid. The segments were further co-cultured in the dark at 25 °C with the bacteria on B5 solid medium with 3% sucrose. Five days later, the explants were washed with B5 medium containing cefotaxime (250 mg/l) to kill remaining agrobacteria. Explants were grown in darkness at 25 °C on B5 solid medium containing cefotaxime (250 mg/l) and geneticin (10 mg/l) for the selective growth of transgenic cells. After 4 weeks of selection, somatic embryos resistant to geneticin appeared on the hypocotyl segments. Each hypocotyl usually gave rise to a few embryos. Embryos were subcultured and rooted on selective medium at 25 °C under light. Plantlets with adequate roots were transferred to potting soil and grown in a greenhouse under normal light and humidity conditions. Several lines of transgenic carrots were obtained and further studied.

PCR analysis

Genomic DNA was isolated from both untransformed and transformed plants by macerating frozen leaves (ca. 3 g) in liquid nitrogen. The resulting powder extract was re-suspended in the extraction buffer (100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 100 mM 2-mercaptoethanol and 2% N-cetyl-N,N,N,-trimethylammonium bromide, pH 8.0) and incubated at 60 °C for 30 min. After a chloroform extraction, nucleic acids were precipitated with 0.7 volume of isopropanol and the pellet obtained after centrifugation was resuspended in Tris-HCl (10 mM, pH 8.0)/EDTA (1 mM) buffer. After RNase treatment the DNA was stored at −20 °C until being used. A fragment of the MV-H expression cassette (promotor-MVH-terminator) was specifically amplified with a forward primer (5′-GCAAGACCCTTCCTCTATAT-3′) from the 35S promoter region and a reverse primer (5′-ATCTGGGAACTACTCACAC-3′) from the 35S terminator region. The presence of the *nptII* gene was detected by PCR amplification with a pair of specific primers within the *nptII* gene (5′-TGCTCCTGCCGAGAAAGTATC-3′ and 5′-TCCTGTATCGCAACCGATGGGC-3′). Genomic DNA of untransformed plants was used as negative control.

Southern blotting

Southern blotting with genomic DNA was performed following conventional protocols. Briefly, 30 μg of extracted DNA was digested overnight at 37 °C with *Eco*RI which cuts the recombinant T-DNA at a single position (between the 35S promoter and the TEV leader). After agarose gel (0.8%) electrophoresis, digestion products were transferred overnight onto a nylon membrane (Hybond+, Amersham-Pharmacia

Biotech, UK). The membrane was equilibrated with 20× SSPE prior to immobilization of DNA by UV-cross-linking (UV Stratalinker, Stratagene, Netherlands). For hybridization, a ^{32}P-labelled H-specific cDNA probe, generated by nick translation according to Feinberg and Volgelstein (1983), was incubated with the membrane for 4 h at 65 °C. The membrane was then washed for 20 min at 65 °C successively with 0.1% SDS in 5× SSC, 2× SSC and finally in 0.2× SSC. Hybridized complexes were detected by autoradiography.

RT-PCR

Total RNA from leaves (1 g) of transformed plants was isolated as described by Hughes and Galau (1988). RNA from untransformed plants was used as a negative control template. Reverse transcription (RT) was carried out for 2 h at 40 °C in 50 μl final reaction volume containing 5 μg RNA, 500 ng random hexamers and a mixture of dNTPs (20 mM each), 10 mM DTT, 10 units of RNasin (Promega, Netherlands) and 200 units of M-MLV reverse transcriptase (Superscript II, Gibco Life Sciences, Belgium). After adding 0.1 volume of 10 mM ATP, ligation was carried out by incubation for 45 min at 37 °C in the presence of 2 units of T4 DNA ligase. PCR amplification was performed with two H-specific primers including the first 18 and the last 12 nucleotides of the coding sequence. The pBIN19-MVH vector was used as positive control template. A PCR reaction was also performed directly on the total RNA to confirm the absence of specific DNA in the extract.

