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(54) Title: COMPOSITIONS AND METHODS FOR SUPPRESSION OF INHIBITOR FORMATION AGAINST FACTOR VIII
IN HEMOPHILIA A PATIENTS

(57) Abrégé/Abstract:

Protein replacement therapy for patients with hemophilia or other inherited protein deficiencies is often complicated by pathogenic antibody responses, including antibodies that neutralize the therapeutic protein or that predispose to potentially life -threatening anaphylactic reactions by formation of IgE. Using murine hemophilia as a model, we have developed a prophylactic protocol against such responses that is non-invasive and does not include immune suppression or genetic manipulation of the patient's cells. Oral delivery of coagulation factor expressed in chloroplasts, bioencapsulated in plant cells, effectively blocked formation of inhibitory antibodies in protein replacement therapy. Inhibitor titers were mostly undetectable and up to 100-fold lower in treated subjects when compared to controls. Moreover, this treatment eliminated fatal anaphylactic reactions that occurred after 4 to 6 exposures to intravenous coagulation factor protein.



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(54) Title: COMPOSITIONS AND METHODS FOR SUPPRESSION OF INHIBITOR FORMATION AGAINST FACTOR VIII IN HEMOPHILIA A PATIENTS

(57) Abstract: Protein replacement therapy for patients with hemophilia or other inherited protein deficiencies is often complicated by pathogenic antibody responses, including antibodies that neutralize the therapeutic protein or that predispose to potentially life-threatening anaphylactic reactions by formation of IgE. Using murine hemophilia as a model, we have developed a prophylactic protocol against such responses that is non-invasive and does not include immune suppression or genetic manipulation of the patient's cells. Oral delivery of coagulation factor expressed in chloroplasts, bioencapsulated in plant cells, effectively blocked formation of inhibitory antibodies in protein replacement therapy. Inhibitor titers were mostly undetectable and up to 100-fold lower in treated subjects when compared to controls. Moreover, this treatment eliminated fatal anaphylactic reactions that occurred after 4 to 6 exposures to intravenous coagulation factor protein.



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COMPOSITIONS AND METHODS FOR SUPPRESSION OF INHIBITOR FORMATION AGAINST FACTOR VIII IN HEMOPHILIA A PATIENTS

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FIELD OF THE INVENTION

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The present invention relates to the fields of recombinant plants and the treatment of disorders for which induction of oral tolerance to therapeutically delivered antigens is desired. More specifically, the invention provides compositions and methods for inducing oral tolerance to Factor VIII and other coagulation factors thereby improving therapeutic outcomes.

BACKGROUND OF THE INVENTION

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Hemophilia is the X-linked bleeding disorder caused by mutations in coagulation factor IX (FIX, hemophilia B) or its co-factor, factor VIII (FVIII, hemophilia A). Since the serine protease FIX has very low activity in the absence of FVIII, mutations in either protein can cause the coagulation defect. This disease affects 1 in 7,500 male births worldwide for hemophilia A and 1 in 30,000 for hemophilia B.¹⁻³ Hence, the majority of patients are FVIII-deficient. Current standard treatment is based on intravenous (IV) infusion of plasma-derived or recombinant factor

concentrate. A major complication of this therapy is the formation of inhibitory antibodies (“inhibitors”), which occurs in 20-30% of patients with severe hemophilia A (as defined by <1% coagulation activity) and in ~5% of severe hemophilia B patients.^{1,4-6} Inhibitors seriously complicate treatment and increase morbidity and mortality of this disease. Increased factor doses may be able to restore hemostasis in patients with low-titer inhibitors (<5 Bethesda Units, BU), while bypass factors are required to treat a bleed in the presence of high-titer inhibitors. However, these treatments are expensive and have to be carefully dosed. Clinical protocols for reversal of the antibody response via immune tolerance induction (ITI) consist of frequent high-dose factor administrations for prolonged periods (months to >1 year), are very expensive (> \$1,000,000), and ~30% of FVIII inhibitor patients fail to respond.⁴

SUMMARY OF THE INVENTION

In accordance with the present invention, a composition comprising lyophilized plant material is provided. In a preferred embodiment, the material comprises a coagulation protein, or immunogenic fragments thereof, produced in chloroplasts within said plant wherein the coagulation protein or immunogenic fragment retains immunogenicity in lyophilized form, which upon oral administration to a mammal in need thereof is effective to produce oral tolerance to the protein. The coagulation factors may include for example, F.II, F.III, F.IV, F.V, F.VI, F.VII, F.VIII, F.X, F.XI, F.XII, and/or F.XIII, or a polypeptides having at least 90 percent identity therewith. In a particularly preferred embodiment, the plant material comprises leaves containing transgenic chloroplasts, and the therapeutic protein is a fusion protein comprising Factor VIII and/or at least one immunogenic domain fragment thereof, and cholera non toxic B subunit (CTB), wherein the material is effective to induce tolerance to said protein or fragment in said mammal upon oral administration. The at least one immunogenic domain fragment is a domain of FVIII selected from the group consisting of A1, A2, A3, B, C1, C2 or heavy chain (HC) fragments. In a particularly preferred embodiment, the therapeutic protein is at least one immunological fragment of FVIII consisting of a C2 domain and/or a HC domain, each fused to cholera non toxic B subunit (CTB), wherein the orally administered fragments induce tolerance to said coagulation protein by suppressing inhibitory antibody formation. Also preferred are compositions effective to induce expression of TGF- β producing CD4⁺CD25⁺LAP⁺ regulatory T cells in spleen, MLN, and Peyer’s patches. A variety of plants may be employed in accordance

with the present invention. Such plants include without limitation, lettuce, carrots, cauliflower, cabbage, low-nicotine tobacco, spinach, kale, and cilantro.

In certain embodiments one, two, three, four, five or six domains are administered together. Exemplary compositions and methods include those where a C2-CTB and an HC-CTB fusion protein are administered together. The present inventors have discovered that the compositions disclosed herein are effective to reduce inhibitor formation against FVIII in hemophilia A subjects. Thus, the invention also provides a method for the treatment of Hemophilia A in a subject in need thereof comprising administration of an effective amount of the compositions described above to a subject in need thereof, said composition being effective to suppress formation of inhibitors of FVIII in said subject and induce expression of TGF- β producing CD4⁺CD25⁻LAP⁺ regulatory T cells in spleen, MLN, and Peyer's patches.

Also provided is a method of treating a subject having a genetic disease and at risk for development of an anaphylactic reaction in response to protein replacement therapy, said method comprising administering an effective amount of a composition comprising a lyophilized tolerance factor and a plant remnant, the tolerance factor being a protein or immunological fragment thereof, wherein loss or mutation of said protein is causative of said disease. In certain embodiments, the tolerance factor is coagulation factor, an acid alpha-glucosidase, alpha-galactosidase A, Glucocerebrosidase, alpha-L-iduronidase, or sphingomyelinase, or variants having at least a 90 percent identity therewith. In a preferred embodiment the therapeutic tolerance factor is conjugated to cholera toxin B. Diseases to be treated using the compositions of the invention, include, for example, Hemophilia A, Hemophilia B, Pompe disease, Fabry disease, Gaucher disease, Mucopolysaccharidosis I, or Niemann-Pick disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B: Chloroplast transformation vectors and integration of transgenes into the chloroplast genome. A. Tobacco chloroplast expression vectors. Homologous chloroplast genome flanking sequences comprising of 16S 3', trnI, trnA gene sequences; Prn, ribosomal RNA operon promoter with GGAGG ribosome binding site; aadA, aminoglycoside 3'-adenylyltransferase gene to confer spectinomycin resistance; 5' UTR, promoter and 5' UTR of tobacco *psbA* gene; 3' UTR, 3' UTR of tobacco *psbA* gene; CTB, cholera toxin B subunit. In both vectors a Gly Pro Gly Pro (GPGP) hinge and furin cleavage site (RRKR) is included between

CTB and the FVIII domain sequence. WT, untransformed wild type. Nt, *Nicotiana tabacum*. The restriction site of AflIII and the sizes of Southern blot fragments are indicated. **B.** Southern blot, tobacco CTB-HC, WT (untransformed), 1-3 transplastomic lines. Tobacco total genomic DNA was digested with AflIII and probed with 0.81kb trnI/trnA flanking region fragment. **C.** Southern blot, tobacco CTB-C2, WT (untransformed wild type), 1-4 transplastomic lines.

Figures 2A -2F. Characterization of CTB-HC and CTB-C2 expression in tobacco chloroplasts. **A.** Detection of heavy chain fusion protein probed with the CTB antibody. CTB standard: 6.25ng, 12.5ng, 25ng. WT: untransformed wild type. 1-4, transplastomic lines. Five µg total protein of homogenate fraction per lane was loaded. **B.** Detection of heavy chain probed with the A2 antibody. CTB: 25ng. 1-4, transplastomic lines. Five µg total protein of homogenate fraction per lane was loaded. **C.** Detection of C2 fusion protein probed with the CTB antibody. CTB standard: 5ng, 10ng, 20ng. S: supernatant fraction; H: homogenate fraction. Two µg total protein of supernatant or homogenate fraction per lane was loaded. **D.** Quantitation of CTB-HC and CTB-C2 expression in tobacco chloroplasts. Proteins were extracted from mature leaves at different time points on the same day. TLP, total leaf protein. **E.** Ganglioside GM1 ELISA binding assay. CTB standard (0.1ng); tobacco CTB-HC (5µg); tobacco CTB-C2 (1µg); untransformed tobacco wild type (5µg); BSA, bovine serum albumin (5µg). **F.** Blue Native Gel Electrophoresis and western blot analysis to evaluate pentamer assembly. Pentamer sizes: CTB: 57.5 kDa; CTB-C2: 155 kDa; CTB-HC: 490 kDa. Samples loaded: CTB standard, 100 ng; WT, 40 µg; CTB-HC, 40 µg; CTB-C2, 10 µg.

Figures 3A – 3F. Suppression of inhibitor formation against FVIII in hemophilia A C57BL6/129 mice by oral administration of a 1:1 mixture of bioencapsulated CTB-C2 and CTB-HC FVIII antigens. **A.** Time line of oral antigen administration and intravenous treatment with BDD-FVIII. Number in circle indicates time-point for tail bleed. **B.** Inhibitor titers (in BU/ml) after 4 weekly i.v. injections of FVIII in non-fed animals (“no plant”) or mice fed with WT or FVIII containing plant material. IgG1 (**C**), IgG2a (**D**), IgG2b (**E**) titers against FVIII for the same experimental groups. Data in B-E are shown for individual mice and as averages ± SEM. **F.** Following the blood draw, mice were sacrificed and spleens collected. Splenocyte cultures for individual mice (n= 3 to 5 per group) were stimulated *in vitro* with 10

µg/ml of BDD-FVIII for 48 hours. Subsequently, cells were harvested and subjected to quantitative RT-PCR analysis. “Fold increase” is change in RNA transcripts of FVIII vs. mock-stimulated cultures. The dotted horizontal line indicates the minimally required increase of 2.5 fold for a statistically significant difference. **G:** Spenocytes derived from the same experimental mice were subjected to ELISPOT analysis for frequency of IL-10 secreting cell population. All data are shown for individual mice and as averages $\pm$ SEM. Unpaired two-tailed Student’s T-tests were used to calculate P values (* ** $P<0.01$).

Figures 4A – 4E. Suppression of inhibitor formation against FVIII in hemophilia A BALB/c mice by oral administration of a 1:1 mixture of bioencapsulated CTB-C2 and CTB-HC FVIII antigens. **A:** Feeding and FVIII treatment schedule. Number in circle indicates time-point for tail bleed. **B:** Inhibitor titers (in BU/ml) after 4 weekly i.v. injections of BDD-FVIII in “No plant”, “WT plant”, and “FVIII plant” fed groups. IgG1 (**C**), IgG2a (**D**), IgG2b (**E**) titers against FVIII for the same experimental groups. All data are shown for individual mice and as averages $\pm$ SEM. Unpaired two-tailed Student’s T-tests were used to calculate P values (* $P<0.05$, ** $P<0.01$).

Figures 5A -5F. Long-term control and reversal of inhibitor formation in hemophilia A BALB/c mice. **A:** Feeding (HC and C2 material) and FVIII administration schedule for prevention of inhibitor formation. Numbers in circles indicate time-point for blood collection. Inhibitor titers in BU/ml (**B**) and IgG1 titers against FVIII (**C**) at weeks 8, 12, and 21 of the experiment for FVIII fed mice (n=5, back square symbols) are compared to control mice (which were fed with WT plant material, n=7, gray diamonds). Statistically significant differences between these groups for specific time points are indicated (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$, as calculated by unpaired two-tailed student’s T-test; data are averages $\pm$ SEM). A third group of mice (n=7) was also fed with FVIII material, and FVIII was administered IV once/week starting one month after initiation of the oral tolerance regimen. However, FVIII feeding and treatment was continued for the remaining duration of the experiment (i.e. 20 weeks of FVIII feeding; these mice are labeled as “FVIII continuously fed” and graphed with black triangle symbols and dotted line in panels B and C; data are averages $\pm$ SEM). **D:** FVIII administration and feeding schedule for reversal of inhibitor formation. Inhibitor formation was

induced by repeated weekly IV injections of FVIII as indicated. Mice were divided into 2 groups with similar average inhibitor titers. Control mice (n=5) did not receive any further treatment. The second group ("FVIII fed", n=4) was fed with FVIII plant material twice per week for the following 3 months. Inhibitor titers in BU/ml (E) and IgG1 titers against FVIII (F) are graphed
 5 for weeks 5, 9, 13, and 17 of the experiment as explained above.

