Stability of a soybean seed-derived vaccine antigen following long-term storage, processing and transport in the absence of a cold chain

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Abstract

BACKGROUND: Soybean seeds are rich in natural protein and are favorable environments for targeted protein expression. Soybeans represent an ideal platform for the production of novel vaccines that, in theory, do not require a cold chain. This study investigated the stability of a soybean-derived antigen following long-term storage, formulation, and shipment overseas in the absence of refrigeration.

RESULTS: Transgenic seeds can be stored for more than 4 years at ambient temperature with no detectable loss of FanC antigen stability. Conventional processing methods utilizing heat, mechanical extraction and solvent extraction resulted in practical formulations that could be lyophilized, stored as dried milk powder and rehydrated in water without loss of FanC antigen stability. Overseas shipment of transgenic seed powder and soymilk formulations in the absence of a cold chain had no adverse effects on formulations or the FanC antigen.

CONCLUSION: The feasibility of long-term storage, processing and shipment of transgenic soy products in the absence of a cold chain was demonstrated. Soybeans represent a practical platform for development of novel vaccines to potentially address the worldwide need for vaccines that are cost-effective, easy to formulate and can be manufactured, stored and transported without refrigeration.

 INTRODUCTION

There is an urgent need in both developing and developed countries for safe and effective vaccines that are affordable and easy to deliver. The World Health Organization continues to support the research of new technology and vaccine delivery systems to help in meeting these needs.1,2 Unfortunately, the requirement for refrigeration during storage and transportation adds greatly to the manufacturing cost of a vaccine and places a burden on those in developing countries where the need for immunization and the prevention of disease is the greatest.3 Over the last two decades recombinant DNA technology has made it possible to express heterologous antigens in numerous systems, including plants, thereby increasing the likelihood that high cost and other issues associated with vaccines may be mitigated with novel vaccine platforms that could aid with goals for global immunization.

As a plant-based platform, soybeans offer many unique advantages, including naturally high levels of protein in seeds (~38% dry weight), as well as nutritional and health benefits relative to other plant protein sources.4,5 It is also likely that soybean seeds can be stored and transported without the need for refrigeration. Since soybeans are a globally established staple in the diets of agricultural animals and humans, the consumption of soy-based products should be readily acceptable and well tolerated.

In agricultural use, soybeans are consumed predominantly as processed soy meal. For human consumption, soybeans also undergo processing to capture oil, remove anti-nutritional factors, and generate palatable end-products such as soymilk, soy meal and soy protein isolates. The methods used for soy processing are well established and used throughout the food production industry.4

A major factor contributing to the feasibility of a practical plant-derived vaccine formulation is the potential to generate high levels of antigen within host systems.6 While it is likely that antigen stability is dictated by intrinsic factors that are antigen-specific, it seems logical that a practical host for antigen expression would be one that is naturally rich in protein, such as soybean. With the use of regulatory signals, foreign proteins have been shown to accumulate to levels representing nearly 3% of total soluble seed protein in soybeans.7,8 More recently, accumulation of a transgenic protein was shown to approach levels representing...
7% of soybean seed proteins when crossed into a β-conglycinin suppression background. The capacity to express and accumulate relatively high levels of foreign protein (up to 7%) coupled with a host naturally rich in protein (~38%) can be exploited to generate milligram quantities of a transgenic antigen within a single soybean seed that can further be processed into less than 1 mL of soymilk for oral delivery. With respect to protein content, formulation, delivery and cost, a soy-based platform is not only feasible but arguably the most logical choice for the development of novel vaccines.

Given the great potential for soy-based vaccines, our laboratory has been developing a novel soy-based vaccine to protect cattle from infection by enterotoxigenic Escherichia coli (ETEC). The model antigen used in our studies is FanC, the most abundant and immunogenic subunit protein of ETEC K99 fimbriae. In earlier studies we expressed transgenic FanC in soybeans and showed that the antigen induced both cellular and humoral immune responses in mice following immunization. In this study, we further demonstrate the practicality of a soy-based platform by reducing to practice numerous assumptions made with respect to long-term storage, processing, shipment and refrigeration of transgenic products that, to date, have not been described. The results from these experiments demonstrate the feasibility and practicality of a soy-based platform for the development of novel vaccines.

