Published in final edited form as:
Curr Opin Biotechnol. 2015 April ; 32: 163–170. doi:10.1016/j.copbio.2014.12.008.

The increasing value of plant-derived pharmaceutical proteins

Markus Sack[a], **Anna Hofbauer**[b], **Rainer Fischer**[a,c], and **Eva Stoger**[b,*]

[a]Institute for Molecular Biotechnology, RWTH Aachen, Germany

[b]Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Vienna, Austria

[c]Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Aachen, Germany

Abstract

The production of pharmaceutical proteins in plants is maturing, as shown by the recent approval of innovative products and the latest studies that showcase plant-based production systems using technologies and approaches that are well established in other fields. These include host cell genome engineering, medium optimization, scalable unit operations for downstream processing, bioprocess optimization and detailed cost analysis. Product-specific benefits of plant-based systems have also been exploited, including bioencapsulation and the mucosal delivery of minimally-processed topical and oral products with a lower entry barrier than pharmaceuticals for injection. Early success stories spearheaded by the FDA approval of Elelyso developed by Protalix have revitalized the field and further interest has been fueled by the production of experimental Ebola treatments in plants.

Keywords

recombinant protein; molecular farming; pharmaceutical; platform technology; expression system; commercial production

Introduction

The recent outbreak of Ebola virus disease in western Africa has focused attention on plant-derived pharmaceuticals because the experimental drug ZMapp, a combination of three humanized monoclonal antibodies that recognize an Ebola virus surface glycoprotein, was manufactured by transient expression in *Nicotiana benthamiana* plants by Kentucky BioProcessing under license from Mapp Biopharmaceuticals Inc. [1]. Transient expression is based on the use of *Agrobacterium tumefaciens*, plant viruses, or hybrid vectors with components of both, and exploits the abilities of these plant pathogens to infect plant tissues, spread systemically and/or achieve high protein yields in a short time. Indeed, transient expression systems using fresh leaves can yield grams of recombinant protein within a few weeks, as previously shown for influenza vaccine candidates [2]. This is a convenient niche for products that must be manufactured rapidly in response to an emergency, such as a

*Corresponding author: Eva Stoger, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria. Telephone +43 1 47654 6366, Fax +43 1 47654 6392, eva.stoger@boku.ac.at.

Europe PMC Funders Author Manuscripts

bioterrorist threat or epidemic. Plant-based production systems also have lower upfront investment costs, making them particularly suitable for deployment in developing countries where infrastructure costs present an entry barrier for research, development and manufacturing [3]. ZMapp is a suitable candidate for plant-based production but global manufacturing capacity is still limited. Therefore, existing manufacturing capacity based on mammalian cells will need to be leveraged in parallel.

The outbreak of Ebola virus disease has highlighted not only the potential advantages of plant-based production systems, but also the limited capacity and the lack of defined regulatory pathways for the development of plant-derived pharmaceutical proteins. Only a handful of manufacturing facilities around the world are approved to produce recombinant proteins in plants in accordance with good manufacturing practice (GMP), which is mandatory for pharmaceutical proteins administed to humans. Kentucky BioProcessing (Owensboro, Kentucky, USA), Icon Genetics (Halle, Germany), the Fraunhofer Center for Molecular Biotechnology (Newark, Delaware, USA), the Fraunhofer Institute for Molecular Biology and Applied Ecology (Aachen, Germany) and Medicago/Mitsubishi Tanabe Pharma (Quebec, Canada, and North Carolina, USA) each have facilities approved for the production of GMP-grade proteins in leafy crops such as tobacco. Further facilities are being constructed by Texas A&M University (College Station, Texas, USA) and G-CON. Ventria Bioscience (Fort Collins, Columbia, USA) can manufacture GMP-grade proteins expressed in rice seeds, Protalix Biotherapeutics (Israel) has an approved facility for carrot cell suspension cultures, Synthon/Biolex has a facility approved for duckweed, and in 2014 the Pharmaceutical Affairs and Sanitation Council in Japan awarded manufacturing and marketing approval to the Advanced Industrial Science and Technology (AIST) for interferon alpha produced in strawberries for the prevention of periodontal disease in dogs.

Although tobacco leaves have been adopted widely, the diversity of plant-based systems contrasts with the small number of microbial and animal-cell platforms regarded as industry gold standards. This can be regarded as a strength in terms of innovation for product-specific requirements, but also as a drawback in terms of standardization and regulatory harmonization. The limited number and capacity of GMP-compliant production facilities correlates with the relatively small number of plant-derived pharmaceutical products that are currently on the market or undergoing clinical development (Table 1). However, several companies with downstream processing (DSP) facilities have established commercial platforms for the production of non-pharmaceutical products to generate revenue in the interim without the lengthy and costly regulatory procedures required for clinical studies. The palette ranges from veterinary pharmaceuticals, technical enzymes and research reagents to media ingredients and cosmetic products (Table 2).

