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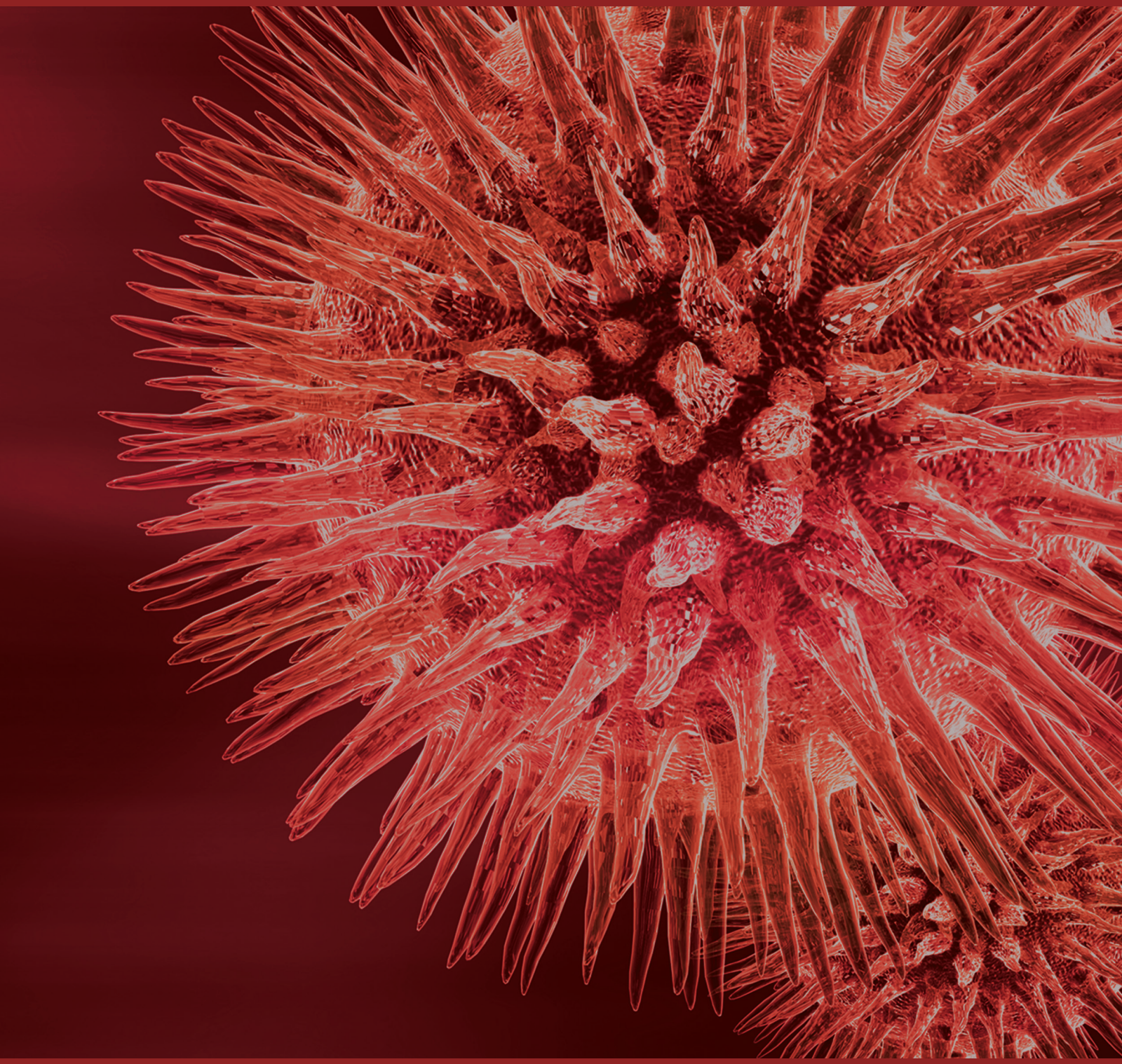
Plant-Made Biologics

Guest Editors: Qiang Chen, Luca Santi, and Chenming Zhang



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Editorial
Plant-Made Biologics

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The increasing world demand for human biologics cannot be met by current production platforms based primarily on mammalian cell culture due to prohibitive cost and limited scalability [1]. Recent progress in plant expression vector development, downstream processing, and glycoengineering has established plants as a superior alternative to biologic production [2–4]. Plants not only offer the traditional advantages of proper eukaryotic protein modification, potential low cost, high scalability, and increased safety but also allow the production of biologics at unprecedented speed to control potential pandemics or with specific glycoforms for better efficacy or safety (biobetters) [5, 6]. The approval of the first plant-made biologic (PMB) by the United States Food and Drug Administration (FDA) for treating Gaucher's disease heralds a new era for PMBs and sparks new innovations in this field [7, 8].

This special issue aims to showcase the recent developments and application of PMBs in areas of plant host systems, expression vectors, novel vaccine candidates, glycoengineering and posttranslational modification, and economic impact and evaluation. Eight original research and review articles among submissions are selected for this special issue.

Manufacturing costs are a prime determinant of the market acceptability, availability, and profitability of the product for its manufacturer. One of the potential traditional advantages of plant-based systems is their ability to lower the production cost of recombinant biologics. Lower manufacturing costs have been widely assumed as an innate feature of plant-based production platforms because they forego the need for capital investments to build sophisticated cell culture facilities and expensive culture media for biomass generation. However, information on the actual costs of producing PMB at industrial scale is not readily available and reports of serious studies in this area are scarce in the scientific literature. Thus, accurately documenting such an advantage is crucial for plant-based systems to be recognized as a serious platform for manufacturing protein biologics. Tusé et al. provided such an important study in this issue. They reported two case studies on plant-made enzymes. One focused on human butyrylcholinesterase (BuChE) produced in greenhouse-cultivated *Nicotiana* plants for use as a medical countermeasure and the other on cellulases produced with plants grown in the field for ethanol production as a fuel extender. Using reported data and SuperPro Designer modeling software, the authors examined process unit operations and estimated the bulk active product and per-dose or per-unit costs. Their analyses demonstrate that a plant-based platform can substantially reduce the cost of these enzymes compared with traditional platforms. For example, the unit production costs for the plant-made BuChE are calculated to be approximately \$234 or \$474 per dose, respectively, dependent on whether or not facility dependent costs are included in the estimation. This is in stark contrast to the ~\$10,000/dose production cost estimated for blood-derived BuChE. Similarly, the study concludes that for the cellulase enzyme, using the plant-based system may result in a >30% reduction in unit production costs and an 85% reduction in the required capital investment compared with the current fungal-based system. The authors did caution that the cost advantages of PMBs are molecule/product-specific

and dependent upon the cost improvement of alternative production platforms. This report presents case studies of PMBs for diverse applications and provides urgently needed technoeconomic evaluations of the current PMB platform.

Two of the original research papers report on the development of plant-made vaccines against infectious diseases. He et al. report their findings of using the domain III (DIII) of West Nile virus (WNV) envelope protein as a vaccine candidate for WNV. They found that *N. benthamiana* plants could produce this antigen efficiently. They also showed the advantage of plant-derived DIII in downstream processing; unlike the insoluble WNV DIII produced in *E. coli*, plant-derived DIII is soluble and readily enriched to high purity without the need for denaturing and refolding. Furthermore, plant-produced DIII was shown to evoke a potent DIII-specific humoral response in mice. No vaccine against WNV is currently available for human use and this study presents an effort towards developing efficacious vaccines against this virus. Another paper in this category investigated the possibility of developing a plant-based vaccine against norovirus Narita 104 (Na) using virus-like particles (VLPs) assembled from the capsid protein (NaVCP). The results showed that expression of NaVCP caused severe leaf necrosis that limited its accumulation in plants. However, plant-produced VLPs were observed by microscopy and induced mucosal and serum antibody responses in mice when delivered intranasally. The authors proposed that Narita 104 VLPs could be a component of a multivalent subunit vaccine.

Downstream processing of target proteins represents a major cost for the overall cost of goods in PMB production. Therefore, reducing cost of PMB extraction and purification will facilitate the commercialization of plant-based production platform and products. A paper in this issue reports an alternative way to extract protein from plants. Instead of homogenization of plant tissue, the authors used a technique called vacuum infiltration-centrifugation (VI-C) to isolate recombinant proteins that are targeted for secretion. Their results indicated that three rounds of VI-C recovered 97% of the secreted proteins accessible to the procedure. While the VI-C procedure was successful for a truncated E1 endoglucanase, the full length E1 enzyme was not recovered as efficiently by the same procedure, indicating the method's limitation on the size of target proteins or the need for technical optimization. However, this study does represent an alternative downstream process for recovering secreted proteins from plant tissue (apoplast) that can potentially drastically reduce the production cost.

Several review articles are also included in this special issue. Two of them discuss the new plant hosts and expression strategies for PMB production and compare them with other alternative manufacturing systems. Hudson et al. report the use of soybean seeds for the expression of a nontoxic form of *S. aureus* enterotoxin B (mSEB). As a natural protein source, soybean seeds allow for an extended storage time under ambient conditions and, thus, can facilitate a more flexible processing schedule. The study results demonstrated an impressive production of ~76 theoretical doses of human vaccine per single soybean seed. Merlin et al. present a comprehensive review of different production strategies applied to four well-characterized, yet very diverse PMBs. The authors emphasize that plant-based production platforms represent a whole array of different strategies that need to be carefully evaluated, in terms of not only mere product yield, but also product quality, production scalability, costs, and cGMP compliance. For a given PMB, the optimal pairing of a plant production host with the most appropriate expression and/or downstream processing strategy often determines its ultimate success. The four case studies center on four different classes of biologics: (i) human glutamic acid decarboxylase (hGAD65), a promising candidate for treating autoimmune type 1 diabetes, (ii) Norwalk VLPs assembled from the Norwalk virus coat protein VP1 for vaccine development, (iii) monoclonal antibody (mAb) 2G12, an anti-HIV-1 human IgG1, a potential human therapeutics against HIV, and (iv) human interleukin-6 (hIL-6), a secreted glycoprotein belonging to the cytokine family. Production of these biologics with various plant systems and expression strategies is examined. The plant systems range from leaf-based production (tobacco, *N. benthamiana*, lettuce, and *Arabidopsis*) to seeds (tobacco, *Arabidopsis*, maize, and petunia), fruits (tomato), and tubers (potato), with both stable and transient expression approaches. Moreover, the production of the fourth-mentioned biologics in plant systems is compared with that of traditional fermenter-based systems such as *E. coli*, yeast, mammalian, and insect cells. The authors highlight the advantages of plant-based systems over fermenters, particularly for certain niche markets. They conclude that plant-based platforms are most beneficial for the production of biologics that require high quantity, rapid production speed, complex post-translational modifications, or oral delivery.

Recent vector development for PMBs is also discussed. Recognizing the potential pitfalls of recombinant protein production in stably transformed plants, a concise review on the novel transient expression systems based on the use of plant virus expression vectors is presented. The author concludes that transient expression systems can overcome the challenges associated with systems based on transgenic plants such as low protein accumulation and long development time, and they can reduce the potential risk of transgene spread from transgenic plants to other plants in the environment and thus alleviate the public concerns.

Overall, the papers in this special issue highlight the recent progress in the PMB field. It is our hope that these papers will provide pertinent information for not only the PMB community, but also the overall scientific and business community for the further consideration and acceptance of plant-based systems as a viable platform for the development and manufacture of human biologics.

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Research Article

Manufacturing Economics of Plant-Made Biologics: Case Studies in Therapeutic and Industrial Enzymes

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Production of recombinant biologics in plants has received considerable attention as an alternative platform to traditional microbial and animal cell culture. Industrially relevant features of plant systems include proper eukaryotic protein processing, inherent safety due to lack of adventitious agents, more facile scalability, faster production (transient systems), and potentially lower costs. Lower manufacturing cost has been widely claimed as an intuitive feature of the platform by the plant-made biologics community, even though cost information resides within a few private companies and studies accurately documenting such an advantage have been lacking. We present two technoeconomic case studies representing plant-made enzymes for diverse applications: human butyrylcholinesterase produced indoors for use as a medical countermeasure and cellulases produced in the field for the conversion of cellulosic biomass into ethanol as a fuel extender. Production economics were modeled based on results reported with the latest-generation expression technologies on *Nicotiana* host plants. We evaluated process unit operations and calculated bulk active and per-dose or per-unit costs using SuperPro Designer modeling software. Our analyses indicate that substantial cost advantages over alternative platforms can be achieved with plant systems, but these advantages are molecule/product-specific and depend on the relative cost-efficiencies of alternative sources of the same product.

1. Introduction

This study represents original research on the manufacture of plant-made biologics (PMB) and plant-made industrial products (PMIP) through application of analytical modeling tools *in silico*. The main goal of this study was to evaluate unit operations in two plant-made biomanufacturing processes and estimate the cost of goods of the active ingredient (AI) and the impact of those costs on the cost of the final product. A secondary but equally important goal was to compare the manufacturing cost of plant-produced AI to the cost of the same AI manufactured by predecessor technologies.

Much progress has been made towards the development of manufacturing infrastructure for plant-made pharmaceuticals (PMP), which typically consist of recombinant proteins applied as vaccine antigens, therapeutic enzymes, or monoclonal antibodies. Progress has also been made in the manufacture of plant-based biologics, biochemicals, and biomaterials for industry, food, and other applications. Significant and industrially relevant advances in gene expression and bioprocessing methods have been achieved during the past two decades, as reviewed in several prior studies [1–7]. Yet, to date, only three PMP products have been approved by regulatory agencies for commercial sale, including an anti-caries antibody (Planet Biotechnology, USA), an animal health vaccine (Dow AgroSciences, USA), and a therapeutic enzyme to manage a metabolic disorder (Protalix Biotherapeutics, Israel) [5]. This relative scarcity of PMP products reflects the magnitude of the challenges in creating a new manufacturing industry. The development of the plant-based platform has slowly progressed through a multinational "labor of love" in the absence of the levels of investment originally made by the biopharmaceutical industry (with significant help from the venture capital community), which resulted in elevation of fermentation-based systems to their current level of dominance.

Interestingly, beginning in 2009, the US Defense Advanced Research Projects Agency's (DARPA) Blue Angel program made several multimillion dollar investments at various sites with the goals of accelerating the scale-up of the PMP infrastructure and assessing production of relevant volumes of pandemic influenza candidate antigens as a model product to test the plant-based platform (http://www.darpa.mil/NewsEvents/Releases/2012/07/25.aspx). This was a shared investment initiative, and as a result of federal and state government and private investments, the expanded PMB manufacturing capacity should now support production of at least several of the many plant-made vaccines, biotherapeutics, biomaterials, and biocatalysts that are under development by companies and institutions worldwide (most recently reviewed by Gleba et al. [5]). Although capacity expansion helped companies that would manufacture their own or partnered products (e.g., Caliber Biotherapeutics, Bryan, Texas, USA; Medicago Inc., Research Triangle Park, North Carolina, USA), these investments also helped expand capacity at PMP contract development and manufacturing organizations (CDMO) such as Kentucky BioProcessing (Owensboro, Kentucky, USA). This was important to our modeling because the decision to construct a new dedicated manufacturing facility versus contracting services from a CDMO could yield very different cost-of-goods projections.

Fundamental to the commercial introduction of PMB products is the availability of an efficient plant-based manufacturing infrastructure that is at a minimum competitive with and ideally superior to traditional animal cell and microbial fermentation systems as well as to extraction from raw materials from natural sources. The cost to manufacture any product is of paramount importance to its market acceptability, availability to those who need it most, and to the profitability of the product for its manufacturer. While plant-based technologies are often assumed to offer significant cost advantages relative to cell-based fermentation, such assumptions are based on the lower upstream capital investments required for plant growth, lower cost of media, no adventitious agent removal, and other factors [8–13]. However, few of these studies have listed engineering process assumptions or analyzed unit operations adequately; reports such as those of Evangelista et al. [14] and Nandi et al. [15] are exceptions. Therefore, results of recent technoeconomic evaluations for PMP/PMB/PMIP have not been widely available in the public literature.

To analyze and quantify the cost efficiency of plant-based manufacturing, we chose two enzymes representing active ingredients (AI) for diverse product classes and derived for each AI the bulk product (i.e., bulk active) and per-dose or per-unit costs. The first target analyzed is human butyrylcholinesterase (BuChE), an enzyme that can act as a bioscavenger to counteract the effects of cholinesterase inhibitors such as sarin and that is a candidate for biodefense countermeasures in several countries. While this product would encounter market dynamics that are different from other commercial products, it is nevertheless designed to satisfy an important component of public safety and merits review. Currently, BuChE is extracted from outdated human blood supplies, but it can also be made recombinantly in cell culture, transgenic animals, and plant systems.

The second case study focuses on the cellulase complex, a mixture of 4–6 enzymes used to saccharify cellulosic feedstocks for the production of ethanol as a fuel extender. This target was selected for study because, for more than 30 years, the cost of cellulases has been a major impediment to the economic viability of cellulosic ethanol programs. Cellulases were also selected because they represent an extremely cost-sensitive product class on which to conduct case studies. We reasoned that if plant-based manufacturing showed economic promise for this class, then the economically advantageous production of less cost-sensitive biotherapeutics and other products might also be anticipated. In contrast to BuChE, which consists of a purified molecule, the cellulase complex would be expressed in plants that are cultivated near the cellulosic feedstock and the bioethanol refinery and stored as silage without purification; the semidried catalyst biomass is mixed on demand with the cellulosic feedstock to initiate saccharification followed by fermentation. This approach varies significantly from previous approaches in which cellulase enzymes are produced via fermentation processes using native or engineered microorganisms. For the cellulase case study, the plant-based cellulase production process is compared with a recent technoeconomic analysis of cellulase enzymes produced from *Trichoderma reesei* fermentation using steam-exploded poplar as a nutrient source [16].

2. Materials and Methods

2.1. Modeling Software. The technoeconomic modeling for both case studies was performed using SuperPro Designer, Version 9.0 (Intelligen, Inc., Scotch Plains, NJ; http://www.intelligen.com/), a software tool for process simulation and flowsheet development that performs mass and energy balances, equipment sizing, batch scheduling/debottlenecking, capital investment and operating cost analysis, and profitability analysis. This software has been used to estimate cost of goods in a variety of process industries including pharmaceuticals produced by fermentation [17] and plant-made pharmaceuticals [14, 18]. It is particularly useful at the early, conceptual plant design stage where detailed engineering designs are not available or warranted. SuperPro Designer was chosen because it has built-in process models and an equipment cost database for typical unit operations used in the biotechnology industry, such as bioreactors, tangential flow ultrafiltration and diafiltration, chromatography, grinding/homogenization, and centrifugation. There are some unit operations and processes used in the case studies that are currently not included in SuperPro Designer, such as indoor or field plant cultivation, plant harvesting, vacuum agroinfiltration, and screw press/disintegrator. For the butyrylcholinesterase case study, SuperPro Designer's "Generic Box" (bulk flow, continuous) unit procedure was used to model these unit operations. For the cellulase case study, the indoor unit operations were modeled with the same software while the field production calculation and costs were

tracked in Microsoft Excel spreadsheets. Unless otherwise noted, the costs of major equipment, unit operation-specific labor requirements and costs (e.g., operators, supervisors), pure components, stock mixtures, heat transfer agents, power and consumables (e.g., filter membranes, chromatography resins) used in the analyses were determined using the SuperPro Designer built-in equipment cost model and default databanks. For the cellulase case study, the program's parameters such as water costs and total capital investment distributed cost factors were set to be the same as those used in the model described in Klein-Marcuschamer et al. [16]; this SuperPro Designer model is also available at the Joint Bioenergy Institute (JBEI) technoeconomic analysis wiki site (http://www.lbl.gov/tt/techs/lbnl2678.html).

Additional case study specific design parameters were selected based on experimental data from journal articles, patent literature, the authors' laboratory, interviews with scientists and technologists conducting the work cited, technical specification sheets or correlations, heuristics, or assumptions commonly used in the biotechnology and/or agricultural industry. The case study models were based on a new "greenfield" facility, operating in batch mode, although annual production costs neglecting the facility dependent costs were also determined to predict annual production costs using an existing facility. For the butyrylcholinesterase case study, annual operating time of 7920 hours (330 days, 24-hour operation, or 90% online) for the facility was used with indoor grown *Nicotiana benthamiana* plants. It was assumed that the plants would be grown continuously throughout the year (8760 hours, or 365 days, 24-hour operation, or 100% online). For the cellulase case study, since the tobacco plants are grown in the field, it is assumed that plant growth occurs for 215 days of the year (in North America, seeding begins at the end of March and final harvest is at the end of October; 59% online) and the indoor facility is in operation for 127 days per year (35% online). For comparative purposes in the cellulase case study, the laboratory/QA/QC costs were neglected since they were neglected in the JBEI model and such costs are likely to be a minor component for the industrial enzyme case study. The following items were also neglected in both case studies: land costs, upfront R&D, upfront royalties, and regulatory/certification costs as these can vary widely. SuperPro Designer files (*.spf) for the case studies can be downloaded from http://mcdonald.ucdavis.edu/biologics.html, and require SuperPro Designer software to run/view. An evaluation (demo) version of the software can be downloaded from the website: http://www.intelligen.com/downloads.html to view and run the case study files. For the butyrylcholinesterase case study, the process flowsheet was split into separate modules to better understand the contributions of various process segments.

2.2. Modeling Protocol. Process flow and unit operations were derived from published methods and results from a number of sources as indicated in each case study, and from interviews with leading gene expression, agronomy, and manufacturing scientists and engineers who have participated in the development and scale-up of the processes described. On the basis of this information, the SuperPro Designer software was applied to calculate material inputs and outputs, bulk, and per-dose or per-unit costs.

2.3. Host Plant Species Selection and Justification. The two AI classes evaluated in these studies are produced in *Nicotiana* host plants. *Nicotiana* species, notably *N. tabacum, N. excelciana,* and *N. benthamiana,* are preferred hosts for PMB manufacture due to their metabolic versatility, permissiveness to the propagation of various viral replicons, and high expression yields achievable with a wide range of targets, as reviewed by Pogue et al. [19], De Muynck et al. [20], Thomas et al. [1], Gleba et al. [5], and others. Use of these hosts for production of clinical trial materials is also familiar to FDA and other regulatory agencies, thus facilitating *Nicotiana's* acceptance in regulation-compliant manufacturing [5, 21–24].

2.4. Modeling Production of Butyrylcholinesterase

2.4.1. Product Selection and Justification. The enzyme is a globular, tetrameric serine esterase with a molecular mass of approximately 340 kDa and a plasma half-life ($t_{1/2}$) of about 12 days; the plasma $t_{1/2}$ is largely a function of correct sialylation [25, 26]. BuChE has several activities, including the ability to inactivate organophosphorus (OP) nerve agents before they can cause harm. With the recent use of chemical nerve agents such as sarin, there is continued interest on the part of many governments in stockpiling BuChE as a countermeasure. Currently BuChE is purified from outdated blood supplies; however, the high cost of this route (~$20,000 per treatment with 400 mg enzyme [27]) and its low supply limit its utility [28]. It has been estimated that extraction of BuChE from plasma to produce 1 kg of enzyme, which would yield small stockpile of 2,500 400-mg doses, might require extraction of the entire US blood supply [29]. Large amounts of the enzyme are required for effective prophylaxis because of the 1 : 1 enzyme/substrate stoichiometry needed for protection against OP agents. Not surprisingly, recombinant routes have been explored and the enzyme can in fact be produced by microbial fermentation [30], animal cell culture [31, 32], and transgenic goats [33] and stably or transiently expressed in *Nicotiana*, albeit at modest levels of 20–200 mg/kg fresh weight (FW) biomass [29, 34, 35], with yield improvements being the target of ongoing research. The bacterial product is nonfunctional and the mammalian cell culture products do not have the plasma $t_{1/2}$ needed for prophylaxis and may be difficult and expensive to scale, as discussed by Huang et al. [33]. Goat-milk produced BuChE can be obtained at 1–5 g/L milk [33], but consists mostly of dimers, is undersialylated and has short plasma $t_{1/2}$. While expression yields are impressive, transgenic animal sources face challenges of herd expansion to satisfy emergency demand, as well as potential adventitious agent issues, and these challenges need further definition. Furthermore, of these options, only plant-based biosynthesis

yields an enzyme that is sialylated (as described below) and appears to reproduce the correct tetrameric structure of the native human form in sufficient yield to be commercially attractive [29, 36]; hence, the plant-based route became the basis for our modeling exercise. Not surprisingly, the plant route for BuChE manufacture is also the subject of continued DARPA interest and support [27, 37].

2.4.2. Gene Expression Options. BuChE can be produced stably in recombinant plants or transiently in nonrecombinant plants by viral replicons delivered by agrobacterial vectors introduced into the plants via vacuum-assisted infiltration. Relative to stable transgenic plants, the advantages of speed of prototyping, manufacturing flexibility, and ease of indoor scale-up are clearly differentiating features of transient systems and explain why this approach has been widely adopted in the manufacture of many PMP (recently reviewed by Gleba et al. [5]). In our analysis of BuChE, we used expression yields from several sources that evaluated various *Agrobacterium*-mediated expression systems, including Icon Genetics' magnICON expression technology ("magnifection") [29, 34–36]. Magnifection should be familiar to most readers of this volume as it has been applied in R&D programs throughout the world and its features have been the topic of multiple original studies and reviews (see, e.g., Marillonnet et al. [38]; Giritch et al. [6]; Gleba and Giritch [39], Klimyuk et al. [4], and Gleba et al. [5]); therefore, the method is not described here in further detail. Likewise, the process of vacuum-assisted infiltration has been described in detail by Klimyuk et al. [4], Gleba et al. [5], and others and is not further explained here.

2.4.3. Plant Host and Upstream Process. For BuChE, we modeled the use of an *N. benthamiana* transgenic line modified to express the mammalian glycosylation pathway, beginning with a mutant host lacking the ability to posttranslationally add plant-specific pentoses (ΔXF) but with the ability to add galactosyl and sialic acid residues to polypeptides, based on work recently reported by Schneider et al. [36]. Use of this host obviates the need to enzymatically modify the plant-made polypeptide *in vitro* after recovery to ensure the presence of correct mammalian glycan, a procedure that could substantially increase the cost of the AI [40]. A glycan-engineered host can be produced in two ways, by stable transformation or via use of multigene agrobacterial vectors. The feasibility of sialylation via the latter approach was shown recently by Schneider et al. [36] for BuChE. Although there is an extra element of time required to develop a stable transgenic host compared to the transient modification of a pathway, the availability of a transgenic plant obviates the need to manufacture several *Agrobacterium* vectors carrying the genes for the product and two (or more) binary vectors carrying genes for the sialylation pathway; a procedure that would require additional capital and operational investments to generate multiple inocula in large scale. Therefore, for modeling upstream processes, we assumed that transgenic seed was available and that the resultant BuChE would have mammalian glycans and form tetrameric structures [29], and hence its biological activity and plasma half-life would be comparable to the native human enzyme [29, 36].

2.4.4. Downstream Purification. To model downstream purification of BuChE, we assumed harvest and extraction at 7 days after inoculation. Biomass disruption was by homogenization, followed by filtration and clarification, as generally described [28, 34], but with modifications required for scale-up as indicated in Results and Discussion. Purification of the enzyme was by procainamide affinity chromatography [28]. In the overall process, plant growth, inoculation, and product accumulation steps occur indoors in controlled environments, and extraction, clarification, and final purification of BuChE take place in classified suites, so that manufacturing and release of the enzyme can be compliant with FDA cGMP guidance for human therapeutics. Design premises for this process, specific assumptions used in modeling, and resultant cost calculations are presented (see Section 3.1, Tables 1, 2, 3, and 4, and Figures 1, 2, and 3).

2.5. Modeling Production of Cellulases

2.5.1. Product Selection and Justification. Cellulases currently under evaluation in bioethanol programs are all produced by microbial fermentation. Despite decades of research on lowering cellulase manufacturing costs, these enzymes still account for 20–40% of cellulosic ethanol production costs [41, 42]. Hence, lowering the cost of the biocatalyst is critical to the eventual adoption of biofuel processes that utilize renewable plant biomass feedstocks without competing with food or feed supplies. An alternative to fermentation-produced cellulases is the production of these enzymes in crop plants, with the ultimate goal of producing cellulases at commodity agricultural prices. This process concept was modeled to estimate enzyme and ethanol costs produced by this approach. Should such a process for cellulases prove economically viable, it might encourage the production of other cost-sensitive PMB as well as biomaterials, food additives, and industrial reagents.

2.5.2. Gene Expression Options. Scale requirements and cost limitations of cellulases for biofuel applications constrained us to model production to open fields, with minimal indoor operations. We initially surveyed two scenarios for inducing production of cellulases in field-grown plants. The first was adaptation of the typical agroinfiltration method. Nomad Bioscience (Nomad Bioscience GmbH, Halle, Germany) has reported successful substitution of the agroinfiltration step with "agrospray," a technique in which a suspension containing the *Agrobacterium* inoculant is admixed with a small amount of surfactant and sprayed onto the leaves of host plants [5, 43]. This approach eliminates the necessity to grow plants in containers (e.g., trays or carriers), a requirement imposed by the mechanics of the vacuum infiltration treatment in current procedures. Concomitantly, it also eliminates the cost of setting up and operating commercial-scale vacuum chambers, robotic tray manipulators, biomass conveyer

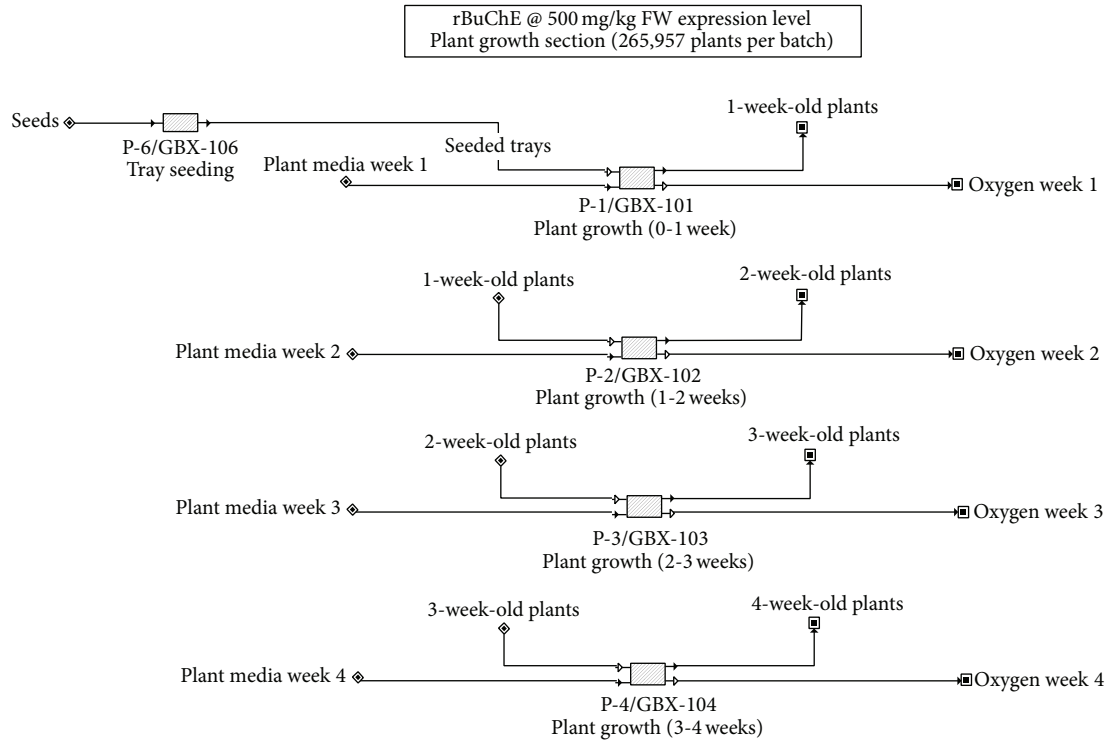

Figure 1: Indoor growth of *Nicotiana benthamiana* plants.

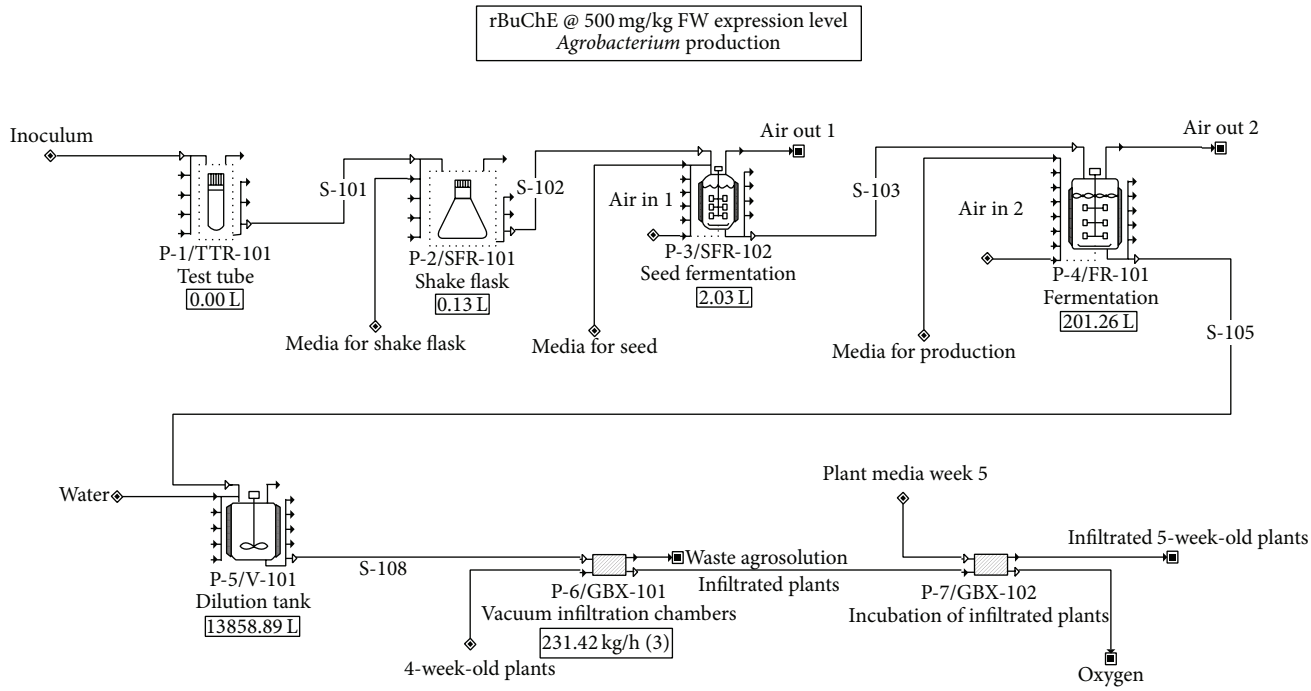

Figure 2: Agrobacterial growth, vacuum infiltration, and incubation.

systems, and so forth. Thus, this new approach should enable large-scale field inoculation of plants with agrobacteria and the production of biologics with more favorable economics. While we modeled the costs of producing cellulases via the agrospray approach, the sheer volume of enzymes needed for commercial-scale cellulosic ethanol processes necessitated a large investment in inoculum production infrastructure, including multiple fermentation trains and associated processing equipment. Further, the most efficient method of inoculating large areas was by aerial spraying, a procedure

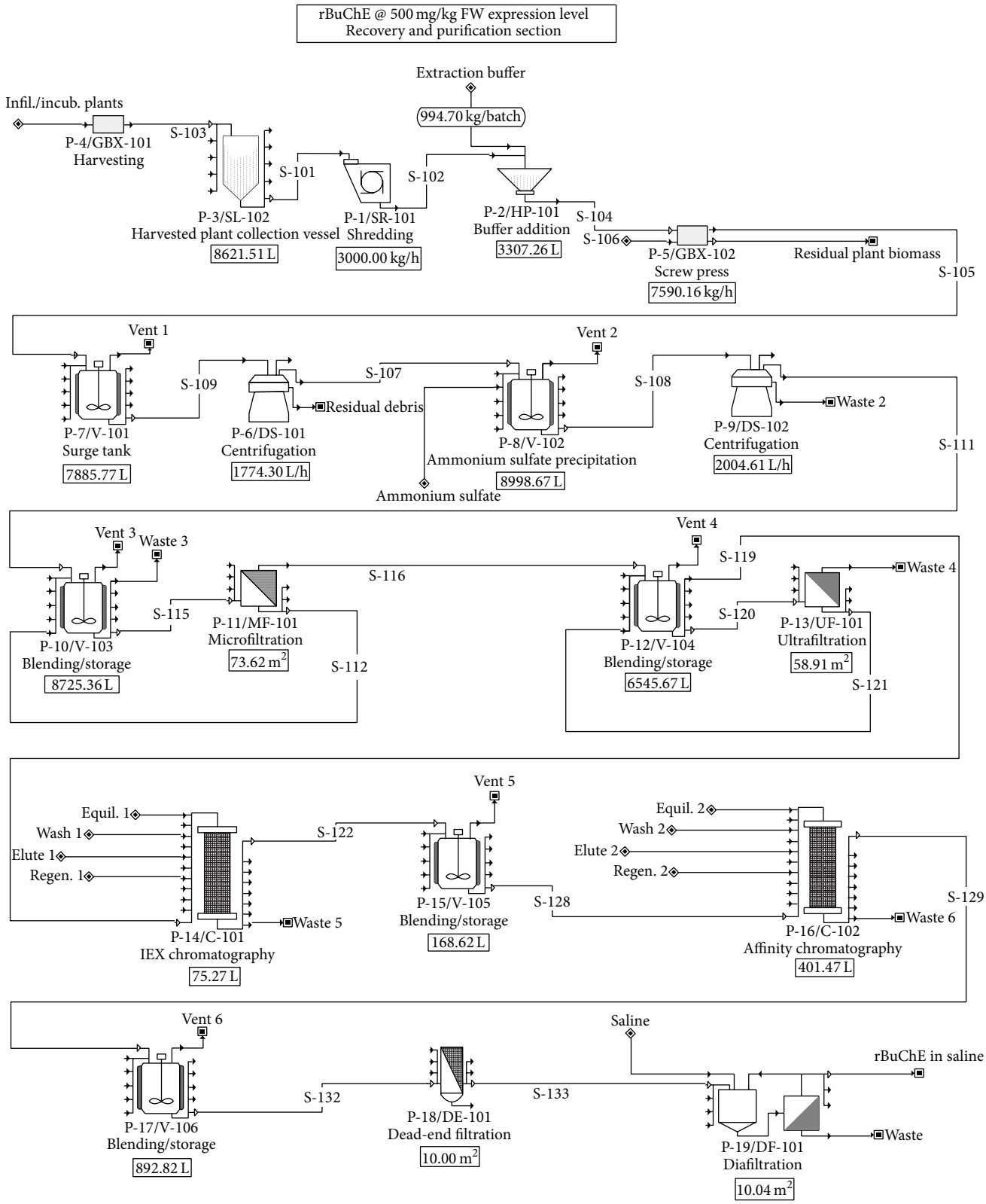

Figure 3: Downstream processing: recovery and purification of rBuChE.

that not only entailed higher cost but that would also face regulatory uncertainties over spraying GM bacteria.

We opted instead for an alternative model using transgenic *N. tabacum* plants, each line of which carries an ethanol-inducible gene for one component enzyme of the cellulase complex. Synthesis of the cellulase is triggered by application of a dilute solution of ethanol (e.g., 2.5% v/v) onto the leaves, a process that has been demonstrated in small scale using a double-inducible viral vector [7]. We assumed that the dilute ethanol solution would be applied via ground irrigation systems that are currently used in agricultural practices, instead of aerial tankers. It was also assumed that the ethanol would be taken off as a side stream from the associated ethanol production facility that uses the cellulase enzymes. In so doing, we obviated the need to produce multiple inocula of GM bacteria and deliver them via aerial spraying. We were also able to model higher biomass density as well as higher expression yields of the enzymes *in planta*. These changes resulted in multiple economic benefits and were therefore adopted in our calculations.

2.5.3. Plant Host and Upstream Process. Issues that are important in PMP, such as mammalian-like glycosylation or other posttranslational modifications, high purity, or specific formulation, are not relevant in the manufacture of cellulases and hence we modeled the use of conventional *Nicotiana* species in the production of the several enzymes necessary for complete saccharification of feedstock. The use of agricultural crops to produce enzymes at low cost has been suggested [5, 41]. In this case study, we modeled the use of stable transgenic *N. tabacum* varieties, each modified to express one cellulase protein upon induction with dilute ethanol. The process is based on inducible release of viral RNA replicons from stably integrated DNA proreplicons. A simple treatment with ethanol releases the replicon leading to RNA amplification and high-level protein production. To achieve tight control of replicon activation and spread in the noninduced state, the viral vector has been deconstructed, and its two components, the replicon and the cell-to-cell movement protein, have each been placed separately under the control of an inducible promoter [7]. In greenhouse studies, recombinant proteins have been expressed at up to 4.3 g/kg FW leaf biomass in the ethanol-inducible hosts [7], but seed lines for field application have yet to be developed. In our modeling, we assumed that each transgenic line would have been already field tested and available for implementation. We also assumed that large-scale stocks of each transgenic seed would need to be produced and have included this unit operation in our cost calculations.

Because cellulases are needed in different ratios to effect saccharification of different feedstocks, we assumed that seeds would be mixed at the appropriate ratios (considering expression levels in each host) and that the seed mixtures would be planted directly in the field. At maturity, what one would expect is a field of plants representing all the needed cellulase classes in the appropriate ratio for the intended feedstock. The current method of hydroponic cultivation of seedlings for transplantation to open fields, a common commercial tobacco cultivation practice to ensure germination and plants with good leaf size and quality, was substituted by direct seeding for more favorable economics. For example, traditionally tobacco may be grown at 12,000–16,000 plants/ha depending on variety [44, 45]. Higher-density seedling production for nontraditional uses of tobacco has been reported, targeting planting densities of over 86,000 plants/ha [44]. While transplanting ensures germination and quality, there is an economic limit to the scale at which it can be deployed with highly cost-sensitive AI, leading to interest in direct seeding practices. Experimental high-density cultivation studies via direct seeding have reported 400,000 to over 2 million plants/ha and biomass yields exceeding 150 mt/ha [46–48]. Our modeling included these higher-density practices to determine economic impact.