**MATERIALS AND METHODS**

**Transgenic soybean material**

Construction of the FanC binary vector and characterization of transgenic lines 485-1 and 485-10 were previously described by our laboratory. Briefly, Agrobacterium-mediated transformation events were selected and grown to maturity under restricted conditions in the transformation and greenhouse laboratories at the University of Nebraska, Lincoln (UNL). Seeds from transgenic line 485-10 were propagated through natural self-crossing in the UNL greenhouses beginning in 2003 and homozygous lines were identified. Transgenic lines were taken through a total of six generations between the years 2004 and 2006. Following harvest in 2004, seed lots (20 seeds per lot) were shipped from UNL to the University of North Carolina, Charlotte (UNCC), under a restricted USDA license. Seeds were then stored in brown envelopes at UNCC under ambient laboratory conditions (35% relative humidity, 21–24 °C) until use in 2008. T6 seeds were shipped from UNL to UNCC in 2006 and also stored under ambient conditions. In 2008, T6 seeds were germinated and taken to maturity in a growth room at UNCC. T7 seeds were harvested in 2008 approximately 1 month prior to experimentation for comparison with seeds harvested in 2004.

**Preparation of soymilk using various methods**

Soymilk was prepared using one of three methods: laboratory extraction, solvent extraction or machine extraction. Unless indicated otherwise, the laboratory extraction method was used for all experiments. For routine laboratory extraction of soymilk, soybean seeds were ground to a fine powder using a coffee mill and mixed with either phosphate-buffered saline (PBS) or soy extraction buffer (SEB; 25 mmol L−1 Tris-HCl, pH 8.0, 1 mmol L−1 ethylenediaminetetraacetic acid (EDTA), 10 mmol L−1 β-mercaptoethanol, 0.1% sodium dodecyl sulfate (SDS) and 0.1% Triton X-100) using a 3:1 buffer-to-seed powder ratio. Samples were then sonicated for 15 s × 3 pulses using a Sonics Vibrio cell ultrasonic processor (Sonics & Materials Inc., Newton, CT, USA) and clarified by centrifugation (16 000 × g for 15 min at 4 °C) to remove insoluble debris. For laboratory extraction of seed chips, thin slivers of seed were cut with a razor blade and processed as described above. For soymilk preparation using the solvent extraction method, seed powder was incubated with 10 volumes of hexane at 65 °C for 10 min with occasional rocking. The extraction process was repeated two additional times using fresh hexane. Seed material was collected by centrifugation (1200 × g for 5 min at 23 °C) and washed three times in 100% ethanol. Defatted seed material was dried for 30 min in a Centrivap concentrator (Labconco, Kansas City, MO, USA) at 60 °C and then used to prepare defatted soymilk using the laboratory extraction method. For soymilk preparation using the machine method, 80 g soybean seeds were soaked in tap water for 16 h and processed with 0.8 L water in a Hurricane Soymilk Maker (www.soynkits.com, Springfield, UT, USA). Following the 15 min cycle, machine-prepared soymilk was cooled to room temperature and filtered through four layers of cheesecloth to remove insoluble debris. For protein determination, soymilk preparations were diluted 1:15 with PBS, and 10 μL of diluted samples were incubated with 200 μL of Bradford Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA, USA) for 5 min at room temperature. Serial dilutions (0–1.0 mg mL−1) of purified BSA (Sigma-Aldrich, St Louis, MO, USA) were also incubated with the Bio-Rad dye reagent for 5 min. The optical density of duplicate samples was measured at 595 nm and the average optical density of each BSA standard was plotted as a function of protein concentration to generate a linear curve. The total protein concentration in soymilk preparations was then determined by extrapolation from the standard curve.

**Densitometry analysis**

Quantification of FanC in transgenic T2 seeds was carried out by densitometry using Quantity One 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA, USA). Total soluble seed protein was subjected to western blot analysis using rFanC as a standard. X-ray films were scanned using the Gel Doc 2000 imaging system (Bio-Rad Laboratories), followed by densitometry analysis according to the manufacturer’s directions. Based on analysis of pixel volume of known standards and unknown samples, unknown quantities were extrapolated from the generated linear regression curve. Densitometry measurements were performed in triplicate.

