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Review article

Commercial-scale biotherapeutics manufacturing facility for plant-made pharmaceuticals

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Summary

Rapid, large-scale manufacture of medical countermeasures can be uniquely met by the plant-made-pharmaceutical platform technology. As a participant in the Defense Advanced Research Projects Agency (DARPA) Blue Angel project, the Caliber Biotherapeutics facility was designed, constructed, commissioned and released a therapeutic target (H1N1 influenza subunit vaccine) in <18 months from groundbreaking. As of 2015, this facility was one of the world's largest plant-based manufacturing facilities, with the capacity to process over 3500 kg of plant biomass per week in an automated multilevel growing environment using proprietary LED lighting. The facility can commission additional plant grow rooms that are already built to double this capacity. In addition to the commercial-scale manufacturing facility, a pilot production facility was designed based on the large-scale manufacturing specifications as a way to integrate product development and technology transfer. The primary research, development and manufacturing system employs vacuum-infiltrated *Nicotiana benthamiana* plants grown in a fully contained, hydroponic system for transient expression of recombinant proteins. This expression platform has been linked to a downstream process system, analytical characterization, and assessment of biological activity. This integrated approach has demonstrated rapid, high-quality production of therapeutic monoclonal antibody targets, including a panel of rituximab biosimilar/biobetter molecules and antiviral antibodies against influenza and dengue fever.

Introduction

In recent years, the plant-made pharmaceutical (PMP) community has made significant progress in bringing therapeutics to the clinic: Elelyso™ (Pastores *et al.*, 2014; Zimran *et al.*, 2015) (Protalix, Carmiel, Israel) has been given market registration; non-Hodgkin's lymphoma vaccines (Bendandi *et al.*, 2010; McCormick *et al.*, 2008; Tusé *et al.*, 2015) (Large Scale Biology, Icon Genetics/Bayer, Halle, Germany), interferon-α2b (De Leede *et al.*, 2008), CaroRx (Weintraub *et al.*, 2005) and RhinoRx (Planet Biotechnology, Hayward, CA), lactoferrin (Laffan *et al.*, 2011) (Ventria, Fort Collins, CO), anti-HIV antibody (Rosenberg *et al.*, 2013) (Pharma-Planta Consortium, EU-South Africa), edible vaccines (Tacket *et al.*, 2000; Thanavala *et al.*, 2005; Yusibov *et al.*, 2002) (Arizona State University, Tempe, AZ; Thomas Jefferson University, Philadelphia, PA), the influenza serotype H1N1 and H5N1 vaccines (Cummings *et al.*, 2014; Landry *et al.*, 2010, 2014) (Fraunhofer CMB, Newark, DE, and Medicago, Quebec, QC, Canada) and more recently the ZMapp™ cocktail (Qiu *et al.*, 2014) (Mapp Biotherapeutics, San Diego, CA) have demonstrated the potency of plant-made therapeutics in clinical trials. The development of such plant-made pharmaceuticals was accompanied over the years with the design and construction of manufacturing facilities of different capacities to accommodate the production of clinical material under current Good Manufacturing Practice (cGMP) standards (Wirz *et al.*, 2012). The first cGMP manufacturing facility producing PMPs was designed by Large Scale Biology Corporation (LSBC) in Owensboro, KY, USA (now Kentucky BioProcessing) and opened in the year 1999 using the plant-virus transient expression system Geneware® (Pogue *et al.*, 2002, 2010). Later, new plant viral-based vectors were modified to address different expression strategies and better controlled processes making use of *Agrobacterium tumefaciens* and a vacuum infiltration procedure (Gleba *et al.*, 2005; Huang *et al.*, 2010; Marillonnet *et al.*, 2005; Roy *et al.*, 2010; Sainsbury *et al.*, 2009).

In 2007, the Defense Advanced Research Projects Agency (DARPA) sponsored the development and validation of a pilot-scale cGMP manufacturing facility using vacuum agroinfiltration of *Nicotiana benthamiana* capable of processing 100 kg plant biomass per batch at the Fraunhofer CMB, Newark, DE. The goal of this initial project was to demonstrate the feasibility of the plant transient expression system to rapidly manufacture vaccines in response to epidemics and outbreaks. In 2009 and based on the success of this proof-of-concept project, DARPA selected PMPs as a platform of choice for rapid development and low-cost, global-scale rapid manufacturing of vaccines and biotherapeutics needed for national biosecurity. DARPA separately sponsored three additional PMP manufacturing facilities: Kentucky BioProcessing (Owensboro, KY), Medicago (Durham, NC) and Caliber Biotherapeutics (Bryan, TX). Each participant had individual prerequisites for performance and technology development. Caliber was tasked with building a highly automated, hardened facility that would use hydroponics to assure commercial-scale

production without concerns surrounding contamination due to soil. Being a harden facility, damage by natural forces such as storms and tornados which would severely impair greenhouse structures is less likely. Caliber also used a mobile biomanufacturing technology (G-Con Manufacturing) to assure that very rapid changeover could be accomplished to accommodate multiple downstream process trains for different biological products. The objective was to build a cGMP ready facility that could rapidly develop cGMP processes and produce products under fully compliant conditions. The DARPA Blue Angel programme set an objective to perform the research and development required to deliver a recombinant protein antigen to be used in a candidate influenza serotype H1 vaccine (hemagglutinin (HA) antigen), expressed in hydroponically grown *Nicotiana benthamiana* plants at a scale of 1 kg of purified vaccine protein per month. The product was required to meet all FDA requirements for purity, quality and cGMP.

The DARPA challenge consisted of the following:

1. Designing a commercial biotherapeutics facility using a plant-made pharmaceutical platform that was hardened, provided high containment, and self-sufficient.
2. Building and commissioning a facility, developing a candidate protein antigen, and demonstrating process robustness by delivering a target protein at commercial scale.
3. Completing the objectives in <18 months.

The Caliber facility was built as a cost-sharing project with DARPA. The Technology Investment Agreement (TIA) was part of Project Blue Angel, a vaccine production acceleration project recommended by the US President's Council of Advisors on Science and Technology (https://www.whitehouse.gov/sites/default/files/microsites/ostp/PCAST-Influenza-Vaccinology-Report.pdf). Caliber was tasked to show that PMPs can be scaled up to a robust, large-scale manufacturing platform that can rapidly deliver biotherapeutic proteins at a low cost. One of the stated goals of the agreement was to address the 'insufficient capability to provide vaccines against pandemics caused by new strains, as well as infections caused by intentional biothreats...' (http://www.defense.gov/News/NewsArticle.aspx?ID=61520). Given the defence implications and lack of an official government response to the H1N1 crisis, project execution, project innovation, equipment innovation and facility integration were all paramount. The development of the proof-of-concept accomplished in this project is expected to have a substantial positive impact on US military readiness, with significant additional benefits to the private sector. The production and scale-up of influenza vaccine currently relies on the 1950s technology that has been in continual use since that time. While there are strong research data supporting the use of plant-based technology, this project represented the first demonstration that plant-based technology can be scaled up to the levels required to support the US military and government customers. As a result of the successful proof-of-concept achieved by Caliber, this programme is now well positioned to respond to a need to make a wide range of protein therapeutics. The project was accomplished using an advance design/build that allowed the parallel construction of the building and the downstream processing facilities utilizing G-Con PODSTM (autonomous self-contained bioprocess environments). Caliber was operational as of April 2011 when preliminary process implementation and testing started.

