A plant-based cholera toxin B subunit–insulin fusion protein protects against the development of autoimmune diabetes

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Oral administration of disease-specific autoantigens can prevent or delay the onset of autoimmune disease symptoms. We have generated transgenic potato plants that synthesize human insulin, a major insulin-dependent diabetes mellitus autoantigen, at levels up to 0.05% of total soluble protein. To direct delivery of plant-synthesized insulin to the gut-associated lymphoid tissues, insulin was linked to the C-terminus of the cholera toxin B subunit (CTB). Transgenic potato tubers produced 0.1% of total soluble protein as the pentameric CTB–insulin fusion, which retained GM₁-ganglioside binding affinity and native antigenicity of both CTB and insulin. Nonobese diabetic mice fed transformed potato tuber tissues containing microgram amounts of the CTB–insulin fusion protein showed a substantial reduction in pancreatic islet inflammation (insulinitis), and a delay in the progression of clinical diabetes. Feeding transgenic potato tissues producing insulin or CTB protein alone did not provide a significant reduction in insulinitis or diabetic symptoms. The experimental results indicate that food plants are feasible production and delivery systems for immunotolerization against this T cell-mediated autoimmune disease.

Keywords: vaccine development, insulin-dependent diabetes mellitus, potato, oral tolerance

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease leading to the destruction of insulin-producing pancreatic cells^{1,2}. Both humoral and cellular mechanisms of immunity are involved in disease pathogenesis, although destruction of pancreatic cells appears to be mediated predominantly by the activity of CD4⁺ and CD8⁺ T cells. While there are several autoantigens recognized by the T-cell repertoire, insulin appears to have a critical role in the diabetogenic response, and it has been demonstrated that a major subset of T cells infiltrating the islet respond to insulin in the nonobese diabetic (NOD) mouse³.

Oral administration of pancreatic tissue-specific autoantigens may provide a clinical approach for prevention of spontaneous autoimmune diabetes^{4–7}; however, the therapeutic potential is limited by the requirement for repeated administration of large amounts of autoantigen, and tolerization is usually less efficient in previously sensitized hosts³. The nontoxic B subunit of cholera toxin (CTB) was used to overcome such limitations by serving as a carrier molecule for chemically conjugated autoantigens for the induction of oral tolerance⁸. Therapeutic application of the CTB carrier has been demonstrated for prevention and treatment of autoimmune diseases in animals^{9,10}. Conjugation with CTB may greatly facilitate antigen delivery and presentation to the gut-associated lymphoid tissues (GALT) due to its affinity for the cell surface receptor GM₁-ganglioside located on cells of the GALT including the membranous cells as well as enterocytes for increased uptake and immunologic recognition^{8,11}.

The development of recombinant protein production in plants has allowed the use of genetically modified edible plants for the production and delivery of vaccine antigens^{12–14}. We show that transgenic potato plants synthesizing human insulin and its CTB conjugate suppress insulinitis and clinical symptoms of diabetes in NOD mice.

Results

Construction of transgenic potato plants. Potato plants (*Solanum tuberosum* var. Bintje) transformed with the expression vector pPCV701luxF containing either the human insulin cDNA (INS) or the CTB–INS fusion gene were generated by an *Agrobacterium tumefaciens*-mediated transformation method (Fig. 1). Regenerated kanamycin-resistant putative transformants displayed luxF marker gene activity detected by low light image analysis methods (data not shown). Transformed plants were analyzed for presence of the human insulin gene, its mRNA, and protein product. Presence of the human insulin gene was confirmed in transformed plant genomic DNA (Fig. 2A). A full-length preproinsulin mRNA was detected in total RNA preparations by reverse transcription (RT) PCR amplification using the insulin gene-specific DNA primers (Fig. 2B). Direct PCR amplification of total RNA preparations without the RT step indicated no amplification of this fragment, confirming that the amplification product was not due to DNA contamination of the RNA preparation. Untransformed potato plants did not show the presence of the insulin gene or its mRNA. The amount of insulin protein synthesized in transformed tuber tissue was measured by chemiluminescent ELISA methods, and was found to be approximately 0.05% of total soluble tuber protein (data not shown).

