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Synthesis of an HIV-1 Tat transduction domain-rotavirus enterotoxin fusion protein in transgenic potato

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Abstract A DNA fragment encoding a 12-amino acid (aa) HIV-1 Tat transduction peptide fused to a 90-aa murine rotavirus NSP4 enterotoxin protein (Tat-NSP4$_{90}$) was transferred to *Solanum tuberosum* by *Agrobacterium tumefaciens*-mediated transformation. The fusion gene was detected in the genomic DNA of transformed plant leaf tissues by PCR DNA amplification. The Tat-NSP4$_{90}$ fusion protein was identified in transformed tuber extracts by immunoblot analysis using anti-NSP4$_{90}$ and anti-Tat as the primary antibodies. Enzyme-linked immunosorbent assay results showed that the Tat-NSP4$_{90}$ fusion protein made up to 0.0015% of the total soluble tuber protein. The synthesis of Tat-NSP4$_{90}$ fusion protein in transformed potato tuber tissues demonstrates the feasibility of plant cell delivery of the HIV-1 Tat transduction domain as a carrier for non-specific targeting of fused antigens to the mucosal immune system.

Keywords NSP4 · Nonstructural protein · Diarrhea · Enteric disease

Abbreviations *APC:* Antigen-presenting cells · *BA:* Benzyladenine · *BSA:* Bovine serum albumin · *CT:* Cholera toxin · *CTB:* Cholera toxin B subunit · *CTL:* Cytotoxic T lymphocytes · *2,4-D:* 2,4-Dichlorophenoxyacetic acid · *ELISA:* Enzyme-linked immunosorbent assay · *HIV-1:* Human immunodeficiency virus type 1 · *MHC:* Major histocompatibility complex · *IAA:* Indole-3-acetic acid · *NAA:* α-Naphthaleneacetic acid · *NPT II:* Neomycin phosphotransferase II · *NSP4:* Rotavirus enterotoxin non-structural protein · *PBS:* Phosphate-buffered saline · *PBST:* Phosphate-buffered saline containing 0.05% Tween-20 · *PTD:* Protein transduction domain

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Introduction

Throughout the last decade, genetically engineered plants have been increasingly used as vehicles for the production of edible vaccines for protection against a wide variety of human infectious and autoimmune diseases (Mason et al. 1992, 1996; Haq et al. 1995; McGarvey et al. 1995; Thanavala et al. 1995; Arakawa et al. 1998,1999; Modelska et al. 1998; Tacket et al. 1998). However, the expression level of vaccine protein antigens in genomically transformed plants is only 0.001–0.3% of the total soluble plant protein (Yu and Langridge 2000), potentially limiting the extent of the immune response. To generate greater levels of immunity, alternative strategies must be developed, such as the use of adjuvants to stimulate immune responses to the antigen or the targeting of available antigen molecules to the mucosal immune system. Antigen targeting has been accomplished by the fusion of antigen protein to ligands that bind to and efficiently enter gut epithelial cells (Arakawa et al. 1998, 1999, 2001; Yu and Langridge 2001).

In contrast to available bacterial toxin B subunit ligands, which target antigen to the Gm1 ganglioside receptor on enterocyte cells, human immunodeficiency virus type 1 (HIV-1) Tat protein enters the cytosol of most mammalian cell types through the plasma membrane by an as yet unidentified mechanism (Frankel and Fabo 1988; Fawell et al. 1994). Tat transduction peptide fusion with ovalbumin, β-galactosidase, and horseradish peroxidase have been shown to retain their enzymatic activity following entry into mammalian cells (Fawell et al. 1994; Watson and Edwards 1999). A sequence of basic amino acids from HIV-1 Tat (RKKRRQRRR), called the protein transduction domain (PTD), has been identified to be linked to the direct uptake of heterologous proteins into mammalian cells (Nagahara et al. 1998; Vocero-Akbani et al. 1999). When incubated with antigen-presenting cells

(APCs), Tat-conjugated peptides were presented to T cells in association with major histocompatibility complex (MHC) class I receptors, resulting in the stimulation of antigen-specific cytotoxic T lymphocyte (CTLs) responses in vitro. Further, mice immunized with dendritic cells exposed to tat-ovalbumin conjugates generated antigen-specific CTL responses (Kim et al. 1997).