Veterinary pharmaceuticals are useful from a developmental perspective because they have a lower regulatory burden compared to human medicines. The demand for veterinary products has also increased because of the One Health Initiative, which aims to reduce the use of antibiotics in livestock production and thus the emergence of antibiotic-resistant and potentially zoonotic pathogens [http://www.onehealthinitiative.com/about.php]. These factors have encouraged the development of cost-effective, efficient and scalable production and delivery platforms for veterinary pharmaceuticals. Plant-based production systems

Europe PMC Funders Author Manuscripts

satisfy these demands, and plant tissues also offer unique opportunities for oral delivery thus removing the need for expensive processing [4–7]. This has resulted in the emergence of novel niche applications for plant-derived recombinant proteins, as discussed in the following sections.

Plant-derived pharmaceuticals for mucosal delivery

Mucosal delivery is beneficial for the administration of both human and veterinary pharmaceuticals and many candidates have been produced in plants to allow the delivery of either unprocessed or partially-processed plant tissues [8]. For example, passive immunization has been confirmed recently by the mucosal delivery of plant-derived antibodies against gastro-intestinal pathogens [9]. Furthermore, active immunity has been promoted by the delivery of plant-derived vaccines to mucosal surfaces, because these induce the production of pathogen-specific secretory IgA (sIgA) at the infection site [10].

Oral delivery is also preferable to daily subcutaneous injections of drugs indicated for autoimmune and inflammatory diseases, including functional peptides such as insulin and autoantigens that induce tolerance. The delivery of plant-derived autoantigens to the gut, the largest organ of the immune system, shows promise for the treatment of such diseases and also for the induction of tolerance to allergies[8,10–15]. Finally, it may even be possible to treat brain diseases by the oral delivery of plant-derived neurotherapeutic proteins fused with the transmucosal cholera toxin B (CTB) subunit, because such fusion proteins have been shown to cross the blood-brain and blood-retinal barriers in a mouse model [16].

One of the drawbacks of oral delivery is that proteins must withstand the harsh conditions in the gastrointestinal tract to reach their effector sites, i.e. the mucosal surface and gut-associated lymphoid tissue. This can be achieved by the encapsulation of drugs in protective coatings, but plants provide a natural counterpart in which proteins are stored within cells or organelles that resist digestion, prolonging the opportunity for interactions with the immune system. Any plant tissue matrix may be suitable for bioencapsulation (Table 3). For example, Protalix Biotherapeutics is exploring the use of lyophilized carrot cells for the oral delivery of taliglucerase alfa in phase II clinical studies (www.protalix.com). Freeze-dried tobacco, Arabidopsis and lettuce cells have also proven effective [8,10]. The plant cell wall is difficult to digest and provides an initial defensive barrier, but even when this is breached proteins can be further protected by ensuring they accumulate in subcellular compartments such as plastids or seed storage organelles, the latter being able to withstand chemical, thermal and enzymatic degradation[17]. This strategy is advantageous for companies and costumers. The use of edible plant tissues allows the protein to be administered as minimally-processed plant material, thereby removing the need for expensive DSP steps, and the pharmaceutical can be stored for prolonged periods at ambient temperatures, e.g. in the form of unmilled grains. Such formulations create new regulatory challenges but these can be addressed by molecular profiling of the production host, including whole-genome sequencing as well as transcriptomic, proteomic and metabolomic analysis [18,19]. It may also be possible to market some products as non-pharmaceutical or health-promoting nutraceutical products, e.g. cereals with hypocholesterinemic activity [20] or improved nutritional properties (see Farré et al., this issue).

Europe PMC Funders Author Manuscripts

Improving product quality and quantity by host cell engineering – new targets and technologies

The economic viability of a production process depends on the yields of the product and this is particularly important for topical applications where higher and more regular dosing schedules are necessary. Many factors affect intrinsic product yields including the control of transgene expression and protein targeting, biological properties of the host and the environment. However, the optimal combination of these factors must be established empirically, often based on high-throughput screening and modeling methods and large-scale statistical experimental designs [21]. This also requires high-throughput cloning techniques for construct optimization [22,23]. The genetic background of the production host is an important factor that can be influenced by crossing, breeding and selection [24], but targeted engineering of the host genome can also be used to reduce the accumulation of endogenous storage proteins, thus providing further capacity for the storage of recombinant proteins [25]. A deeper understanding of recombinant protein storage and protein quality control in the endomembrane system will promote strategies such as the induction of ectopic storage organelles [26–29].

The accumulation of target proteins can be limited by post-translational degradation and product quality can be compromised by proteolysis. Several groups are currently charting the proteolytic activities of plant cells and developing strategies to suppress them [30–34]. The inhibition of specific proteases by host cell engineering may enhance product accumulation, quality and overall recovery. However, these advantages have to be weighed against the potential disadvantages, such as the prohibitive cost of protease inhibitors in large-scale processes and the potential impact on protein quality control if endogenous proteases are inactivated.

The targeted modification of glycosylation pathways has been used to manufacture glycoproteins whose function can be enhanced by specific glycoforms [35]. Further targets for host genome engineering include enzymes involved in modifications other than glycosylation, such as the synthesis of hydroxyproline, which is required for the production of collagen. Future targets may include non-product-related properties such as the polyphenol or fiber content, to reduce the level of contaminants released during DSP. The use of new genome engineering techniques, such as zinc finger and TALE nucleases and the recent CRISPR/Cas9 system, promise to simplify and enhance the development of pharmaceutical crops even further by providing the means to introduce transgenes at permissive sites and achieve homozygosity in one generation (see Hartung et al. and Kamoun et al., this issue).

