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Expression of a Ricin Toxin B Subunit: Insulin Fusion Protein in Edible Plant Tissues

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Abstract

Onset of juvenile Type 1 diabetes (T1D) occurs when autoreactive lymphocytes progressively destroy the insulin-producing beta-cells in the pancreatic Islets of Langerhans. The increasing lack of insulin and subsequent onset of hyperglycemia results in increased damage to nerves, blood vessels, and tissues leading to the development of a host of severe disease symptoms resulting in premature morbidity and mortality. To enhance restoration of normoglycemia and immunological homeostasis generated by lymphocytes that mediate the suppression of autoimmunity, the non-toxic B chain of the plant AB enterotoxin ricin (RTB), a castor bean lectin binding a variety of epidermal cell receptors, was genetically linked to the coding region of the proinsulin gene (INS) and expressed as a fusion protein (INS–RTB) in transformed potato plants. This study is the first documented example of a plant enterotoxin B subunit linked to an autoantigen and expressed in transgenic plants for enhanced immunological suppression of T1D autoimmunity.

Keywords

Ricin toxin B chain; Insulin; Type 1 diabetes; Autoimmune; Oral tolerance; Transgenic plant; Potato

Introduction

Mucosal delivery of autoantigens has long been pursued as a means of prevention, suppression, and treatment of autoimmune disorders through stimulation of immune tolerance (for a recent review, see Faria and Weiner [1]). For Type 1 diabetes (T1D), the autoantigen of choice for generation of oral tolerance is frequently insulin [2–7]. Immune tolerance generated toward an autoantigen by oral delivery of the antigen was shown to reduce disease symptoms in several animal models of autoimmune disease [8–18]. Further, it was shown that autoantigen dosage can be reduced substantially when the autoantigen is linked to a bacterial A–B enterotoxin adjuvant or co-delivered with an adjuvant [8, 11, 19]. In many cases, the adjuvant used to stimulate immune responses has been the cholera toxin B protein chain (CTB). The CTB oligomer exists in nature as a pentameric protein consisting of five identical subunits that bind for the most part to G_{M1} ganglioside—a glycolipid found in apical membranes of all epithelial cells and especially the intestinal epithelium [20]. Genetic linkage of the CTB gene at either its N or C terminus with genes encoding a diabetes autoantigen such as insulin or glutamic acid decarboxylase (GAD), can be expressed and retained in the cytoplasm of transgenic plant cells for large-scale production of the immunomodulated autoantigen. The purified fusion protein is assembled

in the plant into pentamers consisting of five CTB-autoantigen subunits arranged in a donut shaped configuration that, when delivered to susceptible animals displaying new onset autoimmune diabetes, were shown to suppress the development of autoreactive autoantigen-specific inflammatory T lymphocytes. A recent strategy for prevention of autoimmune destruction of the pancreatic beta cells was shown to involve exposure of mucosal epidermal cell receptors to self-reactive proteins (autoantigens) such as insulin, which results in the recruitment of T helper cells (Th2) and regulatory T cells (Treg) that retard the destructive process [21]. Because large amounts of insulin are needed to treat an estimated 65,000 diagnosed cases of new onset T1D worldwide per year (International Diabetes Federation, www.idf.org), edible plants were assessed as a potentially inexpensive production and delivery system for the synthesis of human insulin [8]. The biological mechanisms that underlie bacterial exotoxin B subunit protein stimulation of inflammatory or immune suppression responses remain incompletely understood. However, it has been suggested that low doses of antigen favor active suppression of inflammation, while higher doses cause deletion or inactivation (anergy) of antigen-specific lymphocytes [22]. Major players identified among lymphocyte classes activated for immune suppression include T-helper 2 (Th2) cells, Th3 cells, Tr1 cells, CD4⁺CD25⁺ regulatory cells, FoxP3[?], and latency-associated peptide (LAP⁺) regulatory T cells. For a review of the roles that these lymphocytes are known to play in down-regulation of inflammatory responses that form the basis for oral tolerance (see Faria and Weiner [23]).