Figures 6A and 6B. Active suppression of antibody formation against FVIII by induction of regulatory T cells. A. Adoptive transfer experiments. CD4⁻, CD4⁺CD25⁻, and CD4⁺CD25⁺ cells were purified via magnetic sorting from spleens and mesenteric lymph nodes (MLN) of FVIII fed mice (n=3) at time-point 3 indicated in Fig. 5A and pooled (with a final ratio of
 10 approximately 30% spleen and 70% MLN-derived CD4⁺ T cells). Cells (10⁶ per mouse) were adoptively transferred into naïve BALB/c mice via tail vein injection. Control cells were from unchallenged naïve mice of the same strain. Twenty-four hours later, all recipient mice (n=5 per group) were challenged with 1 IU FVIII in adjuvant via subcutaneous injection. IgG titers
 15 against FVIII were determined 3 weeks later. All data are shown as averages ± SEM; * *P*<0.05, ** *P*<0.01. B. Frequencies of Treg subsets in FVIII fed and control hemophilia A BALB/c mice. Cells derived from spleens, mesenteric lymph nodes (MLN), inguinal lymph nodes (ILN), and Peyer's patches (PP) were isolated from mice that had either been fed with FVIII (HC+C2, "FVIII fed") or WT plant material ("control") followed by IV treatment with FVIII ("FVIII fed").
 20 Stained cells were first gated for live CD4⁺ cells (positive CD4-eFluor 450 and negative viability dye eFluor 506 staining). The frequencies of CD4⁺CD25⁻LAP⁺ cells, CD4⁺CD25⁺Foxp3⁺ cells, and Tr1 cells (CD4⁺LAG-3⁺CD49b⁺) were calculated using flow cytometric analysis. Data for individual animals as well as averages ± SEM are shown (n=3-5/group). Unpaired two-tailed Student's T tests were used to calculate P values for all panels.

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Figures. 7A-7D. Delivery of FVIII antigen to the GALT and into circulation. A-C: Immunostains (original magnification 200X) of ileum cryo-sections from unfed (A, negative control) or CTB-C2 fed (B: lamina propria, C: Peyer's patch) BALB/c hemophilia A mice. Stains are for C2 domain of FVIII (green), CD11c (red), and nuclei (DAPI; blue). D: Human
 30 FVIII antigen levels were measured in plasma or liver protein extract of the CTB-HC fed

C57BL6/129 and BALB/c hemophilia A mice and WT fed control mice of the same strain using HC-specific ELISA. All data are shown for individual mice and as averages $\pm$ SEM.

Figures 8A-8C: Antibody responses against CTB protein in hemophilia A mice fed with FVIII chloroplast transgenic plant material. IgG1 (A), IgG2a (B), and IgG2b (C) serum titers against CTB following 8 weeks of feeding with a mixture of CTB-HC and CTB-C2 transgenic tobacco material. Sera from mice of the same strain 2 weeks after challenging with 20 μ g CTB in adjuvant served as positive controls. Unchallenged naïve mice of the same strain served as negative controls. The dotted horizontal line indicates the background of the assay (average readings for negative controls). All data are shown for individual mice and as averages $\pm$ SEM.

Figures 9A – 9G Generation and characterization of CTB-C2 transplastomic lettuce plants

A: Lettuce CTB-C2 expression vector and a portion of native chloroplast genome. 16S trnI and trnA 23S, lettuce homologous chloroplast genome flanking sequences comprising of 16S 3' (or 23 5') end sequences and complete trnI, trnA genes; Prn, ribosomal RNA operon promoter with GGAGG ribosome binding site; aadA, aminoglycoside 3'-adenylytransferase gene for spectinomycin resistance; 5' UTR, promoter and 5' UTR of *psbA* gene from lettuce; 3' UTR, 3' UTR of *psbA* gene from lettuce; CTB, cholera toxin B subunit. A Gly Pro Gly Pro (GPGP) hinge and furin cleavage site (RRKR) is included between CTB and the C2 sequence. WT, wild type.

B: Southern blot. WT (untransformed wild type), 1-5: transplastomic lines, lettuce genomic DNA was digested with AflIII and probed with 1.12-kb lettuce flanking region. The 7.4-kb positive band contains the CTB-C2 insertion fragment in the transplastomic lines. The absence of WT fragment (4.8-kb) in transplastomic lines 1 and 4 clearly demonstrate homoplasmy. **C: Western blot.** Probe, anti-CTB pAb. CTB standard: 5ng, 10ng, 20ng. S: supernatant fraction; H: homogenate fraction. Molecular weight of CTB-C2: 31 kDa. CTB: 12 kDa. 2.5 μ g total protein of supernatant or homogenate fraction per lane was loaded. **D: Ganglioside GM1 ELISA binding assay.** CTB standard (0.1ng); CTB-C2 (1 μ g); untransformed lettuce wild type (1 μ g); BSA, bovine serum albumin (5 μ g). **E: CTB-C2 expression levels (Average $\pm$ STDEV) in μ g/g of fresh and lyophilized leaves.** Lyophilized leaf materials from T0 plants: 2004 $\pm$ 136; Fresh: 94 $\pm$ 6. **F: Comparison of protein concentrations between the lyophilized leaf and fresh leaf**

samples. Equal amount of lyophilized or fresh leaf material was used for this analysis . 1, undiluted CTB-C2 extract (60 µg leaf powder); 1:10, 10 times dilution (6 µg leaf powder); 1:20, 20 times dilution (3 µg leaf powder). WT, wild-type lettuce. CTB standard: 7.5 ng and 15 ng per lane loaded for quantitation. **G: Preparation of capsules:** lyophilized and powdered lettuce

5 leaves expressing CTB-C2 fusion protein for use as oral antigen.

Figures 10A and 10B. Screening for CTB-HC lettuce plants (T1) (A); by western blot analysis (B). Probe: anti-CTB ; CTB-HC = 97.7 kDa. All individual plants except plant #14 showed expression of CTB-HC fusion protein.

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Figure 11. Screening for CTB-C2 lettuce plants (T1) by western blot analysis. All individual plants showed expression of CTB-HC fusion protein.

DETAILED DESCRIPTION OF THE INVENTION

15 Hemophilia A is the X-linked bleeding disorder due to deficiency of coagulation factor VIII (FVIII). To address serious complications of inhibitory antibody formation in current replacement therapy, we created tobacco transplastomic lines expressing FVIII antigens, heavy chain (HC) and C2, fused with the transmucosal carrier, cholera toxin B subunit (CTB). CTB-HC and CTB-C2 fusion proteins expressed up to 80 or 370 µg/g in fresh leaves, assembled into

20 pentameric forms, and bound to GM1 receptors. Protection of FVIII antigen through bioencapsulation in plant cells and oral delivery to the gut immune system was confirmed by immunostaining. Feeding of HC/C2 mixture substantially suppressed T helper cell responses and inhibitor formation against FVIII in hemophilia A mice of two different strain backgrounds. Prolonged oral delivery was required to control inhibitor formation long-term. Substantial

25 reduction of inhibitor titers in pre-immune mice demonstrated that the protocol could also reverse inhibitor formation. Gene expression and flow cytometry analyses showed up-regulation of immune suppressive cytokines (TGF-β/LAP and IL-10). Adoptive transfer experiments confirmed an active suppression mechanism and revealed induction of CD4⁺CD25⁺ and

CD4⁺CD25⁻ T cells that potently suppressed anti-FVIII formation. In sum, these data support plant cell-based oral tolerance for suppression of inhibitor formation against FVIII.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of molecular biology.

5 Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein.

In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only
10 and are not intended to be limiting.

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques, encompassed by the present invention. See, for example, Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (1982) and Sambrook et al., *Molecular Cloning: A Laboratory*
15 *Manual*, Cold Spring Harbor Laboratory Press, New York (1989); *Methods in Plant Molecular Biology*, Maliga et al, Eds., Cold Spring Harbor Laboratory Press, New York (1995); *Arabidopsis*, Meyerowitz et al, Eds., Cold Spring Harbor Laboratory Press, New York (1994) .

Methods, vectors, and compositions for transforming plants and plant cells are taught for
20 example in WO 01/72959; WO 03/057834; and WO 04/005467. WO 01/64023 discusses use of marker free gene constructs.

Proteins expressed in accord with certain embodiments taught herein may be used in vivo by administration to a subject, human or animal, in a variety of ways. The pharmaceutical compositions may be administered orally or parenterally, i.e., subcutaneously, intramuscularly or
25 intravenously. Thus, this invention provides compositions for parenteral administration which comprise a solution of the fusion protein (or derivative thereof) or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycerine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional,
30 well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH

adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of fusion protein (or portion thereof) in these formulations can vary widely depending on the specific amino acid sequence of the subject proteins and the desired biological activity, e.g., from
5 less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Therapeutic compositions produced by embodiments of the present invention can be administrated by the consumption of the foodstuff that has been manufactured with the
10 transgenic plant producing the therapeutic protein. The edible part of the plant is used as a dietary component while the therapeutic protein is administrated in the process.

Thus, in one embodiment, the invention pertains to an administrable tolerance inducing composition that comprises an oral tolerance factor, such as an coagulation factor, having been expressed by a plant and a plant remnant. A plant remnant may include one or more
15 molecules (such as, but not limited to, proteins and fragments thereof, minerals, nucleotides and fragments thereof, plant structural components (such as cellular compartments), etc.) derived from the plant in which the antigen was expressed. Accordingly, a vaccine pertaining to whole plant material (e.g., whole or portions of plant leafs, stems, fruit, etc.) or crude plant extract would certainly contain a high concentration of plant remnants, as well as a composition
20 comprising purified antigen that and one or more detectable plant remnant.

The tolerance inducing compositions of certain embodiments of the present invention may be formulated with a pharmaceutical vehicle or diluent for oral, intravenous, subcutaneous, intranasal, intrabronchial or rectal administration. The pharmaceutical composition can be formulated in a classical manner using solid or liquid vehicles, diluents and additives appropriate
25 to the desired mode of administration. Orally, the composition can be administered in the form of tablets, capsules, granules, powders and the like with at least one vehicle, e.g., starch, calcium carbonate, sucrose, lactose, gelatin, etc. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, e.g., water, saline, dextrose,
30 glycerol, ethanol or the like and combination thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH

buffering agents, or adjuvants which enhance the effectiveness of the vaccines. The preparation for parental administration includes sterilized water, suspension, emulsion, and suppositories. For the emulsifying agents, propylene glycol, polyethylene glycol, olive oil, ethyloleate, etc. may be used. For suppositories, traditional binders and carriers may include polyalkene glycol, triglyceride, witpsol, macrogol, tween 61, cocoa butter, glycerogelatin, etc. In addition, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like can be used as excipients.

Oral tolerance factors may be administered by the consumption of the foodstuff that has been manufactured with the transgenic plant and the edible part of the plant expressing the antigen is used directly as a dietary component while the vaccine is administrated in the process.

Examples of readily edible plants that may be transformed to express the constructs described herein include, but are not limited to, apple, berries such as strawberries and raspberries, citrus fruits, tomato, banana, carrot, celery, cauliflower; broccoli, collard greens, cucumber, muskmelon, watermelon, pepper, pear, grape, peach, radish and kale.

The tolerance inducing proteins may be provided with the juice of the transgenic plants for the convenience of administration. For said purpose, the plants to be transformed are preferably selected from the edible plants consisting of tomato, carrot and apple, which are consumed usually in the form of juice.

Those skilled in the art will appreciate that active variants of the genes specifically disclosed herein may be employed to produce plant derived therapeutic compositions. J Exp Med. 1997 May 19;185(10):1793-801 provides some specific examples of fragments of known antigenic proteins and genes coding therefor.

According to another embodiment, the subject invention pertains to a transformed chloroplast genome that has been transformed with a vector comprising a heterologous gene that expresses a coagulation factor. In a related embodiment, the subject invention pertains to a plant comprising at least one cell transformed to express a coagulation factor.

In one embodiment, coagulation factor polypeptides according to the invention comprise at least 12, 15, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250 or 265 contiguous amino acids of a known coagulation factor sequence. A coagulation factor polypeptide of the invention therefore can be a portion (or fragment) of a coagulation factor, a full-length coagulation factor protein, or a fusion protein comprising all or a portion of coagulation factor protein. Those skilled in the art,

equipped with the teachings herein, will be enabled to express and utilize other known coagulation factors. Examples of other coagulation factors that may be used with the present invention include, but are not limited to, those polypeptide sequences associated with the following accession nos. NG_009258.1; NG_008953.1; NG_008107.1; NG_008051.1;

5 NM_001993.3 and NM_00128.3, as provided in the related databases as of the filing date of the present invention. Also, Appendix A shows representative examples of sequences relating to noted coagulation factors. The interactive regions and protease regions of these sequences are known.

Coagulation factor polypeptide variants which are biologically active, i.e., confer an
10 ability to increase tolerance against the corresponding factor upon oral administration also are considered coagulation factor polypeptides for purposes of this application. Preferably, naturally or non-naturally occurring coagulation factor polypeptide variants have amino acid sequences which are at least about 55, 60, 65, or 70, preferably about 75, 80, 85, 90, 96, 96, or 98% identical to the amino acid sequence of the known coagulation factor sequence. Percent identity
15 between a putative coagulation factor polypeptide variant and a known amino acid sequence may be determined, for example, by using the Blast2 alignment program (Blosom62, Expect 10, standard genetic codes).

Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid
20 replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid
25 residues can be substituted, inserted, or deleted without abolishing biological or immunological activity of an coagulation factor polypeptide can be found using computer programs well known in the art, such as DNASTAR software. Whether an amino acid change results in a biologically active coagulation factor polypeptide can readily be determined by assaying for coagulation factor activity, as described for example, in the specific Examples, below.

30 A coagulation factor polynucleotide can be single- or double-stranded and comprises a coding sequence or the complement of a coding sequence for an coagulation factor polypeptide.