Here, we discuss the design of a commercial-scale PMP manufacturing facility integrating a plant-based transient expression system and the application of its pilot-scale facility which is a direct scale-down based on the large-scale manufacturing format designed to help study and validate process change at low risk/low cost.

Facility design and technology

Nicotiana benthamiana (*N. benthamiana*) is the bioreactor of choice for the transient expression of recombinant protein in a manufacturing setting because of its high susceptibility to *Agrobacterium* transfection and plant viruses (Goodin *et al.*, 2008), as well as its growth pattern and high leaf:plant biomass ratio. The facility has the capacity to grow over 4 million *N. benthamiana* plants at a single time in a highly automated multilevel growing environment under proprietary LED lighting fixtures. The facility was designed for facile expansion to increase biomass production per batch from approximately 3500 kg (current) to 7000 kg (expanded). This plant-based system offers substantial technical advantages over mammalian cell-based systems, including high-throughput development and screening of candidate product molecules, ease of both primary amino acid sequence manipulation and glycosylation engineering through either introducing mutations in the target molecule to add or reduce the number of N-glycosylation sites or modifying the glycosylation machinery of the host, plus low-cost scale-up to >150 kilograms of purified product annually. The Caliber design build team included Beck Construction, EEA Engineering, Malin, Rockwell Automation and Banks Engineering. The building was designed in a parametric building information management (BIM) and estimation model using advanced three-dimensional computer-aided design (CAD) systems. The facility was completely modelled in a 3D BIM model and all systems were deconflicted '*in silico*' before construction (Figure 1). Software systems such as (i) D-Profiler, a parametric 3D CAD-based estimating tool, (ii) Revit object-based 3D CAD design system, (iii) Navisworks for '*in silico*' design conflict resolution and (iv) Synchro, 4D CAD and timeline planning were all used to design and manage the build. The walls of all plant grow rooms employed computational flow dynamic modelling to design very specific perforation patterns and provide laminar air flow for temperature control.

Several proprietary improvements were made to the plant platform process including the following:

1. Design and development of proprietary LED illumination systems
2. Very large-scale automated hydroponic growth systems
3. High-throughput infiltration system

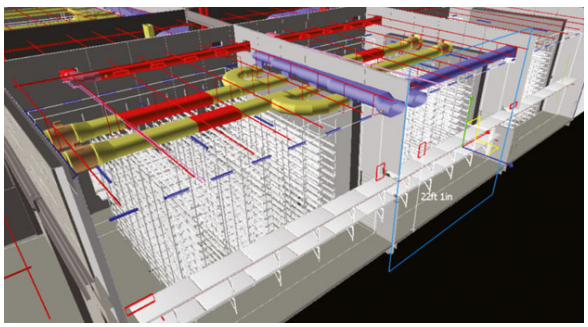

Figure 1 3D Parametric BIM model of the facility. Only rooms containing the grow racks are shown.

4. Modular, flexible downstream process modules to allow rapid response and/or configuration to a new protein therapeutic target.

The facility leverages G-Con Manufacturing's (www.gconbio.com) core technology—the design and construction of mobile biomanufacturing facilities (or PODSTM) (Jornitz, 2014). The PODSTM give Caliber the flexibility to rapidly reconfigure the downstream processes without disruption of the facility. The facility itself is scalable, creating a sustainable competitive advantage and the ability to produce and process thousands of kilograms of plant biomass per day. It was designed with the intention of scaling up capacity without interruption of ongoing operations, achieved by the simple addition of additional plant growth rooms and systems (*i.e.* racks, watering systems, trays, etc.) which can be built for the same price per square metre as a traditional automated warehouse. With this completely automated system, the upstream capital cost is 10% of an equivalent tank-based bioreactor facility containing a three 2000-L bioreactor production line at a 2 g/L yield producing 150 kg of monoclonal antibodies (mAb) per year. By having the downstream processes housed in proprietary PODSTM, additional process space can be added as necessary to accommodate any process modifications that are related to the scale-up. The design/build team broke ground to build the Caliber facility on 9 April 2010. Commissioning under cGMP guidelines began on 1 November 2010 and the fully functional building was occupied on 22 March 2011, with the first product released and shipped on 24 December 2011. Execution of an 18-month build demonstrated design innovation, rapid facilities systems integration and highly efficient construction management. A schematic representation of the facility production flow is presented in Figure 2.

The facility is designed around the concept of overlapping utility capacity in which the largest single utility unit can be down for maintenance and/or repairs and the utility loads can be maintained with redundant equipment. The facility has three hot-water boilers with a heating capacity of 2 million BTU/h each. The chilled water cooling capacity consists of 2 independent chillers at 400 tons each. The existing purified water (PW) system is capable of generating 150 LPM with a storage capacity of 7500 L and is used for the extraction buffer preparation. In addition, water for injection (WFI) is used for the preparation of the remaining process buffers. The facility also has a liquid-handling biowaste inactivation system where heat is utilized to inactivate all BSL-1 designated liquid waste streams from two 19 000-litre holding tanks. Security and reliability were important factors in the design. The building, including the hydroponic facility, was constructed using precast concrete and rated at Seismic 4. Emergency power is provided by a 2-megawatt diesel-powered generator capable of powering all processes in the facility.

The DARPA demonstration project was run under 'cGMP-like' conditions and documentation. This approach was implemented solely due to the short period of time allocated for process development, which was insufficient for full process validation. A full cGMP 'readiness' audit was completed by a DARPA-selected consulting firm at the end of the demonstration project. Caliber was found to be designed and built to achieve full cGMP compliance when a product and process were developed under the normal guidelines.

Bioprocessing clean rooms

G-Con Manufacturing developed the autonomous PODSTM that have been implemented in the Caliber facility. Each PODTM in the facility is 5.5 × 12.8 metres. The PODSTM were manufactured and fully commissioned at G-Con's 5100-m^2 facility in College Station, Texas, while construction of the shell facility was underway and transported to the facility site by flatbed truck. When the shell was essentially complete, the PODSTM were moved in and connected to the building in a matter of hours, whereas typically clean room infrastructure takes days or weeks. The simultaneous building of shell and PODTM had an enormous effect on G-Con's capacity to meet the aggressive deadlines imposed by DARPA. Each PODTM contains a working space of over 42 m^2. The PODSTM are equipped with air bearings allowing them to be moved into place effortlessly as they 'float' on a layer of compressed air. The installation required no special rigging to move the PODSTM into place within the grey space at Caliber, and the PODTM air bearings installation was designed to operate as a general installation mechanism at any other facility. Process piping was completed prior to installation, and process equipment can be pre-installed at the factory or at the site. PODTM utilities are connected via umbilicals with quick connectors to a service chase. The clean room PODSTM are attached to a class D access corridor providing an interface to the building and an additional level of pressure cascade for containment. The PODSTM have on-board inlet and exit filtration in addition to high-