Our previous laboratory efforts have focused on the development of immunomodulated autoantigens synthesized in edible plants for enhanced prevention of infectious disease or immunological suppression of autoimmunity [8, 24–34]. The major goal of the study presented here was to determine whether plant synthesized ricin enterotoxin B subunit linked to diabetes autoantigens for example insulin could be synthesized in transformed food plants. Establishment of transformed edible plant tissues expressing significant amounts of INS–RTB fusion protein could establish a basis for assessment of INS–RTB maintenance of euglycemia and durable immunological homeostasis in new onset T1D. Earlier experiments showed that the CTB chain when conjugated to insulin, was able to reduce the effective autoantigen dose approximately 5,000-fold for prevention of diabetes onset in the well-known non-obese diabetic (NOD) mouse mammalian model of T1D [15]. While the non-toxic B subunit lectin portion of bacterial A–B toxins was shown to be an effective adjuvant in previous mammalian experiments, several obstacles were encountered that could limit the usefulness of these A–B toxins when linked to antigens or autoantigens. For example, all the previously mentioned toxin B subunit protein chains obtained from bacteria require subunit pentamerization for significant binding to cell-surface G_{M1} carbohydrate receptors. Further, large immunomodulator–autoantigen fusion proteins may generate steric hindrance thereby limiting pentamer formation, resulting in a reduced transfer of antigenic proteins across intestinal cell membranes to underlying immune cells in the lamina propria. An additional concern is that many individuals have had prior exposure to diarrhea-inducing exotoxins and may possess memory T and B cells that could rapidly generate a neutralizing antibody response to these proteins if re-encountered as part of a vaccine therapy. Therefore, based on its properties as a homologous plant protein, we selected the ricin toxin B chain (RTB) from the castor bean plant (*Ricinus communis*) to assess its potential application as an immunomodulatory molecule capable of enhancing immunosuppression to a linked T1D autoantigen. It is known that RTB, a galactose-containing (lectin) molecule is capable of binding epidermal cell glycolipids and glycoproteins with its terminal galactose residues. Since the ricin B chain is monomeric, it is capable of binding a wider variety of host membrane receptors than bacterial toxin B chains. In addition, the RTB chain is taken up into epidermal cells by multiple mechanisms including endocytosis and since it is of plant origin, it is more likely to fold properly to form biologically active immunomodulatory molecules in plant cells. Since consideration of RTB for application as an immunomodulator

and carrier molecule, several reports have demonstrated the expression of properly folded RTB in transgenic plants [35, 36]. Further, the efficacy of RTB enhancement of vaccines against infectious disease has also been demonstrated [26, 37, 38].

Recent experiments in our laboratory have compared the protective effects of different diabetes-specific autoantigens such as human proinsulin (INS) and GAD, following conjugation to lectin adjuvants [39]. These adjuvants included the B chains of cholera toxin—CTB, the heat-labile enterotoxin—LTB from enterotoxigenic *Escherichia coli*, the enterotoxin B chain from *Shigella*—STB, and the plant toxin ricin B subunit—RTB. Each enterotoxin B subunit fusion protein was expressed in transgenic *E. coli* and purified extracts were delivered to NOD mice by oral gavage as a widely accepted T1D animal model. All fusion proteins tested generated a significant increase in autoantigen-mediated immune suppression of pancreatic inflammation (insulinitis). However, the insulin–RTB fusion protein monomer was shown to contain an unusually high number of cysteine residues (15 total). This large number of cysteine residues we found reduced correct RTB protein folding to less than 8% of the purified fusion protein refolded in an optimal buffer for protein refolding (unpublished data). Interestingly, the predominantly denatured form of RTB linked to insulin retained its immunosuppressive activity in comparison with mice fed unconjugated insulin or buffer only. However, the maximum level of RTB immunomodulation could not be adequately assessed since the improperly folded RTB molecule lacked receptor-binding ability. Therefore, to obtain a population of properly folded ricin–autoantigen fusion proteins, the INS–RTB fusion protein DNA construct was introduced into a eukaryotic protein processing system through construction of transgenic potato plants capable of producing the natively-folded insulin–RTB fusion protein (see Materials and Methods). In transformed plants, in addition to the INS and RTB genes, a beta-phaseolin (P) signal sequence from *Phaseolus vulgaris* was inserted immediately in front of the insulin N-terminus to facilitate synthesis and storage of P–INS–RTB in the endoplasmic reticulum.

Plant expression systems offer unique advantages for the production of pharmaceutical compounds. Plant transformation, regeneration of transformed plants, and selection for foreign gene expression can be both time consuming and technically demanding. However, once transformed plants have been selected they can harvest light energy to produce kilograms of therapeutic protein for a fraction of the cost of biopharmaceutical products isolated from transgenic microorganisms [40]. Since plants are eukaryotes, their cells are capable of synthesizing proteins with post-translational modifications and protein processing characteristic of eukaryotes often providing the correct pattern of protein folding required for human immuno-therapy. The construction of plant-produced immunomodulated vaccines for protection against autoimmunity, antibodies, and other medically or agriculturally important drugs have been extensively reviewed [41–43].

Materials and Methods

Construction of Plant Expression Vector pPCV701_P–INS–RTB

Oligonucleotides containing suitable restriction endonuclease sites were introduced 5' and 3' to the genes encoding P, INS, and RTB by routine polymerase chain reaction (PCR) methods. Following PCR amplification, the isolated gene-containing DNA fragments were ligated to create the fusion product *XbaI*–P–*SacI*–INS–*HindIII*–RTB–*SmaI*. The resulting DNA fusion sequence was inserted into the multiple cloning site of pPCV701_MCS, a modified plant expression vector previously described by Koncz et al. [44] as seen in Fig. 1. The modified plant expression vector contains a microsomal retention signal encoding sequence—SEKDEL [45–47], located immediately downstream from the multiple cloning site. The SEKDEL sequence causes the P–INS–RTB fusion protein to be sequestered in the

