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PROTEIN EXPRESSION USING PLANTS AS BIOREACTORS

A VIABLE APPROACH TO SUSTAINABLE AND LOW-COST BIOPHARMACEUTICAL MANUFACTURING

A SwiftPharma whitepaper
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EXECUTIVE SUMMARY

The past decade has seen a significant shift in the nature of the products being manufactured and sold by the innovative biopharmaceutical (biopharma) industry. The global biopharmaceutical portfolio of today reflects increased therapeutic competition, a greater prevalence of large molecule drugs, expansion in the number of personalized or targeted products, and a rise of treatments for many orphan diseases. These trends have given rise to biopharmaceutical products with extremely limited production runs, highly specific manufacturing requirements, and genotype-specific products.

This fundamental shift in the overall product mix and a focus on continuing to improve the efficiency and effectiveness of production is spurring an evolution in the technologies and processes needed to support advanced biopharmaceutical manufacturing. Innovation in manufacturing technology is helping to drive improved economics, flexibility and quality while potentially benefiting patients both directly and indirectly. Biopharmaceutical manufacturers are generally making investments in the following areas:

- Continuous manufacturing to improve scalability and facilitate time to market, while lowering capital and operating costs and enhancing quality

- New process analytical tools to improve process robustness, accelerate scale-up to commercial production and drive more efficient use of resources

- Single-use systems to increase flexibility and reduce production lead times, while lowering capital investment and energy requirements

- Alternative downstream processing techniques to improve yields while lowering costs, green chemistry to reduce waste, and new vaccine and therapy production methods to increase capacity, scalability, and flexibility.

The changes in biopharmaceutical portfolios and the rise of advanced manufacturing technologies have impacts both inside and outside of biopharma companies.

First, they are driving biopharma companies to seek increasingly specialized workers who possess needed experience and skills. As a result, organizations are helping to design training programs at university biomanufacturing centers devoted to teaching relevant skills to students and employees. Second, the changes are causing biopharma companies to work collaboratively on manufacturing innovation through partnerships with academic institutions, diagnostics developers, production equipment manufacturers, and medical device manufacturers. Third, the new portfolios and technologies required are giving biopharma companies more reasons to consider location, ecosystem, and expression platform advantages in their strategic decisions around manufacturing. Finally, the rise of biopharmaceutical advanced manufacturing technologies is positively impacting society by benefiting patients, and the environment.

While Chinese hamster ovary (CHO) cells are the workhorse of biologics production, other cell expression systems are starting to gain ground, such as plant-based expression systems. Though an older technology in and of itself, plant-based manufacturing has recently been validated in the mainstream biopharmaceutical industry, but there is still a lack of biomanufacturers using such platforms for good manufacturing practice (GMP) production.

That said, the recent development of a successful plant-produced COVID-19 vaccine has proved that the industry is open to change and recognizes the need for a variety of production platforms in order to adapt to our growing and changing world.

This whitepaper provides a general and comprehensive view on using plants as a fast, scalable, and low-cost biopharmaceutical manufacturing platform and how it can offer solutions to high unmet needs.

Jeroen Hofenk
Chief Science Officer

PLANTS AS BIOREACTORS

The application of plant-based systems to produce biopharmaceuticals for human and veterinary indications is a vast and rapidly expanding field. Available systems range from stable transgenic root-cell culture to transient expression in whole plants in which tobacco plants are the most popular ones.

Products that have been expressed include monoclonal antibodies (MAbs) **(1)**, subunit vaccines **(2)**, virus-like particles (VLPs) **(3)**, specialty enzymes **(4)**, and structural proteins such as collagen or elastin **(5)**.

Conventional bioproduction platforms such as Chinese Hamster Ovary (CHO) cells, Escherichia coli (E. coli), and Pichia pastoris (P. pastoris) have long histories of patient safety and regulatory acceptance. Newer systems based on insect cells (transfected with baculovirus), transgenic animals, and plants are quickly accumulating the clinical safety data required to provide similar confidence for use **(6)**.

In fact, On the regulatory front, both FDA and the European Medicines Agency are well informed about plant-made products and have been supportive and helpful in evaluating clinical trials and manufacturing systems, the source adds. Moreover, few, if any, clinical trials involving plant-made products have been put on clinical hold for toxicity.

However, at this point in time, there are few contract development and manufacturing organizations that currently manufacture biologics for human clinical trials under current GMP compliance.

The COVID pandemic has played a role in bringing new technological platforms to the forefront, including a plant-made vaccine, baculovirus vaccines, and mRNA vaccines. In this relatively short period, these three platforms have gained a more mainstream prominence. The hope is that this attention will result in investment and subsequent development of these systems for routine development and production of biologics.

THE CURRENT STATE-OF-ART

One of the first approved plant-produced biologics, was a subunit vaccine against Newcastle disease in chickens **(7)**. The vaccine was derived from plant cell culture and was approved by the US Department of Agriculture (USDA) in 2006.