Examples of other coagulation factor polynucleotides that may be used with the present invention include, but are not limited to, those polynucleotide sequences associated with the following accession nos. NG_009258.1; NG_008953.1; NG_008107.1; NG_008051.1; NM_001993.3 and NM_00128.3, as provided in the related databases as of the filing date of the present invention.

FVIII is a highly immunogenic molecule that can cause potent antibody responses in hemophilia A patients and in experimental animals at low antigen doses. Under some circumstances, auto-antibodies against FVIII can form in non-hemophilic individuals, resulting in acquired hemophilia A. The majority of inhibitors bind to A2, A3, or C2 domain. These highly immunogenic sequences also contain several CD4⁺ T cell epitopes. Animal studies suggest that inhibitor formation against FVIII can be prevented by tolerization to parts of the molecule, such as combination of A2 and C2 domains, while a single domain may not be sufficient. It is shown herein that FVIII heavy chain and C2 domain can be expressed as cholera toxin B subunit (CTB) fusion proteins in tobacco. Oral delivery of a mixture of these bioencapsulated antigens suppresses inhibitor formation in hemophilia A mice.

Accordingly, in view of the teachings herein, it is to be appreciated that the full length coagulation factor sequence may be utilized or portions thereof that preserve the epitope peptides. Portions may include polypeptide fragments of at least 15 amino acids with the goal of including and preserving the immunogenic potential of at least one peptide epitope of the sequence. However, ideally, the sequence administered includes as many epitopes as possible, as this would increase the likelihood of successful tolerization. For example, with respect to the FVIII sequence, the full sequence may be expressed, or portions thereof, typically as a fusion protein with CTB. Van Haren et al., *Mol Cell Proteomics*, 2011)Jun;10(6): M110.002246, teaches several sequences pertaining to epitopes (see, e.g., Fig 2B). Other references teaching the location of different epitopes of the FVIII sequence include Jones et al., *Journal of Thrombosis and Haemostasis* 2005, 3:991-1000; Reding et al., *Journal of Thrombosis and Haemostasis*, 2003, 1:1777:1784; Pratt et al., *Thromb Haemost* 2004: 92:522-8; and Hu et al., *Journal of Thrombosis and Haemostasis* 2004, 2:1908-1917. FVIII comprises multiple domains: A1, A2, A3, B, C1 and C2. In certain embodiments, the sequence pertains to the full sequence of at least one of the domains and optionally at least a portion or all of two, three, four, five or six domains. The following is an amino acid sequence of FVIII (homo sapiens), which can be

used in a plastid expression scheme (such as by implementing a polynucleotide that codes the full amino acid sequence or portions thereof) or plastid based compositions as taught herein:

MQIELSTCFLLCLLRFCFSATRRYYLGAVELSWDYMQSDLGELP
 VDARFPPRVPKSFPFNTSVVYKKTLFVEFTDHLFNIAPRPPWMGLLGPTIQAEVYDT
 5 VVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSSHTYVWQV
 L KENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLF
 AVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYW
 HV IGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLLMDLGQFLLFCHISSH
 QHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNPSFIQIRS
 10 VAKKHPKTWVHYIAAEEEDWDYAPLV LAPDDRSYKSQYLNNGPQRIGRKYKKVRFMA
 Y TDETFKTREAIQHESGILGPLLYGEVGD TLLIIFKNQASRPYNIYPHGITDVRPLYSR
 RLPKGVKHLKDFILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSFVNMERDLASGL
 IGPLLCYKESVDQRGNQIMSDKRNVLFSVFDENRSWYLTENIQRFLPNPAGVQLED
 PEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMV
 15 YEDTLTLFPFSGETVFMSEMPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYED
 SYEDISAYLLSKNNAIEPRSFQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTPM
 PKIQNVSSSDLLMLLRQSPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSSEMTHF
 RPQLHHSGDMVFTPESGLQLRLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLA
 AGTDNTSSLGPPSMPVHYDSQLD TTLFGKKSSPLTESGGPLSLSEENND SKLLESGLM
 20 NSQESSWGKNVSTESGRLFKGKRAHGPALLTKDNALFKVSISLLKTNKTSNNSATNR
 KTHIDGPSLLIENSPSVWQNILESDTEFFKVTPLIHDRMLMDKNATALRLNHMSNKTT
 SSKNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPESARWIQRT HGKNSLNSGQGSPK
 QLVSLGPEKSVEGQNFLSEKNKVVGKGFTKDVGLKEMVFPSSRNFLTNLDNLHEN
 NTHNQEKKI QEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLFLSTRQNVEGSYDGA
 25 YAPVLQDFRSLNDSTNR TKKHTAHFSKKGEEENLEGLGNQTKQIVEKYACTTRISPNT
 SQQNFTVQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTLTQIDYNE
 KEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL P
 AASYRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGLGTSATNSVTYKKV
 ENTVLPKPDLPKTSKGKVELLPKVHIYQKDLFP TETSNGSPGHLDLVEGSLLQGTEGAI
 30 KWNEANRPGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEK SPEKTA
 FKKKDTILSLNACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCSQNPPVLKRHQRE

ITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLW
 DYGMSSSPHVLNRNAQSGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRA
 EVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPKKNFVKPNETKTYFWKVQHMM
 APTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFT
 5 IFDETKSWYFTENMERNCRAPSNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQR
 IRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPKAGIWR
 VECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHY
 SGSINAWSTKEPFSWIKVDLLAPMIIHGKTQGARQKFSSLYISQFIIMYSLDGKKWQ
 TYRGNSTGTLMVFFGNVDSSGIKHNIENPPIIARYIRLHPHTHSIRSTLRMELMGCDL
 10 NSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEW
 LQVDFQKTMKVTGVTQGVKSLTSMYVKEFLISSQDGHQWTLFFQNGKVKVVFQGN
 Q DSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDL

Degenerate nucleotide sequences encoding coagulation factor polypeptides, as well as
 homologous nucleotide sequences which are at least about 50, 55, 60, 65, 60, preferably about 75,
 15 90, 96, or 98% identical to the nucleotide sequence encoding a coagulation factor also are
 coagulation factor polynucleotides. Percent sequence identity between the sequences of two
 polynucleotides is determined using computer programs such as ALIGN which employ the
 FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension
 penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of
 20 coagulation factor polynucleotides which encode biologically active coagulation factor
 polypeptides also are coagulation factor polynucleotides.

It will be apparent to those skilled in the art that such substitutions can be made outside
 the regions critical to the function of the molecule and still result in an active polypeptide.
 Amino acid residues essential to the activity of the polypeptide encoded by an isolated
 25 polynucleotide of the invention, and therefore preferably not subject to substitution, may be
 identified according to procedures known in the art, such as site-directed mutagenesis or alanine-
 scanning mutagenesis (see, e.g., Cunningham and Wells, 1989, Science 244: 1081-1085). In the
 latter technique, mutations are introduced at every positively charged residue in the molecule,
 and the resultant mutant molecules are tested for activity to identify amino acid residues that are
 30 critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be
 determined by analysis of the three-dimensional structure as determined by such techniques as

nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (see, e.g., de Vos et al., 1992, Science 255: 306-312; Smith et al., 1992, Journal of Molecular Biology 224: 899-904; Wlodaver et al., 1992, FEBS Letters 309: 59-64).

TABLE 2: Amino Acid Substitutions

Original Residue	Exemplary Conservative Substitutions, others are known in the art.
Ala;	ser
Arg;	lys, gln
Asn;	gln, his
Asp;	glu
Cys;	ser
Gln;	asn, lys
Glu;	asp
Gly;	pro
His;	asn, gln
Ile;	leu, val
Leu;	ile, val
Lys;	arg, gln
Met;	leu, ile
Phe;	met, leu, tyr
Ser;	thr
Thr;	ser
Trp;	tyr
Tyr;	trp, phe
Val;	ile, leu

- 5 Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The
- 10 substitutions which in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an
- 15 electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (c) by increasing the number of sites for sulfation and/or glycosylation.

For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For

20 example, a conservative substitution would be replacing one hydrophobic residue for another,

or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

5 Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

10 Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and asparyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups
15 of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, *Proteins: Structure and Molecular Properties*, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

 As this specification discusses various proteins and protein sequences it is understood
20 that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out
25 herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence.

 Variants and homologs of the coagulation factor polynucleotides described above also are coagulation factor polynucleotides. Typically, homologous coagulation factor polynucleotide sequences can be identified by hybridization of candidate polynucleotides to known coagulation
30 factor polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions: 2 X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS,

room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50°C. once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes each homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Species homologs of the coagulation factor polynucleotides disclosed herein also can be identified by making suitable probes or primers and screening cDNA expression libraries. It is well known that the T_m of a double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner et al., J. Mol. Biol. 81, 123 (1973)). Variants of coagulation factor polynucleotides or polynucleotides of other species can therefore be identified by hybridizing a putative homologous coagulation factor polynucleotide with a polynucleotide having a nucleotide sequence of SEQ ID NO: 1 or the complement thereof to form a test hybrid. The melting temperature of the test hybrid is compared with the melting temperature of a hybrid comprising polynucleotides having perfectly complementary nucleotide sequences, and the number or percent of basepair mismatches within the test hybrid is calculated.

Nucleotide sequences which hybridize to coagulation factor polynucleotides or their complements following stringent hybridization and/or wash conditions also are coagulation factor polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed., 1989, at pages 9.50-9.51.

Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20°C below the calculated T_m of the hybrid under study. The T_m of a hybrid between a coagulation factor polynucleotide having a nucleotide sequence shown in SEQ ID NO: 1 or the complement thereof and a polynucleotide sequence which is at least about 50, preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences can be calculated, for example, using the equation of Bolton and McCarthy, Proc. Natl. Acad. Sci. U.S.A. 48, 1390 (1962):

$T_m = 81.5^\circ\text{C} - 16.6(\log_{10} [\text{Na}^+]) + 0.41(\%G+C) - 0.63(\%\text{formamide}) - 600/l$, where l = the length of the hybrid in basepairs.

Stringent wash conditions include, for example, 4 X SSC at 65° C, or 50% formamide, 4 X SSC at 42°C, or 0.5 X SSC, 0.1% SDS at 65°C. Highly stringent wash conditions include, for example, 0.2 X SSC at 65°C.

In alternative embodiments, the invention pertains to a method of treating a subject
 5 having a genetic disease prone and at risk to experiencing an anaphylactic reaction responsive to protein replacement therapy. The method comprises administering to the subject an effective amount of a composition comprising a tolerance factor and a plant remnant, and administering a therapeutically effective amount of a protein corresponding to a defect or deficiency associated with said disease. Typically, a tolerance factor pertains to a coagulation factor (see above), an
 10 acid α -glucosidase (accession nos. NM_001079803.1, NM_001079804.1, NM_0001152.3), α -galactosidase A, (accession no. NM_000169.2) Glucocerebrosidase (accession nos. J03059, J03060), α -L-iduronidase (accession no. NM_000203.3), or sphingomyelinase (accession nos. NM_000543.3, NM_001007593.1. The principles described above with respect to coagulation factor polypeptides and polynucleotides and variants in the preceding eleven paragraphs also
 15 apply to the sequences associated with the accession nos. provided in this paragraph. Also, the disease treated typically pertains to Hemophilia A, Hemophilia B, Pompe disease, Fabry disease, Gaucher disease, Mucopolysaccharidosis I, or Niemann-Pick disease. The tolerance factors may be conjugated to a CTB protein (see, e.g., Lai, CY, Journal of Biological Chemistry, (1977) 252:7249-7256, or accession no. DQ523223, also see ref 39) to enhance oral tolerance potential.

20 The following examples are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

EXAMPLE I

While there are currently no prophylactic protocols against inhibitor formation in patients, preclinical experiments in murine models of hemophilia A have provided proof-of-principle that
 25 preventive immune tolerance to FVIII can be established.⁶⁻¹¹ However, such protocols utilize genetic manipulation or immune suppressive drugs, raising safety concerns for translation to human treatment. In contrast, oral tolerance could be a more readily acceptable form of prophylactic tolerance induction and may be more readily tested in clinical trials.^{12,13} However, effective tolerogenic delivery of coagulation factor antigen to the gut-associated lymphoid tissue
 30 (GALT) is a challenge.¹⁴ In accordance with the present invention, we have developed a cost-effective system for production of high levels of protein in chloroplasts of transplastomic plant

cells, which provide bioencapsulation of the antigen through the cellulose containing cell walls.^{15,16} Because of the high number of chloroplast genomes per cell and our optimized expression system, transgenic proteins can accumulate in green leaves at much higher levels than this is the case for more traditional transgenic plant technologies.^{17,18} Oral delivery of

5 transplastomic plant cells has been effective in prevention of insulinitis in non-obese diabetic mice and of inhibitor formation in hemophilia B mice.^{19,20}

For FIX inhibitors, ITI is often not sustainable because of anaphylactic reactions and development of nephrotic syndrome. In hemophilia B mice, we demonstrated that repeated oral delivery of bioencapsulated FIX prevented inhibitor formation and fatal anaphylaxis in

10 subsequent replacement therapy.²⁰ Encouraged by these results, we sought to develop a protocol for hemophilia A. FVIII is a large protein, comprised of a signal peptide and a 2332 amino acid polypeptide. Structurally, FVIII contains 6 distinct domains, which are organized in the following order: A1-A2-B-A3-C1-C2.²¹ The large, central B domain is highly glycosylated and aids in secretion of the molecule.²²⁻²⁴ However, recombinant B domain deleted (BDD) FVIII is

15 biologically active, and represents one of the products currently used in the clinic. FVIII is secreted as a heterodimer following at least two intracellular cleavages within the B domain. Consequently, circulating FVIII is comprised of a heavy chain (containing A1-A2-B domains) and a light chain (A3-C1-C2 domains), which are non-covalently linked.^{21,23}

FVIII is a highly immunogenic molecule that can cause potent antibody responses in

20 hemophilia A patients and in experimental animals at low antigen doses.^{6,25} The majority of inhibitors bind to A2, A3, or C2 domain.^{6,26-28} These highly immunogenic sequences also contain several CD4⁺ T cell epitopes.^{6,29,30} Animal studies suggest that inhibitor formation against FVIII can be prevented by tolerization to parts of the molecule, such as combination of A2 and C2 domains, while a single domain may not be sufficient.^{14,31} Here, we demonstrate that FVIII

25 heavy chain and C2 domain can be expressed as cholera toxin B subunit (CTB) fusion proteins in tobacco chloroplasts. Oral delivery of a mixture of these bioencapsulated antigens suppressed and also reversed inhibitor formation in a mouse model of hemophilia A.