2.5.4. Downstream Recovery. In contrast to typical PMP products, the cellulases would not be extracted after accumulation; rather, the plants would be mechanically harvested and transported to a centralized facility for silaging and storage. Since the cellulase enzymes need to be continuously supplied to the saccharification process in the bioethanol plant and the harvested tobacco is only available for a limited period during the year, the silage inventory would increase during the tobacco-harvesting period and would decrease during the fall/winter. Cellulase activity in the ensilaged biomass is expected to be stable during the off-season storage [43]. For feedstock conversion, cellulase-containing biomass would be mixed with pretreated lignocellulosic feedstock (corn stover in our model) under controlled conditions to effect saccharification. Although not considered in this economic analysis, this feedstock replacement could also reduce corn stover feedstock requirements and associated costs. After separation of solids, the sugar solution would be fermented conventionally into ethanol, followed by distillation. The overall process we modeled is based on the US National Renewable Energy Laboratory (NREL) process described by Humbird et al [49], with substitution of fungal cellulase production in the NREL model by the cellulases stored as silage described herein. Design premises for this process, specific assumptions used in modeling, and the resultant cost calculations are presented (see Section 3.2, Tables 5 and 6, and Figures 4 and 5).

3. Results and Discussion

3.1. Butyrylcholinesterase Process Design Premises and Assumptions. The following premises and assumptions were used for evaluation of rBuChE biomanufacturing (Table 1). Although we calculated the construction of a new dedicated manufacturing facility, we also calculated operating costs if a facility with the required capacity were to be already available for toll-manufacturing of the enzyme; results are reported for both scenarios. The overall process is broken into three components: (1) indoor growth of *Nicotiana benthamiana* (Figure 1); (2) *Agrobacterium* growth, vacuum infiltration, and *N. benthamiana* incubation (Figure 2); and (3) rBuChE

Table 1: Recombinant butyrylcholinesterase (rBuChE) design premises and key assumptions.

Parameter	Value
General assumptions for the facility	
rBuChE production level	25 kg rBuChE/year (bulk)
rBuChE doses per year	62,500 doses/year at 400 mg/dose
Downstream Recovery/purification yield	20%
Annual operating days	330 days
rBuChE production in plants following infiltration/incubation	125 kg rBuChE/year
Batch cycle time (time between start of new infiltration batches)	7 days
Batches per year	47
Base case rBuChE expression level	500 mg/kg FW at ~7 days after infiltration
Facility lifetime	15 years
Depreciation	Straight line over 10 years, 5% direct fixed capital salvage
Working capital	30 days of labor, materials, utilities, waste treatment
Lab/QC/QA costs	2% of total labor costs for plant growth and agroinfiltration sections, 15% of total labor costs for recovery and purification section
Start-up/validation costs	5% direct fixed capital
Assumptions for indoor plant growth section	
Mass per plant at 5 weeks	0.02 kg FW/plant
Tray size	4 ft × 4 ft
Number of plants per tray	256
Tobacco seed cost	\$0.001/seed
Age at infiltration	4 weeks
Total number of plant batches in inventory	5 batches (just seeded, 1 wk old, 2 wk old, 3 wk old, 4 wk old—ready to infiltrate, 5 wk old—infiltrated, incubated and ready to harvest)
Total number of plants in inventory	~1.3 million
Total plant growth area	83,320 ft^2 total, or 10 levels with 8,332 ft^2 footprint
LED fixture costs	\$40/ft^2 plant growth area (including capital cost factors for plant growth area as shown in Table 2)
LED energy costs	20 W/ft^2
Assumptions for *Agrobacterium* production, vacuum infiltration, and plant incubation section	
Agro "loading"—mass of recombinant *Agrobacterium* to mass of plant tissue	0.00001 kg dry weight (dw) bacteria/kg FW plant biomass
Agrobacterium biomass density at 12 hours of culture	2.6 g dw/L = 0.0026 kg dw/L
Inoculum density used in seed train	1% V/V
Dilution factor between agroinfiltration solution and agrobacterial production fermentor	78
Percent weight change in plant tissue following vacuum agroinfiltration	30%
Trays processed per vacuum chamber (30 ft) per day	336 trays/chamber/day
"Excess" *Agrobacterium* solution used	87% of total infiltration solution
Incubation time for infiltrated plants	~7 days
Assumptions for the rBuChE recovery and purification section	
Overall yield in downstream processes	20%
Harvesting rate	3 trays/minute
IEX chromatography	
Binding capacity	20 mg/mL
Resin cost	\$1,839/L
Number of reuse cycles	100
Affinity chromatography	
Binding capacity	3 mg/mL
Resin cost	\$10,000/L
Number of reuse cycles	30

Table 2: Capital cost factors for rBuChE case study.

Capital cost factors						
	Estimated based on PC (listed equipment PC plus unlisted equipment PC)					
Unlisted equipment	0.2 listed purchased equipment cost					
Direct costs	Plant growth		Agroproduction/infiltration		rBuChE recovery/purification	
Piping	0.1	PC	0.35	PC	0.35	PC
Instrumentation	0.2	PC	0.4	PC	0.4	PC
Insulation	0.01	PC	0.03	PC	0.03	PC
Electrical facilities	0.1	PC	0.1	PC	0.1	PC
Building	0.2	PC	0.45	PC	3	PC
Yard improvement	0.15	PC	0.15	PC	0.15	PC
Auxiliary facilities	0.1	PC	0.4	PC	0.4	PC
UE installation	0.5	UEPC	0.5	UEPC	0.5	UEPC
Direct costs multiplicative factor	**2.35**		**2.88**		**5.43**	
Engineering	0.25	DC	0.25	DC	0.25	DC
Construction	0.35	DC	0.35	DC	0.35	DC
Indirect costs multiplicative factor	**1.41**		**1.73**		**3.26**	
Contractors fee	0.05	DC + IC	0.05	DC + IC	0.05	DC + IC
Contingency	0.1	DC + IC	0.1	DC + IC	0.1	DC + IC
Other costs multiplicative factors for DFC	**0.56**		**0.69**		**1.30**	
Total multiplicative factor for DFC	**4.33**		**5.30**		**9.99**	
Total multiplicative factor for TCI (except working capital)	**4.54**		**5.56**		**10.49**	

PC: purchase cost; DC: direct cost; IC: indirect cost; UEPC: unlisted equipment purchase cost; DFC: direct fixed capital; TCI: total capital investment.

Table 3: rBuChE facility cost summary (in millions of US dollars).

	Plant growth	*Agrobacterium* inoculum growth/infiltration/incubation	Recovery/purification	Totals
Total capital investment	\$16.1	\$19.6	\$56.7	\$92.4
Annual operating costs **excluding** facility dependent costs	\$2.8	\$0.89	\$10.9	\$14.6
Annual operating costs **including** facility dependent costs	\$4.3	\$4.5	\$20.7	\$29.5

recovery and purification (Figure 3). Table 2 shows the capital cost adjustment factors used for each section of the facility.

Process flowsheets for rBuChE production are shown. The seeding and indoor growth of *N. benthamiana* is shown in Figure 1. Each batch of plants (~266,000 plants comprising 1,039 4 ft × 4 ft trays per batch) will be grown indoors under LED lighting for 4 weeks prior to vacuum infiltration. Figure 2 shows the agrobacterial seed train and production fermentor (200 L with 160 L working volume), the vacuum infiltration system (3 vacuum chambers, each 6 ft diameter × 30 ft length), and the plant incubation facility for the infiltrated plants (6.8 days). The oxygen output streams indicated in Figures 1 and 2 represent net oxygen production by the plants due to photosynthesis. However, oxygen production was not included as part of the model since it does not impact the economics of the process. Figure 3 shows the downstream processes for recovery and purification of the rBuChE, which was modeled after the rBuChE lab purification scheme from vacuum infiltrated *N. benthamiana* described by Hayward [34] and the purification methods described by Lockridge et al. [28]. Major operations include plant harvesting, shredding, screw press/disintegration, ammonium sulfate precipitation, centrifugation, tangential flow microfiltration, tangential flow ultrafiltration, ion exchange chromatography, affinity chromatography, and diafiltration.

3.1.1. Manufacturing and Economic Calculations. Table 3 shows the total capital investment and annual operating costs for the plant-made rBuChE facility at an expression level of 500 mg/kg FW plant biomass (vacuum infiltration of 4-week

Table 4: rBuChE production cost summary (in US dollars).

	Plant growth	*Agrobacterium* inoculum growth/infiltration/incubation	Recovery/purification	Totals
Cost per dose **excluding** facility dependent costs	\$45	\$14	\$175	\$234
Percentage of cost	19.2	6.0	74.8	100.0
Cost per dose **including** facility dependent costs	\$70	\$72	\$332	\$474
Percentage of cost	14.8	15.2	70.0	100.0

old plants and 7 days after infiltration). The annual operating costs are shown with and without facility dependent costs (e.g., depreciation) to simulate a new facility and use of an existing facility, respectively. Table 4 shows the resulting rBuChE cost per dose for both cases.

Table 3 shows the breakdown of the capital investment and operating costs for the plant-made rBuChE and indicates that the unit production costs are estimated to be about \$234/dose if facility dependent costs are not included in the annual operating costs or about \$474/dose if these costs are included. Most of the capital cost (~60%) and a significant portion of the operating costs (>70–75%) are associated with the recovery and purification of rBuChE. Our base case assumed rBuChE expression of 500 mg/kg FW because that is a target expression level in ongoing research at several institutions. If a currently achievable level of 100 mg/kg FW is used instead (reported expression range is 20–200 mg/kg FW [29, 34, 35]), the costs increase to \$1,210/dose and \$430/dose when including and excluding facility dependent costs, respectively. In any scenario examined, the production costs in plants are significantly lower than the estimated production costs for blood-derived BuChE (~\$10,000/dose).

We recognize that additional modification or formulation of the plant-produced enzyme might be necessary or desirable prior to adoption for human use and that such additional modifications would increase the cost of the AI. For example, Geyer et al. [29] reported improved pharmacokinetics of PEGylated plant-produced BuChE relative to the nonmodified enzyme. However, because consensus on the preferred options for modification has not yet been reached, we omitted these additional steps from our calculations.

3.2. Cellulases Process Design Premises and Assumptions. The following premises and assumptions were used for evaluation of cellulase biomanufacturing in open fields. Due to the fact that this process is specialized and due to the scale and input requirements of a modern biofuels operation, our analysis included the construction of a new, dedicated manufacturing facility to provide the required cellulase enzymes for a large-scale (61 million gallons per year) cellulosic ethanol facility (Table 5).

Figure 4 shows the process operations required for cellulase enzyme production on a per-batch basis. The flowsheet on the top shows the blending tank needed for preparation of the ethanol induction solution to be applied in the field, and the flowsheet on the bottom shows the transport and storage operations following harvest of the transgenic tobacco.

3.2.1. Manufacturing and Economic Calculations. Table 6 shows the total capital investment and annual operating costs for the production of 2.87 million kg of cellulase enzymes per year (unpurified) at an expression level of 4 g cellulase/kg FW tobacco biomass and a plant density of 130 metric tons of biomass per hectare per year. The table also indicates the corresponding costs obtained from the JBEI model for fungal fermentation-based production of approximately the same amount of cellulase enzymes per year (2.82 million kg cellulases/year).

For the base case study, the plant-based system results in a >30% reduction in unit production costs for the cellulases as well as an 85% reduction in the required capital investment. For the plant-based cellulase production system, the major contributors to the unit production cost were the costs associated with tobacco cultivation (70%), the costs associated with ethanol spraying (20%), followed by the costs associated with ethanol dilution, transporting and storage (8%), and seed costs (4%). The differences in total capital investment and annual operating costs for the two cellulase production platforms are not surprising, since the fungal fermentation area alone requires twelve 288,000-L fermenters along with the seed train necessary to provide the inoculum for the production fermenters. The differences between the two systems would be expected to be even larger if the total capital investment included additional factors for associated piping, instrumentation, insulation, electrical facilities, buildings, yard improvements, and auxiliary facilities (these were not included in the plant-based model since they were neglected in the JBEI model) because these would be reflected in the facility dependent component of the annual production costs.

Figure 5 shows the effect of biomass density on the unit production costs for cellulase enzyme using the ethanol-induced tobacco system and indicates, as expected, that the cost of goods decreases as tobacco biomass density increases. In agronomic studies with field-seeded tobacco cultivated at high density, biomass yields exceeding 150 mt/ha have been achieved [47, 48]; higher field densities may be possible with selected varieties and specialized agronomic practices.

Table 5: Open field cellulase manufacturing design premises and key assumptions.

Parameter	Value	Source
	Cellulosic ethanol facility assumptions	
Cellulosic ethanol facility capacity	61 million US gallons/year	Humbird et al., 2011 [49]
Cellulosic feedstock	700,000 metric tonnes (dry) corn stover per year, 2,000 metric tonnes/day	Humbird et al., 2011 [49]
Land area required for corn stover feedstock	2,034,000 hectares/year	Humbird et al., 2011 [49]
Annual operating hours	8,410 hours/year	Humbird et al., 2011 [49]
Conversion	87 gallons ethanol/metric tonne corn stover @76% conversion	Humbird et al., 2011 [49]
Enzyme loading	20 mg enzyme "protein mixture" per gram cellulose in feedstock (2% wt/wt)	Humbird et al., 2011 [49]
Enzyme mixture required	4,100,000 enzyme "protein mixture"	Humbird et al., 2011 [49]
Cellulase enzyme required	2,870,000 kg/year	About 2% higher than in Klein-Marcuschamer et al., 2012 [16]
Cellulase in enzyme mixture	70.0% total soluble protein	Calculated from above
	Base case tobacco agronomic and cellulase enzyme production assumptions	
Base case tobacco biomass production	130 metric tonne fresh weight (FW)/ha year	
Mass of a full grown tobacco plant growth at high density	1.0 kg fresh weight (FW)/plant	
Number of plants per hectare	130,000 plants/hectare	Calculated
Tobacco seed cost	\$0.001/seed	
Tobacco growth cycle	82 days from seed to induction, harvest at 7 days after induction spray	
Tobacco planting season (US Midwest/South)	Late March to late October	
Land reuse during growing season	Based on a total of 127 plant batches per year, land recycling can start with batch 94, so land requirement is only 0.74 of that required if no land was reused	Calculated
Tobacco production cost (labor and machinery for seeding, harvesting)	\$1,000/hectare	
Number of tobacco batches per year	127 batches/year	Calculated
Cellulase expression level	4 g cellulase/kg FW tobacco at 7 days after induction, with 2 applications of ethanol induction solution	Werner et al., 2011 [7]
Land area required	5,519 hectares/year	Calculated
Tobacco land area required as a fraction of corn stover land area required	0.27%	Calculated
Ethanol induction	Foliar application of aqueous solution of 2.5% (v/v) ethanol, 2 applications using ground irrigation/sprinklers (central pivot, traveler, or side roll), at 500 L/hectare	

Table 5: Continued.

Parameter	Value	Source
Cost of ethanol for induction	\$0.73/kg	Humbird et al., 2011 [49]
Percentage of ethanol drawn off from biorefinery for induction	0.12%	Calculated
Capital cost of ethanol spray irrigation system	\$2,223/hectare	http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs141p2_023892.pdf
Annual operating cost of ethanol spray irrigation system	\$988/hectare year	http://www.caswcd.org/Irrigation%20guide/Sec7.pdf
Base case assumptions for plant-based cellulase production facility		
Construction period	12 months	Klein-Marcuschamer et al., 2012 [16]
Start-up period	18 months	Klein-Marcuschamer et al., 2012 [16]
Project lifetime	25 years	Klein-Marcuschamer et al., 2012 [16]
Income tax rate	40%	Klein-Marcuschamer et al., 2012 [16]
Working capital	30 days of labor, raw materials, utilities, waste	Klein-Marcuschamer et al., 2012 [16]
Start-up cost	5% direct fixed capital investment, not depreciable	Klein-Marcuschamer et al., 2012 [16]
Depreciation	Straight line over 10 years, salvage value 5% direct fixed capital	Klein-Marcuschamer et al., 2012 [16]
Unlisted equipment	5% of the major purchased listed equipment	Klein-Marcuschamer et al., 2012 [16]

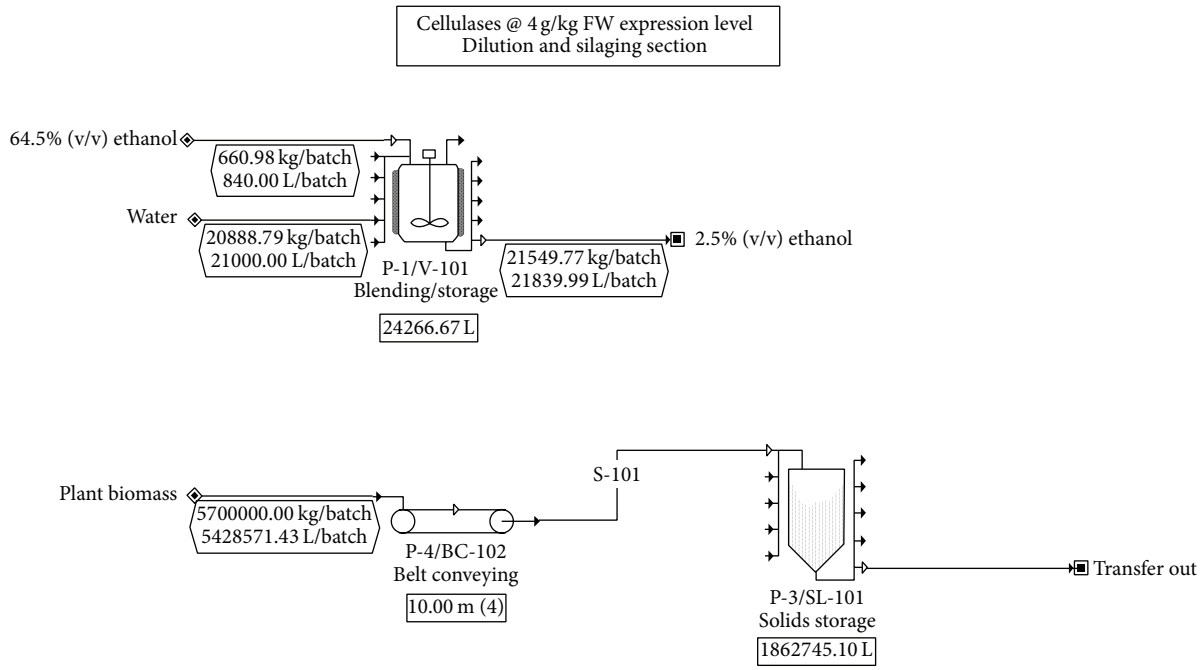

Figure 4: Process operations in the manufacture of cellulases in tobacco biomass.

Table 6: Capital investment and operating costs for manufacturing of cellulases in field-cultivated plants (in 2013 US dollars).

	Plant-based cellulase production process	Fungal-based cellulase production process
Total capital investment (millions of US dollars)	$11.5	$81.5
Total annual operating costs per unit of cellulase production (millions of US dollars)	$20.0	$29.9
Unit production cost ($/kg cellulase)	$6.98	$10.6

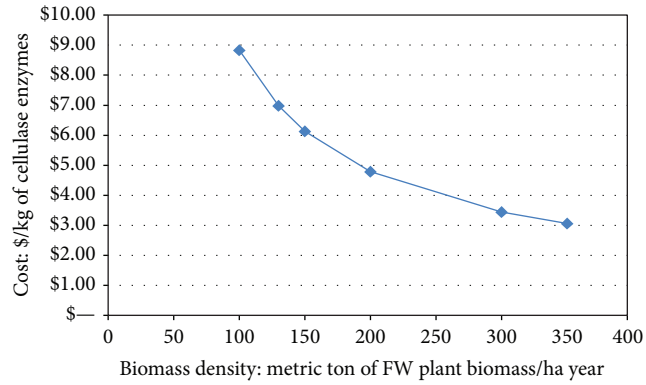

Figure 5: Cost of cellulase enzymes as a function of plant biomass.

4. Conclusions

With hundreds of candidate biologics in development, traditional protein-manufacturing practices may face a major global capacity shortage for the production of new and off-patent biotherapeutics. Worldwide, there are approximately three dozen facilities capable of very large-scale biotherapeutics manufacturing; thus, traditional methods may not produce sufficient quantities of products to meet patient population needs. The challenge is compounded when food additives, industrial products, and biomaterials are added to the capacity estimates. The addition of plants as a biomanufacturing platform could help alleviate this shortage.

Several advantageous features of plant-based systems continue to support interest in plant-based PMB manufacturing. Among them is the potential to scale upstream expression with considerably lower capital requirements compared to traditional cell culture processes. Plant viral replicons delivered within agrobacterial vectors have shown superior speed relative to transgenic plants and proven robust when scaled to industrially relevant settings [2, 4–6, 20, 23]. Conversely, if a large and continuous supply of a consistent AI is needed at low cost for industrial applications, the use of inducible promoters in transgenic plants grown in the field can offer advantages in obviating the production of very large volumes of agrobacterial inoculum and its application over large areas [5, 7]. Plants are also free of adventitious agents that can infect humans and animals (a concern in cell-based systems and transgenic animals) and this inherent safety feature pays dividends by enabling the streamlined purification of the final product without the need for adventitious agent removal steps. Plants' eukaryotic protein processing enable them to synthesize complex classes of biomolecules, such as monoclonal antibodies, therapeutic

enzymes, and multiepitope vaccines that are at the forefront of pharmaceutical interventions. Recent advances in glycoengineering of host plants have enabled the production of human- and mammalian-identical (or at least mammalian-similar) molecules that exhibit comparable or even superior pharmacology to their cell culture-derived counterparts [5, 50, 51]. Inescapably, the growth of the population in developing world regions, the aging of the population in industrialized countries, population displacement due to political turmoil, degradation of environmental quality, and depletion of nonrenewable resources are serious challenges that have not been and likely cannot be readily met only by the existing product manufacturing platforms. This creates new opportunities for plant-based systems to yield lower cost and more widely accessible biopharmaceuticals, food, feed, fuels, and industrial materials.

Here we analyzed the technoeconomics of plant-based manufacture for two active ingredients, both of them enzymes, under development for widely different markets: butyrylcholinesterase for use as a medical countermeasure and a cellulase complex for the production of cellulosic ethanol. In the first case study on BuChE, we modeled transient vectors encoding the protein of interest introduced into glycan-engineered *N. benthamiana* host plants via vacuum-assisted agroinfiltration, followed by disintegration of the plant biomass and extraction and purification of the AI. This route was taken because we anticipate exploratory modifications to the composition of the AI during its development cycle, and transient expression enables the most facile and economic prototyping of the product with direct scalability to commercial production. In contrast, in the second case study on cellulases, we modeled the use of transgenic host plants carrying the genes for each of the enzymes in the cellulase complex under the control of an ethanol-inducible promoter element. Harvest of the biomass is followed by partial drying to produce silage without further purification of the AI. This route was taken to obviate the cost of inoculum manufacture and aerial application, considering the vast areas of land that would need to be dedicated to cellulase biosynthesis. The penalty we accepted is the time to develop each transgenic line.

In both evaluations, we applied the SuperPro Designer modeling tool to generate discrete input and output data for each unit operation, from which we derived bulk AI as well as per-unit/per-dose costs. The calculated costs for these products made in plants were compared to publicly available costs for the same AI produced through predecessor technologies.

4.1. Butyrylcholinesterase. With the assumptions and process parameters adopted for this case study, our results show that rBuChE could be manufactured in plants using transient expression for approximately $234 per 400-mg dose if an existing toll-manufacturing facility were available to accommodate production of 25 kg/year of purified enzyme (equivalent to 62,500 doses/yr). If a new facility with that capacity needs to be built, the cost per dose is projected to increase to approximately $474. Further economic gains could be possible if capacity were to be increased to 100 kg of enzyme per year or more (data not shown), which, in a toll-manufacturing scenario, could reduce the cost of rBuChE to below $200/dose. Even with conservative assumptions, these costs are dramatically below the costs obtainable with blood-extraction processes for this enzyme and may be substantially lower than those for transgenic approaches. In addition, the combination of speed of product prototyping enabled by transient expression, the superior quality and functionality of the rBuChE obtained, lack of adventitious agents, and the rapid scalability of plant systems should make plants the preferred platform for the rapid and cost effective production of this and similar products.

4.2. Cellulases. With the assumptions and process parameters adopted for this case study, our results show that high-density field cultivation of tobacco induced to synthesize several enzymes of the cellulase complex could be competitive with fungal cellulases produced by fermentation for the saccharification of biomass in the production of cellulosic ethanol. Our model adopted many of the process parameters from published studies on the conversion of lignocellulosic feedstocks (in our case corn stover); we replaced the unit operations for the fungal-sourced enzymes with the unit operations for the plant-sourced catalyst and compared operating costs and cost per kg of cellulase blend. Using 130 mt/ha of transgenic tobacco biomass as our base case, our model suggests that plant-sourced cellulases could be produced for just under $7/kg. Even when using a more conservative biomass yield of 100 mt/ha, plant-sourced cellulases could be produced for under $9/kg. These costs compare favorably to the more than $10.6/kg for the fungal-sourced product (all costs adjusted to 2013 US dollars). In a high-volume industry such as biofuels manufacturing, these differences would be significant. These estimates could change depending on how closely empirical results from field trials compare to the modeled assumptions (e.g., expression yields in *N. tabacum* or *N. excelciana* versus those in *N. benthamiana*; length of growing season; weather and other environmental variables, etc.). Conversely, because some of the process assumptions were derived from nonoptimized pilot studies, significant further improvements might be possible in agronomic output, gene expression yield, and cellulase processing efficiency, potentially resulting in even more favorable economics for cellulases and other cost-sensitive, high-volume PMIP.

4.3. Concluding Remarks. The SuperPro Designer modeling software used in these case studies accommodated all major process unit operations in two widely different PMB manufacturing approaches. The program is flexible and allows adaptation through user-definable functions to complement its existing equipment and cost database. Future work will include refinement of the model with specific focus on PMP/PMB/PMIP unit operations and application of the refined model to technoeconomic studies of other plant-made products. It is our hope that wider adoption of evaluations such as the ones presented here will assist decision-makers in early stage product target selection. Doing so

would enable the best match to be found between a product's features and its preferred manufacturing platform early enough in the process to avoid costly mistakes in later stages of development.

Disclosure

Karen A. McDonald is a co-founder of Inserogen, Inc., a plant-based biotechnology company with a focus on the development of orphan drugs for replacement therapy. Tiffany Tu is a graduate student in the McDonald laboratory. Daniel Tusé is a co-founder of Intrucept Biomedicine LLC, a plant-based biotechnology company developing antimicrobial therapeutics. No financial support was received for this study from any of the organizations mentioned in the text.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Quantitative Evaluation of E1 Endoglucanase Recovery from Tobacco Leaves Using the Vacuum Infiltration-Centrifugation Method

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As a production platform for recombinant proteins, plant leaf tissue has many advantages, but commercialization of this technology has been hindered by high recovery and purification costs. Vacuum infiltration-centrifugation (VI-C) is a technique to obtain extracellularly-targeted products from the apoplast wash fluid (AWF). Because of its selective recovery of secreted proteins without homogenizing the whole tissue, VI-C can potentially reduce downstream production costs. Lab scale experiments were conducted to quantitatively evaluate the VI-C method and compared to homogenization techniques in terms of product purity, concentration, and other desirable characteristics. From agroinfiltrated *Nicotiana benthamiana* leaves, up to 81% of a truncated version of E1 endoglucanase from *Acidothermus cellulolyticus* was recovered with VI-C versus homogenate extraction, and average purity and concentration increases of 4.2-fold and 3.1-fold, respectively, were observed. Formulas were developed to predict recovery yields of secreted protein obtained by performing multiple rounds of VI-C on the same leaf tissue. From this, it was determined that three rounds of VI-C recovered 97% of the total active recombinant protein accessible to the VI-C procedure. The results suggest that AWF recovery is an efficient process that could reduce downstream processing steps and costs for plant-made recombinant proteins.

1. Introduction

Plant-based production platforms can produce a wide variety of functional proteins including cell wall degrading enzymes [1], biopharmaceuticals [2, 3], and vaccines [4]. Advances in *Agrobacterium*-mediated gene transfer [5] have offered a popular and facile way to express these proteins of interest (POI) in a variety of species of plants by both stable transformation methods and through transient expression in wild-type plants in contained facilities [6]. Protein manufacturing in plants has advantages such as low cost of production, scalability, and good mimicry of native human glycosylation [7–9]. However, the cost of recovering and purifying POI from the plant tissue can account for up to 90% of the total cost since alkaloids, phenolics, fiber, proteases, and contaminating proteins all need to be removed for most applications [10, 11].

These components are present in leaf extracts because they are typically prepared by homogenization, an extraction method performed with buffer that destructively grinds the whole leaf. This process liberates most of the soluble protein in the leaf, but the extract includes not just the POI but also contaminating proteins from every subcompartment of the cell. Fortunately though, understanding of the biological mechanisms of the plant secretion pathway [12] has enabled the engineering of gene constructs which target secretion of POI outside the cells. The dissolved proteins secreted to the aqueous phase of the extracellular space, called the apoplast, allow for techniques that recover POI without breaking open cells. Recovering the apoplast has been used for collecting

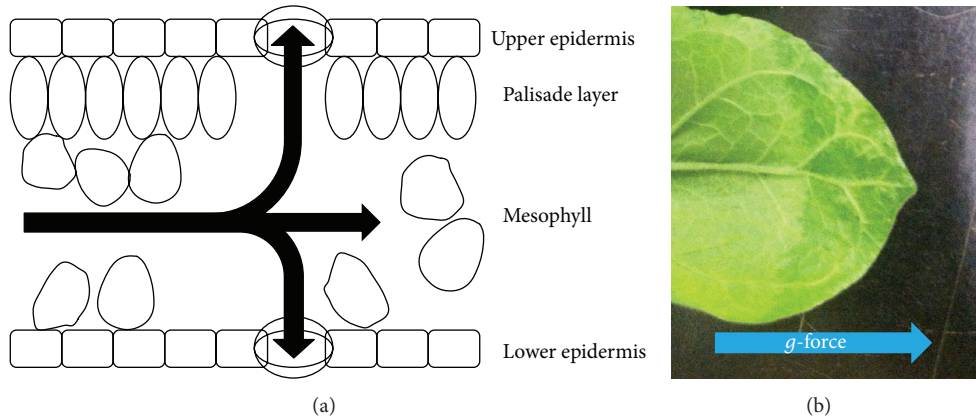

Figure 1: (a) A schematic of the cross-section of a tobacco leaf. Arrows represent the bulk flow of the apoplast wash fluid through the leaf. The flow is in the direction of the centrifugal force as fluid is forced out through the stoma. (b) The centrifugation step to recover AWF from this *Nicotiana benthamiana* leaf was interrupted. The bulk flow of the infiltrated fluid moves evenly through the leaf over time in the direction of the centrifugal force (arrow).

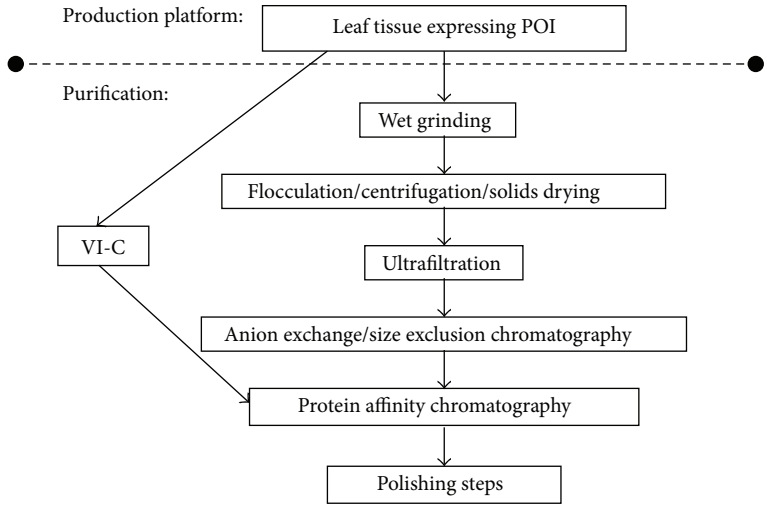

Figure 2: A typical protocol for recovering and purifying recombinant proteins of interest (POI) from leaf tissue, also depicting the steps that could be circumvented by employing the vacuum infiltration-centrifugation (VI-C) method.

secreted recombinant proteins for two decades [13], and it continues to be a desirable goal [14, 15]. The vacuum infiltration-centrifugation method (VI-C) is the most common method of recovering the apoplast, and it is potentially feasible at large scales [16, 17]. During the vacuum infiltration step, the leaf is submerged in buffer. Air is pulled through the leaf stoma during vacuum application and the buffer floods the extracellular space after vacuum release, dissolving the apoplast and increasing the overall weight of the leaf tissue [18]. The centrifugation is employed to force this fluid out of the leaf through the stoma, as seen in Figure 1. This restores the leaf to near its initial or fresh weight and yields an extract called the apoplast wash fluid (AWF). The AWF only draws from the extracellular space, so it is already clarified from the vast majority of the contaminants that makes purification from homogenate extracts (HE) so prohibitive. The large number of downstream processing steps required for most industrial applications [6, 10, 15] may be circumvented by switching the recovery method from homogenate extraction to VI-C, as seen in Figure 2.

In this study various efforts to maximize the yields of POI in the AWF by improving the VI-C method was achieved at bench scale. In its origins, VI-C had been used by plant pathologists to study the leaf apoplast as a substrate for the growth of infectious bacteria [19]. It has continued to be used by plant physiologists studying the native composition of the apoplast [20–25]. In such efforts, obtaining concentrated fluid as free as possible from intracellular contaminants is the major goal. However, the technique has only been used to a limited extent as a means for larger scale recovery of recombinant proteins. To learn how VI-C could be applied for maximal recovery of apoplast components, we utilized the colorimetric marker indigo carmine to enable clear visualization of the blue dye before and after AWF recovery. To

totally clear any visible indigo carmine from the leaf required applying two additional rounds of VI-C, the increasing centrifugal force to ensure recovery of the greatest volume of AWF, and using three applications of vacuum pressure per round of vacuum infiltration, which maximized buffer uptake by the leaf tissue. For further studies, the selection of stable and nonbinding POI was desired for examining whether expressed secreted proteins in AWF could be recovered as effectively and if not what fraction of the protein was inaccessible to the VI-C technique. For this, a native protein, peroxidase, as well as a thermophilic heterologous protein, E1 endoglucanase from *A. cellulolyticus,* were selected. In the E1 study, both the catalytic domain (E1cd) and the full-length holoenzyme (E1holo, possessing both the cellulose binding domain and the catalytic domain) were transiently expressed in *N. benthamiana* to demonstrate the effect of the additional domain on endoglucanase yield by AWF recovery.

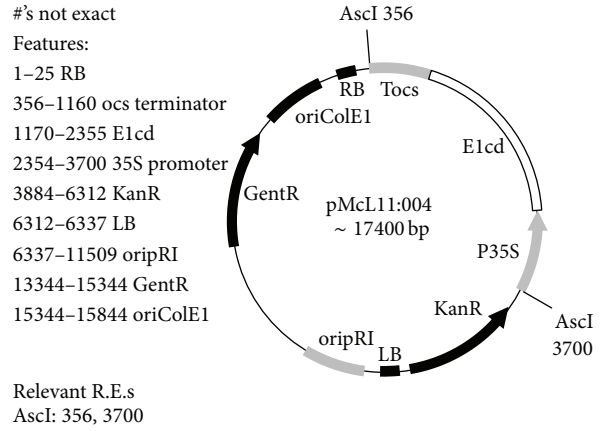

Figure 3: Plasmid pMcL11:004, the binary plasmid for transformation into *A. tumefaciens*, created by cutting the backbone plasmid pDU97:1005 with AscI and insertion of the 35S-E1cd-Tocs gene.

2. Materials and Methods

2.1. Plant Material. Nicotiana benthamiana var. TW16 (National Germplasm Resources Laboratory, Accession #: PI 555478, Beltsville, MD) and *Nicotiana tabacum* var. Xanthii (B. Falk Lab, UC Davis Plant Pathology, Davis, CA) were grown from seed in a greenhouse with a temperature range of 21°C–34°C, on average 28°C ± 3°C, and an observed average absolute humidity of 12 ± 2 g/m^3. Two weeks after seeding, seedlings were transplanted three in a 6″ pot with Sunshine Mix #1 soil (Sun Gro Horticulture, Vancouver, BC). A 6″ pot with three five-week-old *N. tabacum* plants were transplanted together a second time into 12″ pots. Pots were watered twice a day by an automated irrigation system and a custom fertilizer injection system comprising twelve essential plant nutrients.

Twelve- or thirteen-week-old *N. tabacum* leaves were excised by cutting the petiole from the stem about 30 cm above the soil level and were brought from the greenhouse to the lab in sealed plastic containers kept humid with moist paper towels. Five-week-old *N. benthamiana* plants were brought to the lab whole, and prior to the incubation or agroinfiltration the most recent mature leaves (between the third to the fifth leaf from the meristem) were excised.

Prior to apoplast wash fluid recovery the midrib and edges of each leaf were removed and strips of lamina tissue were excised from the intercostal regions between the veins, strip width not exceeding 1 cm, similar to Rathmell's technique [25]. In some experiments, each leaf was then partitioned into two identical strip sets, in which case one set was selected for apoplast wash fluid recovery (the "washed" set) while the other identical set went unprocessed (the "unwashed" set), incubating in a humidity chamber throughout the experiment until homogenization. Each strip set was cut to weigh 700–800 mg.

2.2. E1 Constructs. The gene for full-length E1 endoglucanase (E1holo) from *Acidothermus cellulolyticus* (NCBI Accession #: P54583) was codon-optimized using GeneDesigner software (version 1.1.4.1, DNA 2.0, Burlingame, CA) and the codon usage table for *N. benthamiana* [26]. The 41 amino acid native signal peptide was removed from the N-terminus and replaced with the RAmy3D signal peptide from the α-amylase gene from *Oryza sativa* (NCBI Accession #: M59351.1). To the C-terminus, a 6-His tag was added. The construct was placed under the control of the CaMV 35S promoter and octopine synthase terminator (Tocs). The sequence and the gene in entirety were submitted to GenBank (Accession #: HQ541433). The method for construction for the truncated E1 (E1cd) was the same as for the full-length protein except with the cellulose binding domain and linker region between domains removed. DNA 2.0 (Menlo Park, CA) synthesized, verified, and inserted the optimized genes for both E1cd and E1holo into pJ201, one of its standard cloning vectors, prior to propagation in *E. coli*. The genes were then ligated into a binary vector, pDU97.1005 (modified by SL Uratsu and AM Dandekar, unpublished, from pCGN1547 [27]) for transformation into *Agrobacterium tumefaciens* EHA105 pCH32 (Figure 3).

2.3. Agroinfiltration. Transformed *A. tumefaciens* was thawed from glycerol stocks and grown in small volumes of Luria-Bertani (LB) medium in round-bottom 10 mL tubes at 28°C in an incubator shaking at 250 rpm. Cultures were then inoculated 1% v/v into 200 mL LB medium and incubated again for 18–22 h at 28°C and 250 rpm. After growth, the bacteria were centrifuged for 20 min at 3,200 g. The pellet was resuspended in activation solution consisting of 10 mM 2-(*N*-morpholino) ethanesulfonic acid (pH = 5.6), 10 mM $MgCl_2$, and 150 μM acetosyringone (3′,5′-Dimethoxy-4′-hydroxyacetophenone; Aldrich Chemicals, Milwaukee, WI) to an optical density at 600 nm of 0.5, as measured by a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). In the activation solution, the bacteria cultures were incubated in the dark for two to five hours and just prior to agroinfiltration 0.02% v/v Silwet L-77 (Lehle Seeds, Round Rock, TX) was added.

Detached plant leaves were held submerged into activated *Agrobacterium* solution in a plastic container by a plastic lined

wire mesh. Three rounds of vacuum infiltration were applied in a 5-gal Nalgene vacuum chamber, where for each round the pressure was allowed to reach an absolute pressure 30 kPa held at that pressure for at least 30 s. Leaves were then patted dry with paper towels and allowed to dry on a rack for an hour prior to incubation.

2.4. Incubation. Incubation was performed in a $19'' \times 14'' \times 7''$ air-tight plastic storage box. Perlite soil additive (E.B. Stone Organics, Suisun City, CA) was submerged in DI water for at least three hours and poured into the box to create a layer about 4 cm thick to maintain humidity throughout the incubation. Plastic lined steel mesh was fit into the box to suspend the leaves about 4 cm above the Perlite layer. The box with detached leaves was incubated in the dark at 20°C.

2.5. Apoplast Wash Fluid Recovery. Plant strips were rinsed to remove any debris from their surfaces, blotted dry with paper towels, and weighed to obtain their fresh weight. Strips were then placed in 50 mL Falcon tubes and submerged in 20°C–25°C harvest buffer at a buffer to biomass ratio ranging from 10 to 25 mL/g FW. The harvest buffer consisted of 50 mM sodium acetate (pH = 5.5), 100 mM NaCl, and 0.02% Silwet L-77. In the indigo carmine experiments, leaf strips were vacuum infiltrated with 1900 μg/mL indigo carmine (Sigma, St. Louis, MO), dissolved in 50 mM sodium acetate (pH = 6.2), 100 mM NaCl, and 0.02% Silwet L-77. Three rounds of vacuum application were performed in a Nalgene container and brought to an absolute pressure of 30 kPa and held for 30 s prior to release for at least 30 s between rounds. Strips were removed from the buffer, and the buffer in the tubes was capped, refrigerated, and kept as "Rinse Fluid" (RF) samples. Strips were blotted dry with paper towels and weighed again to monitor the volume of buffer infiltrated into the strips.