Currently, one product has received full approval from the US Food and Drug Administration (FDA) for human use: Elylys (Taliglucerase Alpha), a mitochondrial human glucocerebrosidase enzyme deficit therapy for Gaucher disease produced in carrot-root cell culture by Protalix Biotherapeutics **(8)**.

A monoclonal antibody cocktail for Ebola (Zmapp) was produced in Nicotiana benthamiana (N. benthamiana) **(9)** by Icon Genetics. The drug was approved for compassionate use during the 2014 outbreak in Western Africa. A subsequent clinical trial demonstrated its safety and therapeutic benefit, however it failed to reach the specified statistical threshold for efficacy, largely due to the low number of enrolled patients **(10)**.

Recently, Medicago Inc. reported their results of a phase 3 trial for a VLP influenza vaccine produced in N. benthamiana. The study demonstrated the product's safety and efficacy as well as the consistency of its production process **(11)**. Early in 2022, Medicago's Covifenz vaccine against SARS-CoV-2, based on plant-derived VLPs, received approval from Health Canada for use in adults 18–64 years old **(3, 12)**.

Regenerative medicine firm CollPlant has secured CE mark approval for its Vergenix STR soft tissue repair matrix to treat tendinopathy. Vergenix STR aggregates cross linked rhCollagen produced in N. tabacum with autologous platelet-rich plasma (PRP), which is a concentrated blood plasma derived from the patient's own blood that contains high levels of platelets containing growth factors.

The fact that only a few plant-produced therapeutic products have been clinically translated thus far may be more related to industrial and regulatory inertia than to product inadequacy, especially given substantial evidence of functional equivalency.

PLANT SYSTEM RATIONALE

Plant-based biopharmaceutical production offers numerous advantages such as speed in development, scalability, lower production costs, and inherent safety. In terms of production parameters, the upstream part of the company's process (i.e., growing and transfecting the plants with the protein of interest) is much simpler and more robust than cell culture-based systems because fewer components are required, there is no need to maintain sterile boundaries, and there is no risk of exogenous viral contamination. Growth of plants requires only air, light, water, and fertilizer salts (though plant cell cultures in bioreactors requires addition of a suitable carbon source such as glucose to support growth).

Thus, plant-based systems eliminate the need for costly supplements extensive cloning selection, and master- and working cell bank maintenance required for mammalian cell culture. Using whole plants as hosts are also far less capital intensive (e.g., equipment, technologies, facility, personnel) for the upstream processing portion of plant-based biologics production, compared to investment needed to set up comparable upstream processing for mammalian cell-based systems (both single-use and traditional stainless-steel reactor facilities) **(13)**.

The ability to grow plants as reactors with high precision in automated and vertical hydroponic systems have been well developed. Such plant manufacturing systems have no animal/human inputs or intervention, and thus there are no concerns for adventitious virus contamination in these systems.

The upstream capital expenditure for a plant system is much lower than that of a traditional staged bioreactor train, whether single-use or stainless-steel based. Furthermore, scale-up is not incremental in plant-based systems as additional capacity does not require a linear increase in robotics, and personnel, or associated infrastructure (steam, water, and sanitary pipework). Increasing manufacturing scale requires only that more plants are grown.

Other advantages of the plant-made system include much lower energy consumption, lower water usage through recycling, and extremely low waste treatment costs.

Furthermore, when comparing manufacturing with single-use systems, one must be mindful of the environmental impacts. Specifically, while single-use systems are considered by some to be more environmentally friendly than the cleaning associated with stainless-steel, at the end of the day, single-use systems use a significant amount of plastic disposables. This significant use of plastics likely contributes to the biopharmaceutical industry being 55% more emissions-intensive than the automotive industry.

Conversely, the key raw materials with a plant-based manufacturing system are considered “all-natural”: purified water, stone wool or another biodegradable substrate for growing the plants, and seeds.

Meanwhile, the downstream processes (DSPs) are comparable between plants and traditional bioreactors and typically amount for around 75% of the production costs. The major cost-drivers during DSP are (i) the high particle burden of primary extracts, requiring extensive clarification, (ii) the large amounts of host cell proteins (HCP) impurities which had to be separated from the product, and (iii) the presence of plant secondary metabolites, including pigments and phenols, that might permanently bind to, and thus alter, the product. Particles, HCPs, and metabolites are typically released due to the thorough homogenization required to extract the product from plant tissues.

AVAILABLE HOSTS

A plethora of recent reviews summarize the state-of-art of plant-based expression systems for the production of biopharmaceuticals **(14-16).** Many such systems are available depending on the plant being used such as rice **(17),** tobacco and potato **(18),** and lettuce **(19).** Which option to select depends upon the characteristics required for the final product. E.g., a protein might be selectively localized and expressed through the cytoplasm, apoplast, chloroplast, or vacuole **(20).**

For example, MAbs typically are secreted into the extracellular space (apoplast) via the endoplasmic reticulum (ER)/Golgi secretory system, and then recovered from plant extracts using industry-standard (ultra)filtration and chromatographic approaches that are commonly shared with conventional expression platforms.