Protein extraction and western blotting

Plant tissues were homogenized on ice in PBS containing 5 mM EDTA and protease inhibitors (10 μg/ml Aprotinin, 1.8 mg/ml Iodoacetamide, 10 μg/ml leupeptin, 1 mM PMSF). The mixture was centrifuged at 1700 × *g* (10 min, 4 °C) to remove insoluble debris. The membrane fraction was sedimented by ultracentrifugation at 100 000 × *g* (45 min, 4 °C), and re-suspended in PBS containing 0.5% NP-40. Protein concentration was determined with the DC Protein Assay kit (BioRad, Belgium). One gram of wet weight of carrot plant gave about 100 μg of membrane protein.

Proteins of the membrane fraction were separated by 12% SDS-PAGE under reducing and denaturing conditions (100 mM DTT, 4 M urea, 2% SDS). Proteins were blotted onto nitrocellulose membrane in Tris-glycine buffer for 2 h at 250 mA. The membrane was blocked for 1 h at room temperature with 5% non-fat dehydrated milk in PBS containing 0.1% Tween-20. For detection of the MV-H protein two specific monoclonal antibodies (MABs; BH47 and BH195, 1:1000 dilution) and goat anti-mouse IgG-conjugated horseradish peroxidase secondary antibodies (1:5000 dilution) were used. Bound antibodies were detected by enhanced chemiluminescence (ECL kit, Amersham-Pharmacia Biotech).

Enzyme-linked immunosorbent assays (ELISA)

To characterize recombinant H protein in leaf and root extracts microtiter plates (Maxisorb, Nunc, Denmark) were coated with increasing concentrations of plant extract (in PBS) and revealed with H-specific MABS (dilution 1:1000). BH47 recognizes the sequential helix-forming epitope H236-350 (Fournier *et al.*, 1997; Deroo *et al.*, 1998); BH67 and BH81 are conformation-dependent antibodies (unpublished); BH1 binds only denatured protein (Ziegler *et al.*, 1996); BH216 binds to the hemagglutinin noose epitope (HNE) H386-400 (Ziegler *et al.*, 1996). Mouse sera (1:250 to 1:32000) were titrated against immobilized purified H protein (BHK-H) produced in BHK-21 cells (50 ng/well; Bouche *et al.*, 1998b). The coated antigens were washed and blocked with 1% BSA in Tris-buffered saline (15 mM, pH 7.4). After addition of MABs or mouse serum, alkaline phosphatase-conjugated goat anti-mouse IgG (1:750; Southern Biotechnology Association, USA) and *p*-nitrophenylphosphate (Sigma, USA) were used for detection. Optical density (OD) was measured after 90 min at 405 nm. Data were expressed as net OD values after subtracting either the absorbance of the negative control antigen (e.g. extract of wild-type roots or leaves, or BHK-0 antigen; Figure 4) or the absorbance of the conjugate without mouse serum (<0.150 OD). H-specific antibody isotypes and subclasses were determined in mouse sera (1:500) by ELISA with specific conjugates and substrate of a commercial kit (BioRad). Data were expressed as OD at 415 nm after 30 min as recommended.

Immunizations

Groups of four SPF BALB/c mice were primed by intraperitoneal injection of with 500 μg of leaf or root extracts from transgenic or wild-type (wt) plants or 100 μg of BHK-H or BHK-0 antigens emulsified (1:1) in Freund's complete adjuvant (Sigma). By

comparison with the ELISA signal of mammalian-expressed purified H protein, the plant extract and the BHK extract was estimated to contain about 10 and 20 μg of the specific protein, respectively. Mice were boosted on days 14, 28 and 42 with the same antigen preparation emulsified in Freund's incomplete adjuvant (Sigma). Sera were drawn 7 days after boosting and were tested as pooled sera (after the second boost) or as individual sera (after the third boost).