**Dehydration of soymilk**

Soymilk preparations were adjusted to a concentration of 18 mg mL−1 and 1 mL aliquots were placed in 15 mL conical tubes, covered with parafilm (perforated with small holes) and dehydrated in a Centrivap concentrator for up to 4 h. Immediately following dehydration, one set of aliquots were returned to their hydrated form using 1 mL dH2O, while the other sets were stored at 23, 37 and 45 °C for 7 days prior to rehydration.

**Heat treatment of transgenic soymilk and ground seed**

Aliquots of soymilk (200 μL) at a concentration of 0.2 mg mL−1 were placed in 1.5 mL Eppendorf tubes, covered with one drop of mineral oil (to prevent evaporation), capped and placed inside a boiling water bath. Aliquots were removed every 10 min and placed on ice. For toasting, seed powder was spread evenly in a glass dish and placed inside a 74 °C dry heat incubator. A portion of the material was removed after toasting for 0.3, 1.5, 6 and 20 h and...
allowed to cool to 23 °C. Soymilk was prepared from the toasted seed powder and protein was quantified using the Bradford assay. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were performed using either 10 µg protein (boiling experiment) or 5 µg protein (toasting experiment).

Partial purification of soybean-derived FanC
Separation of the 7S, 11S and whey protein fractions in soymilk was completed using an isoelectric fractionation method described by Thanh and Shibasaki.13 Total soluble protein was prepared from solvent-treated powder by incubating with buffer containing 300 mmol L⁻¹ Tris-HCl (pH 8.0) and 10 mmol L⁻¹ β-mercaptoethanol (20:1 buffer to powder ratio) at 23 °C for 1 h. Following centrifugation (10 000 × g for 20 min at 4 °C) the soymilk was transferred to a new tube and the pH was adjusted to 6.4 using 2 mol L⁻¹ HCl to precipitate the 11S fraction. The 11S fraction was collected by centrifugation (10 000 × g for 20 min at 4 °C) and the pH of the supernatant was adjusted to 4.8 with 2 mol L⁻¹ HCl to precipitate the 7S fraction, which was also collected by centrifugation (10 000 × g for 20 min at 4 °C). The 11S and 7S pellets were resuspended in 2 mL of 300 mmol L⁻¹ Tris-HCL (pH 7.8) and the pH of the whey supernatant was raised to 8.0. Protein concentrations were determined using the Bradford assay, and 20 µL protein from each fraction was subjected to SDS-PAGE and western blot analysis.

For salt precipitation, finely ground ammonium sulfate powder was added to 10 mL soymilk over a 5 min period to bring the final salt concentration to 56 mg mL⁻¹ (10%). The solution was allowed to rock for 30 min and precipitated proteins were collected by centrifugation (12 000 × g for 15 min at 4 °C). The volume of the supernatant was determined and ammonium sulfate powder was added to bring the total salt concentration to 114 mg mL⁻¹ (20%). The above steps were repeated three times, adding ammonium sulfate powder to fractions that precipitated proteins at 156 mg mL⁻¹ (30%), 183 mg mL⁻¹ (40%) and 204 mg mL⁻¹ (50%). Precipitated proteins were resuspended in PBS to 10 mL, and 20 µL of each fraction was subjected to SDS-PAGE and western blot analysis.

Protease digest of FanC
Protease (0.00375 U, Sigma, St Louis, MO, USA) was added to 75 µL PBS containing 0.6 µg rFanC. Aliquots (15 µL) were removed at 2, 4, 6, 8 and 15 min time points, mixed with SDS-PAGE loading buffer, boiled for 10 min and subjected to SDS-PAGE and western blot analysis.

Western blot analysis
Seed proteins were separated in 12% SDS-PAGE and transferred in 10 mmol L⁻¹ CAPS buffer (pH 11) to Immobilon-P membrane (Millipore, Bedford, MA, USA) as previously described.12 Membranes were blocked overnight at 23 °C in PBS containing 50 g L⁻¹ non-fat powdered milk. Rabbit anti-FanC serum (1:5000) was added to fresh block solution and incubations were carried out for an additional 2 h at 23 °C. Membranes were washed three times in PBS containing 0.05% Tween (PBST) for 10 min at 23 °C. Membranes were then incubated with a goat anti-rabbit immunoglobulin antibody conjugated with horseradish peroxidase (Cell Signaling Technology, Danvers, MA, USA) in block solution. Following three additional washes in PBST, immunodetection was carried out using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) and bands were visualized with Blue Devil film (Genesee, San Diego, CA, USA).