Immunoblot analysis of transgenic potato plants transformed with the CTB–INS fusion gene revealed an oligomeric CTB–INS fusion protein with higher molecular weight than either bacterial CTB or plant-synthesized CTB pentamer¹⁵ (Fig. 3A). The oligomeric fusion protein, which dissociated into monomers by boiling transgenic plant homogenates for 5 min, showed an apparent molecular weight of 30 kDa (Fig. 3B). The oligomeric CTB–INS fusion protein retained specific affinity for GM₁-ganglioside and both CTB and insulin native antigenicity. The amount of total CTB–INS

fusion protein construct was estimated to be approximately 0.1% of the total soluble protein based on the number of relative light units (RLU) generated from known amounts of bacterial pentameric CTB used as a standard (Fig. 3C). A concentration-dependent increase in the RLU signal was observed only when GM₁-ganglioside was used as the capture molecule, indicating that the fusion protein exists as a pentamer since only pentameric CTB can bind GM₁-ganglioside. Similar to bacterial CTB and plant-synthesized CTB, heat treatment dissociated the pentameric form of the CTB-INS protein into monomers that completely lost affinity for GM₁-ganglioside (Fig. 3D). The CTB-INS fusion protein derived from potato leaf tissues exhibited identical biochemical and antigenic properties to the tuber-derived fusion protein.

Figure 1. Plant expression vector pPCV701luxF. RB and LB: the right and left T-DNA borders; mas P1, P2: the mannopine synthase bidirectional promoters for bacterial luciferase (*luxF*) and IDDM autoantigen gene expression respectively; Bla: β -lactamase for ampicillin resistance in *Escherichia coli* and for carbenicillin resistance in *Agrobacterium tumefaciens*; NPT II: neomycin phosphotransferase II gene provides kanamycin resistance in plants; pNOS: nopaline synthase promoter; g7pA, g4pA, and OcsipA: polyadenylation signals from the *A. tumefaciens* T₁-DNA, gene 7, and gene 4, the octopine synthase gene, respectively; Ori pBR: pBR322 origin of replication. (1) human preproinsulin cDNA, (2) CTB-INS fusion gene.

Humoral immune response in mice fed CTB-INS potato tissues. We previously demonstrated that feeding plant tissues synthesizing CTB induced both mucosal and serum antibody responses¹⁴. Feeding CTB-INS potato tuber tissues to NOD mice induced both serum and intestinal anti-CTB antibodies (Fig. 4A). In addition, serum anti-insulin IgG levels were substantially elevated in animals fed CTB-INS potato tissues in comparison with animals fed potato tissues synthesizing insulin or untransformed potato tissues (Fig. 4B). As TH₁ lymphocyte-mediated oral toler-

Figure 2. Human insulin biosynthesis in potato tuber tissue. (A) PCR detection of insulin cDNA. Lane 1: 1 kb molecular weight marker; lane 2: preproinsulin cDNA from plant expression vector pPCV701luxF-INS; lane 3: in the absence of the plant expression vector; lane 4: genomic DNA from untransformed potato leaves; lane 5: genomic DNA potato leaf tissues transformed with preproinsulin cDNA. (B) RT-PCR detection of insulin mRNA. Lane 1: 1 kb molecular weight marker; lanes 2 and 3: total RNA preparations from untransformed potato leaf tissues; lanes 4 and 5: total RNA preparations from preproinsulin gene transformed potato leaf tissues. Lanes 2 and 4: the PCR reaction only, minus the reverse transcription step.

Figure 3. Synthesis of CTB-INS fusion protein in potato tuber and leaf tissues. (A) Immunoblot detection of the pentameric CTB-INS fusion protein. Lane 1: bacterial CTB pentamer; lanes 2 and 4: total soluble protein from leaf and tuber tissues of potato plants transformed with vector minus the CTB-INS fusion gene respectively; lanes 3 and 5: CTB-INS pentamer from leaf and tuber tissues respectively. One hundred grams of total soluble potato protein were loaded per lane. (B) Immunoblot detection of the monomeric CTB-INS. Lane 1: bacterial CTB monomer; lane 2: total soluble tuber protein from a potato plant transformed with vector without CTB-INS gene; lanes 3 and 4: CTB-INS monomers from transgenic potato leaf and tuber tissues, respectively. One hundred grams of total soluble protein were loaded per lane. (C) Quantitative analysis of the binding affinity for GM₁-ganglioside pentameric CTB-INS fusion protein by chemiluminescent GM₁-ELISA. (D) Heat-induced pentamer dissociation into monomers. Approximately equal amounts of the three different constructs indicated were used to measure RLU signal levels.