Flow cytometry

The reactivity of immune sera (1:50) with native H protein or MV was tested by flow cytometry as described before using permanently H-transfected Mel-Juso cells expressing the recombinant protein at their surface (Mel-Juso/H, gift of R. de Swart, Rotterdam, Netherands; de Swart *et al.*, 1997) or MV-superinfected EBV-transformed human B cell line (MV-WMPT; gift of B. Chain, London, UK; Muller *et al.*, 1995). For both assays wt or uninfected cells (Mel-Juso/wt or WMPT) were used as negative control cells. Cells were incubated on ice (30 min) with diluted serum of individual mice. After washing, FITC-conjugated goat anti-mouse Fc-specific antibody (1:200, Sigma) was used for detection. FITC-conjugate alone, naïve serum on positive and negative cells or test serum on negative cells served as negative controls. BH26 (1:500; Ziegler *et al.*, 1996) served as an antibody positive control. Dead cells were excluded by propidium iodide staining (1 μg/ml).

Plaque reduction neutralization assay (PRN)

To test MV-neutralizing activity, mouse sera were heated for 30 min at 56 °C to inactivate complement. Duplicates of two-fold serial dilutions (1:64 to 1:32768) of heat-inactivated serum were mixed with an equal volume of medium containing 35 plaque-forming units of Edmonston strain MV. After 2.5 h of pre-incubation at 37 °C, the mixture was added to a subconfluent Vero cell culture in 24-well plates (2.5×10^5 cells per well). After one hour unbound virus was removed and cells were covered with a carboxymethylcellulose (4%) overlay. After four days of incubation under tissue culture conditions, a 0.2% neutral red solution was added. On day 5, the overlay was removed and cells were fixed with a 10% formaline solution. The 50% neutralization titre, defined as the reciprocal of the dilution that reduces the number of plaques by 50%, was calculated according the Spearman-Kärber method. Titres <64 were considered to be negative.

Results

Production and genetic analysis of transformed plants

The transformation of carrot plants was mediated by recombinant *Agrobacterium* infection using the pBIN19-MVH plasmid (Figure 1). The regenerated transgenic plants showed no morphological changes in comparison to wt carrot plants (data not shown). About 30 plants resulting from independent transformation events were selected and grown in the greenhouse. Ten of them were analysed further.

The presence of the MV-H expression cassette in transgenic plants was confirmed by PCR followed by gel electrophoresis of the amplified fragments (Figure 2A). From all transformed plants tested a product of the expected size (2.2 kb) was amplified (lanes 3–12). The same size was obtained with pBIN19-MVH vector as a positive control template (lane 1). This product was absent in DNA of untransformed plants (lane 2). Similarly, the presence of the *nptII* gene was confirmed as a specific product of 360 bp in all geneticin-resistant plants (Figure 2B, lanes 3–12). This product was absent from untransformed plants (lane 2).

Copy numbers of the transgene and the integration pattern were determined by Southern blot by digesting genomic DNA of the 10 transformed plants (Figure 2C) with *Eco*RI (which has a unique restriction site in the T-DNA, see Figure 1). Hybridization with a MV-H-specific probe showed in every transgenic plant a unique restriction pattern (lanes 3–12) indicating that insertions occurred at random sites throughout the genome. All plants appeared to have integrated a single copy of the transgene, as illustrated by the presence of a single hybridization band. The insert corresponding to the radioactive probe was used as positive control template (lane 1). No signal appeared when genomic DNA of wt plants was used as a template (lane 2).