Enteric diseases causing dehydration through the effects of diarrhea claim the lives of millions of people annually, most of whom are children in economically developing countries. Rotaviruses are the single most important cause of virus-based severe diarrheal illness in infants and young children in industrialized and developing countries (Kapikian and Chanock 1996). Mammalian rotaviruses belong to the family *Reoviridae* (Jawetz et al. 1989) and are spherical, 70-nm particles first characterized in 1973 (Bishop et al. 1973). The virus genome contains 11 segments of double-stranded RNA, each encoding a viral capsid or nonstructural protein (Kapikian and Chanock 1996).

Identification of a rotavirus non-structural protein gene (NSP4) encoding a peptide of 175 amino acids, which functions both as a viral enterotoxin and a factor involved in the acquisition of host cell membrane during virus budding into the endoplasmic reticulum (ER) and from cells, has provided a new approach for mucosal immunization (Ball et al. 1996; Newton et al. 1997). Johansen et al. (1999) showed that the induction of antibodies to a 22-amino acid (aa) immunodominant epitope of the non-structural protein ($NSP4_{22}$) generated protective humoral and cellular immune responses in human subjects, thereby providing protection from clinical disease without the need for the induction of antibodies to viral capsid or other structural proteins. Arakawa et al. (2001) detected the synthesis and oligomer assembly of the CTB (cholera toxin B subunit)-rotavirus enterotoxin NSP4 22-aa immunodominant epitope fusion protein in transformed potato plants. Further, the $NSP4_{22}$ epitope was found to generate protective antibodies in rotavirus-challenged, orally immunized mice (Yu and Langridge 2001). The CTB-$NSP4_{22}$ mucosal vaccine also provided a significant reduction in diarrhea symptoms in passively immunized mouse neonates challenged with simian rotavirus SA-11. Therefore, the plant-synthesized mucosal vaccination approach is clearly promising with respect to the protection of infants and young children**.**

To enhance the mucosal immune response to NSP4, we constructed a plant expression vector containing the $NSP4_{90}$ gene encoding a 90-aa peptide that omits the membrane destabilizing domain. The larger antigen protein could generate additional linear and possibly conformational NSP4 epitopes that might be expected to stimulate an increased and potentially more diverse immune response that may provide more complete protection of immunized mice against rotavirus infection. An HIV-1 12-aa protein transduction domain (Tat), known to present fusion proteins by MHC class I receptors on APCs, was fused to $NSP4_{90}$ and the construct transferred into potato explants. Here, we demonstrate for the first time that the Tat-$NSP4_{90}$ fusion gene is transcribed and translated correctly in regenerated transgenic plants. Transformed tuber tissues can be used in future oral immunization experiments to determine whether the Tat protein transduction peptide, and the additional $NSP4_{90}$ amino acid sequence, can provide more complete protection against rotavirus infection in preclinical animal trials.

Materials and methods

Construction of plant expression vector pPCV701Tat-NSP4

A Tat-$NSP4_{90}$ fusion gene encoding the 90-aa NSP4 peptide lacking membrane-destabilizing activity was constructed using routine PCR cloning methods. The oligonucleotide 5′ primer (5′-GG**CCATGG**CCAAAGAGCAGATAACT-3′) and the 3′ primer (5′-GC**GAATTC**AGTCAACTTATCGTAAAT-3′) synthesized in the Loma Linda University Core Facility were used for amplification of the $NSP4_{90}$ gene from plasmid pCR2.1-NSP4 containing the simian virus SA 11 gene 10 encoding the full-length $NSP4_{175}$ protein (provided by Dr. M. Estes, Baylor School of Medicine, Houston, Tex.). Briefly, the PCR conditions included 30 cycles of PCR amplification (DNA strand denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and complementary strand synthesis at 72°C for 30 s). The PCR products were digested with *Nco*I and *Eco*RI, and the digested PCR products were ligated with pHA-Tat (Nagahara et al. 1998) previously digested with *Nco*I and *Eco*RI. One additional PCR reaction was conducted to amplify the Tat-$NSP4_{90}$ fusion gene from ligated DNA using the 5′ primer (5′-GC**TCTAGA**GCCACCATGGGCCGCAAGAAACGC-3′) and the 3′ primer (5′-GC**AGATCT**AGTCAACTTATCGTAAAT-3′). The oligonucleotide sequence surrounding the translation initiation codon of the Tat PTD was converted to a preferred nucleotide context (ACCATGA) for more efficient translation in eukaryotic cells (Kozak 1981). The amplified Tat-$NSP4_{90}$ fusion gene fragment was inserted into plant expression vector pPCV701FM4;SEKDEL, which contains a DNA sequence encoding the ER retention signal (SEKDEL), under control of the *mas* P2 promoter (Velten et al. 1984). Plasmid DNA in the ligation mixture was transferred into *Escherichia coli* strain HB101 by electroporation (Arakawa et al. 1997), and ampicillin-resistant colonies were isolated after overnight culture at 37°C on LB plates containing 100 μg ml^{-1} ampicillin. To confirm the presence of the correct Tat-$NSP4_{90}$ fusion DNA sequence in transformed *E. coli* cells, we isolated plasmid DNA from individual colonies of transformants and subjected them to DNA sequence analysis in a model 373A DNA Sequencer (Applied Biosystems, Foster City, Calif.). The plasmid with the correct DNA sequence was designated as pPCV701Tat-NSP4 (Fig. 1). The plant expression vector was transferred into *Agrobacterium tumefaciens* strain GV3101 pMP90RK by electroporation and the plasmid DNA in the transformed *Agrobacterium* cells checked for spontaneous deletions by restriction endonuclease digestion prior to transformation of potato stem explants (Arakawa et al. 1997).