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Figure 4. Anti-CTB and anti-insulin antibody titers in mice fed transgenic potato tissues. Prediabetic 5-week-old NOD mice were fed transgenic potato tissues containing insulin, CTB-INS, or untransformed tuber tissues once per week until 10 weeks of age. (A) CTB-specific serum and intestinal antibodies and (B) serum anti-insulin IgG1 and IgG2a antibodies determined by the chemiluminescent ELISA method. Serum and intestinal washings were pooled from five individual animals within the same group. The titer was defined as the reciprocal of the highest dilution of the sample to give an RLU signal above the background RLU levels. Data are an average of six measurements.

Figure 5. Level of insulinitis in NOD mice. Five-week-old female NOD mice were fed 3.0 g of potato tuber tissues. The animals were killed at 10 weeks of age for histopathological examination of pancreatic islet tissues. (A) Pancreatic islet from an animal fed CTB-INS potato tissues (histopathologic score, 0). (B) An islet from an animal fed CTB-INS potato tissues (histopathologic score, 2). (C) An islet from an animal fed untransformed potato tissues (histopathologic score, 4). (D) An islet from an animal fed untransformed potato tissues (histopathologic score, 5). Open arrows indicate areas of lymphocyte infiltration. (E) Semiquantitative insulinitis score. Data are expressed as the mean score of each group SEM ($p = 0.001$ for the group fed CTB-INS potato tubers in comparison with the group fed untransformed potato tubers). Insulinitis scores were compared by the student's *t* test for unpaired samples.

ance results in predominantly an IgG1 rather than IgG2a isotype induction, we measured the serum levels of anti-insulin IgG1 and IgG2a antibodies¹⁶. The experimental results indicated a increase in serum IgG1 anti-insulin antibody titers in NOD mice fed transgenic potatoes producing the CTB-INS fusion protein, in comparison with antibody titers detected in the serum of mice fed either untransformed potatoes or transgenic potatoes producing insulin alone (Fig. 4B). All three groups of mice showed similar levels of serum anti-insulin IgG2a.

Suppression of insulinitis. To determine the effects of feeding transgenic potato tissues producing insulin and CTB-INS on the reduction of insulinitis, female NOD mice were fed autoantigen containing potato tuber tissues once per week for 5 weeks starting at 5 weeks of age. The mice were killed at 10 weeks of age for histopathological analysis of pancreatic tissues. At the time of death, all the mice including the animals fed untransformed potato tissues had not yet developed any diabetic symptoms as determined by urine and blood glucose analysis. A normal pancreatic islet showed no signs of lymphocyte infiltration (insulinitis score, 0; Fig. 5A). Pancreatic islets from an animal fed CTB-INS potatoes had an insulinitis score of 2 (Fig. 5B) and an animal fed untransformed potato tissues had an insulinitis score of 4 (Fig. 5C). Islets were heavily infiltrated in a mouse fed untransformed potato tissues (insulinitis score, 5; Fig. 5D). The Student's *t* test revealed a significant reduction in insulinitis in mice fed CTB-INS potato tissues compared with those fed untransformed potato tissues (1.9 ± 0.5 standard error of means [SEM] versus 3.9 ± 0.4 SEM, $p = 0.001$; Fig. 5E). Although there was a detectable reduction in insulinitis severity in NOD mice fed transformed potato tissues containing 30 μ g of insulin (3.8 ± 0.4

Figure 6. Effect on diabetes in NOD mice by feeding CTB-INS potato tuber tissues. Five-week-old female NOD mice (10 animals per group) were fed with either transgenic potato tuber tissue producing CTB or CTB-INS once per week for a total of five weeks. Animals were monitored weekly for diabetes (>250 mg/dl blood glucose) beginning at 10 weeks of age. The results were expressed based on Kaplan-Meier analysis of the control group fed transgenic potatoes producing CTB, and a group fed transgenic potatoes synthesizing CTB-INS ($p < 0.01$, Cox-Mantel log rank statistics).

SEM) compared with animals fed untransformed potato tissues, the reduction in severity was statistically insignificant.