Claimed yields have reached up to four grams of product per kilogram of plant biomass **(15).** In other cases, product proteins can be retained within plant tissue — e.g., encapsulated in chloroplasts. Minimal processing of the plant biomass yields a feed additive that can be formulated for oral delivery**.**

There are two basic approaches to heterologous protein expression in plant cells: transient and stable transgenic expression. Transient systems often use the common soil bacterium Agrobacterium tumefaciens for gene delivery, whereby rapid expression can be achieved over a relatively short time before natural plant responses reduce expression from foreign DNA sequences. Transgenic systems rely upon the integration of recombinant DNA into the host genome for long-term expression of a product. Transgenic plants are created using A. tumefaciens infection or DNA-coated particle bombardment and subsequent regeneration of plantlets from infected cells (calli). These plantlets are bred to homozygosity for active DNA inserts (which, by definition, are relatively benign in terms of plant physiology and development). This is similar to the process for creation of production cell lines for mammalian expression systems — and likewise can be time consuming.

In the case of parenteral (injectable) drug products, or when rapid response is needed, transient expression systems using N. benthamiana generally are preferred because they generate high yields and use an industry-standard non-food-crop host **(21).** To produce encapsulated drugs destined for oral delivery, a stable transgenic food-crop platform might be preferable in which the product gene is included in the plant (or plastid) genome.

A transient system depends upon the ability of A. tumefaciens to transfer genes into a plant host through a tumor-inducing (Ti) plasmid vector **(22).** There are many variants of this system, depending on what specific elements are included in the vector along with the gene(s) of interest.

In general, the choice can be reduced to use of viral components for in situ replication and systemic distribution of the genes **(23)** or use of nonviral methods **(24)**. Herein, we focus on transient expression platforms using N. benthamiana as a host and with a nonviral system based on a binary transfer DNA (T-DNA) vector.

In summary, the gene for the protein of interest is cloned into a T-DNA vector and transformed into a suitable strain of A. tumefaciens (e.g., GV2260, EHA105 and LBA4404). In a process known as agroinfiltration, the transformed bacteria are introduced to plant tissue to transfer the product gene into the plant cells (Figure 1). After incubation, plants are harvested and homogenized. Proteins are extracted by physical (filtration) and chemical (affinity chromatography) methods to provide a drug substance with the required purity. In practice, each component of the overall process has multiple requirements and presents opportunities for optimization during scale-up from lab to large-scale manufacturing, as outlined in the following sections.