Improving product quality and quantity by engineering at the protein level

Protein yields can be improved by the addition of stabilizing domains and fusion sequences, although such modifications may not be compatible with clinical applications and the removal of fusion partners during processing can be inefficient and expensive [36]. The formation of protein bodies derived from the endoplasmic reticulum (ER) can be induced in tissues that are not adapted for storage functions (e.g. leaves) by adding polypeptide

Europe PMC Funders Author Manuscripts

sequence tags derived from cereal prolamins, elastin-like polypeptides (ELPs) or fungal hydrophobins, thus increasing the stability of stored proteins and allowing them to remain intact after harvest. Hydrophobins and ELPs can also facilitate protein purification because they allow the solubility of the recombinant protein to be controlled [37–39]. The performance of vaccine candidates can also be enhanced using this strategy, e.g. by expressing virus-like particles (VLPs) or zein bodies as antigen-presenting formulations. Fusion proteins may also function as integrated adjuvants to increase vaccine efficacy at lower costs, e.g. the CTB and zein domains can both act as adjuvants [8,40].

Upstream and downstream processes engineering

Several production and processing facilities for plant-derived pharmaceuticals are now up and running, and technologies established in other fields to promote the transition from development to commercial manufacturing are beginning to emerge in plants and are likely to follow the same trends observed for other platforms such as mammalian cells. In this context, there have been several recent developments in bioprocess engineering [41,42], particularly in terms of medium optimization, standardization and streamlining of development and production, the increasing use of process analytical technologies (PAT) and online monitoring, automation, predictive scale-down models, disposable technologies, continuous production and purification processes and cost models to determine economic feasibility.

Each unit operation within a bioprocess typically has a number of input parameters that affect the desired output parameters of product quantity/quality and process time/cost. PAT can be used to gain a deeper insight into the unit operations required for the development of efficient and robust processes [43] but it also supports the optimization of processes using statistical approaches such as design-of-experiments (DoE) and multivariate analysis (MVA). These are widely used in the development of processes based on microbial and mammalian cells but are now becoming accepted as a strategy to optimize production using plant cells and even intact plants [44].

Scale-down models are important during bioprocess development because the individual upstream and downstream processing steps are interdependent and thus overall process optimization requires integrated analysis which is difficult to achieve at full production scale. Scale-down models are used to support the integrated analysis of multiple consecutive processing steps, which in the case of plants must include growth, development and harvesting as well as the traditional extraction, processing and purification stages [45,46]. One of the impacts of modeling is that it enables the costs of manufacturing to be evaluated with greater accuracy, which is necessary for the translation of laboratory processes into a commercial environment. Several costing studies have been reported recently, including those based on top-down analysis [47] and bottom-up approaches [48,49].

Conclusions and outlook

Plants have several key advantages for the production of recombinant pharmaceutical proteins in niche markets, including rapid production and scalability, the ability to produce

unique glycoforms, and the intrinsic safety of food and feed crops which allows the delivery of topical and mucosal products in unprocessed or partially processed tissues. Several recent reports discuss favorable head-to-head comparisons with plants outperforming other platforms [50–52]. These factors are drawing the attention of the pharmaceutical industry and a second wave of pharmaceutical products is now progressing through the clinical development pipeline.

The outlook for molecular farming is favorable because several alternative paths are available for product development. In addition to the typical ‘produce and purify’ route for biopharmaceutical products, there is also the potential for the oral administration of whole tissues, the topical or oral administration of partially-purified tissues such as flour pastes and juices, the topical use of crude extracts that are more similar to herbal products than conventional pharmaceuticals and may even constitute a new product category, and the combination of traditional medicinal plants with biotechnology-derived pharmaceutical extracts. As the interest in such products increases, we are likely to see more large-scale production facilities therefore increasing the global production capacity. With the rise of disposable equipment for DSP, it is even possible that inexpensive production infrastructure can be installed in developing countries for the rapid production of plant-based pharmaceutical products in the region for the region. The construction of further manufacturing facilities will allow more accurate cost analysis at larger scales, providing the information necessary to investigate the feasibility of pharmaceutical production on the scale of commodity products.

Acknowledgements

The authors would like to acknowledge financial support by the Austrian Science Fund FWF (I1103).