Vacuum infiltrated leaf strips were loaded into perforated 50 mL Falcon tubes with no particular orientation and centrifuged to obtain the AWF. Each Falcon tube possesses 8–15 circular perforations about 3 mm in diameter each. The perforated tubes with the leaf strips were transported to the centrifuge in a humid box. It was found that fixed angle centrifuges damaged the leaf strips unacceptably, observed as green coloration in the AWF, so a Beckman GS-6KR (Beckman Coulter, Inc., Brea, CA) centrifuge with swing-out wells was used. Collection caps were fashioned from the bottom halves of 50 mL Falcon tubes and they were positioned in the centrifuge under the perforated tubes to catch the recovered AWF. The centrifuge was run at 25°C for 10 minutes at 3,200 g, and the AWF was recovered from the collection cap, the volume was measured and recorded, and the AWF sample was refrigerated prior to assaying performed the same day. The perforated tubes with the centrifuged leaf strips inside were brought back to the lab in a humid box and the new tissue weight was measured prior to the next round of vacuum infiltration. Vacuum infiltration and centrifugation steps of subsequent rounds were performed the same as the first, and changes in the weight of the strips were monitored throughout the experiment (Supplemental Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/483596).

2.6. Homogenate Extraction. Leaf strip sets were homogenized with liquid nitrogen or wet grinding after each experiment. Powder from liquid nitrogen extraction was resuspended in 10 mL/g FW ice cold harvest buffer in a 15 mL Falcon tube. The powder was allowed to incubate in the tube in an ice bath for ten minutes prior to centrifugation for 10 min at 4°C and 6,000 g. Exactly 1 mL of the supernatant was decanted into 1.5 mL Eppendorf tubes and centrifuged again for 20 min at 4°C and 20,000 g (Eppendorf Centrifuge 5403, Hauppauge, NY) prior to assaying. For the results in the E1cd and E1 holoenzyme studies, a GrindoMix GM 200 (Retsch Technology, Haan, Germany) was used to homogenize leaf strip sets. Strips were homogenized with 50 mL of ice cold harvest buffer at 8,500 rpm for two rounds of 15 s each.

2.7. Quantitative Analysis. Indigo carmine was dissolved in 50 mM sodium acetate 100 mM NaCl and 0.02% Silwet L-77. A linear standard curve was developed from 0–333 μg/mL indigo carmine by direct measurement of 50 μL per well at an absorbance of 608 nm by a SpectraMax 340pc (Molecular Devices, Sunnyvale, CA).

The total soluble protein assay was performed by the method of Bradford [28] using Coomassie Brilliant Blue G-250 dye (Bio-Rad, Hercules, CA). A standard curve was produced from bovine serum albumin (BSA) (Fisher, Pittsburgh, PA) diluted in harvest buffer. Sample, diluted sample, or standard measuring 10 μL was added to 90 μL harvest buffer in a 96-well plate. Bradford dye measuring 200 μL was added to each well and color was developed for five minutes prior to the measurement of absorbance at 590 nm by a SpectraMax 340pc.

Malate dehydrogenase (MDH) activity assay for measurement of intracellular contamination in apoplast wash fluid or rinse fluid was performed as described [24, 29]. A standard curve was produced from 0.75 mM β-nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH) (Sigma-Aldrich, St. Louis, MO) diluted in 50 mM phosphate buffer (pH = 7.5), 200 μL per well. Then, 10 μL sample or diluted sample was added to 90 μL phosphate buffer in a 96-well plate at room temperature. The reaction was started when 50 μL 1.5 mM and 50 μL of 2 mM oxaloacetic acid (OAA) (Sigma-Aldrich, St. Louis, MO) were added to each sample or diluted sample well. The decrease in absorbance at 340 nm in the sample wells, corresponding to the conversion of NADH to NAD^+ by MDH in a reversible redox reaction that also converts OAA to malate, was monitored for three minutes and compared to the NADH standard curve by a SpectraMax 340pc.

Peroxidase activity was measured by monitoring the conversion of pyrogallol to purpurogallin in the presence of hydrogen peroxide [30]. Horseradish peroxidase powder (Sigma, St. Louis, MO) dissolved to 1.5 Units per mL was used to generate a standard curve. To each well of a 96-well plate, 10 μL of sample, diluted sample, or horseradish peroxidase standard was added to 190 μL of 200 mM KH_2PO_4 (pH = 6.0) buffer. A 1 : 1 mixture of 0.3% hydrogen peroxidase and 50 mg/mL pyrogallol were prepared just before assaying and 100 μL was added to each well. Change of absorbance

at 420 nm corresponding to the production of purpurogallin was measured over a 40 s duration by a SpectraMax 340pc.

The activity of E1 endoglucanase was measured fluorometrically using methylumbelliferyl-β-D-cellobioside (MUC) as a substrate as described previously [31, 32]. E1 converts the MUC substrate, which is not fluorescent, to 4-methylumbelliferone (MU), and 3 μM MU diluted in acetate buffer (50 mM acetate, 100 mM NaCl, pH = 5.5) was used to generate a standard curve. Samples were diluted to 600 μL in acetate buffer and incubated with 200 μL of 500 μM MUC or 125 μM MUC in 1.5 mL Eppendorf tubes at 65°C for 30 min. Before and after the reaction, 200 μL of the reaction volume was transferred to 800 μL stop buffer (150 mM glycine buffer, pH = 10.0) and the change in fluorescence (λ_{ex} 360 nm/λ_{em} 460 nm) was measured with a VersaFluor fluorometer (Bio-Rad, Hercules, CA). As an alternative to using the VersaFluor, some samples were read by adding 50 μL of reaction volume to 50 μL stop buffer in triplicate wells of an opaque 96-well plate and read with a SpectraMax M2 (Molecular Devices, Sunnyvale, CA). A unit of activity (U) was defined as the generation of one nanomole of MU per minute [32]. Recombinant E1cd has been previously purified from *Streptomyces lividans* [33] and its specific activity has been previously reported at 40 U per microgram [32]. E1holo is estimated to have a specific activity of 20 U per microgram based on assuming that it has the same molar specific activity as E1cd and accounting for the ratio of their molecular weights.

2.8. Calculations. The percent yield of a component may be calculated by the formula

$$\%\ \text{yield} = 100\% * \frac{\sum_{i=1}^{n} \text{AWF}_i + \sum_{i=1}^{n} \text{RF}_i}{\text{total}}, \tag{1}$$

where the "total" is the amount of the component in the leaf prior to recovery, assumed to be the sum of the yields from all the AWF and all the RF from every round of recovery as well as the yield in WHE (the homogenized tissue from which the AWF had been recovered).

The purity fold improvement of a component calculated for a given sample (AWF, RF, or WHE) was calculated as the ratio of its specific yield relative to the specific yield in the total. The concentration fold improvement of a component in a sample was calculated by dividing that component's concentration in the sample by that of the total, standardizing it to a 10 mL/g FW buffer to biomass ratio. The purity and concentration fold improvements in volume V of component k can be given as follows:

$$\begin{aligned} \text{Purity fold improvement} &= \frac{[k]_V/[\text{TSP}]_V}{[k]_{\text{total}}/[\text{TSP}]_{\text{total}}}, \\ \text{Concentration fold improvement} &= \frac{[k]_V}{[k]_{\text{total}}}. \end{aligned} \tag{2}$$

In indigo carmine experiments, the dilution factor (F_{dil}) was calculated to make predictions for how much the product would be diluted in subsequent washes. It describes the ratio between total extracellular space and the volume of the liquid apoplast phase to show theoretically how much an apoplast component would be diluted per round of vacuum infiltration and AWF recovery (3). The volume of the apoplast can be measured by infiltrating a leaf with a marker, k, impermeable to cell membranes (such as indigo carmine) and observing the decrease in its concentration from the infiltration solution to the recovered AWF (4) [34]. An n-number of rounds of vacuum infiltration-centrifugation washes is theorized here to have an exponential effect on the concentration of the marker in the nth recovered yield (5). Equation (5) assumes that there are no changes in the volumes of either the total leaf extracellular volume or its apoplast between rounds of recovery. It assumes complete mixing of the infiltrated buffer with the latent liquid phase prior to centrifugation, and it also assumes the marker or POI is completely soluble and does not interact with the extracellular matrix. Equation (5) does not account for loss of the component into the RF during the vacuum infiltration step, so experimentally observed yields were calculating by adding the content in the AWF with the content in the RF during comparison with model predictions. Equation (6) describes the maximum yield from the summation of theoretically infinite rounds of VI-C:

$$F_{\text{dil}} = \frac{V_{\text{air}} + V_{\text{apo}}}{V_{\text{apo}}}, \tag{3}$$

$$V_{\text{apo}} = V_{\text{air}} * \frac{[k]_{\text{buffer}}}{[k]_{\text{AWF}}} - V_{\text{air}}, \tag{4}$$

$$\text{Yield}_n = \frac{\text{Yield}_1}{F_{\text{dil}}^{n-1}}, \tag{5}$$

$$\sum_{n=1}^{\infty} \text{Yield}_n = \text{Yield}_1 * \frac{F_{\text{dil}}}{F_{\text{dil}} - 1}. \tag{6}$$

Statistical analysis was performed using Microsoft Excel software. Standard deviations reported throughout this paper were calculated just from sample-to-sample variability as other possible sources of uncertainty such as assaying, volume, or leaf weight measurements were considered negligible compared to this. Whether values were statistically different was determined using two-tailed paired student's t-tests using a 95% confidence interval as the threshold for significance.

3. Results and Discussion

3.1. Recovery of Indigo Carmine. Indigo carmine dye was very thoroughly removed from the *N. tabacum* leaf tissue after three rounds of vacuum infiltration-centrifugation. The first AWF recovered from the tissue infiltrated with indigo carmine was dark blue, visually very similar in shade to the infiltration buffer. Subsequent rounds of AWF recovery on the same tissue produced increasingly dilute extracts. Furthermore, the tissue was losing the blue hue it acquired after its infiltration with the dye, becoming indistinguishable from a control set infiltrated with clear buffer by the third round of VI-C.

The visual observations were confirmed by analysis of the samples' absorbance at 608 nm and plotted on Figure 4.

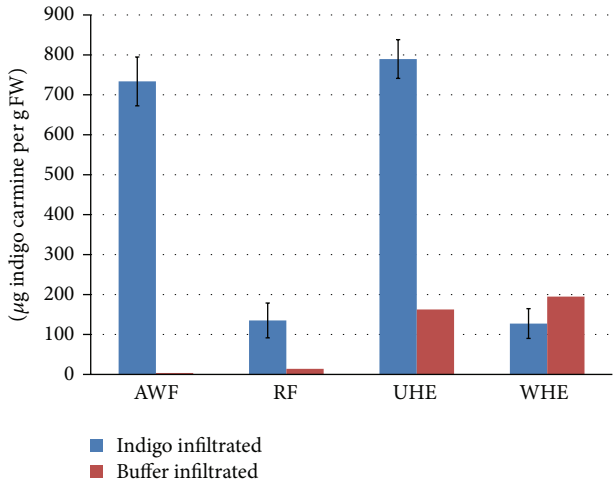

Figure 4: Yields in various extracts from 12-week-old *N. tabacum* leaf strips vacuum infiltrated with 1900 μg/mL indigo carmine (n = 3). Chlorophyll produces the background absorbance observed in buffer infiltrated samples (n = 1). AWF = apoplast wash fluid; RF = rinse fluid; UHE = unwashed homogenate extract; WHE = washed homogenate extract.

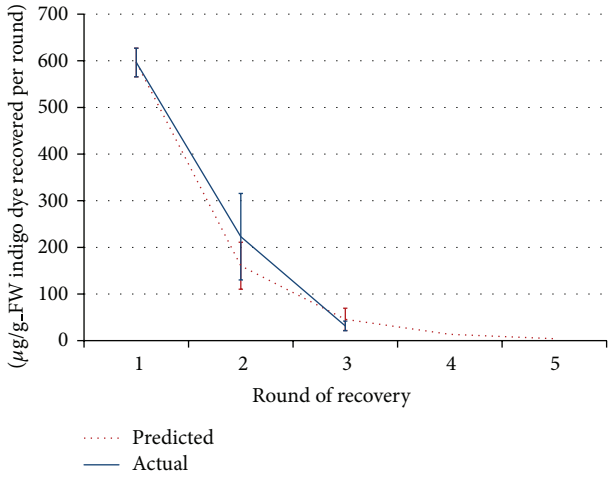

Figure 5: Yields per round of recovery (AWF and RF combined) of infiltrated indigo carmine from 12-week-old *N. tabacum* leaf strips (n = 3), showing the experimental results alongside the results predicted from the first round of recovery. Only three rounds of recovery were performed in experiment, while predicted results are shown extrapolated for additional rounds. Error bars in the predicted data reflect variation in *N. tabacum* dilution factors that were observed.

The absorbance of the indigo carmine in the homogenate from tissue extracted prior to AWF recovery (unwashed homogenate extract; UHE) was much higher than the background from chlorophyll, whose absorbance was observed in the UHE of clear buffer infiltrated tissue. Meanwhile, after three rounds of AWF recovery, the washed homogenate extract (WHE) of the indigo infiltrated leaf tissue had an absorbance that was statistically indistinguishable from the WHE of clear buffer infiltrated leaves (which in turn had a similar absorbance value to the same control's UHE). Meanwhile, the amount of dye recovered in the AWF and the RF was approximately equal to the indigo content in the UHE, determined after adjusting the absorbance reading against background absorbance. These results suggested almost complete recovery of the infiltrated indigo carmine from the tobacco leaf strips in this experiment.

The concentration of the apoplast component recovered during each round of VI-C was demonstrated to be predictable, as seen in Figure 5. By performing one round of AWF recovery, the dilution factor (F_{dil}) was calculated in this experiment to be 4 ± 1 (n = 3), and this value was used to predict the dilution of indigo carmine in AWF recovered in subsequent rounds. The dilution of indigo in these later AWF extracts was more than expected by the model because of the dye leaching from the leaf interstitial space into the acetate buffer during vacuum infiltration. However, by pooling the yield of AWF with the yield of its corresponding RF, the actual observed recovery of indigo showed no statistical difference with predicted values, and so the data was interpreted as in support of the model.

Using the model and (6), it was postulated that for the F_{dil} found for this experiment, 97.5% of the total theoretically recoverable indigo carmine was obtainable in three rounds of VI-C. Infinitely more rounds of VI-C would have only improved yields of indigo carmine by an additional 2.5%, so future experiments were limited to three rounds.

3.2. Recovery of Peroxidase. Five rounds of VI-C performed on strips of *N. tabacum* leaves in triplicate showed that up to 94% of the peroxidase activity could be recovered in AWF (19, 2.6, and 1.4 U/g FW measured in the accumulated AWF, accumulated RF, and residual homogenate extract resp.) (Figure 6(a)). Up to 71% of the activity was recovered during the first round of recovery and activity was up to 16 times more concentrated than in homogenate extracts (37 versus 2.3 U/mL, normalizing to a buffer to biomass ratio of 10 mL per g FW). Average yields and concentrations during subsequent rounds of average were diluted by a factor of 4, in agreement with the dilution factor of exogenously infiltrated indigo carmine.

Despite the high yields of peroxidase, only a very small percentage of the total soluble protein was recovered, about 2%. While the peroxidase activity was diluted upon each additional round of recovery, TSP levels remained relatively stable. Therefore, while the first recovered AWF and RF had up to 95-fold improvement in purity over homogenate extracts (400 versus 4.2 U/mg TSP), this value decreased as more extracts from the same tissue were collected (Figure 6(b)). The malate dehydrogenase recovered throughout the experiment for each tissue sample was <1%, and since MDH is found typically only in the cytosol or intracellular organelles, this underscores that recovery was highly selective for secreted proteins.

3.3. Recovery of Recombinant E1 Catalytic Domain. From *N. benthamiana* tissue agroinfiltrated to express the truncated

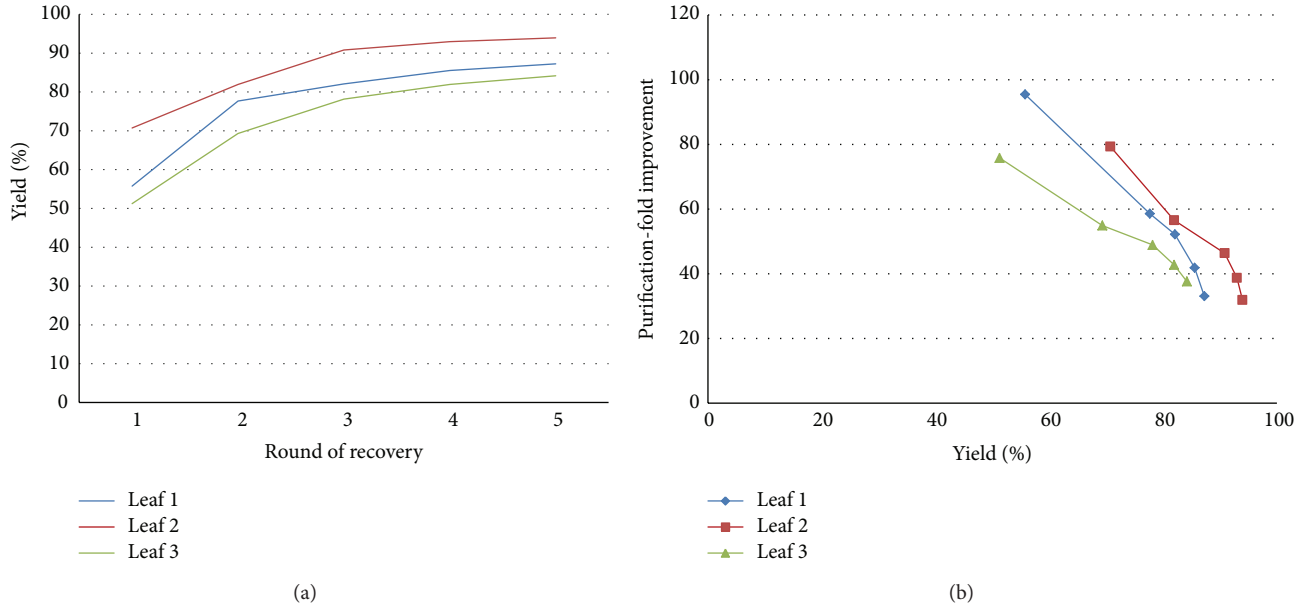

Figure 6: The accumulated percent yield and pooled purification-fold improvement of peroxidase recovered in RF and AWF from tissue excised from triplicate 12-week-old *N. tabacum* leaves over five rounds of vacuum infiltration-centrifugation. (a) Accumulated percent yield after each round of recovery. (b) Purity versus yield chart over five rounds of recovery, depicted for each leaf sample.

catalytic domain (E1cd), apoplast wash fluid (AWF) and rinse fluid (RF) samples were collected by three rounds of vacuum infiltration-centrifugation. The total E1cd activity was 2800 ± 1300 units (70 ± 33 μg, based on estimated specific activity) per kg fresh weight of leaf tissue, of which only 23% ±4% remained residually in the WHE (see Supplemental Table 1). In contrast, the WHE retained 86% ±4% of the total soluble protein and 98% ±1% of MDH. As a result of this selective recovery, VI-C produced an extract that is 9 ± 4 times more pure than an extract produced by homogenization.

More than half of the total E1cd activity, 61% ±14%, was recovered during the first round of VI-C, followed by 13% ±4% in the second round and 4% ±1% in the third (See Supplemental Table 2). The observed dilution factor averaged across the three rounds of recovery was therefore 4 ± 1, similar to the result calculated for *N. tabacum* in the indigo carmine and peroxidase studies, and suggested another 4% of the expressed E1cd activity may have been recoverable from additional rounds of recovery. It is therefore predicted of the E1cd expressed, 18% was in regions inaccessible to AWF recovery such as inside the cells or in between tightly packed epidermal cells.

Recovering recombinant protein using consecutive rounds of VI-C may or may not be desirable because a decrease in purity is observed alongside an increase in yield. For each round of recovery the specific activity, or purity, of E1cd went down, dropping from an 8-fold improvement to a 6-fold improvement when the three AWFs and three RFs were combined as seen on Figure 7. When considering just the AWFs, which almost always had the higher specific activity compared to the corresponding RF while also being by far more concentrated, the purity still dropped from a 9- to a 7-fold improvement. The drop in purity

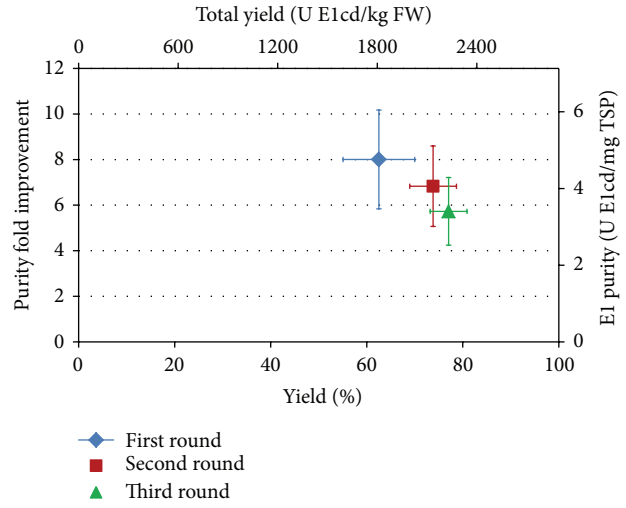

Figure 7: Average accumulated recovery of truncated E1 collected after each of three rounds of infiltration-centrifugation (pooled AWF and RF). Purity is graphed as an improvement over the total expression on one axis and its purity per milligram of total soluble protein on the other axis. Yield is graphed both as percent yield and also as the units of enzyme recovered per kg fresh weight of leaf tissue.

between rounds was because E1cd yield was dramatically diluted while the amount of intracellular contamination, as measured by MDH yields, was stable between rounds of recovery. The trend for TSP recovery was in between these two extremes: while the first round yielded significantly more TSP than any subsequent round, after that there were no distinguishable differences (see Supplemental Tables 1 and 2).

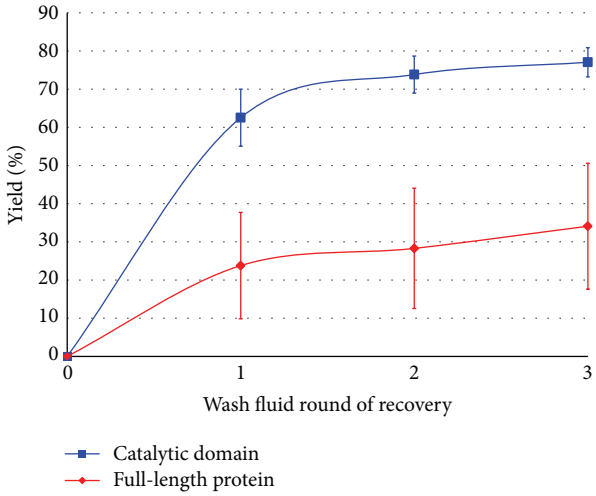

Figure 8: The accumulated recovery (combined AWF and RF) of truncated E1 (catalytic domain) and the full-length E1 endoglucanase after each round of infiltration-centrifugation. Differences in yield between catalytic domain and full-length protein were significant at each round.

This underscored the diversity of the leaf's native apoplast proteins, which would have a range of sizes, solubility, and affinity to the cell wall. Since percent yield in the recovered AWF of TSP was always at least three times greater than that for MDH (in the RF, MDH was too diluted to be accurately assayed), it is also concluded that most of the contaminating proteins in these extracts were apoplast localized. In addition to native apoplast proteins, the purity of E1cd may also be negatively affected by the presence of secreted inactive E1cd. Since in these studies E1cd was quantified only by its activity, any inactive E1cd would be included in the overall TSP levels.

3.4. Recovery of Recombinant E1 Full-Length Enzyme. The holoenzyme of E1 endoglucanase (E1holo) has a cellulose binding domain, which increases its affinity for the cell wall matrix while also making it substantially bigger than the truncated version (126 kDa versus 51 kDa as visualized on a Western blot, not shown). Because of this, it was expected that the percent yield of the full-length enzyme would be less than that of the truncated isoform. This hypothesis was supported by the experimental results, as seen in Figure 8. The percent yield from three rounds of VI-C was 34% ±17%, substantially less than for the truncated version, which was 77% ±4%. The values for TSP and MDH obtained and their trend over multiple rounds of VI-C however largely matched the results obtained for the sample set expressing E1cd, enabling a fair comparison between sample sets based on the characteristics of the recombinant proteins expressed.

Besides percent yield, there were other differences between the two sample sets. There was an overall lower expression level of E1holo versus E1cd, and it is possible that this would have a negative effect on the percent yield recovered as well. The theory that the cell wall impedes flow of the larger enzyme however is supported by how the dilution factor for this experiment could not even be calculated because in some samples the yields from the third round of VI-C were actually higher than those from the second round. This could be explained by considering the effect of applying centrifugal force on the tissue repeatedly during the course of the experiment, and how this might compromise cell wall integrity and increase its effective porosity. Also, recovery of E1holo, as seen from its relatively larger error bars in Figure 8, was more prone to sample variability, perhaps suggesting that free movement of large recombinant proteins through the apoplast is more sensitive to subtle differences in cell wall morphology. Therefore, it is theorized that the differences observed comparing the recovery of the E1holo and its truncated version were in contrast because of differences in how they interact with the cell wall matrix as AWF is pulled out of the leaf tissue.

4. Conclusions

By implementing measures to maximize the amount of buffer infiltrated into the tissue and performing centrifugation until the tissue is restored to its fresh weight, we show for the first time that three rounds of VI-C are sufficient for recovering almost all of the activity of secreted proteins into AWF extracts characterized by remarkable purity and concentration improvements. Over 90% of native peroxidase was recovered from mature wild-type *N. tabacum* leaf strips. From agroinfiltrated *N. benthamiana* leaves, as much as 81% of the expressed E1cd was recovered. Analysis of the dilution factor suggests that recovery of the residual activity within extracted leaf tissue was impeded either by incomplete secretion or by interaction with the cell wall matrix.

These were the highest yields ever achieved from the apoplast of leaf tissue using a nondestructive extraction technique, demonstrating that, if the experiments were repeated at a higher scale, they may be employed to increase extract purity and concentration without enduring major losses in yield. Preliminary tests could be performed with industrial vacuum chambers and centrifuges to scale up recovery of exogenous infiltrated chemicals or native secreted proteins from kilograms of wild-type plant tissue, and knowledge gained from these efforts would directly apply to recovery of heterologous proteins from agroinfiltrated or transgenic tobacco. To conform to the bench scale experiments, the applied vacuum would need to result in the saturation of the leaf tissue with infiltration buffer and the centrifuge step would need to restore the tissue to its original fresh weight without damaging it. As long as these conditions are met, we hypothesize that the AWF extracted at large scale will be relatively pure and enriched compared to conventional large scale homogenization methods.

Other future work explores the use of cell wall degrading enzymes to treat the leaf prior to AWF recovery to improve the percent yield of larger recombinant proteins. Another technique we are developing employs AWF recovery periodically over the entire transient expression phase. This method has the potential to further improve AWF purity while also increasing overall yields of protein per unit weight of leaf tissue.

Nomenclature

AWF: apoplast wash fluid
E1cd: the catalytic domain of E1 endoglucanase from *A. cellulolyticus*
E1holo: the full-length holoenzyme of E1
F_{dil}: dilution factor
HE: homogenate extract
POI: protein of interest
RF: rinse fluid, spent vacuum infiltration buffer
TSP: total soluble protein
UHE: unwashed homogenate extract (no VI-C performed on tissue)
VI-C: vacuum infiltration-centrifugation
WHE: washed homogenate extract (tissue after rounds of VI-C).

Disclosure

N. Kingsbury is currently affiliated with the University of Massachusetts, Amherst and formerly in the laboratory of K. McDonald. K. McDonald is a cofounder of Inserogen, Inc., a plant-based biotechnology company with a focus on the development of orphan drugs for replacement therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

N-Glycosylation Modification of Plant-Derived Virus-Like Particles: An Application in Vaccines

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Plants have been developed as an alternative system to mammalian cells for production of recombinant prophylactic or therapeutic proteins for human and animal use. Effective plant expression systems for recombinant proteins have been established with the optimal combination of gene expression regulatory elements and control of posttranslational processing of recombinant glycoproteins. In plant, virus-like particles (VLPs), viral "empty shells" which maintain the same structural characteristics of virions but are genome-free, are considered extremely promising as vaccine platforms and therapeutic delivery systems. Unlike microbial fermentation, plants are capable of carrying out *N*-glycosylation as a posttranslational modification of glycoproteins. Recent advances in the glycoengineering in plant allow human-like glycomodification and optimization of desired glycan structures for enhancing safety and functionality of recombinant pharmaceutical glycoproteins. In this review, the current plant-derived VLP approaches are focused, and *N*-glycosylation and its in planta modifications are discussed.

1. Plant-Derived Virus-Like Particle (VLP)

Viruses are able to form the quaternary structure of viral capsids through molecular self-assembly of repetitive building blocks [1, 2]. Plant viruses can be easily multiplied, which are structurally uniform, robust, and biodegradable with a size particularly suitable for nanoscale applications. Virus-like particles (VLPs) are multimeric self-assembled protein complexes mimicking the organization and conformation of native viruses but lack the viral genome making them replication-deficient and noninfectious [3]. VLPs consist of protein shells (termed as capsids), and the capsids are typically composed of identical coat protein subunits. Peptide-based vaccines are in general poorly immunogenic and for this reason they require multiple injections and adjuvants in order to increase their effectiveness. VLPs offer a promising approach to the production of vaccines against many diseases, because their repetitive, high density display of epitopes is potentially highly effective in eliciting strong immune responses [4]. VLPs lacking viral nucleic acid are noninfectious. Nevertheless, they are self-assembled protein structures mimicking infectious viruses and thus constitute a safe and effective approach for the induction of neutralizing antibodies to surface proteins, where soluble forms of their protein subunits have failed. It has been also reported that viral structures are regarded as a vaccine platform to display foreign epitopes [5].

In general, bacteria, yeast, insect, and animal cells have been applied as cell-based systems to produce VLPs. The bacterial cell cultures have been explored as a VLP production platform with advantages in terms of scalability and production cost [6]. However, bacteria are prokaryotes which lack glycosylation process essential for proper immunogenicity and antigen stability when VLPs are applied as vaccines. In contrast to bacteria, yeast cells have glycosylation apparatus [7]. However, their glycoforms are mainly high mannose type, which is not desirable for the most therapeutic glycoproteins [8]. The matured glycoforms in baculovirus-insect cell system also are mainly high mannose type [9]. The glycosylation of envelope proteins affects their folding and thus is essential for formation and immunogenicity of VLPs [10, 11]. In glycosylation process, bacteria, yeast, and

insect cells have fundamental limitations. The mammalian cells have proper glycosylation apparatus and ability to fold the envelope proteins of virus, which facilitate functional VLPs production. However, the mammalian cell-based systems require manufacturing facilities including fermentation bioreactors for large-scale upstream processing, which is too expensive to establish. This high production cost is a major disadvantage of the mammalian cell-based system. Plants do not need such expensive facilities to produce biomass. Thus, plants are considered as a potential bioreactor system for VLPs with advantages such as low cost of upstream biomass process, flexible production scalability, and the lack of human pathogen contaminants [12, 13]. Nevertheless, plants for VLP production platform are not perfectly acceptable due to relatively lower VLP production level than animal systems and plant-specific *N*-glycosylation of glycoproteins [14, 15]. Development of new plant expression system and advanced *N*-glycosylation engineering overcome such hurdles.

2. Virus-Like Particles in Plant Expression Systems

VLPs can be generated through different types of viral vectors and expression strategies in plants [16, 17]. The plant-derived viral vectors used for VLP expression can be classified into full virus vectors such as the potato virus X (PVX) [18, 19] and the cowpea mosaic virus (CPMV) [20] and the deconstructed vectors such as bean yellow dwarf virus (BeYDV) [21, 22] and MagniCON based on tobacco mosaic virus (TMV) [23, 24]. The earlier plant VLPs were Hepatitis B core antigen (HBcAg) VLPs [25] and Hepatitis B surface antigen (HBsAg) VLPs fused to soybean vegetable storage protein vspA (VSPαS) in transgenic tobacco leaves obtained by *Agrobacterium*-mediated transformation [25, 26]. PVX and CPMV based viral vectors were applied to generate HBcAg VLPs [27]. Transgenic plants using *Agrobacterium*-mediated DNA transfer have been used for the stable gene expression system for VLPs; however the VLP expression level is low [10~24 μg/g fresh leaf weight (FLW)] [27, 28]. The human papilloma virus (HPV) L-1 based VLP vaccines were also successfully expressed and assembled in stable transgenic potato and tobacco [29–31]. Unlike stable transformation, the transient expression with deconstructed geminiviral vectors showed 80 times higher accumulation of HBcAg VLPs in *N. benthamiana* compared to both PVX and CPMV vectors [32]. Another transient expression system using deconstructed TMV-based MagnICON vector agroinfiltration has allowed the production of HBcAg VLPs with a yield of 2.38 mg/g (FLW), almost 3 times higher compared to the geminiviral vector within a short period time [17, 32–35]. The intraperitoneally injected HBcAg VLPs obtained from the MagnICON system efficiently induced immune responses generating HBcAg specific IgGs in mice. These results indicate that, among currently available VLP expression systems, the production of large quantities of VLPs for vaccine applications is more feasible using MagniCON systems. In many cases genetic manipulation of plant-derived VLPs has been performed to modify the external surface of the particle. To this end, the heterologous polypeptide has been fused at the N- or C-terminus of the CP. VLPs can also be exploited as "platforms" for the presentation of foreign epitopes and/or targeting molecules on chimeric VLPs (cVLPs) [1, 2, 6]. Indeed, the VLPs can display multicomponent vaccine candidate epitopes as a fusion form between two different proteins [5]. For instance, the green fluorescent protein (GFP) and the HB surface antigen (HBsAg) S-protein were transiently expressed and heterodimerized with the native HBsAg sequentially forming chimeric VLPs (cVLPs) in *N. benthamiana* [36]. The HBsAg fusion with GFP was showed to be more stable and immunogenic than native HBsAg in *in vivo* mice experiment, indicating that cVLPs can be applied to display heterologous antigens to generate more immunogenic vaccines [5]. The fusion proteins between domain III (DIII) of West Nile virus (WNV) and HBcAg were expressed and displayed as cVLPs with geminiviral transient expression vectors in *N. benthamiana* [37]. In addition, the influenza virus M2 epitope [38] or HPV16 epitopes [27, 39, 40] individually were fused to HBcAg induced strong immune responses generating specific antibodies. The cVLPs displaying both HPV16 E6 and E7 proteins triggered their specific antibodies, respectively [39]. In general, vaccines are administered through intramuscular, subcutaneous, and intravenous injections. In addition, vaccines can be orally or nasally applied to induce mucosal immune responses [17]. Indeed, various results indicate that VLPs can be applied safely as oral vaccines carrying multiple epitopes without needle injection. For example, oral delivery of purified Norwalk virus CP (NVCP) VLPs produced in tobacco and tomato stimulated mucosal and serum immune responses to produce IgA and IgG [41] and oral administration with HBsAg displaying HIV-1 ENV and GAG epitopes provoked strong serum and mucosal antibody responses in mice [42]. These results indicate that VLPs can be applied safely as oral vaccines carrying multiple epitopes without needle injection.

3. Glycosylation of VLP Vaccines

Even though virus-like particles- (VLPs-) based vaccines have shown promising results, commercial production systems are currently limited to eukaryotic cells such as yeast, insect, and mammalian [14]. For instance, Lassa virus (LASV) VLPs cannot be easily produced in bacterial cell systems, because bacteria are incapable of performing glycosylation and other posttranslational protein modifications which are a key feature in most VLP-based proteins [14]. The glycosylation pattern of GP1 and GP2 glycoproteins of Lassa virus (LASV) has been shown to play a critical structural and functional role in preserving protein stability and allowing binding and fusion to host cells [43]. The glycosylation of VLP proteins has major impact on their structure and function, and thus it is important to determine the choice of platforms for their production. As the viral glycoproteins localize, guide, and potentiate the process of enveloped virus assembly, it becomes important to study their individual and combined behavior upon expression in both animal and plant cells, in order to identify domains within the glycoproteins

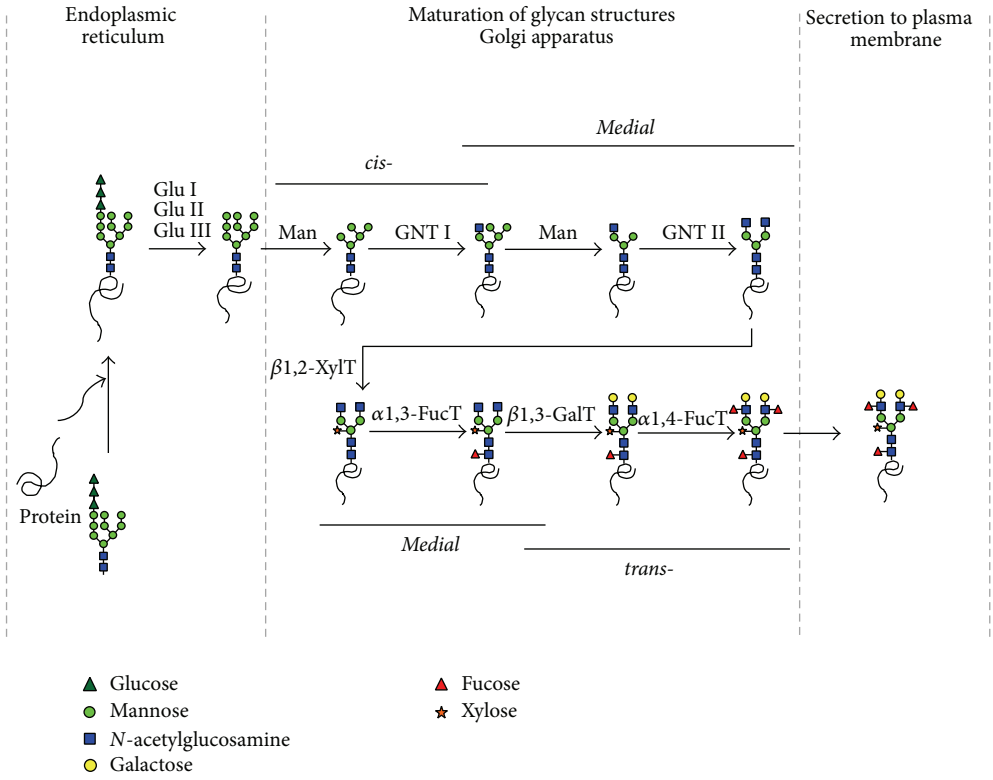

Figure 1: *N*-glycosylation pathway in plant. The primary glycosylation pathways with consequent series of steps occur in different subcellular compartments, ER, Golgi complex within the plant cell. During the pathway, glycosidase digestion and additional glycosyltransferase result in additional different branches and terminal glycan residues. GuI: glucosidase I, GuII: glucosidase II, GuIII: glucosidase III, Man: mannosidase, GNT I: *N*-acetylglucosaminyltransferase I, GNT II: *N*-acetylglucosaminyltransferase II, β1,2-XylT: β1,2-xylose transferase, α1,3-FucT: α1,3-fucose transferase, β1,3-GalT: β1,3-galactosidase, α1,4-FucT: α1,4-fucose transferase.

responsible for the critical differences between the intracellular targeting in either cell system. The large structural protein of lettuce necrotic yellow virus was glycosylated with complex oligosaccharides containing *N*-acetylglucosamine *N*-linked to asparagine residues [44]. The potato virus X CP and PPV CP were also glycosylated [45]. Glycosylated CP of beet western yellows virus plays a role in the virus/aphis interaction and promotes the aphid transmission of the virus [46]. Reviewed earlier, plants offer an attractive alternative system for VLP vaccine production with cost-effective, scalable, versatile, appropriate glycosylation, efficient assembly of VLP, and safety from adventitious human pathogens [12].