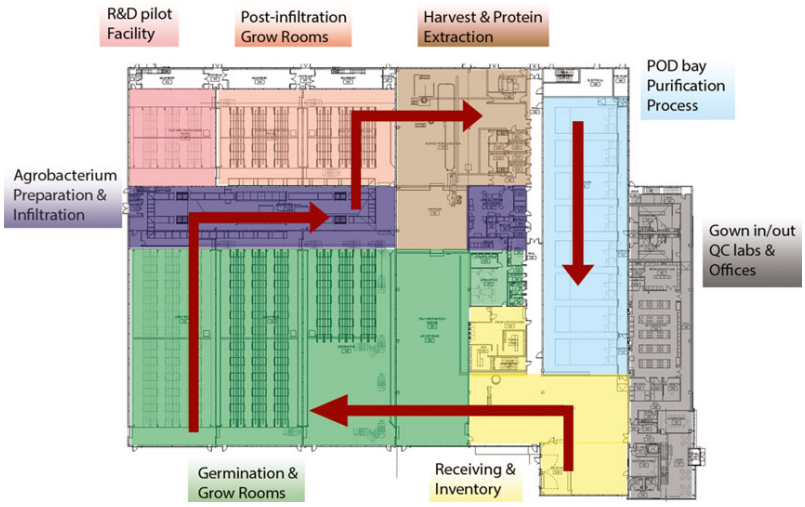

Figure 2 Facility production flow diagram.

efficiency particulate arrestance (HEPA) filtration in the workspace. This effectively isolates the clean room from the grey space and allows the PODTM to be used in either positive or negative pressure modes and in constant volume or variable volume modes. PODSTM have 'on-board' internal fire suppression so that hard connections to building sprinkler systems are not necessary. Materials of construction are consistent with and resistant to the major disinfectants and decontamination systems, for example vapour-phase hydrogen peroxide (VHP). PODSTM are also equipped with a complete and robust control system featuring Rockwell ControlLogix programmable logic controllers (PLCs) with additional digital input/outputs. All sensors and control systems are Internet protocol (IP) addressable, meaning that they can be controlled remotely over the Internet using secured protocols. The PODTM is connected to the local area network via Ethernet and a single cable connection. Input/output protocols are provided to the client and facilitate the integration of the environmental monitoring and control. Each PODTM is served by its own redundant heating, ventilating and air conditioning (HVAC) system, with two air handlers located in the mechanical space of the PODTM. Each HVAC system has an independent controller with a data/controls interface to the facility building automation system. These systems receive chilled water from an outside source through an umbilical cord connection. Each air handler is sized to provide the required air turnover rate to continue operations inside the PODTM. Pressure cascades can be maintained as a three-zone system with a minimum of 0.045 inch of water differential pressure. The system, as designed, recirculates air internally in the PODTM or provides a 'single-pass' air flow. Bag-in/Bag-out HEPA filters are provided on the exhaust air streams, with each HVAC system having independent supply HEPA filters and a shared exhaust HEPA filter. If one air handler fails, the operating unit adjusts to compensate for the lost airflow without a loss of operational integrity.

Integration of process and facility

Systems integration is one of the key elements in the start-up and ongoing development of any facility. Bank integration provided the control systems for individual process components, including the infiltrator and cutter as well as data monitoring and control graphical user interface for the PODSTM and building management system. This system completely monitors and fully controls processes from seeding and germination, to purified products and fill/finish into bulk vials. Such processes include all LED lights, air flow, temperature control, infiltrations, harvesting, centrifuges, and all modular clean room PODSTM plus their respective downstream processing equipment. There are multiple servers including a historian server that provides data for historical reference and trending analysis. This system is integrated into the G-Con data network storage area network (SAN), which is a RAID 50 array. This system takes full advantage of the Rockwell ControlLogix PLC system and Factory View software. The Control Net and D Net architecture services the PODSTM and their attendant process skids and systems that are reporting data and need to integrate with the overall process control. Multiple user interfaces or human–machine interfaces (HMIs) and data manipulation can be performed in any location via thin client terminals in the facility or over the Internet via a secured authenticated logon. A current historian server is on line to visualize trending and other process data. An archival historian is used to back up all data from process runs and batch releases.

As this facility was the first of its kind, Caliber needed a facility-wide system that was practical, reliable and able to interact with all the PLCs on the various pieces of process equipment as well as communicate with and accept information from the building automation system. The facility also needed a solution that was modular, flexible and able to interface with major networks such as ControlNet, DeviceNet and Ethernet. While initially limited to the upstream process equipment, the process control systems (PCS) had to be scalable such that any future downstream recovery equipment could be easily integrated into the control system. The automation core of the PCS relies on the Integrated Architecture approach from Rockwell Automation. The Rockwell systems are built on modules of prevalidated software and are configurable to the multiple disciplines and needs of the bioprocess industry. The PlantPAx solution supplied by Rockwell Automation was comprised of an Allen-Bradley 1756-L73 ControlLogix Programmable Automation Controller (PAC), backed up with an HMI server running FactoryTalk View SE and several thin clients. The solution included the use of Rockwell Automation's Global Engineering Modularity Standards (GEMS) control modules as the basis of the control architecture. The GEMS modules were implemented in ControlLogix add-on instructions with associated HMI faceplates for the supervisory control and data acquisition (SCADA) system. The process sequencing was programmed using Rockwell Automation's Phase Manager Software and a custom sequence manager, which followed the ANSI/ISA-88.01 standard for modular programming. The engineering and quality team managed and coordinated all the activities for the entire life cycle of the project, from design documentation, programming, and start-up through commissioning, resulting in numerous engineering turnover packages (ETOPs). Subsequently, the Caliber team was honoured at the 2014 Interphex Meeting with the Pharmaceutical Processing Facility Excellence Award for process innovation.

The process

Plant biomass production

The hydroponic plant production system at Caliber grows *N. benthamiana*. This begins by presoaking rockwool Kiem® plugs in 240-count styrofoam trays with pH-adjusted hydroponic nutrient solution. Seeds are then distributed onto rockwool plugs via a Bouldin & Lawson compact needle seeder, set to one to two seeds per plug. Seeding rate is 210, 240-count plug trays/hour. Four seeded 240-count plug trays are then placed into a custom-designed and fabricated aluminium germination tray (total 960 plants per tray). Germination trays are transported by automated conveyor to the germination room (Figure 3a) and placed on the proper flow rack by a wire-guided forklift (Figure 3b–c). The trays are fitted with a watering channel, for use with an ebb and flow subirrigation system, delivering a recirculating hydroponic solution that provides a balance of essential plant growth nutrients. Nutrient solution is supplied every 12 h via an automated irrigation system controller. Germination racks rise 14 levels high (15 metres) and extend 18 metres long. Grow racks extend 36 metres long in pre-infiltration grow rooms and extend 27 metres long in the postinfiltration rooms. A laminar flow wall disburses high volumes of conditioned air to control the environment to a constant temperature of 27 ± 2 °C. Germination trays are placed under constant LED lights. In germination rooms, as well as grow and postinfiltration rooms, a constant relative humidity is maintained at approximately 50%. High humidity is controlled by

Figure 3 Illustration of semi-automated plant growth and infiltration procedure. (a) Germination room showing laminar flow walls, plant growth flow racks and the robotic transplanter (in the foreground); (b) *N. benthamiana* growing under LED lights in the germination room; (c) forklift operation for tray racking; (d) plant grow trays conveyed into the infiltration room; (e) plant trays entering custom-designed infiltrator; (f) plant trays exiting the infiltrator, inverted backup and conveyed to the postinfiltration rooms.

condensation/reheat in the air handler, while low humidity is controlled by steam injection into the air handler. Both modes are humidistat controlled. Under these conditions, germination typically occurs within 3–5 days and plants remain in the germination grow room for approximately 21 days. The total capacity of the germination room is 1680 germination trays.