Abbreviations

GMP	good manufacturing practice
DSP	downstream processing
DoE	design-of-experiments
PAT	process analytical technologies

References

1. Qiu X, Wong G, Audet J, Bello A, Fernando L, Alimonti JB, Fausther-Bovendo H, Wei H, Aviles J, Hiatt E, et al. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. Nature. 2014; 514:47–53. [PubMed: 25171469]
2. D'Aoust MA, Couture MM, Charland N, Trepanier S, Landry N, Ors F, Vezina LP. The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. Plant Biotechnol J. 2010; 8:607–619. [PubMed: 20199612]
3. Vamvaka E, Twyman RM, Christou P, Capell T. Can plant biotechnology help break the HIV-malaria link? Biotechnol Adv. 2014; 32:575–582. [PubMed: 24607600]
4. Duvenage L, Hitzeroth II, Meyers AE, Rybicki EP. Expression in tobacco and purification of beak and feather disease virus capsid protein fused to elastin-like polypeptides. J Virol Methods. 2013; 191:55–62. [PubMed: 23578703]

5. Kolotilin I, Kaldis A, Devriendt B, Joensuu J, Cox E, Menassa R. Production of a subunit vaccine candidate against porcine post-weaning diarrhea in high-biomass transplastomic tobacco. PLoS One. 2012; 7:e42405. [PubMed: 22879967]
6. Virdi V, Coddens A, De Buck S, Millet S, Goddeeris BM, Cox E, De Greve H, Depicker A. Orally fed seeds producing designer IgAs protect weaned piglets against enterotoxigenic Escherichia coli infection. Proc Natl Acad Sci U S A. 2013; 110:11809–11814. [PubMed: 23801763]
7. Clarke JL, Waheed MT, Lossl AG, Martinussen I, Daniell H. How can plant genetic engineering contribute to cost-effective fish vaccine development for promoting sustainable aquaculture? Plant Mol Biol. 2013; 83:33–40. [PubMed: 23729352]
8. Kwon KC, Verma D, Singh ND, Herzog R, Daniell H. Oral delivery of human biopharmaceuticals, autoantigens and vaccine antigens bioencapsulated in plant cells. Adv Drug Deliv Rev. 2013; 65:782–799. [PubMed: 23099275]
9. Virdi V, Depicker A. Role of plant expression systems in antibody production for passive immunization. Int J Dev Biol. 2013; 57:587–593. [PubMed: 24166441]
10. Lee G, Na YJ, Yang BG, Choi JP, Seo YB, Hong CP, Yun CH, Kim DH, Sohn EJ, Kim JH, et al. Oral immunization of haemaggulutinin H5 expressed in plant endoplasmic reticulum with adjuvant saponin protects mice against highly pathogenic avian influenza A virus infection. Plant Biotechnol J. 2014
11. Lakshmi PS, Verma D, Yang X, Lloyd B, Daniell H. Low cost tuberculosis vaccine antigens in capsules: expression in chloroplasts, bio-encapsulation, stability and functional evaluation in vitro. PLoS One. 2013; 8:e54708. [PubMed: 23355891]
12. Shenoy V, Kwon KC, Rathinasabapathy A, Lin S, Jin G, Song C, Shil P, Nair A, Qi Y, Li Q, et al. Oral Delivery of Angiotensin-Converting Enzyme 2 and Angiotensin-(1-7) Bioencapsulated in Plant Cells Attenuates Pulmonary Hypertension. Hypertension. 2014
13. Sherman A, Su J, Lin S, Wang X, Herzog RW, Daniell H. Suppression of inhibitor formation against FVIII in a murine model of hemophilia A by oral delivery of antigens bioencapsulated in plant cells. Blood. 2014; 124:1659–1668. [PubMed: 24825864]
14. Shil PK, Kwon KC, Zhu P, Verma A, Daniell H, Li Q. Oral Delivery of ACE2/Ang-(1-7) Bioencapsulated in Plant Cells Protects against Experimental Uveitis and Autoimmune Uveoretinitis. Mol Ther. 2014
15. Iizuka M, Wakasa Y, Tsuboi H, Asashima H, Hirota T, Kondo Y, Matsumoto I, Takaiwa F, Sumida T. Suppression of collagen-induced arthritis by oral administration of transgenic rice seeds expressing altered peptide ligands of type II collagen. Plant Biotechnol J. 2014; 12:1143–1152. [PubMed: 24989432]
16. Kohli N, Westerveld DR, Ayache AC, Verma A, Shil P, Prasad T, Zhu P, Chan SL, Li Q, Daniell H. Oral delivery of bioencapsulated proteins across blood-brain and blood-retinal barriers. Mol Ther. 2014; 22:535–546. [PubMed: 24281246]
17. Saeki M, Nishimura T, Kaminuma O, Mori A, Hiroi T. Oral immunotherapy for allergic diseases using transgenic rice seeds: current state and future prospects. Int Arch Allergy Immunol. 2013; 161(Suppl 2):164–169. [PubMed: 23711869]
18. Kawakatsu T, Kawahara Y, Itoh T, Takaiwa F. A whole-genome analysis of a transgenic rice seed-based edible vaccine against cedar pollen allergy. DNA Res. 2013; 20:623–631. [PubMed: 23956243]
19. Juarez P, Fernandez-del-Carmen A, Rambla JL, Presa S, Mico A, Granell A, Orzaez D. Evaluation of unintended effects in the composition of tomatoes expressing a human immunoglobulin A against rotavirus. J Agric Food Chem. 2014; 62:8158–8168. [PubMed: 25065456]
20. Cabanos C, Kato N, Amari Y, Fujiwara K, Ohno T, Shimizu K, Goto T, Shimada M, Kuroda M, Masuda T, et al. Development of a novel transgenic rice with hypocholesterolemic activity via high-level accumulation of the alpha' subunit of soybean beta-conglycinin. Transgenic Res. 2014; 23:609–620. [PubMed: 24676962]
21. Vasilev N, Gromping U, Lipperts A, Raven N, Fischer R, Schillberg S. Optimization of BY-2 cell suspension culture medium for the production of a human antibody using a combination of fractional factorial designs and the response surface method. Plant Biotechnol J. 2013; 11:867–874. [PubMed: 23721307]