The following materials and methods are provided to facilitate the practice of the present invention.

Design and construction of chloroplast expression vectors

Because efficient delivery of bioencapsulated antigen to the GALT is required for tolerance induction and is facilitated by transmucosal carriers, human FVIII antigens were expressed as CTB fusions, a successful strategy for tolerogenic delivery of FIX and pro-insulin.^{20,32} Because of the large size of the FVIII molecule and the need for CTB fusions to form pentamers to bind to the GM1 receptor on gut epithelial cells, two separate FVIII chloroplast transformation vectors were constructed to include either the heavy chain (abbreviated as HC, with identical amino acid sequence as in recombinant BDD-FVIII and therefore containing A1 and A2 domains and 5 amino acids of B domain) or the C2 domain. The cDNA fragment of human FVIII-HC was amplified by PCR. PCR products, flanked with a furin cleavage and suitable restriction sites, were cloned into the pCR BluntII Topo vector (Invitrogen), and the sequence was verified. Then, the HC DNA fragment was ligated with pLD-Ctv-5CP chloroplast transformation vector containing the CTB and GPGP hinge sequences to create the pLD-CTB-HC expression vector.^{19,33} An analogous pLD-CTB-C2 expression vector was also constructed. Chloroplast vectors pLD-CTB-HC and pLD-CTB-C2 (**Fig. 1A**) contain homologous flanking sequences *16S/trnI* and *trnA/23S* from tobacco chloroplast genome to facilitate recombination with the native chloroplast genome. Expression of CTB-HC and CTB-C2 is regulated by the highly expressed tobacco chloroplast *psbA* 5'UTR-promoter and 3'UTR. The CTB-HC and CTB-C2 expression cassettes contain a glycine-proline-glycine-proline (GGPG) hinge between CTB and the HC or C2 element to prevent steric hindrance of the fusion proteins. In addition, a furin cleavage site, Arg-Arg-Lys-Arg, was created at the junction region of the fusion proteins to efficiently release the FVIII domains after internalization by epithelial cells.³⁴ The expression cassettes include the *aadA* (aminoglycoside 3' adenylyltransferase) selection marker gene with a GGAG ribosome binding site, driven by a tobacco plastid ribosomal operon promoter (Prn), to confer spectinomycin resistance. The final chloroplast transformation vectors pLD-CTB-HC and pLD-CTB-C2 (**Fig. 1A**) were sequenced and used for transformation.³³

Regeneration of transplastomic tobacco plants

Tobacco chloroplast transformation vectors pLD-CTB-HC and pLD-CTB-C2 (**Fig. 1A**) were used to transform tobacco (*Nicotiana tabacum*) via particle bombardment with gold particles coated with the plasmid DNA.³³ The bombarded leaves were then transferred to

selection/regeneration medium. Regeneration of FVIII transplastomic tobacco plants was performed as described earlier.^{33,34,35}

Characterization of FVIII expression in leaf tissues of transplastomic plants

- 5 Immunoblot analysis and quantitation of the CTB-HC and CTB-C2 fusion proteins were performed by previously reported protocols.^{18,34} GM1-ganglioside receptor binding assay was performed as reported earlier.³⁴ The Bis-Tris 3-12% gradient native gel electrophoresis followed by immunoblot analysis was carried out by following the instruction manual of the NativePage Novex Bis-Tris Gel System (Life Technologies).
- 10 The cDNA fragment of human FVIII heavy chain (A1-A2 domains plus first 5 amino acids of B domain) is amplified by PCR. PCR products, flanked with a furin cleavage and suitable restriction sites, are cloned into the pCR BluntII Topo vector (Invitrogen), and the sequence is verified. Lettuce expression vector pLsDV-CTB.C2 containing the CTB-C2 DNA fragment was amplified from pLD-CTB.C2 by PCR, and the sequence of the PCR product was
- 15 confirmed after cloning into pCR BluntII Topo vector. Lettuce expression vector pLs-CTB.C2 was created by subcloning the Ls 5' UTR/CTB-C2/Ls 3' UTR cassette into the pLsDV vector.^{34,35} Regeneration of lettuce plants was performed as described earlier.^{33,35}

Characterization of the F.VIII transplastomic lettuce plant

- 20 To evaluate transgene integration and homoplasmy of the transplastomic lines, PCR and Southern blot analyses were carried out. Immunoblot analysis and quantitation of the CTB-HC and CTB-C2 fusion proteins were performed by previously reported protocols. GM1-ganglioside receptor binding assay was performed as reported earlier. The Bis-Tris 3-12% gradient native gel electrophoresis followed by immunoblot analysis was carried out by following the instruction
- 25 manual of the NativePage Novex Bis-Tris Gel System (Life Technologies).

Lyophilization and storage

- Lettuce leaves expressing CTB-C2 was lyophilized in Freezone Benchtop Freeze Dry Systems (Labconco) as previously described.³⁶ Lyophilized leaves were stored at room
- 30 temperature under vacuum for a few weeks and ground to fine powder in Warring blender. The lyophilized lettuce fine powder was stored dry at room temperature for several months.

Mouse strains and experiments

Male hemophilia A mice with targeted deletion of *F8* exon 16 (*F8e16^{-/-}*) on a mixed C57BL6/129 or on a pure BALB/c background were housed under special pathogen-free conditions and were ~2 months of age at the onset of experiments.^{9,36} Leaf material was ground in liquid nitrogen and stored at -80°C. A mixture of CTB-HC and CTB-C2 material (total of 125 mg per mouse per dose) was suspended in sterile PBS (200 µl/dose), homogenized, and delivered via oral gavage using a 20-G bulb-tipped gastric gavage needle. For antigen challenge, mice were administrated 1 IU BBD-human FVIII (Xyntha, Pfizer, New York, NY) into the tail vein once a week. Plasma samples were obtained by tail bleed as published.³⁷ ELISA for FVIII antigen and anti-FVIII, Bethesda assays, and lymphocyte assays were as published.^{9,36,37}

Characterization and quantitation of the CTB-HC and CTB-C2 fusion proteins

Tobacco leaves were collected from mature plants at different time points (10 am, 2 pm and 6 pm) on the same day.^{1,2} In brief, the leaf samples were frozen and ground in liquid nitrogen. The ground leaf powder was suspended in an extraction buffer (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 100 mM DTT, 0.1% SDS, 400 mM sucrose, 0.05% Tween 20, 2 mM PMSF and proteinase inhibitor cocktail). The leaf suspension (*i.e.*, homogenate) was either directly used for western blot or underwent an additional centrifugation to separate the supernatant portion. Bradford assay was performed using protein assay dye reagent (Bio-Rad) to determine the protein concentration of the total leaf protein in homogenate fraction and the protein concentration of the total soluble protein in the supernatant portion. To detect the fusion protein expression level in both homogenate fraction and supernatant portion, western blot analysis was performed with anti-CTB antibody as a probe using known concentration of purified CTB protein (Sigma) as the standard. Then, the concentration of CTB-HC or CTB-C2 fusion protein was quantitatively measured by densitometry analysis using Alpha imager 2000. The percentage of fusion protein and amount of transgenic protein (µg/g or mg/g) is calculated according to the formula published earlier.³

Lymphocyte assays

Murine splenocytes were isolated by standard methods and cultured in RPMI 1640 media containing 5 µM β-mercaptoethanol, 100 mM insulin / transferrin / selenium, glutamine and

pencillin / streptomycin with or without 10 µg/ml of FVIII for 48 hours at 37 °C and 5% CO₂. Cells were then harvested and RNA was extracted using Qiagen RNeasy isolation kit (Valencia, CA, USA) according to manufacturer's protocol. Quantitative RT-PCR was performed as previously described using SABiosciences kit.⁴ Frequencies of IL-10 secreting cells in

5 hemophilia A C57BL6/129 mice in each group were determined by ELISpot assay (R&D System, Minneapolis, MN, USA) according to manufacturer's protocol. Spots were counted using the CTL-ImmunoSpot^H S5 UV analyzer (Cellular Technology, Shaker Heights, OH, USA). Each sample was run in duplicate. CD4⁺, CD4⁺CD25⁺, and CD4⁺CD25⁻ cells were purified from spleens and MLN using a magnetic isolation kit (Miltenyi Biotech, Bergisch Gladbach,

10 Germany). The cells were then pooled for each experimental group and adoptively transferred to naïve BALB/c mice at 10⁶ cells per mouse via tail vein injection. Recipient mice were challenged 24 hours later via subcutaneous injection of 1 IU FVIII in adjuvant (Sigma Adjuvant System, Sigma, St. Louis, MO, USA).

15 **Antibody and antigen assays**

Antibody responses to FVIII were measured via ELISA for anti-FVIII IgG1, IgG2a, and IgG2b , and by Bethesda assay for inhibitor formation as described before.⁴⁻⁶ Human FVIII HC antigen in circulation was detected via ELISA. Mice were fed with CTB-HC (250 mg) twice per day for 2 days, and bled and sacrificed 5 hours after the last gavage. Harvested liver was washed

20 with cold PBS and homogenized in RIPA buffer (PBS with 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH 7.5) with complete protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN, USA) for 30 min on ice. After centrifugation for 20 min at 14,000 × g at 4°C, the supernatant was collected. For HC-specific ELISA, plate was coated with 2 µg/ml GMA-012 (Green Mountain Antibodies, Burlington, VT, USA) overnight. BBD-FVIII (Xyntha,

25 Pfizer, New York, NY, USA) was used as the standard. Sheep anti-human FVIII (Haematologic Technologies Inc, Essex Junction, VT, USA) was diluted to 0.3 µg/ml, and HRP-rabbit anti-sheep IgG (Invitrogen, Camarillo, CA, USA) was used at 1:2000 dilution. Antibody responses to CTB were measured via ELISA as published.⁷

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Flow cytometry

Spleens, mesenteric lymph nodes, Peyer's patches, and inguinal lymph nodes were harvested, and single cell suspensions were prepared using 70- μ m cell strainer in cold PBS buffer, followed by Fc blocker for 5 min at room temperature. Cells were surface stained for CD4-eFluor 450, CD25-PE, LAP-Perp at 4°C for 30 min in PBS, followed by viability dye eFluor 506 stain at 4°C for 30 min in PBS. Fixation and Foxp3 Alexa Fluor 647 stain was performed using the transcriptional factor staining buffer set. Other cells were surface stained with CD4-eFluor 450, CD49b-APC, and LAG3-PE (Tr1 staining) at 4°C for 30 min in PBS, followed by viability dye eFluor 506. Controls included isotype control, single positive, and unstained cells. All kits and antibodies were purchased from eBiosciences (San Diego, CA). Flow cytometry was performed on a LSR II system (BD Bioscience, San Jose, CA), and data were analyzed with FCSEXPRESS software (De Novo Software, Los Angeles, CA).

Immunohistochemistry

Mice were fed with CTB-C2 (250 μ g) twice per day for 2 days and sacrificed 5 hours after the last gavage. Tissue was collected, frozen, cryo-sectioned, and stained as described.¹⁹ The following antibodies were used: rabbit anti-FVIII light chain (1:200, Santa Cruz Biotechnology, Dallas, TX); biotin anti-mouse CD11c (1:200, BD Biosciences, San Jose, CA; Alexa Fluor-488 donkey anti-rabbit IgG (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA), and streptavidin Alexa Fluor-568 (1:400, Invitrogen, Grand Island, NY). Images were captured using Nikon Eclipse 80i fluorescence microscope, Retiga 2000R digital camera (QImaging, Surrey, BC, Canada), and Nikon Elements software.

RESULTS

Characterization of FVIII-transplastomic lines

Putative transplastomic tobacco lines obtained after bombardment of chloroplast vectors were first screened by PCR analysis. Site-specific transgene integration into the chloroplast genome was confirmed with two specific primer sets 3P/3M and 5P/2M, which anneal specifically to complementary sequences of transgene cassette and the chloroplast genome.³³ Three independent tobacco lines from pLD-CTB-HC transformation and four independent lines from pLD-CTB-C2 transformation showed positive PCR products of correct sizes (data not

shown). The CTB-HC- and CTB-C2-transplastomic tobacco lines were further examined by Southern blot analysis for site-specific stable integration and homoplasmy. Homoplasmy is achieved when all copies of the chloroplast genomes have stably integrated transgenes. The results showed that all 3 tested lines of CTB-HC transplastomic lines had integrated transgenes at specific sites and were homoplasmic, showing only the larger genome fragment (8.6kb) with the transgene insert when compared with the 4.4kb fragment in the untransformed control genome (**Fig. 1B**). The CTB-C2-transplastomic tobacco lines also showed integration of transgenes into the chloroplast genome and homoplasmy (**Fig. 1C**).