endoplasmic reticulum via golgi cisternae recycling, which causes the fusion protein to build up in concentration within the cell. The source of the INS gene came from plasmid pPCV701_CTB-INS, a construct designed in our lab for expression in plants that contains human proinsulin linked to the C terminus of the CTB chain [8]. The phaseolin-RTB plasmid template DNA was obtained from Dr. Lynne M. Roberts, University of Warwick, UK and is described in Frigerio et al. [48]. All restriction enzymes, ligase, polymerase, and accompanying buffers were purchased from New England Biolabs, Ipswich, MA. All PCR conditions used in the cloning experiments were identical: 32 cycles, 94°C initial DNA melting for 5 min (30 s for subsequent cycles), 55°C for 30 s of primer annealing, and 72°C for 1 min of DNA replication of the complementary strand and polymerization. The completed pPCV701_P-INS-RTB plasmid (Fig. 1) was transferred into *Agrobacterium tumefaciens* strain GV3101 pMP90RK for transformation of potato plants after verification of the correct DNA sequence. The correct DNA sequences were confirmed by PCR amplification of P-INS-RTB gene fusion and restriction enzyme digestion of the plasmid DNA with XbaI/SmaI to produce the anticipated 1.14 kb P-INS-RTB fragment. In addition, DNA nucleotide sequencing was performed on regions that span upstream and downstream to the P and RTB genes, respectively, to check for correct gene insertion into the plasmid.

In vivo Plant Transformation

Potato plants (*Solanum tuberosum* cv. Bintje) were grown in Magenta GA-7 culture boxes (Sigma, St. Louis, MO) on Murashige and Skoog (MS) basal medium [49] containing 3.0% sucrose and 0.22% Gelrite at 20°C in a light room under cool white fluorescent tubes (12 μ E) set on a 16 h photoperiod regime. *A. tumefaciens* colonies that contain the pPCV701_P-INS-RTB plasmid were selected on yeast extract broth (YEB) solid media with antibiotics, prepared as follows: 5.0 g/l beef extract, 1.0 g/l Bacto™ yeast extract, 5.0 g/l Bacto™ peptone, 5.0 g/l sucrose, 0.1 g/l MgSO₄·7H₂O, pH = 7.2; 18 g/l agarose, autoclaved then cooled to 55°C before adding 100 mg/l carbenicillin, 25 mg/l gentamycin, 50 mg/l kanamycin, and 50 mg/l rifampicin. Plant stem sections (~1 cm) were excised from ~1 month old plants with a sterile scalpel, and immersed in 10 ml of liquid MSO media (Table 1) containing ~0.6 OD culture of transformed *A. tumefaciens* and 250 μ l of 14.8 mM acetosyringone to stimulate *Agrobacterium* infection. After incubation with the *Agrobacterium*, the stem cuttings were placed on P1 solid medium (Table 1) for 2 days in the light room to continue *Agrobacterium*-mediated T-DNA integration into the plant genomic DNA. The *Agrobacterium*-inoculated stem explants were briefly washed free of excess bacteria by brief vortexing in MSO media containing 100 mg/l erythromycin to prevent bacterial growth prior to incubation of the explants on P1-S plates (Table 1) in the light room. The stem explants were transferred to fresh P1-S plates every 7–10 days to prevent bacterial growth. After 1 month on callus formation medium, the stem explants were transferred to P2-S plates (Table 1) to initiate shoot development. New shoots (~1 cm) were removed from the callus with a sterile scalpel and placed upright into P3-S solid medium (Table 1) for root induction. Putative transformed plants were vegetatively propagated in Magenta boxes (Sigma, St. Louis, MO) under sterile conditions until sufficient plant shoot material could be obtained for analysis of transgene expression.

Plant DNA Extraction

Genomic DNA was extracted from >2 cm-wide sterile transgenic plant leaves using the Qiagen DNeasy plant mini kit (Qiagen #69104, Valencia, CA) with the following adaptations to the kit protocol: (1) Leaves from the putatively transformed plants were homogenized to a fine powder in liquid N₂ with a mortar and pestle. (2) After suspension of the powdered leaf material in the lysis buffer included in the Qiagen kit, two rounds of centrifugation at 20,000×g for 5 min at 4°C were performed in a Sorvall RC-5B refrigerated centrifuge to eliminate plant debris. (3) Approximately 40–50 mg of leaf material was used

per QIA shredder mini spin column (half-capacity) to reduce impurities and improve DNA isolation. (4) The spin columns were washed three times with 100% ethanol to thoroughly remove plant compounds that inhibit PCR. (5) The plant genomic DNA was eluted twice from the columns using 50 μ l each of 65°C DNase-free sterile water (100 μ l final volume).

The plant DNA concentration was measured by UV spectrophotometry at 260 nm. The DNA concentration from each plant sample was normalized to 20 ng/ μ l and then separated by electrophoresis on a 0.8% agarose gel that was subsequently stained with ethidium bromide (EtBr). The EtBr-stained DNA bands were visualized by UV light fluorescence to verify the integrity of the extracted genomic DNA.