Rockwool plugs with fully germinated seedlings are then transplanted using a Tuinbouw robotic transplanter to a perforated solid inert medium of support placed in specially designed aluminium grow trays, each with a total capacity of 320 plants. Grow trays are transported by automated conveyor to the grow room and placed on the proper flow rack by a wire-guided forklift where a drip irrigation system is used to provide recirculating hydroponic nutrient solution delivered every 8 h via an automated water conditioning and delivery system. Each tray is fitted with synthetic growing medium that provides nutrient solution distribution via a nutrient film technique (NFT) delivery system. Transplanted trays are placed in a controlled grow room that maintains a constant temperature of 24 ± 2 °C. Plants reach optimum biomass in 10–12 days. The number of plants per grow tray was set to 320 to optimize plant biomass per footprint and generate about 3 kg of biomass per tray for infiltration. The weight of a typical plant at infiltration reaches 12 g to 15 g with an overall plant average calculated at 8 g to 10 g. However, when calculating the average plant weight, one has to consider a percentage of seeds not germinating or germinating slower leading to smaller plants with competition disadvantage in the growth period and no selective infiltration or harvest under this manufacturing format (320 plants per tray). The total maximum capacity of the grow rooms is 5040 trays.

LED lighting for plant germination and growth was selected for multitude of advantages this technology provides. Standard fluorescent lighting does not provide sufficient photic energy and is broad spectrum, leading to substantial inefficiencies for plant growth and/or energy usage. Both fluorescent lighting and metal halide lighting are energy-intensive and present localized heat load issues. Higher environmental temperature uniformity is achieved with LED lighting at a much lower energy input. Computational flow dynamic modelling showed that <1.5 °C of temperature variation across the entire plant production area is required to meet standards for plant uniformity. Caliber jointly designed an LED-based lighting array fixture with an LED lighting provider. Data from the DARPA production campaign demonstrated that plant growth rate more than doubled during the germination period under red and blue LED lights (Figure 4), which then in and of itself increased the output of product for any given vaccine campaign by simple growth of biomass. The LED lighting system has the following characteristics:

-25 ° half-angle optics with a 2 : 1 uniformity ratio over this half-angle from each individual array (to focus photons on the plants)

-Composite blue/red spectrum: 25% 450 ± 10 nm wavelength/ 75% 660 ± 10 nm wavelength (to provide optimum wavelengths for plant growth, alleviate dark spots and provide crossover effects to maintain uniformity across all plants)

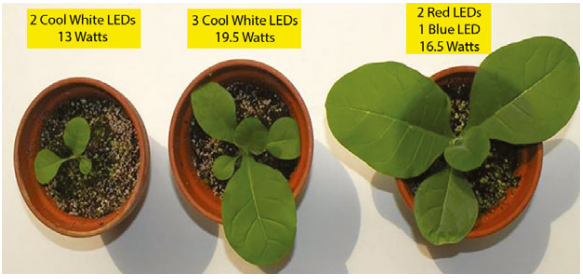

Figure 4 *N. tabacum* plants grown under different lighting conditions. All seeds were germinated using identical soil and fertilizer at 26.6 °C. All light treatments were provided 24 h/day, 7 days/week.

-Minimum 50 000 h life at 70% output
-Custom fixture that is 9′ long × 2″ wide extruded aluminium frame with integral cooling fins and Lexan lens (to focus photons as well as dissipate heat on the back side of the fixture)
-Custom easy mount brackets and pigtail connectors
-Fully cleanable components designed to withstand oxidant treatment
-Modular 'plug-and-play' power supply boards for LED drivers incorporated into the grow rack structure and operating at 27 V
-IP65 hose-proof rated components

This novel LED lighting system produced grow trays of up to 3 kg of plant biomass when continuous lighting was provided and reduced germination and pre-infiltration growth time from 6 weeks to 5 weeks (Figure 4). The system also reduced energy costs by 44%. For the production of therapeutics, at 5 weeks post-seeding, plants are released as raw material to the infiltration area under a series of strict quality control/assurance criteria. Plant release criteria include plant size, mass and phenotypical characteristics such as chlorotic conditions. Strict insect and fungus controls are in place, any of which could constitute grounds for rejection for drug production.

Infiltration

The infiltrator was custom-designed and fabricated for this facility (Figure 3e). It is an 18.3-metre-long vacuum vessel holding 7000 L of infiltration solution. Trays are conveyed from the grow room (Figure 3d) and properly oriented for infiltration two at a time by an inverter (Figure 3f). A single infiltration cycle encompasses eight trays that are charged into the vacuum infiltrator. A roller section holds the trays in an inverted position over an infiltration solution of *Agrobacteria* containing product expression construct(s). *Agrobacteria* containing expression construct(s) for the protein(s) to be produced are grown in single-use bioreactors in a fermentation laboratory in the facility and pumped to the infiltration area staging tank for inoculation. Infiltration solution is prepared to maintain an *Agrobacterium* titre that has been optimized for the construct being used and monitored for pH and temperature prior to initiating the infiltration sequence. Additional infiltration solution is stored in a 750-L tank and is gradually used during the infiltration sequence to maintain the total volume of infiltration solution as the infiltrated plants exit the infiltrator. Doors to the infiltration vessel close on a gasket, during which time the plants are lowered into the solution by pneumatic actuators. Vacuum is then quickly evacuated using a vacuum accumulator system and automatic controls. As infiltrated plants exit the infiltrator, they are rinsed with water to reduce the amount of infiltration solution carryover and inverted back to their upright position to then be transported by the automated conveyor system to the post-infiltration grow racks for a period of 6–10 days depending on the product being made. The total capacity of the postinfiltration rooms is 2520 trays.

Post-Infiltration and harvest

During the postinfiltration incubation period, a constant temperature of 22 ± 2 °C and a humidity of approximately 50% are maintained. During this time period, the inserted T-DNA hijacks native plant protein expression machinery to drive product expression. Post-infiltration conditions including temperature, age and morphology of plants, predetermined light dark cycle, and time of harvest are optimized to ensure consistent expression and recovery of high-quality product. The total upstream plant-based production process can range from 37 to 43 days depending on the plant growth protocol chosen for a particular recombinant protein to be produced.

When the appropriate postinfiltration incubation time for quality product has been reached, grow trays are conveyed to the harvest area where plants are cut and then mechanically homogenized with 2 to 3 volumes of buffer (plant:buffer w:v) using a two-stage, precision-controlled homogenization system. Homogenate is then clarified by dick stack centrifugation and/or multiple depth filtration regimes. Filtrate is then pumped to the downstream processing area for purification.

Downstream processing

All downstream processing occurs in G-Con PODSTM. Each PODTM is currently configured to house downstream processing units. The first PODTM houses a UF/DF skid, appropriate support filtration equipment, and buffer storage tanks for molecular weight separation, concentration (when appropriate), and buffer substitution of the process stream, prior to capture chromatography. The second PODTM houses two GE ÄKTA high-capacity chromatography skids that can be configured for capture (isocratic or gradient elution), or primary polishing chromatography. Both of these skids are supported by disposable buffer/product storage and portable clean-in-place systems. The third PODTM houses a chromatography skid that performs the final chromatography polishing step. A supporting UF/DF system performs product concentration and final buffer exchange prior to sterile fill and cryostorage. The fourth PODTM houses the M & O Perry sterile vial fill system with an automated Newman labeller. PODTM five is used for cryostorage and houses the vapour-phase liquid nitrogen tanks for final product storage and a series of freezers for reagent and sample storage. The sixth PODTM provides buffer preparation and storage to support the chromatography processes and UF/DF steps. Buffers are received in disposable systems ready for use or appropriate sterile dilution. A seventh PODTM is used for glassware and other utensil cleaning following vector deactivation in an on-board autoclave unit and glassware washer. An eighth PODTM is configured as a negative pressure, exit filtered BSL-2 laboratory to provide in-process assessment of biological activity (*e.g.* cell surface binding, cytotoxic/cytostatic activity) in cell-assay-based formats to support potency and quality measurements.

