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Current Status and Perspectives of the Molecular Farming Landscape

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Abbreviations

CHO Chinese hamster ovary, CPMV Cowpea mosaic virus, CRISPR clustered regularly interspersed palindromic repeats, CTB cholera toxin B-subunit, EMA European Medicines Agency, FDA Food and Drug Administration, GMP good manufacturing practice, HBV Hepatitis B virus, HIV Human immunodeficiency virus, HSV Herpes simplex virus, ICM immune complex mimic, IgA immunoglobulin A, IgG immunoglobulin G, PMP plant-made pharmaceutical, RNAi RNA interference, scFv single-chain variable fragment, TALEN transcription activator-like effector nuclease, TMV Tobacco mosaic virus, USDA US Department of Agriculture, VLP virus-like particle.

1.1 Introduction

Molecular farming refers to the use of plants for the production of recombinant proteins. Plants are often presented as more scalable and less expensive than the current industry standards (microbial and animal cells in fermenters) (Stöger et al., 2014). In the case of pharmaceutical products, where the alternative term molecular pharming is often applied, plants are often considered to be safer too. However, plants are unlikely to displace industry stalwarts such as Escherichia coli and Chinese hamster ovary (CHO) cells, which are considered gold standards for protein manufacturing, at least when competing in areas where these established platforms are strongest. Plants cannot yet match the yields of these competitors, and adopting plants would require the biomanufacturing industry to introduce new practices and technologies for both upstream production and downstream processing. Plants have a limited track record with the pharmaceutical regulators because manufacturing that complies with good manufacturing practice (GMP) is in its infancy (Fischer et al., 2012). In contrast, the industry
favorites have a long and successful history, and the regulatory framework has been built up around them. Success has resulted in the selection of a small number of high-performance platform technologies that are widely used in commercial processes, whereas molecular farming is known for the diversity of expression strategies and production systems, making it difficult to establish standardized processes. This diversity is on one hand an advantage because it means that a suitable platform can be found for each product and application (e.g. edible crops for oral vaccines); but the absence of standard platforms makes the existing regulations more difficult to apply and this dissuades industry players from investing in long-term production capacity. This chapter provides an overview of the current molecular farming landscape in terms of the most prevalent platforms, products, and downstream processing strategies based on an analysis of the literature published between 2010 and 2016, and discusses the perspectives for this technology and likely future developments.

1.2 Brief History of Molecular Farming

Molecular farming differs from other applications of plant biotechnology in that the recombinant protein itself is the desired product rather than the effect it has on the performance or activity of the plant host (Ma et al., 2003; Stöger et al., 2014). The first deliberate use of plants as a production host involved the expression of a recombinant antibody in transgenic tobacco plants (Hiatt et al., 1989); this was swiftly followed by the production of human serum albumin in tobacco and potato plants and cell suspension cultures (Sijmons et al., 1990). The fact that these initial products were human proteins with medical relevance immediately established the possibility of using plants for the production of protein biopharmaceuticals, which became known as plant-made pharmaceuticals (PMPs). The resulting gold rush of researchers looking to express diverse pharmaceutical proteins in plants led to many proof-of-principle studies that were published in the 1990s and early 2000s (reviewed by Fischer and Emans, 2000; Ma et al., 2003; Twyman, 2005). These early studies shared three main characteristics. First, there was no universal agreement on the ideal host platform, leading to the development of an extremely diverse array of production systems (Twyman et al., 2003). The diversity embraced different species of whole plants (tobacco, cereals, legumes, oilseeds, leafy edible crops, potato, tomato, and various aquatic and unicellular species), various tissue and cell culture systems (hairy roots, teratomas, and cell suspension cultures), and a bewildering array of expression strategies (transgenic plants, transplastomic plants, various transient expression systems, inducible expression, and different protein targeting strategies). Second, and in contrast to the diversity of expression hosts, three main product classes emerged: antibodies, vaccine candidates, and replacement human proteins. Third, and perhaps most importantly in the context of future events, very few of these studies were concerned with anything further than establishing that the recombinant proteins could be expressed. The commercial potential of molecular farming was touted on the basis that plants were safe, scalable, and economical compared to existing platforms, but without the translational research to show whether or not these promises could be fulfilled. Many small start-up companies were established to promote specific host systems for molecular farming, but without the ability to translate such early-stage research they soon went out of
business. The big industry players, which had initially expressed cautious interest in this emerging technology, eventually withdrew their support (Fischer et al., 2014).