Identification of INS–RTB Gene in Transformed Plants by PCR

Two sets of optimized primers were constructed to amplify segments of DNA spanning the INS–RTB gene fusion and the kanamycin-resistance neomycin phosphotransferase selectable marker gene (NPT), respectively. The NPT forward primer (5'-GAACAAGATGGATTGCACGCAG GT-3') was complementary to nucleotides at position 7–30 of the Tn5 NPT gene. The NPT reverse primer (5'-AT GTTTCGCTTGGTGGTCGAATGG-3') was complementary to position 394–417. The INS forward primer (5'-ACG AGGCTTCTTCTACACACCC-3') was complementary to position 63–86 of the human proinsulin gene. The RTB reverse primer (5'-ACTGTTCCCTGATGTCGCTGC-3') was complementary to position 319–342 of the ricin toxin B chain gene. Independent PCR reactions for each set of primers was performed using 10 pmol/primer (2 μ l each), 100 ng plant DNA template (5 μ l each), 2.5 mM deoxynucleotidetriphosphates (5 μ l), and 1 μ l of REDTaq polymerase (Sigma #D-8187, St. Louis, MO) in 5 μ l of the supplied 10 $\times$ buffer and brought up to 50 μ l with DNase-, RNase-, and protease-free water. Each 50 μ l reaction mixture was initially melted at 95°C for 5 min, followed by 40 cycles of melting for 30 s at 95°C, 90 s annealing at 59°C, and 45 s of polymerization at 72°C. PCR products were analyzed by 2% agarose gel electrophoresis and bands visualized by UV illumination of EtBr-stained DNA.

Verification of Amplified Sequences From Transformed Plants by Restriction Endonuclease Digestion

PCR products generated from transformed plant DNA using INS–RTB primers were cleaned up with the QIA-quick® nucleotide removal kit (Qiagen #28304, Valencia, CA). The eluted genomic DNA concentration was normalized to 40 ng DNA/ μ l per sample. The samples were subjected to *HindIII* digestion for 2 h at 37°C to cleave the 5'-A[^]AGCTT-3' site located between INS and RTB gene segments. Undigested versus digested PCR products were visualized by UV light fluorescence in an EtBr-stained 2% agarose gel following electrophoretic separation of the DNA bands.

Tissue Print Immunoblot

Tubers grown from genetically transformed plants were initially screened for protein expression by “stamping” potato slices onto a nitrocellulose membrane, followed by standard immunoblot protein identification techniques. Transformed potato plants were maintained in sterile Magenta boxes on P3 solid media until tuber formation (~3–4 months). Three separate tubers from each of three transformed plants (Q, S, and U) were cut ~1 cm from the tuber ends, then placed cut-side down onto 100 $\times$ 25 mm culture plates containing solid P4 protein induction media (Table 1). After 1 week of incubation on P4 media, the tuber slices were gently dabbed onto Kimwipe tissues to remove excess moisture and media. Following this step, the incubated tuber surfaces were firmly and evenly pressed onto a sheet of dry nitrocellulose paper (NitroBind 0.45 micron transfer membrane, MSI, Westboro, MA #EP4HY00010), to transfer in register, proteins from the tuber slice to the membrane. Prior

to protein transfer to increase adsorption of soluble proteins onto the membrane, the nitrocellulose sheets were soaked for 15 min in 200 mM CaCl_2 and allowed to air dry prior to tissue printing. Transformed and control tuber slices were stamped onto the membrane in triplicates using three different tubers per transformed plant. The tuber tissue stamped membrane was blocked for 1 h at room temperature (R/T) by incubating with 5% non-fat dry milk in Tris-buffered saline (TBS)—20 mM Tris, 500 mM NaCl, pH = 7.5. Following a 5 min wash in TBST (TBS + 0.05% Tween-20), the membrane was incubated overnight at 4°C with 1:500 monoclonal mouse anti-human insulin antibody (Advanced ImmunoChemical Inc, Long Beach, CA #HG1-2I1, clone 7F8) diluted with 1% non-fat milk in TBST. The membrane was then washed 3×, 5 min each with TBST and incubated for 2 h at R/T with 1:2,000 goat polyclonal anti-mouse IgG conjugated to alkaline phosphatase (Sigma #A-7686). Finally, the membrane was washed 3×, 5 min each with TBST, 1× with TBS, then soaked in Lumiphos Plus chemiluminescent substrate (Lumigen, Southfield, MI) for 5 min. Luminescence from the tissue blotted membrane was detected with a Hamamatsu low light imaging system equipped with MetaMorph™ software to quantify the luminescence signal emitted from the print of each tissue slice.