22. Engler C, Youles M, Gruetzner R, Ehnert TM, Werner S, Jones JD, Patron NJ, Marillonnet S. A Golden Gate Modular Cloning Toolbox for Plants. ACS Synth Biol. 2014
23. Sarrion-Perdigones A, Palaci J, Granell A, Orzaez D. Design and construction of multigenic constructs for plant biotechnology using the GoldenBraid cloning strategy. Methods Mol Biol. 2014; 1116:133–151. [PubMed: 24395362]
24. Hood EE, Devaiah SP, Fake G, Egelkrout E, Teoh KT, Requesens DV, Hayden C, Hood KR, Pappu KM, Carroll J, et al. Manipulating corn germplasm to increase recombinant protein accumulation. Plant Biotechnol J. 2012; 10:20–30. [PubMed: 21627759]
25. Takaiwa F. Increasing the production yield of recombinant protein in transgenic seeds by expanding the deposition space within the intracellular compartment. Bioengineered. 2013; 4:136–139. [PubMed: 23563599]
26. De Meyer T, Depicker A. Trafficking of endoplasmic reticulum-retained recombinant proteins is unpredictable in Arabidopsis thaliana. Front Plant Sci. 2014; 5:473. [PubMed: 25309564]
27. Kudo K, Ohta M, Yang L, Wakasa Y, Takahashi S, Takaiwa F. ER stress response induced by the production of human IL-7 in rice endosperm cells. Plant Mol Biol. 2013; 81:461–475. [PubMed: 23371559]
28. Feeney M, Frigerio L, Kohalmi SE, Cui Y, Menassa R. Reprogramming cells to study vacuolar development. Front Plant Sci. 2013; 4:493. [PubMed: 24348496]
29. Arcalis E, Ibl V, Peters J, Melnik S, Stoger E. The dynamic behavior of storage organelles in developing cereal seeds and its impact on the production of recombinant proteins. Front Plant Sci. 2014; 5:439. [PubMed: 25232360]
30. Niemer M, Mehofer U, Torres Acosta JA, Verdianz M, Henkel T, Loos A, Strasser R, Maresch D, Rademacher T, Steinkellner H, et al. The human anti-HIV antibodies 2F5, 2G12, and PG9 differ in their susceptibility to proteolytic degradation: down-regulation of endogenous serine and cysteine proteinase activities could improve antibody production in plant-based expression platforms. Biotechnol J. 2014; 9:493–500. [PubMed: 24478053]
31. Mandal MK, Fischer R, Schillberg S, Schiermeyer A. Inhibition of protease activity by antisense RNA improves recombinant protein production in Nicotiana tabacum cv. Bright Yellow 2 (BY-2) suspension cells. Biotechnol J. 2014; 9:1065–1073. [PubMed: 24828029]
32. Robert S, Khalf M, Goulet MC, D'Aoust MA, Sainsbury F, Michaud D. Protection of recombinant mammalian antibodies from development-dependent proteolysis in leaves of Nicotiana benthamiana. PLoS One. 2013; 8:e70203. [PubMed: 23894618]
33. Goulet C, Khalf M, Sainsbury F, D'Aoust MA, Michaud D. A protease activity-depleted environment for heterologous proteins migrating towards the leaf cell apoplast. Plant Biotechnol J. 2012; 10:83–94. [PubMed: 21895943]
34. Pillay P, Schluter U, van Wyk S, Kunert KJ, Vorster BJ. Proteolysis of recombinant proteins in bioengineered plant cells. Bioengineered. 2014; 5:15–20. [PubMed: 23778319]
35. Strasser R, Altmann F, Steinkellner H. Controlled glycosylation of plant-produced recombinant proteins. Curr Opin Biotechnol. 2014; 30C:95–100.
36. Piron R, De Koker S, De Paepe A, Goossens J, Grooten J, Nauwynck H, Depicker A. Boosting in planta production of antigens derived from the porcine reproductive and respiratory syndrome virus (PRRSV) and subsequent evaluation of their immunogenicity. PLoS One. 2014; 9:e91386. [PubMed: 24614617]
37. Phan HT, Hause B, Hause G, Arcalis E, Stoger E, Maresch D, Altmann F, Joensuu J, Conrad U. Influence of elastin-like polypeptide and hydrophobin on recombinant hemagglutinin accumulations in transgenic tobacco plants. PLoS One. 2014; 9:e99347. [PubMed: 24914995]
38. Gutierrez SP, Saberianfar R, Kohalmi SE, Menassa R. Protein body formation in stable transgenic tobacco expressing elastin-like polypeptide and hydrophobin fusion proteins. BMC Biotechnol. 2013; 13:40. [PubMed: 23663656]
39. Reuter LJ, Bailey MJ, Joensuu JJ, Ritala A. Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells. Plant Biotechnol J. 2014; 12:402–410. [PubMed: 24341724]