RESULTS
Characterization of FanC in transgenic soybeans stored more than 4 years at ambient temperature
In 2004, the FanC model antigen was expressed in soybean using the constitutive CaMV 35S promoter, and two independent transgenic lines (485-1, 485-10) were shown to accumulate FanC to levels of approximately 4 ng µg⁻¹ of total soluble seed protein in both leaves and seeds.12 T1 and T2 seeds from these lines were harvested in 2004 and 20 seeds from each transgenic line were stored in paper envelopes at 23 °C during the interim. Seeds of individual lines were taken to homozygosity and propagated through a total of six generations to create a substantial (kg) supply of seed for use in these experiments. To examine whether FanC was still present in transgenic seeds following long-term storage, seed chips from 2004 T2 seeds, and T7 seeds harvested the month prior, were subjected to confocal microscopy using immunofluorescence to detect and visualize FanC protein. Nucleic acids (ds DNA) within the chips were counterstained with DAPI. Figure 1(A) shows fluorescence in 2004 seeds chips and 2008 seed chips, but not in the non-transgenic controls. The abundance and
distribution of fluorescent signal were consistent with expression and accumulation of transgenic FanC targeted to the cytoplasm. The similarity of signal intensity between the 2004 and 2008 material suggested that a similar level of FanC immunogenic epitopes was still present in seeds that were stored for more than 4 years.

To determine whether FanC epitopes remained intact following long-term storage, seed protein extracts were generated from individual T2 seeds and proteins were subjected to SDS-PAGE and western blot analysis. Figure 1(B) shows that a 18.5 kDa protein, consistent with the predicted size of FanC, was recognized by polyclonal antibodies raised against recombinant FanC. Notably, there were no fragments smaller than 18.5 kDa in size that would have been diagnostic of FanC degradation. To determine whether protease degradation of FanC could be detected in these assays, rFanC was incubated with a protease cocktail in a time course experiment. As shown in Fig. 1(B) (right panel) protease digestion of rFanC resulted in the disappearance of the full-length protein with a concomitant increase in proteolytic fragments over time. The fact that protease-degraded FanC was not detected in seeds stored for more than 4 years demonstrates the long-term stability of FanC within the context of native seed storage proteins.

To measure the level of FanC in seeds stored for more than 4 years, T2 seed protein extracts were quantified against known amounts of rFanC protein standard. Figure 1(C) reveals that 4 µg soymilk extract contained ~18 ng FanC, translating to approximately 4 ng µg⁻¹ of total soluble protein. The fact that the rFanC migrates slightly slower than soy-derived FanC is attributed to the 6× histidine tag present on the rFanC standard. The bands in Fig. 1(C) were further quantified by densitometry (Fig. 1(D)), which revealed that the percentages of FanC in 2, 4, and 8 µg samples of soymilk were 8, 14 and 34 ng, respectively. These quantification and densitometry results further confirmed that seeds stored for more than 4 years maintained levels of antigen that were previously reported (~4 ng µg⁻¹) with no significant degradation or loss of immunogenic epitopes.

**Stability of FanC following formulation into soymilk**

Soymilk is routinely prepared in our laboratory using brief sonication in a mild buffer followed by centrifugation to remove insoluble debris. Since commercial processing involves solvent extraction, mechanical disruption and heat, we prepared soymilk formulations using commercial conditions and examined these formulations for stability of the FanC antigen. Figure 2(A) outlines the solvent and machine extraction methods that were mimicked. The western blot in Fig. 2(B) (top panel) shows that FanC is stable when exposed to heat, mechanical extraction and extraction with hexane and ethanol. Furthermore, FanC degradation products were not detected on any of the western blots, demonstrating that FanC remains intact when formulated into soymilk using large-scale commercial conditions. Following western blot analysis, the membrane was stained with Coomassie Blue to verify protein loading and visualize protein compositions in the different formulations. With the exception of a 22 kDa protein present in machine-extracted soymilk, the overall protein composition appeared to be relatively similar (Fig. 2(B), bottom panel).
revealed the disappearance of endogenous soymilk proteins with increased boiling time (bottom panel). Despite the degradation of native milk proteins, FanC remained intact and immunogenic, as demonstrated by antibody binding in the western blot analysis.

