Plant Molecular Farming: Much More than Medicines

Marc Tschofen,[1] Dietmar Knopp,[2] Elizabeth Hood,[3] and Eva Stöger[1]

[1]Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, 1190 Vienna, Austria; email: eva.stoeger@boku.ac.at

[2]Institute of Hydrochemistry, Chair for Analytical Chemistry, Technische Universität München, 80333 Munich, Germany

[3]Arkansas State University Biosciences Institute, Jonesboro, Arkansas 72467

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Abstract

Plants have emerged as commercially relevant production systems for pharmaceutical and nonpharmaceutical products. Currently, the commercially available nonpharmaceutical products outnumber the medical products of plant molecular farming, reflecting the shorter development times and lower regulatory burden of the former. Nonpharmaceutical products benefit more from the low costs and greater scalability of plant production systems without incurring the high costs associated with downstream processing and purification of pharmaceuticals. In this review, we explore the areas where plant-based manufacturing can make the greatest impact, focusing on commercialized products such as antibodies, enzymes, and growth factors that are used as research-grade or diagnostic reagents, cosmetic ingredients, and biosensors or biocatalysts. An outlook is provided on high-volume, low-margin proteins such as industrial enzymes that can be applied as crude extracts or unprocessed plant tissues in the feed, biofuel, and papermaking industries.

INTRODUCTION

Molecular farming is the production of recombinant proteins in plants with the intention to use the protein itself as the product, in purified form, crude extracts, or in planta, rather than seeking a change in phenotype, performance, or metabolism. The first examples of molecular farming involved the production of an antibody and human serum albumin in transgenic plants and plant cell suspension cultures (1, 2). This led to the rapid exploration of many different plant species as hosts for the production of recombinant pharmaceutical proteins, a field sometimes described as molecular pharming (3, 4). Many proof-of-principle studies were published in the following years, but the breakthrough to commercial success only came once a defined regulatory framework was accepted for plant-derived biologics, culminating in 2012 with the approval of taliglucerase alfa, a recombinant form of human glucocerebrosidase developed by Protalix Biotherapeutics for the treatment of the lysosomal storage disorder Gaucher's disease (5).

Although pharming applications have taken the limelight, another quieter revolution has been under way in the area of plant-derived nonpharmaceutical proteins, which were first successfully commercialized by the US biotechnology company ProdiGene, Inc., in the late 1990s (6). Other companies have since taken up this mantle, including those with a clinical development program, in the realization that nonpharmaceutical products can reach the market more quickly because of the much lower regulatory burden. The product portfolio ranges from technical enzymes and research-grade reagents to cosmetic products (3, 7). There are more nonpharmaceutical products already on the market than there are pharmaceutical proteins undergoing clinical development (**Table 1**). Veterinary products produced in plants are also gaining attention, reflecting imminent regulatory changes enforcing the reduction of antibiotic use in food and dairy animals (8–10).

The benefits of molecular farming for pharmaceutical products are often described in terms of costs, scalability, and safety. Nonpharmaceutical products also benefit from the low costs and greater scalability of plants, but the benefits are magnified because downstream processing and purification does not have to meet the strict criteria enforced for pharmaceutical good manufacturing practice (GMP). A number of molecular farming products, including cell culture components, feed/food supplements, and cosmetic ingredients, attract a premium because the manufacturers can claim animal- and endotoxin-free production. There are also products not needing to be purified, such as enzymes that conditionally digest lignocellulose, that can be used to facilitate papermaking, biofuel production, and the manufacture of animal feed by avoiding the need for expensive additives and environmentally damaging pretreatment processes.

In this review, we discuss the nonpharmaceutical products of molecular farming in more detail, focusing on commercialized products such as antibodies, enzymes, and growth factors that are used as research-grade or diagnostic reagents, cosmetic ingredients, biosensors, and biocatalysts (including the conversion of plant biomass into sugars) and to facilitate bioremediation. Most commercial nonpharmaceutical products of molecular farming are currently produced on a small to medium scale, making it possible to rely on contained growing facilities rather than field cultivation, which attracts additional regulatory scrutiny. However, for low-margin products with a large potential market, the full potential of molecular farming will only be realized if large-scale production can be achieved.

THE BENEFITS OF PLANTS AS EXPRESSION HOSTS

The benefits offered by plants as expression hosts are highlighted in several recent reports containing favorable head-to-head comparisons with other platforms (11–13). One of the key advantages of all plant-based systems is that plants are much less expensive than mammalian cells but have

Table 1 Commercial development of nonpharmaceutical proteins produced in plants

Product	Company	Application	Plant species	Development stage	Country	Processing degree	Advantage	Source
Trypsin, avidin, endo-1,4-β-D-glucanase	ProdiGene/ Sigma-Aldrich	Technical reagents	Maize seeds	Commercialized	United States	Purified	Cost, animal-free	**http://www.sigmaaldrich.com**
Cellobiohydrolase I	Infinite Enzymes/ Sigma-Aldrich	Technical reagent	Maize seeds	Commercialized	United States	Purified	Cost, integrated production	**http:///www.sigmaaldrich.com**
Growth factors, cytokines, thioredoxin, TIMP-2	Agrenvec	Research reagents	Tobacco leaves, transient	Commercialized	Spain	Purified	Cost, animal-free	**http://www.agrenvec.com**
Growth factors, cytokines	ORF Genetics	Research reagent	Barley seeds	Commercialized	Iceland	Purified	Cost, animal-free	**http://www.orfgenetics.com**
Epithelial growth factor	Sif Cosmetics	Cosmetics	Barley seeds	Commercialized	Iceland	Purified	Cost, animal-free	**http://www.sifcosmetics.com**
Albumin, lactoferrin, lysozyme, transferrin, insulin	Ventria Bioscience/ InVitria	Research reagents	Rice seeds	Commercialized	United States	Purified	Cost, animal-free	**http://www.invitria.com**
Aprotinin	Kentucky Bio-Processing	Research reagent	Tobacco leaves, transient	Commercialized	United States	Purified	Cost	**http://www.kbpllc.com**
Collagen	CollPlant	Research reagent, tissue culture, health applications	Transgenic tobacco	Commercialized	Israel	Purified	Cost, animal-free	**http://www.collplant.com**

(*Continued*)

Table 1 (*Continued*)

Product	Company	Application	Plant species	Development stage	Country	Processing degree	Advantage	Source
Trypsin, enterokinase, growth factors, cytokines	Natural Bio-Materials	Research reagents, cosmetic ingredients	Rice cell suspension	Commercialized	South Korea	Purified	Cost, animal-free	**http://www.nbms.co.kr**
Antibody	Center for Genetic Engineering and Biotechnology	Purification of a hepatitis B vaccine	Transgenic tobacco	Commercial application	Cuba	Purified	Cost	**http://gndp.cigb.edu.cu**
α-Amylase	Syngenta	Bioethanol production	Maize seeds	Commercialized	United States	Biomass extract	Cost, integrated production	**http://www.syngenta.com**
Phytase	Origin Agritech	Feed	Maize seeds	Commercialization pending	China	Delivered in biomass	Increased mineral availability, integrated production	**http://www.originseed.com.cn**
Growth factors	NexGen	Tissue culture reagent	Tobacco leaves, transient	Commercialized	South Korea	Purified	Cost, animal-free	**http://www.nexgen.com**

a similar secretory pathway. This allows them to fold and assemble complex proteins efficiently due to the presence of chaperones and protein disulfide isomerases that catalyze the formation of disulfide bonds, a capability not shared by bacterial production systems. This has proved invaluable for the production of pharmaceuticals, particularly multimeric proteins such as antibodies but also complex technical proteins such as collagen and spider silk (14–17). The secretory pathway is also where posttranslational modifications (PTMs) are carried out, including glycosylation, γ-carboxylation, β-hydroxylation, amidation, proline hydroxylation, and sulfation (18, 19). Glycosylation has received the most attention because there are differences in N-glycan and O-glycan structures between plants and mammals, and even between different plant and mammalian species, which can affect protein structure, biological activity, and stability when the protein is injected as a drug (20, 21). The precise control of glycosylation has allowed the production of plant-derived glycoproteins with humanlike or human-compatible glycans, as well as biobetters in which the glycan profiles have been tweaked to improve efficacy or longevity, or to simplify downstream processing (5, 22, 23). Glycosylation is less relevant for nonpharmaceutical proteins, but the other forms of modification listed above are necessary for some products to assemble properly; for example, proline hydroxylation is required for the assembly of collagen, and this capability can be conferred on plants by genetic engineering of the production host (17).

OPTIMIZING THE YIELDS OF RECOMBINANT PROTEINS IN PLANTS

As with all expression systems, the yields of any given product of molecular farming are not predictable, because this depends on a combination of the intrinsic properties of the protein, the host, and the production strategy. Standardized approaches have been developed to improve the expression construct and this can be combined with various forms of strain and process optimization to ensure the greatest synergy between the host and its environment (24, 25). Strain optimization strategies are platform-specific. Plant cell suspension cultures can be improved by high-throughput screening to identify the most productive cells and use them to produce high-yielding monoclonal cell lines (26). In contrast, the yields in whole plants can be increased by breeding and selection among the best performing primary transformants, which identifies those with stable transgenes at permissive integration sites and pairs them with the optimal genetic background (27, 28). Process optimization strategies are similarly diverse. In cell cultures, this involves medium optimization and the testing of different bioreactor designs and process parameters, which has been accelerated recently by the use of statistical experimental designs to test multiple parameters simultaneously (29). For transgenic plants and transient expression platforms, the environment of the plant can have a substantial impact; for example, differences in temperature of only 1–2°C can change the yields of a recombinant protein by up to 15% (30). The combination of good construct design, optimal genetic background, and a supportive environment has achieved extraordinary yields of up to 10.6% of the total soluble protein (TSP) for human serum albumin expressed in rice seeds (31), 30% TSP for industrial enzymes expressed in maize seeds (32), 36% TSP for a murine antibody expressed in *Arabidopsis* (33), and more than 70% TSP for proteins expressed in tobacco chloroplasts (34).