Plant transformation

Potato plants *Solanum tuberosum* cv. Bintje were grown under sterile conditions in Magenta GA-7 culture boxes (Sigma, St. Louis, Mo.) on MS basal medium (Murashige and Skoog 1962) containing 3.0% sucrose and 0.2% Gelrite at 20°C in a light room under a 16/8 (day/night) photoperiod regime with light provided by cool-white fluorescent tubes at an intensity of 12 μmol photons m^{-2} s^{-1}. Stem explants were excised from the plants and immersed in a culture dish containing an overnight suspension culture of exponential-

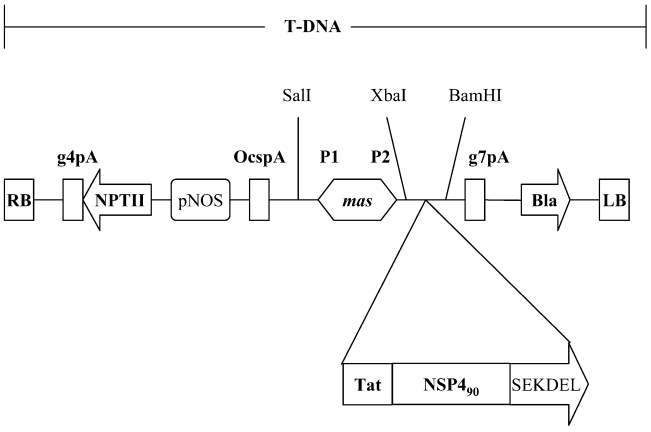

Fig. 1 Plant expression vector pPCV701Tat-NSP4. The genes located within the T-DNA sequence flanked by the right and left border (*RB* and *LB*, respectively) are: the Tat-NSP4$_{90}$ antigen fusion gene under the control of the *mas* P_2 promoter, an *NPT II* expression cassette for kanamycin selection of transformed plants, a beta-lactamase (*bla*) gene for detection of ampicillin resistance in *E. coli* and carbenicillin resistance in *A. tumefaciens*. The g7pA polyadenylation signal is from gene 7 in the *A. tumefaciens* T_L-DNA; the OcspA polyadenylation signal is from the *A. tumefaciens* octopine synthase gene; the Pnos promoter is from the *A. tumefaciens* nopaline synthase gene; the g4pA polyadenylation signal is from gene 4 in the T_L-DNA