Suppression of diabetes. The plants producing CTB-INS that provided effective suppression of insulinitis were further analyzed by testing the effects of their tuber tissues on suppression of diabetic symptoms using Kaplan-Meier method (Fig. 6). For the time interval between 16 and 24 weeks of age, the incidence of diabetes was substantially lower in the group fed CTB-INS than in the control group fed transgenic potato tubers producing only CTB ($p < 0.01$, log rank test).

Discussion

A major autoantigen of IDDM, glutamic acid decarboxylase, was recently produced in plants and successfully prevented development of diabetic symptoms in NOD mice¹⁶. Oral immunotolerization against autoimmune diseases, however, usually requires repeated administration of large (milligram) amounts of autoantigens^{3,5,16}. We expressed a different major IDDM autoantigen, insulin, and its CTB conjugate protein for enhanced immunologic tolerance⁸. The pentameric structure of the CTB fusion protein not only facilitates site-specific delivery and presentation of conjugated proteins to the GALT, it also increases the molar concentration of the antigen per molecule of CTB pentamer. This increase in antigen concentration in the GALT may substantially reduce the traditional requirement for high levels of antigen biosynthesis in the food plant for effective vaccine production.

The proinsulin was conjugated with CTB via the flexible hinge peptide, which may reduce steric hindrance between the CTB and insulin moieties facilitating CTB subunit assembly in plant cells¹⁷. The application of less frequently used codons in plants within the hinge peptide may promote translational arrest during the protein elongation process, facilitating CTB subunit folding prior to translation of the insulin message¹⁸. The CTB leader peptide and the microsomal retention signal permit sequestration of recombinant proteins in the plant endoplasmic reticulum, increasing protein concentration for more effective subunit interactions (i.e., CTB fusion protein pentamerization)^{12,15,19,20}. The fusion protein retaining GM₁-ganglioside binding affinity indicates that it exists predominantly as a pentamer as only the pentameric CTB configuration can bind to the receptor. The presence of the pentameric CTB-INS chimera as the predominant protein species in plant tissues suggests efficient pentamerization of the fusion protein within plant cells.

Induction of both systemic and intestinal anti-CTB antibodies in NOD mice fed transformed potato tubers synthesizing the CTB-INS fusion protein suggests that the fusion protein was effectively delivered to the intestinal immune system. This finding was further reinforced by the detection of increased serum anti-insulin IgG antibody titer. The humoral response mounted against both CTB and insulin proteins may be a collateral effect occurring in response to stimulation of Th₁ cells by oral tolerance. There is a decreasing gradient of lymphocyte sensitivity to the induction of oral tolerance with Th₁ cells being the most susceptible to tolerization followed sequentially by Th₂ cells and B cells, it is possible that induction of systemic T cell tolerance may occur simultaneously with systemic and mucosal B cell priming²¹⁻²⁴.

Induction of predominantly serum anti-insulin IgG1 rather than IgG2a antibody in mice fed transgenic potato tissues synthesizing the CTB-INS fusion protein indicates that the immune response was biased toward an insulin-specific Th₂ lymphocyte response, suggesting that the observed oral tolerance was mediated by active suppression²⁵. Feeding autoantigens that stimulate the GALT preferentially generates a Th₂-lymphocyte response including secretion of lymphokines such as interleukin (IL)-4, IL-10, and transforming growth factor (TGF)- β ^{6,7,16}. In addition, recombinant CTB subunits without the CTA induce mucosal antibody production mediated by TGF- β , a major inducer of oral tolerance^{6,26}. Therefore, it is possible that plant-synthesized CTB-INS fusion protein delivered to the GALT preferentially generates insulin-specific Th₂ lymphocytes for induction of systemic tolerance as well as concurrent induction of both mucosal and systemic humoral responses against CTB and insulin.