DIFFERENT SYSTEMS FOR DIFFERENT APPLICATIONS

The biopharmaceutical industry has consolidated around a small number of microbes and mammalian cell types grown in fermenters, but there is much more diversity in molecular farming, where different platforms offer overlapping and complementary benefits. Three major strategies

GENE TRANSFER STRATEGIES

Molecular farming involves several distinct strategies for gene transfer that may involve either stable transformation or transient expression of a nonintegrated transgene. Stable transformation usually involves the integration of exogenous DNA into the nuclear genome, achieved using either *Agrobacterium tumefaciens* or a physical delivery technique such as particle bombardment. This can be applied to plant cells in culture to generate transgenic cell lines, or to callus tissue that can regenerate into whole transgenic plants. Alternatively, cell lines can be derived from transgenic plants directly. It is also possible to introduce transgenes into the plastid genome of certain species to generate transplastomic plants (119). There are several different transient expression methods. One approach is agroinfiltration, in which *A. tumefaciens* is injected or vacuum infiltrated into leaves so that many leaf cells take up but do not integrate the T-DNA and milligrams of protein can be produced in a few weeks (120–122). Other transient expression systems are based on systemically spreading plant RNA viruses (123–128). These approaches are combined in the Magnifection and CPMV-HT (*Cowpea mosaic virus* hypertranslatable) platforms, in which deconstructed viruses that cannot spread systemically are instead delivered to many cells by agroinfiltration (129–132).

have emerged: cell suspension cultures, transgenic plants, and transient expression (7, 35) (see the sidebar Gene Transfer Strategies).

One of the consequences of this diversity is that the platform can be matched to the requirements of the product rather than the product being modified to suit the platform, the latter being the typical approach in the biopharmaceutical industry. Plant cell cultures are grown in chemically defined sterile medium in fermenters and are particularly suitable for cosmetic ingredients and research/diagnostic reagents required in small quantities. The transient expression system can produce large amounts of protein in a short time, which is ideal for products with irregular demand and unstable markets. Transgenic plants have a long development process, but ultimately they are the most scalable and can be used to produce proteins required in the largest amounts. They are also the most suitable platform for in situ products, such as enzymes for the degradation of lignocellulose. In the special case of products that need to be stockpiled in an environment with no cold chain, transgenic cereal plants are ideal because recombinant proteins expressed in seeds have been shown to remain stable and active for several years without significant degradation (36).

PROTEIN TARGETING

Recombinant proteins can be selectively expressed in particular plant organs by using restrictive rather than constitutive promoters. This strategy is chosen for two reasons. First, restrictive promoters often achieve higher yields in their target tissues than constitutive promoters (e.g., the promoters of endogenous storage protein genes in cereals are usually more active in seeds than promoters for housekeeping genes such as those encoding actin and ubiquitin). Second, it is often beneficial to target recombinant proteins to sink tissues, where they are more stable and do not interfere with vegetative growth (37).

Proteins can also be targeted to specific cellular compartments such as the cytosol, apoplast, or plastids or the lumen of the endomembrane system, using specific peptide tags. The endoplasmic reticulum (ER) is the first part of the secretory pathway, and its oxidizing environment supports protein folding, assembly, and PTM to a much greater extent than the cytosol. Proteins are directed to the ER by including an N-terminal signal peptide (38). By default, these pass through the Golgi body and are secreted to the apoplast, but other signals can be added to retrieve proteins to the

ER or divert them to the vacuole (39–41). The secretory pathway in seeds is ideal for molecular farming because it includes specialized storage organelles that allow proteins to accumulate and become encapsulated in a protective matrix (37, 42, 43). These storage organelles can even be induced ectopically in tissues that are not adapted for storage functions and provide a simple strategy for producing active insoluble enzyme polymers (44). For pharmaceutical applications, the inclusion of additional peptide tags changes the nature of the product and attracts additional regulatory scrutiny, but this is not an issue for nonpharmaceutical products and protein targeting can therefore be exploited fully to maximize yields and to accumulate a recombinant protein in a suitable compartment (**Figure 1**).

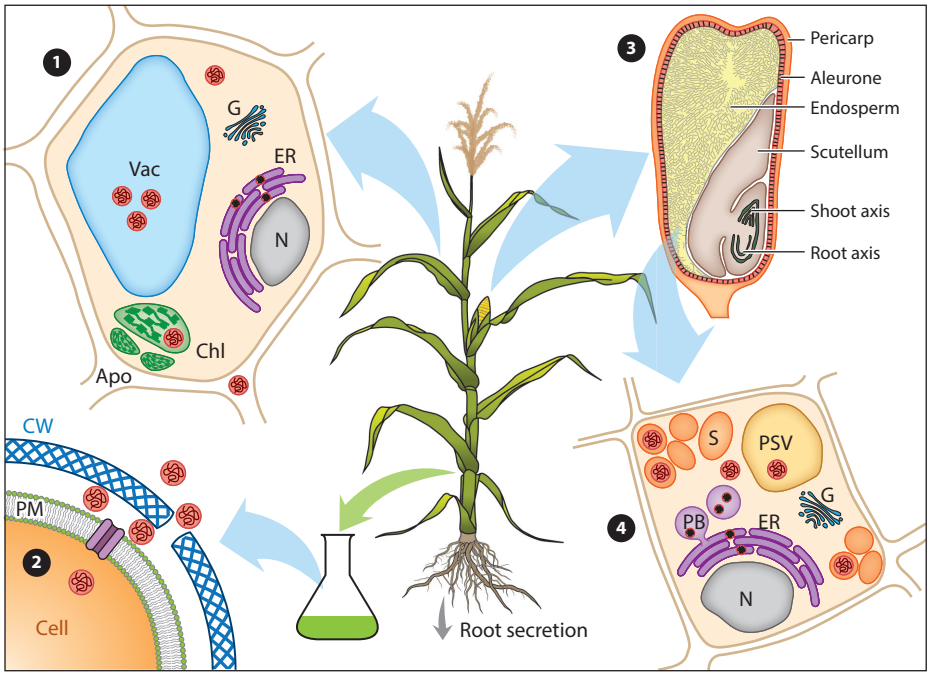

Figure 1

Targeting strategies for the expression of recombinant proteins. Recombinant proteins can be selectively expressed in particular plant organs such as leaves, roots, seeds or in cell cultures derived from one of these organs. Proteins can also be targeted to specific cellular compartments. In vegetative cells (❶), proteins (*red circles*) can be targeted to the apoplast (Apo), the vacuole (Vac), or the cytosol, or they can be retained in the endoplasmic reticulum (ER). Recombinant proteins can also be targeted to the chloroplasts (Chl) by means of a signal peptide. Alternatively, the recombinant gene can be inserted in the chloroplast genome and expressed directly in the plastids. This approach often results in high yields of recombinant protein but does not support certain posttranslational modifications such as glycosylation. When plant cells are cultivated in a suspension culture, it is often beneficial to secrete the protein to the apoplast, because this facilitates purification from the culture medium (❷). In some cases, digestion of the cell wall (CW) is an option to efficiently release protein trapped between the plasma membrane (PM) and the cell wall. Seeds (❸) are a very attractive site for the accumulation of recombinant proteins due to their native storage capabilities. Within the seed, recombinant proteins may be directed to either the endosperm or the embryo. The unique storage organelles of the seed cells (❹) include protein storage vacuoles (PSV), ER-derived protein bodies (PB), and starch granules (S), all of which have been used for protein accumulation. Finally, proteins can be produced in roots and secreted to the rhizosphere. This strategy is often preferred for phytoremediation purposes. Abbreviations: G, Golgi body; N, nucleus.

DOWNSTREAM PROCESSING AND PURIFICATION

Downstream processing is the set of physical and chemical steps used to purify recombinant proteins from the biological starting material. Recombinant proteins produced in microbes or mammalian cells are usually secreted into the medium, and downstream processing follows a standardized procedure involving centrifugation and/or filtration to remove cells and debris, then chromatography steps tailored to each product. The diversity of molecular farming platforms means that the equivalent downstream processes for plants must cater to the different properties of each host system at least during the early steps. Whereas plant cell suspension cultures, aquatic plants, and some hydroponic whole-plant systems can secrete recombinant proteins into the medium, most products are retained within the plant tissue and must be released by disruption, which introduces numerous soluble and insoluble process-related contaminants (30).

The basic downstream process for whole-plant tissues therefore begins with extraction, which is achieved by homogenization or milling to disrupt cells and release the product into a buffer. For small-scale processes, the buffer may contain additives such as protease inhibitors to protect the target protein, but this becomes too expensive at larger scales and different strategies, such as flocculation, heat precipitation, or direct capture using robust affinity media, are required to rapidly remove impurities (30). Insoluble debris can be removed by centrifugation, but again this is difficult to scale up, so filtration is preferred when process scalability is important. Several in-line depth filters may be necessary to progressively remove coarse and fine debris, followed by a membrane filter to remove fines. Once the extract is clarified, further downstream processing is based on chromatography steps tailored to the product and is therefore similar to all other platforms.