Expression of the transgene in transgenic plants

The transcription of the MV-H gene was analysed by RT-PCR on total RNA extracted from transgenic plants (Figure 3A). Untransformed plants served as a negative control (lane 3). All tested plants produced a specific major transcript of the expected size

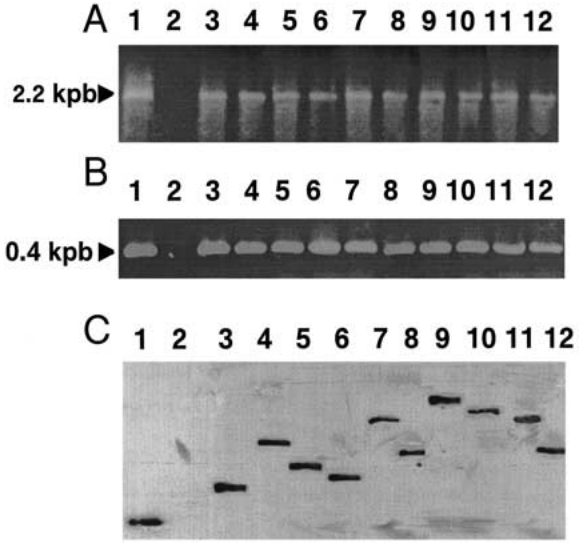

Figure 2. Genetic analysis of 10 independent transgenic plants transformed with pBIN19-MVH plasmid. Genomic DNA was isolated from carrot leaves (lanes 3–12) and PCR was performed with primer pairs specific of (A) the MV-H expression cassette and (B) the *nptII* gene. Genomic DNA of untransformed plant (lanes 2) and of the pBIN19-MVH vector (lanes 1) served as negative and positive control templates respectively. (C). Southern blotting of the same genomic DNA (lanes 3–12) digester with *Eco*RI and hybridized with a ^{32}P-labelled specific MV-H probe. The DNA fragment specific of the probe (lane 1) and DNA from wt plants (lane 2) were used as positive and negative control DNA targets respectively.

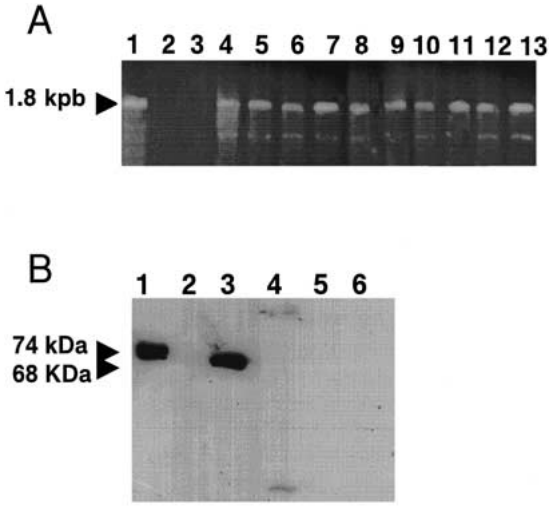

Figure 3. Transcriptional (A) and translational (B) activity of transgenic clones. A. Total RNA was isolated from carrot leaves (lanes 4–13) and RT-PCR was performed with a pair of MV-H-specific primers. Lane 2 shows the PCR directly performed on the extracted RNA, to show the absence of specific endogenous plant DNA; lane 3 corresponds to the RNA of wt plants as a negative control. The pBIN19-MVH plasmid served as the positive control of the PCR (lane 1). B. Western blotting of MV-H expressed in carrots compared with the recombinant protein produced in mammalian cells. A 500 ng portion of H leaves (lanes 3, 6) or of wt leaf extract (lanes 2, 5) or 50 ng of BHK-H (lanes 1, 4). A mixture of the specific MABs BH47 and BH195 (lane 1, 2, 3), an irrelevant MAB (lanes 4, 5, 6).

of the transgene (lanes 4–13). The minor unspecific, smaller band was also found when other transgenes were tested. No amplified DNA was detected when the PCR was directly performed on the RNA preparations, confirming the RNA-specificity of the reaction (lane 2).