While the molecular farming pharma bubble expanded and then collapsed, other researchers were considering the industrial potential of the technology. The major player was Prodigene Inc. (College Station, TX, USA), which was investigating the use of maize as a platform for the production of research-grade reagents and industrial enzymes in addition to pharmaceuticals. Importantly, the research carried out by Prodigene looked into the economic viability of molecular farming at an early stage. The key aspect was that they considered not only upstream production but also downstream processing, and they were the first to develop a commercial process which took into account the upstream yield, the downstream recovery and purity, and compared the overall costs to existing production methods (Hood et al., 1999; Kusnadi et al., 1998). Accordingly, they found that maize-derived recombinant avidin was commercially competitive with the existing commercial avidin product derived from hens’ eggs (Hood et al., 1999) and that maize-derived β-glucuronidase was commercially competitive with the existing commercial enzyme isolated from bacteria (Witcher et al., 1998). Many of the downstream processing concepts developed by Prodigene provided the foundations of more recent processes for the isolation of PMPs (Menkhaus et al., 2004; Nikolov and Woodard, 2004; Wilken and Nikolov, 2012). These methods have also been adopted by the next generation of companies using cereals for commercial molecular farming, including Ventria Bioscience (Fort Collins, CO, USA) which produces various pharmaceutical and cosmetic products in rice seeds (Wilken and Nikolov, 2006, 2010) and ORF Genetics (Kopavogur, Iceland) which produces diagnostic and research reagents as well as cosmetic products in barley.

The pioneers of pharmaceutical molecular farming learned their lessons from the early failures and looked at the Prodigene story with renewed interest. Success in their own field would require more focus on the downstream elements of the production process as well as translational research to make the leap from proof-of-principle studies to commercial reality. One more lesson was also taken from Prodigene, which eventually went out of business not because its products were unprofitable but due to cumulative fines levied against them for breaching environmental regulations (the adventitious growth of some of their transgenic maize plants in a neighboring soybean field). The molecular farming community now generally avoids using field grown plants unless they are well isolated and there is minimal risk of outcrossing or admixture. Ventria Bioscience grows rice in Colorado, well away from rice crops destined for the food chain. Other than this atypical exception, molecular farming is mostly carried out in contained facilities, attracting a lower regulatory burden and avoiding the associated negative public perception issues.

The next wave of pharmaceutical molecular farming therefore focused on several issues that were not addressed in the 1990s and early 2000s: the ability to develop entire manufacturing processes that were economical at the industrial scale, the ability to harmonize molecular farming with existing regulations covering pharmaceutical products, and the ability to compete with the existing industry platforms. This resulted in the consolidation of molecular farming technology around a smaller number of the most promising production systems, namely transgenic tobacco and cereal crops, transient expression in leafy crops such as tobacco and its close relative Nicotiana benthamiana,
and contained fermenter-based platforms such as plant cell suspension cultures and clonally-propagating aquatic plants (moss and duckweed).

Although transgenic plants were favored during the early development of molecular farming because of their scalability, all of the commercial breakthroughs in the pharmaceutical sector were achieved with plant cell suspension cultures or similar contained systems because they were easier to accommodate under existing GMP regulations. Early development focused on tobacco cells, which were easy to handle, and rice cells, which have a sugar-dependent promoter system that allows growth and product accumulation to be separated. These two systems are still widely used today. In 2006, tobacco cells were used by Dow AgroSciences (Zionsville, IN, USA) to produce the first veterinary vaccine from plants to be granted approval by the US Department of Agriculture (USDA), although it was never commercialized (Schillberg et al., 2013). In 2012, carrot cells were used by Protalix Biotherapeutics (Karmiel, Israel) to manufacture the first pharmaceutical recombinant protein derived from plants to be approved for human use by the US Food and Drug Administration (FDA) (Mor, 2015). Other important contributors include the moss system developed by Greenovation GmbH (Heilbronn, Germany) and the duckweed system developed by the now disbanded Biolex Therapeutics (Pittsboro, NC, USA), both of which have been used to manufacture pharmaceutical proteins for phase I and II clinical trials.

Transient expression refers collectively to several different approaches based on the introduction of bacteria (*Agrobacterium tumefaciens*) and/or viruses into plants, with the plants then used as an incubator to accumulate recombinant proteins produced by the genetically engineered microbes. In some transient systems, bacteria are infiltrated into leaf spaces (agroinfiltration) and the surrounding cells are transfected with T-DNA, allowing the production of recombinant protein in these infiltrated patches of cells for a few days or weeks. In other systems the vector is a recombinant virus, and the infection (and the production of recombinant protein) is systemic. In still other cases, deconstructed viruses usually based on *Tobacco mosaic virus* (TMV) are combined with the agroinfiltration system so that a large number of cells are initially transfected with a T-DNA copy of the RNA-based virus genome. When the T-DNA copy is transcribed, the RNA genome replicates in the cell and spreads locally, thus increasing the number of gene copies and the yield of recombinant protein. Several related platforms have been developed using deconstructed TMV vectors including the Launch Vector system (Fraunhofer CMB, Newark, DE, USA) and the Magnifecition system (Icon Genetics, Halle (Saale), Germany). Similarly, the *Cowpea mosaic virus* (CPMV) hypertranslatable vector is a deconstructed version of CPMV comprising only the translation enhancer sequences from RNA-2 and the *Tomato bushy stunt virus* p19 suppressor of posttranscriptional gene silencing. This non-replicating vector achieves yields of up to 1.5 g recombinant protein per kg wet biomass. A similar system called INPACT (IN Plant ACTivation) has been developed based on the rolling circle replication mechanism of *Tobacco yellow dwarf virus* (Dugdale et al., 2013; Dugdale et al., 2014). Several companies have developed agroinfiltration-based transient expression platforms in *N. benthamiana* that have been used to produce vaccine candidates on a much shorter timescale than can be achieved using standard approaches, for example, influenza vaccines produced by Medicago (Québec, Canada) can be produced in a few weeks compared to six months or more using chicken eggs. The potential of this rapid-response strategy was thrown into sharp focus when ZMapp™, an experimental cocktail of three chimeric monoclonal antibodies against Ebola
virus, was produced by transient expression and received FDA approval for emergency use during the 2014 West Africa Ebola virus outbreak (Na et al., 2015).