Although it yielded encouraging results, expression of VLPs expressed in plants suffers from plant-specific glycosylation of glycoproteins [14, 15]. Most proteins in eukaryotic multicellular organisms including plants are synthesized as glycoproteins with *N*- and *O*-glycosylation, which are important posttranslational protein modifications [47]. *N*-glycans attached to proteins are crucial for protein folding, assembly, and their stability but also involved in cell to cell adhesion, protein targeting, and immune responses as biological activity [48, 49]. During the glycoprotein transportation through the secretory pathway, the oligosaccharide *N*-linked to the asparagine residue (Asn) undergoes several maturation steps involving the removal of glucose and mannose residues by different exoglycosidase to generate high mannose type *N*-glycan in the endoplasmic reticulum (ER) and the Golgi apparatus and, eventually, it is characterized by the addition of new oligosaccharide residues in the Golgi apparatus to form the matured complex type *N*-glycan (Figure 1). In the ER, the first step of *N*-glycosylation of plant proteins is the transfer of the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ from a dolichol lipid to specific Asn residues on the nascent polypeptide chain [50]. Processing of this oligosaccharide into high mannose, complex, hybrid, or paucimannosidic type *N*-glycan occurs during the secretory pathway. Particularly in plant, the β-mannose is substituted by a bisecting β1,2-xylose that is not found in mammalian *N*-glycans, and the proximal *N*-acetylglucosamine of the core is substituted by an α1,3-fucose, instead of an α1,6-fucose in mammalians. In addition, β1,3-galactose and fucose that are α1,4-linked to the terminal *N*-acetylglucosamine of plant *N*-glycans form Lewis a (Le^a) glycosylation (Figure 1) [51]. These modifications are not present in mammalian. Many mammalian complex *N*-glycans have an α1,6-fucose on the first core *N*-acetylglucosamine of *N*-glycan and are characterized by terminal β1,4-galactose and sialic acid which are not observed in plants (Figure 1) [52–54]. Most plant-derived therapeutic proteins are complex glycoproteins requiring posttranslational modifications. The β1,2-xylose,

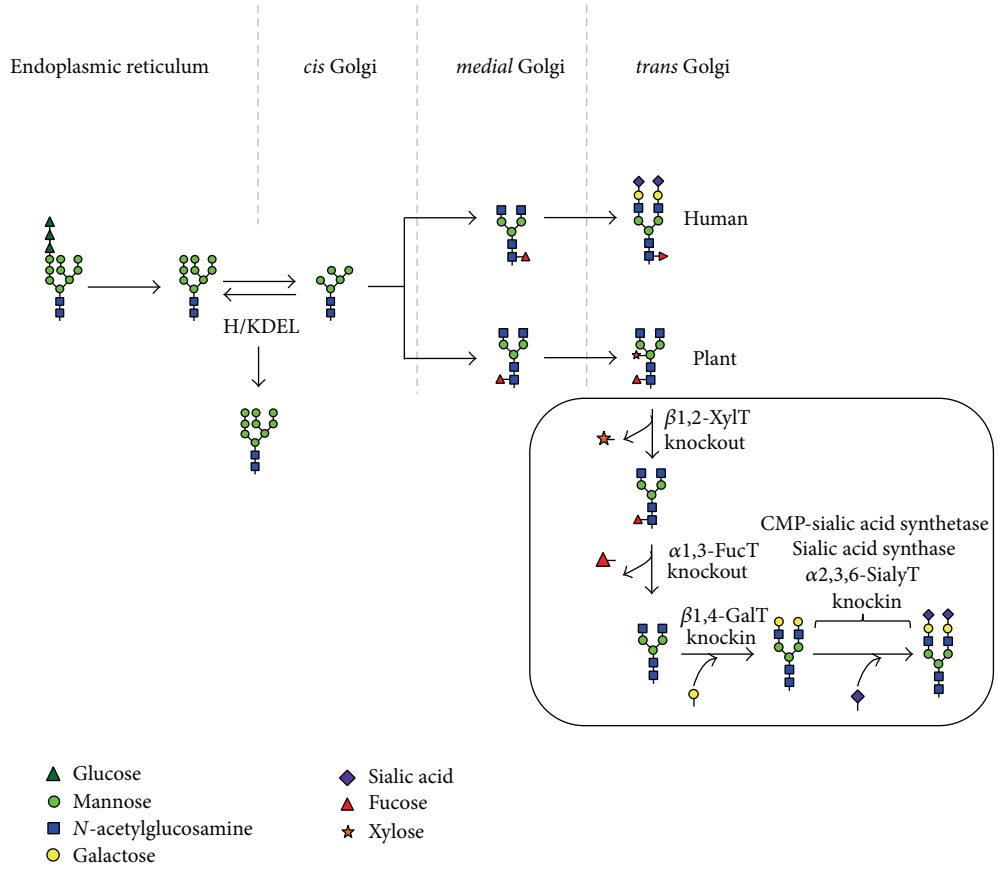

Figure 2: Schematic diagram of humanization of the glycosylation pathway in plant. In ER, protein is glycosylated and three glucoses are removed from the attached glycan. The glycoproteins then are transferred to the Golgi complex where mannoses are trimmed, and the glycoresidues are sequentially attached. When the ER retention signal KDEL sequence is attached to the C-terminal of glycoproteins, glycoproteins are retained and accumulated in the ER. Plant glycans carry β1,2-xylose and α1,3-fucose residues attached to the *N*-acetylglucosamine whereas human glycans contain α1,6-fucose, β1,4-galactose, and α2,3,6-sialic acid. In humanization glycoengineering process the β1,2-XylT and α1,3-FucT should be knocked out to remove xylose and fucose, respectively. The β1,4-GalT should be knocked in to add β1,4-galactose. Furthermore, finally CMP-sialic acid synthetase, sialic acid synthase, and α2,3,6-sialic transferase should be knocked in to attach α2,3,6-sialic acid to the terminal galactose. KDEL: ER retention motif (Lys-Asp-Glu-Leu), β1,2-XylT: β1,2-xylose transferase, α1,3-FucT: α1,3-fucose transferase, β1,4-GalT: β1,4-galactosidase, α2,3,6-SialyT: α2,3,6-sialic transferase.

core α1,3-fucose, and Le^a containing epitopes have been considered as immunogenic glycan epitopes found in plant-specific *N*-glycans. Such glycan residues are not present in humans, and thus proteins could cause immune rejection inducing plant-glycan specific antibodies causing protein clearance in blood stream as well as potential allergenic effects [54–56]. These hurdles can be overcome by recent progress in plant glycoengineering. The plant expression with glycoengineering will allow the novel application of plant-made VLPs, including vessels for the delivery of small therapeutics, DNA fragments, and adjuvants (Figure 2).

4. *N*-Glycomodification in Plants

4.1. Targeted Expression to the ER. *N*-glycan structures influence biofunctionality and stability of therapeutic proteins and even directly affect immunogenicity of glycosylated subunit vaccines displayed on VLP surfaces. In plants, thus, *N*-glycosylation pathway has been modified in order to humanize the glycan structures of glycoproteins [57, 58].

A commonly used approach to express recombinant glycoproteins in plants is their accumulation in ER by addition of C-terminal signal H/KDEL ER retention motif [59]. The ER-retained proteins contain high mannose type *N*-glycans structurally similar between plant and mammalian cells [58, 60, 61]. The high mannose type *N*-glycans are oligosaccharide structures that mammals and plants have in common and thus are probably not immunogenic [62]. This strategy is largely devoid of plant-specific, immunogenic β1,2-xylose and core α1,3-fucose. Additionally, some studies have reported enhanced accumulation of KDEL-tagged proteins in the ER. Such ER retention of proteins usually increases the production level compared to that without KDEL in plant [63,

64]. Plant-derived monoclonal antibody (mAb) with high mannose *N*-glycan structure has shorter half-life than that of the mammalian-derived mAb with mammalian specific glycan structures [65]. However, mAb with high mannose glycans had relatively similar biological activities compared to the mammalian-derived mAb overcoming concerns about plant-specific glycoepitopes expressed by others [58]. In addition, the high mannose type glycan structure would be expected to cause an enhanced immune response through the mannose receptor (MR) on macrophages and dendritic cells recognizing the oligomannose of glycoproteins [66], which is an advantage for vaccine development. According to a previous study [67], the high mannose glycans on antigenic protein can render the protein more immunogenic, producing IgG against the high mannose glycosylated protein.

4.2. Knockout of Plant-Specific Glycosyltransferases. Gene inactivation or silencing may be used to reduce or eliminate the activity of plant- specific glycosyltransferases. In a plant cell, the specific enzymes are β1,2-xylosyltransferase and core α1,3-fucosyltransferase, which are responsible for transfer of the plant-specific xylose and fucose onto the attached *N*-glycan. Such glycan residues are not present in humans and are thus unwanted on proteins intended for therapeutic use. The knockout of the genes that are responsible for the synthesis of these glycan epitopes β1,2-xylosyltransferase and core α1,3-fucosyltransferase provides an easy strategy to solve this problem. The feasibility of this strategy has been proven by the generation of knockout *Arabidopsis thaliana* plant lacking xylosyltransferase and fucosyltransferase [68, 69]. In addition, biological activity assays of such glycoengineered mAbs showed that their antigen binding activity was not altered but significantly enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) effect [70, 71]. Therapeutic antibodies without fucosylation have higher binding affinity for FcγRIIIa than for fucosylated human serum IgG, which is desirable to overcome the interference by human plasma IgG. Thus, the therapeutic antibodies without fucosylation can avoid the inhibitory effect of human plasma IgG on ADCC through their high FcγRIIIa binding affinity.

4.3. Humanization of Plant N-Glycosylation. The immunogenic and allergenic reactions of the β1,2-xylose and core α1,3-fucose *N*-glycan epitopes on plant-derived glycoproteins have been a problem for application of therapeutic proteins produced from plant expression system [72]. Glycoengineering strategies using transgenic plants and the availability of mutant plants lacking xylosyltransferase and fucosyltransferase genes for humanization of *N*-glycosylation allow producing recombinant proteins with more mammalian-like *N*-glycan structures in plant expression system. Most proteins used for therapy of human diseases are glycosylated, and the glycan structures have been shown to affect safety and efficacy of therapeutic glycoproteins [73]. Particularly, nonsialylation significantly causes shorter *in vivo* half-life of circulating glycoproteins, because exposed galactose glycan residues are recognized and captured by asialoglycoprotein receptors resulting in internalization of the glycoproteins in hepatocytes [74]. Terminal acid residues in *N*-linked glycans of most therapeutic glycoproteins affect important roles in *in vivo* physical stability, immunogenicity, and enzymatic activity [68, 70, 71, 74, 75]. Previous studies have demonstrated the importance of fully sialylated *N*-linked glycans and of consistency of homogeneous *N*-linked glycan structures on therapeutic glycoproteins in heterologous expression systems [75–77]. For instance, the sialylated recombinant erythropoietin (EPO) had longer plasma half-life (5-6 h) compared to that (2 min) of desialylated EPO [78]. This beneficial effect of sialic acid on protein stability likely explains why knockin strategies for plant glycoengineering in glycosylation have mainly focused on the addition of terminal β1,4-galactose and sialic acid residues to humanize *N*-glycan in mutant plants lacking plant-specific *N*-glycan residues [75]. It was claimed that plant virus-based transient expression systems can be applied as the knockin strategy of β1,4-galactose and sialic acid transferring genes in the mutant plants, allowing the generation of abundant amount of therapeutic proteins within 1 week after virus infection, provide a feasible advantage over existing glycoprotein expression systems [75].

5. Conclusions

Taken together, plant-derived VLPs are considered safe because plants do not bear human pathogens and promising in terms of cost-effective scalability and speed of production. In fact, as far as upstream and downstream processing are concerned, plant-derived VLPs can take advantage of what has been done so far in the broader field of plant-made pharmaceuticals. Also compared to prokaryotes host cells, plants host guarantees the appropriate posttranslational modifications, such as glycosylation, often needed for proper protein function.

In plants, glycoengineering has been improved to create plants able to perform the ideal glycosylation enhancing efficacy and potency of VLPs-based therapeutics. As described in this review, several strategies, focused on the inactivation and/or addition of key enzymes, can be adopted to decorate tailor-made glycoforms of VLPs in plants. Thus, plant expression systems will be further improved for production of VLPs-based vaccines with respect to their proper glycomodification and the rapid and cost-effective expression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Norovirus Narita 104 Virus-Like Particles Expressed in *Nicotiana benthamiana* Induce Serum and Mucosal Immune Responses

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Narita 104 virus is a human pathogen belonging to the norovirus (family Caliciviridae) genogroup II. Noroviruses cause epidemic gastroenteritis worldwide. To explore the potential of developing a plant-based vaccine, a plant optimized gene encoding Narita 104 virus capsid protein (NaVCP) was expressed transiently in *Nicotiana benthamiana* using a tobacco mosaic virus expression system. NaVCP accumulated up to approximately 0.3 mg/g fresh weight of leaf at 4 days postinfection. Initiation of hypersensitive response-like symptoms followed by tissue necrosis necessitated a brief infection time and was a significant factor limiting expression. Transmission electron microscopy of plant-derived NaVCP confirmed the presence of fully assembled virus-like particles (VLPs). In this study, an optimized method to express and partially purify NaVCP is described. Further, partially purified NaVCP was used to immunize mice by intranasal delivery and generated significant mucosal and serum antibody responses. Thus, plant-derived Narita 104 VLPs have potential for use as a candidate subunit vaccine or as a component of a multivalent subunit vaccine, along with other genotype-specific plant-derived VLPs.

1. Introduction

Noroviruses (NoVs), which belong to the family Caliciviridae, are the leading causes of acute viral gastroenteritis in humans worldwide. NoVs are approximately 38 nm icosahedral viruses and contain a positive-sense, single-stranded, nonenveloped RNA genome of ~7.5 kb [1]. The open reading frame (ORF) 1 encodes nonstructural proteins required for viral replication. ORF2 codes for the capsid protein and the ORF3 encodes a small basic protein that is a minor structural protein included in the virion [2–4]. The major capsid protein can diverge by as much as 60% between genogroups and 20–30% between genotypes within a genogroup. Human NoVs are classified into two distinct genogroups (GI and GII) [5] which are further subdivided into more than 25 different genotypes. The majority of norovirus outbreaks currently are caused by the GII.4 genotypes, which cause significant morbidity and mortality [6]. Several viruses in this category have emerged since 1990s worldwide [7–15]. Thus, we studied the development of a plant-based vaccine against Narita 104 virus (NaV) (NCBI database, accession number AB078336), a GII.4 strain of norovirus [16].

Heterologous expression of recombinant Norwalk virus capsid protein (NVCP) in insect and plant systems showed that the capsid protein can self-assemble into virus-like particles (VLP) that are morphologically and antigenically similar to native NV particles [17–19]. Recombinant Norwalk virus VLP (rNV VLP) elicited VLP-specific systemic (serum IgG) and mucosal (vaginal and fecal IgA) antibodies in mice when administered orally in presence and in the absence

of an adjuvant [18, 20, 21]. The plant-based expression of native NaV capsid protein was poor due to incorrect mRNA processing, but a plant optimized gene enhanced expression [22]. Also, new approaches to achieving high levels of protein using rapid virus-vectored transient expression have been developed, including the tobacco mosaic virus (TMV) system (ICON system) [23–25].

A commercial vaccine to prevent norovirus infection is not currently available. For the successful design of a norovirus vaccine, variability of capsid protein in different genotypes must be taken into account [16, 17, 26–28]. Plants are an economical and safe platform for vaccine development due to ease of scalability and lack of mammalian pathogens [29, 30]. The ICON system allows for rapid and scalable production of various antigens. Thus, within a short period of time multiple vaccine candidates can be expressed and tested for their efficacy [21, 31–34].

In this study, we performed plant-based expression and partial purification of Narita VLP (NaVLP) and further evaluated these VLPs as a candidate vaccine by testing the immunogenicity and induction of GII.4-specific VLP antibody in mice.

2. Materials and Methods

2.1. Construction of Plant Expression Vector. A plant-optimized sNaVCP gene (Genbank accession number GQ389627) from pCRblunt-sNaVCP [22] was introduced into pICH10990 (ICON Genetics, Halle, Germany) to obtain pICHsNaV. The coding sequence in pCRblunt-sNaVCP was end-tailored to create an EcoRI site at the 5′ end using a high-fidelity PCR kit (Roche) with primers sNaCP-eco (5′-GACGAATTCAACAATGAAGATGGCTTCTAATG) and M13RHT (5′-GGAAACAGCTATGACCATG). The resulting PCR product was digested with EcoRI-SacI and the fragment was ligated into pICH10990 digested likewise to yield pICHsNaV (3′ module). The plasmid was sequenced to assure fidelity and mobilized into *Agrobacterium tumefaciens* GV3101. The two modules containing the integrase (pICH14011) and the 5′ module (pICH15879) that mediates cytosolic accumulation were coinfiltrated along with the 3′ module [24]. For expression of NVCP and GFP, the 3′ module vectors pICH-sNVCP and pICH-GFP [21] were used.

2.2. Agrobacterium Delivery Using Vacuum Infiltration. Equal volumes of overnight-grown *Agrobacterium* cultures (3′ module, integrase, and 5′ module), $OD_{600} \approx 1.8$, were mixed and sedimented at 6,000 ×g for 3 min. The pellet was resuspended in 15 mL of a solution containing 10 mM MES (pH 5.5) and 10 mM $MgSO_4$ in order to obtain the final combined OD600 of ~0.1. Four-week-old greenhouse grown *N. benthamiana* plants were submerged upside down in polystyrene cylindrical flasks (6 cm height, 7 cm diameter) containing 2.5 L of the bacterial suspension and transferred to a desiccator. Vacuum (23 in. Hg) was applied for 2 min using a pump (Welch model number 2546B-01) and gently released. Gentle shaking of the desiccator was performed during vacuum application to remove air bubbles. After confirming the success of the infiltration by visual inspection (infiltrated areas of the leaf become translucent when observed against a source of light), plants were placed in the growth room to recover.

2.3. RNA Extraction and Hybridization. Total RNA was extracted from leaves of tobacco (Trizol reagent, Invitrogen) and residual DNA removed by DNA-free system (Ambion). RNA was fractionated on formaldehyde agarose gels and blotted to nylon membranes as described [35]. The membrane was hybridized with digoxigenin-labeled probe synthesized by PCR using primers 3′UTR-F (GAGCTCCCGGGGATCCTCTA) and 3′UTR-R (TTAGGGAGGATTCGAACCTCTCAC) and pICHsNaV as template, as described in the product literature (Roche).

2.4. Protein Extraction. Four days postinfiltration (DPI), *N. benthamiana* leaves were harvested, weighed, flash-frozen in liquid N_2, and stored at −80°C. For total protein extraction, frozen leaves were homogenized by blending in extraction buffer [25 mM sodium phosphate (pH 6.6), 100 mM NaCl, 1 mM EDTA, 50 mM sodium ascorbate, and 10 μg/mL leupeptin] at the ratio of 10 mL per gram of fresh weight of leaf. Homogenates were then immediately filtered through Miracloth (Calbiochem) and centrifuged at 11,300 g for 20 min at 4°C. The supernatant was passed through 0.22 μm filter (Millipore) and kept at 4°C before concentrating using stirred cell (Amicon, model number 8200, Millipore) with 300 kDa cut-off membrane (Ultracel PL30000; Millipore) followed by further filtration through a 30 kDa cut-off membrane (Biomax PB 300,000; Millipore). The retained concentrated extract was used for further analysis.

2.5. SDS-PAGE and Western Blot. Samples were denatured by boiling in SDS-PAGE buffer and resolved using 4–20% gradient polyacrylamide gels and followed by either Coomassie brilliant blue G-250 staining with PageBlue protein staining solution (Fermentas) or electrophoretically transferred to Hybond P membrane (Amersham Pharmacia) for Western blot. Membranes were probed with rabbit polyclonal anti-NaV antibody conjugated to HRP diluted 1 : 5000. The membranes were developed by chemiluminescence using the ECL plus detection reagent (Amersham Pharmacia).

2.6. NaVCP ELISA and Bradford. Plant extracts were analyzed by NaVCP sandwich ELISA. Recombinant NaVCP derived from insect cells using baculovirus system (i-NaV, gift of N. Takeda) served as reference standard. Briefly, rabbit anti-NaV 104 serum was diluted to 1 : 10,000 in 0.01 M phosphate-buffered saline (PBS; 50 μL per well) and coated on the 96-well plate using carbonate high binding buffer for 4 h at 23°C. The plates were then blocked with 5% nonfat dry milk in PBS with 0.05% Tween 20 (DM/PBST) for 1 h at 23°C. After washing the wells once with 1X PBST, samples (50 μL per well) diluted in 1%DM/1X PBST were added and incubated for 1 h at 37°C. The wells were washed three times and incubated for 2 h at 37°C with rabbit anti-NaV104

IgG-horseradish peroxidase conjugate, diluted 1 : 20,000 in 1%DM/PBST. The plate was developed with TMB detection kit (Amersham Biosciences) for 8–10 min at 23°C and the reaction was ended by adding 1 M H_3PO_4. Absorbance was read at 450 nm. For standard curve, i-NaV was diluted serially twofold in 1%DM/1X PBST from concentrations starting at 12.5 ng/mL and processed as above. The leaf supernatants were tested for total soluble protein (TSP) by the Coomassie blue dye-binding assay [36] using reagent (Bio-Rad) with BSA as a standard.

2.7. Sucrose Gradient Sedimentation. The sucrose gradient was performed as described [21] with modifications. Briefly, gradients were generated using 60, 50, 40, 30, 20, and 10% sucrose prepared in 0.01 M PBS and incubated for at least 2 h at 4°C to allow for formation of continuous gradient. The sample (either crude leaf extract or previously purified NaVLP) was layered on the top of the gradient, and tubes were centrifuged at 151,000 g for 2.5 h at 4°C. Fifteen fractions were generated and analyzed by ELISA.

2.8. Electron Microscopy. Pooled sucrose gradient fractions were analyzed by antibody immobilization negative staining and examination by transmission electron microscopy (TEM). Formvar coated Ni grids were coated with rabbit polyclonal anti-NaV antibody (1 : 10 dilution) for 10 min at 23°C. Samples were applied to antibody-coated grids for 10 min. After washing, grids were stained using 2% uranyl acetate for 1 min and examined with a Philips CM-12 TEM.

2.9. Anion Exchange Chromatography. Anion exchange chromatography was performed as described [37] with slight modifications. The concentrated sample was applied to packed columns (1.5 × 12 cm, Bio-Rad) containing DEAE Sepharose Fast flow (GE Healthcare) that was preequilibrated with buffer (25 mM sodium phosphate, 100 mM NaCl, pH 5.8). The flow-through containing NaVCP products were collected separately after washing five times with the same buffer. Final elution was done with high salt buffer (2 M NaCl in 25 mM sodium phosphate buffer, pH 5.8) and collected separately. The flow-through fractions containing most of the NaVCP products were pooled and then concentrated again using 30 kDa cut-off membrane (Centriprep YM-30, Millipore) and analyzed by SDS-PAGE gel and ELISA. The partially purified sample was used for immunization studies.

2.10. NaVCP Immunization in Mice. All animals were housed in American Association for Laboratory Animal Care, approved quarters, and were provided unlimited access to food and water. All procedures were approved by the ASU IACUC and performed in accordance with the Animal Welfare Act. Female 5-week-old BALB/c mice (Charles River, San Diego, CA) were distributed randomly and acclimated for at least 1 week prior to any procedures or treatment. Mice (N = 10/group) were immunized intranasally with NaVCP VLPs (25 μg) alone on days 0 and 21 and compared to mock-vaccinated (PBS alone) controls. Mice were not anesthetized for nasal immunization. Intranasal immunization was performed by using a 20 μL pipet to instill half of the vaccine into each nare (~5–10 μL/nare). Serum was collected during the entire timecourse (days 0, 12, 21, 42, and 56) as previously described [38]. After the animals were humanely euthanized, nasal lavages were collected and processed as previously described [38]. All samples were stored at −80°C for future analysis of antibody titers. Serum and nasal samples were evaluated by ELISA as previously described [38] and outlined above. Sample dilutions included the following ranges: serum (1 : 100–1 : 10,000,000) and nasal samples (1 : 2–1 : 5000). An absorbance value of 0.1 or higher was considered to be positive and the geometric mean titer (GMT) was calculated as the reciprocal of the highest dilution tested that provided a positive absorbance value.

2.11. Statistical Analyses. Statistical analysis was performed using Prism software (GraphPad; San Diego, CA). Geometric mean titer (GMT) values were evaluated statistically at each time point using the Mann-Whitney nonparametric test. Statistical comparisons between the NaVLP vaccination group and the PBS delivery (mock-vaccinated) group were completed and displayed in Figure 4. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Transient Expression of NaVCP in Nicotiana benthamiana. A TMV-based ICON expression vector was utilized for efficient expression of NaVCP. This TMV vector is based on a deconstructed viral system for expression of gene of interest [21, 24, 25, 34]. The NaVCP coding sequence was cloned into the 3′ module, and when delivered in combination with 5′ module and integrase module, the system drives the expression of NaVCP in the cytoplasm. Three days postinfiltration (DPI), total RNA was extracted from the *N. benthamiana* leaf and RNA blot was performed with 3′UTR specific probe. Both NaVCP transcripts (genomic RNA, ~8 kb and subgenomic RNA, ~2 kb) at expected sizes (Figure 1(a)) were detected. The same RNA blot was restriped and reprobed with NaVCP specific probe (data not shown) to confirm the results. Although high expression of foreign proteins has been obtained using the ICON system, NaVCP expression in plants promoted the rapid onset of tissue necrosis at ~5-6 DPI (Figure 1(d)). In spite of the need to harvest at 4 DPI (much earlier than NVCP, [21]), approximately 0.28 mg of NaVCP per g fresh leaf weight was obtained, as quantified by NaVCP ELISA (Table 1). Plant-derived NaVCP was also detected in the Coomassie stained SDS-PAGE gels loaded with crude protein extracts from *N. benthamiana* (Figure 1(b), lane 4) and was confirmed by Western blot probed with polyclonal rabbit anti-NaVCP antibody (Figure 1(c), lane 3). The plant-derived NaVCP comigrated with insect cell-derived NaVCP at the expected size of 58 kDa (Figure 1(b) lane 2 and Figure 1(c) lane 1) and was absent in leaves infiltrated with an empty vector (Figure 1(b) lane 3 and Figure 1(c) lane 2). The insect-derived VLP sample also had a smaller ~50 kDa protein, which is consistent with insect-based expression of various

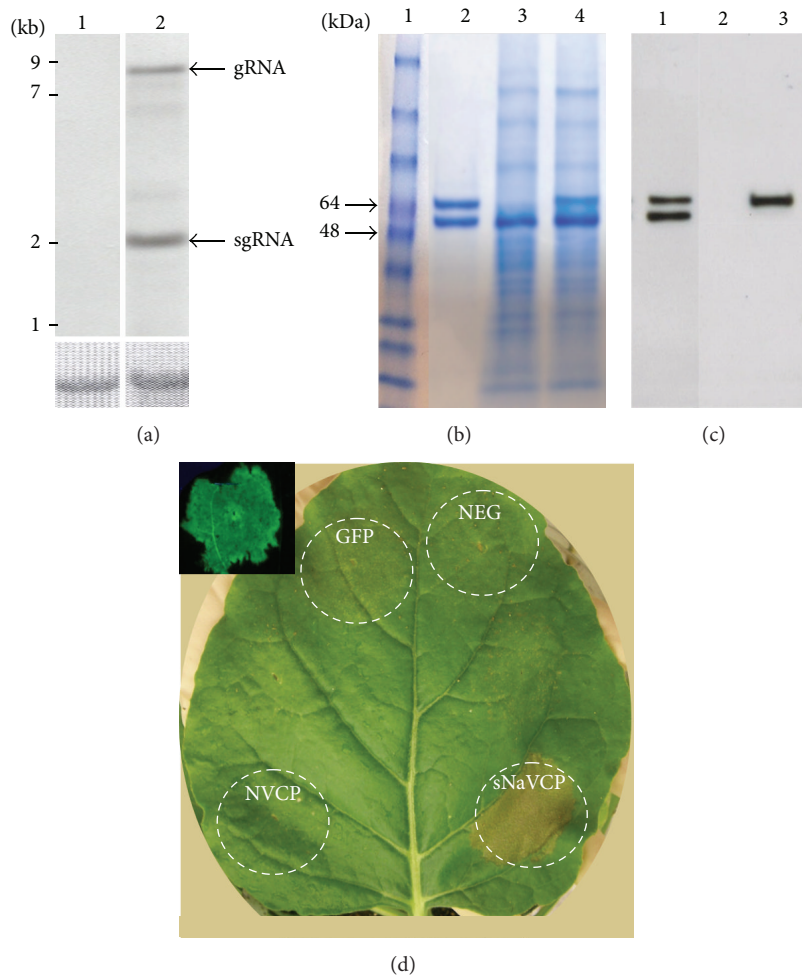


Figure 1: Initial characterization of transient expression of NaVCP. (a) Northern blots of RNA from leaves infiltrated with pICHsNaV (lane 2) showing genomic RNA (gRNA) and subgenomic RNA (sgRNA) of NaVCP after 3 DPI. Lane 1 is noninfiltrated leaf as negative control. Two μg of total RNA was loaded and probed with probe specific to TMV 3′UTR. Ribosomal RNA loading is shown at the bottom. (b) Coomassie stained SDS-PAGE of NaVCP showing presence of NaVCP at expected size ~58 kDa. Lane 1: protein molecular mass markers; lane 2: insect-derived NaVCP (1 μg); lane 3: crude protein extract (15 μg) from leaves infected with empty vector; lane 4: crude protein extract (15 μg) from leaf samples infiltrated with pICHsNaV and harvested at 4 DPI. The 64 kDa and 48 kDa molecular mass markers are indicated by arrows at left. (c) Western blot of SDS-Page of sNaVCP showing presence of NaVCP at expected size ~58 kDa. Lane 1: insect-derived NaVCP (25 ng); lane 2: crude protein extract (10 μg) from leaves infected with empty vector. Lane 3: crude protein extract (10 μg) from leaf samples infiltrated with pICHsNaV and harvested at 4 DPI. (d) Hypersensitive response on pICHsNaV infiltrated region. Part of the leaf that was infiltrated with pICHsNaV displayed cell death 5 DPI whereas such symptoms are absent in infiltrated areas of GFP, NVCP, and empty vector (Neg). Inset shows GFP fluorescence at 10 DPI.

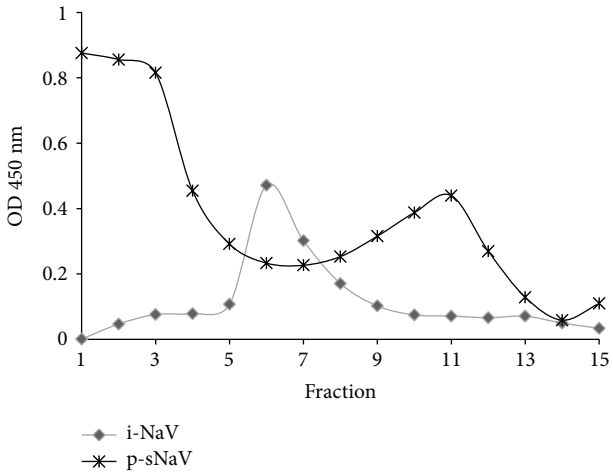
norovirus capsids and attributed to either an alternative translation initiation site or to proteolytic cleavage [39]. Thus, the plant-based system provided an advantage in that only full-length NaVCP was observed.

3.2. Plant-Derived NaVCP Assembles VLPs. Sucrose gradient sedimentation was performed to confirm VLP assembly of plant-derived NaVCP. Crude protein extracts from *N. benthamiana* and insect derived NaVCP were separately layered on 10–60% sucrose gradients. The gradient fractions (1–15) from top to bottom were analyzed by NaVCP ELISA and also confirmed by Western blot on selected fractions (data not shown). The profiles revealed two peak fractions (~2-3 and ~10–12) for plant-derived NaVCP (Figure 2(a)). Interestingly, plant-derived NaVCP showed a faster sedimenting peak at fraction 11 whereas insect-derived NaVCP exhibited a peak at fraction 6. To examine VLP, pooled faster sedimenting

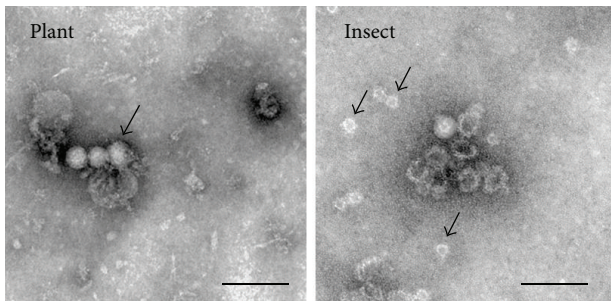
Table 1: NaVCP accumulation in *N. benthamiana* leaf at different times after infiltration with *Agrobacteria*.

DPI	μg/g fresh wt.	% NaVCP/TSP
3	198 ± 11	1.30 ± 0.10
4	285 ± 3	1.89 ± 0.25
5	Necrosis observed	

DPI: days postinfiltration.



(a)



(b)

Figure 2: NaVLP assembly and electron microscopy. (a) Sucrose gradient sedimentation for evaluation of VLP assembly for plant- and insect-derived NaVLP. Crude extracts from *N. benthamiana* infiltrated with pICHsNaV or purified insect-derived NaVLP were sedimented separately on 10–60% sucrose gradients and 15 fractions were collected. Distribution of NaVCP across the gradient fractions was determined by ELISA. The top of the gradient is at left. Fractions 5 to 14 were pooled for EM studies. (b) Visualization by electron microscopy of NaVLP. Left: plant-derived NaVLP partially purified by sucrose gradient, immunocaptured on grids, and negatively stained; arrow indicates 33 nm VLP. Right: insect-derived NaVLP; arrows indicate small ~20 nm VLP. Bars = 100 nm.

fractions (5–14) from plant samples were immobilized using rabbit anti-NaVCP antibody followed by negative staining and visualized by electron microscopy. Both plant and insect-derived samples showed particles that were approximately 33 nm in diameter (Figures 2(b)) and appeared similar to Norwalk VLP [19, 21, 40]. Both samples but especially the insect-derived sample also contained smaller VLP with a diameter of ~20 nm, consistent with the 23 nm Norwalk VLP obtained from insect cells [19]. The slower sedimenting fractions in the plant samples most likely represent VLP assembly intermediates.

3.3. Partial Purification of NaVCP from N. benthamiana. In order to conduct immunization studies, various methodologies for purifying plant-derived NaVLP were investigated. In a previous study [19, 21, 40], pH 5.7 precipitation of

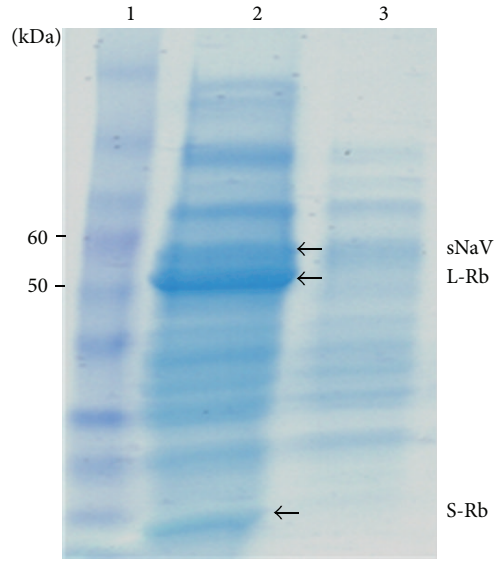


Figure 3: Partial purification of NaVCP expressed in *N. benthamiana* leaf. Coomassie stained gel showing partially purified NaVCP after DEAE chromatography. Lane 1: protein molecular mass markers; lane 2: concentrated plant extract before DEAE chromatography; lane 3: DEAE chromatography flow-through containing NaVCP. sNaV: Narita virus capsid protein; L-Rb: large subunit of Rubisco; S-Rb: small subunit of Rubisco.

Table 2: Extraction efficiency of NaVCP using different pH for buffer.

pH	μg/g fresh wt.	% NaVCP/TSP
5.7	53 ± 18	0.7 ± 0.15
6.6	133 ± 22	1.4 ± 0.16

plant proteins, especially ribulose bisphosphate carboxylase (Rubisco), was used in an attempt to partially purify NVCP. However, utilizing a similar strategy resulted in substantial loss of NaVCP as compared to NVCP. When the pH of the extraction buffer was raised from pH 5.7 to pH 6.6, the concentration of extracted NaVCP was much higher in the soluble fraction (Table 2) and minimal loss (less than 10%) was observed in the insoluble pellet (data not shown). The supernatant was concentrated by filtering through a 300 kDa cut-off membrane to remove some of the plant endogenous proteins followed by 30 kDa cut-off membrane to concentrate the extracted NaVCP.

The final concentrated extract was further fractionated using anion exchange chromatography to yield partially purified NaVCP extract. The majority of the NaVCP was contained in the flow-through fraction (Figure 3, lane 3), while the two major endogenous plant proteins, large and small subunit of Rubisco, which comprise ~50% of the leaf protein, were lacking in the flow-through fraction. Rubisco subunits were finally eluted in the high salt buffer. The flow-through fractions were pooled, concentrated further, and used for immunization studies. The DEAE purified NaVCP used for animal studies was ~5% of total soluble protein (TSP) as measured by ELISA.

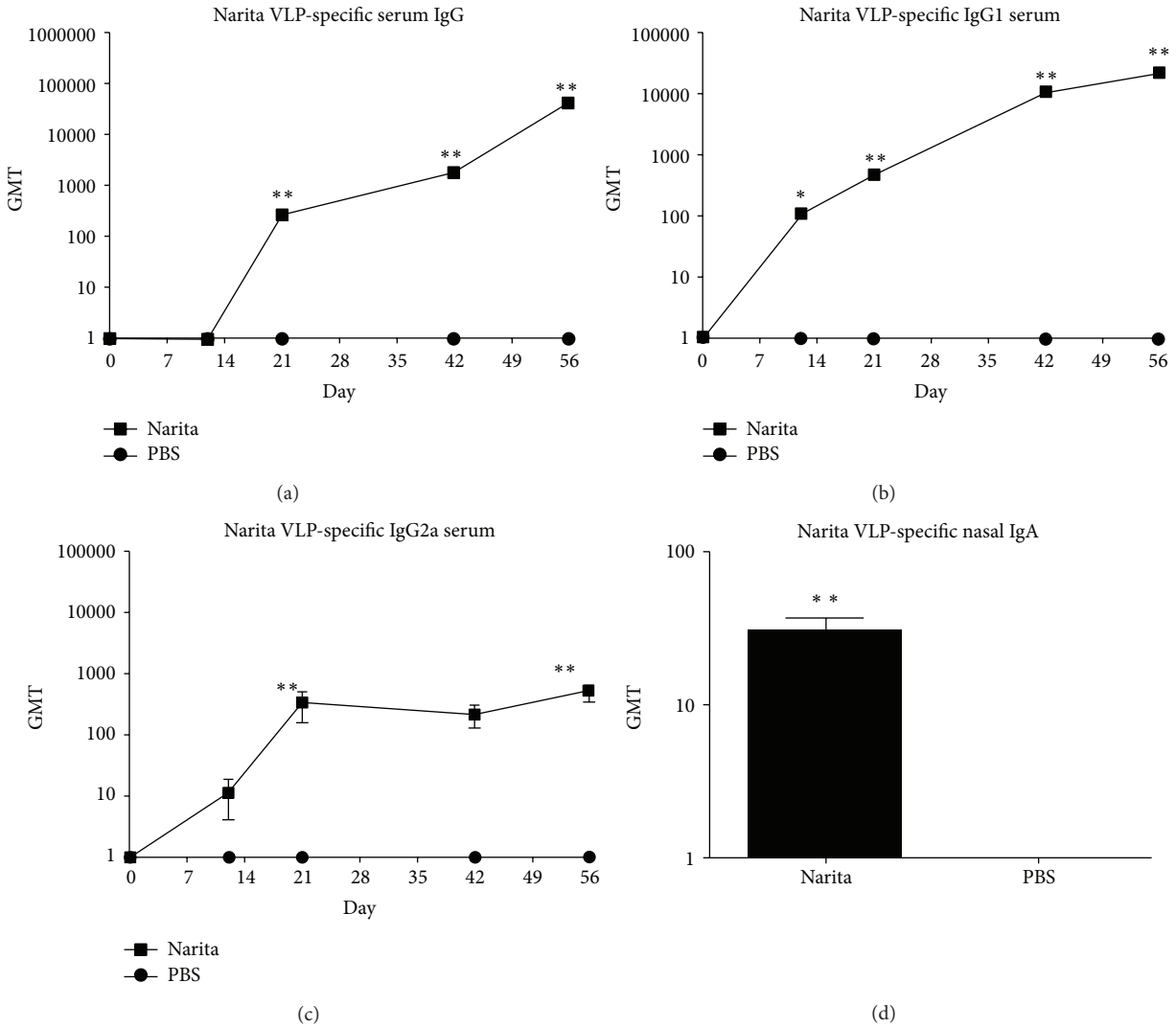


Figure 4: Intranasal immunization with NaVLP induced significant serum and mucosal VLP-specific antibody production in mice. Female BALB/c mice ($n = 10$ per group) received intranasal delivery of plant-derived NaVCP [25 μg] on day 0 and day 21. Time course analysis of VLP-specific serum IgG and IgG isotypes ((a)–(c)) and nasal IgA (d) responses in mice following intranasal delivery of plant-derived NaVLP. Serum IgG, IgG isotypes, and nasal IgA were measured by ELISA. The y-axis shows the geometric mean titers (GMTs) and the error bars show the standard error of the mean. A P value of <0.05 was considered significant. $^{*} < 0.01$ and $^{**} < 0.001$.

3.4. Intranasal Vaccination with NaVCP Induces Serum and Mucosal VLP-Specific Antibody Responses. To evaluate the immunogenicity of the plant-derived NaVCP, female mice were intranasally vaccinated with NaVLP (25 μg) or mock-vaccinated (PBS treated) on days 0 and 21. Serum was collected on days 0, 12, 21, 42, and 56 after vaccination and evaluated for VLP-specific antibody production. Nasal washes were collected at the end of the study (day 56) following euthanasia. By day 21, a significant amount of Narita VLP-specific antibodies was produced in the antigen-containing group relative to the mock PBS controls (Figure 4(a)). The amount of VLP-specific IgG and IgG isotype antibody in the serum continued to rise over the period of the study through day 56 (Figures 4(a)–4(c)). Consistent with studies using NV VLP alone (without adjuvant), IgG1 levels were much higher relative to the IgG2a isotype indicating a predominately Th2 response [38, 41]. A significant amount of VLP-specific nasal IgA production was observed in the NaVCP vaccinated mice relative to the mock-vaccinated controls (Figure 4(d)), indicating a robust local response. Thus, NaVLP delivered intranasally resulted in significant levels of VLP-specific antibody at serum and mucosal sites (Figure 4).

4. Discussion

VLPs have been used as vaccines as a safer alternative to attenuated live or inactivated killed viruses. VLP-based vaccines have been successful in the clinic; for example, there are two commercially available HPV VLP-based vaccines [42]. Recombinant Norwalk (rNV) VLPs are being investigated as vaccine candidates in the clinic as the VLPs are morphologically and antigenically similar to Norwalk virus as

demonstrated by electron microscopy and ELISA [17, 20, 43, 44]. Initial studies using insect-derived rNV VLP expressed via baculovirus expression system were immunogenic in mice [17, 20, 45]. Using adjuvants and formulations can significantly enhance the mucosal immunogenicity of plant-based VLP vaccines as recently shown [38, 41, 46].

For the last decade, plant-based vaccines have gained steady acceptance in the scientific community. Plant-based expression is a cost-effective and convenient system for antigens to be used in mucosal delivery. Plants represent relatively safe platforms to express vaccines since they are free of mammalian pathogens that affect other production systems such as transgenic animals and cell lines [29, 30]. Norwalk VLPs have been previously shown to accumulate in transgenic potato [18], tobacco [18, 21], and tomato [40, 47]. Very high expression (0.8 mg/g leaf) of NV VLPs was attained in transient assay using viral vectors TMV-based expression system in *N. benthamiana* [21]. In this study, feasibility of high-level expression of norovirus Narita 104 was explored for use as subunit vaccine. Narita 104 virus is a GII.4 strain of norovirus similar to those responsible for recent outbreaks [48].