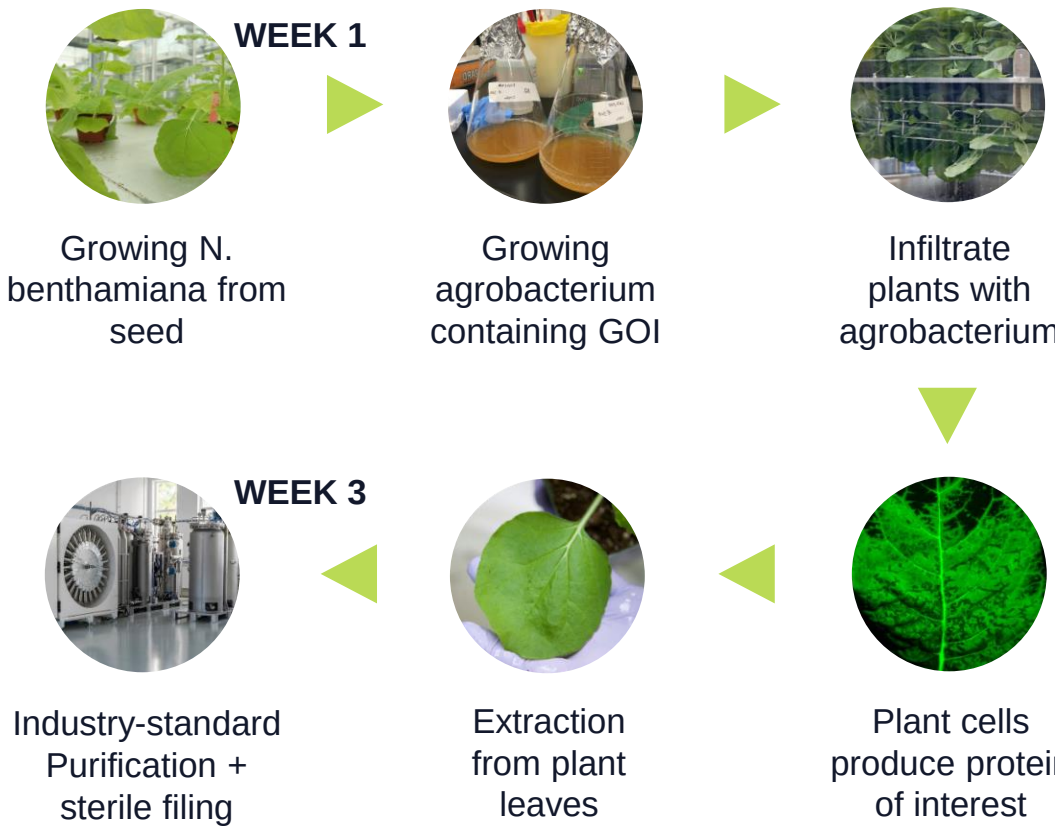

Figure 1: Plant-based production process using transient expression

VECTOR CONSTRUCTION

Depending on the source of the original DNA sequence for a protein of interest, it is usually advantageous to modify codon use to mimic that of the host plant. Codon usage regulates the speed of translation elongation, resulting in non-uniform ribosome decoding rates on mRNAs during translation that is adapted to co-translational protein folding process. Several online tools apply codon bias for N. benthamiana to create a synthetic DNA sequence for any protein. The novel sequence can be scanned to eliminate potential intron splice sites, micro-RNA (miRNA) motifs, and polyadenylation sequences — among other problematic sequences that are context specific.

However, a gene's intron structure can be designed specifically to provide for intron-mediated enhancement of expression **(25)**. In the case of proteins that will be secreted from plant cells, native or plant-preferred variations in signal peptide can be used, with the option of targeting proteins to specific compartments such as vacuoles **(26)**. Designed genes are cloned into the T-DNA region of a suitable binary vector in the context of control elements including a promoter, 5′ and 3′ untranslated regions (UTRs), and a terminator, each of which will dramatically affect final product yield the good or the better **(27, 28)**.

Once the sequence is verified, a vector construct is transformed into A. tumefaciens using standard chemical methods and/or electroporation. Stocks of the strains to be used for manufacturing are maintained according to a formal cell banking system that complies with regulatory guidelines.

GROWTH OF A. TUMEFACIENS

The Agrobacterium strain(s) to be used for expression of a protein of interest typically are grown in a standard bacterial culture medium to a stage at which they are stable and infective **(29)**.

Industrial processes normally use well-controlled bioreactors, although laboratory-scale developmental studies routinely use shake flask systems **(30)**. In processes destined for production of human therapeutics, culture media should be formulated to contain no animal-derived components **(31)**.

Plant-derived compounds, such as phenolics produced by wounded tissue or their analogs (e.g., acetosyringone), frequently are used in growth media to enhance bacterial infectivity, but they are not essential **(21)**. Conditions for growth such as pH, temperature, and dissolved oxygen as well as time of harvest will influence product yield from the plant tissue **(32)**.

GROWTH OF N. BENTHAMIANA

Plant growth conditions before exposure to Agrobacterium strains are critical to the production process. Parameters such as the choice of growth substrate (nutrient content, pH levels, and salinity), incident light, photoperiod, leaf temperature, atmospheric CO^2 concentration, humidity, and air-flow patterns will all affect plant health and morphology — which, in turn, will affect a plant's interaction with infective Agrobacterium and consequently influence protein yield **(33)**.

Early-stage development work is regularly conducted in greenhouse or vertical farming systems with limited environmental control. They can be subject to uncontrolled variability, which affects yield and quality of recombinant proteins (produced either transiently or from transgenic plants) **(34)**. In an industrial setting, and especially for biopharmaceuticals produced under current good manufacturing practice (CGMP) conditions, plants typically are grown in highly controlled contained systems based on multilayer hydroponic stacks. Lighting is provided by controlled low-temperature light-emitting diodes (LEDs) to enable zoned definition of parameters of intensity and spectral quality depending on plant age and needs. Such facilities can achieve high productivity in a small footprint and are constructed for cleanability and controlled access. The use of robotic systems to move trays of plants onto and out of the stacks provides further opportunity for control and efficiency.

A particular advantage of transient expression is that commitment to manufacturing a specified product is made at the pre-infiltration stage of plant growth. The plants, in effect, are product agnostic until they are exposed to the A. tumefaciens carrying the product vector(s). That provides for exceptional flexibility in a manufacturing facility.

AGROINFILTRATION

The process for placing Agrobacterium in contact with host plant cells first involves dilution of high-density bacterial cultures in a suitable buffer. Although plants can withstand surprisingly high concentrations of Agrobacterium infiltration cocktails, the optimal number of viable, infective cells required in a cocktail to maximize the number of gene copies transferred to each cell should be identified by quantitive design of experiments. In the case of multigene systems, the individual components can be delivered through single strains containing many genes **(35)**; multiple strains, each containing a single vector **(36)**; or some combination of those. In effect, the decision is both practical (“What is more productive?”) and economic (“What is less costly?”). For example, multigene vectors might exhibit fragility during gene transfer from bacterium to plant cell, and use of several individual gene strains will require multiple bioreactors, which will impose an increase in process costs.