40. Whitehead M, Ohlschläger P, Almajhdi FN, Alloza L, Marzábal P, Meyers AE, Hitzeroth II, Rybicki EP. Human papillomavirus (HPV) type 16 E7 protein bodies cause tumour regression in mice. BMC Cancer. 2014; 14:367. [PubMed: 24885328]
41. Vazquez-Villegas P, Espitia-Saloma E, Rito-Palomares M, Aguilar O. Low-abundant protein extraction from complex protein sample using a novel continuous aqueous two-phase systems device. J Sep Sci. 2013; 36:391–399. [PubMed: 23255256]
42. Gecchele E, Schillberg S, Merlin M, Pezzotti M, Avesani L. A downstream process allowing the efficient isolation of a recombinant amphiphilic protein from tobacco leaves. J Chromatogr B Analyt Technol Biomed Life Sci. 2014; 960:34–42.
43. Holland T, Blessing D, Hellwig S, Sack M. The in-line measurement of plant cell biomass using radio frequency impedance spectroscopy as a component of process analytical technology. Biotechnol J. 2013; 8:1231–1240. [PubMed: 24039008]
44. Buyel JF, Fischer R. Characterization of complex systems using the design of experiments approach: transient protein expression in tobacco as a case study. J Vis Exp. 2014:e51216. [PubMed: 24514765]
45. Hassan S, Keshavarz-Moore E, Ma J, Thomas C. Breakage of transgenic tobacco roots for monoclonal antibody release in an ultra-scale down shearing device. Biotechnol Bioeng. 2014; 111:196–201. [PubMed: 23860965]
46. Buyel JF, Fischer R. Scale-down models to optimize a filter train for the downstream purification of recombinant pharmaceutical proteins produced in tobacco leaves. Biotechnol J. 2014; 9:415–425. [PubMed: 24323869]
47. Tuse D, Tu T, McDonald KA. Manufacturing economics of plant-made biologics: case studies in therapeutic and industrial enzymes. Biomed Res Int. 2014; 2014:256135. [PubMed: 24977145]
48. Walwyn DR, Huddy SM, Rybicki EP. Techno-Economic Analysis of Horseradish Peroxidase Production Using a Transient Expression System in Nicotiana benthamiana. Appl Biochem Biotechnol. 2014
49. Buyel JF, Kaever T, Buyel JJ, Fischer R. Predictive models for the accumulation of a fluorescent marker protein in tobacco leaves according to the promoter/5'UTR combination. Biotechnol Bioeng. 2013; 110:471–482. [PubMed: 22948957]
50. Avesani L, Merlin M, Gecchele E, Capaldi S, Brozzetti A, Falorni A, Pezzotti M. Comparative analysis of different biofactories for the production of a major diabetes autoantigen. Transgenic Res. 2014; 23:281–291. [PubMed: 24142387]
51. Merlin M, Gecchele E, Capaldi S, Pezzotti M, Avesani L. Comparative evaluation of recombinant protein production in different biofactories: the green perspective. Biomed Res Int. 2014; 2014:136419. [PubMed: 24745008]
52. Baeshen NA, Baeshen MN, Sheikh A, Bora RS, Ahmed M, Ramadan HI, Saini K, Redwan EM. Cell factories for insulin production. Microb Cell Fact. 2014; 13:141. [PubMed: 25270715]
53. Pastores GM, Petakov M, Giraldo P, Rosenbaum H, Szer J, Deegan PB, Amato DJ, Mengel E, Tan ES, Chertkoff R, et al. 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. 2014; 53:253–260. [PubMed: 24950666]
54. Magnusdottir A, Vidarsson H, Bjornsson JM, Orvar BL. Barley grains for the production of endotoxin-free growth factors. Trends Biotechnol. 2013; 31:572–580. [PubMed: 23849675]
55. Alfano R, Youngblood BA, Zhang D, Huang N, MacDonald CC. Human leukemia inhibitory factor produced by the ExpressTec method from rice (Oryza sativa L.) is active in human neural stem cells and mouse induced pluripotent stem cells. Bioengineered. 2014; 5:180–185. [PubMed: 24776984]
56. Youngblood BA, Alfano R, Pettit SC, Zhang D, Dallmann HG, Huang N, Macdonald CC. Application of recombinant human leukemia inhibitory factor (LIF) produced in rice (Oryza sativa L.) for maintenance of mouse embryonic stem cells. J Biotechnol. 2014; 172:67–72. [PubMed: 24380819]
57. Wakasa Y, Takagi H, Hirose S, Yang L, Saeki M, Nishimura T, Kaminuma O, Hiroi T, Takaiwa F. Oral immunotherapy with transgenic rice seed containing destructed Japanese cedar pollen

allergens, Cry j 1 and Cry j 2, against Japanese cedar pollinosis. Plant Biotechnol J. 2013; 11:66–76. [PubMed: 23066780]