To examine the effects of dry heat on the FanC antigen, soymilk formulations were prepared from soy powder that was toasted at 74 °C for up to 20 h. As shown in Fig. 4(C) (top panel), toasting at 74 °C had no detrimental effect on the FanC antigen. Following overexposure of the western blot signal, FanC degradation products could still not be detected (middle panel). In contrast to the boiling experiment where degradation of endogenous soymilk proteins could be visualized on Coomassie Blue-stained membranes, degradation of soymilk proteins following toasting at 74 °C for up to 20 h was not detected (bottom panel). Thus FanC appears to be a robust antigen that would likely survive formulation using current large-scale processing methods.

Stability of FanC in lyophilized and rehydrated soymilk formulations

The ability to lyophilize a soymilk vaccine for reconstitution at a later time would be an extremely attractive feature for advanced vaccine formulations. To examine whether FanC remains stable following dehydration and rehydration, transgenic soymilk was prepared using various extraction methods, and aliquots of each were lyophilized as outlined in Fig. 5(A). Following lyophilization, one set of samples was rehydrated with water, while other sets were stored for 1 week at various elevated temperatures prior to rehydration. The samples prepared using the machine extraction method had an appearance similar to powdered milk purchased in a grocery store, and was immediately rehydrated with water. Samples prepared using the laboratory extraction took 2–3 min to go back into solution, while lyophilized samples prepared from solvent-extracted soy powder required brief sonication. As shown by western blot analysis in Fig. 5(B–D), FanC remained stable following lyophilization and storage at elevated temperatures, demonstrating the feasibility of a soymilk dried powder vaccine formulation.

FanC is stable following overseas shipment of transgenic seed powder and soymilk in the absence of refrigeration

To characterize the stability of FanC following transportation in the absence of a cold chain, aliquots of transgenic seed powder and soymilk formulations were placed in 0.5 mL Eppendorf tubes and shipped in a padded envelope to Chandigarh, India, as outlined in Fig. 6(A). As controls for this experiment, aliquots of transgenic seed powder and machine-derived soymilk were stored at 4 °C in our laboratory. Following 30 days in transit, both transgenic soy powder and soymilk appeared visually identical to the samples stored at 4 °C. Furthermore, the shipped transgenic soymilk samples did not exhibit signs of spoilage, such as curdling or foul odor. Soymilk was prepared from the shipped and stored soy powder samples, and the stability of FanC was analyzed using western blot analysis as shown in Fig. 6(B). Shipment of transgenic soy powder or soymilk formulations in the absence of refrigeration did not affect FanC stability, demonstrating the flexibility of the soybean platform with respect to storage and shipment.

DISCUSSION

Factors such as temperature, moisture, pest pressure, storage system and rotation are all critical factors for maintenance of...
seed protein extractability, end-product appeal (color, flavor, texture) and protection from microbial infections. Of these factors, moisture (relative humidity, RH) and temperature appear to be most important with respect to the quality of soybeans during long-term storage.\textsuperscript{14–16} For example, Lui et al. showed that under adverse storage conditions (88% RH, 20 °C) the protein content in crude soy flour, defatted soy flour and soy protein isolate decreased by up to 50% during storage over an 8-month period. In contrast, storage under mild conditions (55% RH, 20 °C), cold conditions (55% RH, 20 °C) and/or uncontrolled ambient garage conditions (60–88% RH, 7–31 °C) resulted in little to no loss of protein in the same preparations.\textsuperscript{17} In other studies Hou and Chang showed that soybeans stored for up to 18 months under mild, cold, or ambient conditions did not exhibit significant changes in the structural characteristics of glycinin and β-conglycinin seed storage proteins.\textsuperscript{18,19} In the present study we demonstrate that a transgenic seed-accumulated protein remains stable and intact following storage for 52 months under controlled ambient laboratory conditions (\textasciitilde35% RH, 21–24 °C). Furthermore, native seed proteins derived from 2004 seeds appeared virtually identical to seed proteins derived from 2008 seeds when visualized on SDS-PAGE gel blots stained with Coomassie Blue (data not shown). Our findings are therefore consistent with those previously reported for soybean storage proteins and further demonstrate that soybeans
appear to provide a favorable environment that can sustain protein stability for extended periods of time when stored under non-adverse conditions.