Results from the DARPA blue angel project

The total production of HA antigen during the DARPA Blue Angel programme was eight batches (two per week) over a 4-week period. This production was performed with a process 'in development' that was researched and transferred to production in <6 months. Caliber accomplished this much-abbreviated process development effort within the time constraints of the government agreement. In summary, a new commercial-scale facility was constructed, commissioned, and ran to produce and release the therapeutic target in <20 months. The released product was sterile, clear, colourless and free of visible particles with a purity >97%. It passed a battery of 11 release assays which included identification by both molecular weight and tryptic peptide MALDI-TOF mass spectrometry.

Caliber has generated considerable historical data on the costs associated with producing plant-made therapeutics. Using real operating data from commercial-scale runs for the DARPA Blue

Angel project, Caliber demonstrated that production of pandemic flu subunit vaccine bulks drug cost between $0.10 and $0.12 per 50 μg dose on an annualized cost basis.

Production of monoclonal antibodies for preclinical and clinical studies in Caliber's pilot-scale facility

Expression of full-length monoclonal antibodies (mAbs) in plants has been extensively documented since the first report of antibody production in transgenic plants 25 years ago (Hiatt *et al.*, 1989). In our experience, the clinical development of therapeutic mAbs needs to address manufacturing constraints and capabilities as early as possible to allow for facile and rapid production scale-up. As more plant-made therapeutics are expected to enter clinical trials soon, in-depth product characterization during process development in a scaled-down manufacturing setting allows for the integration of quality control and industrialization aspects as early as preclinical studies. Here, we discuss the process flow for the production and characterization of mAbs and their release as preclinical and clinical materials using Caliber's PMP platform at the pilot scale.

Pilot-scale R&D facility

Caliber's pilot-scale research and development (R&D) facility was built as a true 'scale-down version' of the full-scale manufacturing facility. The R&D facility employs five 3.6 metres long × 2.1 metres wide growth chambers (GreenPODSTM; G-Con, LLC) engineered to mimic all germination and growth conditions found within the large manufacturing facility. All plant growth protocols, tray configurations, and infiltration procedures utilized at full manufacturing capacity described earlier were scaled down to the pilot-scale R&D facility, which allows for a rapid and seamless transfer of processes between scales. One GreenPODTM is designated for germination of *N. benthamiana* seeds before they are ready for transplantation. Once ready, they are transferred into one of two GreenPODSTM where they remain for another 2 weeks. Within each GreenPODTM, there are a series of stacked grow trays (to mimic the conditions in the larger manufacturing facility) each of which can hold up to 60 plants or 500–600 grams of biomass. In total, the pilot-scale R&D facility produces up to 24 trays per week (12–14 kg biomass).

The pilot-scale facility infiltrator was engineered to reproduce conditions used in the manufacturing infiltrator and to accommodate the smaller pilot grow trays (Figure 5a). Two additional GreenPODSTM are used to house plants postinfiltration. After approximately 5–10 days (length of time is highly dependent on the vector and the protein of interest) in postinfiltration, plants are harvested and delivered for downstream processing within the same pilot-scale facility. The plant biomass is homogenized, and the protein of interest is extracted into an aqueous buffer via dual-stage mechanical disruption. Subsequently, the homogenate is centrifuged and subject to depth filtration to clarify the extract. If necessary, tangential flow filtration (TFF) is employed to concentrate the extract prior to chromatography. A mobile clean room PODTM within the R&D pilot facility containing GE HiScale columns at various diameters and heights is available to custom-pack chromatography resin. High-throughput, small-scale screening (96-well format) of resins or chromatography conditions can also be performed. For conventional column chromatography, four ÄKTA fast protein liquid chromatography systems (AVANT, PURE, PILOT and Process) are used for optimization and automation (Figure 5c). Postchromatography product concentration and buffer exchange are performed by TFF, followed by formulation and aseptic fill/finish.

Antibody expression and downstream process development

Dual viral-based vector systems have been the method of choice for the expression of antibody light and heavy chains in which the light-chain gene is usually introduced into a potato X virus (PVX)-based vector and the heavy-chain gene cloned into a tobacco mosaic virus (TMV)-based vector (Giritch *et al.*, 2006; Roy *et al.*, 2010). To date, Caliber has used PVX- and TMV-based vectors for the expression of over 17 monoclonal antibodies at a 10–12 kg plant biomass scale using the pilot-scale facility. Results from the production of rituximab (original sequence and a selection of amino acid substitution in the heavy-chain sequence affecting the final antibody activity without modifying N-glycosylation profiles) and antiviral antibodies (targeting influenza and dengue viruses) are presented below as illustration of the process development and characterization phases before the release of preclinical and clinical material by the quality control laboratory (Figure 6a–c). Design of experiments (DOE) analysing combinations of expression vectors, *Agrobacterium* cultures and concentrations, *Agrobacterium* clone ratio, infiltration vacuum strength, and postinfiltration conditions allowed for optimization and adaptation of laboratory-based vacuum infiltration protocols to manufacturing scale where the infiltration volume varies from 100 L (pilot scale) to 7000 L (manufacturing scale). Under pilot-scale conditions, the expression of plant-made biobetter rituximab candidates reached between 100 and 650 mg/kg of plant biomass depending on the protein modification made. Other mAbs tested, including anti-influenza virus and anti-dengue virus candidates, had expression levels of approximately 300 mg/kg of plant biomass before expression optimization with a final yield between 62% and 68% depending on the process chosen and a final purity of 95% to 98%.

As an initial step towards creating Caliber's plant-made mAb process development platform, iterative DOE with fractional factorial design examining pH, conductivity, detergents, antioxidants and protease inhibitors was utilized to screen homogenization/extraction conditions. Postextraction, antibody concentrations were measured by biolayer interferometry (BLI) utilizing protein A biosensors (BLItz system, Pall), while SDS-PAGE was utilized for qualitative visualization of fully assembled antibody product and host plant proteins (HPPs). Quantitative antibody extraction titre was statistically analysed against extraction variables to generate desirability profiles and ultimately formulate extraction conditions to maximize product extraction, while minimizing extract complexity through reduction of HPP extraction/solubilization.