However, a number of novel purification strategies have been developed in plants based on the use of fusion tags that encourage partitioning (see the sidebar Protein Tags for Purification). The disruption of plant tissues introduces process-related contaminants that are not found in bacteria, yeast, or mammalian cells (e.g., fibers, oils, and superabundant plant proteins such as RuBisCO) and these may require specific early processing steps to ensure removal (45–47). As discussed above, for upstream process optimization, downstream processing in molecular farming is now being addressed using statistical experimental designs and multivariate statistics to build quality into the process and ensure robust purification. Several costing studies have been reported recently using either top-down (48, 49) or bottom-up (50, 51) analytical approaches. Although plants do not produce endotoxins, downstream processing must nevertheless cater for their removal because the bacteria that colonize plants do produce endotoxins, and large amounts are produced during agroinfiltration. Similarly, plants do not support mammalian viruses but virus removal is

PROTEIN TAGS FOR PURIFICATION

Several protein sequence tags have been used to simplify the downstream processing and purification of plant-derived recombinant proteins, including common affinity tags such as His_6 for immobilized metal affinity chromatography and more sophisticated tags for specialized separation processes that are less expensive and more scalable. The latter include elastin-like polypeptide tags that allow purification by temperature-dependent inverse transition cycling (133), hydrophobins that promote hydrophobic phase partitioning (134, 135), and oleosins that target proteins to the oil bodies of seeds, which can be separated in an oily phase by flotation centrifugation (136–138). Like the tags used for protein targeting, purification tags attract regulatory scrutiny for pharmaceutical products, but this is not an issue when the product is not intended for medical use.

still necessary under GMP conditions to prevent contamination with adventitious viruses during production, although the risk of contamination is acknowledged to be much lower than in mammalian systems (45). The costs of downstream processing for injectable pharmaceutical proteins are therefore similar across all platforms and can account for up to 80% of overall production costs, severely compromising any cost savings achieved by upstream production in plants. These restrictions do not apply to nonpharmaceutical products, and processing therefore represents a much smaller proportion of overall costs, even when the products are purified for use as technical reagents (52–54).

OPPORTUNITIES FOR PURIFIED PRODUCTS

Diagnostic and Technical Reagents

Bioanalytical methods rely on specific, high-affinity binding between a detection reagent and its target, for example, an enzyme and substrate or an antibody and antigen. Whereas enzymes are mostly used for process control and clinical diagnosis (e.g., the measurement of blood glucose), antibodies are used in a wider range of applications (see the sidebar Analytical Applications of Antibodies Produced by Molecular Farming). These include immunological assays for the detection of protein biomarkers, food allergens, toxins, chemical contaminants in food (e.g., pesticides, adulterants, and packaging derivatives), persistent organic pollutants in the environment, and pathogens for the purposes of disease monitoring and containment (55). Sensitive and cost-effective analytical methods are required for high-throughput screening when many samples need to be tested, and this approach is also useful for screening drug and catalyst libraries and for chemical reaction discovery and development (56, 57).

Antibodies were originally isolated from animals and were used as full-size molecules or enzymatic cleavage products such as Fab and $F(ab)_2$ antigen-binding fragments. The ability to produce antibodies as recombinant proteins and create libraries for screening against target antigens has enabled the development of smaller derivatives such as single-chain variable fragment(s) (scFv) and nanobodies [also known as variable heavy-chain antibody fragments (VHHs)] that facilitate screening for improved specificity, affinity, and stability (58, 59). Alternative binding scaffolds such as affibodies and anticalins can also be produced as recombinant proteins (60–62). Recombinant antibodies, fragments, and novel binders can also be produced as fusion proteins that extend their

ANALYTICAL APPLICATIONS OF ANTIBODIES PRODUCED BY MOLECULAR FARMING

Many antibodies have been produced by molecular farming and some have already been tested for a wide range of analytical applications, including immunoaffinity solid-phase extraction and immunoextraction, which remove analytes without contaminants even when the analytes are present at low concentrations. The antibodies can be immobilized onto solid support materials like agarose, silica, polystyrene-divinylbenzene, and glass, as well as monolithic materials. Further applications include other immunoassay formats such as lateral flow devices, which are popular in food chemistry for on-site detection of allergens, pathogens, and toxins and for food authentication by key biomarkers (139, 140). More recent microfluidic platforms and bead-based methods reflect advances in nanoparticle technology and the synthesis of antibody-functionalized detection probes (141–143). In addition to the diagnostic use of antibodies in different immunoassay formats, there is an increasing interest in developing highly selective sorbents for efficient sample preparation.

functionality, for example, by combining a binding domain with a visual marker protein or with a toxin to allow the selective killing of cancer cells (58, 63).

Small antibody derivatives such as scFv are routinely produced in bacteria because they fold spontaneously and do not usually require glycosylation. In contrast, full-size antibodies contain multiple disulfide bonds and N-glycans, and although they can be produced in bacteria (64), mammalian cells are preferred. Chinese hamster ovary cells are the favored platform for pharmaceutical antibodies because they produce high titers. However, this platform is among the most expensive and is not suitable for low-margin antibodies used as technical reagents. Plants offer the necessary economy and scalability for technical reagents, so they have been widely used to produce antibody variants. The first demonstration of molecular farming was the production of a full-size IgG in tobacco (1), and many different formats and fragment types have been produced since then (65). Plants have also been used to produce a full-size secretory IgA, which normally requires two different types of mammalian cell (14) and an IgM, which is among the most complex of human proteins (15).

In a particularly innovative use of plants, Julve et al. (66) cloned an entire VHH library in deconstructed *Tobacco mosaic virus* vectors and introduced them into *Nicotiana benthamiana* leaves by agroinfiltration. Mosaic patterns of leaf cells, each infected with one particular strain of the virus (and hence one VHH coding sequence), were formed due to the phenomenon of superinfection exclusion, where two versions of the same virus cannot exist in the same cell, because one is always outcompeted. Whole-leaf extracts thus represented a polyclonal mixture of antibodies, whereas individual infection zones were monoclonal.

There are currently no plant-derived pharmaceutical antibody products on the market, although several have progressed to clinical development (45) and a tobacco-derived antibody against *Streptococcus mutans* has been approved as a medical device for the treatment of dental caries. A tobacco-derived antibody was also approved by Cuban authorities in 2006 as an affinity purification reagent for a hepatitis B vaccine produced in yeast (67). Although this antibody is not used as a pharmaceutical, it nevertheless had to meet GMP standards as part of the vaccine manufacturing process. The production of nonpharmaceutical antibodies in various formats has been demonstrated for applications in diagnostics, food processing, and quality validation, but none of these products have yet reached the market (58, 68).

Although plant-derived antibodies have not yet been marketed as diagnostics or research-grade reagents, other proteins have been produced in plants for this purpose. ProdiGene began this process by developing maize as a commercial platform for the production of enzymes and technical reagents, including avidin and β-glucuronidase (GUS), both of which are important molecular biology research tools (52, 53). An important principle demonstrated by these case studies was that molecular farming can be economically viable even when the natural source of a protein is abundant (e.g., egg whites for avidin and *E. coli* for GUS) and where a market is already established. The average yield of avidin in maize was 0.5% of the dry seed weight. A typical egg contains 1.5 mg of avidin, so 800 kg of eggs but only 20 kg of corn would be required to produce 20 g of avidin (69). The yield of *E. coli* GUS in maize was 80 mg/kg dry seed, and the maize product was identical in size and almost identical in functional parameters (pI, K_m, V_{max}, and K_i) to its *E. coli* counterpart (53). Both GUS and avidin from maize have been distributed as research reagents by Sigma-Aldrich, and avidin is currently available.

Biocatalysts

Trypsin is a serine protease found in the digestive system of many vertebrates and it can hydrolyze almost any protein at the C-terminal side of the basic amino acids lysine or arginine. In mammals,

pancreatic cells produce and store the inactive precursor trypsinogen and secrete it to the duodenum, where enteropeptidases as well as already-cleaved trypsin molecules activate it by cleaving off the propeptide Val-Asp-Asp-Asp-Asp-Lys (70).

The broad activity of trypsin makes it useful for many applications, ranging from food processing and leather tanning to specialized functions such as cleaving biopharmaceutical proteins and digesting proteins into peptides for proteomic analysis. Industrial trypsin is usually isolated from bovine or porcine pancreas, but these sources are deemed unsafe for pharmaceutical applications due to the risk of contamination with viruses and prions (71). However, the production of recombinant trypsin is challenging because the autoactivation of trypsinogen disrupts the host cell. In *E. coli*, this has been circumvented by periplasmic targeting or induction during the late logarithmic growth phase of a high-density, fed-batch culture, resulting in a yield of 56 mg/L (72). In yeast, cultivation can be carried out below the optimal pH range of trypsin to maintain its inactive state (73, 74). Alternatively, a mutation was introduced to prevent self-cleavage of the propeptide, resulting in yields of up to 40 mg/L, but the product must then be cleaved with a different enzyme in vitro (75).

In plants, these issues have been addressed by expressing inactive trypsinogen in maize seeds under the control of the embryo-preferred globulin-I promoter and the optimized barley α-amylase signal sequence, resulting in a yield of 3.3% TSP (76). The progeny of this maize line produced 58 mg of trypsin per kilogram of seed. The trypsinogen was fully converted into active trypsin upon extraction apparently by autocatalytic processing and/or endopeptidases in the seeds. The maize-derived trypsin was functionally identical and physically similar to native bovine trypsin (V_{max}, K_m, pH optimum, stability, and inhibition by aprotinin and benzamidine), although unlike the native protein, it was also glycosylated (76). Further characterization revealed an unusual nonconsensus N-glycosylation site at Asp77 (77). This product was marketed as TrypZean™ and has been distributed since 2002 by Sigma-Aldrich (product no. T3568, T3449) as a reagent to dissociate adherent cells from vessel surfaces. TrypZean is covered by a number of patents and patent applications (78). Recombinant bovine trypsin was also produced in rice cell suspension cultures under the control of the sucrose starvation–inducible rice α-amylase 3D promoter achieving yields of 68 mg/L of medium (79). This product is currently marketed by Natural Bio-Materials, South Korea (**Table 1**).

Only a few manufacturers can offer recombinant animal-free trypsin at a price that is competitive with maize-derived trypsin. TrypZean usually sells at approximately $10/mg whereas trypsin from *Escherichia coli* costs more or less the same and that from *Pichia pastoris* is twice as expensive. Despite all efforts, none of these systems achieves the economy of trypsin from bovine or porcine pancreas, which sells at approximately $100/g (78). However, certifiable animal- and endotoxin-free products are quality attributes that are valued in the market for tissue culture components and cosmetics (**Table 1**).