The infiltration process itself requires a system that efficiently introduces bacterial strains to the interior of a leaf or to a whole plant (via stomata). Whole plants in a tray are transferred from a growth chamber to a vacuum pressure vessel containing the bacterial suspension, typically using conveyor systems to limit manual labor. The plants are inverted and immersed in the bacterial suspension, and chamber atmospheric pressure is reduced to force out air from the leaves. After a short period, pressure is returned to atmospheric, and the leaves are filled with bacterial suspension to begin the process of horizontal gene transfer into the plant cells through the bacterium’s type 4 secretion systems **(37)**. Infiltrated plants are removed from the infiltration module and transferred to a second set of growth racks for post-infiltration incubation.

POST-INFILTRATION PERIOD

This is the period where the plants infected with foreign DNA are allowed to incubate under appropriate conditions in a contained environment while they accumulate product. The incubation conditions (including time) that support highest yields of recoverable product of appropriate quality might be different from those that were best for preincubation growth, so such conditions should be determined empirically.

For example, environmental temperature or light intensity might need to be reduced to limit accumulation of nonproductive (noninfected) biomass. However, care must be taken to prevent stress or necrotic responses, which can significantly reduce yield or quality of a heterologous protein. A CGMP-compliant facility will feature a linear process flow with increasing environmental containment from pre-infiltration plant growth through agroinfiltration to plant harvest and transfer to downstream product recovery.

PRODUCT RECOVERY

There is little practical difference between product recovery from plant-based systems and that from other, well-established product expression platforms.

Product-containing biomass is homogenized in an appropriate extraction buffer (with protease inhibitors and/or antioxidants, depending on the requirements, to maintain product integrity through processing) and first filtered to remove insoluble components and debris before entering a series of diafiltration steps. The process stream may be ultrafiltered and/or concentrated before the first chromatography step, all under conditions that are designed to prevent contamination with adventitious agents (bacteria and fungi) **(38)**. For any given protein, a suitable chromatographic process is outlined before proceeding to large-scale production to ensure a maximal positive COGS outcome.

SPECIAL CONSIDERATIONS

Plant-based protein production systems as explained above require a few considerations. One involves plant responses to the pathogen insult inherent to transient Agrobacterium-mediated infection. A plant will generate a response to limit damage by the foreign DNA that has been introduced and/or the presence of bacteria in the leaf tissue. That response can take several forms, including production of inhibitors of bacterial integrity such as salicylic acid or phenolic compounds.

The most significant response of concern is that against the foreign DNA inside infected plant cells. This is similar in effect to the antiviral response termed posttranslational gene silencing (PTGS), whereby molecular mechanisms are assembled to detect and destroy the mRNA driving heterologous protein production **(39)**.

Plant cells use small RNA molecules created from the mRNA (through activities of systems such as Dicer endoribonuclease) and incorporated into the RNA-induced silencing complex (RISC) to direct degradation of the parent molecule. Further systems act on the DNA to depress activity at the promoter regions by methylation **(40)**.

A solution to that problem lies in the means used by viruses to counter the plant defense. Several viral proteins are known to interfere with PTGS responses. For example, the P19 protein of tomato bushy stunt virus is among the most efficient, widely used, and studied suppressors of PTGS **(41)**.

Host plants might also respond to foreign proteins by producing proteases, which can reduce protein yield significantly and/or affect product quality **(42)**. Protease responses can be addressed either by using inhibitors or by deleting specific genes from the plant genome. N. benthamiana which has a unique characteristic: it sacrifices viral defense for rapid growth — which makes the species particularly useful for biotechnology applications **(43)**.

PLANTS VS. MAMMALIAN

An important criterion for any expression system used to manufacture biologics products is the comparability of the end product with a validated expression system that is well known and widely used in the industry, such as mammalian CHO cells.

Plant cells are highly developed eukaryotic cells; each cell has the same machinery as a mammalian cell to express heterologous protein sequences accurately. Plants do, however, have slightly different post-translational modifications such as minor glycoform differences.

If the glycoform on a particular therapeutic is a key to functionality, the glycoform can now be modified in the plant cell expression system to be as fully functional as the mammalian counterpart. This has been demonstrated in the literature for over a decade. One advantage in the plant system seems to be the consistency of the glycoform pattern expressed.

Plants also tend to produce a more homogenous product with post-translational modifications. Mammalian cells produce multiple glycoform patterns, which can be affected by culture conditions, including scale-up to larger reactor systems. Many biologics do not have any post-translational modifications.

For most recombinant proteins, a plant-based expression system yields products that are highly comparable to mammalian/CHO systems, particularly in regard to monoclonal antibody (mAb) development, plants can deliver a more homogeneous N-linked glycosylation pattern. This characteristic provides greater assurance that the glycoform of choice is appropriately represented in the final product, likely improving efficacy.