Crude membrane preparations of leaves (500 ng protein) and of BHK-H cells (50 ng protein) gave specific bands of similar intensities in western blots (Figure 3B). Based on ELISA with purified H protein the content of specific protein was estimated at about 2% and 20% of specific protein in membrane fraction. The H-specific monoclonal antibodies BH47 and BH195 that bind to two non-overlapping epitopes revealed a strong under reducing conditions with an estimated molecular mass of about 68 kDa (lane 3). Under the same conditions, H protein produced in mammalian cells (BHK-H) migrated with an apparent size of ca. 74 kDa as reported earlier (lane 1; Bouche *et al.*, 1998b). This difference in mass may correspond to different levels of glycosylation of the monomer, but this was not further explored (Vialard *et al.*, 1990). Negative controls included an irrelevant MAB (lanes 4–6) as well as a crude membrane preparation of wt leaves (lanes 2).

Antigenicity of the recombinant H protein

The western blot suggested that there may be differences in glycosylation. Glycosylation is well known to be important for the proper folding of the H protein (Hu *et al.*, 1994). Several monoclonal antibodies were used to investigate the conformational integrity of the transgenic protein (Figure 4A). Microtitre plates were coated with increasing concentrations (62.5–1000 ng/well) of membrane fractions from both transgenic leaves and roots. High net signals were obtained with two conformational-dependent MABs (BH67 and BH81), and BH47 which recognizes the sequential epitope H236-250 (Fournier *et al.*, 1997) with a putative helical conformation (Deroo *et al.*, 1998). In contrast, BH1, which binds denatured protein only, showed essentially no reactivity above the background of wt plants. BH216 binds to the hemagglutinin noose epitope (HNE, H386-400) with its oxidized cysteine bridge (Ziegler *et al.*, 1996). The

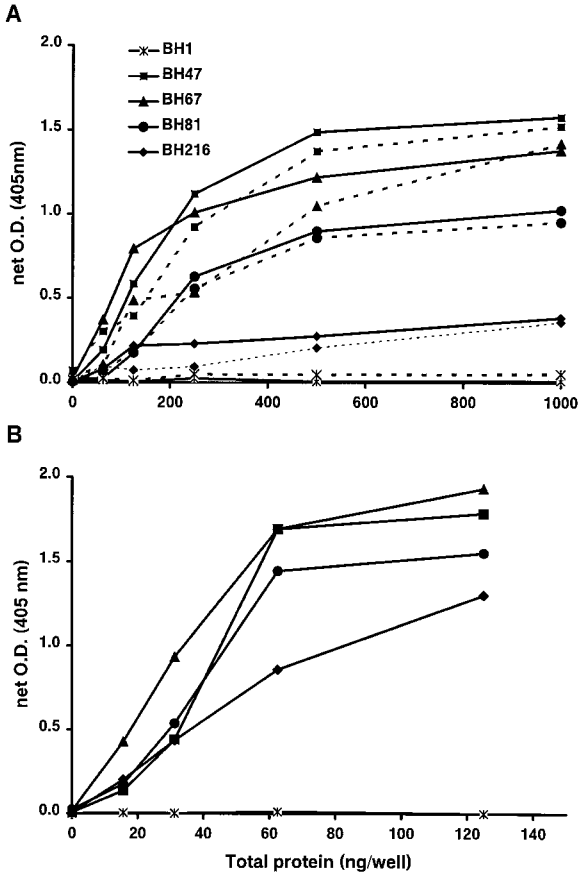

Figure 4. (A) Antigenicity of H leaves (solid line) and H roots (dashed line). (B) BHK-H antigen after coating of increasing concentrations of antigen and detection by H-specific MABs. Data are shown as net OD after subtraction of the corresponding wt antigen.