58. Juarez P, Presa S, Espi J, Pineda B, Anton MT, Moreno V, Buesa J, Granell A, Orzaez D. Neutralizing antibodies against rotavirus produced in transgenically labelled purple tomatoes. Plant Biotechnol J. 2012; 10:341–352. [PubMed: 22070155]
59. Lindh I, Bråve A, Hallengärd D, Hadad R, Kalbina I, Strid Å, Andersson S. Oral delivery of plant-derived HIV-1 p24 antigen in low doses shows a superior priming effect in mice compared to high doses. Vaccine. 2014; 32:2288–2293. [PubMed: 24631072]
60. Karimi F, Mousavi A, Salmanian A, Alizadeh H, Rafati S. Immunogenicity of EIT chimeric protein expressed in transplastomic tobacco plants towards development of an oral vaccine against Escherichia coli O157:H7. Plant Biotechnology Reports. 2013; 7:535–546.
61. Kwon KC, Nityanandam R, New JS, Daniell H. Oral delivery of bioencapsulated exendin-4 expressed in chloroplasts lowers blood glucose level in mice and stimulates insulin secretion in beta-TC6 cells. Plant Biotechnol J. 2013; 11:77–86. [PubMed: 23078126]
62. Choi J, Diao H, Feng ZC, Lau A, Wang R, Jevnikar AM, Ma S. A fusion protein derived from plants holds promising potential as a new oral therapy for type 2 diabetes. Plant Biotechnol J. 2014; 12:425–435. [PubMed: 24373324]
63. Chan HT, Chia MY, Pang VF, Jeng CR, Do YY, Huang PL. Oral immunogenicity of porcine reproductive and respiratory syndrome virus antigen expressed in transgenic banana. Plant Biotechnol J. 2013; 11:315–324. [PubMed: 23116484]
64. Tokuhara D, Álvarez B, Mejima M, Hiroiwa T, Takahashi Y, Kurokawa S, Kuroda M, Oyama M, Kozuka-Hata H, Nochi T, et al. Rice-based oral antibody fragment prophylaxis and therapy against rotavirus infection. J Clin Invest. 2013; 123:3829–3838. [PubMed: 23925294]
65. Pepponi I, Diogo GR, Stylianou E, van Dolleweerd CJ, Drake PM, Paul MJ, Sibley L, Ma JK, Reljic R. Plant-derived recombinant immune complexes as self-adjuvanting TB immunogens for mucosal boosting of BCG. Plant Biotechnol J. 2014; 12:840–850. [PubMed: 24629003]
66. Gorantala J, Grover S, Rahi A, Chaudhary P, Rajwanshi R, Sarin NB, Bhatnagar R. Generation of protective immune response against anthrax by oral immunization with protective antigen plant-based vaccine. J Biotechnol. 2014; 176:1–10. [PubMed: 24548460]
67. Mathew LG, Herbst-Kralovetz MM, Mason HS. Norovirus Narita 104 virus-like particles expressed in Nicotiana benthamiana induce serum and mucosal immune responses. Biomed Res Int. 2014; 2014:807539. [PubMed: 24949472]
68. Suzuki K, Kaminuma O, Yang L, Takai T, Mori A, Umezu-Goto M, Ohtomo T, Ohmachi Y, Noda Y, Hirose S, et al. Prevention of allergic asthma by vaccination with transgenic rice seed expressing mite allergen: induction of allergen-specific oral tolerance without bystander suppression. Plant Biotechnol J. 2011; 9:982–990. [PubMed: 21447056]
69. Hongli L, Xukui L, Ting L, Wensheng L, Lusheng S, Jin Z. Transgenic tobacco expressed HPV16-L1 and LT-B combined immunization induces strong mucosal and systemic immune responses in mice. Hum Vaccin Immunother. 2013; 9:83–89. [PubMed: 23108357]

Table 1
Examples of plant-derived pharmaceutical products assessed in clinical studies

Company	Products	Main Application	Current Status
Protalix	Elelyso	Gaucher's ERT	FDA-approved for the US, but not for Europe
	PRX-102 (alpha galactosidase)	Fabry ERT	Phase I/II
	PRX-12 (oral glucocerebrosidase)	Gaucher's ERT	Phase II
Ventria	VEN100(lactoferrin)	Antibiotic-associated diarrhea, anti-inflammatory	Phase II
	VEN120	Inflammatory bowel disease	Phase I
	VEN130	Osteoporosis	Phase I
Biolex (now Synthon)	Locteron™	HCV	Phase II / IIb
Icon Genetics	NHL vaccine MAPP66	HSV/HIV	Phase I/II Phase I
Medicago	H5	Pandemic influenza vaccine	Phase II/III, approved for emergency use
	H5 intradermal		Phase I
	Seasonal influenza vaccine		Phase I
Planet Biotechnology	CaroRX	Anti-caries antibody	Approved as medical device
Fraunhofer IME	HIV Antibody	Microbicide	Phase I
Fraunhofer CMB	HA vaccine	Vaccine	Phase I
VAXX/ Arizona State University	NoroVAXX	Vaccine	Phase I
MAPP	ZMapp	Ebola antibody cocktail	Emergency use Phase I expected soon
Greenovation		Fabry ERT	Scheduled for Phase I