Immunoblot Detection of P-INS-RTB in Purified Extracts of Plant Tissues

Tubers were sliced (~1 mm), under sterile conditions and incubated on P4 induction media (Table 1) for 72 h to induce protein expression by auxin (2,4 D) stimulation of the *mas* promoters. Following auxin upregulation of protein synthesis, the tissue slices were stored frozen at -80°C. The frozen tissues were homogenized in liquid N_2 using a mortar and pestle. Excess tissue homogenate was stored at -80°C for further analysis by Asialofetuin ELISA (see below). The homogenized tissue was mixed 1:1 (w/v) with chilled denaturing extraction buffer—DEB (8 M urea, 100 mM NaCl, 100 mM Tris, and 0.1% Tween-20, pH = 8.0). The mixture was vortexed briefly, then centrifuged at 14,000 rpm for 15 min at 4°C to pellet plant debris. The supernatants containing soluble plant proteins were mixed 1:2 with denaturing Laemmli buffer containing 5% β -mercaptoethanol and boiled for 10 min. The samples were then separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gel electrophoresis was run for 1.5 h at 100 V in Tris-glycine buffer (25 mM Tris-Cl, 250 mM glycine, 0.1% SDS, pH = 8.3). Immunoblot transfer of the plant proteins onto nitrocellulose membrane with a semi-dry electrotransfer apparatus (Sigma) was performed at 70 mA for 2 h using standard techniques [50]. The membrane was blocked with 40 ml of 5% non-fat milk in TBST overnight at 4°C. Mouse anti-RTB monoclonal antibodies (clones RB127, RB691, and RB732—Santa Cruz Biotechnology #sc-52191, #sc-52194, and #sc-52195, respectively) were diluted 1:500 each (400 ng/ml final) in 15 ml TBST plus 1% non-fat milk and applied to the membrane overnight at 4°C. Unbound antibodies were washed from the membrane with ~30 ml of TBST 5× (5 min each wash). Goat anti-mouse polyclonal antibodies conjugated to horseradish peroxidase—HRP (Pierce #1858413) were diluted 1:5,000 (2 ng/ml final) in 15 ml TBST plus 1% non-fat milk and incubated with the membrane for 1 h at R/T with gentle shaking. The membrane was washed 5× with TBST (5 min each) and 1× with TBS (10 min). A total of 4 ml Super Signal West Femto chemiluminescent substrate (Pierce #34094) containing 2 ml of Luminol/enhancer plus 2 ml stable peroxide solution was applied by surface tension onto the membrane and incubated for 5 min in the dark. Chemiluminescence was detected on Classic Blue Autoradiography Film BX (MIDSCI #BX57, St. Louis, MO).

Quantitation of RTB Lectin Activity in P-INS-RTB Plants by Asialofetuin-ELISA

Wells of an irradiated polystyrene (high binding) opaque white, flat-bottom Costar® microplate (Corning Inc. #3922, Lowell, MA) were incubated O/N at 4°C with 100 μl /well of 10 $\mu\text{g}/\text{ml}$ asialofetuin (Sigma #A-4781) prepared in bicarbonate buffer, pH = 9.6. Unbound well surfaces were then blocked with 300 μl /well of 5% non-fat milk O/N at 4°C.

Previously prepared frozen tuber homogenates (see Immunoblot section under Materials and Methods) were mixed 1:1 (w/v) with chilled plant extraction buffer—PEB (200 mM Tris base, 1% citric acid, 1% PVP-10, 0.1% Tween-20, pH = 7.7) containing 2% protease inhibitor cocktail (Sigma P-8340) and centrifuged at 14,000 rpm, 15 min at 4°C. Supernatants containing crude soluble protein extracts were applied in triplicate (100 µl/well) to the asialofetuin-coated 96-well microtiter plate and incubated for 1 h at R/T with mild shaking at ~45 rpm on a G2 Gyrotory® shaker (New Brunswick Scientific Co., Inc., Edison, NJ). The wells were washed 4×, 5 min each with 300 µl/well phosphate-buffered saline + 0.05% Tween-20 (PBST) to remove non-specific plant proteins. A mixture of mouse anti-RTB monoclonal antibodies from clones RB127, RB257, RB363, RB691, RB732, RB944, and RB999 (Santa Cruz Biotech #sc-52191~7, respectively) was diluted 1:200 (1.0 ng/ml final) in 1% non-fat milk/PBST and added to the microplate at 100 µl/well. After 1-h incubation with the primary antibodies, the wells were washed 4× with PBST (5 min each). A 1:5,000 dilution (2 ng/ml final concentration), of goat polyclonal anti-mouse IgG-HRP (Pierce #1858413) prepared in 1% non-fat milk/PBST was added at 100 µl/well, and incubated for 1 h at R/T with shaking. Wells were washed 4×, 5 min each with PBST followed by a final rinse with PBS (300 µl/well). SuperSignal Pico West (Pierce #34079) chemiluminescent substrate was prepared following the manufacturers instructions and added to wells at 100 µl/well for 1 min with shaking in the dark. Light emission at 425 nm was measured within 5 min of applying substrate in a microtiter plate luminometer (Berthold Technologies Micro LumatPlus LB 96 V). Relative light units were determined by subtracting background signal from PBS control wells. Untransformed Bintje negative control plant extracts were compared to P-INS-RTB-expressing plants by evaluating mean relative light units (RLU) ± 95% confidence intervals (CI).

Statistics

In Figs. 3 and 5, the mean ± the 95% CI is given. A probability value of $P < 0.05$ was considered to be significant, as represented by non-overlapping standard error bars between sample means.

Results

Detection of the P-INS-RTB Gene Fusion in Transformed Potato Plants

Approximately 30–40 ng/µl of genomic DNA was isolated from 40 to 50 mg of frozen transformed potato leaf homogenate. Mature leaves (>2 cm wide) were selected for DNA extraction because they were found to have less PCR inhibitors than immature leaves (unpublished data). Nine putative transformed plants were analyzed by PCR, labeled as plants A, J, K, L, P, Q, R, S, and U. Three plants (Q, S, and U) consistently yielded amplified products of the anticipated correct molecular weight when using both NPT primers and INS-RTB primers (Fig. 2a, b). When digested with *HindIII* restriction endonuclease (Fig. 2c), the INS-RTB PCR product for each of the three transformed plants was cleaved into the 197 and 347 bp DNA fragments expected for RTB and insulin, based on cleavage of the single *HindIII* site located between INS and RTB gene fusion products (refer to Fig. 1).