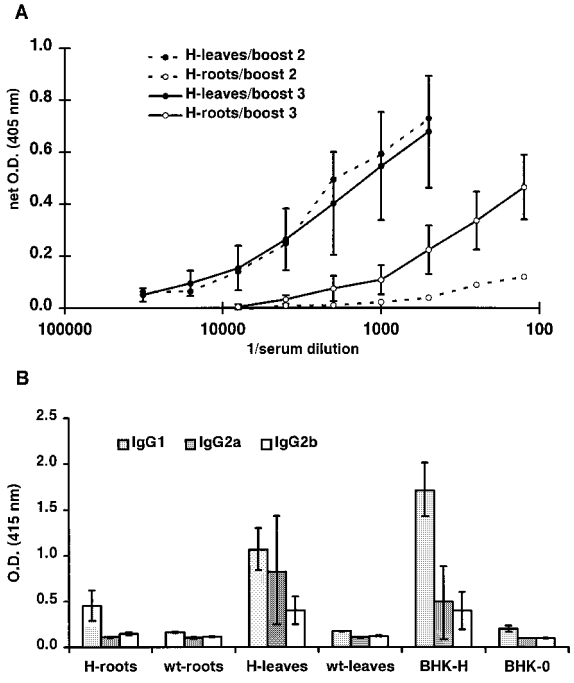

Figure 5. (A) Reactivity with purified H protein of mouse sera induced by H leaves (closed symbols) or H roots (open symbols). Data are expressed as net OD values of pooled sera after boost 2 (dotted lines, pooled serum) and after boost 3 (solid line, mean ± SD of individual sera). (B) Isotype determination of the H-specific antibodies after boost 3 (mean ± SD of individual sera; 1:500 dilution).

antibody binding pattern was essentially the same in root and leaf extracts and resembled that of purified H protein of mammalian origin (Figure 4B). In general, signals tended to be higher in younger than in older tissues (data not shown).

Immunogenicity of transgenic protein

Since the transgenic H protein seemed to be antigenically conserved, its immunogenicity was tested in mice. After 2 or 3 boosts with transgenic plant extracts all animals showed reactivity with purified H protein produced in mammalian cells (Figure 5A). After the third boost, average antibody levels obtained with leaf extracts were about 4 times higher than after immunization with root extracts. Similar levels were found after boost 2 for leaves, whereas antibodies still increased between boost 2 and 3 with root extracts. Sera of control mice immunized with wt plant extract showed no cross-reactivity with the H protein (net OD <0.03; data not shown). Interestingly, antibodies generated with the transgenic leaves were of both the IgG1 and IgG2a subclass, whereas the immune response against the H protein produced in BHK-21 cells was essentially restricted to IgG1, suggesting a difference in the Th1/Th2 balance (Figure 5B). No H-specific IgG2b, IgG3, IgA and IgM were detected.

To exclude that the reactivity may be due to partially denatured recombinant protein, the sera were further tested for antibodies against the intact native protein expressed on MV-superinfected WMPT cells (Figure 6A) and H-transfected Mel-Juso cells (Figure 6B). The flow cytometry data showed that all mice vaccinated with transgenic leaf or root extracts produced high levels of antibodies cross-reacting with the native protein independently whether virus-infected or H-protein transfected cells were used. Antibody levels were similar to those of mice immunized with H protein produced in mammalian cells, while wt plants induced no cross-reactive antibodies.