phase *A. tumefaciens* (1×10^{10}cells ml^{-1}). The explants were incubated in the bacterial suspension for 15 min, blotted on sterile filter paper, and transferred to MS basal solid medium, pH 5.7, containing plant growth regulators, 0.4 μg ml^{-1} IAA and 2.0 μg ml^{-1} BA. The stem explants were incubated for 2 days at 20°C on MS basal solid medium containing IAA and BA to permit T-DNA transfer into the plant genome. For selection of transformed plant cells and for counter selection against continued *Agrobacterium* growth, the explants were transferred to MS solid medium containing kanamycin (100 μg ml^{-1}) and cefotaxime (300 μg ml^{-1}). Transformed plant cells formed calli on the selective medium during continuous incubation for 2–3 weeks. When putative transformed calli grew to 5–10 mm in diameter (3–4 weeks), they were transferred to MS basal solid medium containing 2.0 μg ml^{-1} BA and 0.1 μg ml^{-1} gibberellic acid, 100 μg ml^{-1} kanamycin and 300 μg ml^{-1} cefotaxime for shoot induction. After 3–6 weeks of further incubation in the light room, regenerated shoots were excised from the calli and transferred to MS basal solid medium with antibiotics and without growth regulators to stimulate root formation. After the putative transformed potato plantlets formed roots (3–6 weeks), they were transferred to pots in the greenhouse and grown to maturity (2–3 months) at natural day length.

Detection of the Tat-NSP4$_{90}$ fusion gene in transformed plants

Genomic DNA was isolated from transformed potato leaf tissues using the DNeasy Plant Mini kit (Qiagen, Valencia, Calif.). The concentration of genomic DNA was measured in a UV spectrophotometer (at 260 nm). The presence of the Tat-NSP4$_{90}$ fusion gene in transformed potato DNA (400 ng) was determined by PCR analysis using the 5′ Tat primer and the 3′ NSP4 primer under the same conditions as those for the subcloning described above. In addition, transgenic potato genomic DNA extracts were subjected to PCR analysis with primers (5′-CACCCAAGACGCCGGAGC-3′ and 5′-GGGCGGAAACCCTTGCAA-3′) specific for the plasmid region outside the T-DNA portion to address the possibility that PCR products can come from the presence of contaminating *Agrobacterium* plasmid DNA.

Detection of Tat-NSP4$_{90}$ fusion protein in transformed potato tissues

Transformed potato tuber tissues were analyzed by immunoblot analysis for the presence of Tat-NSP4$_{90}$ fusion gene expression. Tuber tissues were surface-sterilized with a 20% solution of commercial bleach containing 2–3 drops of Tween-80. The sterile tubers were sliced and incubated for 5 days on MS basal solid medium containing 5.0 mg l^{-1} NAA and 6.0 mg l^{-1} 2,4-D to activate the *mas* dual promoters. The auxin-activated tissues were homogenized by grinding in a mortar and pestle at 4°C in extraction buffer (1:1, w/v) (200 m*M* Tris-Cl, pH 8.0, 100 m*M* NaCl, 400 m*M* sucrose, 10 m*M* EDTA, 14 m*M* 2-mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride, 0.05% Tween-20). The tissue homogenate was centrifuged at 17,000 *g* in a Beckman GS-15R centrifuge for 15 min at 4°C to remove insoluble cell debris. An aliquot of supernatant containing 100 μg of total soluble protein, as determined by the Bradford protein assay (Bio-Rad, Hercules, Calif.), was separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 1.5–2 h in Tris-glycine buffer (25 m*M* Tris-Cl, 250 m*M* glycine, pH 8.3, 0.1% SDS). Prior to electrophoresis, the samples were loaded on the gel after boiling for 5 min to ensure protein denaturation. The separated protein bands were transferred from the gel to approximately 80-cm^2 Immun-Lite membranes (Bio-Rad) by electroblotting on a semi-dry blotter (Sigma) for 90 min at 30 V and 70 mA. Nonspecific antibody binding was blocked by incubation of the membrane in 25 ml of 5% non-fat dry milk in TBS buffer (20 m*M* Tris-Cl, pH 7.5, and 500 m*M* NaCl) for 1 h with gentle agitation on a rotary shaker (40 rpm), followed by washing in TBS buffer for 5 min. Primary anti-NSP4 antibody was generated in a rabbit with NSP4$_{90}$ protein expressed and purified from *E. coli* BL21 cells, and primary anti-Tat was obtained from Dr. Andreas Gruber, Harvard University. The membrane was incubated overnight at room temperature with gentle agitation in a 1:2,000 dilution of rabbit anti-NSP4 antiserum in TBST antibody dilution buffer (TBS with 0.05% Tween-20 and 1% non-fat dry milk) followed by three washes in TBST washing buffer (TBS with 0.05% Tween-20). The membrane was incubated for 1 h at room temperature with gentle agitation in a 1:7,000 dilution of mouse anti-rabbit IgG conjugated with alkaline phosphatase (Sigma A-2556) in antibody dilution buffer. The membrane was washed three times in TBST buffer as before and then incubated in 10 ml of BCIP/NBT alkaline phosphatase substrate (Sigma B-5655) for 15 min at room temperature with gentle agitation on a rotary shaker.