Biopolymers

Plants are the main source of the world's most abundant natural polymers—lignocellulose and starch—both of which offer sustainable alternatives to nonrenewable, fossil-derived fuels and materials. Molecular farming has a role to play in this context because it also allows plants to be developed as a source of fibrous animal proteins such as collagen, keratin, silk, and elastin, which have remarkable strength, toughness, elasticity, and biocompatibility and can therefore be used to produce novel and sustainable biopolymers that could replace oil-based plastics (80).

Collagens are the main structural proteins in the extracellular matrix of mammals. The most abundant form is type I collagen which is a helical heterotrimer composed of two α1(I) and

one α2(I) polypeptide chains. The heterotrimeric helix represents procollagen which then polymerizes with other helices to form fibrils and fibers of indeterminate length with elaborate three-dimensional structures (81). The Israeli biotechnology company CollPlant has developed a tobacco line producing fully functional recombinant human collagen. They achieved this by expressing procollagen α1(I) and α2(I) along with a human proline-4-hydroxylase to allow the PTM of proline residues that is required for structural stabilization, and a human lysyl hydroxylase 3 that affects fibril diameter. By targeting the protein to the vacuole, they obtained a yield of 2% TSP, which translates to 200 mg/kg leaf material (17). CollPlant now markets its recombinant human collagen as Collage™ for tissue repair and wound management applications. Conventional medical collagen is sourced from animals and human cadavers with the consequent risk of infection. These collagens also need to be processed to remove cross-links before use, whereas the plant-derived collagen is pathogen free and has no cross-links, so it can be modified to meet the demands of any given application. Collagen is also used in cell culture, cosmetics, and adhesives, as well as in the food industry in the form of gelatin.

Many insect and spider species produce silk, which is a proteinaceous fiber with two main components: the structural protein fibroin and the adhesive protein sericin. The physicochemical properties of silk proteins differ from species to species and even within a species. The strength, elasticity, and stiffness can vary depending on the amino acid sequence and arrangement of fibers. The orb web spider *Nephila clavipes* produces different kinds of silk for webs, cocoons, and draglines. The dragline silk is five times stronger by weight than steel and three times tougher than *p*-aramid (Kevlar®), one of the strongest man-made fibers (16). Silk proteins are challenging to express in microbes and mammalian cells because they are several hundred kilodaltons in size and cannot be secreted. Scheller et al. (82) were the first to produce spider silk proteins in plants, achieving yields of up to 2% TSP in tobacco leaves and potato tubers. Various strategies have been developed to ensure that recombinant silk proteins are produced at the correct native size, including dimerization by nonrepetitive C-termini, cross-linking by transglutaminase, and intein-mediated multimerization (16, 83, 84).

PRODUCTS WITHOUT THE NEED FOR PURIFICATION

Feed Additives

Feed additives improve the quality of animal feed by providing direct nutrition (e.g., milk proteins) or by improving the digestibility of existing components. The latter category, known as feed enzymes, is generally divided into two classes: those required to break down indigestible fibers (e.g., hemicellulases) and those required to remove antinutritional factors (e.g., phytases). Feed additives are routinely produced in microbes and then added to animal feeds as supplements. This is an expensive process and the combined market for feed enzymes is projected to reach $727 million in 2015 (85). A more efficient and less expensive strategy would be to express these enzymes directly in the feed crops as shown in the following three examples.

Maize kernels are a good source of phosphorus, but the element is stored primarily as a complex with phytate, which cannot be digested by monogastric animals, because they lack the enzyme phytase. Phytate also sequesters other essential mineral nutrients such as iron, zinc, and calcium (86). These issues are currently addressed by supplementing animal feed with expensive recombinant phytase produced in *P. pastoris*, but the costs could be reduced by expressing phytase directly in feed crops. The endosperm-specific overexpression of *Aspergillus niger* phytase in maize produced an enzyme that was functionally equivalent to the commercial supplement, and the enzyme activity was sufficient to achieve adequate phosphorus release from normal maize by adding just 5 g

of transgenic seeds to 1 kg of the regular diet. The enzyme was also resistant to gastric digestion allowing it to act on phytate in the gut. Following successful field trials in chickens, phytase-enhanced maize was released as China's first transgenic crop in 2009, and the biosafety certificate was renewed in 2015 (87).

Like phytase, the enzymes β-mannanase and β-glucanase are often added to animal feeds to eliminate antinutritional factors. In this case, the targets are mannans and glucans, which absorb water and increase the viscosity of chime, thus restricting access to the intestinal surface and reducing the efficiency of nutrient uptake. Maize lines expressing *Bispora* sp. MEY-1 β-mannanase under the control of an embryo-specific promoter were tested because *Bispora* is an acidophile and the enzyme should therefore remain functional in the low-pH gastric environment. The enzyme was found to remain active under the same conditions as the commercial supplement from *P. pastoris* but was more thermotolerant during pelleting, with an activity of 10,000 U/kg (87). A feeding trial of β-mannanase supplements in pigs revealed that 400 U/kg feed achieved the greatest feed efficiency, so 40 g of the transgenic seeds per kilogram of conventional diet is sufficient to achieve the anticipated effects (88). The same group also expressed an acidic endo-β-1,3-1,4-glucanase in maize embryos with an activity of 170,000 U/kg and stability over the pH range 1.0–8.0 (89).

Biofuel

The United States is currently the largest producer of maize in the world. Historically, maize has been used primarily for animal feed with only 20% used for food, seed production, and industrial applications. More recently, the growth of the biofuel industry has seen this profile change radically, and today the proportion of maize used for feed and bioethanol production is roughly equal at 44% each, with the remainder used for food, seed, and other (90). The 13.3 billion gallons of bioethanol produced in the United States every year represent 55% of global production.

Maize as food and feed is a good source of carbohydrates, primarily in the form of starch, and this polymer is also the feedstock required for bioethanol production, resulting in competition among producers of feed, food, and bioethanol for agricultural space. However, bioethanol can also be produced from cellulose and hemicellulose, which have nutritional value only for ruminants, are much more abundant than starch and sugar, and are often found in the residue or bagasse from food/feed crops. Biofuels derived from cellulose and hemicellulose would therefore be more sustainable and would not compete with food and feed. Cell wall polymers such as lignocellulose and hemicellulose are abundant sources of fermentable sugars, but the sugars are not released as readily as they are from starch because the polymers tend to be cross-linked, structurally heterogeneous, and pseudocrystalline, making them recalcitrant to enzymatic hydrolysis without harsh and expensive pretreatments. A combination of several enzymes is then required to yield sugars from the recalcitrant biomass, and even conservative estimates suggest that up to 0.6 tons of pure enzyme would be needed to generate the 20 billion gallons of bioethanol that the United States has mandated for 2022, with an estimated infrastructure cost of nearly $30 billion to achieve this output using microbial fermentation (91).

The expression of polymer-degrading enzymes in plants would be less expensive than in microbes, and the enzymes could be purified, used as crude extracts, or preferably expressed in the biofuel crop directly. The complete hydrolysis of cellulose to glucose requires three enzymes (an exo-1,4-β-glucanase, an endo-1,4-β-glucanase, and a β-D-glucosidase), and a second stream of fermentable pentoses can be derived from xylan using the enzymes endo-1,4-β-D-xylanase, β-xylosidase, and α-glucuronidase; most of these enzymes have already been successfully expressed in plants (92). Maize lines individually expressing endo-β-1,4-glucanase and endo-β-1,4-xylanase

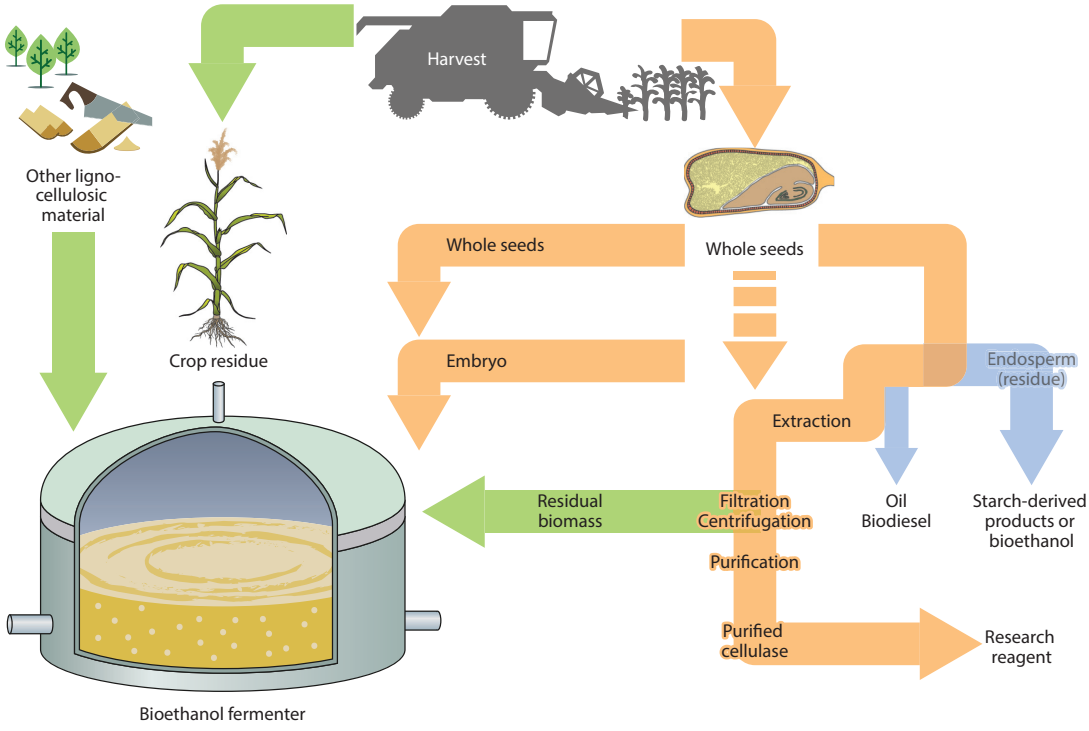

Figure 2

Plant-derived recombinant enzymes for biofuel production. Cellulases produced in maize seeds can be purified as research reagents or used in the bioethanol production process either as crude extract or enzyme-containing biomass. The residues arising from the processing steps provide additional biomass or valuable side products. Biomass flows are represented by green, added value product streams by blue, and cellulase-containing fractions by orange arrows. If the cellulase is produced in the embryo, then endosperm and embryo can be separated to reduce the enzyme-to-biomass ratio and the residual endosperm can be used for starch-derived products. Similarly, the embryo can be defatted to obtain oil as a valuable side product.

were produced by the US company Agrivida and were shown to increase bioethanol production from 42% to 65% theoretical ethanol yield (93). This group also engineered an improved thermostable xylanase containing a bacterial self-splicing intein that prevents autohydrolysis during growth but can be induced by heat after harvest (94).