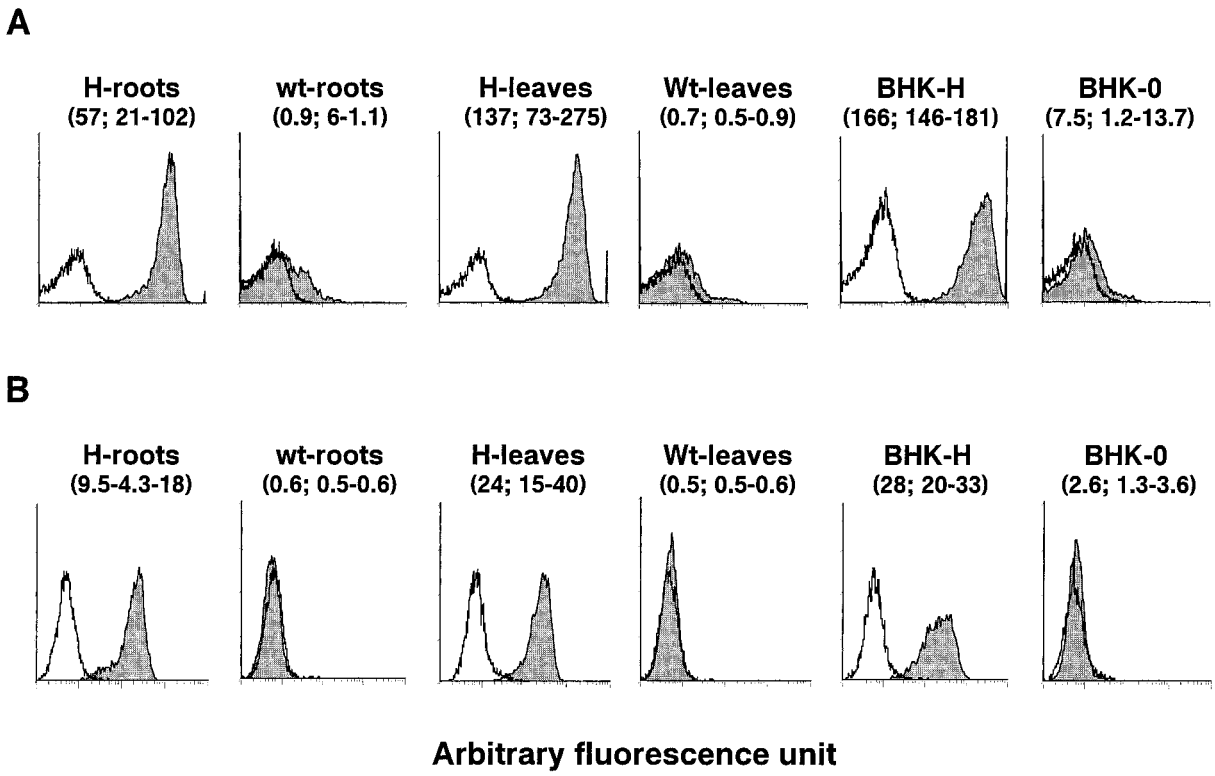

Figure 6. Flow cytometry analysis of mouse sera (1:50) on (A) MV-superinfected EBV-transformed human B cell line or (B) H-transfected Mel-Juso cells after immunization with plant-derived antigen. Median and range of arbitrary fluorescence units are shown for each group of mice. Uninfected or untransfected cells were used as negative cells control (open histograms).

In addition to a strong virus cross-reactivity, all sera showed high levels of neutralizing antibodies in a standard plaque reduction neutralization assay. In the group immunized with leaf extracts, mean neutralizing titres of 10 700 (range 5500–22 500) were observed, while root extracts induced significantly lower titres (3000; range 800–7400). All sera from mice given extracts of untransformed plants had titres <64 and were considered negative.

In general, both cross-reactive and neutralizing titres were higher in leaves than in roots. This could reflect a difference in expression or in the yield of the extraction procedure.

Discussion

Although edible vaccines seem to be feasible, very few antigens have been expressed in plants fit for human consumption (Modelska *et al.*, 1998; Kapusta *et al.*, 1999; Sandhu *et al.*, 2000). For instance, potatoes have been used to express antigens of human pathogens (Mason *et al.*, 1996; Arakawa *et al.*, 1998a, b; Richter *et al.*, 2000; Kong *et al.*, 2001). However, raw potatoes are not very appealing, and cooking can drastically reduce the immunogenicity of the vaccine (Kong *et al.*, 2001). This is the first report of the expression of an antigenic protein as a transgene in mature carrots (which can be eaten raw by human beings) and of the antigenic and immunogenic properties of the heterologous protein in mice. Genomic integration of the MV-H gene under the CaMV 35S double promoter was obtained using *Agrobacterium tumefaciens*. Southern blot analysis showed that all regenerated transgenic plants analysed integrated a single copy of the transgene and that integration occurred randomly in the carrot genome. In all clones a specific transcript of the expected size could be amplified by RT-PCR.