Expression of CTB-HC and CTB-C2 fusion proteins in protein extracts from leaves of transplastomic tobacco plants was evaluated by western blot analysis. Under fully denatured and reducing conditions, blots probed with anti-CTB polyclonal antibody revealed full-length CTB-HC fusion protein with the expected molecular mass of 98 kDa (**Fig. 2A**) in all transplastomic lines. No cleaved products were observed even after solubilization of pentamers and destabilization of disulfide bonds with reducing agents. A similar banding pattern was observed in a parallel blot probed with an anti-A2 domain specific monoclonal antibody. In addition, there was no cross-reactivity of CTB standard protein or any other plant protein in untransformed leaf extracts (both used as negative controls) with the anti-A2 antibody (**Fig. 2B**). Quantitation of the fusion protein was performed by densitometry on western blots of leaf extracts using known amounts of purified CTB protein as the standard. The CTB-HC fusion protein was found to accumulate up to 0.8% total leaf protein or 80 µg/g fresh leaf tissue (**Fig. 2A and D**). The CTB-C2 fusion protein was similarly analyzed with anti-CTB polyclonal antibody in tobacco plants. As shown in **Fig. 2C**, a 31 kDa polypeptide representing the correct size of CTB-C2 fusion protein was detected in both fractions (supernatant and homogenate) of independent transplastomic lines. The CTB-C2 fusion protein accumulated up to 4.2% in the homogenate (*i.e.*, 4.2% TLP or 370 µg per g of fresh leaf, **Fig. 2D**).

Pentamer assembly of CTB-HC and CTB-C2 in transgenic chloroplasts

A plasma membrane receptor (GM1-ganglioside) binds CTB *in vivo*, and a pentameric structure is required for binding to GM1 receptor.³⁸⁻⁴⁰ To evaluate receptor binding ability of CTB-HC and CTB-C2 fusion proteins produced in tobacco chloroplasts, GM1-binding ELISA was performed. As observed in **Fig. 2E**, CTB-HC and CTB-C2 fusion protein extracts along

with purified CTB protein showed strong binding affinity to GM1. Therefore, CTB-HC and CTB-C2 fusion proteins assembled properly to form pentameric structures within transformed chloroplasts. To further evaluate the pentamer assembly directly, we ran blue native gels, and the blots were probed with anti-CTB polyclonal antibody. These results indicate that the pentameric structure (CTB-HC, 490 kDa; CTB-C2, 155 kDa) was formed in both CTB-HC and CTB-C2-transformed tobacco chloroplasts. In addition, other oligomeric forms larger than pentamers were also observed (**Fig. 2F**). Lack of cleaved products confirmed stability of assembled pentamers or multimers within transformed chloroplasts.

10 **Oral delivery of bioencapsulated FVIII suppresses inhibitor formation in hemophilic mice**

Plant leaf materials were ground in liquid nitrogen as published.¹⁹ CTB-HC and CTB-C2 materials were mixed and suspended in PBS buffer so that the final product contained approximately equal amounts of both fusion proteins (~5µg HC/6µg C2 per dose/mouse). Male hemophilia A mice (F8c16^{-/-}) on C57BL6/129 genetic background received oral gavage of 125 mg mixed material per dose, twice per week for 2 months (**Fig. 3A**). During the second month, FVIII concentrate (recombinant BDD-FVIII) was given IV once per week at 1 IU/mouse. As expected based on prior findings, control mice that received no gavage (n=9) or were fed with WT plant material (n=6) formed very high-titer inhibitors (50-391 BU/ml; **Fig. 3B**).^{9,36} These were predominantly IgG1 with substantially less IgG2a and IgG2b formation (**Fig. 3C-E**). In contrast, inhibitor formation was significantly suppressed (on average 7-fold) in those mice that had been fed with FVIII plant material (n=6). These differences in BU correlated with the level of suppression of FVIII-specific IgG1 formation (**Fig. 3C**). IgG2a and IgG2b anti-FVIII became undetectable in FVIII-fed mice (**Fig. 3D-E**).

To address the effect of oral antigen delivery on T cell responses to FVIII, we harvested splenocytes from C57BL6/129 F8c16^{-/-} mice that had been fed with WT or FVIII expressing plant material and treated with FVIII. *In vitro* re-stimulation with FVIII induced expression of several cytokines associated with different T helper cell responses in cultures from WT fed mice (**Fig. 3F**). IL-6 was the most highly and consistently expressed cytokine, which we have previously shown to be expressed by CD4⁺ T cells of this strain in response to FVIII.⁹ These control mice lacked expression of immune suppressive cytokines or Treg markers. In contrast, splenocytes from FVIII-fed did not show expression of IL-6 or other cytokines associated with

Th1 (IL-2, IFN- γ), Th2 (IL-4, IL-13), or Th17 (IL-17) responses. Instead, up-regulation of Treg markers (CD25, FoxP3, CTLA-4) and, more markedly, of suppressive cytokines IL-10 and TGF- β was observed. Hence, the response was shifted from an effector to a suppressive/regulated response. These results were further supported by an increase in IL-10 producing splenocytes in ELISpot assay (**Fig. 3G**).

Suppression of inhibitor formation is successful in different strain backgrounds

The identical experiment was performed in hemophilia A mice with the same *F8* mutation but backcrossed on a BALB/c background. Inhibitor formation in this strain is not as brisk.^{36,41} Nonetheless, control mice (n=8-11/group) invariably formed high-titer inhibitors (8-200 BU/ml) after 4 weekly IV injections of FVIII (**Fig. 4A-B**). The response was again dominated by IgG1, albeit that IgG2a and IgG2b responses were also observed at substantial titers in some of the animals (**Fig. 4C-E**). Among FVIII-fed mice, 7 had undetectable inhibitors and 4 formed low-titer inhibitors (1-4 BU/ml), indicating more complete suppression in this strain by oral antigen administration (**Fig. 4B**). Total IgG formation was suppressed by approximately 1 log, with absent IgG2a and IgG2b and IgG1 reduced to low-titer (**Fig. 4C-E**). Next, we extended weekly IV administration of FVIII (without additional feeding) for another month in 5 animals previously fed with FVIII plant material (3 of which had initially undetectable inhibitors). All 5 mice showed an increase in Bethesda titers to 35-138 BU/ml after 1 month (**Fig 5B**). As expected, inhibitor and anti-FVIII IgG titers further increased in control animals treated with FVIII in parallel, reaching levels substantially higher (on average 9-fold) than those in initially FVIII-fed mice (445-998 BU/ml, **Fig. 5B**). Control mice were subsequently fed with WT plant material for 2 months without further exposure to FVIII (**Fig. 5A**). These animals maintained their Bethesda titers and showed a modest decline in IgG1 anti-FVIII (**Fig. 5B-C**). In animals initially tolerized to FVIII, further oral delivery of FVIII plant material for 2 more months reversed inhibitor titers to an average of 11 BU/ml, ranging from undetectable to 20 BU/ml, which correlated with a reversal of IgG1 formation (**Fig. 5A-C**). An additional experimental group (n=7) was orally tolerized, again followed by weekly IV injections of FVIII starting 1 month after initiation of oral tolerance. However, in this case the oral tolerance regimen was continued along with replacement therapy ("FVIII continuously fed" group in **Fig. 5B,C**), which resulted in further suppression of the average inhibitor titer at time point #2, (1.7-

fold compared to mice with discontinued oral delivery and 15-fold compared to control mice), and suppression was again sustained (Fig. 5B). Reminiscent of our published data on FIX, binding antibodies against FVIII remained detectable by ELISA in this group (Fig. 5C).²⁰

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Reversal of inhibitor formation

To test whether the oral protocol is effective in pre-immune mice, we treated hemophilia A BALB/c mice with FVIII and divided them into two groups (n=4-5) with similar average Bethesda titers (~60 BU). One group (control) was not further exposed to FVIII antigen, while the other group was subjected to the oral tolerance regimen (Fig. 5D). Inhibitor titers in control animals spontaneously rose further to an average of nearly 150 BU and eventually contracted to the original titer of ~60 BU (Fig. 5E). IgG1 anti-FVIII titers showed a substantial further increase to a level that was subsequently maintained (Fig. 5F). In contrast, oral FVIII delivery slowed and then reversed inhibitor formation, resulting in a 3- to 7-fold decrease compared to controls after 2-3 months of feeding (Fig. 5E). IgG1 formation was also significantly decreased (by ~2.5-fold, Fig. 5F).

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Oral antigen delivery induces a Treg response against FVIII

Lymphocyte assays in the C57BL/6/129 strain suggested Treg induction. We sought to obtain more direct evidence for induction of active immune suppression using adoptive transfer studies, which was possible in the pure BALB/c background. Lymphocytes were isolated from spleens and mesenteric lymph nodes (MLN) of FVIII-fed hemophilic BALB/c mice at the end of the experiment outlined in Fig. 5A. Upon adoptive transfer to naïve mice of the same strain, CD4⁺CD25⁺ T cells, and even more so CD4⁺CD25⁻ T cells (but not CD4⁻ cells) were able to significantly suppress antibody formation to FVIII (Fig. 6A). From previous studies, it has become clear that CD4⁺CD25⁺FoxP3⁺ Treg are critical in tolerance induction to coagulation factors.^{8,42} In order to identify potential suppressor cells in the CD4⁺CD25⁻ T cell population, we performed flow cytometric analyses of various lymphatic tissues in tolerized vs control mice (Fig. 6B). We found significant induction of CD4⁺CD25⁻LAP⁺ T cells (which express high levels of TGF-β) in spleens, MLN, and Peyer's patches, but no induction of type 1 regulatory T (Tr1) cells (which express high levels of IL-10 and are LAG-3⁺CD49b⁺).^{12,13,43} Consistent with *in vitro* RT-PCR array data and our previous findings, overall frequencies of CD4⁺CD25⁺FoxP3⁺ Treg

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showed only a subtle increase as antigen-specific cells of this subset function at low cell numbers.^{42,44}

Local and systemic delivery of bioencapsulated FVIII

Delivery of FVIII antigen to the GALT was demonstrated by immunostaining, which
 5 showed presence of fed FVIII antigen in epithelial cells and delivery to dendritic cells (DC) in
 the lamina propria and Peyer's patches of the small intestine (**Fig. 7A-C**). Presence of a furin
 cleavage site between CTB and FVIII sequences should facilitate systemic delivery of FVIII
 antigen following uptake in the gut. Indeed, we found HC antigen in plasma samples and liver
 protein extracts from samples obtained from hemophilia A mice 5 hrs following the last gavage
 10 (**Fig. 7D**). Delivery of CTB-HC/CTB-C2 only infrequently elicited systemic antibody responses
 to CTB, and there was no correlation between anti-CTB and FVIII inhibitor titers (**Figure 8** and
 data not shown). The reason for anti-CTB formation in a subset of C57BL6/129 mice is unclear
 but may relate to processing of the receptor-bound CTB antigen (that is cleaved off FVII
 sequences) or strength of B or T cell epitopes for this antigen/strain combination.

15 In order to facilitate clinical translational studies, antigens should be developed in an
 edible crop. Therefore, we first generated lettuce (*Lactuca sativa*) CTB-C2 transplastomic plants.
 The lettuce chloroplast expression vector pLs-CTB.C2 was created by introducing lettuce
 flanking sequences *16S/trnI* and *trnA/23S*, and 5' UTR and promoter of *psbA* gene from lettuce
 (**Fig. 9A**). Transplastomic plants were obtained after 3 – 4 months of selection and regeneration
 20 following bombardment of lettuce leaves. Southern blot analysis indicated that homoplasmy was
 achieved in most transplastomic lines (**Fig. 9B**). Up to 1.7% TLP (or 102 µg/g fresh leaves) of
 CTB-C2 fusion protein was expressed in lettuce chloroplasts as observed in western blot analysis
 (**Fig. 9C**). The pentamer form of lettuce-made CTB-C2 was also verified by GM1 binding assay
 and by blue native gel electrophoresis/immunoblot analysis (**Fig. 9D-E**). No cleaved products
 25 were observed even after dissolution of pentamers and destabilization of disulfide bonds by
 reducing agents (**Fig. 9C**). The C2 antigen concentration per gram of leaf tissue increased 20
 fold after lyophilization. Up to 2004 µg CTB-C2 protein per gram of lyophilized leaf materials
 was observed (**Fig. 9F-G**) when compared to 102 µg/g in fresh leaves, which was detected with
 a parallel immunoblot assay. The lyophilized and ground lettuce CTB-C2 powder can be
 30 prepared as capsules (or in a different formulation more suitable for pediatric patients) and stored
 at room temperature for long periods to facilitate oral delivery in clinical studies (**Fig. 9H**).

In order to scale up biomass of CTB-HC leaf materials, more than 200 T1 plants from the CTB-HC transplastomics were transplanted into soils in the greenhouse. Western blot analysis to screen the positive plants was first performed (**Figure 10**). Lettuce leaves with high-level expression of CTB-HC fusion protein were harvested. More than 10 kg of fresh CTB-HC lettuce leaves have been collected. Approximately 200 g (dry weight) of lyophilized CTB-HC lettuce leaf powders were obtained from 4 kg of fresh CTB-HC leaves. The concentration of CTB-HC protein in the lyophilized leaf powders was 101 μ g per g of dry weight.

In the same way (**Figure 11**), approximately 5 kg of CTB-C2 lettuce fresh leaves have been harvested. A total of 150 g of lyophilized CTB-C2 leaf materials have been obtained From 3 kg of fresh leaves. The CTB-C2 concentration in the lyophilized leaf materials was 3.32 mg per g of dry weight.