Another company, Edenspace Systems Corporation, has patented transgenic plants expressing cellulase, hemicellulase, ligninase, or combinations thereof for improved saccharification (95). Currently, the only commercially available plant-derived hydrolase is an exo-1,4-β-glucanase from *Trichoderma reesei* produced in maize by Infinite Enzymes, LLC as a purified protein (Sigma-Aldrich product no. E6412). The pricing includes the costs for extraction and purification making it unsuitable for large-scale applications. Even so, the enzyme is expressed under the control of the embryo-preferred globulin-1 promoter, so defatted germ formulations can be used for industrial saccharification (91) as shown in **Figure 2**. Expression levels of >6% TSP were achieved (96) corresponding to 0.5% of the dry seed weight after seven backcrosses and two generations of self-pollination (91). Assuming that expression levels can be improved to 1% of the dry seed weight and that saccharification requires 30 g of enzyme per gallon of biofuel, a cultivation area of only 64 square miles for enzyme production would be sufficient to achieve an output of 20 million gallons of biofuel.

The production of biofuels from lignocellulosic biomass will take time to establish on a global scale, and in the meantime it may be possible to optimize the production of bioethanol production from maize starch by improving the efficiency of amylases, the enzymes that digest starch into sugars. The current conversion process involves the addition of α-amylase and/or β-amylase. Enogen® is a maize line expressing a thermostable α-amylase, which was developed by Syngenta and approved by the US Department of Agriculture in 2011. If conventional maize feedstock is mixed with Enogen at a 3:1 ratio, production costs fall by $0.04 per gallon, water usage by 7.7%, and natural gas consumption by 8.9% (97). Amylase-expressing plants could also be used for malting, baking, and the production of glucose and fructose syrup from starch (98).

Paper Manufacturing

In the paper manufacturing industry, the raw wood pulp undergoes a delignification process to separate the lignin from the cellulose fibers and the cellulose-rich pulp is often bleached to produce white paper. Both processes require harsh physical and chemical treatments that generate environmental pollution. Laccase is a copper-containing oxidoreductase found in white rot fungi that can oxidize phenolic compounds such as lignin, and this can be incorporated into almost every part of the papermaking process to reduce the use of chemicals and energy, avoid chemical waste, and improve paper strength and quality (99). As in the other cases discussed above, the main factor that prevents the adoption of laccase-based pulping is the cost of the microbial recombinant enzyme. Similarly, molecular farming would allow the enzyme to be applied as crude extracts or to be expressed in the trees used for the raw material as long as it remained inactive prior to cropping. Laccase from the white rot fungus *Trametes versicolor* has been produced in maize and some detrimental effects were observed even when expression was controlled by an embryo-preferred promoter with subcellular targeting to the cell wall (100). The yield was 2.0% TSP or 50 ppm of seed weight in the fifth generation (101). Xylanase can also be used to process pulp (102). The sequential treatment of pulp with xylanase and laccase resulted in a 50% reduction in postcolor number, a 15.71% increase in the tear index, and lower levels of absorbable organic halogens (34%) in the bleach effluents (103). Laccase could also be used to treat textile mill effluents, remove phenolic compounds from beverages, remove sulfur from fossil fuels, and develop biosensors (104). Plants that are engineered to secrete laccase from their roots could also be used to remove pesticides and xenohormones from the soil (104–106).

Future Products

Enzymes are advantageous for industrial processes because they yield specific products (thus reducing toxic residues) and operate at low temperatures using water as a solvent (thus reducing the need for harsh chemicals). The current industrial enzyme market is worth approximately $8 billion with a compound annual growth rate of 7% (107). Plants are ideal for the production of enzymes because they are inexpensive and highly scalable, which means it is much less expensive to expand production capacity than would be the case for any fermenter-based system (108). Plants can also produce enzymes that are toxic to microbes and animal cells. The applications of molecular farming in the field of industrial enzymes will depend on the ability to produce cost-effective alternatives for recombinant enzymes currently produced in microbes.

Enzymatic biodiesel is one of the most lucrative new applications of biotechnology-derived enzymes because it is a nonpolluting and carbon-neutral fuel (109). Biodiesel can be manufactured from numerous oils and fats, including virgin vegetable oils and waste cooking oils and fats. Europe produces the most biodiesel in the world with the United States in second place, and the global

production volume exceeds 6 billion gallons per year (110). Recombinant enzymes for biodiesel production are generally too expensive when produced in microbes, but plants could be used for the inexpensive production of lipases and phospholipases that can be purified, used as crude extracts, or expressed in oil crops directly. Phospholipase can also be used to remove gum from vegetable oils to prevent the formation of emulsions that limit the yield of pure oils for food and biodiesel applications (111).

The biorefinery concept will also be important in future developments because this will allow the design of low-waste processes in which all raw materials are converted into useful products, for example, sugar cane processes that also process the bagasse and wood pulping processes that also utilize the tree bark (112). Xylanase and oxidation/reduction enzymes such as laccase and peroxidase are suitable for these applications because the reactions are difficult to achieve through nonenzymatic means, offering a large potential market for low-cost recombinant enzymes produced on a large scale in plants rather than microbes (114). Enzymes such as manganese peroxidase and laccase have been produced in maize seeds (100, 113). The manganese peroxidase accumulated to high levels without detrimental effects when the enzyme was targeted to the embryo cell wall, whereas laccase lignified the embryo and inhibited germination suggesting it should be produced in an inactive form to allow normal vegetative growth.

Economic Considerations

The economic principles of molecular farming depend not only on the actual costs of the process but also the development time, which can range from 1 year for a product required in small amounts produced using a transient expression system to 7–10 years for a product required on a large scale produced in transgenic crop seeds. The scale of production provides the greatest benefit compared to fermenter-based systems because it costs much more to scale up fermenter infrastructure than to grow additional hectares of a production crop, even if the crop is grown in greenhouses rather than open fields (108). We consider here the development of transgenic maize lines expressing cellobiohydrolase (CBH1) as a case study (115–117).

The transformation and regeneration of transgenic maize lines takes approximately 1 year. When the T1 seeds are available, they are screened to identify the best-performing lines. The best T1 plants are crossed with elite inbred germplasm, backcrossed over six generations, and self-crossed for a further two generations to generate parental transgenic lines in an elite background that can be used to generate hybrids for enzyme production. This process takes 4 years if a winter nursery is used to double the generations achieved per year but is worth the effort because large increases in yield are achieved. In the case of the CBH1 lines, the production hybrids achieved a twentyfold increase in yield over the original T1 seeds (116). During the breeding process, excess grain was used to optimize extraction and purification methods for product characterization and to test downstream applications in biomass conversion (117).

The most challenging stage of the process is scaling up production to meet customer demand. The initial laboratory-scale process was suitable for the purification of approximately 1 g of enzyme per month from 2 kg of grain and could be achieved using bench-scale equipment. In contrast, a typical pulp and paper mill would require approximately 12 kg of enzyme per week. Infinite Enzymes (**http://www.infiniteenzymes.com**) is currently working with an agricultural engineer to design a process that will meet this demand by supplying 1,900 L of extract containing the enzyme at a concentration of 6 g/L. Assuming an enzyme yield of 0.5% dry seed weight, this would require the processing of 2.5 tonnes of seed per week. This is well beyond laboratory scale but far below the commercial scale of most US agricultural processing companies (e.g., ADM, which processes more than 76,000 tonnes of grain per day). The development of a processing facility is

therefore challenging because most equipment is designed for smaller laboratory scale or larger industrial scale operations. Pilot plant equipment can be used to provide standard apparatus such as mixing tanks, but more specialized equipment such as wet or dry milling units to separate germ and endosperm and continuous flow filtration units adequate for removing flour solids are more difficult to source. The cost of scale-up can approach $500,000 to $1 million, although one way to lower this cost is to lease the equipment.

The upstream production scale required to meet demand can be calculated by using conservative yield estimates of 1 tonne of grain per acre. Approximately 730 hectares (1,825 acres) would therefore be needed to produce enough grain to meet the annual demand of one pulp and paper mill. In the United States, this scale of production could be carried out under permit without deregulating the product as long as sufficient isolation could be achieved. Although this is possible, it is not ideal, because the cost of custom grain production is relatively high at $1,200 per acre. Profit can be made if the sales volume is high and processing is efficient, but this clearly demonstrates why it is important to optimize the enzyme yield to maximize production per hectare. In other countries, the regulatory burden of medium- to large-scale production (118) is circumvented by growing the production crop in greenhouses, for example, ORF Genetics in Iceland.