Transient expression systems based on infiltration are also compatible with glyco-engineering to achieve the precise control of protein glycosylation. Even though plant-specific glycans do not have proven adverse effects in humans (Shaaltiel and Tekoah, 2016), glyco-engineering can be used to design product-tailored glycan profiles that increase efficacy or longevity, or simplify downstream processing (see Chapters 4 and 8). For example, six genes have been co-expressed to achieve human-like glycan modifications in plants (Castilho et al., 2013). The trend is now moving towards the infiltration of stably engineered host plants with customized post-translational modification capacity to simplify production and improve batch-to-batch product consistency.

In addition to the transgenic rice and barley platforms discussed above, transgenic tobacco plants have continued to feature in the development of molecular farming. The role of tobacco plants in the current molecular farming landscape was strongly influenced by the success of the EU Pharma-Planta project, a publicly-funded international research program launched in 2004 aiming to take a candidate molecular farming product all the way through development culminating in a phase I human clinical trial (Ma et al., 2015; Sack et al., 2015b). After selecting the Human immunodeficiency virus (HIV)-neutralizing human monoclonal antibody 2G12 as a primary target, the consortium developed an entire GMP production process in concert with the European Medicines Agency (EMA) and tested the resulting product in a phase I safety trial. The negotiations with the regulators produced new guidelines for the manufacturing of pharmaceuticals in transgenic tobacco and paved the way for additional projects using this production host (Sparrow et al., 2007).

1.3 Recent Developments in R&D and Commercialization

As stated above, the molecular farming landscape is characterized by diverse host species, platforms, and technologies but most products fall into one of three categories, which are explored in the following sections. Covering every single product would require an entire book in itself so we have elected to focus on a smaller number of relevant case studies. However, Figure 1.1 provides an overview of the current state of play and identifies which platforms are primarily associated with which types of product.

1.3.1 Antibodies

Molecular farming began with the expression of a recombinant antibody in tobacco (Hiatt et al., 1989) and many of the early molecular farming studies considered different types of antibodies including whole immunoglobulins, antibody fragments, and various antibody fusion proteins (Fischer et al., 2003). Antibodies provided a useful foundation for technology development because researchers were reasonably assured by earlier studies that the expression of most antibodies would be successful, and this allowed the exploration of parameters such as protein targeting, different antibody formats, and different applications ranging from pharmaceutical production to the use of antibodies to prevent plant diseases (Safarnejad et al., 2011). A decade elapsed before any antibody PMPs reached clinical development and the first three product
candidates enjoyed mixed success. The first product candidate was Avicidin, a full-length IgG recognizing the colorectal cancer marker EpCAM. This was produced in transgenic maize and developed as a cancer treatment by Monsanto (Creve Coeur, MO, USA) but was withdrawn from phase II trials in 1998 due to side effects, which were unrelated to its production in plants (Fischer et al., 2013). The second candidate was CaroRX, a chimeric secretory IgA/G produced in transgenic tobacco plants indicated as a prophylactic for the prevention of dental caries. The antibody recognizes Streptococcus mutans adhesin, which is required for the bacteria to colonize the tooth surface. Because this product was developed for topical oral application (in toothpaste or mouthwash) the easiest regulatory path was to register it as a medical device rather than a pharmaceutical product (Ma et al., 1998). Finally, the former Large Scale Biology Company (Vacaville, CA, USA) produced a series of single-chain variable fragment (scFv) products in tobacco using TMV vectors. These were developed as personalized therapies for patients with non-Hodgkin’s lymphoma. When administered to mice, the scFvs stimulated the production of anti-idiotypic antibodies capable of recognizing individual lymphomas, and on that basis 12 such personalized antibodies were developed for human patients in the early clinical trial. Although Large Scale Biology Company has ceased trading, the anti-idiotypic scFvs are still under
development along with related products by Icon Genetics in concert with Bayer Pharma AG (Wuppertal, Germany).