Advantages of the plant-based platform for oral tolerance in hemophilia

An oral tolerance protocol would be ideal for induction of antigen-specific tolerance while avoiding use of genetic manipulation of patient cells or of immune suppressive drugs, which have undesired side effects, increase the risk of infection, and may impact development of the immune system.^{12,14,16,20} Therefore, oral delivery of FVIII antigen may be an acceptable form of prophylactic tolerance induction in pediatric patients. Our current study demonstrates that multiple domains of FVIII can be expressed in plant chloroplasts. Moreover, oral administration of a mixture of bioencapsulated HC and C2 domain antigens substantially suppressed inhibitor formation in subsequent replacement therapy or animals with pre-existing response to FVIII infusion.

Oral delivery of plant-made pharmaceutical proteins is emerging as an effective approach. Bioencapsulation of therapeutic proteins within plant cells protects them from harsh environment of the gastrointestinal tract.^{15,45,46} In addition, elimination of highly expensive purification, cold storage, transportation and sterile injections significantly reduces their costs. Although we have not optimized the codons for FVIII expression in the current investigation, it has been reported that protein expression can reach up to 70% of the total leaf protein, under optimal conditions.¹⁸ Thus, the constructs described herein can be constructed using the humanized versions of FVIII, thereby further increasing efficacy. Multigene engineering is especially relevant for studies with

FVIII because all domains may be simultaneously expressed in a single transformation cassette.⁴⁷

Clinical implications

5 We provide proof-of-principle for suppression of FVIII inhibitors by oral delivery of transplastomic plant material. Transgene codon optimization can be employed to increase expression, as observed for several other human genes in chloroplasts.^{18,32} The C2 domain is expressed 3.6-fold higher than the heavy chain because of higher codon compatibility, underscoring the need for codon optimization to achieve higher levels of expression in

10 chloroplasts. Using a combination of FVIII domains, their ratio becomes important. For example, we observed only a 3-fold reduction in inhibitor titers in hemophilic C57BL/6/129 mice when a ratio of HC-C2 of 1:3 (instead of 1:1) was used (data not shown). The tolerogenic antigen mix may be further optimized by preparing different ratios of domains or subunits or by addition of more domains such as A3. The new data with FVIII demonstrate that the approach can also be

15 applied to reversal of pre-existing inhibitors that have formed during replacement therapy. Thus, new oral tolerance protocols for prevention of inhibitor formation in high-risk patients and as an alternative or an addition to current ITI have been developed.

Induction of suppressive CD4⁺ T cell responses

20 Adoptive transfer studies demonstrate that oral FVIII delivery induced multiple subsets of CD4⁺ T cells that actively suppress antibody formation. Therefore, this mechanism is distinct from immune tolerance induced by hepatocyte-derived antigen, which primarily induces CD4⁺CD25⁺FoxP3⁺ Treg.^{44,48,49} Antigen presented in the GALT additionally induced a strongly suppressive CD4⁺CD25⁻ T cell response. We do not believe this reflects memory effector T cell

25 activity, as transfer of such FVIII-experienced cells from mice that had not received oral delivery increases rather than suppresses anti-FVIII formation (X Wang, unpublished observations). Rather, flow cytometric analyses of CD4⁺ T cells suggests induction of CD4⁺CD25⁻LAP⁺ Treg, which are known to be inducible by antigen presentation in the gut and suppress by expression of large amounts of TGF- β , a cytokine that is also required for peripheral induction of

30 CD4⁺CD25⁺FoxP3⁺ Treg. Consistent with tologenic oral antigen delivery, induction of CD4⁺CD25⁻LAP⁺ was observed in Peyer's patches and MLN, which drain the gut, but not non-

draining lymph nodes. Increased frequency in the spleen is consistent with suppression of a systemic response, which is required to control inhibitor formation against IV delivered FVIII antigen. We found no evidence for induction of Tr1 cells. Nonetheless, there was induction of IL-10, a critical anti-inflammatory cytokine in the GALT. Both FoxP3⁺ Treg and LAP⁺ Treg are potential sources of IL-10 expression. Co-delivery of HC and C2 domain was sufficient to suppress inhibitor formation against the entire FVIII molecule in the BALB/c strain. In humans, additional T cell epitopes in other domains likely exist. However, efficient induction of Treg may provide sufficient suppression so that not all epitopes have to be covered by the orally delivered antigens.

In conclusion, FVIII domains can be produced at high levels in leaves of transplastomic plants. Oral delivery of modified plant cells induces Treg that suppress antibody formation to IV delivered FVIII and therefore represents a promising approach to control formation of inhibitors.

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WHAT IS CLAIMED IS:

1. A composition comprising lyophilized plant material comprising at least one fragment of Factor VIII (FVIII) conjugated to cholera toxin B subunit (CTB), said fragment consisting of a FVIII domain selected from the group consisting of C2 and heavy chain (HC), said fragment conjugated to CTB having been produced in chloroplasts within said plant and retaining immunogenicity in lyophilized form, which upon oral administration to a mammal in need thereof is effective to induce oral tolerance to FVIII.
2. The composition of claim 1, wherein said plant is selected from the group consisting of lettuce, tomato, apple, berries, citrus fruits, banana, carrot, celery, cauliflower, broccoli, collard greens, cucumber, muskmelon, watermelon, pepper, pear, grape, peach, radish and kale.
3. The composition of claim 2, wherein the berries are strawberries or raspberries.
4. The composition of any one of claims 1 to 3, wherein a hinge peptide, GPGP, and a furin cleavage site, RRKR, are present between said CTB and said at least one FVIII fragment.
5. The composition of any one of claims 1-4, wherein the composition is for use in induction of oral tolerance for the treatment of hemophilia A.
6. The composition of any one of claims 1-4, wherein the composition is for use in induction of oral tolerance for the treatment of hemophilia A, wherein C2-CTB and HC-CTB fusion proteins are for administration together, said composition being effective to induce expression of TGF- β producing CD4⁺CD25⁺LAP⁺ regulatory T cells in spleen.
7. The composition of any one of claims 1-4, wherein the composition is for use in induction of oral tolerance for the treatment of hemophilia A, wherein said oral tolerance is caused by suppression of inhibitor formation or reversal of pre-existing inhibitors formed during FVIII replacement therapy.