CONCLUSION

The plant-derived nonpharmaceutical proteins that are commercially available currently outnumber the medical products of molecular farming, reflecting the shorter development times and lower regulatory burden outside the pharmaceutical industry. Most of the nonpharmaceutical proteins are produced on a small to medium scale, which avoids the regulatory burden associated with large-scale agricultural production. However, molecular farming is increasingly being considered as an economic alternative not only for the production of pure diagnostic and technical reagents required in small amounts but also for high-volume, low-margin industrial enzymes that can be applied as crude extracts or unprocessed plant tissues in the feed, biofuel, and papermaking industries, which will require increasingly larger industrial processes.

SUMMARY POINTS

1. Plant-based production systems are established on the market. The first product for human medical use was commercialized in 2012, but currently the nonpharmaceutical products outnumber the pharmaceutical products as they do not require clinical studies and thus generate faster returns.
2. The palette of market sectors comprises research-grade or diagnostic reagents, cosmetic ingredients, biosensors or biocatalysts but also industrial enzymes used in the feed, biofuel, and papermaking industries.
3. Key advantages of plant-derived products are animal- and endotoxin-free production and cost advantages for the raw materials and processing.
4. Large-scale use of technical reagents, antibodies, and industrial applications could become feasible, but scale-up will require some investment to install equipment for these products.

5. Plants are renewable biomass for energy production. Enzymes used in the biomass conversion process could be produced directly in the plant biomass rather than in separate microbial systems.
6. Further development and widespread success of the technology will be strongly influenced by the level of regulation and restrictions applied to genetically modified plants and products derived thereof. In order to directly benefit from the technology, Europe will have to be proactive in the regulatory process.

DISCLOSURE STATEMENT

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LITERATURE CITED

1. Hiatt A, Cafferkey R, Bowdish K. 1989. Production of antibodies in transgenic plants. *Nature* 342:76–78
2. Sijmons PC, Dekker BM, Schrammeijer B, Verwoerd TC, van den Elzen PJ, Hoekema A. 1990. Production of correctly processed human serum albumin in transgenic plants. *Biotechnology* 8:217–21
3. Paul MJ, Teh AY, Twyman RM, Ma JK. 2013. Target product selection—Where can molecular pharming make the difference? *Curr. Pharm. Des.* 19:5478–85
4. Stöger E, Fischer R, Moloney M, Ma JK. 2014. Plant molecular pharming for the treatment of chronic and infectious diseases. *Annu. Rev. Plant Biol.* 65:743–68
5. Tekoah Y, Shulman A, Kizhner T, Ruderfer I, Fux L, et al. 2015. Large-scale production of pharmaceutical proteins in plant cell culture-the protalix experience. *Plant Biotechnol. J.* 13:1199–208
6. Hood E, Witcher D, Maddock S, Meyer T, Baszczynski C, et al. 1997. Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. *Mol. Breed.* 3:291–306
7. Fischer R, Schillberg S, Buyel JF, Twyman RM. 2013. Commercial aspects of pharmaceutical protein production in plants. *Curr. Pharm. Des.* 19:5471–77
8. Kolotilin I, Topp E, Cox E, Devriendt B, Conrad U, et al. 2014. Plant-based solutions for veterinary immunotherapeutics and prophylactics. *Vet. Res.* 45:117
9. MacDonald J, Doshi K, Dussault M, Hall JC, Holbrook L, et al. 2015. Bringing plant-based veterinary vaccines to market: Managing regulatory and commercial hurdles. *Biotechnol. Adv.* 33(8):1572–81
10. Clarke JL, Waheed MT, Lossl AG, Martinussen I, Daniell H. 2013. How can plant genetic engineering contribute to cost-effective fish vaccine development for promoting sustainable aquaculture? *Plant Mol. Biol.* 83:33–40
11. Avesani L, Merlin M, Gecchele E, Capaldi S, Brozzetti A, et al. 2014. Comparative analysis of different biofactories for the production of a major diabetes autoantigen. *Transgenic Res.* 23:281–91
12. Merlin M, Gecchele E, Capaldi S, Pezzotti M, Avesani L. 2014. Comparative evaluation of recombinant protein production in different biofactories: the green perspective. *Biomed. Res. Int.* 2014:136419
13. Baeshen NA, Baeshen MN, Sheikh A, Bora RS, Ahmed M, et al. 2014. Cell factories for insulin production. *Microb. Cell Fact.* 13:141
14. Ma JK, Hiatt A, Hein M, Vine ND, Wang F, et al. 1995. Generation and assembly of secretory antibodies in plants. *Science* 268:716–19

15. Loos A, Gruber C, Altmann F, Mehofer U, Hensel F, et al. 2014. Expression and glycoengineering of functionally active heteromultimeric IgM in plants. *PNAS* 111:6263–68
16. Hauptmann V, Weichert N, Rakhimova M, Conrad U. 2013. Spider silks from plants - a challenge to create native-sized spidroins. *Biotechnol. J.* 8:1183–92
17. Stein H, Wilensky M, Tsafrir Y, Rosenthal M, Amir R, et al. 2009. Production of bioactive, post-translationally modified, heterotrimeric, human recombinant type-I collagen in transgenic tobacco. *Biomacromolecules* 10:2640–45
18. Komori R, Amano Y, Ogawa-Ohnishi M, Matsubayashi Y. 2009. Identification of tyrosylprotein sulfotransferase in Arabidopsis. *PNAS* 106:15067–72
19. Matsubayashi Y. 2011. Post-translational modifications in secreted peptide hormones in plants. *Plant Cell Physiol.* 52:5–13
20. Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, et al. 2010. Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnol. J.* 8:564–87
21. Strasser R, Altmann F, Steinkellner H. 2014. Controlled glycosylation of plant-produced recombinant proteins. *Curr. Opin. Biotechnol.* 30:95–100
22. Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, et al. 2006. Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. *Nat. Biotechnol.* 24:1591–97
23. Forthal DN, Gach JS, Landucci G, Jez J, Strasser R, et al. 2010. Fc-glycosylation influences Fcgamma receptor binding and cell-mediated anti-HIV activity of monoclonal antibody 2G12. *J. Immunol.* 185:6876–82
24. Ullrich KK, Hiss M, Rensing SA. 2015. Means to optimize protein expression in transgenic plants. *Curr. Opin. Biotechnol.* 32:61–67
25. Twyman RM, Schillberg S, Fischer R. 2013. Optimizing the yield of recombinant pharmaceutical proteins in plants. *Curr. Pharm. Des.* 19:5486–94
26. Kirchhoff J, Raven N, Boes A, Roberts JL, Russell S, et al. 2012. Monoclonal tobacco cell lines with enhanced recombinant protein yields can be generated from heterogeneous cell suspension cultures by flow sorting. *Plant Biotechnol. J.* 10:936–44
27. Hood EE, Devaiah SP, Fake G, Egelkrout E, Teoh KT, et al. 2012. Manipulating corn germplasm to increase recombinant protein accumulation. *Plant Biotechnol. J.* 10:20–30
28. Rademacher T, Sack M, Arcalis E, Stadlmann J, Balzer S, et al. 2008. Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. *Plant Biotechnol. J.* 6:189–201
29. Fischer R, Vasilev N, Twyman RM, Schillberg S. 2015. High-value products from plants: the challenges of process optimization. *Curr. Opin. Biotechnol.* 32:156–62
30. Buyel JF. 2015. Process development strategies in plant molecular farming. *Curr. Pharm. Biotechnol.* 16:966–82
31. He Y, Ning T, Xie T, Qiu Q, Zhang L, et al. 2011. Large-scale production of functional human serum albumin from transgenic rice seeds. *PNAS* 108:19078–83
32. Devaiah SP, Requesens DV, Chang YK, Hood KR, Flory A, et al. 2013. Heterologous expression of cellobiohydrolase II (Cel6A) in maize endosperm. *Transgenic Res.* 22:477–88
33. De Jaeger G, Scheffer S, Jacobs A, Zambre M, Zobell O, et al. 2002. Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. *Nat. Biotechnol.* 20:1265–68
34. Oey M, Lohse M, Kreikemeyer B, Bock R. 2009. Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *Plant J.* 57:436–45
35. Twyman RM, Stöger E, Schillberg S, Christou P, Fischer R. 2003. Molecular farming in plants: host systems and expression technology. *Trends Biotechnol.* 21:570–78
36. Doran PM. 2006. Foreign protein degradation and instability in plants and plant tissue cultures. *Trends Biotechnol.* 24:426–32
37. Hofbauer A, Stöger E. 2013. Subcellular accumulation and modification of pharmaceutical proteins in different plant tissues. *Curr. Pharm. Des.* 19:5495–502
38. Martoglio B, Dobberstein B. 1998. Signal sequences: more than just greasy peptides. *Trends Cell Biol.* 8:410–15