Detection of P-INS-RTB Fusion Protein Synthesis in Transformed Potato Tissues by Tissue Immunoprinting

The tissue immunoprints revealed the highest binding of anti-insulin antibody to the P-INS-RTB protein residue from plant U, with somewhat less binding to plant S as compared with the untransformed Bintje tuber tissue prints (Fig. 3). RTB expression levels were the greatest in cell layers immediately underlying the epidermal cell layer. Although levels of bioluminescence characteristic of P-INS-RTB expression were detectable, plant Q was not

considered to be statistically significant in P-INS-RTB expression in comparison with untransformed Bintje tuber prints (Fig. 3).

Immunoblot Detection of P-INS-RTB in Plant Crude Extracts

Crude extracts from putatively transformed plants Q, S, and U all showed anti-RTB specific bands (Fig. 4), although the protein appeared predominantly as large aggregates. Extracts from plants J, L, and P as well as the untransformed Bintje negative control did not react with anti-RTB antibodies. This result was expected based on the lack of a detectable gene fusion product in the PCR results. A plant extract from potatoes expressing phaseolin linked to ricin B chain (P-RTB) previously synthesized in our lab by Dr. Nak-Won Choi was used as a positive control (+), as was commercial RTB (+') purified from castor bean plants (Sigma L-9639). The expected 31.5 kDa band for P-RTB and 29 kDa band for RTB was detected, although aggregated multimers were also observed.

Detection of RTB Lectin Activity in P-INS-RTB Plants by Asialofetuin-ELISA

Crude soluble extracts from plants Q, S and U were analyzed for the presence of P-INS-RTB with properly folded RTB, as indicated by the ability to bind asialofetuin—a galactose-terminating glycoprotein that serves as a ligand for natively folded RTB [51]. All three plants demonstrated a significantly higher signal from crude extracts than did the untransformed potato extract ($P = 0.05$), although plants Q and U were only marginally significant (Fig. 5).

Discussion

Agrobacterium-mediated transformation of potato tissues with T-DNA containing the P-INS-RTB fusion gene resulted in the regeneration of nine putatively transformed (Km^r) plants. PCR evaluation of plant genomic DNA for the presence of integrated P-INS-RTB genes using two different DNA primer sets showed that three of the putatively transformed plants contained amplicons of the correct size. Based on PCR results, the three transgenic plants (Q, S, and U) were analyzed for the presence of the P-INS-RTB fusion protein by tissue immunoprinting with anti-human insulin monoclonal antibodies. The tissue immunoprinting procedure provided supportive *in vivo* data on the location and intensity of INS-RTB fusion protein expression in transgenic tuber slices without resorting to the *in vitro* extraction of soluble protein in experimental buffers that can often lead to protein denaturation or proteolysis of target proteins. Direct application of fresh hand cut transformed tuber sections to the membrane permitted antibody capture of specific folded even improperly folded or insoluble INS-RTB recombinant proteins. Thus, the tissue printing method provided strong immunological confirmation of insulin-RTB protein in the transgenic potato tuber tissues the activity of the fusion protein depending solely upon availability and accessibility of suitable primary and secondary antibodies. The tuber tissue immunoprint data in Fig. 3, demonstrated confirmatory amounts of the P-INS-RTB recombinant protein in tuber tissue slices from plants S and U, even permitting morphological identification of sub-epidermal cell layers generating the highest P-INS-RTB gene expression levels in the live tissues slices. Based on quantification of chemiluminescence from the tuber immunoprints, transformed plants S and U were identified to generate the highest P-INS-RTB fusion protein levels in cells located immediately basal to the tuber epidermis, adjacent to the cambial layer in which cell proliferation appeared to be the highest. This result suggests that recombinant P-INS-RTB and other recombinant proteins may be expressed at their highest levels in rapidly dividing, as yet undifferentiated plant tissues.

Extracts from the transformed and untransformed plants were evaluated by western blotting to confirm the immunospecificity and expected molecular weight of the P-INS-RTB fusion protein. Initial attempts to detect immunospecific protein bands in significant quantities were hampered by the presence of what appeared to be either endogenous protease activity, weak stimulation of the *mas* promoter, or other physical losses that may have occurred during the isolation and purification insulin-RTB protein. The expression level of P-INS-RTB in potato plant extracts was frequently difficult to detect by western blotting using individual monoclonal antibodies. Often, antibodies from several clones had to be combined to generate a detectable signal. The use of anti-RTB polyclonal antibodies proved to be also somewhat problematic based on a large degree of non-specificity that occurred during the performance of western blotting and ELISA techniques. This result may have occurred due to the fact that the commercial antibodies were generated by injecting the ricin A-B enterotoxin isolated from castor bean plants, which may have produced cross-reactive antibodies against common plant proteins or sugar moieties on glycosylated protein structures common to both castor bean and potato plants. A variety of different batches of anti-RTB antibodies were tested with similar results suggesting that the common banding pattern was not due alternative problems such as antibody degradation.