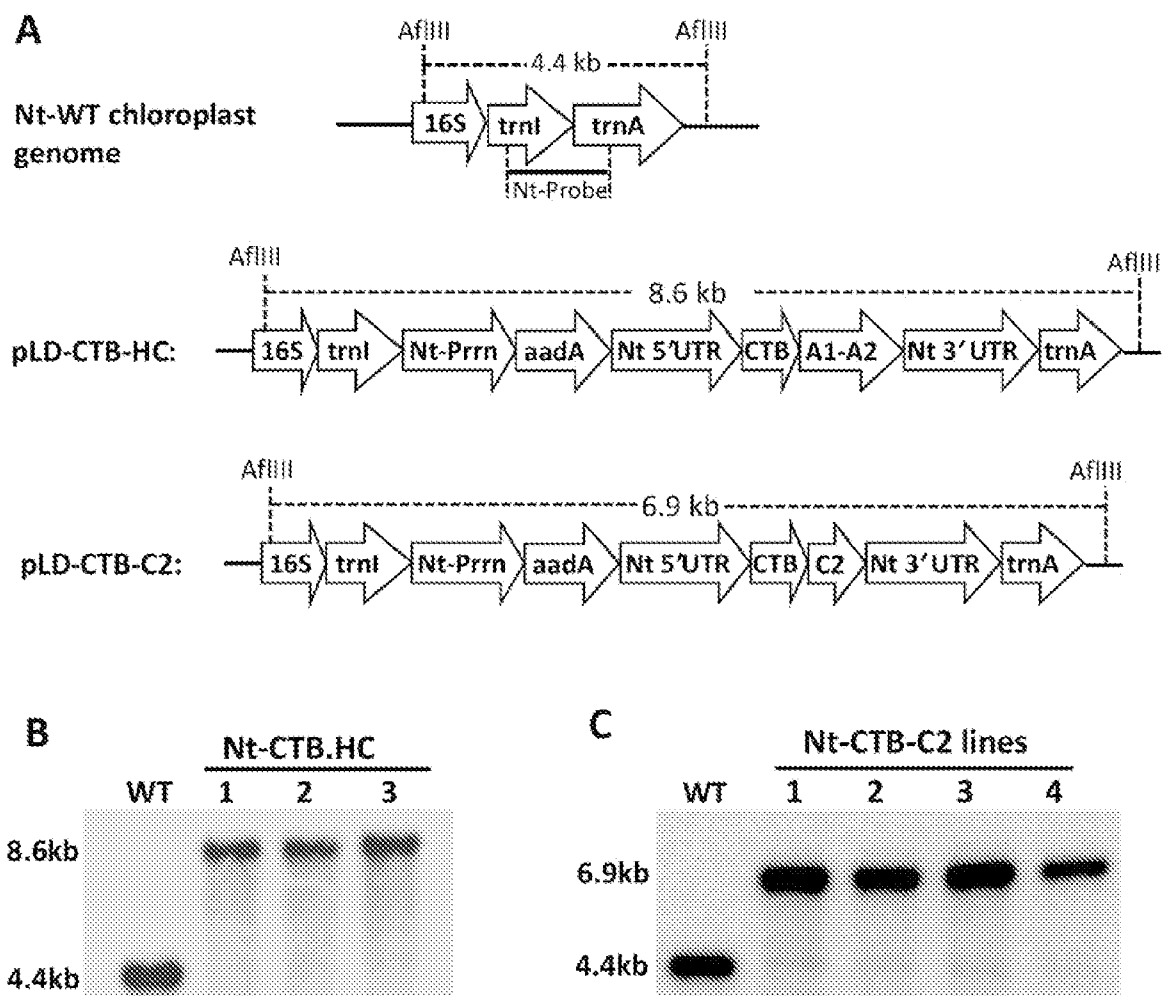


Figure 1

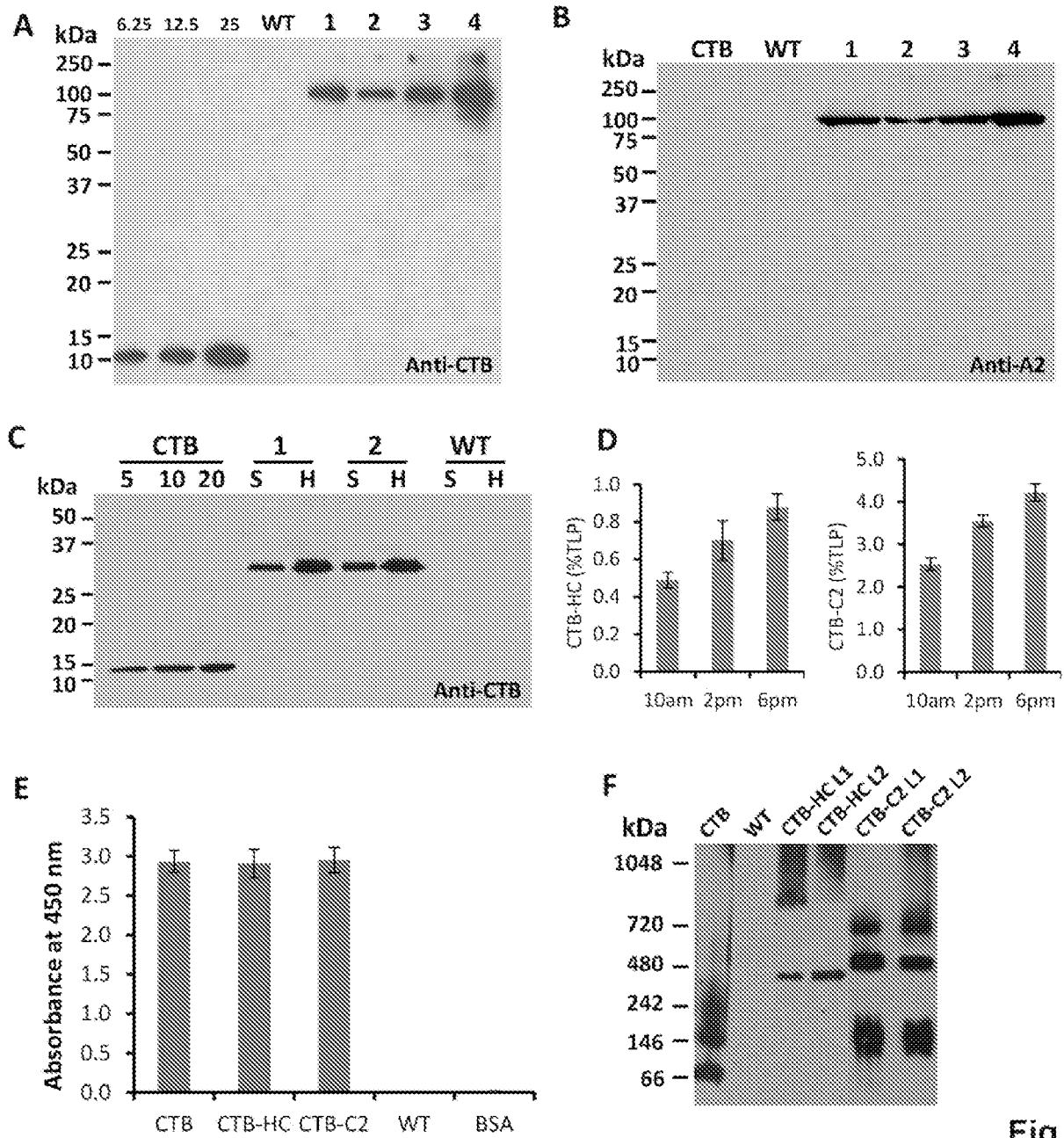


Fig. 2

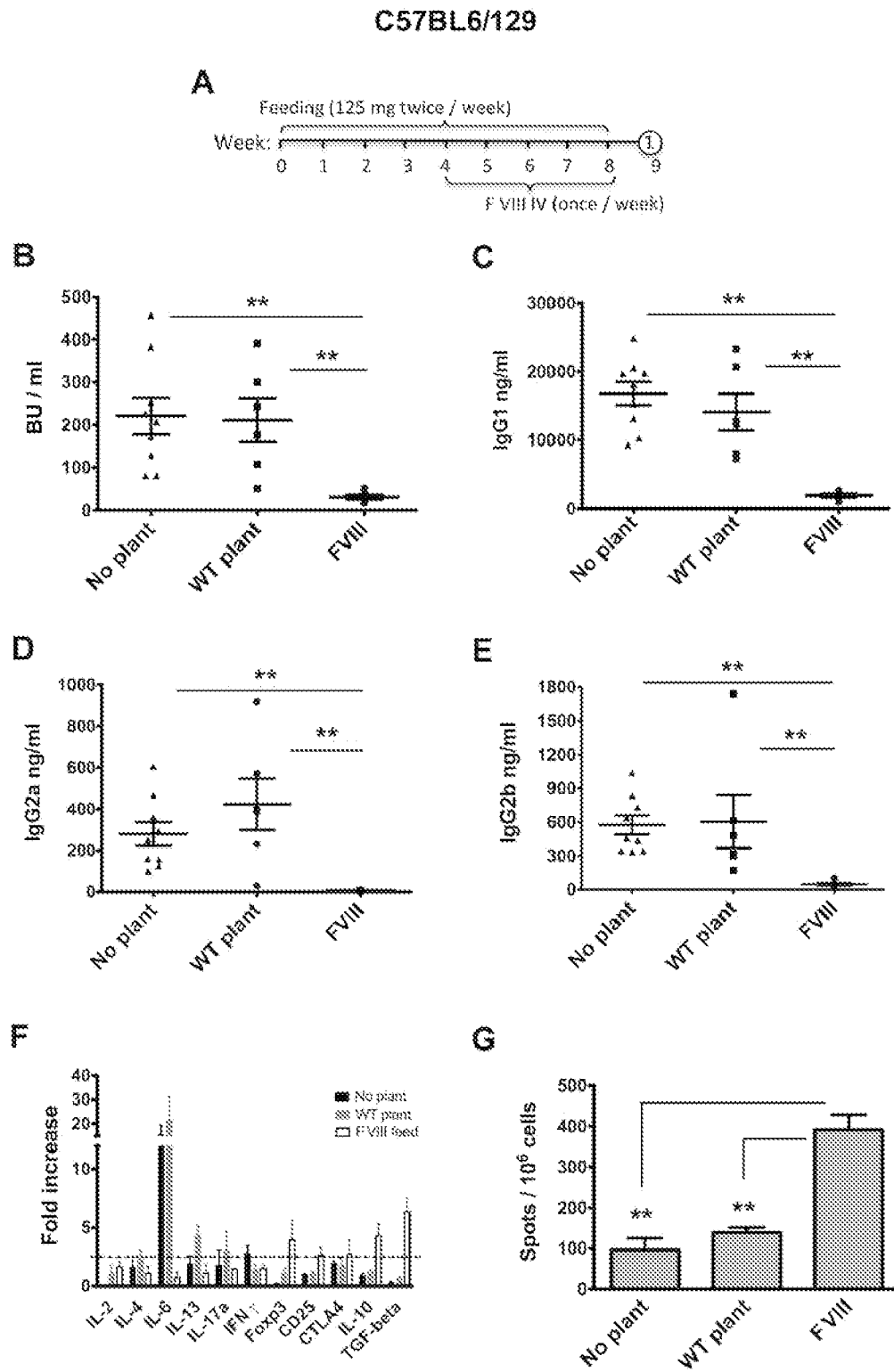


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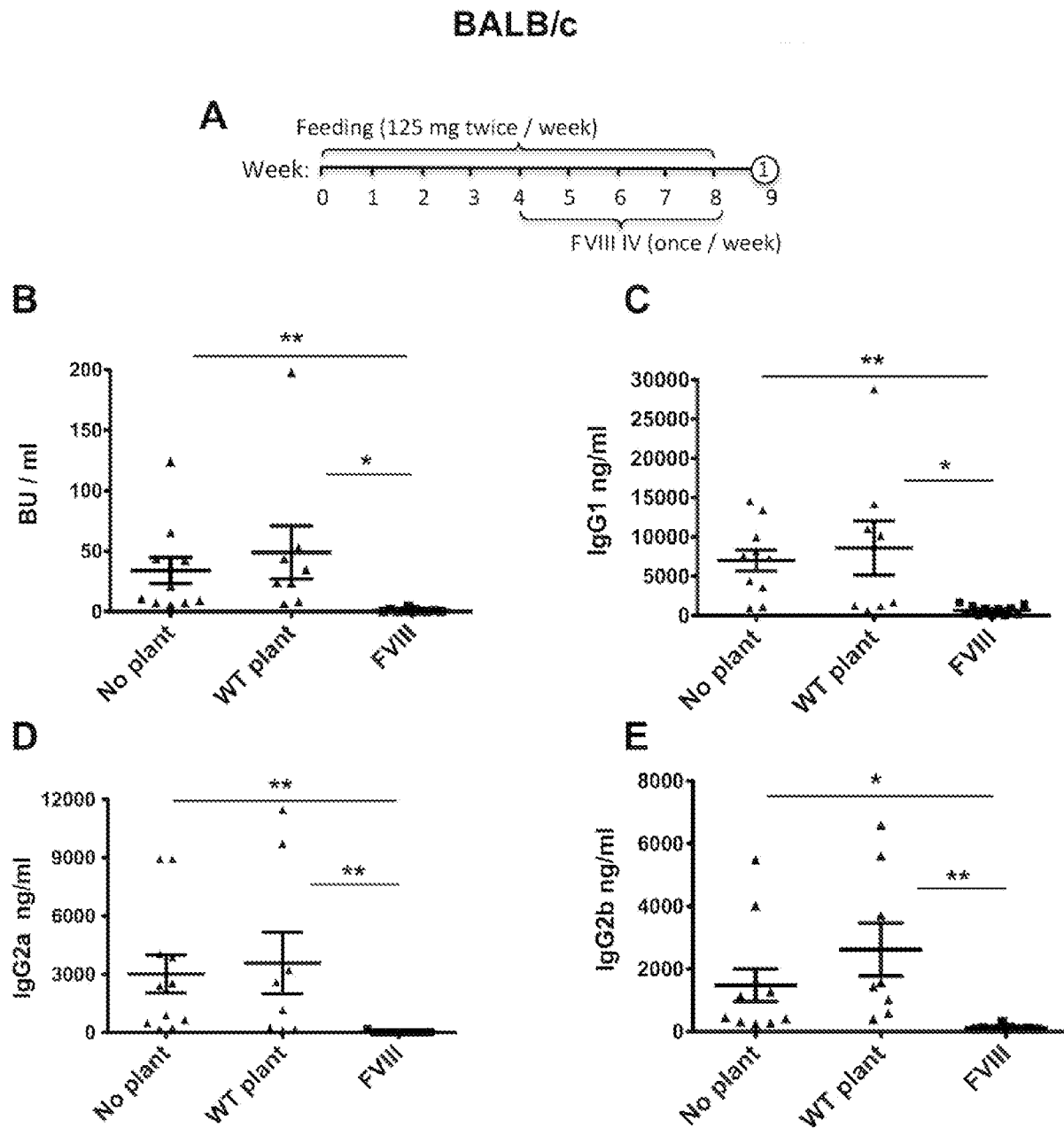


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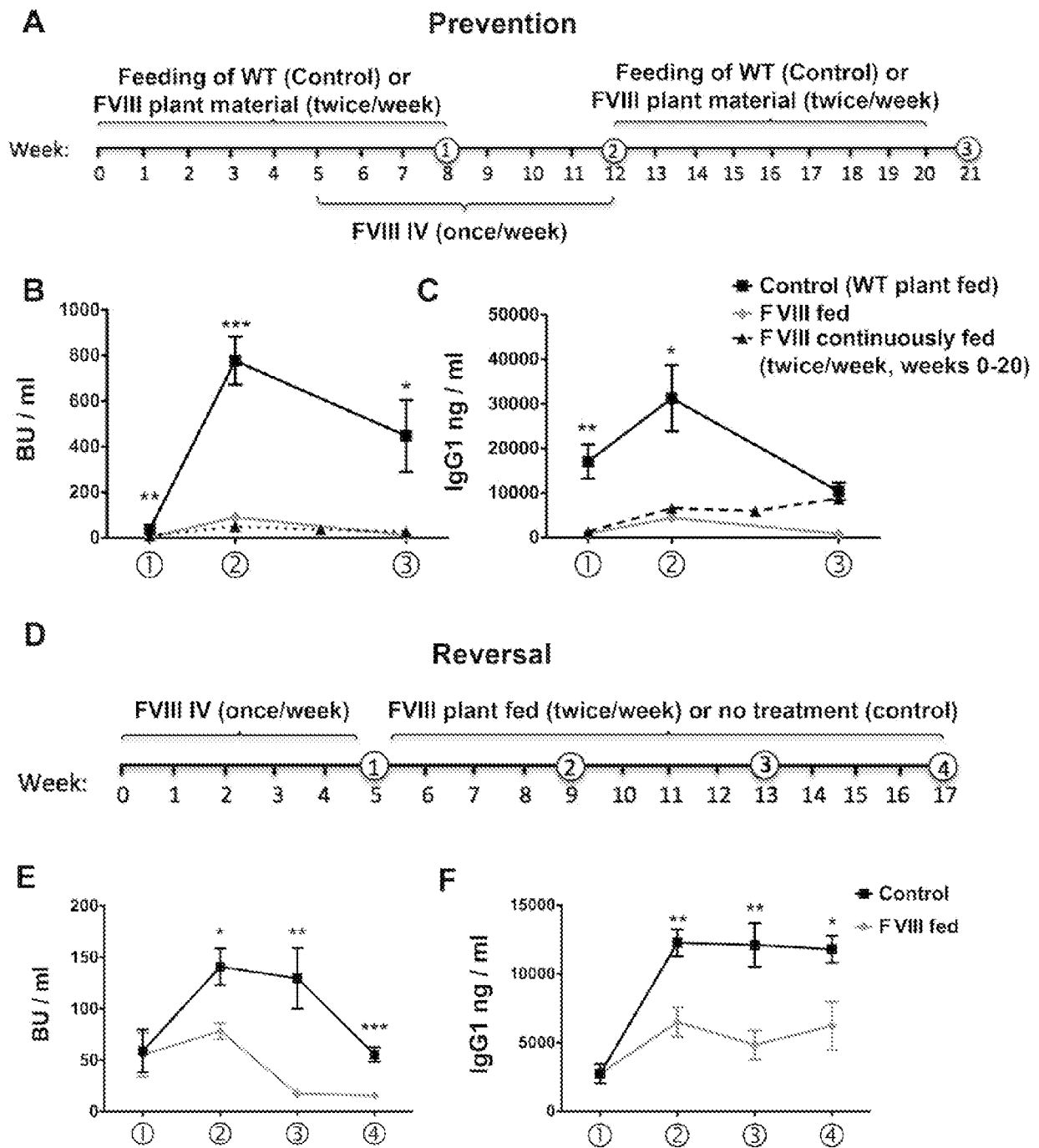
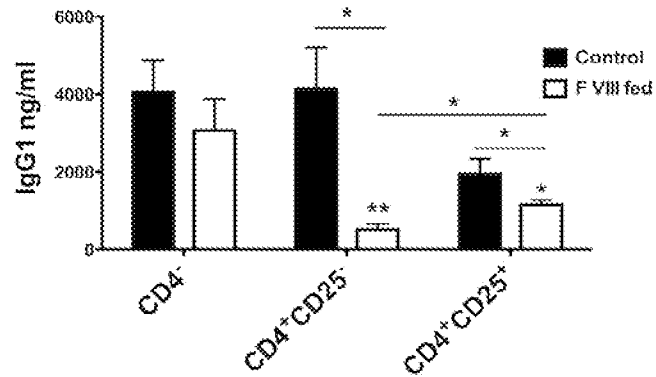
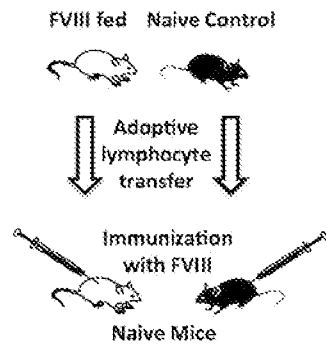
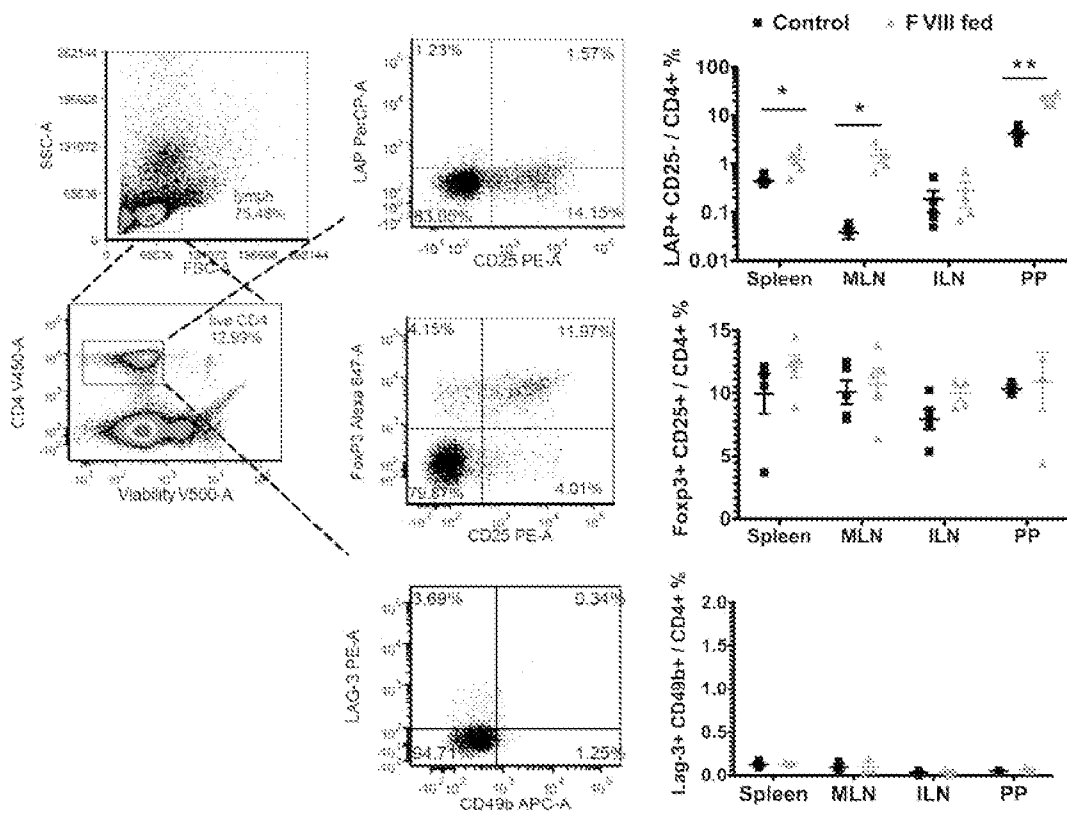