There have been several reports in the literature regarding the stability of transgenic immunomodulatory proteins in other crop seeds following storage at ambient temperature. Using wheat, Stoger et al. reported stability of a single-chain antibody for 5 months in dried seed, while Nochi et al. reported stability of the cholera toxin B subunit antigen for 18 months in rice seed. Using a corn-based system, Lamphear et al. reported that the transmissible gastroenteritis virus antigen was stable for 10 months, while Moravec et al. reported that the B subunit of heat-labile toxin remained stable for up to 3 months in soybean seeds. In the present study we demonstrate that transgenic FanC remains stable and intact following storage for more than 4 years under ambient laboratory conditions. To our knowledge, this is the longest time period that any transgenic seeds have been stored and then analyzed for relative stability and abundance of a transgenic vaccine antigen. The fact that FanC and other antigens can be stored for long periods of time under ambient conditions may be attributed to relatively favorable environments within seeds (high protein, natural antioxidants and stabilizers, low moisture, etc.).

It is critical in any vaccine protocol that processing methods do not affect the immunogenicity of an antigen. For this reason, most efficacy studies involving plant-derived vaccine formulations have utilized native unprocessed material. For agricultural feeding purposes unprocessed formulations may be acceptable, but depending on the system, processing to a palatable product may be essential. For example, raw potatoes were used in the first successful plant-based vaccine clinical trials in humans. In efforts to create more palatable formulations, potato tuber slices expressing the hepatitis B antigen were boiled for 10 min; however, such treatment significantly reduced the immunogenicity of the formulation. Other fruits and vegetables (e.g. bananas, tomatoes, lettuce) can be consumed in raw form and have been used to express heterologous proteins, but in the absence of additional downstream processing these products would have short shelf-lives and would require refrigeration during storage and shipment.

Soybeans can be easily processed into soymilk by a number of methods, and can be packaged for storage without refrigeration. To demonstrate that transgenic soybean is practical for the formulation of subunit vaccines, we compared our unheated laboratory extraction method to common methods used in the processing industry involving solvent extraction (hexane and ethanol) and mechanical disruption (soymilk machine). Subjecting soymilk to such processing conditions resulted in a stable product with no evidence of FanC degradation. Soy milk machines are very simple to use and would be advantageous for areas where on-site preparation of vaccines is necessary; the end-product would not require purification and could be consumed immediately. Also, defatted soymilk prepared using the solvent extraction method has a longer half-life than fatted soymilk. Soy powder could therefore be stored or shipped without refrigeration, and preparation of soymilk vaccines could be made by hand, even in the absence of electricity.

Heat treatments are routinely included in soy processing to eliminate native anti-nutritional factors and increase shelf-life of the product by removal of microorganisms. We subjected soy-derived FanC to several types of heat treatments to determine its stability and potential for processing under multiple conditions. FanC showed no signs of degradation when subjected to dry heat following solvent extraction (Fig. 3(B)), boiling (Fig. 4(B)) or toasting (Fig. 4(C)). The resistance to heat will likely be a unique property of each transgenic protein and therefore may not be applicable to every vaccine candidate. FanC is an adhesion protein on an enteric pathogen, so it is logical to reason that FanC may be more stable in harsh conditions relative to other vaccine candidates. Our lab recently tested the heat stability of a seed-derived antigen to a non-enteric pathogen and found it was stable at 85 °C for up to 15 min (Piller KJ, unpublished results). Future soy-derived antigens that may not be stable under boiling or toasting conditions could be processed using an alternative method that does not require heat, such as a large-scale version of the laboratory extraction method. Given the high protein content of soybean seeds and current expression levels of foreign antigens, typical doses of antigen could be administered in as little as 1 mL of processed soymilk. Thus, even if antigen stability is reduced 10-fold by processing with heat (or alternatively accumulates to levels below 10 ng µg⁻¹ of total soluble seed protein as is the case with FanC), a 10-fold larger dose of soymilk (10 mL vs. 1 mL) would...
still be viewed as practical for administration. In such cases, there should not be adverse host reactions to ingestion of such small amounts (10 mL) of unprocessed soymilk, although this concept remains to be investigated.