The relatively slow uptake of antibody PMPs in part reflects the excellent track record of CHO cells as the gold standard for antibody manufacturing and the understandable reluctance of the biomanufacturing industry to consider an untried technology with an unsure regulatory footing. Until the last few years, only a handful of further antibody PMPs reached clinical development, including one produced in duckweed by Biolex Therapeutics, and the aforementioned Pharma-Planta tobacco-derived 2G12. This unfavorable situation may well have persisted given the hegemony of CHO cells were it not for the leap forward brought about by the realization that transient expression not only allows antibody manufacturing on a much greater scale than CHO cells, but also achieves production-scale manufacturing within a matter of weeks and allows the production of several different antibodies in one greenhouse, providing an economical way to produce antibody cocktails. This was explored with the cocktail MAPP66, a combination of antibodies envisaged as a form of pre-exposure prophylaxis against *Herpes simplex virus* (HSV) and HIV, produced by Magnifection in tobacco by Icon Genetics and Bayer Pharma AG. As stated above, however, the breakthrough came with ZMapp, the three-antibody cocktail for the post-exposure treatment of Ebola virus disease. This was produced by transient expression as an emergency response because no other platform was quick enough, and was administered to seven patients, five of whom survived. The life-saving capabilities of molecular farming have thrust the technology into the spotlight. Current R&D activities focus on the expression of secretory IgAs and the production of inhibitory antibodies against challenging pathogens such as Ebola virus, dengue virus, West Nile virus, poliovirus, rabiesvirus and *Plasmodium falciparum*, the parasite responsible for the most severe form of malaria. These antibodies are intended as emergency treatments against emerging or multidrug-resistant strains, or for post-exposure therapy, or short-term prophylaxis. Another recent development is the expression of antibodies in the context of novel immune complex mimics (ICMs), a strategy discussed in more detail below.

### 1.3.2 Vaccines

Whereas antibodies share a similar basic structure which ensures a reasonable likelihood of successful expression in plants and allows the use of generic purification strategies (at least for full-size variants that retain the constant region), vaccine candidates are highly diverse and have to be engineered individually for each pathogen, not only to present protective epitopes, but also to be stable and sufficiently immunogenic. In this context, the variety of different molecular farming hosts and expression strategies is an advantage. For example, the multivalent presentation of antigens on plant virus-like particles (VLPs) can enhance immunogenicity, the accumulation of vaccine candidates in seeds provides a cost-efficient solution for long-term storage, and the accumulation of antigens in the subcellular compartments of edible tissues achieves bioencapsulation, thus delaying digestion and prolonging contact between antigens and gut-associated lymphoid tissues. In contrast to antibodies, which have mostly been expressed in tobacco-based systems, vaccines are much more likely to be expressed in edible tissues, particularly cereal seeds, potato tubers,
fruits, and fresh salad leaves. The first plant-derived vaccine candidates to enter clinical development were transgenic lettuce leaves and potato tubers expressing the *Hepatitis B virus* (HBV) surface antigen (Kapusta et al., 1999; Richter et al., 2000), transgenic potatoes expressing Norwalk virus capsid protein (Tacket et al., 2000), transgenic potatoes and maize expressing the enterotoxigenic *E. coli* labile toxin B-subunit (Tacket et al., 1998; Tacket et al., 2004), and virus-infected spinach producing rabiesvirus glycoprotein (Yusibov et al., 2002). In these early trials, there was no need for the products to meet GMP standards for phase I trials and preparation for clinical testing was therefore more straightforward than it is today. All the above-mentioned trials were successful in that the vaccines were deemed safe and elicited serum or secretory antibody responses against the antigen. Many subsequent vaccine candidates have been produced as fusion proteins with the *E. coli* labile toxin B-subunit or cholera toxin B-subunit (CTB) because these act as inbuilt adjuvants (Chan and Daniell, 2015; Topp et al., 2016).

As discussed previously for antibodies, the use of plants for the production of vaccines has really taken off with the development of transient expression systems. This reflects their ability to reach production scale for new vaccine candidates only weeks after a new pathogen variant is discovered, making them suitable as a response strategy to emerging epidemics and pandemics and even bioterrorist threats (D’Aoust et al., 2010). Medicago has produced vaccines against H1N1 and H5N1 influenza within three weeks of receiving the hemagglutinin and neuraminidase gene sequences (Landry et al., 2010; Pandey et al., 2010), and Fraunhofer CMB has achieved the same objective for vaccines against strains H3N2, H5N1, and H1N1 with yields of 50–200 mg/kg fresh leaves (Shoji et al., 2008; Shoji et al., 2011). Plants may also be ideal for the production of vaccines against poverty-related diseases like malaria, focusing on the expression of pseudovirions and VLPs (Jones et al., 2013; Pillet et al., 2016; Pillet et al., 2015). Another recent development is the co-expression of vaccine antigens and antigen-specific IgGs to generate self-adjuvanting ICMs with superior immunogenicity, which may be particularly suitable for mucosal boosting strategies as well as in primary vaccination scenarios (Pepponi et al., 2014).