Those plant-specific glycan features are potentially immunogenic **(44)** and must be removed from biotherapeutic protein products that are destined for treatment of humans. Removal can be achieved by down-regulating **(45)** or deleting the cognate fucosyl- and xylosyltransferases (e.g., using techniques based on clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein 9 (Cas9)) **(46)**.

REFERENCES

1. Klimyuk V, Pogue G, Herz S, Butler J, Haydon H. Production of recombinant antigens and antibodies in Nicotiana benthamiana using 'magnifection' technology: GMP-compliant facilities for small- and large-scale manufacturing. Curr Top Microbiol Immunol. 2014;375:127-54. doi: 10.1007/82_2012_212. PMID: 22527176.

2. Santoni M, Gecchele E, Zampieri R, Avesani L. Plant-Based Systems for Vaccine Production. Methods Mol Biol. 2022;2412:95-115. doi: 10.1007/978-1-0716-1892-9_6. PMID: 34918243.

3. D'Aoust MA, Lavoie PO, Couture MM, Trépanier S, Guay JM, Dargis M, Mongrand S, Landry N, Ward BJ, Vézina LP. Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. Plant Biotechnol J. 2008 Dec;6(9):930-40. doi: 10.1111/j.1467-7652.2008.00384.x. PMID: 19076615.

4. Alkanaimsh S, Karuppanan K, Guerrero A, Tu AM, Hashimoto B, Hwang MS, Phu ML, Arzola L, Lebrilla CB, Dandekar AM, Falk BW, Nandi S, Rodriguez RL, McDonald KA. Transient Expression of Tetrameric Recombinant Human Butyrylcholinesterase in Nicotiana benthamiana. Front Plant Sci. 2016 Jun 16;7:743. doi: 10.3389/fpls.2016.00743. PMID: 27379103; PMCID: PMC4909763.

5. Ruggiero F, Exposito JY, Bournat P, Gruber V, Perret S, Comte J, Olagnier B, Garrone R, Theisen M. Triple helix assembly and processing of human collagen produced in transgenic tobacco plants. FEBS Lett. 2000 Mar 3;469(1):132-6. doi: 10.1016/s0014-5793(00)01259-x. PMID: 10708770.

6. Schillberg S, Finnern R. Plant molecular farming for the production of valuable proteins - Critical evaluation of achievements and future challenges. J Plant Physiol. 2021 Mar-Apr;258-259:153359. doi: 10.1016/j.jplph.2020.153359. Epub 2021 Jan 5. PMID: 33460995.

7. https://www.chemistryworld.com/news/plant-derived-drug-approved-in-us/3003199.article

8. Pfizer and Protalix's Elelyso receives FDA approval to treat type 1 Gaucher disease - Pharmaceutical Technology (pharmaceutical-technology.com)

9. Budzianowski J. Tobacco against Ebola virus disease. Przegl Lek. 2015;72(10):567-71. PMID: 26946569.

10. PREVAIL II Writing Group. A Randomized, Controlled Trial of ZMapp for Ebola Virus Infection. N. Engl. J. Med. 375(15) 2016: 1448–1456; https://doi.org/10.1056/NEJMoa1604330.

11. Ward BJ, et al. Phase III: Randomized Observer-Blind Trial to Evaluate Lot-to-Lot Consistency of a New Plant-Derived Quadrivalent Virus Like Particle Influenza Vaccine in Adults 18–49 Years of Age. Vaccine 39(10) 2021: 1528–1533; https://doi.org/10.1016/j.vaccine.2021.01.004.

12. Medicago Covifenz COVID-19 Vaccine. Health Canada: Ottawa, Ontario, 31 March 2022; https://www.canada.ca/en/health-canada/services/drugs-health-products/covid19-industry/drugs-vaccines-treatments/vaccines/medicago.html.

13. Chen Q, Davis KR. The potential of plants as a system for the development and production of human biologics. F1000Res. 2016 May 19;5:F1000 Faculty Rev-912. doi: 10.12688/f1000research.8010.1. PMID: 27274814; PMCID: PMC4876878.

14. Powell JD. From Pandemic Preparedness to Biofuel Production: Tobacco Finds Its Biotechnology Niche in North America. Agriculture 4(5) 2015: 901–917; https://doi.org/10.3390/agriculture5040901.

15. Schillberg S, Finnern R. Plant Molecular Farming for the Production of Valuable Proteins: Critical Evaluation of Achievements and Future Challenges. J. Plant Physiol. 258–259, 2021: 153359; https://doi.org/10.1016/j.jplph.2020.153359.

17. Swope K, et al. Reproducibility and Flexibility of Monoclonal Antibody Production with Nicotiana benthamiana. mAbs 14(1) 2022: e2013594; https://doi.org/10.1080/19420862.2021.2013594.