NaVCP was expressed using a replicating plant virus based strategy to yield high levels of protein expression [24]. In this strategy, NaVCP was inserted among viral replicating elements, amplified episomally, and subsequently translated in the cytosol of plant cell. Expression in *N. benthamiana* required partial purification of the protein prior to using it in immunization studies. A high level of expression of foreign protein (0.5–5 mg recombinant protein per g of leaf biomass) has been obtained with the TMV based system with no signs of necrosis on the infiltrated plants [49]. NVCP, which is similar to NaVCP, was expressed at 0.8 mg/g leaf in *N. benthamiana* at 12 DPI and necrosis was not observed even after 22 DPI [21]. However, expression of NaVCP was limited due to rapid emergence of a hypersensitive response-like symptom followed by cell death by 5 DPI. In spite of this shorter infection time, expression of NaVCP was reasonably high, attaining 0.3 mg/g leaf. A similar response was observed when expression of NaVCP was driven by a geminiviral vector system [39] in *N. benthamiana* (Mathew, LG., and Mason HS., unpublished data). In spite of the cell death response, expression observed with the TMV system was approximately 55-fold higher than the transient expression in *N. benthamiana* leaf using a nonreplicating vector system (Mathew, LG., and Mason, HS. unpublished data).

The rapid induction of a leaf cell death response by NaVCP is interesting, since the related NVCP showed no such response [21]. The capsid proteins of Norwalk and Narita 104 viruses are substantially divergent, with amino acid sequences 45% identical and 60% similar over the entire length. The capsid proteins have similar protein structures comprising shell (S) and protruding (P) domains [50]. The S domains are more highly conserved, and the P1 subdomains are moderately well conserved. However, the most surface-exposed P2 subdomains are the most variable, and NaVCP has a 10-aa insertion "QTGQNTK-FTP." We performed P2 subdomain swapping experiments; however, when the chimeric capsid proteins were expressed in *N. benthamiana,* the results were inconclusive and failed to show convincing evidence of the direct involvement of P2 in the induction of cell death by NaVCP (Mathew and Mason, unpublished data). Nonetheless, it seems likely that the sequence divergence is related to the great difference in induction of the cell death response and could be mediated by host cell defense responses that recognize a particular pattern in NaVCP.

The evidence that plant-derived NaVCP assembles into NaVLP strengthens the finding that plant-derived NaVCP can be used as a vaccine candidate. The observation that plant- and insect-derived NaVLP showed different sucrose gradient sedimentation profiles may be explained in several ways. The electron micrograph of insect-derived NaVLP showed that most particles appeared to be either partially assembled, characterized by a deeper electron dense stain, or small 20 nm VLP (Figure 2(b) right). The plant-derived NaVLP appeared to be mostly fully assembled with rare occurrence of 20 nm VLPs (Figure 2(b) left). The partially assembled and smaller 20 nm VLPs were likely slower sedimenting than the plant-derived VLPs. Another possibility is that plant-derived VLP formed aggregates, perhaps by interaction with endogenous cellular material and, therefore, sedimented faster. Further studies need to be conducted in order to understand the difference in the sedimentation pattern from insect-derived and plant-derived NaVLP.

In spite of the relatively shorter infection period due to induction of hypersensitive response, useful yields of NaVCP were obtained. The crude extract had a significantly large amount of endogenous plant protein, including Rubisco, which constitutes ~50% of the total leaf protein [51]. A strategy using pH 5.7 extraction buffer [21], resulted in substantial loss (almost half) of the NaVCP protein in the supernatant (Table 2). The size exclusion concentration aided in the removal of plant endogenous protein and some partially assembled particles. However, the final concentrate did show a substantial amount of Rubisco, both large and small subunits (Figure 3, lane 2). Most of the Rubisco was removed in the final anion exchange chromatography by maintaining the buffer at acidic pH (Figure 3, lane 3).

The partially purified NaVLPs were intranasally delivered to mice to determine the level of immunogenicity both systemically and at the local mucosal immunization site. In the absence of adjuvant, nasal immunization resulted in the significant induction of VLP-specific antibodies in serum and nasal secretions. Therefore, this GII.4-specific vaccine candidate was immunogenic, thus validating the use of plant-derived NaV as a norovirus vaccine or component for a multivalent formulation. Further, we have seen no difference in antibody responses produced by insect cell-derived and plant-derived norovirus VLP prepared similarly and delivered at comparable doses (data not shown). Another indication of the biosimilarity of plant and insect cell-derived VLP is that mice immunized with tomato-derived Norwalk VLP were strongly boosted using insect cell-derived VLP [39].

Although the VLP sample was relatively impure (Figure 3), the robust immune responses in vaccinated mice

suggest that contaminating leaf proteins did not impair the immunogenicity of VLP. Additional studies will need to be conducted to optimize the purification, formulation, delivery route, and immunogenicity of this VLP-based vaccine candidate. There is no small animal model or in vitro propagation assay for human norovirus; therefore, it would be difficult to determine if the antibody levels generated in this study would correlate to protection from challenge. For the purpose of this study we aimed to determine the immunogenicity of the GII-specific norovirus VLP vaccine candidate. Future studies will optimize this formulation and test for blockade of VLP binding to histoblood group antigens specific for this genogroup of norovirus.

In conclusion, our plant-based expression technology provided a convenient platform for expedient and cost-effective production of immunogenic recombinant NaVLPs for vaccine studies. Future preclinical studies that include plant-derived GII-4-specific VLP within a multivalent vaccine will be required to determine if this approach can be exploited for generating a protective norovirus vaccine formulation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Lolita George Mathew and Melissa Herbst-Kralovetz equally contributed to this study.

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Research Article

Soybean Seeds: A Practical Host for the Production of Functional Subunit Vaccines

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Soybean seeds possess several inherent qualities that make them an ideal host for the production of biopharmaceuticals when compared with other plant-based and non-plant-based recombinant expression systems (e.g., low cost of production, high protein to biomass ratio, long-term stability of seed proteins under ambient conditions, etc.). To demonstrate the practicality and feasibility of this platform for the production of subunit vaccines, we chose to express and characterize a nontoxic form of *S. aureus* enterotoxin B (mSEB) as a model vaccine candidate. We show that soy-mSEB was produced at a high vaccine to biomass ratio and represented ~76 theoretical doses of human vaccine per single soybean seed. We localized the model vaccine candidate both intracellularly and extracellularly and found no difference in mSEB protein stability or accumulation relative to subcellular environment. We also show that the model vaccine was biochemically and immunologically similar to native and recombinant forms of the protein produced in a bacterial expression system. Immunization of mice with seed extracts containing mSEB mounted a significant immune response within 14 days of the first injection. Taken together, our results highlight the practicality of soybean seeds as a potential platform for the production of functional subunit vaccines.

1. Introduction

The use of transgenic plants to express recombinant proteins has gained popularity over the past decade and represents a growing segment in the pharmaceutical industry. Currently, the bulk of biopharmaceuticals are produced in recombinant microbe expression systems or insect and mammalian cell cultures. However, as with all protein expression systems, there are advantages and disadvantages to these systems which are described in several review articles [1–3]. Some of these limitations include the types of proteins that can be produced and in the posttranslational processing that can be achieved making production costs prohibitively high. Based on these limitations, an increased demand for biopharmaceuticals will require improved and cost effective manufacturing practices and practical transportation methods for a global community.

As an alternative to traditional systems, a number of pharmaceuticals have been successfully produced in various plant-based expression systems. Although these plant systems offer great potential, they too present several challenges. Many crop systems used to date have a low protein content which can increase the overall production costs since purification expenses are typically inversely proportional to final target protein concentration in plant biomass. Therefore, crops with higher protein content and a compact biomass are more cost effective for molecular farming. When it comes to express large amounts of a pharmaceutical protein in a plant host, soybean should be considered as a practical alternative. The soybean system has many distinct advantages when compared with existing expression systems. For example, soybeans contain ~40% protein by dry mass and therefore represent one of the richest natural sources of protein known. Given this high protein content, it is possible to express large amounts of transgenic protein in a single soybean seed. Furthermore, with typical transgenic expression levels of 1–4% of total soluble protein (TSP), there are few, if any, host systems that can produce such levels of foreign

protein based on weight. Second, soybean is a relatively easy and inexpensive plant to grow making the production of biopharmaceuticals in soybeans extremely cost effective. Another advantage of soybean is the proven stability of proteins in dry mass over extended periods of time, suggesting that pharmaceuticals could be shipped as crushed seed or processed powder and stored under ambient conditions, thus eliminating any requirement for a cold chain. Soybean also possesses the necessary machinery for eukaryotic post-translational modification [4] and is capable of generating large and complex recombinant proteins (>600 kDa) that are often recalcitrant to expression in traditional expression systems [5]. Given these advantages, soybean represents a practical host for the production of proteins for numerous applications.

Soybean-based vaccines, in particular, offer specific advantages over vaccines produced in other, more conventional systems. For example, soy-based vaccines could either be formulated into consumables for oral delivery or purified for injection or other downstream uses. In an effort to demonstrate the practicality of soybean seeds as a host system for manufacturing protein-based vaccine candidates we chose to express a nontoxic form of Staphylococcal enterotoxin B (SEB) as a model vaccine candidate. SEB is a well-characterized, superantigen-like exotoxin produced by the bacteria *Staphylococcus aureus*. SEB mediates its toxicity by linking MHC class II molecules with T cell receptors outside of the antigen binding site [6]. Clinical symptoms of SEB poisoning include anorexia, nausea, vomiting, and diarrhea. Three-dimensional structures of SEB and its complex with MHC class II molecules have been elucidated [7, 8] and several biochemical studies have offered clues to the biologically important regions of this protein [9–12]. While SEB remains a CDC Category B toxin, there is still no vaccine for SEB poisoning in the market.

Due to the inherent superantigen properties of SEB, the native toxin cannot be used as a practical vaccine antigen. However, mutated forms of the protein that remove superantigenicity while leaving immunogenic capacity intact should serve as a viable vaccine option. Such alterations can be accomplished using chemical treatment or genetic manipulation to introduce site specific mutations [13, 14]. Various mutagenesis studies have identified important mutations that reduce or eliminate biological activity of the wild-type toxin while retaining immunogenic epitopes that elicit protective antibody responses [10, 12, 15]. Specifically, single mutations of key residues in the hydrophobic binding loop (L45R), polar binding pocket (Y89A), and disulfide loop (Y94A) in recombinant forms of SEB eliminated binding to the MHC class II receptor [15] but did not disrupt native structure and generated effective immune responses. This triple mutant form of SEB (mSEB) possessed greatly diminished biological activity and was reported to be tolerated as a vaccine in both mice and nonhuman primates. The triple mutant also offered protection to immunized animals when challenged with native SEB (nSEB) [14–16]. Synthetic mSEB has also been used as a model antigen and overexpressed in tobacco (NT1) cells using a geminivirus-based replicon system [17].

In this study we engineered a model vaccine candidate to demonstrate the practicality of soybean as a platform for the production of vaccine candidates and other therapeutics. Two well-characterized plant promoters were used to target expression to seeds, and different signal peptides were included to evaluate accumulation in different subcellular locations. We found that all recombinant forms of the model mSEB vaccine were expressed at a high vaccine to biomass ratio and were accurately processed by the plant machinery. We show that a soy-mSEB vaccine candidate was biochemically equivalent to, and exhibited immunological properties that were analogous to, those exhibited by *E. coli*-derived mSEB and native SEB. Functionality of soy-mSEB was shown in groups of mice immunized with the model vaccine candidate. Taken together, these findings demonstrate the practicality of soybean as a cost-effective host for the production of important vaccine candidates.

2. Materials and Methods

2.1. Seed-Specific SEB Expression Cassette Design and Construction. Soybean codon optimized mutant SEB genes containing sequences encoding the native SEB N-terminal signal peptide sequence and the native soybean glycinin N-terminal signal peptide sequence were synthesized from GeneArt (Life Technologies Carlsbad, CA) and DNA 2.0 (Menlo Park, CA), respectively.

Restriction endonuclease NcoI and XbaI sites were engineered on the 5′ and 3′ termini to facilitate subcloning. Following digestion with NcoI and XbaI the synthetic genes were isolated from an agarose gel and ligated into linearized pPTN200 [19]. The resulting pPTNST108 construct contained the 7S β-conglycinin promoter, Tobacco Etch Virus (TEV) translational enhancer, native SEB signal peptide, mutant (L45R, Y89A, Y94A) SEB open reading frame (ORF), and 35S terminator. The construct pPTN764 contained the soybean 11S glycinin promoter and signal peptide sequence, an identical mutant SEB ORF, and 35S terminator elements. Both constructs included a cassette encoding for phosphinothricin acetyltransferase (bar gene) under the control of the nopaline synthase (nos) promoter and terminator elements. Following subcloning, the identity of both constructs was confirmed using multiple restriction digestion analyses. Integrity of the mSEB ORF was verified by double-stranded sequencing (Davis Sequencing, LLC, Davis CA). Soybean transformations were carried out as previously described [20–22].

2.2. Preparation of Genomic DNA and PCR. Genomic DNA was prepared from cotyledon tissue using the Maxwell 16 Instrument and the Maxwell Tissue DNA Purification Kit (Promega, Madison, WI). Duplex PCR reactions were carried out using GoTaq Flexi DNA polymerase (Promega, Madison, WI) with the following primers: SEB forward (5′-GGACAAGCGCCTCTTCATCTC-3′), SEB reverse (5′-AGGTACACCTCGATCTTCACG-3′), VSP (vegetative storage protein) forward (5′-GCTTCCACACATGGGAGCAG-3′), and VSP reverse (5′-CCTCTGTGGTCTCCAAGCAG-3′).

Following an initial denaturation step at 95°C for 5 minutes the reactions were subjected to 35 cycles comprising denaturation at 95°C for 30 sec, annealing at 52°C for 45 sec, and extension at 72°C for 1 min. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

2.3. Seed Protein Extracts and Western Blot Analysis. Soluble seed protein was extracted from either seed chips or ground seed powder using an extraction buffer of phosphate-buffered saline (PBS) and sonication for 20 seconds. Samples were clarified from soluble debris by centrifugation and protein concentrations were determined with the Bradford Reagent (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as a standard.

Soluble protein extracts (3 μg) were subjected to 10% SDS-PAGE under nonreducing conditions. Unless noted, SDS sample buffer did not contain β-mercaptoethanol. Samples were transferred in 1x CAPS buffer (N-cyclohexyl-3-aminopropanesulfonic acid, pH 11) containing 10% methanol to Immobilon-P membrane (Millipore, Bedford, MA, USA). Membranes were blocked overnight with 5% nonfat milk in 1XPBS at 4°C, followed by Western analysis with an in-house primary antibody (1 : 5000) and goat anti-rabbit IgG HRP secondary antibody (1 : 5000). Immunodetection was carried out using the SuperSignal West Pico substrate kit (Thermo Scientific, Rockford, IL, USA). For protein visualization, membranes were stained with Coomassie blue for 1 minute followed by destaining.

2.4. Quantification of Recombinant Protein in Seed Extracts. Quantification of recombinant soy-mSEB protein expression within seed extracts were determined by Western blot analysis. Protein extracts from a master mix of seed powder consisting of 100 seeds from the T3 generation of ST108 were compared to known amounts of purified recombinant (*E. coli*) mutant SEB standards by Western blot as described above. X-ray films of the results were scanned for densitometric analysis. Integrated density was determined using ImageJ software. A standard curve was plotted using the integrated densities of known mSEB standards. A best-fit standard curve was used to determine the amount of SEB in seed extracts. Theoretical vaccine yields were estimated based on the amount of soluble protein from a starting biomass of 1 L of soy powder (approximately 800 grams) as previously described [23]. Assumptions included 160 mg dry weight of an average soybean seed, 40% seed protein composition, 1.2% expression level for mSEB, and 10 μg for a single human vaccine dose, which is similar to the dose recommended for recombinant hepatitis B surface antigen immunizations [24].

2.5. Protein Characterization and N Terminal Sequencing of SEB Fragments. Soybean mSEB was immunoprecipitated using anti-SEB antibodies and protein-A agarose beads (Sigma-Aldrich, St. Louis, MO). The immunoprecipitated protein was electrophoresed on a 10% SDS-PAGE preparative gel in the absence of β-mercaptoethanol and soy-mSEB protein was eluted from the gel after Coomassie staining. Eluted protein was dialyzed against PBS and concentrated by using centriplus YM-3 centrifugal filter devices (Millipore, Bedford, MA). Concentrated protein was then mixed with SDS-PAGE sample buffer containing βME, electrophoresed on a 12% SDS-PAGE gel and immobilized onto Immobilon-PVDF membrane. The membranes were stained with Coomassie blue for 1 min, followed by destaining and extensive washes with water. Bands of interest were excised for protein sequencing (Iowa State University protein sequencing facility) of N-terminal amino acids. For signal peptide cleavage prediction, full length amino acid sequences were entered into SignalP 4.1 software [18].

2.6. Confocal Microscopy. Whole seed tissue was imbibed for 12 hours in 1XPBS and fixed as described previously [5, 22, 25]. Briefly, sections were permeabilized with 1XPBS containing 0.2% Tween-20 for 10 minutes, followed by blocking in 1XPBS supplemented by 3% BSA overnight at 4°C. Tissue was incubated with rabbit anti-SEB serum (1 : 200) for 4 h at 23°C, followed by incubation with an Alexa Fluor 594 goat anti-rabbit antibody (1 : 200) for 1 h at 23°C. Lastly, tissue sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 5 minutes at 1 : 500 and cover slips were mounted using Gel/Mount aqueous mounting media. Images were collected with a LSM 710 Spectral Confocor 3 Confocal Microscope (Carl Zeiss, Inc.) under 20x magnification and a 405 nm laser to visualize nuclei stained with DAPI in conjunction with a 561 nm laser to collect emitted fluorescence from the Alexa Fluor 594 antibody. Stacks of images (26 optical sections, 20 nm apart) were collected in the Z plane of the specimens and projected to form a single image using the ZEN Light Edition software.

2.7. ELISAs. Three different antibodies were used for ELISAs: one used a rabbit polyclonal anti-SEB antibody (generated in house against *E. coli*-derived mSEB) at a concentration of 1 : 500; a second used a commercial HRP-conjugated sheep anti-SEB polyclonal antibody (Abcam number ab15925) at a concentration of 1 : 1000; and a third used a mouse monoclonal anti-SEB (Abcam number ab6064) at a concentration of 1 : 1000. Microtiter plates were coated with 100 ng/well of each protein (soy-mSEB, rSEB, nSEB, or cholera toxin as a control) in 100 μL of 0.1 M bicarbonate buffer (Ph 8.0) at 4°C overnight. Plates were washed in 1XPBS, 0.1% Tween-20, and blocked with 2% BSA for 1 hour. After a second wash detection antibodies were added for 2 hours at room temperature. The in-house anti-SEB ELISA was washed and an anti-rabbit IgG-HRP conjugate was added for 2 hours at room temperature followed by another wash and the addition of the TMB substrate. The commercial HRP conjugated polyclonal anti-SEB ELISA was washed and incubated with TMB substrate (BioFX). The commercial monoclonal anti-SEB ELISA was washed and incubated with HRP-conjugated anti-mouse IgG for 2 hours at room temperature followed by a final wash and the addition of TMB. All reactions were stopped using 0.5 M sulfuric acid and absorbance was read at 405 nm. Absorbance values have not been background subtracted for any of the values given and data are represented as mean ± standard deviation.

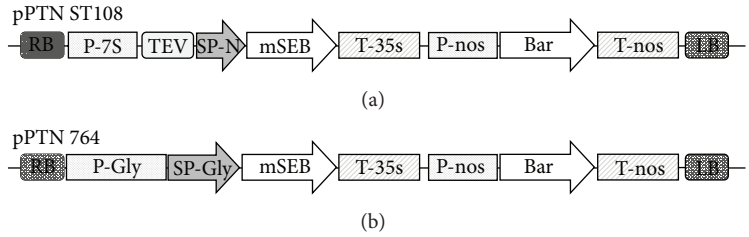

Figure 1: Gene construct design. (a) The pPTN ST108 binary vector used for Agrobacterium-mediated transformation comprising the following regulatory elements: 7S soybean β-conglycinin promoter (P-7S), tobacco etch virus translational enhancer element (TEV), native SEB bacterial signal peptide (SP-N), mutant SEB gene (mSEB), and 35S cauliflower mosaic virus terminator element (T-35s) followed by the selectable marker cassette (nopaline synthase promoter (P-nos), phosphinothricin acetyltransferase gene (bar), and nopaline synthase terminator element (T-nos)). (b) The pPTN 764 binary vector contained soybean 11S glycinin promoter (P-Gly), soybean glycinin signal peptide (SP-Gly), mSEB, and T-35S, followed by the selectable marker cassette. Arrows show orientation of cassettes relative to the right border (RB) and left border (LB) sequences.

2.8. Immunization of Mice and Detection of Antibody Titers. Seed extract containing approximately 10 μg of the target soy-mSEB was emulsified in an equal volume of either Complete Freund's adjuvant (primary immunization) or incomplete Freund's adjuvant (booster immunizations). Preimmune serum was collected prior to the first injection and 1 day prior to each booster immunization from groups ($n = 4$) of 4-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME). Intraperitoneal immunization with seed extract plus adjuvant (10 μg CT) took place on day 0 with boosts on days 14 and 28. To determine anti-SEB titers in sera of immunized mice, microtiter plates were coated with 20 ng/well of native SEB (Toxin Technology, Sarasota Florida) in 100 μL of carbonate buffer at 4°C overnight. Wells were blocked with 3% BSA in PBS. After washing, sera were tested using serial 3-fold dilutions beginning at 1 : 1000 and were incubated for 3 hours at 23°C followed by washes. An HRP-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) was added for two hours at 23°C. Following washes, plates were incubated with TMB substrate (BioFX) and enzymatic reactions were stopped with the addition of 0.5 M sulfuric acid and absorbance was read at 405 nm. Absorbance values represent serum diluted at 1 : 27,000 and have not been background subtracted and data are represented as mean ± standard deviation.

3. Results

3.1. Molecular Characterization of Transgenic Events. A synthetic mSEB gene was codon optimized for expression in *Glycine max* and used to create the binary vectors pPTNST108 and pPTN764 (Figure 1). The pPTNST108 construct contains the native *S. aureus* SEB signal peptide sequence and an open reading frame encoding a triple mutant SEB cloned downstream of the soybean β-conglycinin promoter. The pPTN764 construct contains an identical mutant SEB open reading frame cloned downstream of the native soybean glycinin promoter and signal peptide elements.

Agrobacterium-mediated transformation was used to transform soybean somatic embryos. A total of 25 separate transgenic events were obtained using pPTNST108 and 12 transgenic events were obtained using pPTN764. These events were taken to maturity and all appeared to be phenotypically similar to wild-type nontransgenic control plants. A large-scale molecular screen involving duplex PCR and Western analysis was used to identify specific progeny and lines to be moved forward. A representation of the data generated by the molecular screen is shown in Figure 2.

T1 seeds derived from each transformation event were collected and cotyledon chips were prepared from 8 individual seeds. For duplex PCR, genomic DNA was incubated with primers designed to amplify a diagnostic 796 bp soy-mSEB fragment. Primers were also included to duly amplify a 325 bp vegetative storage protein fragment which served as an internal control. For the characterization of ST108 and 764 transformation events shown in Figure 2, duplex PCR identified the mSEB transgene in 7 of the 8 T1 progenies examined (Figures 2(a) and 2(b)). To identify those progenies with detectable mSEB, seed proteins were extracted from each chip, separated under nonreducing SDS-PAGE conditions, and detected by Western analysis. For the representative samples shown in Figure 2, all 7 of the PCR-positive progenies also accumulated immunoreactive protein that was detected by rabbit sera containing anti-SEB polyclonal antibodies (Figures 2(c) and 2(d)). The immunoreactive protein migrated with a MW of ~28 kDa, consistent with the predicted MW of 28.3 kDa for mSEB. The lack of detectable protein in nontransgenic and wild-type seed extracts (negative control) demonstrated the specificity of the antibody for the mSEB epitopes. Recombinant mSEB protein purified from *E. coli* was included on each gel and served as an internal positive control.

Western analyses resulting from a large scale screen of all events revealed that progeny from 18 of the 25 ST108 transgenic events (72%) and 6 of the 12 764 transgenic events (50%) expressed mSEB protein. Based on mSEB expression levels in these experiments, lead progenies were taken to maturity and characterized over multiple generations. The examples shown in Figure 2 represent some of the highest expressing lines that were propagated over several generations and used for subsequent studies. The stability of soy-mSEB was demonstrated by Western analysis in T2 and

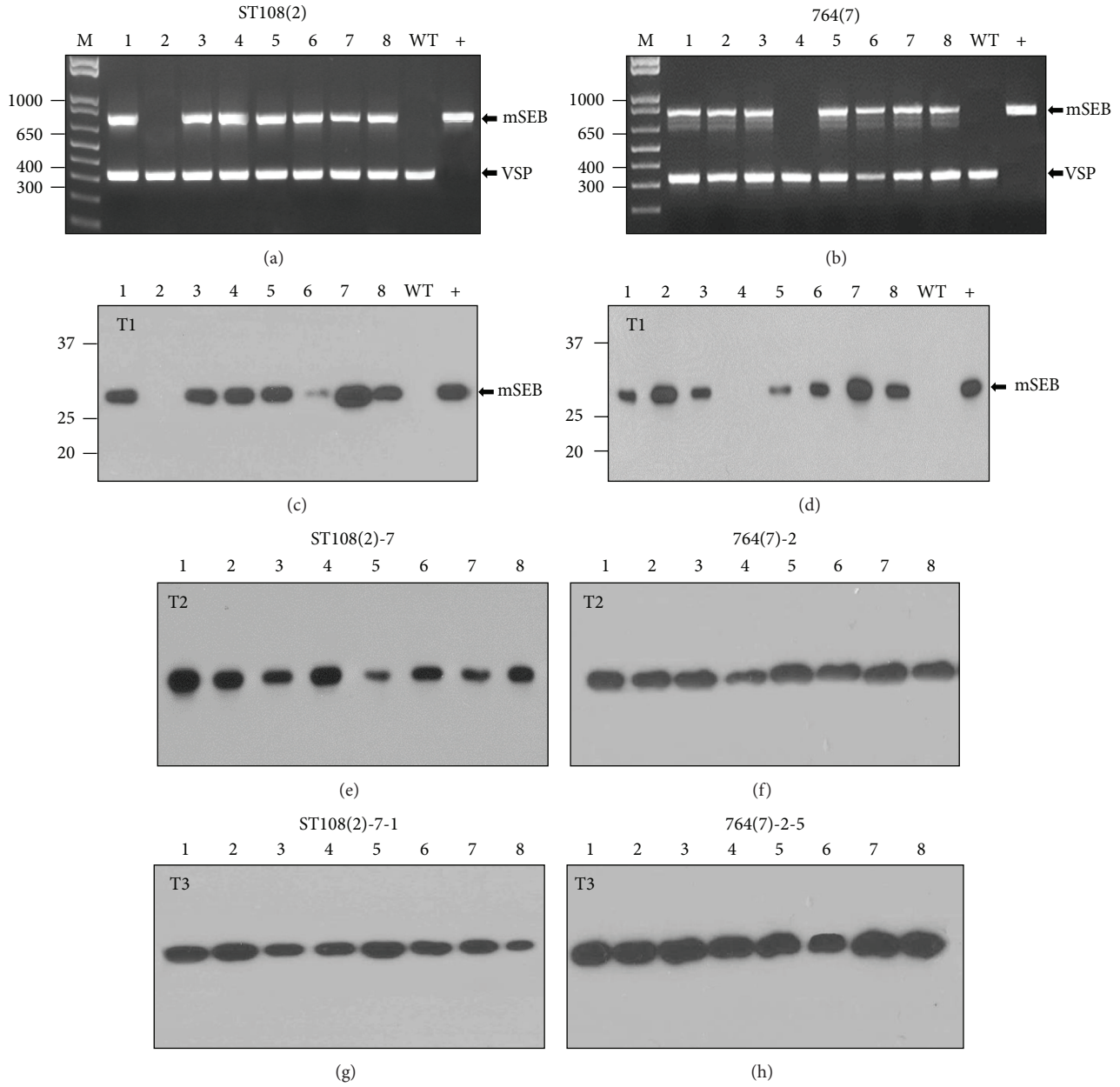

Figure 2: Molecular characterization of soy-mSEB events. (a) and (b) Duplex PCR of 8 T1 progeny from the indicated transformation events. WT: nontransgenic (negative control); +: plasmid DNA (positive control). Arrow shows position of amplified DNA fragments derived from mSEB and vegetative storage protein (VSP). Sizes of molecular weight markers are shown in base pairs. (c) and (d) Western blot of protein derived from the T1 progeny shown in (a) and (b). Arrow indicates soy-mSEB immunoreactive protein. Sizes of molecular weight standards are shown as kDa. (e) and (f) Western blots of T2 progeny from the indicated events. (g) and (h) Western blots of T3 progeny from the indicated events.

T3 generations (Figures 2(e), 2(f), 2(g), and 2(h)) and all subsequent generations (data not shown). Southern results preformed on T1 progeny suggested the presence of up to 3 copies of the transgene present at multiple loci.

All plants propagated and taken to maturity were subjected to foliar spray with Ignite 280 SL herbicide to monitor for the expression of the herbicide selectable marker. There was a direct correlation between plants lacking the transgene and severe leaf chlorosis. All plants that contained the transgene and accumulated mSEB showed no visible signs of chlorosis (data not shown).

The approximate level of soy-mSEB protein expression was determined by semiquantitative Western analysis. Known amounts of seed protein (extracted from a master powder mix of 100 ST108 homozygous T3 seeds) and purified recombinant mSEB (quantification standards) were used in these experiments (Figure 3(a)). X-ray films of the Western blots were subjected to densitometric examination, and

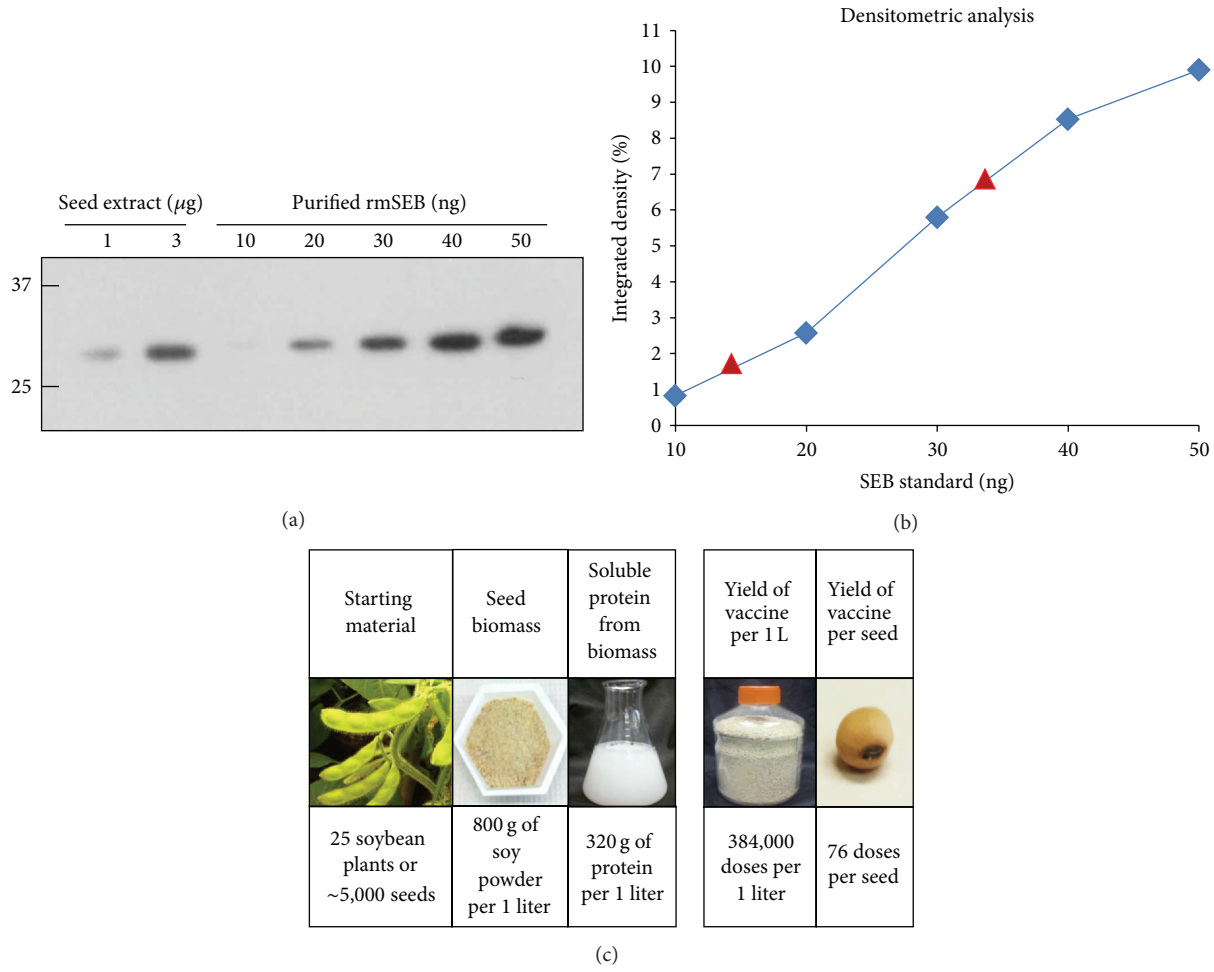

Figure 3: Quantification of soy-mSEB. (a) Known amounts of total seed protein (ST108, T3 generation) and various known amounts of purified *E. coli*-derived mSEB protein (standards) were separated under nonreducing SDS-PAGE and subjected to Western analysis. (b) A standard curve generated from the five known standards following densitometric analysis of the film shown in (a). (c) Chart showing theoretical number of vaccine doses present within a single transgenic soybean seed and in a 1 liter volume of crushed soybean powder. Calculations assume 200 soybeans per plant, 160 mg average seed weight, 40% seed protein content, 1.2% mSEB expression, and a 10 μg human vaccine dose, which is similar to the dose recommended for recombinant hepatitis B surface antigen immunizations [18]. The calculations above do not account for any losses during the purification procedures.

a standard curve was generated. Extrapolation from this curve indicated 13.7 ng mSEB present in 1000 ng total seed protein (1.37% TSP) and 33.6 ng mSEB present in 3000 ng protein (1.12% TSP). Using an average of these numbers, we determined that soy-mSEB represents ~1.2% of total soluble seed protein (Figure 3(b)). These results were also verified by ELISA and imply that an average ST108 soybean seed (160 mg dry weight) with a protein composition of 40% and transgene expression level of 1.2% contains 768 theoretical micrograms of mSEB or 76.8 10 μg human doses of vaccine. This equates to 384,000 vaccine doses produced in seeds produced by ~25 soybean plants (Figure 3(c)).

3.2. Soy-mSEB Protein Characterization, N Terminal Sequencing, and Signal Peptide Cleavage. Native SEB is a single polypeptide with a known disulfide loop that is essential for mitogenic activity. The cysteines responsible for the disulfide bridge are located at amino acid positions 93 and 113. We noticed that full length soy-mSEB protein could only be detected using nonreducing SDS-PAGE conditions (Figure 2) but not using standard reducing conditions (data not shown). This observation suggested nicking or proteolytic cleavage somewhere within mSEB. To examine this possibility further, soy protein from ST108 and 764 transformation events was compared with *E. coli*-derived mSEB and native SEB protein under reducing and nonreducing conditions. While the inclusion of β-mercaptoethanol as a reducing agent did not significantly alter the mobility of the *E. coli*-derived mSEB or native SEB proteins in SDS-PAGE, the inclusion of β-mercaptoethanol resulted in the detection of two smaller fragments with mobilities of ~12 and 16 kDa in both soy samples (Figures 4(a) and 4(b)). The appearance and sizes of these fragments are consistent with cleavage within the

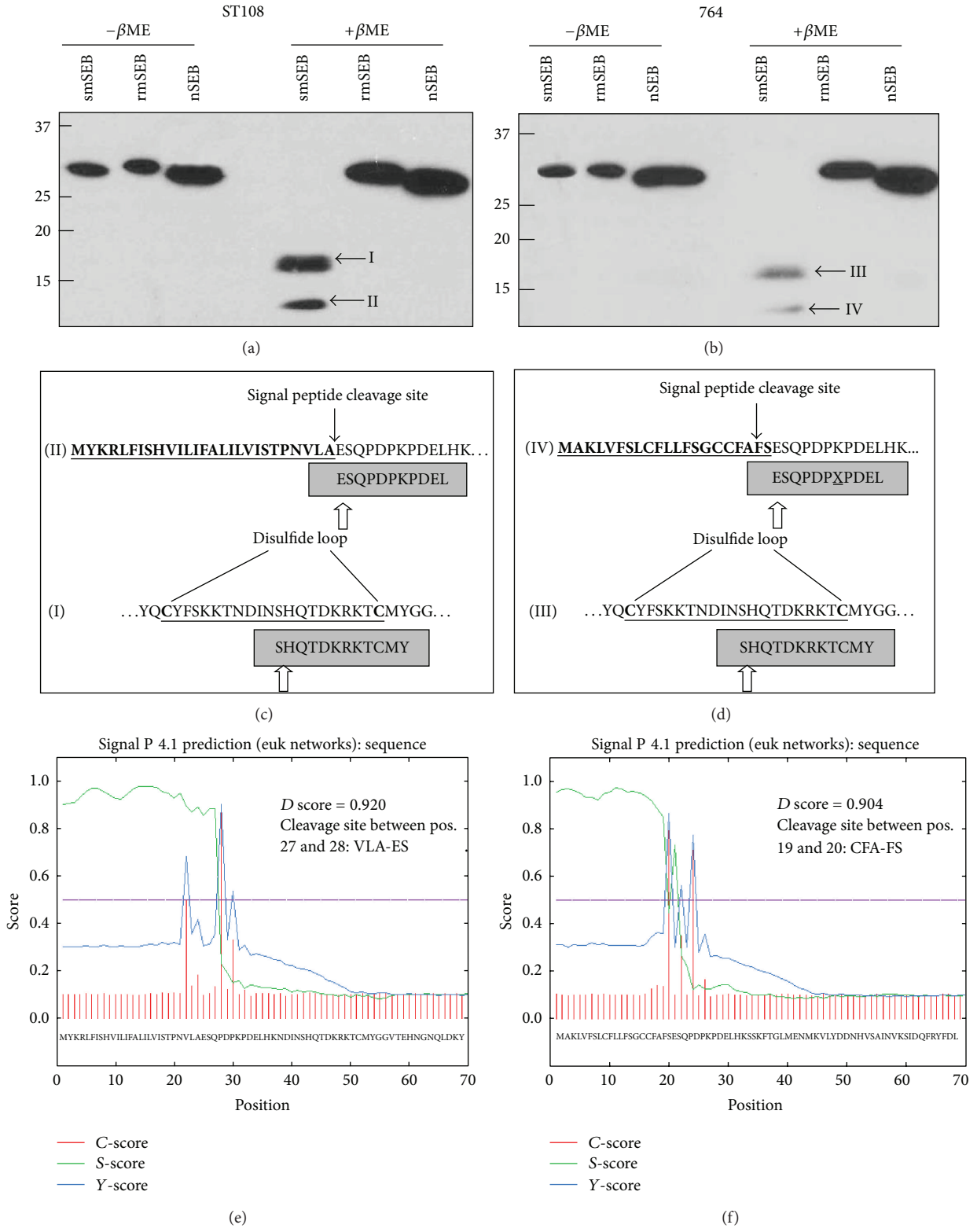

Figure 4: Characterization of soybean-derived mSEB. (a) and (b) Western blot analysis of soy-mSEB, *E. coli*-derived mSEB, and native SEB under nonreducing and reducing conditions. The ST108 soy-mSEB fragments detected under reducing conditions are labeled I and II, while those derived from 764 soy-mSEB are labeled III and IV. (c) and (d) N-terminal sequencing of soy-mSEB fragments detected under reducing conditions. Amino acids identified from N-terminal protein sequencing are shown in shaded boxes and aligned with the relevant portion of the mSEB protein sequence. The bacterial and soybean signal peptide sequences are underlined with bold typeface. Solid arrows indicate the predicted location for signal peptide cleavage and open arrows indicate observed N-termini. (e) and (f) SignalP 4.1 analysis of the ST108 and 764 soy-mSEB amino acid sequences.

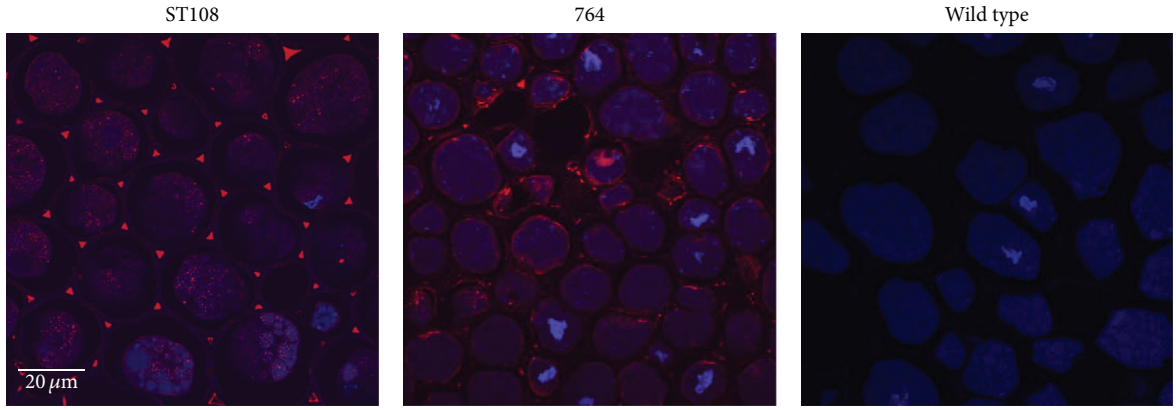

Figure 5: Immunohistochemical detection of soy-mSEB in T2 seeds. (a) ST108 seed section. (b) 764 seed section. (c) Nontransgenic (WT) seed section (control). Red fluorescence represents soy-mSEB protein that is either secreted into apoplastic spaces (ST108) or localized throughout the cell (764). DAPI staining of nuclear material is shown in blue. Samples were viewed at 20x magnification using confocal microscopy, and identical microscope parameters were used for photography of all samples shown.

disulfide loop region. The slight mobility difference observed with the *E. coli*-derived mSEB protein is the result of a C-terminal histidine tag included for purification.