### 1.3.3 Replacement Human Proteins

The third major category of PMPs is replacement human proteins, which can be divided into two groups based on production objectives – those with a high demand because they are blood products (such as human serum albumin) or replacement proteins for fairly common diseases (such as insulin for diabetes and gastric lipase for cystic fibrosis), and those with a low demand because they are required as replacement therapies for orphan diseases (such as glucocerebrosidase for Gaucher’s disease) or they are growth factors/cytokines used in minute amounts (such as interferons). The high-demand proteins are ideal for molecular farming in transgenic plants because there is a large demand and the market would benefit from the promise of large-scale production. Examples that have reached clinical development include gastric lipase and lactoferrin produced in maize by Meristem Therapeutics SA (Clermont-Ferrand, France) and insulin produced in transgenic safflower by SemBioSys Genetics Inc. (Calgary, Canada). Neither company is still trading,
although Meristem’s intellectual property was acquired by Ventria Bioscience, and lactoferrin is now one of their key products. The low-demand products are suitable for production in mammalian cells as well as plants, so the molecular farming products that have reached clinical development and even the market have exploited another benefit of plants, that is, their glycan structures. For example, Biolex Therapeutics produced Locteron, a biobetter version of interferon α2a that is more efficacious due to the presence of plant glycans. Similarly, the first approved PMP for human use (taliglucerase alfa, marketed as Elelyso®), a recombinant human glucocerebrosidase produced in carrot cells by Protalix Biotherapeutics, benefits from the absence of sialic acid residues on the glycans, which allows the direct uptake of the protein by macrophages, the predominant cell type affected in the target disease – type 1 Gaucher disease. In contrast, the recombinant version produced in CHO cells (Imiglucerase) must be trimmed in vitro to remove the sialic acid residues, which increases the costs of production. The approval of taliglucerase alfa was accelerated due to its inclusion under the terms of the Orphan Drug Act 1983.

1.4 Commercial Products and Platforms

Over the last five years, molecular farming has consolidated around three types of platforms, which provide distinct sets of advantages in addition to the general merits of plants. First, cell suspension cultures/aquatic plants in containment are similar in concept to microbial and mammalian cells and have generally the same benefits in terms of controlled production, but plant cells combine the inexpensive media of microbes with the ability to produce complex proteins like mammalian cells. These platforms are ideal when glyco-optimization produces biobetter versions of products already produced in other platforms, such as Elelyso and Locteron. Second, transient expression platforms provide short production timescales and rapid scale-up, making them particularly suitable for the large-scale production of vaccines particularly in the context of an emergency response (such as ZMapp for the treatment of Ebola virus disease), but also for the small-scale production of personalized medicines (such as the scFv anti-idiotype antibodies for non-Hodgkin’s lymphoma) where investment in large-scale facilities would not be feasible. Finally, transgenic plants have the benefit of virtually unlimited scalability (Buyel et al., 2016), particularly when grown in fields like the crops developed by Ventria Bioscience and ORF Genetics. Transgenic plants are ideal for the production of high-volume/low-margin products such as bulk enzymes and hormones (e.g. gastric lipase and insulin) and antibodies and vaccines for diseases with a large affected population, particularly diseases of poverty such as HIV/AIDS, malaria, and tuberculosis. Transgenic plants are also the ideal vehicle for oral vaccines because the antigens can be expressed in edible tissues. As shown in Figure 1.2, which summarizes the distribution of molecular farming activities over the different platform categories and product classes based on the number of publications between 2010 and 2016, transient systems based on N. benthamiana dominate the R&D landscape in the field of vaccines and antibodies, whereas bioreactor-based carrot cells and moss systems focus on enzyme production. Although there are very few products, publications reporting pre-clinical and clinical research on serious
product candidates are distributed over the whole field, indicating a drive toward commercialization for all types of platforms and products.

### 1.5 Downstream Processing and Infrastructure

We conducted a literature search using PubMed (http://www.ncbi.nlm.nih.gov/pubmed/; access date: October 10, 2017) in order to compile the latest developments in the downstream processing of PMPs, from January 2011 to October 2017. The search terms “plant downstream processing” and “plant recombinant protein purification” yielded 627 and 4870 hits respectively, but only 81 were relevant (less than 1.5%). In contrast, the search term “cell culture downstream processing” yielded only 418 hits, but the frequency of relevant articles was much higher (~26%). The discrepancy probably reflects the multiple definitions of plant, which not only refers to crops but also to factories and heavy machinery, as in the term “manufacturing plant”. This dual use provides ample scope for confusion.