An area that distinguishes PMP from pharmaceutical production in other systems (*e.g.* mammalian/insect/bacterial cells) is the extract clarification procedure. Removal of fibrous plant particulates from a column feedstream can be challenging, and many vendors traditionally target filtration products for the production in other expression systems. Multiple extract clarification schemes were examined and included the following: (i) primary clarification through centrifugation followed by secondary clarification by depth filtration, (ii) primary and secondary clarification by depth filtration and (iii) primary clarification by body feed filtration and secondary clarification through depth filtration. Ultimately, conditions for each scheme were identified

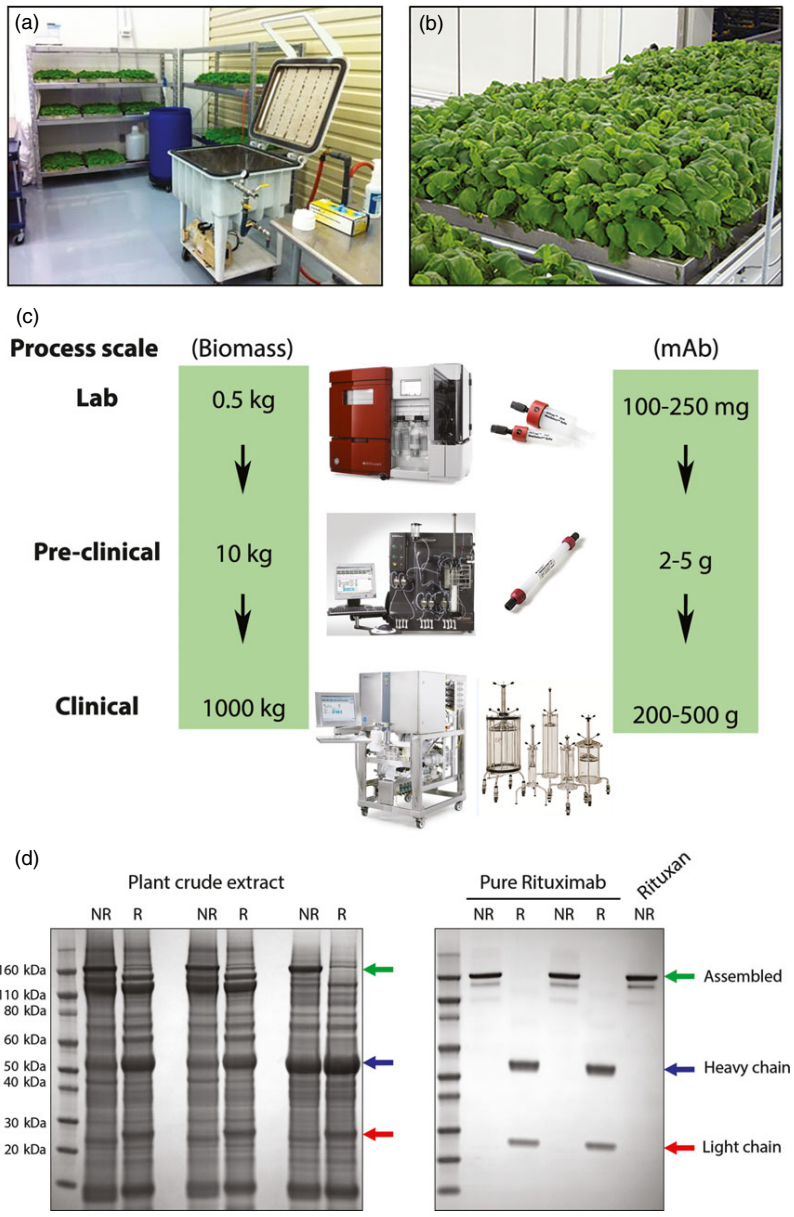

Figure 5 Overview of production scales and final mAb product. (a) Pilot-scale infiltration room; (b) infiltrated plants at harvest during large-scale manufacturing; (c) summary of process scales and mAb yields using the ÄKTA protein purification system; (d) SDS-PAGE analysis of three different batches of plants expressing rituximab (left) and two separate batches of purified plant-made rituximab. Samples were loaded under reduced (R) and nonreduced (NR) conditions.

that produced column-ready extract with minimal product loss (<10%). Taking into account in-house equipment and cost, clarification scheme (i) was selected and employed for all antibody lots produced. Over multiple lots, this clarification scheme was found to be robust and reproducible, yielding 94.4 ± 5.6% product recovery (calculated from multiple lots of rituximab production, n = 6), as determined by quantitation of product in clarified extract by BLI. Once clarified plant extract is generated, downstream processing steps follow a traditional chromatography path. For mAbs, capture chromatography employed MabSelect SuRe alkali-tolerant protein A-derived resin. Initial protein A (PA) chromatography conditions examined the effects of clarified extract pH, conductivity, and residence time on product capture and purification under the identified capture conditions, and product recovery for this stage was found to be 95.7 ± 0.9% (calculated from multiple lots of rituximab production, n = 6). Following PA chromatography, a viral inactivation step was performed by lowering the pH of the protein elution pool. Following viral inactivation, pH and conductivity were immediately adjusted to specifications determined for the second chromatography step. As a polishing chromatography step, multiple cation exchange (CEX) resins were screened for the ability to separate antibody aggregates and degradants from fully assembled full-length product species. As anticipated, the largest product loss occurred during this step, although effective removal of antibody aggregates and degradants was achieved. Product recovery was found to be 71.5 ± 3.1% (calculated from multiple lots of rituximab production, n = 3). The third and final chromatography step was implemented solely to reduce endotoxin levels present in the CEX elution pool. As part of the transient *Nicotiana*-based expression system, Gram-negative *Agrobacterium* were introduced during infiltration and cell wall remnants (*i.e.* endotoxin) must be effectively reduced prior to product concentration and formulation. Single-use membrane chro-

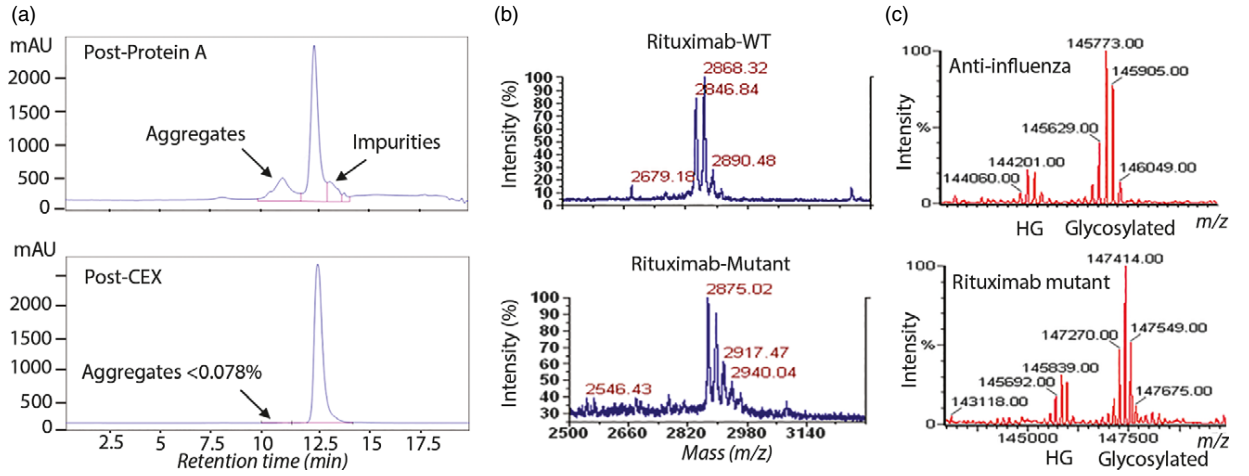

Figure 6 Overview of antibody characterization and release. (a) SE-HPLC of plant-made rituximab postprotein A chromatography displaying the impurities and aggregates, and purified plant-made rituximab post-CEX chromatography; (b) zoomed MALDI-TOF MS spectra of tryptic peptides that differentiate the wild-type and mutant plant-made rituximab. Note that the plant-made rituximab peptide carrying the mutation displays a mass of *m/z* 2875.02 Da, while the corresponding peptide derived from wild-type (WT) rituximab appears at *m/z* 2846.84 Da, resulting in the addition of 28.18 Da in the mutant tryptic peptide fragment; (c) glycoform identification by LC-MS of plant-made anti-influenza and rituximab mutant. HG, hemiglycosylated.