Although distinct bands of P-INS-RTB protein were occasionally identified by western blot using various anti-RTB monoclonal antibodies (Fig. 4), the RTB-autoantigen fusion protein appeared to aggregate into large (>250 kDa) molecular complexes. Further attempts to disassociate the aggregated molecules into the predicted 40.8 kDa band characteristic of P-INS-RTB protein monomers were largely inconclusive, even following incubation of the samples in an SDS detergent sample cracking buffer at different temperatures and with various concentrations of SDS, chaotropes (i.e., 8 M urea), and several reducing agents, including beta-mercaptoethanol, dithiothreitol, and Tris[2-carboxyethyl] phosphine—TCEP (unpublished data). Thus, we suspect the unusually large number of sulfur containing cysteine residues (six residues in insulin and nine cysteine residues in RTB), may have undergone aberrant disulfide bond reformation with partially denatured cysteine-rich proteins in plant crude extracts leading to significant intermolecular disulfide bond formation resulting in the formation of insulin-RTB molecular aggregates. An attempt to decrease protein aggregation achieved minor success through reduction of cysteine disulfide bonds with 100 mM TCEP followed by alkylation of the resultant sulfhydryl groups with 100 mM of iodoacetamide to prevent reestablishment of disulfide linkages (data not shown). Tuber extracts of P-INS-RTB protein prepared from plants “S” and “U” in PEB retained the ability to bind the artificial substrate asialofetuin, which suggests a significant amount of RTB may be folded natively by the plants and may have only aggregated following partial denaturation, as seen in the immunoblot (Fig. 4).

Based on the numerous steps in sample preparation that may have been responsible for partial P-INS-RTB protein denaturation, it remains unclear as to the amount of P-INS-RTB fusion protein that was originally natively-folded in the plant prior to isolation. However, from this study, it appears that transformed edible plants can synthesize significant amounts of soluble, biologically active P-INS-RTB that remains effective for immunological suppression of diabetes symptoms. The results of our earlier experiments using bacterial-synthesized INS-RTB for immune suppression in juvenile NOD mice provided a significant level of prevention of new onset diabetes, even when >90% of the RTB protein was denatured [39]. Thus, bacterial synthesized INS-RTB suppression of T1D demonstrates that the INS-RTB fusion protein synthesized in plants may be even more effective as a vaccination strategy for autoimmune diabetes prevention as it is well-known that plant production systems provide appropriate eukaryotic post-translational modifications that preserve and enhance the biological functions of the recombinant protein. In contrast to other available immunomodulatory systems available to enhance immune responses, RTB

may claim certain advantages: (1) RTB is a plant protein, thus it is more likely to be correctly synthesized and folded by transformable plant systems. (2) Since RTB binds to a variety of receptor types (any glycoprotein or glycolipid with a terminal galactose residue on its sugar groups), there are a great number of opportunities for adhesion and uptake of the fusion protein by cells along the mucosal route. Other enterotoxin B subunits favor only a small subset of receptors, such as Gm1 for CTB or Gb3 for STB. The Gm1 ganglioside receptor also contains galactose. Thus, RTB can bind all the receptors that CTB does and potentially many more. (3) RTB can tolerate large antigen and autoantigen proteins fused to its N terminus (for example, GFP) without disruption of lectin receptor binding activity, unlike CTB which has been shown in our lab to have difficulties forming pentameric oligomers necessary for receptor binding when large autoantigen proteins such as GAD55 are fused to CTB. (4) The general patient population is unlikely to have pre-existing neutralizing antibodies to RTB as previous exposure to ricin is unlikely without being lethal. Hence, RTB-fusion proteins are more likely to be processed efficiently by cells of the immune system. In contrast, many people have been exposed to cholera or cholera vaccines and would therefore be more likely to mount a neutralizing immune response to a CTB-X fusion protein thereby eliminating or reducing immune cell uptake and processing of the antigen resulting in blockage of a significant portion of the toxin carrier effect essential for delivery of antigens to the immune system. (5) RTB fusion proteins used concurrently or sequentially in prime-boost immunizations along with other toxin B subunit-fusion proteins will enhance the immune response toward the fused antigen/autoantigen rather than toward the adjuvant. Thus, the results of this study demonstrate for the first time the abundant opportunities for development of more successful RTB based subunit vaccines for stimulation of immunity and suppression of autoimmunity than have thus far been considered.

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Fig. 1.