We tested our initial search strategy to see if landmark publications in the field were included, and found that several (e.g. Wirz et al., 2012 and Holtz et al., 2015) were not covered by the search terms even though both publications are listed in PubMed and represent major achievements for the PMP community. Looking at the keywords in the two publications, we found that instead of “downstream processing” or “purification” they mentioned “manufacturing” or “plant factory” and indeed “molecular farming/pharming”. Other relevant publications were not found because the journals were not indexed by PubMed (e.g. Buyel and Fischer, 2014) or because they did not mention plants as a generic production platform but stated the species of production host instead. Similar issues have been discussed for the development of strategies to search
online databases for patents relating to secondary metabolites produced in plants (Miralpeix et al., 2014). For these reasons, it is unlikely that a literature search can ever be comprehensive unless the PMP community voluntarily adopts standardized keywords to ensure that relevant articles are captured, for example, the terms “PMP manufacturing” or “PMP downstream processing” may be appropriate. Of course such a discussion will require an easily accessible online forum and the website of the recently

![Figure 1.3](attachment:image.png)

**Figure 1.3** Increase in the number of publications concerning the downstream processing of PMPs and the corresponding increase in manufacturing capacity. (A) The number of total publications (circles) and review articles (open squares) focusing on the downstream processing of PMPs has increased since January 2011 and we expect this trend to continue in the future. (B) Several pilot-scale facilities (<1000 kg biomass output per week) using whole plants were built before 2015, when the first process-scale facility became operational. Several companies have already announced or commenced additional projects to further increase their manufacturing capacity.
founded International Society for Plant Molecular Farming (ISPMF; http://www. societyformolecularfarming.org/) may prove ideal for this purpose.

Based on the literature coverage we achieved, we found that the number of publications in the field of PMP downstream processing (including reviews) has increased since 2011 with an all-time high for reviews in 2015 (Figure 1.3A), which is likely to be exceeded in the coming years. We interpret this trend to represent a continuously growing interest in PMP downstream processing, as well as the corresponding methods and infrastructure. We speculate that the growing interest reflects the maturation of different plant-based production platforms, which has shifted the focus of research from product selection and expression testing to purification and scale-up, facilitating preclinical and clinical development. This speculation is supported by the fact that pilot and process scale manufacturing capacities for PMPs have increased more than sixfold in the last decade (Figure 1.3B), with the first industrial-scale facility (>1000 kg biomass output per week) becoming operational in 2015. The major drivers in capacity building are currently iBio/Caliber Biotherapeutics, Fraunhofer IME/CMB, Kentucky Bioprocessing, and Medicago (mostly owned by Mitsubishi and Philip Morris). The latter has recently announced a ~ $USD 250 million project to build another industrial-scale facility by 2019. Companies such as ORF Genetics and PlantForm Corporation (Canada) may also be interested in similar facilities in the future, along with projects that have partial public funding such as a site being built at the John Innes Centre (UK). A technological cross-fertilization of such facilities dedicated to the production of PMPs with similar sites constructed for food production, for example, by Spread (Japan), seems appealing because PMP manufacturers may learn a lot from routine process and quality control tools that have already been established and well documented in the food industry (Caldwell, 2012; Haley and Mulvaney, 1995; McGrath et al., 1998).

Other indicators for the shift in focus toward effective and scalable process design for actual products may be the type of proteins that are reported in PMP downstream processing publications and the clarification methods used therein (Figure 1.4). When comparing reports from 2011 and 2016, we found that fewer model proteins are discussed in recent publications but more actual products, such as vaccines, enzymes, and monoclonal antibodies. We believe that this trend is highly beneficial for the PMP community. Model proteins are typically expressed at high levels and are highly soluble, whereas real products may be more challenging, with lower yields and the potential to interact with materials typically used during downstream processing, for example, diatomaceous earth in depth filters (Buyel et al., 2015). As a consequence, the efficiency of a downstream process may be overrated in terms of yield and recovery when evaluated using model proteins. Realistic cost estimates and process limitations will thus require the testing of real products. Ultimately, an increasing number of successful purification approaches will accelerate the evolution of PMP downstream processing, improving the economic competitiveness of the associated platforms compared to traditional expression systems.

In this context we found it interesting that filtration is becoming more common as an element of PMP downstream processing. Although filtration was not used as the major clarification step in any 2011 publications, it was the preferred method by 2016, whereas the opposite trend applied to centrifugation (Figure 1.4). Again, we consider these trends to represent the increasing degree of maturity and scale in the corresponding processes. Filtration and centrifugation may generally have an equivalent capacity to
remove dispersed particles from the process feed stream, but centrifugation is typically less prone to the adsorption of a protein product and is compatible with diverse samples. Centrifugation is therefore the method of choice for small-scale processes (laboratory bench scale) but is more difficult to scale up than filtration. The latter also requires less capital investment, has a smaller facility footprint, and importantly is most often a single-use technology, reducing cross contamination risks and cleaning validation costs (Pegel et al., 2011). Filtration is therefore more routinely used for clarification in larger-scale processes, as can also be seen for processes based on the CHO cell expression system. It will be interesting to see where these developments will lead, which other techniques from established expression systems or related industries will be adopted, and the production scales that can be established in the next decade.