Given the mobility of the two fragments detected under reducing conditions, we predicted that the smaller fragment represented an N-terminal mSEB polypeptide while the larger fragment represented a C-terminal mSEB polypeptide. To map the cleavage sites within soy-mSEB, the larger fragment from both ST108 and 764-derived proteins was subjected to N-terminal protein sequencing. Results from the sequencing experiment identified the N-terminal amino acid residues at the site of cleavage as SHQTDKRKTCMY. This sequence is present within the disulfide loop and confirmed that cleavage of mSEB occurred within this conserved loop region (Figures 4(c) and 4(d)).

Our final characterization of soy-mSEB involved the identification of the N-termini of both mature mSEB proteins. Note that the ST108-derived protein was engineered with a 27-amino-acids bacterial signal peptide while the 764-derived protein was engineered with the 21-amino-acid soybean glycinin signal peptide. The ST108 and 764 mSEB ORF sequences were analyzed using SignalP 4.1 software to predict the presence and location of potential signal peptides. This service predicted cleavage of ST108 between amino acids 27 and 28 and cleavage of 764 between amino acids 19 and 20 (Figures 4(e) and 4(f)). To identify the mature N-terminus of both soy-mSEB proteins, the smaller fragments obtained by treatment with β-mercaptoethanol were subjected to N-terminal protein sequencing. In both cases, the N-terminal sequence was identified as ESQPDPKPDEL. This sequence is identical to the N-terminus of mature native SEB. These results verified that the heterologous bacterial signal peptide was accurately recognized and processed by the soybean signal peptidase machinery. This was also the case with the 764 events containing a heterologous glycinin leader peptide sequence.

3.3. Soy-mSEB Protein Cellular Localization. To determine soy-mSEB localization, immunohistochemistry was carried out on cotyledon tissue using an in-house anti-SEB antibody and an Alexa Fluor 594 goat anti-rabbit IgG secondary antibody. Confocal images show that mSEB derived from ST108 transformation events was secreted into apoplastic spaces (Figure 5(a)) while mSEB derived from 764 transformation events remained intracellular and appeared to be associated with the cell membrane (Figure 5(b)). DAPI staining of nuclear material showed that transgenic protein was also excluded from the nucleoplasm. Fluorescence was not observed in control (nontransgenic) tissues prepared using identical conditions (Figure 5(c)).

3.4. Seed Promoter Specificity. Practical use of soybean as a host for recombinant protein production would involve the harvest of seed and disposal of remaining biomass. To verify that soy-mSEB is present only in seed and not in the leftover biomass, protein was extracted from leaf, stem, and root material and compared with protein derived from master seed powder stocks. Western experiments confirmed that mSEB protein was only detectable in mature seed material and not in leaves, stems, and roots (Figure 6). Coomassie staining of the membranes used in these Western experiments confirmed the presence of plant protein on the blot.

3.5. Soy-Derived SEB Is Immunologically Similar to Commercial Forms of SEB. To evaluate immunogenicity of soy-mSEB relative to *E. coli*-derived mSEB and native SEB, an ELISA was performed using three separate anti-SEB antibodies. Soy-mSEB and *E. coli*-derived mSEB were purified as previously described [23] and purified native SEB was purchased commercially (Toxin Technology, Sarasota Florida). Equal amounts of the three purified proteins, along with cholera toxin (negative control), were coated onto ELISA plates and incubated with different anti-SEB antibodies. Absorbance

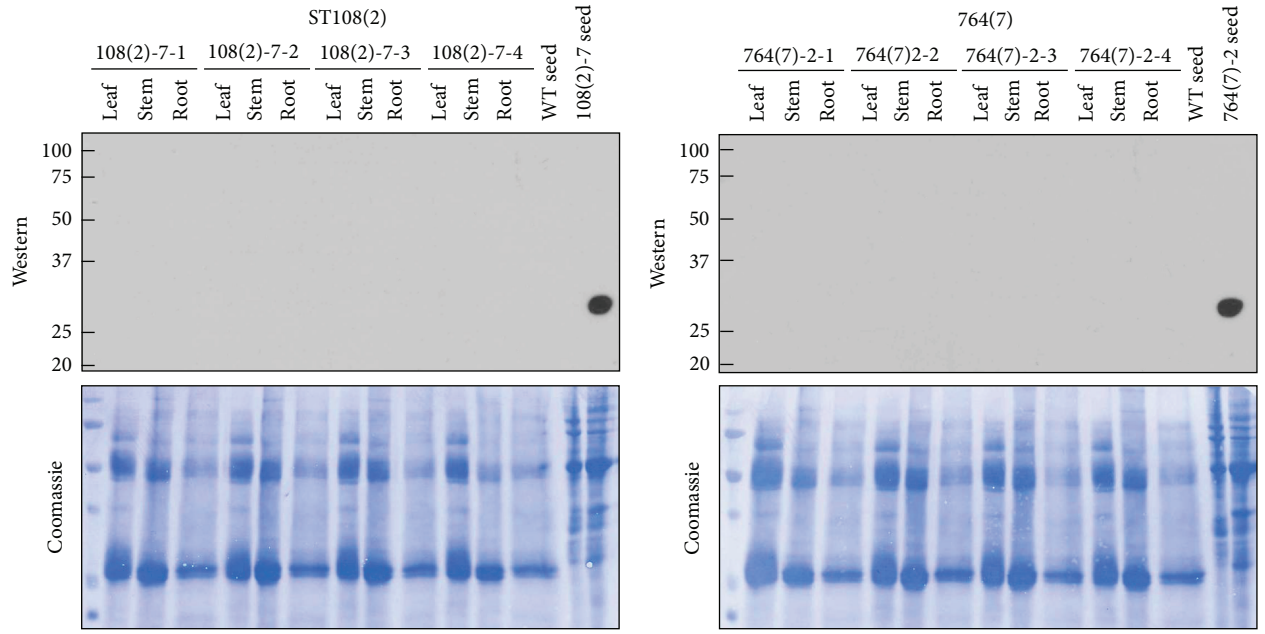

Figure 6: Western blot analysis of promoter specificity. Nonreducing SDS-PAGE conditions were used to separate 10 μg total protein extracted from leaf, stem, and root tissues of the indicated T2 progeny. Equal amounts of T1 seed protein (parent) and nontransgenic (WT) seed protein were also included as controls. Top panels show X-ray film of the resulting Western blots while bottom panels show the blots used in this experiment following staining with Coomassie blue. Sizes of molecular mass standards are shown as kDa. (a) Data for events derived from ST108; (b) data for events derived from 764.

readings from these ELISAs are shown in Figure 7. An in-house rabbit anti-SEB polyclonal antibody recognized all three proteins similarly (Figure 7(a)). Comparable results were observed in the absorbance readings from ELISAs using a commercially purchased sheep anti-SEB polyclonal antibody (Figure 7(b)). Since polyclonal antibodies are likely to bind both linear and conformational epitopes along the entire length of the SEB protein, these results suggested that soy-mSEB epitopes are intact. A third ELISA was performed using a commercial mouse monoclonal antibody which specifically detects one target epitope on the native SEB protein. Results from this ELISA (Figure 7(c)) were consistent with results from the previous two ELISAs in that absorbance readings for all three SEB proteins were similar. The results obtained here are consistent with the notion that nontoxic soy-mSEB protein is immunologically similar to both *E. coli*-derived mSEB and native SEB.

3.6. Immunization of Mice with Soy-mSEB Elicits an Antibody Response. To determine whether soy-mSEB could generate specific immunity, groups of mice were administered intraperitoneal injections of transgenic seed protein containing approximately 10 μg of the soy-mSEB vaccine (along with cholera toxin adjuvant) on days 0, 14, and 28. Blood was taken from each animal prior to immunization, and on day 42, and the presence of serum antibodies against soy-mSEB was detected by ELISA (Figure 8). Mice immunized with soy-mSEB showed significant levels of IgG anti-SEB antibody production 14 days after immunization when compared to the prebleed. Antibody titers continued to increase by days 28 and 42 following booster vaccinations. These results demonstrate that the soy-mSEB vaccine candidate was effective in inducing antibodies which recognized native SEB.

4. Discussion

Over the past two decades, there has been substantial research on the expression of heterologous proteins in plants as a means to produce biopharmaceuticals. While numerous plant systems have been shown to support expression of heterologous proteins, the soybean has enormous potential with distinct advantages over these other systems. To date soybeans have been engineered to express a variety of therapeutic proteins [5, 26–28]. Soybeans have a high protein content (~40%) making them an excellent host for increased expression and storage of recombinant protein. In the present study we report an expression level of 1.2% of TSP. If one assumes a vaccine dose of 10 μg, as is recommended for recombinant hepatitis B surface antigen immunizations [24], this translates into ~76 theoretical doses of human vaccine in a single seed. Although such calculations represent theoretical protein and do not take into account potential loses during purification they nonetheless represent significantly larger recoverable yield based on biomass when compared with other recombinant protein systems. Another important characteristic of soybeans is that these seeds have evolved as specialized compartments that store proteins for embryo nutrition. Therefore, soybeans possess metabolic adaptations

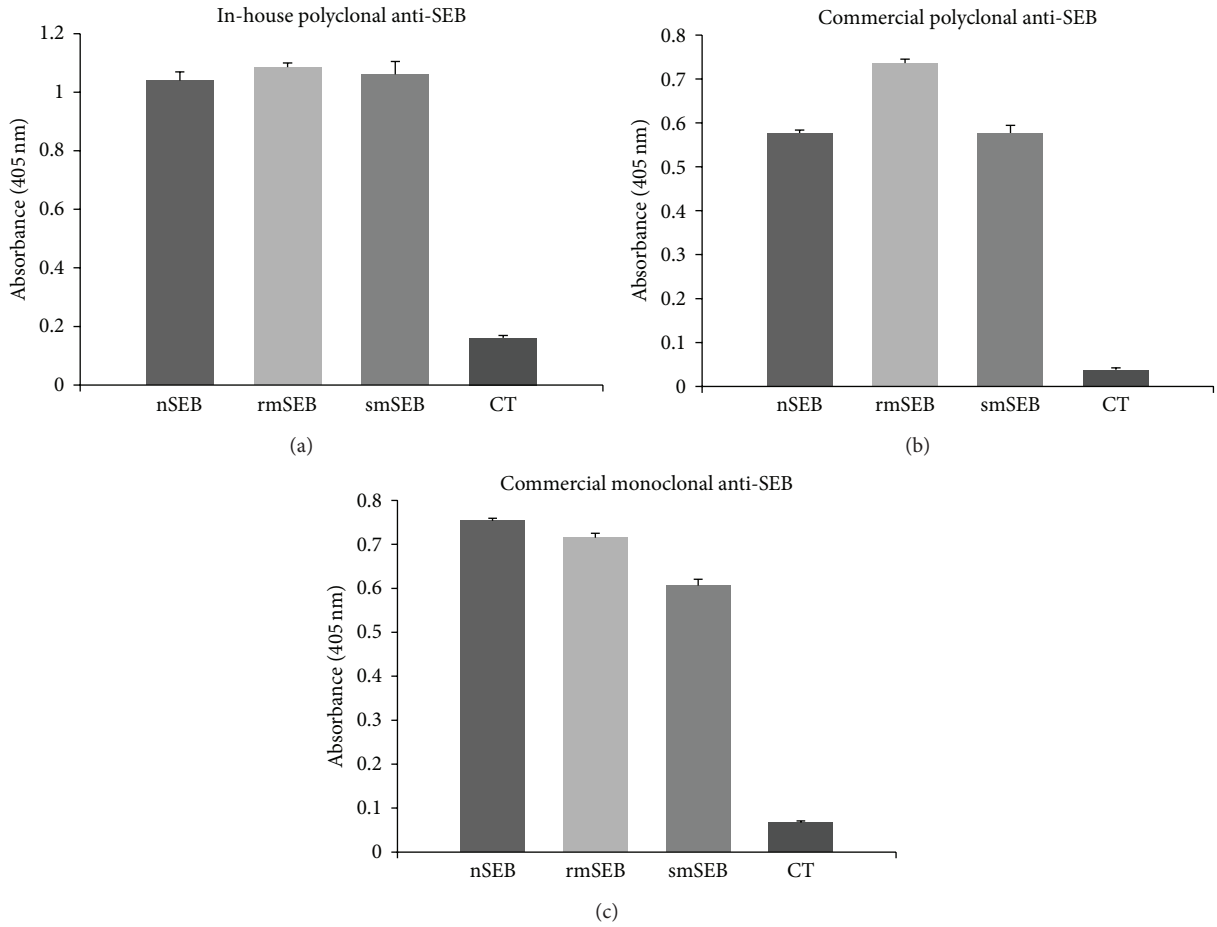

Figure 7: Immunogenicity of SEB proteins. ELISAs were used to determine relative immunogenicities of purified native SEB (nSEB), *E. coli*-derived recombinant mutant SEB (rmSEB), and soy-derived mutant SEB (smSEB) proteins. Cholera toxin (CT) was included as a negative control. 100 ng purified protein was coated in each well. All assays were performed in quadruplicate. (a) ELISA results using an in-house rabbit anti-mSEB polyclonal detection antibody. (b) ELISA results using a commercial sheep anti-SEB polyclonal detection antibody (Abcam number ab15925). (c) ELISA results using a commercial mouse anti-SEB monoclonal detection antibody (Abcam number ab6064). Values shown represent average absorbance values (405 nm). Error bars represent standard deviation.

that permit stable and long-term storage of proteins which in turn reduces the requirement for sophisticated and expensive storage conditions. Recombinant proteins expressed in soybean have proven to be stable for years at ambient temperatures [25, 29]. This feature reduces or eliminates the need for a cold chain and allows for recombinant protein production to be a separate event with purification occurring at a later time if needed. Transgenic soybeans can also be used for production of therapeutic formulations that do not require purification. The efficacy of engineered therapeutics in crude soymilk formulations could lead to oral vaccines and other therapies that require little, if any, purification from other seed proteins. These simplified methods for expression, storage, and administration make soybean a cost-effective alternative to existing systems. Successful expression of the mSEB model vaccine antigen in this study demonstrates the practicality of soybean as a viable host for the expression of a vaccine candidate that is biochemically and immunologically functional.

A critical first step for efficient production of a vaccine protein in a recombinant system is to maximize the level of foreign protein expression in an effort to decrease production costs. Soybean seeds are the richest source of protein known, and while constitutive promoters can direct protein expression in seeds [22, 30], it is likely that higher accumulations of target proteins in seeds can be achieved using seed-specific promoters. In this study we used the soybean 7S β-conglycinin and 11S glycinin seed storage promoters to target mSEB to seeds. These promoters have also been used by others to successfully express foreign proteins in seed [5, 26, 28, 29]. The use of these promoters allowed us to target soy-mSEB expression to the seed and achieve high levels (1.2% of TSP) of recombinant protein over multiple generations.

In this study we utilized different signal peptide sequences to evaluate subcellular targeting. SEB is a secreted protein which encodes a 27-amino-acid bacterial signal peptide sequence. If this signal peptide is also functional in plants, proteins could potentially be secreted to apoplastic spaces.

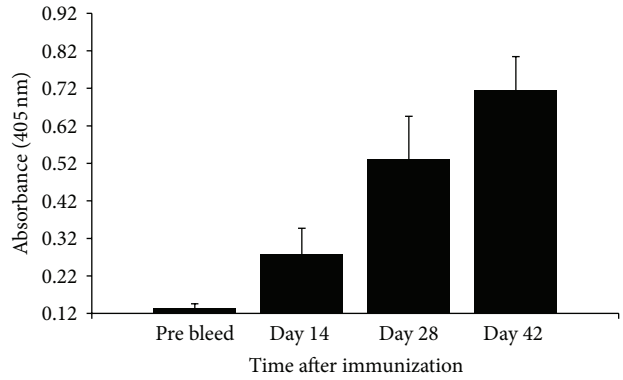

Figure 8: Anti-mSEB titers in mice following immunization. Groups of female BALB/c mice ($n = 4$) were immunized intraperitoneally on day 0 and boosted on days 14 and 28 days with 1 mg transgenic seed extract plus adjuvant. Bleeds were collected just prior to immunization on days 0, 14, and 28, and again on day 42. ELISAs were performed to determine serum IgG anti-mSEB reactivity. Absorbance values (405 nm) represent serum tested at a 1 : 27000 dilution and are presented as mean anti-mSEB titers and error bars represent standard deviation.

This location represents a different biochemical environment than intracellular spaces and therefore may impact foreign protein stability. We found that soy-mSEB containing the bacterial signal peptide was accurately processed by the soybean signal peptidase machinery, resulted in a protein with an N-terminus identical to the native protein, and was localized to apoplastic spaces. Since similar levels of soy-mSEB accumulated both extracellularly (with the bacterial signal peptide) and intracellularly (with the soybean glycinin signal peptide) it appears that mSEB does not have a preference for one subcellular location over the other. This is not surprising given that SEB is a highly stable toxin and has evolved its structure to remain stable under a variety of conditions. However, it is possible that apoplastic spaces are the preferred subcellular location for other recombinant proteins, and to this end we have shown that the bacterial SEB signal peptide may be useful in directing such proteins to those spaces. It is interesting to note that the signal peptide from another bacterial secreted protein (*E. coli* labile toxin subunit B, or LT-B) did not appear to have apoplast-targeting capabilities when tested in plants [31]. In that study, expression of chimeric LT-B genes containing either the native LT-B or maize y-zein signal peptide sequences resulted in the unexpected localization of LT-B to starch granules in maize endosperm [31]. In an effort to learn more about the targeting potential of the SEB signal peptide, we are currently testing whether other heterologous proteins can also be localized to apoplastic spaces when the SEB bacterial signal peptide is utilized [31].

Structural studies of SEB have shown the presence of a protruding disulfide loop in this toxin [32]. In the soybean seed environment it appears that this loop is susceptible to nicking by an unknown mechanism. We hypothesize that this nicking occurs either during or shortly after protein synthesis since extraction of seed protein in the presence of protease inhibitors did not prevent cleaved products from being detected (data not shown). Furthermore, incubation of *E. coli*-derived mSEB and native SEB protein preparations with soybean seed extracts did not induce nicking of those proteins, suggesting that seed proteases may not be involved. Previous reports in the literature describe nicking of native SEB [9, 33] and it has been suggested that this nicking is due to enzymatic or chemical hydrolysis during fermentation or purification. Interestingly, this previous work examined native SEB under reducing and nonreducing conditions and detected smaller fragments of SEB when the protein was exposed to reducing conditions; the site of nicking was mapped to the disulfide loop and occurred within 4 amino acids of the site identified [9]. This study also found that some commercial preparations of native SEB were comprised almost entirely of nicked protein while preparations from other vendors showed no evidence of internal cleavage [9]. Although the data presented in Figure 4 showed no signs of nicking in recombinant and native forms of SEB, when these same X-ray films were examined after extended exposure times there were bands present that indicated low levels of nicked SEB in both recombinant and native forms (data not shown). Thus, nicking within the SEB disulfide loop appears to be related to the SEB protein itself and not a phenomenon specific to any expression system. Importantly, the nicked forms of native SEB have been shown to retain full mitogenic activity as long as the disulfide bridge is intact [9]. A phenomenon involving what appears to be proteolytic cleavage of other plant-derived recombinant proteins has also been reported [28] and may be one reason why many recombinant proteins go undetected and associated experiments are deemed unsuccessful.

In order for a soy-based vaccine to be marketable it must be biologically equivalent to (or preferably superior to) an existing vaccine if one is already present in the marketplace. To date there is no commercial vaccine for SEB poisoning; therefore, the mSEB used as a "model" vaccine in this study could also function as an efficacious vaccine if it is shown to be immunogenic and confers protection following challenge with native toxin. To this end we examined the immunmoreactive profile of soy-mSEB and found it to be similar to that of *E. coli*-derived mSEB and native SEB (Figure 7). These observations suggested that immunogenic epitopes throughout soy-mSEB remain intact. The presence of significant levels of anti-SEB antibodies in blood sera of mice occurring within 14 days of immunization alludes to the efficacy of the soy-based mSEB vaccine.

5. Conclusions

In this study, a mutated nontoxic version of SEB (soy-mSEB) was produced in transgenic soybean seeds as a highly expressed vaccine. Soy-mSEB was specifically expressed within the soybean seed and was shown to be stably expressed over multiple generations. Soy-mSEB was successfully localized both intra- and extracellularly and accumulated equally in both subcellular locations. Soy-derived mSEB was shown to be biochemically and immunologically

similar to recombinant and wild-type commercial forms of SEB. Additionally, functionality of the soy-mSEB as a vaccine antigen was demonstrated using mice which produced anti-SEB titers in blood serum after vaccination with soy-mSEB. Taken together, these results show the efficacy of soy-derived mSEB and demonstrate the potential for soybean as a platform technology to produce pharmaceutical proteins.

To further explore the effectiveness of the soy-mSEB vaccine, current studies are underway to determine whether immunization with purified soy-mSEB confers protection in an animal model when challenged with the native toxin, and if so, whether such protection is comparable to or superior to protection obtained by vaccination with other recombinant forms of mSEB.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Laura C. Hudson and Renu Garg contributed equally.

Acknowledgments

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Research Article

A Plant-Produced Antigen Elicits Potent Immune Responses against West Nile Virus in Mice

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We described the rapid production of the domain III (DIII) of the envelope (E) protein in plants as a vaccine candidate for West Nile Virus (WNV). Using various combinations of vector modules of a deconstructed viral vector expression system, DIII was produced in three subcellular compartments in leaves of *Nicotiana benthamiana* by transient expression. DIII expressed at much higher levels when targeted to the endoplasmic reticulum (ER) than that targeted to the chloroplast or the cytosol, with accumulation level up to 73 μg DIII per gram of leaf fresh weight within 4 days after infiltration. Plant ER-derived DIII was soluble and readily purified to > 95% homogeneity without the time-consuming process of denaturing and refolding. Further analysis revealed that plant-produced DIII was processed properly and demonstrated specific binding to an anti-DIII monoclonal antibody that recognizes a conformational epitope. Furthermore, subcutaneous immunization of mice with 5 and 25 μg of purified DIII elicited a potent systemic response. This study provided the proof of principle for rapidly producing immunogenic vaccine candidates against WNV in plants with low cost and scalability.

1. Introduction

West Nile Virus (WNV) belongs to the *Flavivirus* genus of the Flaviviridae family. It is a positive-stranded, enveloped RNA virus that infects the central nervous system (CNS) of humans and animals. Once a disease that was restricted to Old World countries, it entered into the Western hemisphere through New York City in 1999 and has now spread across the United States (US), Canada, the Caribbean region, and Latin America [1]. The outbreaks of WNV have become more frequent and severe in recent years with 2012 as the deadliest yet with 286 fatalities in the US [1]. WNV infection causes fever that can progress to life-threatening neurological diseases. The most vulnerable human population for developing encephalitis, meningitis, long-term morbidity, and death includes the elderly and immunocompromised individuals [2]. Recent studies also identified genetic factors associated with susceptibility to the disease [3, 4]. Currently, no vaccine or therapeutic agent has been approved for human application. The threat of global WNV epidemics and the lack of effective treatment warrant the development of vaccines and production platforms that can quickly bring them to market at low cost.

The WNV Envelope (E) glycoprotein mediates viral binding to cellular receptors and is essential for the subsequent membrane fusion [5]. It is also a major target of host antibody responses [5]. Studies have shown that WNV E shares a three-domain architecture with E proteins of dengue and tick-borne encephalitis viruses [6]. The domain III (DIII) of WNV E protein contains the cellular receptor-binding motifs and, importantly, the majority of the neutralizing epitopes that induce strong host antibody responses and/or protective immunity are mapped to this domain [7]. As a result, DIII has been targeted as a WNV vaccine candidate [8]. Insect cell and bacterial cultures have been explored to express the WNV DIII protein [9, 10]. However, these culture systems

are challenged by their limited scalability for large-scale protein production. Moreover, DIII expression in bacterial cultures often leads to the formation of inclusion bodies, which requires a cumbersome solubilization and refolding process to yield a recombinant DIII protein that resembles its native structure [10].

Expression systems based on plants may provide solutions to overcome these challenges, because they provide highly scalable production of recombinant proteins at low cost and have a low risk of introducing adventitious human or animal viruses or prions [11, 12]. Stable transgenic plants were first explored to produce subunit vaccine proteins. While feasible, the low protein yield and the long time period are required for generating and selecting transgenic lines hinder a broad application of this strategy [13]. Recently, transient expression systems based on plant virus have been developed to address these challenges. While the infectivity of plant viruses has been eliminated through viral "deconstruction," these vectors still retain the robustness of the original plant virus in replication, transcription, or translation [14]. Thus, deconstructed plant viral vectors promote high-level production of recombinant protein within 1 to 2 weeks of vector delivery [14–16]. The MagnICON system is a popular example of these vectors based on *in planta* assembly of replication-competent tobacco mosaic virus (TMV) and potato virus X (PVX) genomes from separate provector cDNA modules [17, 18]. The 5′ module carries the viral RNA dependent RNA polymerase and the movement protein (MP), and the 3′ module contains the transgene and the 3′ untranslated region (UTR). *A. tumefaciens* strains harboring the two modules are mixed together and coinfiltrated into plant cells along with a third construct that produces a recombination integrase. Once expressed, the integrase assembles the 5′ and 3′ modules into a replication-competent TMV or PVX genome under the control of a plant promoter [18, 19]. This assembled DNA construct is then transcribed and spliced to generate a functional infective replicon. Geminiviral expression system is another example: a DNA replicon system derived from the bean yellow dwarf virus (BeYDV) [20, 21]. Another interesting example is an expression vector system that is based on the 5′ and 3′-untranslated region of Cowpea mosaic virus (CPMV) RNA-2. This vector system does not require viral replication yet allows high-level accumulation of recombinant proteins in plants [22]. Thus, these plant transient expression systems combine the advantages of speed and flexibility of bacterial expression systems and the post-translational protein modification capability and high-yield of mammalian cell cultures. As a result of this development, a variety of protein vaccine candidates have been produced in plants [11, 12, 23–26]. The immunogenicity of a plant-produced vaccine candidate against WNV has not been described.

Here, we described the rapid production of the WNV DIII in *Nicotiana benthamiana* plants using the TMV-based vectors of the MagnICON system. We demonstrated that DIII can be expressed in three subcellular compartments of the plant cell including endoplasmic reticulum (ER), chloroplast, and cytosol, with the highest accumulation level in ER within 4 days after infiltration. Plant ER-derived DIII was soluble and was readily purified to >95% homogeneity. Further analysis revealed that plant-produced DIII was folded properly as it exhibited specific binding to a monoclonal antibody that recognizes a large conformational epitope on WNV DIII. The immunogenicity of plant-derived DIII was demonstrated in mice as subcutaneous immunization elicited a potent systemic response.

2. Results

2.1. Expression of WNV E DIII in ER, Chloroplast, and Cytosol of N. benthamiana Leaves. To demonstrate the feasibility of using plants to produce a candidate vaccine for WNV, we first determined what subcellular compartment was optimal for DIII accumulation. *Agrobacterium tumefaciens* strain containing the 3′ DIII construct module was codelivered into *N. benthamiana* leaves along with the 5′ module and an integrase construct through agroinfiltration [27, 28]. Three different 5′ modules were specifically chosen to target DIII into ER, chloroplast, or the cytosol [24]. Leaf necrosis was observed in the infiltrated area 4 or 5 days post infiltration (dpi) in plants for all constructs, with cytosol-targeted construct causing the most severe symptoms (data not shown). By 6 dpi, necrosis was too extensive to recover significant amounts of live tissue from the infiltrated leaf area. As a result, DIII expression was examined between 2 and 5 dpi by Western blotting. For the construct targeted to accumulate DIII in ER, a positive band with the predicted molecular weight for DIII (13.5 kDa) was detected on Western blot starting 3 dpi (Figure 1, Lanes 3–5). In contrast, no positive band was detected for chloroplast or cytosol-targeted DIII construct even on 5 dpi (Figure 1, Lanes 6 and 7). An *E. coli*-produced DIII was used as a positive control and, as expected, it was detected as a positive band on the Western blot (Figure 1, Lane 8). The *E. coli*-produced DIII appeared to be larger than that from plants (16.9 kDa), because it contained multiple polypeptide tags from the bacterial expression vector pET28a (EMD Milipore). The lack of positive band in the negative control leaf samples (Figure 1, Lane 1) confirmed the specificity of the DIII band. The expression of DIII was quantified by a sandwich ELISA using two WNV specific antibodies (Figure 2). In leaves that DIII was targeted to the cytosol or chloroplast, the maximal levels of accumulation are below 1.16 μg of DIII per gram of leaf fresh weight (LFW) or 0.01% of total soluble protein (TSP), confirming the result of Western blotting. The ER-targeted DIII reached the highest level of production at 4 dpi, with an average accumulation of 73 μg/g LFW or 0.63% TSP, approximately ~63 times more than that in cytosol or chloroplast (Figure 2).

2.2. Purification of DIII from N. benthamiana Plants. The availability of an efficient purification scheme is another essential component for plant-derived DIII to become a viable WNV vaccine candidate. Since DIII was tagged with a His_6 tag, we developed a two-step purification procedure based on acid precipitation and immobilized metal ion affinity chromatography (IMAC). Samples from various purification steps were analyzed by Coomassie blue staining

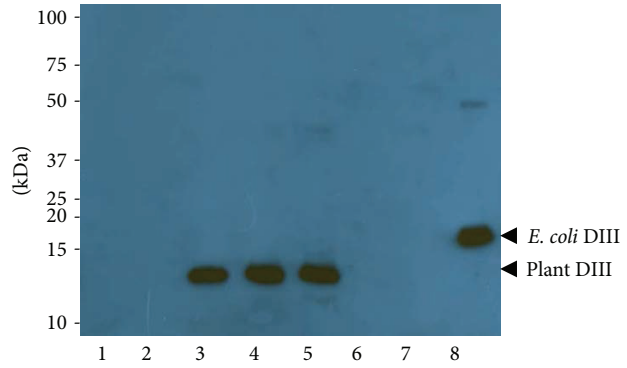

Figure 1: Western blot analysis of DIII expression in *N. benthamiana*. DIII was extracted from *N. benthamiana* leaves and separated on 15% SDS-PAGE gels and blotted onto PVDF membranes. MAb hE16 and a goat anti-human kappa chain antibody were incubated with the membranes sequentially to detect DIII. Lane 1: protein sample extracted from uninfiltrated leaves as a negative control; Lanes 2–5: sample collected 2, 3, 4, and 5 dpi from leaves infiltrated with ER-targeted DIII construct; Lane 6: sample collected 5 dpi from leaves infiltrated with chloroplast-targeted DIII construct; Lane 7: sample collected 5 dpi from cytosol-targeted DIII leaves; Lane 8: *E. coli*-produced DIII as a positive control.

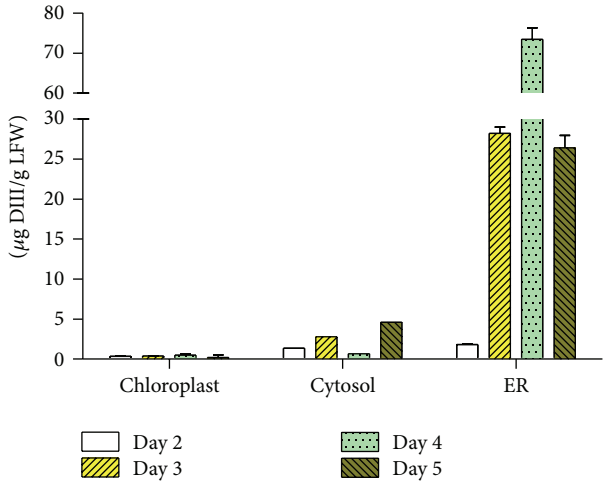

Figure 2: Temporal expression patterns of DIII in chloroplast, cytosol, and ER. Total protein from plant leaves infiltrated with chloroplast, cytosol, or ER-targeted DIII construct was extracted on 2–5 dpi and analyzed by an ELISA with mAb hE16 which recognizes a conformational epitope on DIII and a polyclonal anti-DIII antibody. Mean ± SD of samples from several independent experiments are presented.

analysis of SDS-PAGE (Figure 3(a)) and Western blot analysis (Figure 3(b)). Interestingly, DIII was efficiently extracted in the soluble protein fraction of plant leaves (Figures 3(a) and 3(b), Lane 2), in contrast to the insoluble inclusion body in *E. coli* [29]. Precipitation with low pH (5.0) removed a large proportion of endogenous plant proteins including the most abundant host protein, the photosynthetic enzyme RuBisCo (Figure 3(a), Lane 1), while leaving DIII in the supernatant (Figure 3(b), Lanes 1 and 3). The pH adjustment from pH 5.0 to pH 8.0, which was required for the binding of DIII to the nickel (Ni) IMAC resin, did not cause any significant change in protein profile (Figures 3(a) and 3(b), Lane 4). Ni IMAC efficiently removed the remaining plant host proteins (Figure 3(a), Lanes 5 and 6) and enriched DIII to greater than 95% purity (Figures 3(a) and 3(b), Lane 7). A faint reactive band was detectable in fractions of total soluble protein, pH 5.0 precipitation, and IMAC flow through (Figure 3(b), Lanes 2–5), suggesting a minor DIII degradation product. Only the intact DIII band with the predicted molecular mass was detected in the purified DIII fraction. Approximately 3.2 mg of purified DIII was obtained from 100 g LFW. These results demonstrated that not only can DIII be rapidly produced in plants, but also isolated and purified to high homogeneity using a scalable purification method.

2.3. Plant-Derived DIII Is Specifically Recognized by a Neutralizing Monoclonal Antibody against WNV DIII. To establish a similarity of structural and immunological properties between plant-produced and the native viral DIII, we examined the binding of plant-derived DIII to a monoclonal antibody (mAb) hE16 generated against WNV E. Our previous studies have shown that hE16 not only had potent neutralizing activity, but it also effectively protected mice from a lethal infection of WNV in both prophylactic and postexposure models [30, 31]. Since hE16 binds a conformational epitope that consists of 4 discontinuous secondary structural elements of the native WNV DIII [32], recognition of a recombinant DIII by hE16 will be informative of its proper folding. ELISA results showed that plant-produced DIII demonstrated specific binding to hE16 produced in mammalian cell culture (Figure 4). DIII also specifically bound to a plant-derived hE16 that showed potent therapeutic efficacy in mice (Figure 4) [30]. Similar results were obtained with the sandwich ELISA used for the quantification of DIII in plant extracts (data not shown). These results indicate that plant-produced DIII was folded into a tertiary structure that resembled the native viral DIII on the surface of WNV.

2.4. Plant-Produced DIII Elicits Potent Systemic Immune Response in Mice. To evaluate the immunogenicity of plant-derived DIII, BALB/c mice were injected subcutaneously with four doses of DIII over an 8-week time period (on days 0, 21, 42, and 63). Two dosages of 5 μg and 25 μg of DIII were tested with alum as adjuvant. Mice were divided into 5 groups ($n = 6$ per group), with group 1 as the negative control group injected with alum + saline (PBS), groups 2 and 3 with plant-derived DIII, and groups 4 and 5 with *E. coli*-produced DIII as a control. Individual serum DIII-specific antibody responses were measured by ELISA and Geometric mean titer (GMT) was calculated for each group at various time points (Figure 5). Samples collected from the control PBS group throughout the entire experiment course and preimmune sera for all groups taken prior to the first immunization (day 0) were negative for the presence of anti-DIII IgG (titer < 10) (Figure 5). All mice in groups immunized with 25 μg of DIII responded after the first

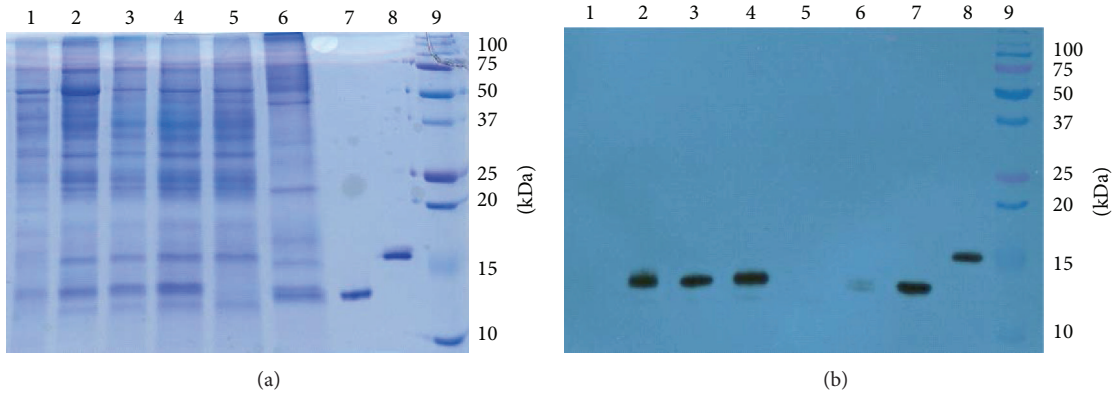

Figure 3: Purification of DIII from *N. benthamiana* leaves. DIII was purified from leaves infiltrated with ER-targeted DIII construct and analyzed on 15% SDS-PAGE gels and either visualized with Coomassie blue stain (a) or transferred to a PVDF membranes followed by Western analysis with hE16 (b). Lane 1: pH 5.0 precipitation pellet; Lane 2: total extracted protein; Lane 3: pH 5.0 supernatant; Lane 4: Ni IMAC loading; Lane 5: Ni IMAC flow through; Lane 6: Ni IMAC wash; Lane 7: Ni IMAC elute; Lane 8: *E. coli*-produced DIII; Lane 9: molecular weight marker.

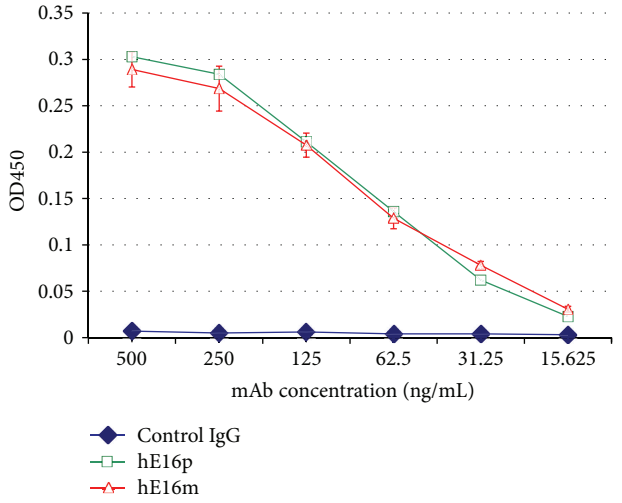

Figure 4: Specific binding ELISA of hE16 to plant-derived DIII. Serial dilutions of hE16 purified from mammalian or plant cells were incubated in sample wells coated with plant-produced WNV DIII and detected with an HRP-conjugated anti-human gamma antibody. A commercial generic human IgG was used as a negative control. Mean ± SD of samples from three independent experiments is presented.

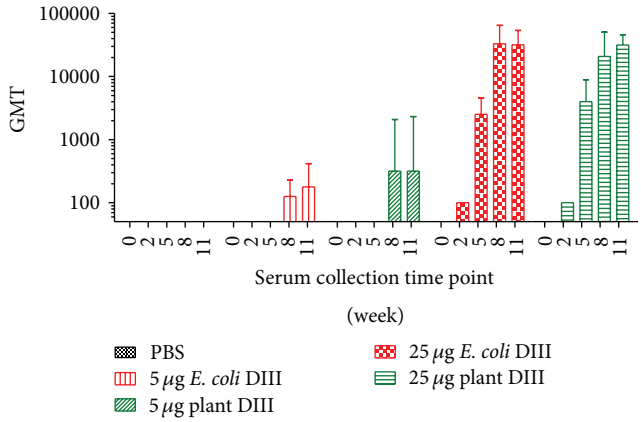

Figure 5: Time course of DIII specific antibody responses in mice upon subcutaneous delivery of plant-derived DIII. BALB/C mice ($n = 6$ per group) were injected on weeks 0, 3, 6, and 9 with the indicated dosage of antigen. Blood samples were collected on the indicated weeks and serum IgG was measured by ELISA. The y-axis shows the geometric means titers (GMT) and the error bars show the 95% level of confidence of the mean.

administration, while a response was only detectable after the third DIII delivery for mice immunized with the lower dosage (5 μg). This dose-dependent trend was also reflected in the amplitude of the response throughout the various time points of the immunization. For groups receiving DIII, IgG titers increased after each of the first three antigen's delivery and reached its peak at week 8, two weeks after the third immunization. Antibody titers at week 11 (two weeks after the fourth dose) were similar to those of week 8 for all groups except the 5 μg *E. coli*-DIII group (Figure 5). This indicated that the last immunization did not significantly further boost the DIII-specific antibody response, especially in mice that received the higher dosage of DIII. Compared with *E. coli*-produced DIII, plant-derived DIII showed at least equivalent potency ($P > 0.5$) in eliciting humoral response against WNV (Figure 5).

In order to evaluate the Th type of response induced by DIII, antigen-specific IgG subtypes IgG1 and IgG2a were evaluated by ELISA for samples collected at week 11 from mice that were immunized with 25 μg of *E. coli*- or plant-derived DIII. As shown in Table 1, >99% of DIII-specific IgG belonged to the IgG1 subtype, indicating an overwhelmingly Th2-type response stimulated by DIII antigen with alum as the adjuvant.

2.5. Characterization of Antiserum against Plant-Derived DIII Antigen. Antisera obtained at week 11 from mice of the 25 μg plant-DIII group were examined in a binding assay with yeast that displayed DIII in its native conformation on its surface. Flow cytometric analysis demonstrated that antibodies in

Table 1: Anti-DIII IgG subtypes (IgG1 and IgG2a) of pooled serum samples.