39. Schouten A, Roosien J, van Engelen FA, de Jong GA, Borst-Vrenssen AW, et al. 1996. The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Mol. Biol.* 30:781–93
40. Neuhaus JM, Rogers JC. 1998. Sorting of proteins to vacuoles in plant cells. *Plant Mol. Biol.* 38:127–44
41. Robinson DG, Oliviusson P, Hinz G. 2005. Protein sorting to the storage vacuoles of plants: a critical appraisal. *Traffic* 6:615–25
42. Takaiwa F. 2013. Update on the use of transgenic rice seeds in oral immunotherapy. *Immunotherapy* 5:301–12
43. Hood E, Cramer C, Medrano G, Xu J. 2012. Protein targeting: strategic planning for optimizing protein products through plant biotechnology. In *Plant Biotechnology and Agriculture*, ed. A Arie, MH Paul, pp. 35–54. San Diego, CA: Academic
44. Llop-Tous I, Ortiz M, Torrent M, Ludevid MD. 2011. The expression of a xylanase targeted to ER-protein bodies provides a simple strategy to produce active insoluble enzyme polymers in tobacco plants. *PLOS ONE* 6:e19474
45. Ma JK, Drossard J, Lewis D, Altmann F, Boyle J, et al. 2015. Regulatory approval and a first-in-human phase I clinical trial of a monoclonal antibody produced in transgenic tobacco plants. *Plant Biotechnol. J.* 13:1106–20
46. Wilken LR, Nikolov ZL. 2012. Recovery and purification of plant-made recombinant proteins. *Biotechnol. Adv.* 30:419–33
47. Buyel JF, Twyman RM, Fischer R. 2015. Extraction and downstream processing of plant-derived recombinant proteins. *Biotechnol. Adv.* 33:902–13
48. Tuse D, Tu T, McDonald KA. 2014. Manufacturing economics of plant-made biologics: case studies in therapeutic and industrial enzymes. *Biomed. Res. Int.* 2014:256135
49. Ou J, Guo Z, Shi J, Wang X, Liu J, et al. 2014. Transgenic rice endosperm as a bioreactor for molecular pharming. *Plant Cell Rep.* 33:585–94
50. Walwyn DR, Huddy SM, Rybicki EP. 2014. Techno-economic analysis of horseradish peroxidase production using a transient expression system in *Nicotiana benthamiana*. *Appl. Biochem. Biotechnol.* 175:841–54
51. Buyel JF, Kaever T, Buyel JJ, Fischer R. 2013. Predictive models for the accumulation of a fluorescent marker protein in tobacco leaves according to the promoter/5′UTR combination. *Biotechnol. Bioeng.* 110:471–82
52. Hood EE, Kusnadi A, Nikolov Z, Howard JA. 1999. Molecular farming of industrial proteins from transgenic maize. *Adv. Exp. Med. Biol.* 464:127–47
53. Witcher D, Hood E, Peterson D, Bailey M, Bond D, et al. 1998. Commercial production of β-glucuronidase (GUS): a model system for the production of proteins in plants. *Mol. Breeding* 4:301–12
54. Howard JA, Hood E. 2005. Bioindustrial and biopharmaceutical products produced in plants. In *Advances in Agronomy*, Vol. 85, ed. D Sparks, pp. 91–124. San Diego, CA: Academic
55. Loos R, Carvalho R, Antonio DC, Comero S, Locoro G, et al. 2013. EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. *Water Res.* 47:6475–87
56. Creminon C, Taran F. 2015. Enzyme immunoassays as screening tools for catalysts and reaction discovery. *Chem. Commun.* 51:7996–8009
57. Collins KD, Gensch T, Glorius F. 2014. Contemporary screening approaches to reaction discovery and development. *Nat. Chem.* 6:859–71
58. De Meyer T, Muyldermans S, Depicker A. 2014. Nanobody-based products as research and diagnostic tools. *Trends Biotechnol.* 32:263–70
59. Geering B, Fussenegger M. 2015. Synthetic immunology: modulating the human immune system. *Trends Biotechnol.* 33:65–79
60. Skerra A. 2007. Alternative non-antibody scaffolds for molecular recognition. *Curr. Opin. Biotechnol.* 18:295–304
61. Richter A, Eggenstein E, Skerra A. 2014. Anticalins: exploiting a non-Ig scaffold with hypervariable loops for the engineering of binding proteins. *FEBS Lett.* 588:213–18
62. Schiefner A, Skerra A. 2015. The menagerie of human lipocalins: a natural protein scaffold for molecular recognition of physiological compounds. *Acc. Chem. Res.* 48:976–85

63. Justino CIL, Duarte AC, Rocha-Santos TAP. 2015. Analytical applications of affibodies. *Trac-Trends Anal. Chem.* 65:73–82
64. Robinson MP, Ke N, Lobstein J, Peterson C, Szkodny A, et al. 2015. Efficient expression of full-length antibodies in the cytoplasm of engineered bacteria. *Nat. Commun.* 6:8072
65. Orzaez D, Granell A, Blazquez MA. 2009. Manufacturing antibodies in the plant cell. *Biotechnol. J.* 4:1712–24
66. Julve JM, Gandia A, Fernandez-del-Carmen A, Sarrion-Perdigones A, Castelijns B, et al. 2013. A coat-independent superinfection exclusion rapidly imposed in *Nicotiana benthamiana* cells by tobacco mosaic virus is not prevented by depletion of the movement protein. *Plant Mol. Biol.* 81:553–64
67. Pujol M, Ramirez NI, Ayala M, Gavilondo JV, Valdes R, et al. 2005. An integral approach towards a practical application for a plant-made monoclonal antibody in vaccine purification. *Vaccine* 23:1833–37
68. Ritala A, Leelavathi S, Oksman-Caldentey KM, Reddy VS, Laukkanen ML. 2014. Recombinant barley-produced antibody for detection and immunoprecipitation of the major bovine milk allergen, beta-lactoglobulin. *Transgenic Res.* 23:477–87
69. Hood E, Howard J. 2014. Commercial plant-produced recombinant avidin. In *Commercial Plant-Produced Recombinant Protein Products: Case Studies*, ed. JA Howard, EE Hood, pp. 15–25. Berlin: Springer
70. Neurath H, Walsh KA. 1976. Role of proteolytic enzymes in biological regulation (a review). *PNAS* 73:3825–32
71. Merten OW. 2002. Virus contaminations of cell cultures—biotechnological view. *Cytotechnology* 39:91–116
72. Yee L, Blanch HW. 1993. Recombinant trypsin production in high cell density fed-batch cultures in *Escherichia coli*. *Biotechnol. Bioeng.* 41:781–90
73. Mattanovich D, Katinger H, Hohenblum H, Naschberger S, Weik R. 2008. Method for the manufacture of recombinant trypsin. US Patent No. 20030157634 A1
74. Muller R, Glaser S, Geipel F, Thalhofer JP, Rexer B, et al. 2010. Method for producing recombinant trypsin. US Patent No. 20080064084 A1
75. Hanquier J, Sorlet Y, Desplancq D, Baroche L, Ebtinger M, et al. 2003. A single mutation in the activation site of bovine trypsinogen enhances its accumulation in the fermentation broth of the yeast *Pichia pastoris*. *Appl. Environ. Microbiol.* 69:1108–13
76. Woodard SL, Mayor JM, Bailey MR, Barker DK, Love RT, et al. 2003. Maize (*Zea mays*)-derived bovine trypsin: characterization of the first large-scale, commercial protein product from transgenic plants. *Biotechnol. Appl. Biochem.* 38:123–30
77. Zhang H, Huang RY, Jalili PR, Irungu JW, Nicol GR, et al. 2010. Improved mass spectrometric characterization of protein glycosylation reveals unusual glycosylation of maize-derived bovine trypsin. *Anal. Chem.* 82:10095–101
78. Krishnan A, Woodard S. 2014. TryZean™: an animal-free alternative to bovine trypsin. See Ref. 69, pp. 43–63
79. Kim NS, Yu HY, Chung ND, Kwon TH, Yang MS. 2014. High-level production of recombinant trypsin in transgenic rice cell culture through utilization of an alternative carbon source and recycling system. *Enzyme Microb. Technol.* 63:21–27
80. Bornke F, Broer I. 2010. Tailoring plant metabolism for the production of novel polymers and platform chemicals. *Curr. Opin. Plant Biol.* 13:354–62
81. Canty EG, Kadler KE. 2005. Procollagen trafficking, processing and fibrillogenesis. *J. Cell Sci.* 118:1341–53
82. Scheller J, Guhrs KH, Grosse F, Conrad U. 2001. Production of spider silk proteins in tobacco and potato. *Nat. Biotechnol.* 19:573–77
83. Hauptmann V, Menzel M, Weichert N, Reimers K, Spohn U, Conrad U. 2015. In planta production of ELPylated spidroin-based proteins results in non-cytotoxic biopolymers. *BMC Biotechnol.* 15:9
84. Weichert N, Hauptmann V, Menzel M, Schallau K, Gunkel P, et al. 2014. Transglutamination allows production and characterization of native-sized ELPylated spider silk proteins from transgenic plants. *Plant Biotechnol. J.* 12:265–75
85. Bhavsar K, Khire JM. 2014. Current research and future perspectives of phytase bioprocessing. *RSC Adv.* 4:26677–91