18. Wakasa Y, Takaiwa F. The Use of Rice Seeds to Produce Human Pharmaceuticals for Oral Therapy. Biotechnol J. 8(10) 2013: 1133–1143; https://doi.org/10.1002/biot.201300065.

19. Scheller J, et al. Production of Spider Silk Proteins in Tobacco and Potato. Nature Biotechnol. 19, 2001: 573–577; https://doi.org/10.1038/89335.

20. Meyers B, et al. Nuclear and Plastid Genetic Engineering of Plants: Comparison of Opportunities and Challenges. Biotechnol. Adv. 28, 2010: 747–756; https://doi.org/10.1016/j.biotechadv.2010.05.022.

21. Conley AJ, Zhu H, Le LC, Jevnikar AM, Lee BH, Brandle JE, Menassa R. Recombinant protein production in a variety of Nicotiana hosts: a comparative analysis. Plant Biotechnol J. 2011 May;9(4):434-44. doi: 10.1111/j.1467-7652.2010.00563.x. Epub 2010 Oct 8. PMID: 21040385.

22. Gelvin SB. Integration of Agrobacterium T-DNA into the Plant Genome. Annu. Rev. Genetics. 51, 2012: 195–217; https://doi.org/10.1146/annurev-genet-120215-035320.

23. Hefferon KL. Plant Virus Expression Vectors Set the Stage As Production Platforms for Biopharmaceutical Proteins. Virology 433, 2012: 1–6; https://doi.org/10.1016/j.virol.2012.06.012.

24. Garabagi F, et al. Utility of the P19 Suppressor of Gene-Silencing Protein for Production of Therapeutic Antibodies in Nicotiana Expression Hosts. Plant Biotechnol. J. 10(9) 2012: 1118–1128; https://doi.org/10.1111/j.1467-7652.2012.00742.x.

25. Laxa M. Intron-Mediated Enhancement: A Tool for Heterologous Gene Expression in Plants? Front Plant Sci. 7, 2017: 1977; https://doi.org/10.3389/fpls.2016.01977.

26. Nakamura K, Matsuoka K. Protein targeting to the vacuole in plant cells. Plant Physiol. 1993 Jan;101(1):1-5. doi: 10.1104/pp.101.1.1. PMID: 8278490; PMCID: PMC158640.

27. Kallolimath S, et al. Promoter Choice Impacts the Efficiency of Plant Glycol-Engineering. Biotechnol J. 13(1) 2018: 1700380; https://doi.org/10.1002/biot.201700380.

28. 28 Diamos AG, et al. 5′ and 3′ Untranslated Regions Strongly Enhance Performance of Geminiviral Replicons in Nicotiana benthamiana Leaves. Front. Plant Sci. 7, 2016: 200; https://doi.org/10.3389/fpls.2016.00200.

29. Leth KI and McDonald KA. Media Development for Large Scale Agrobacterium tumefaciens Culture. Biotechnol. Prog. 33(5) 2017: 1218–1225; https://doi.org/10.1002/btpr.2504.

30. Spiegel H, Boes A, Perales Morales C, Rademacher T, Buyel JF. Ready-to-Use Stocks of Agrobacterium tumefaciens Can Simplify Process Development for the Production of Recombinant Proteins by Transient Expression in Plants. Biotechnol J. 2019 Oct;14(10):e1900113. doi: 10.1002/biot.201900113. Epub 2019 Jul 18. PMID: 31218827.

31. Houdelet M, Galinski A, Holland T, Wenzel K, Schillberg S, Buyel JF. Animal component-free Agrobacterium tumefaciens cultivation media for better GMP-compliance increases biomass yield and pharmaceutical protein expression in Nicotiana benthamiana. Biotechnol J. 2017 Apr;12(4). doi: 10.1002/biot.201600721. Epub 2017 Mar 16. PMID: 28221723.

32. Prudhomme N, et al. Exposure of Agrobacterium tumefaciens to Agroinfiltration Medium Demonstrates Cellular Remodelling and May Promote Enhanced Adaptability for Molecular Pharming. Can. J. Microbiol. 67(1) 2021: 85–97; https://doi.org/10.1139/cjm-2020-0239.

34. Fujiuchi N, Matoba N, Matsuda R. Environment Control to Improve Recombinant Protein Yields in Plants Based on Agrobacterium-Mediated Transient Gene Expression. Front. Bioeng. Biotechnol. 4, 2016: 23; https://doi.org/10.3389/fbioe.2016.00023.

35. Dusek J, et al. Extended Set of GoldenBraid Compatible Vectors for Fast Assembly of Multigenic Constructs and Their Use to Create Geminiviral Expression Vectors. Front. Plant Sci. 11, 2020: 522059; https://doi.org/10.3389/fpls.2020.522059.

36. Irmisch S, et al. Complete Biosynthesis of the Anti-Diabetic Plant Metabolite Montbretin A. Plant Physiol. 184(1) 2020: 97–109; https://doi.org/10.1104/pp.20.00522.