	Group 3			Group 5		
	Concentration (μg/mL)	SEM	Subtype/total %	Concentration (μg/mL)	SEM	Subtype/total %
IgG1	506.33	58.00	99.5%	488.00	48.08	99.8%
IgG2a	2.67	0.70	0.5%	0.98	0.44	0.2%

Serum samples collected at week 11 were pooled for each indicated group and analyzed by ELISA for IgG1 and IgG2a antibody concentration. Mean concentration (μg/mL) of the IgG subtype and the standard error of the mean (SEM) from several independent measurements are presented. Group 3: mice received 25 μg per dosage of plant-derived DIII; Group 5: mice received 25 μg per dosage of *E. coli*-derived DIII.

the anti-DIII sera displayed positive binding to DIII on the surface of the yeast (Figure 6(a)). This indicated that anti-DIII sera contained antibodies that can recognize the native viral DIII protein. Similar positive binding was observed for positive control mAb hE16 (Figure 6(c)), but not for equivalent antisera from mice that were immunized with PBS (Figure 6(b)). To investigate if plant-DIII elicited antibodies that bind to the same epitope as the protective mAb hE16, antisera were further analyzed with a competitive ELISA. Results showed that preincubation of DIII with antisera from immunization of plant-derived DIII significantly inhibited its binding to hE16 (Figure 7). No reduction in DIII binding to hE16 was observed when it was preincubated with preimmune serum. This indicated that plant-produced DIII induced the production of anti-DIII IgGs that bind to the same protective epitope as hE16 or at least to epitopes adjacent to that one. This suggested some of the antibodies in the anti-DIII sera were potentially neutralizing and protective.

3. Discussion

WNV has caused continuous outbreaks in the US since its introduction in 1999. While the number of cases fluctuated and even dropped from 2008 to 2011, the illusion that its transmission would remain at a low rate quickly evaporated as a large WNV epidemic with high incidence of neurological disease broke out in 2012. WNV was also reported to expand into new geographic areas in Europe and other parts of the world. Therefore, the world may face larger and more severe WNV outbreaks associated with human morbidity and mortality. In the absence of an effective treatment, the need for an effective WNV vaccine is more urgent than ever to halt its expansion and to protect human populations that are vulnerable for developing neurological complications.

Previous studies showed that immunization of DIII produced in *E. coli* or insect cell cultures with CpG oligodeoxynucleotide adjuvant or in fusion with bacterial flagellin elicited WNV-neutralizing antibodies in mice and, in certain instances, protected mice from WNV infection [29, 33, 34]. While encouraging, these expression systems may not be able to provide the scale and robustness for WNV manufacturing, as the global threat of WNV epidemics demands a scalable production platform that can quickly produce large quantities of vaccines at low cost. Moreover, DIII is often recovered in the insoluble inclusion bodies in bacterial cultures, thus requiring a cumbersome solubilization and refolding process to yield DIII proteins that resemble their native conformation [29]. The high level of endotoxins in *E. coli*-based expression system also raises biosafety concerns and demands an expensive process of purification and validation for their removal to ensure the safety of the final product [10].

Here, we demonstrated that a transient plant expression system provided a rapid production of WNV DIII in *N. benthamiana* plants. In contrast to forming insoluble aggregates in *E. coli* cultures, DIII was produced as a soluble protein in plant cells. As a result, it can be directly extracted and purified to >95% homogeneity by a simple and a scalable purification scheme without the time-consuming process of denaturing and refolding. This enhanced the likelihood of producing DIII protein that displays its native conformation. Indeed, plant-derived DIII appeared to fold properly as it was specifically recognized by hE16, a protective anti-WNV mAb that binds a large conformational epitope spanning 4 distinct regions of DIII.

Within the three subcellular compartments we tested, DIII accumulated at much higher levels in ER than in chloroplast and cytosol. The highest expression level was achieved rapidly at 4 dpi, with an average accumulation of approximately 73 μg/g LFW. This level is lower than that of other pharmaceutical proteins we have produced with the MagnICON system [24, 30, 35]. The induction of leaf necrosis by DIII may contribute to the lower expression level as it may shorten the window for accumulation. It is not clear if the observed leaf necrosis is caused by an inherent toxicity of DIII or by the employed overexpression system. To our best knowledge, WNV DIII has not been produced in another plant species or with another plant expression system. We also speculate that the 73 μg/g LFW was a conservative estimate from the early small-scale expression experiments, as we routinely obtained 30–70 μg of purified DIII from 1 g of *N. benthamiana* leaves with 30–50% recovery rate in pilot scale experiments (Chen, unpublished data). The underestimation could be partially attributed to the fact that hE16 was used as a capture antibody in the ELISA, as it only detected fully folded DIII that displayed the specific conformational epitope. Regardless, this expression level of WNV DIII is still the highest compared with other plant-produced *Flavivirus* vaccine proteins, including DIII of dengue virus expressed with a TMV-based vector in tobacco [36]. Since the production of DIII was performed under standard conditions, its accumulation level in plants can be further increased by genetic and environmental optimizations.

Production of DIII by using plant-expression systems may also overcome the challenge of limited scalability and cost issues associated with bacterial and insect cell culture

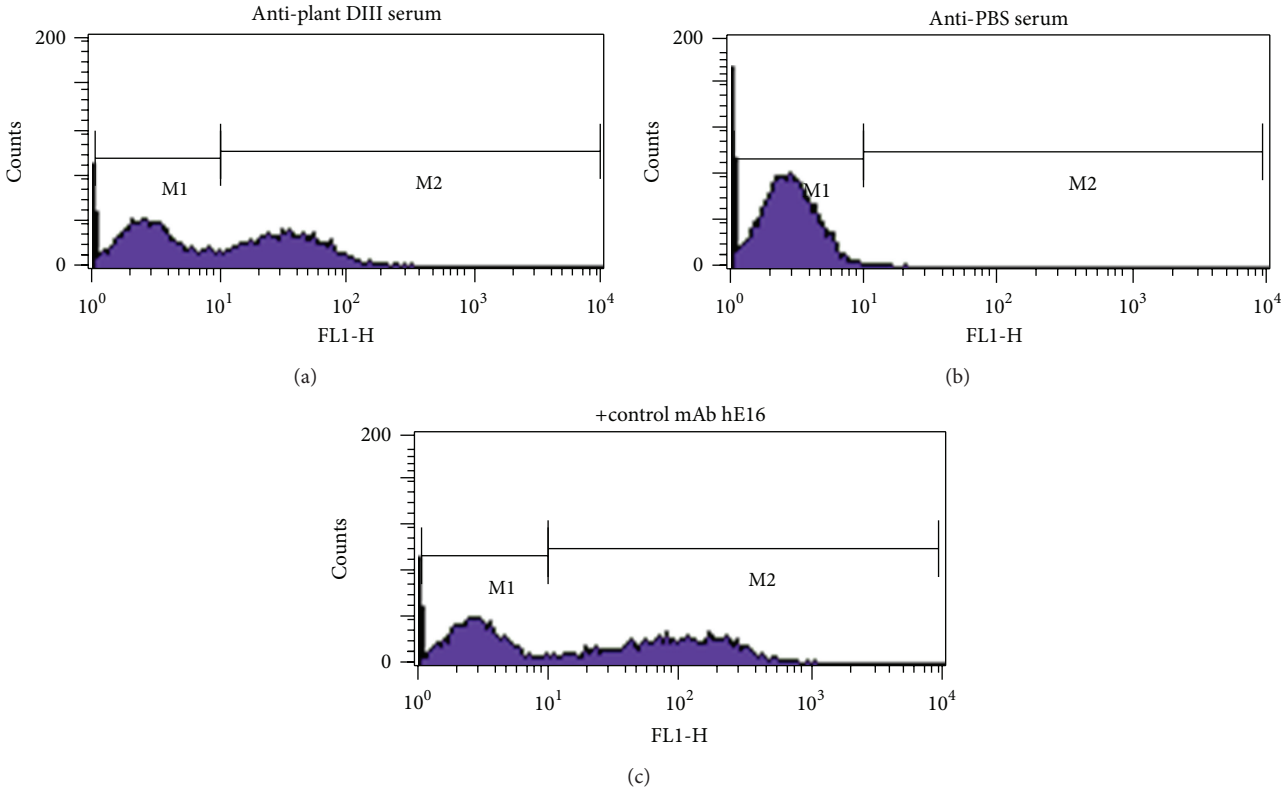

Figure 6: Binding of antibodies in anti-DlII serum to DIII displayed on yeast cell surface. DIII displaying yeast cells were incubated with pooled sera collected on week 11 from mice injected with either 25 μg of plant-produced DIII (a) or PBS (b). hE16 was used as a positive control mAb (c). After incubation, yeast cells were stained with an Alexa Fluor 488-conjugated goat anti-mouse (a and b) or goat anti-human (c) secondary antibody and processed by flow cytometry.

systems. The scalability of both upstream and downstream operations for transient plant expression systems has been recently demonstrated. For example, we used nontransgenic *N. benthamiana* plants for DIII production in this study. As a result, the wild-type plant biomass can be cultivated and produced in large scale with routine agriculture practice without the need to build extraordinarily expensive cell culture facilities [23, 37–39]. We previously demonstrated that commercially produced lettuce could be used as an inexpensive and virtually unlimited source for pharmaceutical protein production [40]. Accordingly, the agroinfiltration process to deliver DIII DNA construct into plant cells has been automated and can be operated in very large scales. For example, several metric tons of *N. benthamiana* plants are regularly agroinfiltrated per hour by using a vacuum infiltration procedure [27, 28]. For downstream processing, our extraction and purification procedure eliminated the hard-to-scale up steps of denaturing and refolding and allowed the recovery of highly purified DIII with a simple two-step procedure of low pH precipitation and IMAC. The scalability of the downstream process, consisting of precipitation and affinity chromatography, has been extensively demonstrated by the pharmaceutical industry and by our studies with other plant-produced biologics [30, 41]. This simple and scalable downstream process from plants will also reduce the costs associated with denaturing and refolding procedures and the overall cost for DIII production. The cost-saving benefit of plant-expression systems was also extensively documented by several case studies.

Our results also indicated that plant-produced DIII showed at least equivalent potency in eliciting humoral response against WNV in mice as *E. coli*-produced DIII. The demonstration of antibodies in anti-plant DIII serum that competed with hE16 for the same DIII epitope indicates the induction of potentially protective antibodies against WNV. It is interesting that both plant- and *E. coli*-produced DIII evoked a Th2-type response with alum as the adjuvant. This is in contrast to a previous report that *E. coli* DIII with CpG adjuvant stimulated a Th1-biased response [33]. This is not totally unexpected, as comparative studies with *Flavivirus* antigens showed that alum tends to induce Th2 type response, while CpG is likely to skew the response toward the Th1 type [42]. Since *E. coli*-produced DIII was shown to be protective in the mouse challenge model [29, 33, 34], the equivalent potency of plant-DIII in generating high IgG titers and the induction of hE16-like antibodies suggest that it is highly likely that plant-DIII will induce protective immunity when a proper adjuvant is used. Overall, the rapidity of DIII expression, the availability of a simple purification scheme, and the low risk of contamination by human pathogen and endotoxin indicate that plants provide a robust and low-cost system for commercial production of subunit vaccines against WNV and other flaviviruses.

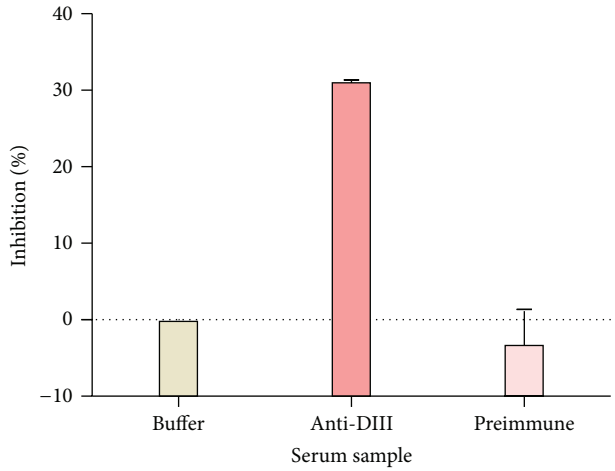

Figure 7: Competitive ELISA of DIII binding by hE16 and antibodies in anti-DIII serum. Plant-derived DIII immobilized in microtiter plate wells was preincubated with 1 : 100 dilution of indicated sera. hE16 was then applied to sample wells to determine its binding to DIII. The inhibition of serum preincubation on the subsequent hE16 binding to DIII is presented as the % of OD_{450} reduction by the preincubation. Mean ± SD of samples from three measurements is presented.

4. Experimental Procedures

4.1. Construction of DIII Expression Vectors. The coding sequence of WNV E DIII (amino acid 296–415, Genbank Acc. number AF196835) was synthesized with optimized *N. benthamiana* codons [43]. An 18 bp sequence coding for the hexa-histidine tag (His_6) was added to the 3′ terminus of the DIII gene and then cloned into the TMV-based expression vector pICl1599 of the MagnICON system [30, 43]. The MagnICON vectors were chosen because they have been demonstrated to drive high-level accumulation of recombinant proteins in *N. benthamiana* plants [30, 31, 38, 41, 43].

4.2. Expression of WNV E DIII in N. benthamiana Leaves. Plant expression vectors were transformed into *A. tumefaciens* GV3101 by electroporation as previously described [24]. *N. benthamiana* plants were grown and agroinfiltrated or coagroinfiltrated with the GV3101 strain containing the DIII-His_6 3′ module (pICH11599-DIII) along with one of its respective 5′ modules (pICH15579 for cytosol targeting, pICH20999 for ER targeting, or pICH20030 for chloroplast targeting) and an integrase construct (pICH14011) as described previously [27, 28, 30, 38, 41].

4.3. Extraction and Purification of DIII from N. benthamiana Leaves. Agroinfiltrated *N. benthamiana* leaves were harvested 2–5 dpi for evaluating DIII expression. Leaves were harvested 4 dpi for other protein analysis. Leaves were homogenized in extraction buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCL, 1 mM PMSF, tablet protease inhibitor cocktail (Sigma, Germany) at 1 mL/g LFW). The extract was clarified by centrifugation at 18,000 ×g for 30 min at 4°C. The pH of the clarified extract was adjusted to 5.0 and subjected to centrifugation at 18,000 ×g for 30 min at 4°C. The supernatant was recovered, pH adjusted back to 8.0, and subjected to another centrifugation. The supernatant was then subjected to Ni IMAC on a 4 mL His. Bind column in accordance with the manufacturer's instruction (Millipore, USA). The purified WNV DIII was eluted with imidazole and the eluate was dialyzed against PBS. The purity of DIII was estimated by quantitating Coomassie blue-stained protein bands on SDS-PAGE using a densitometer as described previously [30].

4.4. SDS-PAGE, Western Blot, and ELISAs. Samples containing DIII were subjected to 15% SDS-PAGE under reducing (5% *v/v* β-mercaptoethanol) conditions. Gels were either stained with Coomassie blue or used to transfer proteins onto PVDF membranes (Millipore, USA). Membranes were first incubated with MAb hE16 [30] and then subsequently with a goat anti-human kappa antibody conjugated with horseradish peroxidase (HRP) (Southern Biotech). Specific bindings were detected using an "ECL plus" Western blot detection system (Amersham Biosciences).

The expression of WNV DIII protein in leaves was determined by a sandwich ELISA. Ninety-six well ELISA microtiter plates (Corning Incorporated, USA) were coated at 1 μg/mL hE16 mAb in coating buffer (100 mM Na_2CO_3, pH 9.6) overnight at 4°C. After washing three times with PBST (PBS containing 0.1% Tween-20), plates were blocked with blocking buffer (PBS containing 5% milk) and incubated with plant extracts. Purified bacterial WNV DIII was used as a positive control to generate the standard curve. Extracts from uninfiltrated plants were used as a negative control. After washing, the plate was incubated with a rabbit anti-WNV DIII polyclonal antibody [43], followed by an HRP-conjugated goat anti-rabbit IgG (Southern Biotech). The plates were then developed with TMB substrate (KPL Inc). Values from negative control leaves were used as "background" of the assay and were subtracted from the corresponding values obtained from DIII construct-infiltrated leaves.

The hE16 recognition ELISA was performed as described previously [30]. Briefly, purified plant-DIII was immobilized on microtiter plates. After incubation with hE16 purified from mammalian cells or from plants, an HRP-conjugated goat anti-human-gamma HC antibody (Southern Biotech) was used to detect bound antibodies. A generic human IgG (Southern Biotech) was used as a negative control.

The titer of DIII-specific IgG in mouse serum was also determined by an ELISA. Microtiter plates were coated with plant- or *E. coli*-derived DIII, blocked with PBS with 1% bovine serum albumin (BSA), and incubated with a serial dilution of serum. After washing with PBST, the plates were incubated with an HRP-conjugated goat anti-mouse IgG (H + L) (Southern Biotech). After further washing with PBST, the plates were developed with TMB substrate (KPL Inc). Geometric mean titer (GMT) was calculated for each group at various time points and was used to express the titer of the DIII specific IgG.

The ELISA for determining the IgG1 and IgG2a subtypes were performed also on plates coated with plant- or *E. coli*-derived DIII as described above. Serial dilutions of serum

were applied to sample wells and incubated for 2 hr at 37°C. After washing with PBST, the plates were incubated with an HRP-conjugated goat anti-mouse IgG1 (Santa Cruz Biotech) or anti-mouse IgG2a (Southern Biotech). In parallel, various dilutions of mouse IgG1 and IgG2a (Southern Biotech) were coated on the same set of plates for generating standard curves. The plates were developed with TMB substrate (KPL Inc.).

A competitive ELISA was also performed on plates coated with DIII purified from plants. After blocking, plates were preincubated with serial dilutions of serum from pooled preimmune serum (Group 3), or pooled serum collected at week 11 (Groups 1 and 3). After thorough washing with PBST, plates were incubated with hE16, subsequently an HRP-conjugated goat anti-human-gamma HC antibody (Southern Biotech), and developed with TMB substrate (KPL Inc). The inhibition of hE16 binding to DIII by preincubation of sera was calculated by $(\text{Binding}_{(\text{no pre-incubation})} - \text{Binding}_{(\text{pre-incubation with serum})}) / \text{Binding}_{(\text{no pre-incubation})}$.

All ELISA measurements were repeated at least three times with each sample in triplicate.

4.5. DIII Expression in E. coli and Yeast Surface Display. The synthesized DIII coding sequence was cloned into the pET28a bacterial expression plasmid (EMD Milipore) with EcoRI and HindIII sites. DIII was expressed in *E. coli* and purified using an oxidative refolding protocol as described previously [44]. Refolded DIII protein was further purified with a Ni His. Bind IMAC as described for plant-derived DIII.

Yeast expressing WNV DIII was generated and stained with mAbs as described previously [30]. Briefly, yeast cells were first grown to log phase and subsequently induced for DIII expression by an additional 24 h culture in tryptophan-free media containing 2% galactose. The yeast cells were then incubated with pooled mice serum collected in week 11 from the DIII immunization experiments or hE16 mAb as a positive control [30]. Serum from the saline mock-immunized mice was used as a negative control. The yeast cells were stained with a goat anti-mouse or goat anti-human secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). Subsequently, the yeast cells were analyzed on a BD FACSCalibur flow cytometer (Franklin Lakes).

4.6. Mouse Immunization. All animal work was approved by the institutional animal care and use committee. Five-week old female BALB/C mice were divided into 5 groups ($n = 6$ per group). Group 1 received saline buffer (PBS) with alum as mock immunized control. Groups 2 and 3 received 5 μg and 25 μg of plant-derived DIII per dosage, respectively. Groups 4 and 5 received 5 μg and 25 μg of *E. coli*-produced DIII per dosage as controls. On day 0, each mouse was injected subcutaneously with 100 μL of material containing saline (Group 1), 5 μg (Groups 2 and 4), or 25 μg (Groups 3 and 5) purified DIII protein in PBS with alum as adjuvant (Sigma, DIII Protein solution: alum volume ratio = 1 : 1). Mice were boosted three times (on days 21, 42, and 63) with the same dosage and immune protocol as in the 1st immunization. Blood samples were collected from the retroorbital vein on day 0 before the immunization (pre-immune sample) and on days 14 (2 week), 35 (5 week), 56 (8 week), and 77 (11 week) after the 1st immunization. Serum was stored at −80°C until usage.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Plant Virus Expression Vector Development: New Perspectives

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Plant made biologics have elicited much attention over recent years for their potential in assisting those in developing countries who have poor access to modern medicine. Additional applications such as the stockpiling of vaccines against pandemic infectious diseases or potential biological warfare agents are also under investigation. Plant virus expression vectors represent a technology that enables high levels of pharmaceutical proteins to be produced in a very short period of time. Recent advances in research and development have brought about the generation of superior virus expression systems which can be readily delivered to the host plant in a manner that is both efficient and cost effective. This review presents recent innovations in plant virus expression systems and their uses for producing biologics from plants.

1. Introduction

It takes just a quick glance at the number and variety of novel biologics that are currently emerging from the commercial sector to realize that the use of plants as production platforms for vaccines and other therapeutic proteins has over time moved from mere theory and into actual practice. Plant-derived biologics are safe, efficacious, and easy to mass produce. They can offer new solutions to the challenge of providing inexpensive medicines that lack cold chain requirements or an established medical infrastructure for the world's rural poor. While assisting developing countries has always been a fundamental driving force for the development of plant-made biologics, other uses have also been presented and range from facilitating the stockpiling of vaccines against pandemic infectious diseases to the applications of plant-made proteins in the field of personalized medicine [1, 2].

Originally, plant-made biologics were engineered to be expressed from stably transformed plants. As this research area progressed to the point of determining plant-based pharmaceutical performance in actual clinical trials, it became increasingly apparent that lengthy tissue culture and plant regeneration procedures and concerns regarding public perception over the use of GM technology were all major stumbling blocks with regard to the use of transgenic plants that needed to be addressed. The design and implementation of novel transient expression systems through the use of plant virus expression vectors has been one technology that circumvents many of these hurdles. Not only are plant viruses able to produce extremely high levels of foreign protein at low cost and in a matter of a few days postinoculation, but also their use as a technology is more appealing to the public because it lacks the negative connotations that are often associated with GM plants. Indeed, removal of the genes encoding virus movement and/or coat proteins prevents plant viruses from moving from plant to plant and as a result reduces concerns about transmission and cross-contamination of pharmaceutical proteins to weedy relatives.

As research and development of plant virus expression vectors progress, the means of introducing them to the host plant has also advanced. Rather than merely infecting plants with the appropriate virus vector, plant leaves can now be inoculated by agroinfiltration, that is, by incorporating the virus vector into Agrobacterium tumefaciens and infiltrating the leaf using a syringe or a vacuum. Both methods have their own advantages, and both are rapid and scalable and lack the requirement of sophisticated equipment. The universality of these technologies makes them amenable for both commercialization and classroom alike [3].

Over the past decade, plant viruses have been developed both as vectors for biopharmaceutical protein production and also as research tools for plant functional genomics studies,

by incorporating virus induced gene silencing (VIGS) as a means to target and downregulate specific host transcripts. The latter technology has great implications for the development of crops with improved characteristics, while the former has the potential to launch a new era for the production of biologics. This review will discuss several of the plant virus expression systems that have been developed to date and will provide selected examples of their application in the field of plant-made biologics.

2. Expression Systems Based on Positive Sense RNA Viruses

2.1. Tombusviruses. Tobacco mosaic virus (TMV) is one of the most well characterized plant viruses and was the first plant virus examined for vector development. While original TMV-based viral vectors depended on usage of the entire viral genome, second generation vectors were "deconstructed" and consisted of the only sections of genomes that were important for replication; these were subcloned into an assortment of plasmid constructs. The first of these new series of vectors, the MagnICON vector system of ICON Genetics, is a core technology of Dr. Gleba and coworkers [4]. A technique for transfecting plants with these recombinant virus vector modules, known as "magnifection," involved the infiltration of a suspension of *A. tumefaciens* using a vacuum into all mature leaves of a tobacco plant, thus infecting the whole plant in its entirety [5]. These constructs are part of the T-DNA of a binary vector, and researchers need to merely mix different Agrobacterium strains which harbour these constructs prior to agroinfecting plants. As an example, yields of recombinant human growth hormone protein (hGH) reaching up to 10% of total soluble protein or 1 mg/g of fresh weight leaf biomass have been achieved using this system of expression [5]. Further modifications, including alteration of transcript splicing sites, modification of codon usage patterns, and the introduction of introns into TMV coding sequences, have improved protein expression further.

The technology has been built upon so that it could be incorporated into the Gateway cloning system, a series of plasmids that are used for a wide variety of expression systems [6]. In this case, the need for traditional cloning has been circumvented by a system that involves site-specific recombination. In this way, foreign genes and other DNA fragments alike can be transferred between plasmids to enable the desired recombination event to take place. Protein expression can also be enhanced 10- to 25-fold by the coexpression of the RNA silencing suppressor gene of Tomato bushy stunt virus known as P19. For example, Lindbo coinoculated plants with a TMV-based vector and a viral suppressor of RNA silencing; this culminated in the production of tremendous levels of recombinant protein (between 600 and 1200 micrograms of GFP per gram of infiltrated tissue) after one week postinfection [7].

Other researchers have found that gene expression can be increased several times by placing the foreign gene open reading frame (ORF) closer to the 3′ end of the TMV RNA. This TRBO (TMV RNA-based overexpression) vector lacks the coat protein coding sequence and can produce 100-fold more recombinant protein in plants than the P19-enhanced transient expression system. Lindbo's group found that this vector could generate 100 times more of a variety of recombinant proteins than their P19 silencer system [8].

The number of examples of the use of TMV-based vector technology as a platform for biopharmaceutical production in plants is rapidly increasing. Since this review cannot adequately describe all in the space constructions allowed, only a few are provided in the following section.

The first example illustrates the use of TMV to generate the broadly neutralizing antibody (bnMAb) known as VRCO1 against HIV-1 [9]. VRC01 binds to the CD4-binding site of gp120 and was isolated recently from a slowly progressing HIV-1-infected donor. Broadly neutralizing antibodies are known for their ability to block infection of a wide number of different strains of a particular virus and thus are considered to be used as part of a topical microbicide to block HIV-1 transmission. Monoclonal antibodies are expensive to manufacture on a large scale, and plants offer an attractive alternative for generating biologically active, inexpensive versions of these much needed pharmaceuticals. *Nicotiana benthamiana* plants were used as the host for a TMV vector which contained a full-length version of the immunoglobulin IgG1 from a single polypeptide. This VRCO1 MAb was produced at approximately 150 mg/kg fresh leaf material within days 5–7 postagroinoculation and could be purified on a protein A affinity column. These plant-made antibodies not only were shown to be biologically active in a neutralization assay but also worked synergistically with other microbicides, demonstrating their potential as part of a topically applied microbicide cocktail to act as a prophylactic against infection by HIV-1 [9].

The use of TMV to produce a vaccine for pandemic influenza virus represents the second example. Seasonal influenza virus has become a serious health threat across the globe. New vaccines must be provided every new year to protect against emerging new virus subtypes that circulate every new flu season. The requirement to supply the demand for flu vaccines worldwide is enormous and cannot currently be met. Recently, Petukhova et al. have used a TMV vector to express three different versions of the epitope M2e of influenza virus [10]. The authors used the CP of TMV as a carrier molecule and expressed the epitope as part of a fusion protein that is exposed on the surface of TMV as a nanoparticle. Antibodies raised in mice against the nanoparticle were specific to M2e and mice immunized with the M2e:TMV recombinant virus were resistant to inoculation with lethal doses of influenza H1N1 virus [10].

More extensive investigation into the utilization of TMV as a feasible vector for influenza vaccine production has been pursued by Yusibov et al.'s research group at Fraunhofer USA. The Fraunhofer group has generated a subunit vaccine based on recombinant hemagglutinin from the 2009 pandemic A/California/04/2009 (H1N1) strain of influenza virus [11]. This TMV-based vaccine was demonstrated to be efficacious and free of adverse effects in human clinical trials in the presence or absence of Alhydrogel as an adjuvant, in a manner

that is highly comparable with the currently used approved vaccine for H1N1 [12, 13]. This represents the first study that demonstrates a plant produced subunit influenza H1N1 vaccine in healthy adults. In a further study, Neuhaus et al., tested the ability of this tobacco produced vaccine in conjunction with a silica nanoparticle-based drug system to induce an antigen-specific recall response at the site of virus entry in human precision-cut lung slices (PCLS) [14]. The authors demonstrated that the plant produced vaccine was capable of reactivating an established antigen-specific T cell response at the site of virus entry [15].

As a third example, Li et al. have used the TMV-based TRBO vector to express the allergen R8 from dust mites in tobacco plants [16]. The authors used murine asthmatic models to investigate the possibility of using these plant-derived antigens for immunotherapy. The plant-derived antigen behaved the same way as the native antigen, offering the possibility that it might be used in the future for the diagnosis of asthma or the production of a candidate vaccine for allergen-specific immunotherapy of asthma [16].

As a final example, MAGNICON vectors were used to compare expression of the highly unstable recombinant protein, human complement factor 5a (C5a) in tobacco plants [17]. Transient expression of C5a that was subcellular targeted to the ER or vacuole using the MagnICON vector was increased from 0.0003 and 0.001% total soluble protein (TSP) to 0.2 and 0.7% of TSP, respectively, demonstrating the utility of this system to produce biopharmaceutical proteins [17].

2.2. Potexviruses. Potato virus X (PVX) and related potexviruses have also been constructed into expression vectors for vaccine production. For example, the L1 protein of canine oral papillomavirus has been expressed in transgenic tobacco chloroplasts using a Potato virus X vector [18]. Similarly, the Human Papillomavirus-16 L2 minor capsid protein has been expressed in plants as part of a fusion protein with the PVX CP [19]. Other potexviruses, including white clover mosaic virus, foxtail mosaic virus, and alternanthera mosaic virus, have also been developed for foreign gene expression. Recently, an expression vector based on plantago asiatica mosaic virus (PlAMV) has been constructed [20]. PlAMV is unique among potexviruses due to an overlap at the third gene of its triple gene block and its CP. As a result, the generation of a recombinant PlAMV expression system resulted in a duplicated CP promoter sequence that is much longer than that generated for other potexviruses. Due to this unusual feature, the PlAMV-based vector was more stable and had the ability to exert a stronger suppression of gene silencing activity than its PVX-based counterpart.

2.3. Cucumovirus. The RNA virus Cucumber mosaic virus (CMV) has also been developed extensively for use in the production of plant-made biologics. Although it has a highly diverse host range, CMV's trimeric RNAs are each required for virus infection to be successful and are packaged into icosahedral capsids, thus enforcing a size limitation to the foreign gene that can be inserted. Recently, Hwang et al. constructed the complete tripartite genome on a binary plasmid and replaced the coat protein gene with one for α-1-antitrypsin [AAT] [21]. The authors placed one of the protein components of the viral replicase (1a) under the control of the XVE chemically inducible promoter in such a way that recombinant viral amplicons could be induced upon addition of the inducer (β-estradiol). This enables all of the components that are required for virus replication to be introduced simultaneously into the same cell, thus improving the efficiency of transgene expression. The authors tried to further improve expression by generating multiple plasmids of reduced size which, when combined, incorporate all of the essential components of the genome that are required for replication. By using both GFP and the *Acidothermus cellulolyticus* endo-1, 4-β-glucanase (E1), a cellulose degrading enzyme, the authors demonstrated that this CMV advanced replication system can effectively achieve recombinant proteins at levels comparable to transgenic plants, but without the same time and effort required [21].

CMV has also been utilized as an antigen presentation system to express epitopes of porcine circovirus type 2 (PCV2) capsid protein [22]. Chimeric CMV:PCV2 particles were injected into mice and pigs and analyzed for their ability to induce a PCV specific antibody response. Furthermore, pigs challenged with the virus were able to demonstrate partial protection against infection, suggesting that generation of a plant virus-based vaccine may be a feasible and affordable approach to combat this animal pathogen [22].

3. Other Plant Viruses Involved in Optimizing Expression

3.1. Cowpea Mosaic Virus. The icosahedral, positive sense RNA virus Cowpea mosaic virus (CPMV) has also been designed for the generation of vaccine and other therapeutic proteins in plants. Initially designed as an epitope presentation system, CPMV chimeric particles have been shown to elicit a robust immune response for a variety of diseases [23]. CPMV has also been used to express entire proteins as fusion products with the capsid protein or movement protein of the virus, which can undergo proteolytic cleavage to release the therapeutic protein.

Medicago, Inc., has also developed their own CPMV vector-based technology which produces virus-like particles (VLPs) carrying influenza virus antigens [24]. These VLPs express a lipid-anchored recombinant HA and are fully protected against lethal viral challenge in both mice and ferrets. This vaccine was placed under further study in a Phase 1 clinical trial of 48 healthy volunteers, who showed that the vaccine was both safe and well tolerated. A Phase 2 clinical trial with over 250 volunteers is now underway. Medicago's production system offers the advantage of inexpensively generating a vaccine within 3 weeks of the release of influenza strain sequence information, with easily adaptable upscaling capacity [24].

Nonreplicating CPMV expression systems have also been developed. These involve the positioning of the foreign gene between the 5′ leader sequence and 3′ untranslated region (UTR) of RNA-2 [25]. Deletion of an in-frame initiation

codon found upstream of the main translation initiation site of RNA-2 brought about a substantial increase in foreign protein production. This new series of vectors based on CPMV, known as the pEAQ vectors, have been explored as a means to mass produce large amounts of pharmaceutical proteins in plants without the need for virus replication. Using a high translational efficiency, the Cowpea Mosaic Virus hypertranslational "CPMV-HT" expression system generates extremely high yields of recombinant protein [23]. Many pharmaceutical proteins have been successfully produced in plants using this system, including anti-HIV-1 monoclonal antibodies and influenza A virus vaccines [25]. Recently, Thuenemann et al. used the nonreplicating CPMV system known as CPMV-HT (Hyper-Trans) to generate the ruminant Bluetongue virus- (BTV-) like particles in benthamiana plants. This required the synchronous expression of four distinct capsid proteins in each cell and enabling them to self-assemble into virus-like particles. These plant-derived VLPs were demonstrated to protect sheep against live virus infection [26].

3.2. Plant DNA Viruses. Plant DNA viruses, including geminivirus and Cauliflower mosaic virus (CaMV), have also been designed as pharmaceutical protein production platforms. Geminiviruses are small single stranded DNA viruses and are named for their twinned capsid morphology; they infect a broad range of plants and can replicate to extremely high copy numbers. Two geminiviruses have been developed for biopharmaceutical production, Bean yellow dwarf virus (BeYDV), a mastrevirus, and Beet curly top virus, (BCTV), a curtovirus [27, 28]. Both viruses have been constructed in such a way that the replication initiator protein (Rep) is expressed independently from the viral genome. A variety of biologics have been produced using this approach, ranging from a vaccine against hepatitis A virus to a monoclonal antibody against Ebola virus [27, 28].

4. Additional Applications for Plant Virus Vectors in Medicine

Besides offering a novel production platform for plant-made biopharmaceuticals, plant viruses have been engineered to provide other medical applications. For example, virus-like particles based on C*owpea mosaic virus* (CPMV) have been designed which can incorporate fluorescent dyes, polyethylene glycol (PEG) polymers, and various targeting moieties on their surfaces for the purpose of creating novel tumour-targeted molecular imaging agents [29, 30]. These CPMV VLPs exhibit high selectivity for molecular targets that are cancer-specific and as a result are effective for in vivo imaging of tumors. CPMV represents an icosahedral nanoparticle and the exterior of its capsid displays 300 accessible lysine residues; each of these can be conjugated to various chemical moieties [30]. Examples of the use of this technology include the construction of CPMV nanoparticles displaying gastrin releasing peptide receptor (GRPR) or vascular endothelial growth factor receptor 1 (VEGFR1) [31].

As another example, Cowpea Chlorotic Mottle Virus (CCMV) has been shown to stably assemble in vitro and package the RNA derived from a mammalian virus, Sindbis virus. The hybrid VLPs were able to deliver and release their RNA contents within the cytoplasm of mammalian cells. The CCMV-based VLP was shown to protect against RNA degradation by cellular nucleases. By conjugating subcellular targeting moieties, these hybrid VLPs could be directed toward distinct sites within the cell [32].

Plant viruses have also been engineered to act as adjuvants to elicit an immune response that is more potent and effective. Recently, nanoparticles that are constructed from the coat protein of the rod-shaped Papaya mosaic virus (PapMV) have been shown to be highly immunogenic and are taken up by dendritic cells [33]. These nanoparticles have been engineered to express an influenza epitope on their surface, and mice and ferrets immunized with these recombinant nanoparticles exhibit an increase in robustness of humoral response to influenza virus infection [34]. TMV has also been demonstrated to stimulate cellular immunity. Incubation of TMV CP fused to T cell tumor-specific epitopes elicited immune responses and protected against tumor challenge in mice, indicating that the virus could act as an antigen carrier and induce an adequate immune response [35].

5. Conclusions

The development of plant virus vectors continues to evolve with respect to their ease and breadth of use in the field of plant-made biologics. Improved expression vectors based on deconstructed viruses will most likely remain a trend in the near future. Efforts will be made to address other challenges including enhanced expression levels, the generation of proteins with more complex quaternary structures, and issues concerning host specificity. With the advancement of techniques to facilitate their introduction and transient expression in plants, plant virus expression vectors and components derived from them offer strategic advantages for the rapid and cost-effective production of biologics for the world's poor. The fact that this technology lends itself to other applications as well, including the development of vaccines to combat global pandemics and even cancer targeting therapeutics, offers much needed vigor to address a broadening spectrum of needs for medical researchers for many years to come.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

Comparative Evaluation of Recombinant Protein Production in Different Biofactories: The Green Perspective

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In recent years, the production of recombinant pharmaceutical proteins in heterologous systems has increased significantly. Most applications involve complex proteins and glycoproteins that are difficult to produce, thus promoting the development and improvement of a wide range of production platforms. No individual system is optimal for the production of all recombinant proteins, so the diversity of platforms based on plants offers a significant advantage. Here, we discuss the production of four recombinant pharmaceutical proteins using different platforms, highlighting from these examples the unique advantages of plant-based systems over traditional fermenter-based expression platforms.

1. Introduction

The market for recombinant pharmaceutical proteins is expanding rapidly. Indeed, nearly all pharmaceutical companies with a market capitalization value of more than $US 10 billion report that their revenue share from such products is growing faster than the share from small-molecule drugs [1]. The industry has focused on a small number of production platforms based on the bacterium *Escherichia coli*, several species of yeast, and a selection of insect and mammalian cell lines, which have been developed and improved in line with current good manufacturing practice (cGMP). However, focusing on a small number of platforms means that the unique requirements of certain target proteins are difficult to meet; this is the case of recombinant proteins that are required in small quantities (e.g., for individual patients) or in massive quantities or that need rapid production scale-up. Plant biotechnology can overcome some of these limitations and the potential of plant-based platforms for the flexible, low-cost production of high-quality, bioactive recombinant proteins is well-documented [2].

Plants successfully perform the majority of posttranslational modifications required for the activity of complex eukaryotic proteins and provide tremendous flexibility in terms of scale, cost, safety, and regulatory issues. For example, cell-based bioreactor systems including plant suspension cells and algae are ideal for lower-volume products, whereas field-grown commodity crops can produce metric tons of recombinant protein at highly competitive costs. Contained production systems based on plants have biosafety advantages over microbial and mammalian production platforms because they neither do produce endotoxins nor do they support the growth of pathogens that infect animals, thus reducing purification costs and minimizing the likelihood of facility shutdowns, decontamination issues, and supply limitations that lead to unmet patient/customer demands. Although the costs of downstream processing and purification are comparable in microbial, mammalian, and plant-based platforms, the lower up-front investment required for commercial production in plants and the potential economy of scale provided by cultivation over large areas are key advantages.

This combination of low capital investment, low-cost of goods, and highly scalable manufacturing means that many proteins that are unsuitable for production in fermenters can be produced commercially using plants. Other proteins can be produced more efficiently by fermentation in plant cells because the posttranslational modifications can be engineered to improve product quality and activity. Not all pharmaceuticals will benefit from plant-based systems but the best production platform should be determined

empirically for each protein using a case-by-case approach. Several recent reviews have discussed the merits of plant-made pharmaceuticals [3], including specific issues related to commercial production [4] and considerations of cGMP issues in plants [5]. This review will focus on four target molecules that highlight different applications across a range of expression systems to illustrate important ways in which plant-based expression platforms are evolving to meet a spectrum of research, development, and commercial needs.

2. Human Glutamic Acid Decarboxylase

The 65-kDa isoform of human glutamic acid decarboxylase (hGAD65) is an enzyme containing the prosthetic group pyridoxal 5′-phosphate (PLP). It forms obligate functional dimers and is localized in pancreatic β-islet cells as well as the brain, where it catalyzes the conversion of glutamate to γ-aminobutyric acid (GABA) and carbon dioxide. In human cells, the major pool of hGAD65 exists as an autoinactivated apoenzyme [6]. The crystal structure provides insight into both the molecular mechanism of catalytic activity and the structural determinants of its antigenicity [6].

The hGAD65 protein functions as an autoantigen in several autoimmune diseases, including autoimmune type 1 diabetes (T1D) and Stiff-Person syndrome. T1D is strongly associated with autoreactivity to hGAD65. Indeed, hGAD65 autoantibodies are present before the clinical onset of the disease and provide a useful marker to predict the likelihood of its development [7]. The relevance of such markers has been confirmed unequivocally in many laboratories that participate in the Diabetes Autoantibody Standardization Program (DASP), which is a collaboration between the US Centres for Disease Control and Prevention and the Immunology of Diabetes Society [8].

The autoantibodies are not directly pathogenic, whereas T cells play a dominant role in the initiation and progression of T1D. T-cell responses against the linear epitopes of hGAD65 can be detected in animal models of the disease and in humans at risk of T1D. Studies in animal models have shown that exposure to hGAD65 may induce immunotolerance [9, 10]. A phase II human clinical investigation, involving genetically predisposed children and young adults with multiple islet cell autoantibodies, is currently exploring whether treatment comprising two injections of 20 μg doses of alum-formulated hGAD65 (the GAD vaccine, Diamyd Medical) prevents the onset of the disease (NCT01122446).

The prevalence of T1D in the general population is currently 0.04%, but this is increasing at 3% per annum in children. If the clinical trial discussed above is successful, then the global demand for recombinant hGAD65 would increase dramatically. GAD65 was initially sourced from porcine brains, although the most abundant source is monkey brain, with a yield of 12 mg/g [11]. These sources are not suitable for therapeutic GAD65 due to the risk of infection with prions and other pathogens, so heterologous production techniques were investigated following the isolation of hGAD65 cDNA [12].

In all heterologous systems, the yield of hGAD65 is reported by measuring its enzymatic and immunochemical activity. Posttranslational modifications occur in the N-terminal region, that is, blockage of the N-terminal amino group, palmitoylation, and phosphorylation, but none of these modifications are necessary for catalytic activity or immunogenicity so in theory the protein can be produced using any expression platform [11]. Furthermore, the abolition of enzymatic activity to generate a mutant protein (hGAD65mut) does not affect the immunoreactivity of the protein and thus its diagnostic and therapeutic potential [13].