### 1.6 Plant Matrix Encapsulation as an Alternative to Purification

Purified PMPs are currently the most advanced product candidates in terms of commercialization and fit more easily into the pre-existing regulatory system for biopharmaceuticals. However, they must compete directly with the equivalent products manufactured using other production systems, and until plants achieve the yields of microbes and mammalian cells they must rely on other advantages for a competitive edge, such as the glyco-optimization seen in the case of Elelyso. Another advantage is the bioencapsulation of PMPs in plant cells or organelles, so they are protected from acids...
and enzymes in the stomach but subsequently released into the gut lumen. Antigens delivered in freeze-dried carrot, lettuce, tobacco, and Arabidopsis cells have proven effective (Sack et al., 2015a). Mucosal delivery using partially processed plant tissues offers benefits for the administration of both human and veterinary pharmaceuticals and avoids the high costs of a complete downstream process (Chan and Daniell, 2015; Kwon and Daniell, 2016; Kwon et al., 2013b). For example, passive immunization has been achieved by the mucosal delivery of plant-derived antibodies against gastrointestinal pathogens (Virdi and Depicker, 2013). Prophylactic oral antibodies with high sporozoite-neutralizing activity against the coccidiosis parasite *Eimeria tenella* have been delivered in pea seeds (Zimmermann et al., 2009), and the protection of weaned piglets against enterotoxigenic *E. coli* infection has been achieved by the oral administration of Arabidopsis seeds producing IgA (Virdi et al., 2013).

Active immunity has been promoted by the delivery of plant-derived vaccines to mucosal surfaces because these can induce an immune response via gut-associated lymphoid tissues, leading to the production of pathogen-specific secretory IgA at the infection site (Lakshmi et al., 2013; Lee et al., 2015). Indeed, one of the first vaccines produced in transgenic plants was a maize-derived spike protein of *Swine transmissible gastroenteritis virus*, which protected pigs against the virulent pathogen (Lamphear et al., 2002). Orally-delivered lyophilized plant cells expressing poliovirus VP1 show promise as a booster vaccine (Chan et al., 2016). The internalization of the vaccine at mucosal surfaces can be achieved using enhanced cellular uptake strategies based on the utilization of receptor-binding proteins or cell-penetrating peptides (Kwon and Daniell, 2016).

Oral delivery can also be used to avoid the need for daily subcutaneous injections when treating autoimmune and inflammatory diseases because the presentation of plant tissues containing autoantigens exposes the antigens to gut associated lymphoid tissues. For example, insulin delivered in seeds can be designed to release functional C-peptides following ingestion (Boyhan and Daniell, 2011), and the glucagon-like peptide-1 agonist exenatide fused to CTB or transferrin can be encapsulated in lyophilized plant cells for oral delivery and this was shown to lower blood glucose levels when fed to mice (Choi et al., 2014; Kwon et al., 2013a). The delivery of heterologous allergens in plant tissues can also induce tolerance to allergies (Iizuka et al., 2014; Shenoy et al., 2014; Sherman et al., 2014; Shil et al., 2014).

Finally, it may even be possible to treat brain and ocular diseases by the oral delivery of plant-derived therapeutic proteins fused to the transmucosal CTB subunit, because such fusion proteins bind to GM1 receptors in the plasma membranes of the nervous system and retina and have been shown to cross the blood–brain and blood–retinal barriers in a mouse model (Kwon and Daniell, 2016).

The oral delivery of PMPs as plant tissues has recently been tested in the clinic. The Protalix Biotherapeutics enzyme Elelyso is an injected product but the same company is exploring the use of lyophilized carrot cells for the oral delivery of taliglucerase alfa in clinical studies because in vitro results demonstrate that the plant cells protect the recombinant protein in the gut and may facilitate absorption. Animal feeding experiments revealed that the active recombinant enzyme was found in the digestive tract and target organs in Gaucher’s disease, although the uptake was inefficient (Shaaltiel et al., 2015).

In a recent study, lettuce plants expressing clotting factor IX fused to CTB were grown under controlled conditions in the Fraunhofer IME GMP-compliant hydroponic facility
(Su et al., 2015). Lettuce cells containing the fusion protein were fed to a mouse model of hemophilia B resulting in the efficient delivery of the antigen to the gut immune system, thus suppressing an inhibitory response and anaphylaxis against factor IX. The Fraunhofer IME facility could yield 24,000–36,000 doses for pediatric care within 3–4 months, making commercial development feasible.