37. Gelvin SB. Traversing the Cell: Agrobacterium T-DNA's Journey to the Host Genome. Front. Plant Sci. 3, 2012: 52; https://doi.org/10.3389/fpls.2012.00052.

38. Buyel JF, et al. Extraction and Downstream Processing of Plant-Derived Recombinant Proteins. Biotechnol. Adv. 33(6) 2015: 902–913; https://doi.org/10.1016/j.biotechadv.2015.04.010.

39. Rössner C, Lotz D, Becker A. VIGS Goes Viral: How VIGS Transforms Our Understanding of Plant Science. Annu. Rev. Plant Biol. 73, 2022 (in press); https://doi.org/10.1146/annurev-arplant-102820-020542.

40. Yoshida T, et al. Genome Defense Against Integrated Organellar DNBA Fragments from Plastids into Plant Nuclear Genomes Through DNA Methylation. Nature Sci. Rep. 9, 2019: 2060; https://doi.org/10.1038/s41598-019-38607-6.

41. Scholthof HB. The Tombusvirus-Encoded P19: From Irrelevance to Elegance. Nat. Rev. Microbiol. 4(5) 2006: 405–411; https://doi.org/10.1038/nrmicro1395.

42. Jutras PV, Dods I, van der Hoorn RA. Proteases of Nicotiana benthamiana: An Emerging Battle for Molecular Farming. Curr. Opin. Biotechnol. 61, 2020: 60–65; https://doi.org/10.1016/j.copbio.2019.10.006.

34. Fujiuchi N, Matoba N, Matsuda R. Environment Control to Improve Recombinant Protein Yields in Plants Based on Agrobacterium-Mediated Transient Gene Expression. Front. Bioeng. Biotechnol. 4, 2016: 23; https://doi.org/10.3389/fbioe.2016.00023.

35. Dusek J, et al. Extended Set of GoldenBraid Compatible Vectors for Fast Assembly of Multigenic Constructs and Their Use to Create Geminiviral Expression Vectors. Front. Plant Sci. 11, 2020: 522059; https://doi.org/10.3389/fpls.2020.522059.

36. Irmisch S, et al. Complete Biosynthesis of the Anti-Diabetic Plant Metabolite Montbretin A. Plant Physiol. 184(1) 2020: 97–109; https://doi.org/10.1104/pp.20.00522.

37. Gelvin SB. Traversing the Cell: Agrobacterium T-DNA's Journey to the Host Genome. Front. Plant Sci. 3, 2012: 52; https://doi.org/10.3389/fpls.2012.00052.

38. Buyel JF, et al. Extraction and Downstream Processing of Plant-Derived Recombinant Proteins. Biotechnol. Adv. 33(6) 2015: 902–913; https://doi.org/10.1016/j.biotechadv.2015.04.010.

39. Rössner C, Lotz D, Becker A. VIGS Goes Viral: How VIGS Transforms Our Understanding of Plant Science. Annu. Rev. Plant Biol. 73, 2022 (in press); https://doi.org/10.1146/annurev-arplant-102820-020542.

40. Yoshida T, et al. Genome Defense Against Integrated Organellar DNBA Fragments from Plastids into Plant Nuclear Genomes Through DNA Methylation. Nature Sci. Rep. 9, 2019: 2060; https://doi.org/10.1038/s41598-019-38607-6.

41. Scholthof HB. The Tombusvirus-Encoded P19: From Irrelevance to Elegance. Nat. Rev. Microbiol. 4(5) 2006: 405–411; https://doi.org/10.1038/nrmicro1395.

42. Jutras PV, Dods I, van der Hoorn RA. Proteases of Nicotiana benthamiana: An Emerging Battle for Molecular Farming. Curr. Opin. Biotechnol. 61, 2020: 60–65; https://doi.org/10.1016/j.copbio.2019.10.006.

43. Bally J, et al. The Extremophile *Nicotiana benthamiana* Has Traded Viral Defence for Early Vigour. *Nature Plants* 1, 2015: 15165; https://doi.org/10.1038/nplants.2015.165.

44. Schoberer J, Strasser R. Plant Glycol-Biotechnology. Semin. Cell Dev. Biol. 80, 2018: 133–141; https://doi.org/10.1016/j.semcdb.2017.07.005.

45. Kaulfürst-Sobell H, et al. Reduced Immunogenicity of Arabidopsis hgl1 Mutant N-Glycans Caused by Altered Accessibility of Xylose and Core Fucose Epitopes. J. Biol. Chem. 286(26) 2011: 22955–22964; https://dx.doi.org/10.1074%2Fjbc.M110.196097.

46. Strasser R, et al. Generation of Glyco-Engineered Nicotiana benthamiana for the Production of Monoclonal Antibodies with a Homogeneous Human-Like N-Glycan Structure. Plant Biotechnol. J. 6(4) 2008: 392–402; https://doi.org/10.1111/j.1467-7652.2008.00330.x.

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