Current commercial platforms for the production of diagnostic and research-grade GAD65 include yeast, baculovirus-infected insect cells, and wheat germ lysates, with costs of €2,000–60,000/mg. The suitability of these different production platforms has been discussed. For example, the expression of GAD65 in bacteria produced a misfolded protein that was primarily localized in inclusion bodies, and it was only possible to produce a soluble and immunogenic product by expressing the protein as an N-terminal fusion with thioredoxin or glutathione S-transferase [14, 15]. As well as making the protein soluble, the fusion partners also facilitated protein isolation, resulting in yields of up to 12.5 g/L.

Recombinant hGAD65 has also been expressed in baby hamster kidney (BHK) cells (Heinaes et al., unpublished data), Chinese hamster ovary (CHO) cells, and mouse myeloma cells, the latter resulting in the highest yield of 1.7 mg/L [16]. Although the overall yield was lower than achieved in bacteria, the recombinant protein was soluble and retained its native structure without a fusion partner. CHO cells have therefore been used to study the subcellular trafficking and localization of hGAD65.

Recombinant hGAD65 has also been expressed in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, both of which produced an active protein with yields of up to 3.52 mg/L [17]. Insect cells infected with baculovirus vectors achieved the highest yields of hGAD65 ever reported, in the best cases reaching 50 mg/L [18], but when hGAD65 was expressed with a C-terminal His_6 tag, the yield dropped to 3–5 mg/L [19].

Several plant-based platforms have also been used to produce hGAD65. *Chlamydomonas reinhardtii* chloroplasts were transformed with an hGAD65 vector and the immunoreactive recombinant protein accounted for 0.3% of the total soluble protein (TSP) in the algal cells [20]. Immunoreactive and enzymatically active hGAD65 has also been expressed in tobacco and carrot plants albeit with disappointing yields; for example, in T1 tobacco plants, the yield was 10.5 μg/g fresh weight (FW) in the leaves [21–23].

The production of hGAD65 in plant- and insect cell-platforms was achieved by expressing the catalytically inactive version, hGAD65mut, which retains its immunogenicity. The mutant protein accumulates to higher levels than its active counterpart, that is, up to 143.6 μg/g FW in tobacco leaves [23]. The hGAD65mut mutant was generated by substituting the lysine residue that binds the cofactor PLP with an arginine residue (K396R). It was proposed that the wild-type version of hGAD65 interferes with plant cell metabolism to suppress its own synthesis, whereas the catalytically inactive

version escapes such feedback and accumulates to higher levels.

Other modified versions of GAD65 have been expressed, including a soluble form generated by substituting the N-terminal domain with the homologous region of the soluble 67-kDa isoform of the protein. This substitution increased the yields of the protein from 3.52 to 12.16 mg/L in *S. cerevisiae* [17] and from 10.5 to 50 μg/g in tobacco leaves [24]. Although differences in the stability of the N-terminal α-helical regions could theoretically account for these differences, there was no improvement to the yield of hGAD65mut in plants when the modification was included [25]. This suggests that abolishing the membrane interactions by removing the N-terminal region does not cause any additional benefit when the biological activity of the protein is eliminated.

Modifying the protein for retention in the endoplasmic reticulum (ER) of plant cells did not increase its yield in transgenic tobacco plants [25]. GAD67/65mut was also expressed in the seeds of three different species (Arabidopsis, tobacco, and petunia) and retained in the ER. The highest yield of 4.5 mg/g dry weight (DW) was achieved in Arabidopsis seeds [26].

The purification of hGAD65 from yeast, insect, and mammalian cells is usually achieved by immunoaffinity chromatography using anti-GAD monoclonal antibodies [16, 27], anion- exchange chromatography [18], or a combination of the two [17]. In bacteria and yeast, higher yields were achieved by expressing tagged fusion proteins and using the tag as the affinity ligand [15, 19, 28]. Although the purification of hGAD65 has not been reported in plants, edible plant tissues containing the protein can be administered by oral delivery such that extensive purification is not required. It has been demonstrated that the oral administration of a crude transgenic tobacco extract containing hGAD65, in combination with interleukin-4 (IL-4), diminished the peripheral immune response to a subsequent systemic challenge with the same autoantigen by inducing oral tolerance [21].

3. Norwalk Virus-Like Particles

Norwalk virus (NV) is the prototype human norovirus (NoV), which contains a single-stranded, positive-sense nonenveloped RNA genome containing three open reading frames and a polyadenylate tail [29]. The NV capsid is a 38 nm icosahedral structure assembled from 90 dimers of VP1, the 58-kDa capsid protein (CP), with $T = 3$ symmetry [30, 31]. NoV belongs to a group of highly infectious viruses that are responsible for more than 95% of epidemic outbreaks of viral gastroenteritis in adults in developed and developing countries [32]. In the USA alone, NoV causes ~21 million infections per year, resulting in 70,000 hospitalizations and 800 deaths, at a cost of $US 5.5 billion [33] (https://www.bcm.edu/molvir). In developing countries, NoV is responsible for up to 1.1 million hospitalizations annually and 218,000 deaths among children [32].

The increasing recognition of NoV as a disease agent, the absence of a specific treatment, and the limited success in preventing disease outbreaks have led to the evaluation of virus-based vaccines [34]. However, the insufficient quantity of virus particles available for analysis has delayed the development of such a vaccine. The only natural source of NV particles is human stools, which characteristically contain very low concentrations of viruses [29].

The successful cloning, sequencing, and expression of the major NV capsid protein VP1 in insect cells were a major breakthrough and showed that recombinant VP1 folds spontaneously into empty Norwalk virus-like particles (NVLPs) that are stable following lyophilization at temperatures of up to 55°C and/or when exposed to acids (pH 3–7) [35]. The recombinant NVLPs remain immunogenic and interact with cellular receptors, eliciting a strong host immune response against the virus [29, 31, 36, 37], and would therefore make ideal NV vaccine candidates [38, 39].

Preclinical studies showed that recombinant NVLPs are immunogenic when administered by the parenteral [29], oral [40, 41], and intranasal routes [42]. Furthermore, a specific formulation for intranasal delivery, comprising NVLP dry powder and a novel plant-derived polysaccharide with gelling properties (GelSite), showed superior immunogenicity in mice than in a liquid formulation including an adjuvant [43].

In phase I studies, orally administered NVLPs were found to be safe but only modestly immunogenic as determined by measuring serum antibody levels and counting specific antibody-secreting cells (ASCs) [44–46]. Conversely, a nasally delivered NVLP formulation including an adjuvant was well tolerated and highly immunogenic [47]. A phase I/II study carried out by LigoCyte Pharmaceuticals showed that two 50 μg intranasal doses of NVLPs protected mice against challenge with a homologous virus [48]. Furthermore, parenteral administration in phase I/II studies demonstrated that two 100 μg intramuscular doses of NVLP vaccine were well tolerated and produced a clinically relevant impact on the incidence of NV after challenge, as well as the severity in breakthrough cases (Takeda Pharmaceuticals USA Inc., 2013). Collectively, these clinical trials indicated that vaccination may be useful to prevent disease caused by the NoV strains most commonly associated with infection in humans. If future clinical trials confirm the efficacy of the NVLP vaccine in humans, large amounts of NVLPs will be needed to facilitate global vaccination campaigns.

The development of an effective NV vaccine has been hindered by the lack of an animal model for virus production and the inability to grow the whole virus in cell culture. Several expression systems have therefore been tested for the production of NVLPs, including baculovirus-infected insect cells, bacteria, yeast, mammalian cells, and plant-based systems. These production platforms have been investigated by electron microscopy to confirm the fact that the Norwalk virus coat protein (NVCP) self-assembles into NVLPs. Furthermore, the immunogenicity of the recombinant NVLPs has been investigated in animals.

The first attempt to produce NVCP in a heterologous system involved baculovirus-infected insect (*Spodoptera frugiperda*) cells (Sf9). NVLPs similar in size and appearance to native capsids were detected and, although no expression data were reported, the yield of purified protein ranged from 65 to 125 mg per liter of infected insect cell cultures [29].

NVCPs representing different NV strains have also been expressed in *E. coli* as fusion proteins with maltose binding protein (MBP) and thioredoxin. The yields of the purified fusion proteins were 26 and 56 mg/L, respectively, but no NVLPs were detected. The unassembled purified capsid proteins were analyzed to determine the possibility of establishing an immunologic detection system for NoV antigens, based on the enzyme-linked immunosorbent assay (ELISA), and confirming the diagnosis of NoV-infected patients using recombinant NVCP [49].

NVCP was successfully expressed in *P. pastoris* system after testing a range of expression vectors and culture conditions. Recombinant NVCP spontaneously formed NVLPs with final yield of 5–10 mg/L after purification. The yeast-derived NVLPs were tested as potential NV oral vaccines by feeding raw yeast extracts to animals. Even at doses as low as 0.1 mg, the yeast-derived NVLPs were able to induce significant systemic and intestinal mucosal responses in the animals [50].

Venezuelan equine encephalitis (VEE) virus replicon particles (VRPs) have been used as vectors to express NVCPs in BHK cells, resulting in the production of NVLPs with yields of approximately 10^{10} partially purified particles per mL [51]. VRPs can be used both as vectors to generate NVLPs in heterologous systems or as a self-replicating vaccine that produces recombinant NVLPs in target cells. Mice inoculated subcutaneously with these particles (two doses, 10^7 infectious units each) developed systemic and mucosal immune responses to NVLPs, as well as heterotypic antibody responses to the major capsid protein from a different NV strain [52].

NVLPs have also been produced in many plant-based systems, with initial experiments focusing on constitutive expression in transgenic plants. Recombinant NVCP self-assembled into NVLPs that accounted for 0.23% of TSP in transgenic tobacco leaves [41] and NVCPs also accumulated to 0.37% of TSP in transgenic potato tubers (34 μg/g of tuber weight) although only ~50% self-assembled into NVLPs. The oral immunogenicity of partially purified NVLPs from tobacco and potato was demonstrated in mice [41], whereas phase I clinical studies in humans demonstrated that the administration of uncooked potatoes containing NVLPs was safe, but only modestly immunogenic [45].

A modified NVCP gene, codon-optimized for plants, was later expressed in tomato and potato, resulting in the accumulation of NVCP at levels of up to 8% TSP in tomato fruits and 0.4% TSP in potato tubers, corresponding to 160 μg/g (100 μg NVLPs/g) in tomato fruits and 120 μg/g (90 μg NVLPs/g) in potato tubers. Freeze-dried potato and tomato tissues were immunogenic when fed to mice, but the delivery of the same doses of air-dried tomato fruit stimulated stronger immune responses. It was proposed that air-drying preserves the stability of NVLPs and the fruit tissue structure, thus conferring greater protection against proteolytic enzymes in the gut [53].

More recently, MagnICON vectors have been used for the rapid and efficient production of NVLPs in *Nicotiana benthamiana* plants. Different subcellular localizations were compared, and the highest yields were achieved by cytosol targeting (860 μg NVCP/g FW in the leaves) at 12 days after infection (dpi). The partially purified recombinant NVLPs were orally immunogenic when fed to outbreed CD1 mice [54].

The agroinfiltration of *N. benthamiana* leaves with an optimized DNA replicon from bean yellow dwarf virus resulted in efficient replicon amplification and robust NVCP production within 5 days. The NVCP yield was ~340 μg/g FW in the leaves and the protein assembled efficiently into NVLPs [55]. The same expression vector was recently used in lettuce, which produces low levels of secondary metabolites. This resulted in average NVCP yield of 200 μg/g FW in the leaves [56]. The production of NVLPs in *N. benthamiana* plants has also been optimized using *Agrobacterium*-mediated transient gene expression for the simultaneous expression of two NV capsid proteins (VP1 and VP2) to increase NVLP stability, along with the *Pepper mild mottle virus* suppressor of viral posttranscriptional gene silencing. This achieved yields of up to 1 mg of partially purified NVLPs per g FW in the leaves [57].

The purification of NVLPs is usually achieved by using ultracentrifugation and density gradient methods that exploit particle size and density regardless of the expression platform [29, 41, 46, 51, 54, 55, 57]. However these methods are technically demanding and difficult to scale up, so alternative processing strategies have been explored [50, 56, 58]. Low-pH precipitation coupled with DEAE anion-exchange chromatography recently allowed the efficient purification of NVLPs from *N. benthamiana* leaves [59]. This was the first report to describe the scaled-up production of a pharmaceutical-grade (cGMP-compliant) NVCP vaccine in plants, and the product is currently being tested in a phase I human clinical trial.

4. Monoclonal Antibody 2G12

The monoclonal antibody (mAb) 2G12 is a broadly neutralizing anti-HIV-1 human IgG1 that recognizes a high-mannose glycan cluster on the surface of the virus glycoprotein 120 (gp120). It was isolated from an asymptomatic HIV-1 infected patient in 1990, and in 1994 its neutralizing activity against HIV-1 strains and its ability to bind with gp120 were described for the first time [60, 61]. The broad biological activity of 2G12 allows it to defend against infection with primary HIV isolates from various clades, either by direct virus neutralization or by combination with other effector cells and complement activation [62].

As well as neutralizing HIV-1 *in vitro*, passive transfer studies in primates demonstrated that 2G12 can control infection and prevent transmission *in vivo* following parenteral or mucosal administration, preferably in combination with other neutralizing antibodies [63, 64]. A phase I study in humans demonstrated the safety of repeated intravenous infusions of 2G12 combined with another broadly neutralizing antibody (2F5) when administered to asymptomatic patients infected with HIV-1 [65, 66]. Moreover, 2G12 combined with two broadly neutralizing antibodies (2F5 and

4E10) was able to delay viral rebound in patients whose infections were fully suppressed by antiretroviral treatment before antibody administration [67]. A phase II trial was then carried out to investigate the pharmacokinetic properties of the antibodies in the cocktail [68]. Such approaches require large doses of recombinant antibody (7–14 g of each antibody per patient) and thus create an immense demand, given that ~35 million people were living with HIV in 2012 (UNAIDS, 2013).

Broadly neutralizing human monoclonal antibodies such as 2G12 can also be applied as a mucosal microbicide to prevent HIV infection [69]. A recombinant form of 2G12 produced in stable transformed tobacco plants has been tested in a phase I clinical trial, based on intravaginal administration of the antibody to healthy female subjects at a dose range of 7–28 mg per individual (NCT01403792). Future trials will test the efficacy of prophylaxis in humans [70].

The complex and glycosylated structure of 2G12 means that it must be produced in eukaryotic expression platforms and then tested in specific assays to confirm its *in vitro* antigen-binding and neutralization capacity. The molecule was initially produced in hybridoma clones, generated by the electrofusion of B-cells and CB-F7 myeloma cells, producing 10 pg of the antibody per cell per day [60]. The mRNA for the 2G12 heavy and light chains was isolated and transcribed into cDNA in 1998 [71]. Large-scale antibody production was then achieved in CHO cells and the antibody was purified by protein A affinity chromatography. For most *in vivo* studies and clinical trials, 2G12 IgG1 was manufactured by Polymun Scientific Immunbiologische Forschung GmbH (Vienna, Austria) under cGMP guidelines, at a cost of €350–500/mg. Uniquely, the 2G12 prepared for clinical trial NCT01403792 was manufactured in tobacco leaves using a novel cGMP process.

HIV microbicides must be effective, safe, user-friendly, and above all economically affordable in the developing world, which has the highest number of HIV patients. Plants are ideal for the production of such low-margin/high-demand antibodies because of the economy of scale offered by agricultural production. This concept was developed in the EU project Pharma-Planta, which achieved the expression of 2G12 in several plant species and the fast-track development of transgenic tobacco as the primary production platform. The use of many different plants showed that the species, tissue, and subcellular compartment could affect the structure and composition of the antibody glycans, but this had no significant impact on the HIV-neutralization capacity of the antibody *in vitro* [72].

A secreted form of 2G12 has been constitutively expressed in the leaves of wild-type Arabidopsis plants [73] and in a mutant strain modified to knock out the genes encoding β1,2-xylosyltransferase (XT) and core α1,3-fucosyltransferase (FT), thus producing complex N-glycans lacking plant-specific residues [74]. The yield of the antibody was 0.05–0.2% of TSP in these young plants. The secreted and ER-retained versions of 2G12 were also produced in Arabidopsis seeds, achieving yields of 3.6 and 2.1 mg/g DW, respectively [75]. In the same series of experiments, the secreted form of 2G12 was also produced in the seeds of the XT/FT knockout line [75].

Cereals are considered more suitable for the production of recombinant proteins in developing countries because dry seeds preserve recombinant proteins in a stable form without a cold chain, and maize has been widely used for the production of pharmaceutical proteins in this context. A secreted form of 2G12 was expressed in the endosperm of the elite maize cultivar M37W and the best-performing line was passed through to a dedifferentiation-regeneration cycle, producing seeds yielding more than 100 μg of the antibody per gram DW and eliminating most of the seed-to-seed variation [76]. HIV-neutralization assays showed that maize-derived 2G12 was nearly three times more potent than its CHO-derived counterpart, probably reflecting the higher proportion of aggregates (which are known to be more efficient than monomeric antibodies in terms of neutralization efficacy). The same antibody has also been retained in the ER of maize endosperm cells by adding a C-terminal KDEL tag to both antibody chains, resulting in its accumulation in ER-derived zein protein bodies [70]. These experiments were carried out using the cultivar Hill, but since this variety has little agronomic relevance, it was backcrossed to elite starch germplasms and a sugar-type sweetcorn background. The average yield in the T3 generation was 38.8 μg/g DW, with a maximum of 60 μg/g DW. As above, the plant-derived antibody was more potent in neutralization assays than the same antibody produced in CHO cells.

The 2G12 antibody has also been produced by transient expression in *N. benthamiana* leaves, initially using three glycoengineered lines in which RNA interference (RNAi) was used to suppress the synthesis of xylosylated and/or core α1,3-fucosylated glycan structures [77]. A binary vector carrying the cDNA sequences of both antibody chains was used for agroinfiltration and the yield was 110 μg/g FW (corresponding to approximately 0.5% TSP) in the leaves. Similarly, *N. benthamiana* leaves were coinfiltrated with two binary vectors, one encoding the two antibody chains and the other carrying the p19 silencing suppressor gene [78]. Secreted and ER-retained forms of the antibody were produced with yields of ~100 μg/g FW in the leaves at 6 dpi, increasing until 18 dpi. There was a small reduction in antigen-binding activity compared to 2G12 from CHO cells, probably reflecting the presence of residual impurities, but as above the HIV-neutralization capacity was higher. The 2G12 antibody has also been transiently expressed in *N. benthamiana* leaves using replicating and nonreplicating systems based on deleted versions of *Cowpea mosaic virus* (CPMV) RNA-2 [79]. In both cases, secreted and ER-retained versions of the antibody were expressed, yielding 14.8 and 37.8 μg/g FW, respectively, using the replicating vector and 66.7 and 123.8 μg/g FW, respectively, using the nonreplicating vector. The resulting antibody once again showed a marginally lower affinity for its antigen but similar or marginally better neutralization activity compared to 2G12 produced in CHO cells.

Tobacco has been used both for the transient and stable expression of 2G12. The secreted and ER-retained forms were transiently expressed in tobacco leaves coinfiltrated with *Agrobacterium tumefaciens* vectors containing expression constructs for the heavy and light chains, achieving yields

of 80–100 μg/g FW in the leaves [78]. The ER-retained form was expressed stably in transgenic tobacco leaves, accounting for 0.4% of TSP in the mature leaves [80]. As above, subsequent assays showed that the plant-derived antibody bound its antigen more weakly but neutralized HIV more potently than the CHO-derived counterpart. The expression of 2G12 in tobacco seeds achieved yields of 0.3% TSP, and immunolocalization studies demonstrated that the antibody accumulated in protein storage vacuoles (PSVs). The seed-derived antibody showed significantly lower antigen-binding activity than the leaf-derived protein, probably reflecting genetic segregation and thus the generation of a significant proportion of seeds expressing the heavy chain alone.

Finally, 2G12 has also been expressed in tobacco cell suspension cultures prepared from cultivar BY-2 [81]. Optimization of the nitrogen supply increased the yield to 12 mg/L by day 7 of the fermentation process. The antibody was secreted into the medium but a proportion also accumulated within the cells. The antigen-binding activity of the fully secreted antibody was 83% compared to the CHO counterpart (set arbitrarily at 100%), whereas that of the intracellular fraction was 40%. This probably reflects the fact that the intracellular antibody is a heterogeneous mixture containing all forms of the antibody at different stages of maturation, folding, and assembly, whereas only the fully folded and assembled version is secreted into the medium.

Several strategies have been proposed to improve the yield and stability of plant-derived recombinant proteins and reduce the costs of processing [2]. For example, the use of elastin-like polypeptides (ELPs) as fusion partners can increase the solubility and stability of recombinant proteins and facilitate purification by a process termed inverse transition cycling (ITC) [82]. Different versions of 2G12 have been expressed constitutively in transgenic tobacco leaves and seeds by fusing one or both antibody chains to ELPs, increasing the yields to 1% TSP [80]. Subsequent characterization of the purified antibodies demonstrated that the ELP fusion does not interfere with antibody assembly in tobacco and endows the recombinant antibody with greater antigen-binding activity albeit at the expense of HIV-neutralization efficacy. In the absence of a convenient fusion partner, the purification of antibodies such as 2G12 usually involves protein A affinity chromatography, an expensive processing option which achieves a recovery of 50–85% depending on the platform but is an expensive processing option. Therefore, additional nonprotein A protocols have been developed based on traditional chromatography methods, and these can achieve a recovery rate of 50–60% and a purity of up to 90% (e.g., [76, 83]).

5. Human Interleukin-6

Human interleukin-6 (hIL-6) is a 26-kDa secreted glycoprotein from the multifunctional cytokine family, which has diverse physiological roles including the induction of the acute phase response and inflammation, the regulation of the immune response, and the promotion of B-cell differentiation into immunoglobulin-secreting cells [84]. The hIL6 protein is also considered a myokine, that is, a cytokine produced by muscle cells in response to muscle contraction and physical exercise, stimulating lipolysis as well as fat oxidation [85, 86]. The overproduction of hIL-6 and other proinflammatory cytokines is associated with severe chronic immune-mediated inflammatory diseases (IMIDs) such as rheumatoid arthritis [87] and atherosclerosis [88]. The disruption of hIL-6 expression also occurs during the neurodegenerative process in Alzheimer's disease [89] and high levels of this molecule are associated with several hyperproliferative diseases and with the progression of cancer [90, 91].

The hIL-6 protein was first isolated from the supernatant of a T-cell line known as TCL-Na1, which is transformed with *Leukemia virus-1* [92], and its biochemical and functional characteristics were subsequently investigated [92, 93]. Native mature hIL-6 has two disulfide bonds and an N-glycosylation site at Asn73, although the glycan appears to be nonessential for biological activity. The activity of the protein can be evaluated *in vitro* by testing the stimulation of IgM production by SKW6-CL4 B-cells transformed with *Epstein-Barr virus* (EBV) [92] or the proliferation of mouse BALB/c lymphocytes or the hIL-6-dependent murine hybridoma cell lines B9 or MH60 [94].

Recombinant antibodies and synthetic peptides that target hIL6 and prevent interaction with its receptor (hIL-6R) are useful therapeutic candidates in rheumatoid arthritis, systemic-onset juvenile idiopathic arthritis (soJIA), and Castleman's disease [95, 96]. The development of such therapeutic molecules requires large quantities of functional hIL-6 but only small amounts of the native protein can be isolated from lymphocyte cultures, that is, ~3 μg of pure protein from 5.7 L of culture medium [92]. The high cost of the recombinant protein produced in *E. coli* (~€10,000–15,000/mg) means that alternative platforms must be considered. Thus far, recombinant hIL-6 has been produced in *E. coli*, *P. pastoris*, baculovirus-infected insect cells, and tobacco plants.

The first attempts to express hIL-6 in *E. coli* involved the use of a pT9-11-derived plasmid with the inducible Trp promoter [97]. In attempting to express the mature protein with no signal peptide, trace amounts of hIL-6 were detected but no significant overexpression was observed. The first 20 amino acids of mature IL-2 (already overexpressed successfully in bacterial cells) were then added to the hIL-6 N-terminus along with a kallikrein cleavage site. The chimeric protein was expressed at high levels within inclusion bodies, with a final yield of 0.4 g/L. The mature form of hIL-6 was subsequently expressed as inclusion bodies using a synthetic gene with a codon-optimized N-terminal portion, with a final yield of 0.55 g/L and an estimated purity of ~60% [98, 99].

Two different approaches have been used to produce soluble hIL-6 in *E. coli*, one using an expression system designed to secrete the protein into the periplasmic space [100] and the other by fusing the protein to the secretion signal of bacterial α-hemolysin signal peptide for secretion into the culture medium [101]. In both cases, the protein was produced in a soluble and active form but with low yields (10 mg/L and 70 μg/L, resp.). Soluble hIL-6 was subsequently expressed in *E. coli* strain BL21 as a fusion with MBP, thioredoxin, ubiquitin, or NusA, although only the MBP and

NusA fusion constructs were successful. The maximum yield was 7.5 g/L in a bioreactor culture optimized for the NusA variant [102]. The authors did not report any attempt to remove the tag from the fusion protein and did not provide any information about the biological activity of the molecule.

More recently, a set of hIL-6 constructs with combinations of N and/or C terminal tags (His_6, T7, GST, and the *E. coli* alkaline phosphatase periplasmic secretion signal) were expressed in different *E. coli* strains with reducing BL21 or oxidizing Origami 2 cytoplasmic environments, at different growth/induction temperatures, in the presence or absence of helper plasmids encoding cytoplasmic chaperones [103]. The highest yield of soluble hIL-6 was 2.6 mg/L and was achieved by expressing cytoplasmic hIL-6 in BL21 cells at 22°C in the presence of chaperones. The recombinant protein was active as shown by its ability to stimulate murine hybridoma cells.

Recombinant hIL-6 has also been expressed successfully in large-scale cultures of the methylotrophic yeast *P. pastoris* [104]. The mature protein cDNA was cloned in frame with the yeast α-factor secretion signal under the control of the inducible *AOX1* promoter (pPICZalphaA vector) and introduced into *P. pastoris* strain X33. Several culture and expression conditions were tested, both in shake flasks and in the large-scale bioreactor. The highest yield was achieved 96 h after induction, reaching 30 and 280 mg/L in the shake flasks and bioreactor, respectively. The molecular mass of the purified hIL-6 was ~20.9 kDa, indicating the absence of glycosylation. The bioactivity of hIL-6 produced in *P. pastoris* was five-fold higher than that of the commercial recombinant hIL-6 produced in *E. coli* when used to stimulate the growth of BALB/c mouse lymphocytes.

The first attempt to produce recombinant hIL-6 in baculovirus-infected insect cells involved the expression of full-length hIL-6 cDNA in a modified *Autographa californica nuclear polyhedrosis virus* (AcNPV) vector. This was used to transfect Sf9 cells, yielding modest amounts of a 22-kDa protein after 72 h, and partial purification of the protein was necessary to establish its biological activity [105]. Functional hIL-6 was subsequently expressed in baculovirus-infected Sf9 cells using a system based on inducible secretion, but the low yields (1 μg/mL) were disappointing [106].

The production of a functional recombinant hIL-6 in transgenic tobacco plants was first reported by [107] but the yield was not determined. More recently, Nausch et al. [108] compared different transient and stable expression strategies for hIL-6 in tobacco and *N. benthamiana*. Stable expression was tested using three different constructs targeting the apoplast, ER, and vacuole, each controlled by the constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter. The ER-retained version of hIL-6 accumulated to much higher levels (an order of magnitude higher) than the proteins targeted to the apoplast and vacuole, and the ER-retention construct was therefore selected. The three best-performing T0, expressing the ER-retained hIL-6, were self-pollinated to obtain T1 and T2 progeny, increasing the yield in the best-performing line to 1397 μg/g TSP (112 μg/g FW in leaves) and 1212 μg/g TSP (303 μg/g DW in seeds), respectively. The same construct was then used for transient expression in two tobacco cultivars and in *N. benthamiana* with the MagnICON system. Two different MagnICON systems were used, one based on the RNA-dependent RNA polymerase from *Turnip vein clearing virus* (TVCV) and the crucifer-infecting tobacco mosaic virus coat protein (cr-TMV/TVCV) and the other based entirely on *Potato virus X* (PVX). By using the cr-TMV/TVCV system, only cell-to-cell movement was observed in all the three host plants, whereas by using the PVX system the infection spreads systemically in *N. benthamiana* [109] but not in the two tobacco cultivars. The highest yields were achieved in *N. benthamiana*, where hIL-6 accumulated to 7.8% of TSP using the cr-TMV/TVCV system and 4.8% of TSP using the PVX system. Significantly lower values were achieved in tobacco, with a maximum yield of ~1% of TSP in the cultivar Virginia. The structural and biological properties of the recombinant proteins were tested by western blot analysis, revealing that plant-derived hIL-6 is present as two glycoforms (26-27 kDa) although no comparisons were made to the glycan structure on the native hIL-6 molecule. The activity of recombinant hIL-6 was tested by applying crude leaf extracts to mouse B-9 cells and performing a hybridoma proliferation assay, indicating that the activity of the plant-derived proteins was equivalent to the aglycosylated commercial standard hIL-6 produced in *E. coli* [94].

The processing strategy for hIL-6 is strongly dependent on the nature of the starting material. The insoluble hIL-6 recovered from *E. coli* inclusion bodies must undergo several solubilization/refolding steps based on the redox couple-assisted oxidation of cysteine residues, followed by a dilution or gel filtration refolding step. Additional ion exchange, reversed phase HPLC, and size exclusion chromatography steps may be used to increase product purity. Although highly pure (up to 99%) active hIL-6 can be recovered using these methods, the efficiency is often low, with a recovery rate of 15–20% [99]. The large number of purification steps and low final yield in these protocols is not cost-effective for industrial manufacturing, so a simpler protocol was developed for the isolation of soluble hIL-6 produced in *P. pastoris* [104]. Here the protein was purified from the culture supernatant by PEG precipitation, followed by anion-exchange and size exclusion chromatography, with a final yield of 56% and a purity of up to 95%. No purification strategy has yet been published for hIL-6 produced in plants.

6. Conclusions

In this review, we discuss the production of four recombinant proteins (hGAD65, NVLPs, 2G12, and hIL-6) which represent heterogeneous pharmaceutical applications, different biochemical features, and a corresponding wide range of production platforms. Table 1 overviews the production of the target proteins in "traditional" heterologous expression systems. We have focused not only on the yields achieved in different production systems but also on the unique properties of the manufacturing process for each protein, thus highlighting the advantages of plant-based systems over fermenters for specific niche markets. This leads to the conclusion that plants are potentially most beneficial for

Table 1: Highest yields of the expression of the four selected recombinant pharmaceutical proteins in "traditional" heterologous production platforms.

Recombinant protein	Heterologous expression system	Highest expression level	Reference
hGAD65	*Escherichia coli*	12.5 mg/mL	[15]
	Saccharomyces cerevisiae	0.46 mg/mL	[27]
	Pichia pastoris	0.42 mg/mL	[27]
	Spodoptera frugiperda cells	0.02 mg/mL	[18]
	Mouse myeloma cells	1.67 mg/L	[16]
NVCP	*Escherichia coli*[*]	56 mg/L	[49]
	Pichia pastoris[*]	10 mg/L	[50]
	Spodoptera frugiperda cells[*]	125 mg/L	[29]
	Baby hamster kidney cells[*]	10^{10} particles/mL	[51]
2G12	Hybridoma clones	10 pg/cell/day	[60]
hIL-6	*Escherichia coli*	7.5 mg/mL	[102]
	Pichia pastoris	0.28 mg/mL	[104]
	Spodoptera frugiperda cells	0.001 mg/mL	[106]

[*] Reported values are the highest yield data of purified or partially purified recombinant protein because of the absence of expression data.

the production of four major categories of pharmaceutical proteins:

(1) pharmaceutical proteins required in large quantities, that is, commodity pharmaceutical proteins such as microbicide components;

(2) pharmaceutical proteins that need to be produced rapidly, that is, rapid-response proteins such as vaccines against rapidly evolving viral strains;

(3) biopharmaceuticals that require complex posttranslational modifications, that is, antibodies and recombinant proteins with specific glycan structures;

(4) biopharmaceuticals intended for oral delivery.

Other plant-derived recombinant proteins, beyond the four targets described here, also fit in these categories, for example, vaccines against seasonal virus strains (e.g., full-length hemagglutinin protein from the A/Wyoming/03/03 (H3N2) strain of influenza, [110]), personalized vaccines (e.g., non-Hodgkin's lymphoma vaccines for individual patients, [111]), and proteins carrying specific glycans that increase their efficacy (e.g., human glucocerebrosidase for enzyme replacement therapy, [112]).

The large-scale production of recombinant pharmaceutical proteins is often hampered by the poor expression of their mature, active forms in prokaryotic hosts such as *E. coli* and by the high costs and the limited scalability of traditional fermenter-based platforms using mammalian cells. One of the most interesting issues that emerge from our case-by-case analysis is the high productivity of plants compared to other platforms when both the intrinsic yield (per unit biomass) and biomass yield (per hectare per year) are taken into account. This advantage is shown in Table 2 for the four case studies considered in this paper, in terms of the number of plants needed to produce 1 g of each target protein. The variety of available plant hosts and expression systems provide diverse toolbox for the manufacture of recombinant proteins. However, it is not a straightforward process to select the ideal plant-based expression system because many aspects need to be considered carefully, including product yields, quality, production scalability, costs, and cGMP compliance.

In our case studies, the highest yields were achieved in transgenic tobacco plants and by transient expression in *N. benthamiana*. For example, hGAD65 is expressed at higher levels in *N. benthamiana* than in tobacco (27.6 versus 10.5 μg/g FW) but stably transformed tobacco plants are more productive overall because they produce much more biomass. Furthermore, the accumulation of hGAD65mut in tobacco leaves exceeds the levels achieved in *N. benthamiana* (143.6 versus 96.6 μg/g FW), thus suggesting that the productivity would be higher in tobacco even without considering the enhanced biomass production [23]. Conversely, the accumulation of hIL-6 in *N. benthamiana* is up to 80-fold more than achieved by stable expression in tobacco leaves and seeds [102].

Meaningful comparisons among different platforms are required for proper evaluation but this is complicated by the diverse units used to report expression data. In our four case studies, yields were reported both in absolute units (mass of recombinant protein per unit of biomass, which allows total productivity to be calculated by factoring in the production scale) and in relative units (%TSP) which is less useful particularly when comparing dissimilar tissues such as leaves and seeds with vastly different water contents. If the costs of downstream processing are assumed to be the same for all platforms, the estimated costs for the manufacture of recombinant proteins in plants are much lower than current fermentation-based technologies because of the lower upstream costs [56]. For example, a 140-fold cost saving was estimated for the production of hGAD65 in transgenic tobacco compared to baculovirus-infected insect cells [23].

In addition to the flexible and cost-effective manufacturing offered by plants generally, transient expression systems offer the further advantage of rapid upscaling due to the short interval between transformation and expression. We used

Table 2: Best-performing plant-based platforms for the production of four selected recombinant pharmaceutical proteins.

Plant host	Plant organ	Recombinant protein	Expression system	Highest expression level	Plants/g recombinant protein	Reference
Tobacco *(Nicotiana tabacum)*	Leaves	hGAD65mut	Transgenic	0.14 mg/g LFW	[1]93	[23]
		NVCP	Transgenic	0.2% TSP	ND	[41]
		IL6_KDEL	Transgenic	0.11 mg/g LFW	[1]119	[108]
		2G12/2G12_KDEL	Transient (binary vector)	0.1 mg/g LFW	[1]133	[78]
	Seeds	hGAD67/65mut	Transgenic	0.4 mg/g DSW	[1]1250	[26]
		IL6_KDEL	Transgenic	0.3 mg/g DSW	[1]1667	[108]
		2G12_KDEL/ELP	Transgenic	1.0% TSP	ND	[80]
Nicotiana benthamiana	Leaves	hGAD65mut	Transient (MagnICON vectors)	0.1 mg/g LFW	[2]3451	[23]
		NVCP (VP1 and VP2)	Transient (binary vector)	1 mg/g FLW	[2]333	[57]
		IL6_KDEL	Transient (MagnICON vectors)	7.8% TSP	ND	[108]
		2G12_KDEL	Transient (viral vector)	0.12 mg/g LFW	[2]2693	[79]
Arabidopsis thaliana	Seeds	hGAD67/65mut	Transgenic	4.5 mg/g DSW	[3]308	[26]
		2G12	Transgenic	3.6 mg/g DSW	[3]385	[75]
	Leaves	2G12	Transgenic	0.2% TSP	ND	[73]
Maize *(Zea mays)*	Seeds	2G12	Transgenic	>0.1 mg/g DSW	ND	[76]
Lettuce *(Lactuca sativa)*	Leaves	NVCP	Transient (viral vector)	0.2 mg/g LFW	ND	[56]
Petunia *(Petunia hybrida)*	Seeds	hGAD67/65mut	Transgenic	0.2 mg/g DSW	ND	[26]
Carrot *(Daucus carota)*	Taproots	hGAD65	Transgenic	0.01% TSP	ND	[22]
Chlamydomonas reinhardtii		hGAD65	Transgenic	0.3% TSP	ND	[20]
Potato plant *(Lycopersicon esculentum)*	Fruits	NVCP	Transgenic	0.16 mg/g fruit weight	[4]0.6	[53]
Tomato *(Solanum tuberosum)*	Tubers	NVCP	Transgenic	0.12 mg/g tuber weight	ND	[53]

The highest expression levels are reported as mass of recombinant protein per unit of biomass (LFW: leaves, fresh weight; DSW: dry seed weight) unless these values were not available, in which case percentage total soluble protein (%TSP) is used instead. The recombinant protein productivity values were calculated by considering the seed or leaf biomass yield per plant ([1][115]; [2][59]; [3] [116]; [4] Mississippi State University website: http://msucares.com/crops/comhort/yield.html). ND: not determined (values were not calculated because of the absence of productivity data or the expression data were reported as %TSP).

the *Norwalk virus* vaccine as a case study to highlight this niche. An effective vaccine needs to be produced quickly after strain identification in order to halt the spread of the new strain. NVLPs produced by transient expression address these issues, allowing the rapid and affordable production of strain-specific vaccines in a timely manner and in relevant locations, including the developing world [54, 55, 59, 110]. However, it should also be borne in mind that plants offer advantages for small-scale expression. For example, the production of personalized vaccines in mammalian cells would require a full production campaign and the "occupation" of a fermenter for each patient requiring a vaccine. In contrast, transient expression in plants would allow many similar vaccines to be prepared by using a small number of plants each enclosed in a protective chamber in a greenhouse. In this manner, the production of a customized idiotype vaccine for non-Hodgkin's lymphoma was achieved in less than two weeks from biopsy by using transient expression in plants [111, 113].

Another key advantage of plant-based expression platforms is their ability to synthesize complex eukaryotic glycan structures. Approximately one-third of approved biopharmaceuticals is thought to be glycoproteins, which favors the use of eukaryotic systems for such products [114]. Although N-glycan synthesis in the ER is conserved among eukaryotes, N-glycan processing in the Golgi body differs among phyla resulting in diverse glycan structures. Plant-derived glycoproteins typically contain non-human-glycans that are added in the Golgi body [114]. Whether these glycans are immunogenic or allergenic is still a matter of debate, but they can be immunoreactive [70].

Two of the target proteins considered in this review are glycosylated: 2G12 and hIL-6. The antibody 2G12 has been expressed in a wide range of plant-based platforms, including wild-type and glycoengineered systems, and has been targeted to different subcellular compartments, thus resulting in a huge variety of glycoforms. The detailed description of these data is beyond the scope of this paper, but overall it was found that the different glycan profiles do not affect the virus-neutralization activity of the antibody *in vitro* [72]. The impact of the different glycoforms *in vivo* should be considered in future studies, but it is likely that Fc-mediated antibody effector functions and antibody-dependent cell-mediated cytotoxicity could be influenced by different glycans and this should be considered in the context of systemic antibody administration [70]. Plant-derived hIL-6 was also produced as a number of different glycoforms, all of which had the same *in vitro* activity as the aglycosylated commercial counterpart produced in bacteria, but similarly the *in vivo* implications need to be evaluated. These considerations are less important if plant-derived pharmaceuticals are intended for topical application [117]. Importantly, plant-specific glycans may also be desirable in specific cases. For example, the presence of terminal mannose residues on plant-derived recombinant glucocerebrosidase was shown to increase its uptake by macrophages and thus its efficacy for the treatment of Gaucher's disease [112].

One final and unique advantage of plant-based systems is the natural "bioencapsulation" provided by edible plant organs when pharmaceuticals are intended for oral administration [45, 118]. The oral delivery of drugs is preferred [119] but unprotected peptides and proteins are exposed to the harsh gastrointestinal environment, which is acidic and rich in proteolytic enzymes. For oral vaccination, several phase I clinical trials have been carried out using edible plants, including those expressing NVLPs as discussed above [45]. These trials confirmed the safety and immunogenicity of NVLPs without the need for a buffer or vehicle other than the plant cell. Transgenic plants expressing antigens may therefore be a significant step towards the goal of developing cost-effective and user-friendly vaccines.

The oral route is also a potentially effective strategy for the prevention of autoimmune diseases by inducing tolerance. For example, the oral administration of plant tissue expressing hGAD65 (in combination with IL-4) to the nonobese diabetic mouse model of T1D effectively prevented the onset of the disease [21]. However, the clinical application of oral tolerance strategies in humans would be challenging because of the immense cost of autoantigen production, particularly if repeated regular doses are required to maintain the beneficial effects. Plants could meet this unprecedented demand for recombinant autoantigens, making such strategies safe, palatable, and economically feasible.

The examples discussed above can be considered proof-of-principle case studies that highlight some of the specific advantages of plant-based production platforms over traditional systems. It is unlikely that plants will completely replace CHO cells and other established systems, but they are now gaining a firm foothold in niche markets where the unique benefits of plants offer the greatest advantages.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Matilde Merlin and Elisa Gecchele are equal contributors to this paper.

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