Quantitation of Tat-NSP4$_{90}$ fusion protein in transformed potato tissues

The expression levels of Tat-NSP4$_{90}$ fusion protein in transformed potato plants were evaluated by quantitative chemiluminescent ELISA methods. The microtiter plate was coated with 100 μl per well of tenfold serial dilutions of a centrifuged plant extract containing total soluble potato tuber proteins in bicarbonate buffer, pH 9.6 (15 m*M* Na_2CO_3, 35 m*M* $NaHCO_3$), covered with Saran wrap, and incubated at 4°C overnight. Following incubation, the wells were blocked by adding 200 μl per well of 1% bovine serum albumin (BSA) in PBS and incubated at 37°C for 2 h, followed by washing three times with PBST (PBS containing 0.05% Tween-20). The plate was washed three times in PBST and the wells loaded with 100 μl per well of 1:8,000 dilution of rabbit anti-NSP4 antibody. The plate was incubated for 2 h at 37°C, followed by washing the wells three times with PBST. The plate was loaded with 100 μl of a 1:20,000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG (Sigma A-2556) per well and incubated for 2 h at 37°C. The plate was washed three times with 300 μl PBST per well, and the plate was finally incubated with 100 μl of Lumi-Phos Plus Chromogenic substrate (Lumigen, Southfield, Minn.) per well for 20 min at 37°C. The enzyme-substrate reaction was measured at room temperature in a Microlite ML3000 Microtiter Plate Luminometer (Dynatech Laboratories, Chantilly, Va.).

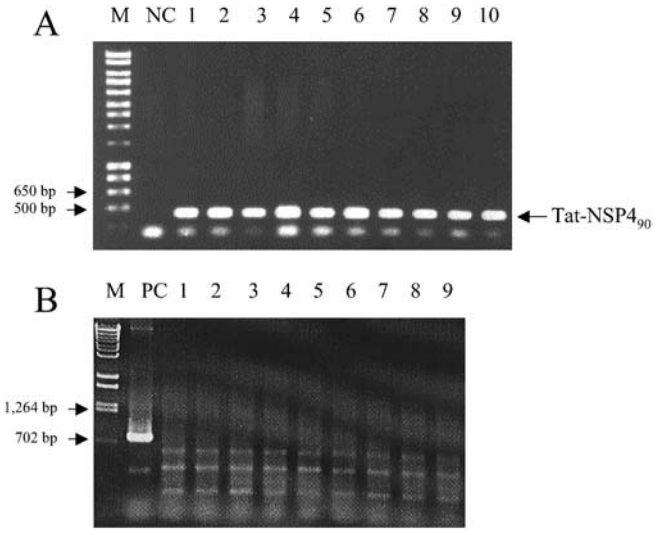

Fig. 2A, B Detection of the Tat-NSP4$_{90}$ fusion gene in transformed potato plant leaf tissues. Genomic DNA (400 ng) from washed transformed potato plant leaf tissues was used to demonstrate the presence of the Tat-NSP4$_{90}$ fusion gene (**A**), and PCR amplification with primers specific for the plasmid region excluding the T-DNA (immediately downstream of the T-DNA right border) showed no *Agrobacterium* contamination (**B**). **Panel A** *Lane M* 1-kb Plus DNA Ladder (Gibco BRL), *lane NC* untransformed plant genomic DNA used as a negative control, *lanes 1–10* transformed plant genomic DNA showing the Tat-NSP4$_{90}$ fusion gene. **Panel B** *Lane M* lambda DNA-*Bst*EII digest molecular-weight markers (New England Biolabs), *lane PC* pPCV701Tat-NSP4 used as a positive control, *lanes 1–9* transformed plant genomic DNA showing no bands of the expected band size

Results

Detection of the Tat-NSP4$_{90}$ fusion gene in transformed potato plants

Ten independent kanamycin-resistant potato plants formed roots on MS medium containing kanamycin (100 μg ml^{-1}). The presence of the Tat-NSP4$_{90}$ fusion gene was detected by PCR analysis of washed young leaf tissue genomic DNA isolated from the putative transformants, and no DNA band corresponding to the Tat-NSP4$_{90}$ fusion gene was detected in untransformed potato leaf genomic DNA (Fig. 2A). PCR amplification with primers specific to the plasmid region excluding T-DNA showed a 760-bp DNA fragment from pPCV701Tat-NSP4 but did not detect any corresponding band from the genomic DNA of transformed potato plants (Fig. 2B).