The generation of milligram quantities of transgenic protein in a seed is quite attainable, considering the high protein content of soybean seed. Thus, fractionation or purification steps would probably not be necessary for soy-based vaccine formulations. However, if there was a need for purification, cost-effective methods could be employed to generate more pure, concentrated or standardized end-product. To determine the stability of soy-derived FanC to fractionation methods, we subjected transgenic soybean seed to a fractional isoelectric precipitation13 and ammonium sulfate precipitation. Though the latter method only allowed for a threefold concentration of total soymilk protein concentration, the former resulted in the purification of FanC away from the major 7S and 11S storage proteins, ultimately yielding a fivefold purification of FanC. The stability of soy-derived FanC recovered in the end-products following both these methods demonstrates the flexibility of the soy-based vaccine platform.

It is estimated that 50% of the vaccines manufactured annually are wasted due to deviations in storage requirements and other handling problems32,33 adding to the overall cost of vaccine manufacturing. These high costs along with the requirement for refrigeration are known prohibitive factors in the availability of vaccines for developing countries.3 A soy-based vaccine delivery system can provide an alternative method for storage and transportation in the absence of cold chain requirements. To demonstrate the feasibility of this concept we shipped transgenic soybean powder and soymilk to India and back to the USA for 30 days with no climate control. Although we anticipated the soy-derived FanC in seed powder would withstand shipping, it was a surprise to find that FanC was also stable in shipped transgenic soymilk formulations. It is likely that the elevated temperature used in the machine extraction method functioned to pseudo-pasteurize the soymilk, while shipment in small autoclaved tubes likely reduced microbial growth.

One can envision that a processed soymilk vaccine could be lyophilized, packaged into individual doses, transported and stored without refrigeration, and then reconstituted on site when needed. We tested the feasibility of this concept and found that transgenic soymilk formulations could indeed be lyophilized, stored at various temperatures, and instantly reconstituted using water, with no adverse effect on the FanC antigen. We are currently exploring the long-term storage effects on lyophilized transgenic soymilk and the ability of reconstituted powder to induce anticipated immune responses in mice following oral administration.

Although soymilk and soy products are generally recognized as safe and well tolerated by most individuals, a small portion of the population (~0.5% in the USA) may not tolerate soy-based formulations due to soybean allergies.34 Since allergens in soybean seeds have been identified, and due to the widespread use of soy products in processed foods, researchers have been developing novel ‘non-allergenic’ soybean lines35 void of soy allergens. Thus it is feasible that traditional laboratory soybean lines expressing a vaccine antigen could be crossed into novel soybean backgrounds that are allergen-free. Furthermore, due to high protein content and foreign transgene expression levels in soybean seeds, a soymilk platform could be used as a bioreactor to produce large amounts of vaccine antigen that could then be purified (e.g. Figure 3) and supplemented into non-soy-based formulations for administration to individuals with soybean allergies.

The production of vaccines in commercial food crops poses certain risks that can be managed and kept to a minimum. Since soybean is a self-pollinating crop, there is little chance for pollen drift and outcrossing generally associated with non-self-pollinating crops. Because of the potential for high antigen yields in seeds, transgenic plants will never need to be grown outdoors in a field, and instead can be propagated in small, enclosed greenhouse systems that are strictly confined and strategically located away from farming regions. To exemplify the enormous potential for antigen production in the soybean system, a 1-acre footprint from an enclosed greenhouse facility can produce 18.6 × 106 g of vaccine antigen (60 Bu × 27.2 kg Bu⁻¹ × 0.38% protein × 3 µg g⁻¹ target vaccine expression) or more than 18 million doses (1 mg per dose) of a vaccine. The 120- to 150-day maturation cycle of soybean would allow for multiple harvests each year, resulting in nearly 50 million doses of vaccine antigen that could be produced within a small, tightly regulated area. Following harvest, seeds could be crushed to render them nonviable, and then processed and formulated within the same facility. In such a scenario, transgenic seeds expressing a vaccine antigen would never need to leave a growth and processing facility.

The results of this present study demonstrate that a model antigen expressed in soybean seeds remains stable when stored for more than 4 years, and processed using commercial methods that employ a variety of temperature conditions. Furthermore, we also demonstrated the practicality of transportation in the absence of the cold chain by shipping transgenic soybean seed powder and transgenic soymilk overseas and back to the USA with no loss in antigen stability. Collectively, these results further support the feasibility of a soy-based vaccine platform for the development of novel and practical vaccines that are cost-effective, stable, easily formulated and shipped worldwide without a requirement for refrigeration.

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