The lack of significant insulinitis reduction in mice fed insulin-producing potatoes does not exclude the possibility that protective T lymphocytes migrate to the pancreas based on a low-antigen dose-mediated Th₂-type response (e.g., active suppression)^{6,10}. In contrast to mice fed CTB-INS potato tissues, anti-insulin humoral

immune responses (e.g., Th₂ lymphocyte-mediated IgG1 induction) were not observed in mice fed potatoes synthesizing only insulin. This experimental result suggests that insulin-producing potatoes did not induce protective (anti-inflammatory) Th₂ cell activation and subsequent migration of Th₂ lymphocytes to the pancreas, probably because the insulin production level in plants was too low to act as an effective tolerogen without the help of the mucosal immune system targeting capability of the CTB carrier molecule. Without induction of low-antigen dose-mediated bystander suppression, one would not expect tolerization against diabetes development. Thus, in our experiments, the failure to reduce lymphocyte infiltration indicates lack of protection against diabetes development.

This study demonstrates that feeding microgram amounts of food plant-produced insulin conjugated with a CTB subunit can effectively suppress development of autoimmune diabetes in NOD mice. The plant-synthesized CTB-INS fusion protein is effective at doses at least 100-fold less than generally reported for unconjugated autoantigens, reinforcing the importance of oral antigen dose and the effect of CTB on oral tolerance induction¹⁰.

Experimental protocol

Plant expression vector. The genes encoding human insulin and its CTB conjugate were inserted into the plant expression vector pPCV701luxF²⁷. Human preproinsulin cDNA was inserted within the multiple cloning site immediately downstream of the mannopine synthase (*mas*) P2 promoter²⁸. Prior to construction of the CTB-INS fusion gene, the oligonucleotide sequence encoding a flexible hinge tetrapeptide (GPGP) was fused at the 3' end of the CTB gene¹⁷. The CTB-hinge fusion was inserted downstream of the *mas* P2 promoter. Less frequently used codons for potato plants were used in the hinge encoding sequence to permit the translation apparatus to reduce the rate of protein elongation, which may facilitate CTB subunit folding¹⁸. The oligonucleotide sequence flanking the CTB translation start codon was modified for efficient translation in eukaryotic cells²⁹. The 21-amino-acid leader peptide of the CTB subunit, which presumably translocates the fusion proteins into the plant endoplasmic reticulum, was conserved¹⁹. The human proinsulin gene was PCR amplified with DNA primers containing the microsomal retention signal (SEKDEL) encoding sequence^{12,30}. Amplified proinsulin:SEKDEL encoding sequence was inserted into the *Sac*I site at the 3' end of CTB-hinge sequence. Following confirmation of the DNA sequences, the plant expression vector was transferred into *A. tumefaciens* strain GV3101 pMP90RK, and potato leaf explant transformation was performed as described³¹.

Plant genomic DNA and total RNA isolation for PCR and RT-PCR analysis. Genomic DNA was isolated from both untransformed and transformed potato leaf tissues³¹. The presence of the insulin gene was determined by PCR analysis using oligonucleotide primers specific for both the pPCV701luxF vector and the insulin gene. Transformed plant genomic DNA (500 ng) was used as a template for detection of the insulin gene under the following PCR conditions: 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s for a total of 30 cycles. Plant total RNA was isolated using a plant RNA isolation kit (Boehringer Mannheim, Indianapolis, IN). RT-PCR analysis was performed to detect the presence of full-length insulin mRNA using an insulin gene-specific oligonucleotide primer set.

Immunoblot analysis. Approximately 1 g of tuber tissue was homogenized on ice in 1 ml of extraction buffer (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Tween-20). The tissue homogenates were centrifuged at 17,000 G for 15 min at 4°C to remove insoluble debris. A 10–20 μ l aliquot of supernatant, containing 50 to 100 μ g of total soluble protein as determined by protein assay (Bio-Rad, Hercules, CA), was analyzed by 10–15% SDS-PAGE. Samples of the plant homogenate were either boiled for 5 min prior to electrophoresis or loaded directly on the gel without heat treatment. Plant homogenates were evaluated for the presence of monomeric or pentameric CTB-INS protein using a rabbit anticholera toxin antiserum and an alkaline phosphatase-conjugated antirabbit IgG (1:5000 and 1:10,000 dilutions, respectively; Sigma, St. Louis, MO).