Figure 5

A**B****Figure 6**

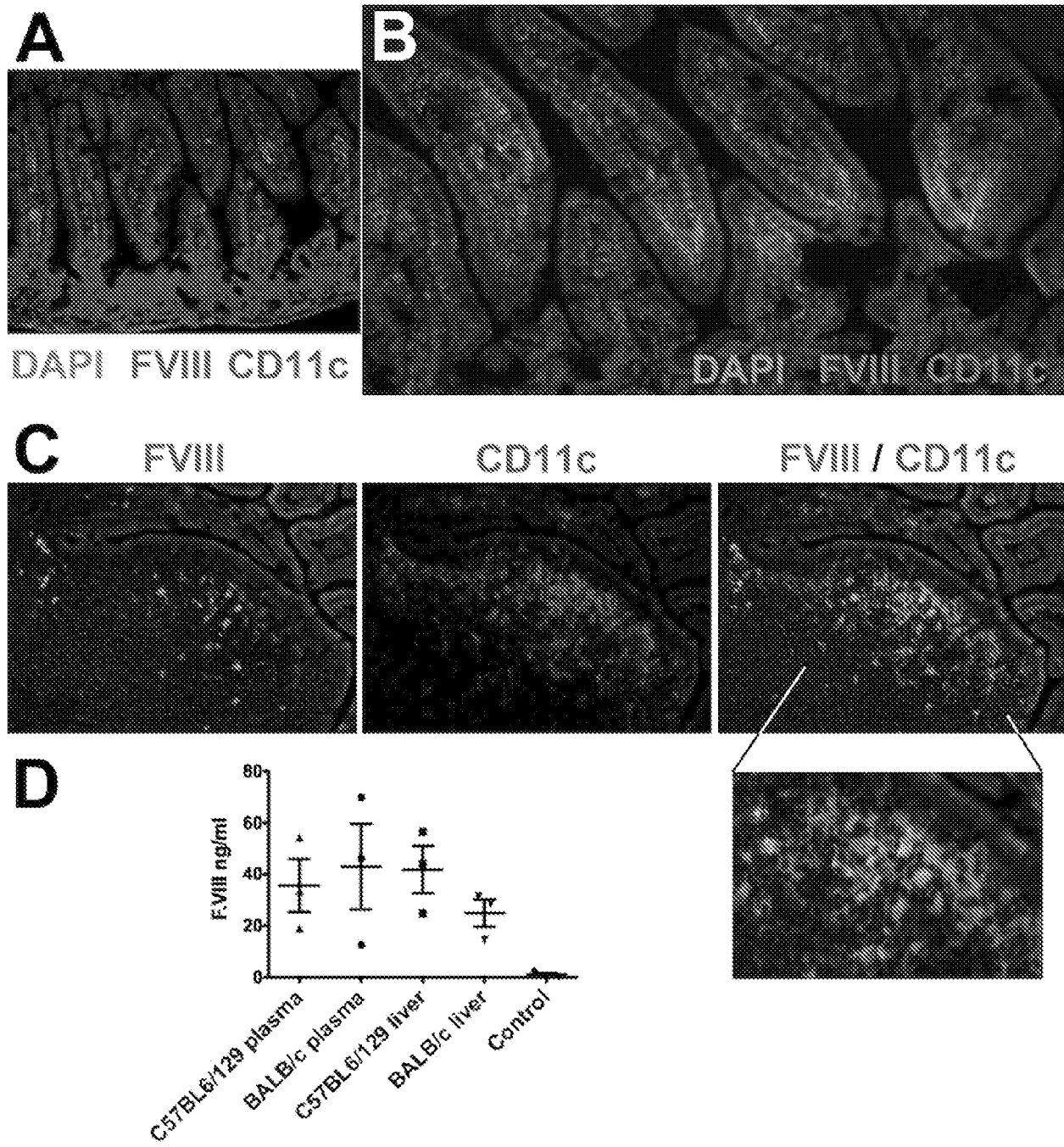


Figure 7

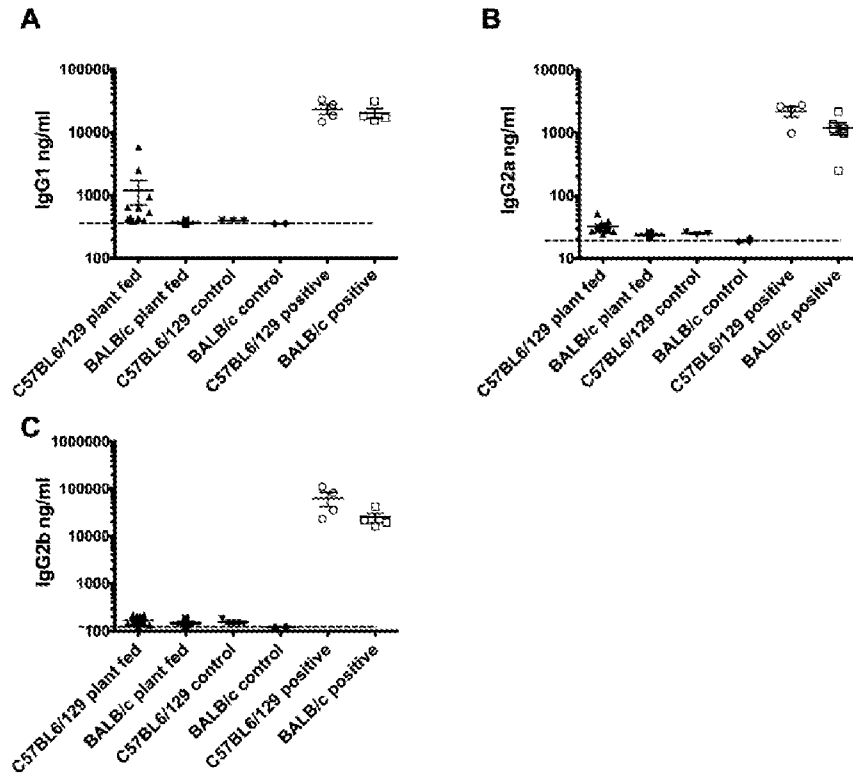


Figure 8

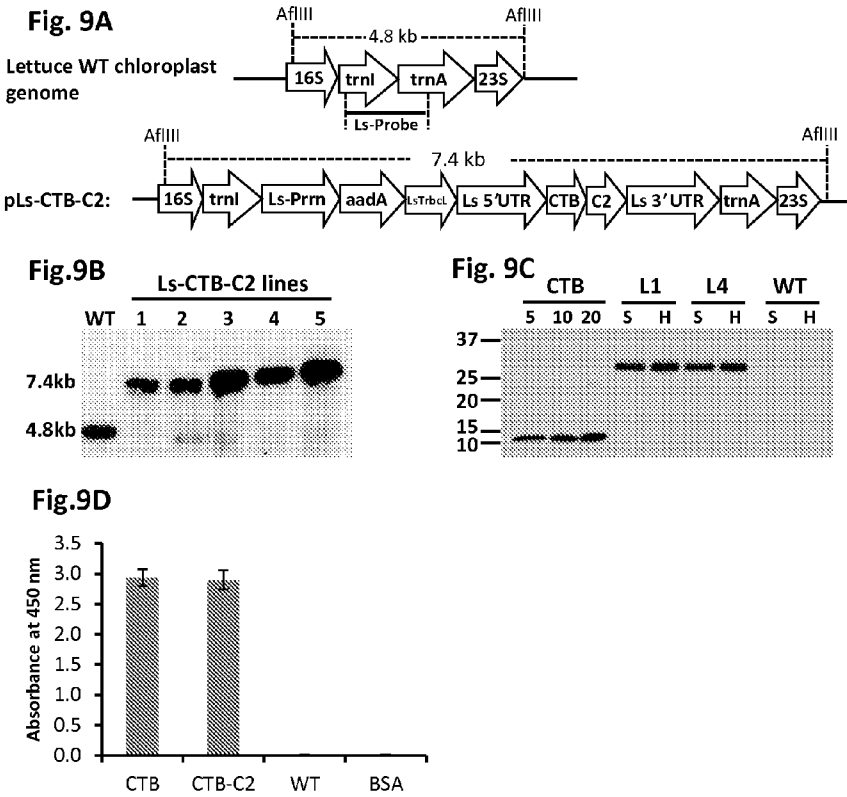


Fig. 9E

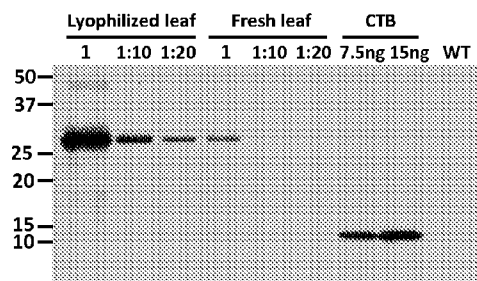


Fig. 9F

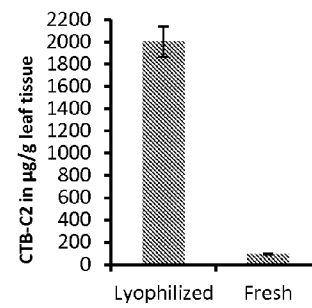


Fig. 9G

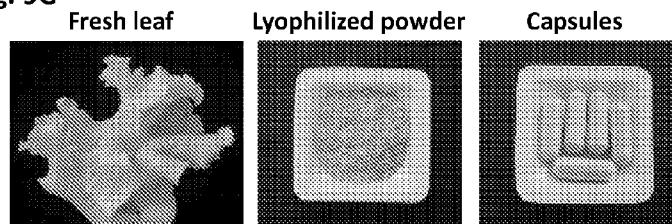


Fig. 10A

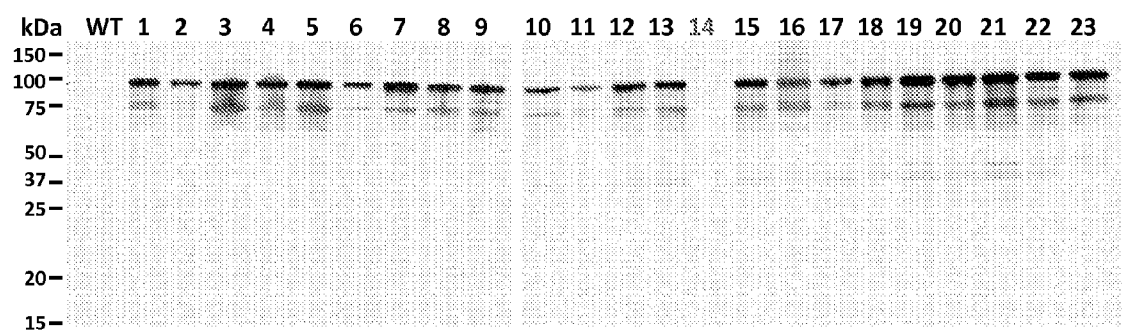


Fig. 10B



Fig .11A

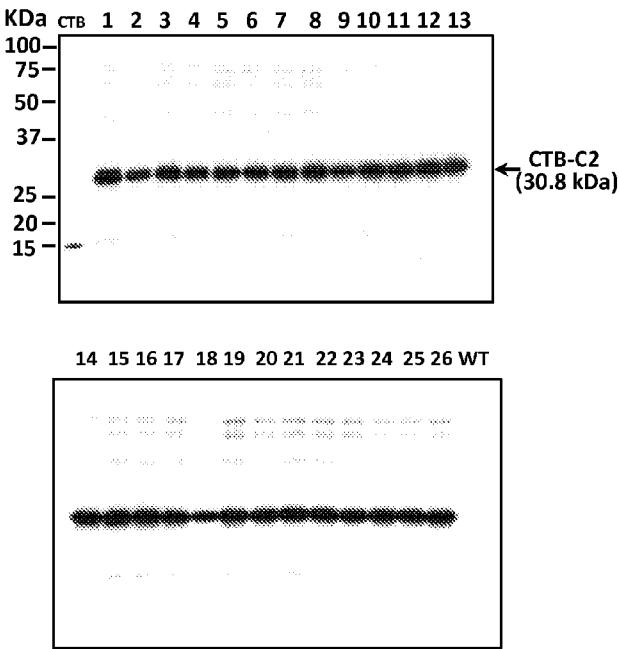


Fig. 11B