The plant cell wall provides an initial barrier to digestion but proteins can be further protected by ensuring that they accumulate in subcellular compartments such as plastids or seed storage organelles (Kwon and Daniell, 2016; Saeki et al., 2013). The process is particularly effective in cereal seeds that contain dedicated organelles for storage proteins, allowing oral vaccines to elicit both systemic and mucosal immune responses (Takaiwa, 2013). The entrapment and subcutaneous delivery of a vaccine antigen within ectopic storage organelles has been shown to increase the immune response (Hofbauer et al., 2016). PMPs tend to be stable when stored at ambient temperatures as lyophilized plant cells or dry seeds, thus avoiding the need for cold storage (Su et al., 2015).

Formulations containing whole cells or parts of genetically modified plants create new regulatory challenges but the first steps towards GMP-compliant production have already been taken. Formulations involving processed plant material such as flour paste or lyophilized preparations from fruits or cells must be established, and adapted quality control protocols are required. The number of oral PMPs is therefore likely to increase in the future because they address expensive purification, cold storage, and transportation costs while increasing the shelf life of current protein-based drugs without compromising safety or efficacy.

1.7 Perspectives and Opportunities for the Future

Recent developments in molecular farming show that the landscape remains dynamic. It features diverse platforms and products, but increasingly the field is focusing on products that can compete on the market. The current status of products that are undergoing preclinical and clinical development shows there is no dominant platform, but rather three broad classes of platforms with advantages and disadvantages, each with many variants that can be exploited to manufacture specific PMP products. Although this diversity is interesting at the R&D level the commercial impact is ambivalent. On one hand the diversity of platforms creates more freedom to operate and provides multiple licensing opportunities, whereas the absence of generic upstream and downstream infrastructure and processes introduces an entry barrier, especially for large pharmaceutical and biomanufacturing companies working with established expression systems.

In the near and mid-term future, the development of new molecular farming products will most probably be initiated by smaller companies addressing niche markets such as orphan diseases, personalized medicine, emergency treatments, and low-budget vaccines using novel approaches such as VLPs and IMCs to optimize or improve mucosal delivery. The option to generate products with minimal processing requirements for mucosal delivery, which increase efficacy or longevity, is another way to exploit the special features of plant systems. GMP-compliant production technology, infrastructure, and downstream processing knowhow are crucial determinants affecting the development of PMPs, defining how quickly they can enter clinical trials. Several molecular farming facilities are already operational or under construction and many
products are already in the pipeline. As is the case for other platforms, the capacity for manufacturing could be expanded by using contract-manufacturing organizations and the trend toward flexible facilities based on single-use equipment will make this more feasible. As the number of PMPs in clinical trials continues to increase, the regulatory authorities will become more familiar with the corresponding upstream production practices as well as downstream processing steps and how these can be transferred into GMP-compliant standardized operations as part of process development. As a result, the regulatory bodies have now issued guidelines for the production of PMPs under GMP conditions, including transgenic plants and transient expression systems, although in the latter case only in the USA.

Another exciting perspective is the rapid development of genome editing tools like zinc finger nucleases, transcription activator-like effector nucleases (TALENs) and the clustered regularly interspersed palindromic repeat (CRISPR)/CRISPR-associated (Cas9) system. These will simplify the knockout of certain unwanted functions and are clearly better than the random mutagenesis approaches and knockdown by RNA interference (RNAi) that have been used in the past. This genome editing toolbox will lead to the development of “off-the-shelf” plant lines and suspension cell cultures, the former particularly suitable for transient expression. These will be used to produce PMPs with defined glycan structures that precisely match requirements to improve safety, efficacy, serum stability, and (in the case of vaccines) immunogenicity.

Genome editing can also be used to generate stable transgenic lines either as production hosts in their own right or for the production of PMPs by transient expression. For example, transgenic plants could be designed to express the invariant secretory component and joining chain of the secretory IgA, and the variable heavy and light chains could be transiently expressed to simplify the production process. A similar strategy could be used to produce vaccine cocktails that require conserved as well as seasonal or strain-dependent components.

1.8 Conclusions

The formerly exotic universe of molecular farming has now become a fascinatingly dynamic and versatile mainstream technology that has the potential to take a significant segment of the market for the production of recombinant proteins, especially pharmaceuticals. This change reflects the recent development of serious production capacities, the greater focus on downstream processing, the emergence of high yielding transient expression platforms, and the targeting of niche products that take advantage of favorable or even unique features of the plant production host. The current pipeline of clinical trials shows that more PMPs are likely to reach the market in the future and that these will probably comprise a selection of recombinant antibodies, vaccines, and human replacement enzymes with a renewed focus on products for oral delivery. In the meantime, molecular farming also remains a profitable enterprise for non-pharmaceutical proteins, such as research-grade reagents and cosmetics ingredients, which enjoy the same diversity of production platforms without the regulatory burden associated with PMPs.
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