Detection and quantification of plant-synthesized Tat-NSP4$_{90}$ fusion protein

The Tat-NSP4$_{90}$ fusion protein (approx. 13 kDa) was detected in three transformed potato tuber tissue extracts out of ten putative transgenic tubers (Fig. 3). No signal corresponding to the Tat-NSP4$_{90}$ fusion protein was detected in untransformed boiled plant extracts.

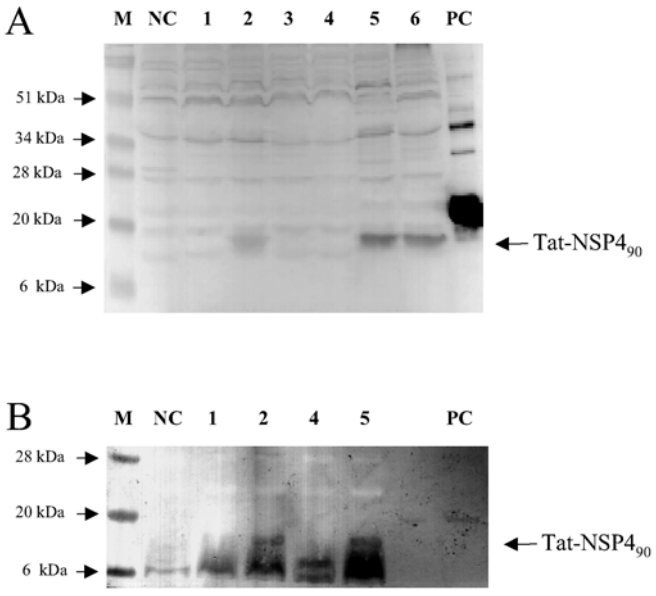

Fig. 3A, B Immunoblot detection of Tat-NSP4$_{90}$ fusion protein in transformed potato tuber tissues. Auxin-induced tuber tissue extracts from transformed potato plants were analyzed for expression of the Tat-NSP$_{90}$ fusion protein using anti-NSP4 (**A**) and anti-Tat antiserum (**B**) as primary antibody. **Panel A** *Lane M* molecular-weight markers (Bio-Rad), *lane NC* negative control extract of untransformed potato tuber tissues (100 μg per lane), *lanes 1–6* extracts of transformed plant tuber tissues (100 μg per lane), *lane PC* positive control is bacterial Tat-NSP4$_{90}$ fusion protein synthesized in and purified from *E. coli* BL21 cells. **Panel B** *Lane M* molecular-weight markers (Bio-Rad), *lane NC* negative control extract of untransformed potato tuber tissues (100 μg per lane), *lanes 1, 2, 4 and 5* extracts of transformed plant tuber tissues (100 μg per lane), *lane PC* positive control, bacterial Tat-NSP4$_{90}$ fusion protein. The additional 4 kDa molecular weight of the bacterial Tat-NSP4$_{90}$ fusion protein is due to the his tag portion of the pRSET vector, which is absent from the plant Tat-NSP4$_{90}$ fusion protein

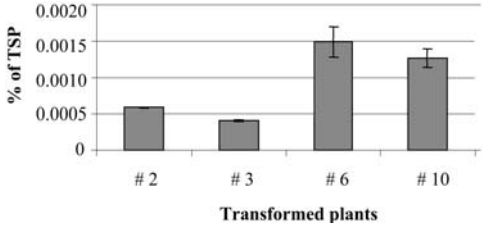

Fig. 4 Tat-NSP4$_{90}$ fusion protein levels in transformed potato tissues. Anti-NSP4 antiserum was used as the primary antibody in the ELISA assay. Serial dilutions of plant extracts containing total soluble potato tuber proteins were used for the ELISA assay. Relative light units generated by the samples were measured and compared with the bacterial Tat-NSP4$_{90}$ standard curve to determine Tat-NSP4$_{90}$ fusion protein expression levels in the transformed tuber tissue extracts

The amount of Tat-NSP4$_{90}$ fusion protein in the transformed tuber tissues was determined on the basis of relative light units (RLU) detected in comparison with the RLU of a bacterial Tat-NSP4$_{90}$ fusion protein-based standard curve. The amount of Tat-NSP4$_{90}$ recombinant

protein as part of total soluble plant protein (TSP) was calculated by dividing the amount of Tat-$NSP4_{90}$ detected based on RLU by the TSP identified in the plant tissue as determined by the Bradford protein assay (Bio-Rad) and found to range from 0.0004% to 0.0015%. Transformed plant no. 6 was found to contain the highest transgenic protein expression level—equivalent to approximately 0.0015% of the total soluble tuber protein (Fig. 4).