Physical map of plant expression vector pPCV701_P-INS-RTB. Genes located within the T-DNA borders flanked by the right and left 25 bp direct repeats (RB and LB) are inserted into the plant genome and include: The *A. tumefaciens mas* P₂ promoter; the beta-phaseolin signal peptide (P), the human proinsulin gene (INS), the ricin toxin B subunit chain (RTB) gene, followed by the SEKDEL endoplasmic reticulum sequestration signal. The g7pA polyadenylation signal is from gene 7 in the *A. tumefaciens* T_L-DNA; the beta-lactamase gene (Bla) for detection of ampicillin resistance in *E. coli* and carbenicillin resistance in *A. tumefaciens*. On the P₁ side of the *mas* dual promoter are located the OcspsA polyadenylation signal from the *A. tumefaciens* octopine synthase gene; the pNOS promoter from the *A. tumefaciens* nopaline synthase gene; a NPTII (neomycin phosphotransferase II) expression cassette for resistance to kanamycin permitting selection of transformed plants. The g4pA polyadenylation signal is from *A. tumefaciens* gene 4 in the T_L-DNA [44]

Fig. 2.

PCR identification of P-INS-RTB DNA in transgenic potato plants. **a** PCR products of plant DNA extracts using primers specific for the kanamycin resistance gene (neomycin phosphotransferase—NPT). The 411 bp NPT amplicon can be seen in all plant DNA PCR products except for potato plants K and R, which appear to contain truncated fragments. **b** PCR detection of the INS-RTB gene fusion with primers that span both genes. Only DNA from transformed potato plants Q, S, and U generated PCR products of the expected 544 bp size, which were further analyzed by digestion with HindIII restriction endonuclease. **c** HindIII digestion of PCR products from plants Q, S, and U showing the expected DNA fragments (347 and 197 bp), when cleaved at the HindIII restriction site between INS and RTB. (-): Untransformed potato (c.v. Bintje) genomic DNA PCR product; (?): PCR product from pPCV701_P-INS-RTB plasmid DNA template extracted from *E. coli* host (strain HB101); *mw* molecular weight markers

Fig. 3.

Tissue immunoprint detection of INS-RTB fusion protein in transgenic tubers. **a** Potato tuber slices were incubated for 1 week on P4 protein induction media and stamped onto a dry nitrocellulose membrane, blocked with non-fat milk, and then stained with mouse anti-human insulin monoclonal antibody (IgG). After washing three times in TBST, the membrane was incubated with goat anti-mouse IgG polyclonal antibodies conjugated to alkaline phosphatase, and visualized by chemiluminescence by adding a small amount of the substrate Lumi-PhosTM to cover the membrane. Presence of the P-INS-RTB fusion protein was detected using the Hamamatsu low light imaging system. *Circled areas* indicate regions analyzed for the presence of luminescence intensity with MetaMorphTM software. **b** The mean light intensity was calculated for three transformed plants (Q, S, and U) in triplicate and compared with untransformed Bintje potato (-)

Fig. 4.

Immunoblot detection of the P-INS-RTB fusion protein in tuber extracts. Anti-RTB specific bands were detected in lanes containing crude homogenate extracts from plants Q, S, and U. In this immunoblot the insulin-RTB fusion protein appears to be aggregated. The negative control untransformed potato plant extract showed no immuno-reactivity to anti-RTB antibodies. Crude extracts from untransformed plants J, L, and P also did not react with the RTB monoclonal antibodies, which confirms the PCR data that these plants are untransformed, possibly chimeras, or partially suppressed transformants generating low INS-RTB yields. Positive controls containing P-RTB (minus the INS gene) from potatoes previously generated in our lab—labeled (+), and commercial RTB (50 ng) purified from castor beans—labeled (+'), both reacted with the anti-RTB antibodies. The expected size of 31.5 kDa for P-INS (+) and 29 kDa for RTB (+') were seen, as were additional bands that appear to be multiples of aggregated RTB due to insufficient reduction of the unusually high number of disulfide bonds present in the insulin-RTB fusion protein

Fig. 5. Asialofetuin-binding ELISA of potato plant extracts. Wells of a 96-well flat bottom microtiter plate were coated with asialofetuin—a glycoprotein capable of binding natively-folded RTB. Crude extracts from potato tubers incubated on P4 protein induction media were applied to the wells and analyzed by detection of chemiluminescence of the anti-RTB labeled protein. Transformed potato plant S demonstrated the highest level of RTB lectin binding activity

Table 1

Formulas for plant growth media

Plant media	Purpose	0.22% gelrite	2.0 mg/l BA	0.5 mg/l IAA	0.5 mg/l GA ₃	100 mg/l Km	300 mg/l Cf	50 mg/l Er	5.0 mg/l NAA	6.0 mg/l 2,4-D
MSO	<i>A. tumefaciens</i> transformation								+	+
P1-(S)	Callus formation	+	+	+			±	±		
P2-(S)	Shoot development	+	+		+	±	±	±		
P3-(S)	Root development	+				±	±	±		
P4	<i>mas</i> promoter activation	+						+	+	+

MSO Murashige and Skoog salt and vitamin mixture (Gibco #10632-057), *S* antibiotic selection (excluded from untransformed control Bintje plants), *BA* benzyl adenine, *IAA* indole acetic acid, *GA* 3 gibberellic acid, *Km* kanamycin—kills untransformed plant cells, *Cefotaxime/claforan*—bactericidal, *Erythromycin*—bactericidal, less toxic to plants, *NAA* alpha-naphthaleneacetic acid—a plant auxin, used to stimulate *mas* promoter, *2,4-D* 2,4-dichlorophenoxyacetic acid—synthetic auxin, more stable than NAA