matography capsules run in flow-through mode were assessed for the ability to successfully retain endotoxin, while permitting product passage. Product recovery was found to be 94.4 ± 3.8% (calculated from multiple lots of rituximab production, $n = 3$), and endotoxin levels were reduced to 0.011 EU/mg. Product was then subjected to tangential flow filtration (TFF) for concentration and buffer exchange with a recovery of 92.4 ± 6.8% (calculated from multiple lots of rituximab production, $n = 3$). In an ISO5 rated class II biosafety cabinet housed within and ISO7 rated G-Con POD™, bulk drug substance was sterile filtered under aseptic conditions (0.2 μm). Final product (Figure 5d) was generally formulated to a final concentration of 10 mg/mL, aseptically transferred to sterile glass vials, stoppered, sealed and submitted to the quality control laboratory for release (Figure 6a–c).

Process conditions for Caliber's plant-made mAb process development platform were then applied, without molecule-specific optimization, for rapid production of preclinical quantities of antiviral mAb therapeutic candidates. The process design and conditions remained robust, permitting facile delivery of high-quality, high-purity material under an aggressive timeline (< 3 months).

Antibody characterization and release

Analytical techniques such as liquid chromatography–mass spectrometry (LC/MS), gas chromatography–mass spectrometry (GC/MS), matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS), size-exclusion high-performance liquid chromatography (SE-HPLC) and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) are used to characterize both in-process and final products as well as evaluate the stability of PMP. For validation purposes, standard references such as Rituxan® (Genentech, South San Francisco, CA, USA) were analysed using the same analytical techniques. Comparative analysis of intact protein molecular weight and tryptic peptide masses was performed by MALDI-TOF-MS to confirm the mutations in the amino acid sequence and identity of the product being made (Figure 6b). In addition, a Waters capillary liquid chromatography (CapLC) system interfaced via electrospray ionization to a Micromass QTOF mass spectrometer was also used for the analysis of molecular isoforms of intact proteins such as Rituxan®, plant-made biobetter rituximab products and plant-made antiviral antibodies. As an example, the deconvoluted electrospray ionization (ESI) mass spectra of fully assembled plant-made anti-influenza and rituximab mutant depicted molecular weights of 145 773 and 147 413 Da, respectively (Figure 6c). Two different glycoforms of the proteins are observed in both deconvoluted ESI spectra. In this particular case, the deconvoluted ESI mass spectra demonstrated the consistency of the glycosylation profile of two independent mAbs produced at different times, from different plant batches. Once characterized and released, Caliber's Analytical and Quality Control Lab initiate a stability evaluation protocol over the course of up to 12 months at various storage temperatures using analyses including SDS-PAGE, MALDI-TOF tryptic peptide mass fingerprinting and size-exclusion chromatography. For candidate molecules, chromatographic profiles, purity, pH, concentration and activity remained within specification at all storage conditions and time points measured. These results are strong indicators that Caliber's plant-made products are highly stable under normal storage conditions (4 °C, −20 °C and −70 °C).

In addition to analytical characterization, cell-based *in vitro* assays have been developed to evaluate antibody activity in comparison with the innovator molecules. For example, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are important effector mechanisms involved in the clinical efficacy of several monoclonal antibody therapeutics (Jiang *et al.*, 2011; Moore *et al.*, 2010). Rituximab binds to CD20 antigen on the surface of lymphoma cells while rituximab's Fc region recruits effector cells to induce lysis of target cells. To compare the efficacy of Caliber's plant-made biobetter rituximab with the commercially available Rituxan®, cytotoxicity activity was tested using the Wil2S cell line, derived from hereditary spherocytosis, and the Daudi cell line, derived from Burkitt's lymphoma (ATCC, Manassas, VA). ADCC efficacy was

determined using the ADCC reporter bioassay with engineered effector cells modified to express FcγRIIIa receptor and NFAT-RE luciferase (Promega, Madison, WI). Plant-made rituximab mutant candidates exhibited a two- to ten-fold increased cytotoxicity when compared with the reference standard (Rituxan®), while the plant-made native sequence and the reference standard showed equivalent cytotoxicity (manuscript in preparation). CDC efficacy using flow cytometry analysis (FACSCalibur) was also performed, which demonstrated enhanced activity of plant-made rituximab candidates in Daudi cells (data not shown). These assays are examples which demonstrate that Caliber's plant-made biobetter rituximab products have enhanced cytotoxicity against cancer cells when compared to the Rituxan® standard.

Conclusion

Plant transient expression is now a recognized flexible and affordable method for drug development which can be scaled up more rapidly than any other recombinant expression systems. The launch of new cGMP manufacturing infrastructures for PMP with the support of the DARPA Blue Angel programme has increased the manufacturing landscape to develop and advance further plant-based treatment to the clinic. With the design and operation of Caliber's manufacturing facility, commercialization of >150 kg/year of plant-made products can now be envisaged and represents a transient expression system capable of ramping drug production to large-scale manufacturing in a short period of time. In addition, with facilities such as Caliber Biotherapeutics, Fraunhofer, Kentucky BioProcessing and Medicago, the opportunity to develop medical countermeasures to rapidly and effectively respond to biothreats and outbreaks such as Ebola can now be realized.

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References

Bendandi, M., Marillonnet, S., Kandzia, R., Thieme, F., Nickstadt, A., Herz, S., Frode, R., Inoges, S., Lopez-Diaz de Cerio, A., Soria, E., Villanueva, H., Vancanneyt, G., McCormick, A., Tuse, D., Lenz, J., Butler-Ransohoff, J.E., Klimyuk, V. and Gleba, Y. (2010) Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. *Ann. Oncol.* **21**, 2420–2427.

Cummings, J.F., Guerrero, M.L., Moon, J.E., Waterman, P., Nielsen, R.K., Jefferson, S., Gross, F.L., Hancock, K., Katz, J.M., Yusibov, V. and Fraunhofer, U.S.A.C.f.M.B.S.G (2014) Safety and immunogenicity of a plant-produced recombinant monomer hemagglutinin-based influenza vaccine derived from influenza A (H1N1)pdm09 virus: a Phase 1 dose-escalation study in healthy adults. *Vaccine*, **32**, 2251–2259.

De Leede, L.G., Humphries, J.E., Bechet, A.C., Van Hoogdalem, E.J., Verrijk, R. and Spencer, D.G. (2008) Novel controlled-release Lemna-derived IFN-alpha2b (Locteron): pharmacokinetics, pharmacodynamics, and tolerability in a phase I clinical trial. *J. Interferon Cytokine Res.* **28**, 113–122.

Giritch, A., Marillonnet, S., Engler, C., van Eldik, G., Botterman, J., Klimyuk, V. and Gleba, Y. (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. *Proc. Natl Acad. Sci. USA*, **103**, 14701–14706.

Gleba, Y., Klimyuk, V. and Marillonnet, S. (2005) Magnifection–a new platform for expressing recombinant vaccines in plants. *Vaccine*, **23**, 2042–2048.