86. Gupta RK, Gangoliya SS, Singh NK. 2015. Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. *J. Food Sci. Technol.* 52:676–84
87. Xu X, Zhang Y, Meng Q, Meng K, Zhang W, et al. 2013. Overexpression of a fungal beta-mannanase from *Bispora* sp. MEY-1 in maize seeds and enzyme characterization. *PLOS ONE* 8:e56146
88. Lv JN, Chen YQ, Guo XJ, Piao XS, Cao YH, Dong B. 2013. Effects of supplementation of beta-mannanase in corn-soybean meal diets on performance and nutrient digestibility in growing pigs. *Asian-Australas. J. Anim. Sci.* 26:579–87
89. Zhang Y, Xu X, Zhou X, Chen R, Yang P, et al. 2013. Overexpression of an acidic endo-beta-1,3-1,4-glucanase in transgenic maize seed for direct utilization in animal feed. *PLOS ONE* 8:e81993
90. Ranum P, Pena-Rosas JP, Garcia-Casal MN. 2014. Global maize production, utilization, and consumption. *Ann. N. Y. Acad. Sci.* 1312:105–12
91. Hood E, Requesens D. 2014. Commercial plant-produced recombinant cellulases for biomass conversion. See Ref. 69, pp. 231–46
92. Li Q, Song J, Peng S, Wang JP, Qu GZ, et al. 2014. Plant biotechnology for lignocellulosic biofuel production. *Plant Biotechnol. J.* 12:1174–92
93. Zhang D, VanFossen A, Pagano R, Johnson J, Parker M, et al. 2011. Consolidated pretreatment and hydrolysis of plant biomass expressing cell wall degrading enzymes. *BioEnergy Res.* 4:276–86
94. Shen B, Sun X, Zuo X, Shilling T, Apgar J, et al. 2012. Engineering a thermoregulated intein-modified xylanase into maize for consolidated lignocellulosic biomass processing. *Nat. Biotechnol.* 30:1131–36
95. Blaylock MJ, Ferguson BW, Lee DA. 2012. Energy crops for improved biofuel feedstocks. US Patent No. 20070250961 A1
96. Hood EE, Love R, Lane J, Bray J, Clough R, et al. 2007. Subcellular targeting is a key condition for high-level accumulation of cellulase protein in transgenic maize seed. *Plant Biotechnol. J.* 5:709–19
97. Urbanchuk JM, Kowalski DJ, Dale B, Kim S. 2009. Corn amylase: improving the efficiency and environmental footprint of corn to ethanol through plant biotechnology. *AgBioForum* 12:149–54
98. van der Maarel MJ, van der Veen B, Uitdehaag JC, Leemhuis H, Dijkhuizen L. 2002. Properties and applications of starch-converting enzymes of the alpha-amylase family. *J. Biotechnol.* 94:137–55
99. Widsten P, Kandelbauer A. 2008. Laccase applications in the forest products industry: a review. *Enzyme Microb. Technol.* 42:293–307
100. Hood EE, Bailey MR, Beifuss K, Magallanes-Lundback M, Horn ME, et al. 2003. Criteria for high-level expression of a fungal laccase gene in transgenic maize. *Plant Biotechnol. J.* 1:129–40
101. Bailey MR, Woodard SL, Callaway E, Beifuss K, Magallanes-Lundback M, et al. 2004. Improved recovery of active recombinant laccase from maize seed. *Appl. Microbiol. Biotechnol.* 63:390–97
102. Juturu V, Wu JC. 2012. Microbial xylanases: engineering, production and industrial applications. *Biotechnol. Adv.* 30:1219–27
103. Sharma A, Thakur VV, Shrivastava A, Jain RK, Mathur RM, et al. 2014. Xylanase and laccase based enzymatic kraft pulp bleaching reduces adsorbable organic halogen (AOX) in bleach effluents: a pilot scale study. *Bioresour. Technol.* 169:96–102
104. Singh Arora D, Kumar Sharma R. 2010. Ligninolytic fungal laccases and their biotechnological applications. *Appl. Biochem. Biotechnol.* 160:1760–88
105. Wang GD, Li QJ, Luo B, Chen XY. 2004. *Ex planta* phytoremediation of trichlorophenol and phenolic allelochemicals via an engineered secretory laccase. *Nat. Biotechnol.* 22:893–97
106. Singh R, Cabrera ML, Radcliffe DE, Zhang H, Huang Q. 2015. Laccase mediated transformation of 17beta-estradiol in soil. *Environ. Pollut.* 197:28–35
107. Li S, Yang X, Yang S, Zhu M, Wang X. 2012. Technology prospecting on enzymes: Application, marketing and engineering. *Comput. Struct. Biotechnol. J.* 2:1–11
108. Howard J, Nikolov Z, Hood E. 2011. Enzyme production systems for biomass conversion. In *Plant Biomass Conversion*, ed. E Hood, P Nelson, R Powell, pp. 227–53. Hoboken, NJ: Wiley-Blackwell
109. Ranganathan SV, Narasimhan SL, Muthukumar K. 2008. An overview of enzymatic production of biodiesel. *Bioresour. Technol.* 99:3975–81
110. Du W, Li W, Sun T, Chen X, Liu D. 2008. Perspectives for biotechnological production of biodiesel and impacts. *Appl. Microbiol. Biotechnol.* 79:331–37

111. Yang B, Wang Y-H, Yang J-G. 2006. Optimization of enzymatic degumming process for rapeseed oil. *J. Am. Oil Chem. Soc.* 83:653–58
112. Bussamra BC, Freitas S, Costa AC. 2015. Improvement on sugar cane bagasse hydrolysis using enzymatic mixture designed cocktail. *Bioresour. Technol.* 187:173–81
113. Clough RC, Pappu K, Thompson K, Beifuss K, Lane J, et al. 2006. Manganese peroxidase from the white-rot fungus *Phanerochaete chrysosporium* is enzymatically active and accumulates to high levels in transgenic maize seed. *Plant Biotechnol. J.* 4:53–62
114. Hayes TL, Zimmerman N, Hackle A. 2014. *World enzymes: industry study with forecasts for 2011 & 2016*. Rep. 2229, Freedonia Group, Cleveland, OH
115. Hood EE, Love R, Lane J, Bray J, Clough R, et al. 2007. Subcellular targeting is a key condition for high-level accumulation of cellulase protein in transgenic maize seed. *Plant Biotechnol. J.* 5:709–19
116. Hood EE, Devaiah SP, Fake G, Egelkrout E, Teoh K, et al. 2012. Manipulating corn germplasm to increase recombinant protein accumulation. *Plant Biotechnol. J.* 10:20–30
117. Hood NC, Hood KR, Woodard SL, Devaiah SP, Jeoh T, et al. 2014. Purification and characterization of recombinant cel7a from maize seed. *Appl. Biochem. Biotechnol.* 174:2864–74
118. Sparrow P, Broer I, Hood EE, Eversole K, Hartung F, Schiemann J. 2013. Risk assessment and regulation of molecular farming—a comparison between Europe and US. *Curr. Pharm. Des.* 19:5513–30
119. Bock R. 2015. Engineering plastid genomes: methods, tools, and applications in basic research and biotechnology. *Annu. Rev. Plant Biol.* 66:211–41
120. Whaley KJ, Hiatt A, Zeitlin L. 2011. Emerging antibody products and *Nicotiana* manufacturing. *Hum. Vaccines* 7:349–56
121. D'Aoust MA, Couture MM, Charland N, Trepanier S, Landry N, et al. 2010. The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. *Plant Biotechnol. J.* 8:607–19
122. Vezina LP, Faye L, Lerouge P, D'Aoust MA, Marquet-Blouin E, et al. 2009. Transient co-expression for fast and high-yield production of antibodies with human-like N-glycans in plants. *Plant Biotechnol. J.* 7:442–55
123. Yusibov V, Rabindran S, Commandeur U, Twyman RM, Fischer R. 2006. The potential of plant virus vectors for vaccine production. *Drugs Res. Dev.* 7:203–17
124. Yusibov V, Rabindran S. 2008. Recent progress in the development of plant derived vaccines. *Expert Rev. Vaccines* 7:1173–83
125. Yusibov V, Streatfield SJ, Kushnir N, Roy G, Padmanaban A. 2013. Hybrid viral vectors for vaccine and antibody production in plants. *Curr. Pharm. Des.* 19:5574–86
126. Canizares MC, Lomonossoff GP, Nicholson L. 2005. Development of cowpea mosaic virus-based vectors for the production of vaccines in plants. *Expert Rev. Vaccines* 4:687–97
127. Lico C, Chen Q, Santi L. 2008. Viral vectors for production of recombinant proteins in plants. *J. Cell Physiol.* 216:366–77
128. Sainsbury F, Lavoie PO, D'Aoust MA, Vezina LP, Lomonossoff GP. 2008. Expression of multiple proteins using full-length and deleted versions of cowpea mosaic virus RNA-2. *Plant Biotechnol. J.* 6:82–92
129. Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y. 2005. Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. *Nat. Biotechnol.* 23:718–23
130. Gleba Y, Klimyuk V, Marillonnet S. 2005. Magnifection—a new platform for expressing recombinant vaccines in plants. *Vaccine* 23:2042–48
131. Gleba Y, Klimyuk V, Marillonnet S. 2007. Viral vectors for the expression of proteins in plants. *Curr. Opin. Biotechnol.* 18:134–41
132. Sainsbury F, Lomonossoff GP. 2008. Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiol.* 148:1212–18
133. Phan HT, Hause B, Hause G, Arcalis E, Stöger E, et al. 2014. Influence of elastin-like polypeptide and hydrophobin on recombinant hemagglutinin accumulations in transgenic tobacco plants. *PLOS ONE* 9:e99347

134. Reuter LJ, Bailey MJ, Joensuu JJ, Ritala A. 2014. Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells. *Plant Biotechnol. J.* 12:402–10
135. Gutierrez SP, Saberianfar R, Kohalmi SE, Menassa R. 2013. Protein body formation in stable transgenic tobacco expressing elastin-like polypeptide and hydrophobin fusion proteins. *BMC Biotechnol.* 13:40
136. van Rooijen GJ, Moloney MM. 1995. Plant seed oil-bodies as carriers for foreign proteins. *Biotechnology* 13:72–77
137. Nykiforuk CL, Boothe JG, Murray EW, Keon RG, Goren HJ, et al. 2006. Transgenic expression and recovery of biologically active recombinant human insulin from *Arabidopsis thaliana* seeds. *Plant Biotechnol. J.* 4:77–85
138. Nykiforuk CL, Boothe JG. 2012. Transgenic expression of therapeutic proteins in *Arabidopsis thaliana* seed. *Methods Mol. Biol.* 899:239–64
139. Anfossi L, Baggiani C, Giovannoli C, D'Arco G, Giraudi G. 2013. Lateral-flow immunoassays for mycotoxins and phycotoxins: a review. *Anal. Bioanal. Chem.* 405:467–80
140. Taranova NA, Berlina AN, Zherdev AV, Dzantiev BB. 2015. 'Traffic light' immunochromatographic test based on multicolor quantum dots for the simultaneous detection of several antibiotics in milk. *Biosens. Bioelectron.* 63:255–61
141. Cho IH, Radadia AD, Farrokhzad K, Ximenes E, Bae E, et al. 2014. Nano/micro and spectroscopic approaches to food pathogen detection. *Annu. Rev. Anal. Chem.* 7:65–88
142. Warriner K, Reddy SM, Namvar A, Neethirajan S. 2014. Developments in nanoparticles for use in biosensors to assess food safety and quality. *Trends Food Sci. Technol.* 40:183–99
143. Knopp D, Tang D, Niessner R. 2009. Review: bioanalytical applications of biomolecule-functionalized nanometer-sized doped silica particles. *Anal. Chim. Acta* 647:14–30