Europe PMC Funders Author Manuscripts

Table 2

Commercially available plant-produced recombinant proteins

Product	Company	Plant system	Application	Advantage	Reference
Elelyso	Protalix	Carrot suspension	Injectable pharmaceutical	Plant-specific glycosylation	www.protalix.com, [53]
Growth factors, cytokines	ORF	Barley seeds	Cell culture supplement	Endotoxin-free	[54]
Growth factors	ORF/SifCosmetics	Barley seeds	Cosmetic ingredient	Animal-free	www.orfgenetics.com
Growth factors, cytokines, antibodies	AgrenVec	Tobacco leaves transient	Research Reagent	Animal-free, Costs	http://www.agrenvec.com
Albumin, transferrin, lactoferrin, lysozyme	Ventria/InVitria	Rice seeds	Cell culture supplement	Animal-free	www.ventria.com/ www.invitria.com/ [55,56]
Aprotinin (non clinical grade)	KBP	Tobacco leaves transient	Research reagent	Cost	
Laccase, trypsin, avidin	ProdiGene/Sigma	Corn seeds	Technical reagent	Cost	www.sigmaaldrich.com
Canine interferon alpha	NAIST	Strawberry fruits	Veterinary pharmaceutical/oral	Cost, minimal processing	
Cellobiohydrolase I	Infinite Enzymes	Corn seeds	Technical enzyme	Cost	www.infiniteenzymes.com/
Antibody	CIGB	Transgenic tobacco	Used for the purification of a HBV vaccine	Cost, animal-free	
Collagen	CollPlant	Transgenic tobacco	Tissue culture, pharmaceutical applications are envisaged	Animal free, "virgin“ collagen	http://www.collplant.com/
Growth factors, cytokines, antibodies	NBM	Rice cell suspension	Bioreagents, Cosmetic ingredients	Animal-free, endotoxin-free	http://www.nbms.co.kr/

Europe PMC Funders Author Manuscripts

Table 3

Recent examples of plant-produced proteins for topical application

Target/product	Application area	Plant species/organ	Encapsulation	Processing degree	Delivery	Reference
Factor VIII antigens	Hemophilia A	Tobacco leaf	Chloroplasts	Homogenized leaf	Oral	[13]
Cry 1 and Cry 2	Cedar pollen allergens	Rice seeds	Protein bodies	Rice grains	Oral	[57]
IgA	Rotavirus	Tomato fruit	Matrix	Fruit derived formulations	Oral	[58]
Angiotensin (ACE2 and Ang 1-7)	Hypertension	Tobacco leaf	Chloroplasts	Lyophilized leaf	Oral	[12]
ACE2 and Ang (1-7)	Uveitis and autoimmune uveoretinitis	Tobacco leaf	Chloroplasts	Leaf powder	Oral	[14]
Antibody 2G12	Immunodeficiency syndrome	Tobacco leaf	None	Purified	Vaginal/mucosal	www.pharma-planta.net
Designer IgA	Enterotoxigenic E. coli infection	Arabidopsis seeds	Seed matrix	Seed	Oral	[6]
Interferon alpha	Periodontal disease	Strawberry fruits	Matrix	Lyophilized fruits	Oral	
HIV-1 p24	Immunodeficiency syndrome	Arabidopsis thaliana and carrot	ER	Fresh and freeze-dried	Oral	[59]
EIT (EHEC)	Hemorrhagic colitis and hemolytic-uremic syndrome	Tobacco leaf	Chloroplast	Freez-dried	Oral	[60]
Exendin-4	Diabetis type 2	Tobacco leaf	Chloroplasts	Lyophilized	Oral	[61]
Exendin-4 (fused with transferrin)	Diabetis type 2	*N. benthamiana* leaf	ER	Partially purified	Oral	[62]
PRRSV envelope glycoprotein	Porcine reproductive and respiratory syndrome virus	Banana leaf	ER	Fresh leaf	Oral	[63]
Typ II collagen (CII256-271 and APL6)	Rhemathoid arthritis	Rice seed	Storage vacuoles	Milled seeds	Oral	[15]
mucoRice-ARP1	Rotavirus-induced diarrhea	Rice seed	PBII	Milled seeds	Oral	[64]
H5	Influenza	arabidopsis	ER	Freeze-dried	Oral	[10]
TB-RICs	Tuberculosis vaccine	Tobacco leaf	None	Purified	Intranasal booster	[65]
Protective antigen	Anthrax vaccine	Tobacco and brassica leaf	Chloroplasts	Fresh leaves	Oral	[66]
Norovirus Narita 104 virus like particles	Gastroenteritis	*N. benthamiana* leaf	VLPs	Purified	Intranasal	[67]
Alpha subunit of soybean beta-conglycinin	Hypercholesterolemia	Rice seed	Storage vacuoles	Ground seed preparation	Oral	[20]
Der p1 Tg rice	Bronchial asthma, allergic rhinitis and atopic dermatitis	Rice seed	Protein bodies	Seeds	Oral	[68]
HPV16-L1 and LT-B	Cervical cancer vaccine	Tobacco leaf	ER	Purified	Oral	[69]
Butyryl Choline Esterase (BchE)	Protection against neurotoxic chemicals	*N.bentamiana* leaf	None	Sabilized plant extract	Spray For inhalation	PlantVax.Inc, Rockville