Goodin, M.M., Zaitlin, D., Naidu, R.A. and Lommel, S.A. (2008) Nicotiana benthamiana: its history and future as a model for plant-pathogen interactions. *Mol. Plant Microbe Interact.* **21**, 1015–1026.

Hiatt, A., Cafferkey, R. and Bowdish, K. (1989) Production of antibodies in transgenic plants. *Nature*, **342**, 76–78.

Huang, Z., Phoolcharoen, W., Lai, H., Piensook, K., Cardineau, G., Zeitlin, L., Whaley, K.J., Arntzen, C.J., Mason, H.S. and Chen, Q. (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. *Biotechnol. Bioeng.* **106**, 9–17.

Jiang, X.R., Song, A., Bergelson, S., Arroll, T., Parekh, B., May, K., Chung, S., Strouse, R., Mire-Sluis, A. and Schenerman, M. (2011) Advances in the assessment and control of the effector functions of therapeutic antibodies. *Nat. Rev. Drug Discovery*, **10**, 101–111.

Jornitz, M.W. (2014) The call for agile, flexible facilities: how podular facilities may answer it. *Pharma. Process.* July/August, 26-27.

Laffan, A.M., McKenzie, R., Forti, J., Conklin, D., Marcinko, R., Shrestha, R., Bellantoni, M. and Greenough, W.B. 3rd. (2011) Lactoferrin for the prevention of post-antibiotic diarrhoea. *J. Health. Popul. Nutr.* **29**, 547–551.

Landry, N., Ward, B.J., Trepanier, S., Montomoli, E., Dargis, M., Lapini, G. and Vezina, L.P. (2010) Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. *PLoS ONE*, **5**, e15559.

Landry, N., Pillet, S., Favre, D., Poulin, J.F., Trepanier, S., Yassine-Diab, B. and Ward, B.J. (2014) Influenza virus-like particle vaccines made in Nicotiana benthamiana elicit durable, poly-functional and cross-reactive T cell responses to influenza HA antigens. *Clin. Immunol.* **154**, 164–177.

Marillonnet, S., Thoeringer, C., Kandzia, R., Klimyuk, V. and Gleba, Y. (2005) Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. *Nat. Biotechnol.* **23**, 718–723.

McCormick, A.A., Reddy, S., Reinl, S.J., Cameron, T.I., Czerwinkski, D.K., Vojdani, F., Hanley, K.M., Garger, S.J., White, E.L., Novak, J., Barrett, J., Holtz, R.B., Tuse, D. and Levy, R. (2008) Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. *Proc. Natl Acad. Sci. USA*, **105**, 10131–10136.

Moore, G.L., Chen, H., Karki, S. and Lazar, G.A. (2010) Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions. *MAbs.* **2**, 181–189.

Pastores, G.M., Petakov, M., Giraldo, P., Rosenbaum, H., Szer, J., Deegan, P.B., Amato, D.J., Mengel, E., Tan, E.S., Chertkoff, R., Brill-Almon, E. and Zimran, A. (2014) A Phase 3, multicenter, open-label, switchover trial to assess the safety and efficacy of taliglucerase alfa, a plant cell-expressed recombinant human glucocerebrosidase, in adult and pediatric patients with Gaucher disease previously treated with imiglucerase. *Blood Cells Mol. Dis.* **53**, 253–260.

Pogue, G.P., Lindbo, J.A., Garger, S.J. and Fitzmaurice, W.P. (2002) Making an ally from an enemy: plant virology and the new agriculture. *Annu. Rev. Phytopathol.* **40**, 45–74.

Pogue, G.P., Vojdani, F., Palmer, K.E., Hiatt, E., Hume, S., Phelps, J., Long, L., Bohorova, N., Kim, D., Pauly, M., Velasco, J., Whaley, K., Zeitlin, L., Garger, S.J., White, E., Bai, Y., Haydon, H. and Bratcher, B. (2010) Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. *Plant Biotechnol. J.* **8**, 638–654.

Qiu, X., Wong, G., Audet, J., Bello, A., Fernando, L., Alimonti, J.B., Fausther-Bovendo, H., Wei, H., Aviles, J., Hiatt, E., Johnson, A., Morton, J., Swope, K., Bohorov, O., Bohorova, N., Goodman, C., Kim, D., Pauly, M.H., Velasco, J., Pettitt, J., Olinger, G.G., Whaley, K., Xu, B., Strong, J.E., Zeitlin, L. and Kobinger, G.P. (2014) Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature*, **514**, 47–53.

Rosenberg, Y., Sack, M., Montefiori, D., Forthal, D., Mao, L., Hernandez-Abanto, S., Urban, L., Landucci, G., Fischer, R. and Jiang, X. (2013) Rapid

high-level production of functional HIV broadly neutralizing monoclonal antibodies in transient plant expression systems. *PLoS ONE*, **8**, e58724.

Roy, G., Weisburg, S., Rabindran, S. and Yusibov, V. (2010) A novel two-component Tobacco mosaic virus-based vector system for high-level expression of multiple therapeutic proteins including a human monoclonal antibody in plants. *Virology*, **405**, 93–99.

Sainsbury, F., Thuenemann, E.C. and Lomonossoff, G.P. (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol. J.* **7**, 682–693.

Tacket, C.O., Mason, H.S., Losonsky, G., Estes, M.K., Levine, M.M. and Arntzen, C.J. (2000) Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. *J. Infect. Dis.* **182**, 302–305.

Thanavala, Y., Mahoney, M., Pal, S., Scott, A., Richter, L., Natarajan, N., Goodwin, P., Arntzen, C.J. and Mason, H.S. (2005) Immunogenicity in humans of an edible vaccine for hepatitis B. *Proc. Natl Acad. Sci. USA*, **102**, 3378–3382.

Tusé, D., Ku, N., Bendandi, M., Becerra, C., Collins, R., Langford, N., Sancho, S.I., López-Díaz de Cerio, A., Pastor, F., Kandzia, R., Thieme, F., Jarczowski, F., Krause, D., Ma, J.K.-C., Pandya, S., Klimyuk, V., Gleba, Y. and Butler-Ransohoff, J.E. (2015) Clinical safety and immunogenicity of tumor-targeted, plant-made Id-KLH conjugate vaccines for follicular lymphoma. *Biomed. Res. Int.* Article ID 648143, in press.

Weintraub, J.A., Hilton, J.F., White, J.M., Hoover, C.I., Wycoff, K.L., Yu, L., Larrick, J.W. and Featherstone, J.D. (2005) Clinical trial of a plant-derived antibody on recolonization of mutans streptococci. *Caries Res.* **39**, 241–250.

Wirz, H., Sauer-Budge, A.F., Briggs, J., Sharpe, A., Shu, S. and Sharon, A. (2012) Automated production of plant-based vaccines and pharmaceuticals. *J. Lab. Autom.* **17**, 449–457.

Yusibov, V., Hooper, D.C., Spitsin, S.V., Fleysh, N., Kean, R.B., Mikheeva, T., Deka, D., Karasev, A., Cox, S., Randall, J. and Koprowski, H. (2002) Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. *Vaccine*, **20**, 3155–3164.

Zimran, A., Gonzalez-Rodriguez, D.E., Abrahamov, A., Elstein, D., Paz, A., Brill-Almon, E. and Chertkoff, R. (2015) Safety and efficacy of two dose levels of taliglucerase alfa in pediatric patients with Gaucher disease. *Blood Cells Mol. Dis.* **54**, 9–16.