Under the fluorescence microscope, a fusion protein of MV-H with the green fluorescence protein (GFP) expressed in tobacco protoplasts showed a predominant association with the plasma membrane (data not shown). The membrane fraction of carrot cells produced a strong signal in western blot correspond-

ing to an estimated 2 μg of specific protein per gram of wet weight. Interestingly, the transgenic protein migrated faster in SDS-PAGE than the same protein produced in mammalian cells, suggesting a size difference of about 6 kDa. A similar observation was made when the rabies virus glycoprotein G was expressed in plants (McGarvey *et al.*, 1995). The expression of the C-terminally fused GFP demonstrates that the H protein is fully translated and that the difference in apparent size is most probably due to post-translational modifications. In the virus, the Edmonston strain H protein undergoes glycosylation at 4 of the 5 predicted N-glycosylation sites (Hu *et al.*, 1994). It has been shown that glycosylation of mammalian proteins is efficient in plants, but normally different carbohydrate side chains are utilized (Bardor *et al.*, 1999). The complex glycans of plants are often smaller than those of animals, partially because they lack sialic acid (Faye *et al.*, 1993). In insect cells, which also lack sialic acid, the reduced size of the recombinant H protein was explained by a difference in glycosylation (Vialard *et al.*, 1990). Authentic post-translational modifications such as glycosylation and cystine-bridge formation are thought to be important for intracellular trafficking (Hu and Norrby, 1995) as well as the antigenic conformation of the H protein (Hu *et al.*, 1994; Hu and Norrby, 1995).

Recently, Huang *et al.* (2001) reported the expression of the H protein in tobacco leaves. In this system, the native H protein was undetectable. After adding a retention signal for the endoplasmic reticulum low levels of protein became detectable by ELISA with polyclonal sera from rabbits and man; the reactivity with MABs was even weaker or negative suggesting that the conformation may have been less than optimal.

The immunogenic integrity of the H protein is critical for the induction of antibodies that protect against virus infection and disease. Conformational dependent MABs confirmed the proper antigenic structure of the transgenic protein produced in carrots; while MAB BH1 which recognizes only denatured protein showed no reactivity.

After immunization with extracts of transgenic carrots high levels of virus-neutralizing antibodies were found, using the WHO recommended plaque neutralization assay (Miller *et al.*, 1995). These titres were comparable to those obtained with the mammalian cell-derived H protein extract. The antibody isotype subclasses suggested that in contrast to the H protein of mammalian origin, which produced primarily a Th2 (or antibody-dominated) response, the plant protein induced a Th1/Th2-balanced response, indicative of both a humoral and cellular response. This may be important to safeguard against atypical measles when such a vaccine is used in unprimed individuals.

In many countries carrots are components of the diet of both adults and children and a stable antigenic transgene in an edible plant that can be consumed raw without further processing could bring a vaccine within reach of the most destitute. Although plants can be used as efficient bioreactors for producing antigens, the full potential of plant-based vaccines becomes apparent when immunogenicity can be demonstrated after oral ingestion. A frequent problem of plant-based vaccines is that the response after oral delivery is inconsistent and variable. Sometimes even low levels of antigen can give an effective response after oral administration in mice (Mason *et al.*, 1996; Wigdorovitz *et al.*, 1999) and man (Kapusta *et al.*, 1999). In other cases mucosal immunity was improved by mucosal adjuvants (Arakawa *et al.*, 1998b; Kong *et al.*, 2001). In the study by Huang *et al.* (2001), the immune response after oral administration of transgenic tobacco was very weak even when exceedingly high levels (3 times 1 mg) of CTB where co-administered. However, when experimental oral vaccines were given after parenteral priming, results were more consistent than after oral immunization alone (Kong *et al.*, 2001; Mantis *et al.*, 2001). Therefore, a prime booster schedule with an oral vaccine could be an attractive alternative when immunity to the current (injectable) live-attenuated measles vaccine wanes in adults. Further studies are required to confirm the efficacy of the carrot plant expressed measles antigen in such a scenario.

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