Discussion

The rotavirus non-structural protein enterotoxin (NSP4) has strong antigenic properties and has been implicated in the cytopathic effects of rotavirus in mammalian cells (Tian et al. 1994; Hoshino et al. 1995; Ball et al. 1996). Expression of the NSP4 gene in insect (*Spodoptera frugiperda*) cells by recombinant baculovirus showed that the polypeptide induced a rise in the concentration of intracellular free calcium (Ca2+) (Tian et al. 1994) and enhanced membrane-destabilizing activity in *E. coli* and mammalian cells typical of viral enterotoxins (Newton et al. 1997; Browne et al. 2000). In earlier studies, the amount of $NSP4_{22}$ immunodominant epitope required to induce diarrhea in mice was found to be considerably higher than the effective dosage of full-length $NSP4_{175}$ (Ball et al. 1996). Thus, the 22-aa immunodominant peptide may represent only one of several available epitopes in the active toxin. It is likely that additional portions of the toxin molecule may be required to generate full toxicity and maximum antigenicity. The NSP4 enterotoxin contains a region that has a direct membrane destabilization activity that can cause ER membrane damage (Tian et al. 1994). An NSP4 peptide containing residues 48–91 was found to contain a membrane-destabilizing domain that was lethal when expression was attempted in *E. coli*, which has a membrane structure similar to that of the ER (Browne et al. 2000). Therefore, an NSP4 peptide of 90 amino acids ($NSP4_{90}$ containing residues 86–175) but excluding the membrane-destabilization domain was used in our study to increase the number of epitopes available for generation of an immune response. Young CD-1 mice immunized with CTB-$NSP4_{90}$ including the $NSP4_{22}$ epitope synthesized and purified from *E. coli* generated higher serum IgG antibody titers against the $NSP4_{90}$ peptide than mice immunized with CTB-$NSP4_{22}$ (unpublished data). These results suggest that $NSP4_{90}$ contains additional linear and conformational epitope(s) available to enhance the protective efficacy of the enterotoxin-stimulated immune response.

Although the *mas* dual promoters remain largely inactive under conditions of normal plant growth, small amounts of fusion protein may be synthesized locally in response to endogenous levels of auxin (Langridge et al. 1989). The small amounts of Tat-$NSP4_{90}$ fusion protein that may be synthesized in transformed plants do not appear to adversely affect morphology of the transformed plants.

CTLs play an important role in protection against viral infection of the host. Virus-specific CTLs can be detected prior to the appearance of the neutralizing antibody, as early as 4 days after infection with viruses such as ectromelia or influenza (Blanden 1974; Yap and Ada 1978). Activated CTLs can eliminate virus-infected host cells prior to the release of progeny virus particles into circulation, resulting in the effective limitation or early clearance of viral infection (Yap et al. 1978; Zinkernagel and Althage 1977). Passive transfer of immune CTLs has been shown to protect against acute rotavirus-induced diarrhea in suckling mice (Offit and Dudzik 1990) and to clear chronic rotavirus infection from adult severe combined immunodeficiency mice (Dharakul et al. 1990). A cellular immune response to NSP4 was detected in naturally infected adults, indicating that NSP4 may stimulate a cellular immune response, possibly including activated CTLs (Johansen et al. 1999). Antigen presentation in association with MHC class I receptors on APCs is required to induce an antigen-specific CTL response. The HIV-1 Tat transduction domain was shown to be processed and present on APCs MHC class I receptors stimulating antigen-specific CTL activation in vivo in immunized mice (Kim et al. 1997). Transgenic potato tubers containing Tat-$NSP4_{90}$ fusion proteins may be used to generate increased number of CTLs for protection of host cells against rotavirus infection and will be the subject of further analysis in future animal mucosal immunization experiments.

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