Chemiluminescent ELISA. Production levels of recombinant insulin and its CTB conjugate in transgenic potato tissues were determined by chemiluminescent ELISA. For quantitation of human insulin in the potato homogenate, a 96-well microtiter plate (Dynatech Laboratories, Chantilly,

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VA) was loaded with serial dilutions of induced leaf or microtuber tissue homogenate in bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. The wells were blocked by 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The plate was incubated in a 1:7000 dilution of anti-insulin antibody (Sigma) in 0.5% BSA in PBS overnight at 4°C, followed by incubation with alkaline phosphatase-conjugated secondary antibodies (Sigma) for 2 h at 37°C. The wells were washed twice with PBST (PBS containing 0.05% Tween-20) and once with PBS after each step. Chemiluminescent substrate Lumi-Phos Plus (Lumigen, Southfield, MI) was added to wells and the plate was incubated for 30 min at 37°C. Chemiluminescence was measured in a Microplate ML3000 Microtiter Plate Luminometer (Dynatech Laboratories). For quantitation of CTB-INS fusion protein, the microtiter plate was coated with 3.0 g/ml of GM₁-monosialo-ganglioside (Sigma) in bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The wells were loaded with various dilutions of plant tissue homogenates diluted in PBS (pH 7.2) and incubated overnight at 4°C. For detection of the CTB moiety of the fusion protein, a rabbit anticholera toxin primary antibody and an alkaline phosphatase-conjugated antirabbit IgG secondary antibody (1:5000 and 1:50,000 dilution, respectively; Sigma) were added¹⁸. For detection of the insulin moiety of the CTB-INS fusion protein, the same set of primary and secondary antibodies previously used for insulin detection was used.

Induction of oral tolerance. Four-week-old female NOD mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the Loma Linda (CA) University animal facility. The mice were divided into the following groups: group 1, fed untransformed potato; group 2, fed transgenic potato synthesizing insulin; and group 3, fed transgenic potato synthesizing CTB-INS fusion protein. At 5 weeks of age, each mouse was fed 3 g of potato tubers once per week until reaching 9 weeks of age (a total of five feedings). Each feeding of transgenic potato tuber tissues was found to deliver approximately 30 µg of insulin, or 20 µg of insulin as the CTB-INS conjugate. The animals were either killed at 10 weeks of age for antibody titer assay and histopathological analysis of pancreatic tissues, or monitored for 6 months for diabetes development.

Antibody titer. The serum and intestinal washings (five animals in each group) were assayed for anti-CTB and anti-insulin antibody isotypes using a chemiluminescent ELISA method. Human insulin or CTB (Sigma) was used for the well coating antigens (500 ng/well), and serial dilutions of pooled serum or intestinal washings were added to the coated microtiter plate wells. Alkaline phosphatase-conjugated anti-mouse IgG1, IgG2a, or IgA antibodies (Zymed, San Francisco, CA) were used as the secondary antibodies. The microtiter plate wells were washed twice with PBST and once with PBS after each step. Chemiluminescence RLU was measured in the Microplate ML3000 Microtiter Plate Luminometer. The titer was defined as the reciprocal of the highest dilution of the sample to give an RLU signal above background RLU levels, which were individually determined for each sample.

Histopathological analysis of pancreatic islets. Insulinitis levels were arbitrarily measured based on the extent of lymphocyte infiltration of the islets of Langerhans. Each group consisted of five mice. At 10 weeks of age the mice were killed and the pancreas removed. Pancreatic tissues were fixed in Bouin's fluid and stained with hematoxylin and counterstained with eosin. The degree of insulinitis was scored based on a seven-level semiquantitative scale ranging from 0–6, where score 0 is normal islet morphology with no sign of T-cell infiltration and scores 1–3 and 4–6 indicate increasing levels of peri-insulinitis and intra-insulinitis, respectively¹⁹. At least 10 islets were scored for each animal. The student's *t* test was used for statistical analysis of the data.

Assessment of diabetic symptoms. The incidence of diabetic symptoms was compared among mice fed transgenic potatoes synthesizing CTB and potatoes synthesizing CTB-INS fusion protein. The feeding schedule was the same as described above. Each group consisted of 10 mice. Starting at 10 weeks of age the mice were monitored on a weekly basis with urinary glucose test strips (Clinistix and Diastix, Bayer) for development of diabetes. Glycosuric mice were bled from the tail vein to check for glycemia using a glucose analyzer (Boehringer Mannheim). Diabetes was confirmed by hyperglycemia (>250 mg/dl) for two consecutive weeks²⁰. The Cox-Mantel log rank test was performed for comparison between the life table (Kaplan-Meier analysis) of two groups.

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