Editor Allison R. Kermode
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# MOLECULAR PHARMING

## Applications, Challenges and Emerging Areas

WILEY Blackwell

**Molecular Pharming**

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Applications, Challenges, and Emerging Areas

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# Preface

This book is of value to researchers that are in the field of plant molecular pharming, as well as those conducting basic research in gene expression, protein quality control, and other subjects relevant to molecular and cellular biology. The contribution conveys the excitement surrounding the present status of the field of plant-made pharmaceuticals and industrial proteins. Indeed this represents a time of increasing momentum after about a five-year hiatus, during which time strategies were being developed to overcome some of the technical hurdles of recombinant protein production in plants. The US Federal Food and Drug Administration approved the first plant-made pharmaceutical glycoprotein intended for human parenteral administration in 2012. The considerable strides toward overcoming the challenges associated with plant-based production of recombinant protein therapeutics have culminated in several plant-derived pharmaceutical proteins (antibodies, vaccines, human blood products, and growth regulators) reaching the stage of preclinical studies or commercial development. The target proteins of interest go beyond therapeutics, as plant hosts have advantages for the production of other valuable targets, such as food industry enzymes and other proteins of industrial relevance. The first part of this book introduces advances in different plant platforms and in strategies for improving the yields and controlling the post-translational modification of plant-made recombinant proteins. This logically leads into chapters that consider some of the high-value proteins that are being successfully made in plant hosts. This includes recombinant antibodies of diagnostic or therapeutic value, oral vaccines for protection against key human pathogens, and enzymes to treat rare childhood genetic diseases. These chapters include a consideration of plant-based platforms that are well established, as well as those which have recently emerged; it underscores both the key remaining challenges, as well as recent landmark successes. A chapter on regulatory issues of plant-based platforms completes the first two-thirds of the book. The remaining part of this book further earmarks this contribution as unique. Here the focus transitions toward small molecule therapeutics, drug screening, and plants as model organisms to study human disease processes. Mammalian neurotransmitters, receptor homologs, and certain early-stage biomarkers of disease processes are present in plant cells. Thus, the concept that plants are appropriate and important models for understanding some aspects of the pathophysiology of human diseases is advanced. This part of the book also emphasizes plant secondary metabolism as the basis for generating rich resources of small molecule therapeutics. There is a diversity of cellular proteins in humans that can be modulated by plant phytochemicals; these molecules are effective regulators of the immune response, signal transduction, mitosis, and

apoptosis (cell death). Flux through pathways of plant secondary metabolism can be manipulated, genetically or by other means, to up-regulate target bioactive molecules so that they are produced at higher levels in plants of importance for their pursuit as "leads". Small molecule libraries have been generated from plants and plant cells are viable systems for drug discovery. Thus, this book is a unique contribution that goes well beyond the use of transgenic plants as vehicles to host the production of recombinant proteins to cover some of the interesting new endeavors in the area of plant biotechnology.

# Part One

# The Molecular Farming/Pharming Landscape

# 1

# 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

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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 Magnifection 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


**Figure 1.1** The diversity of molecular farming platforms and products. This graphical overview shows the most important platforms and corresponding product categories based on an analysis of the literature from January 2011 to June 2016.

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-idiotype 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-idiotype 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

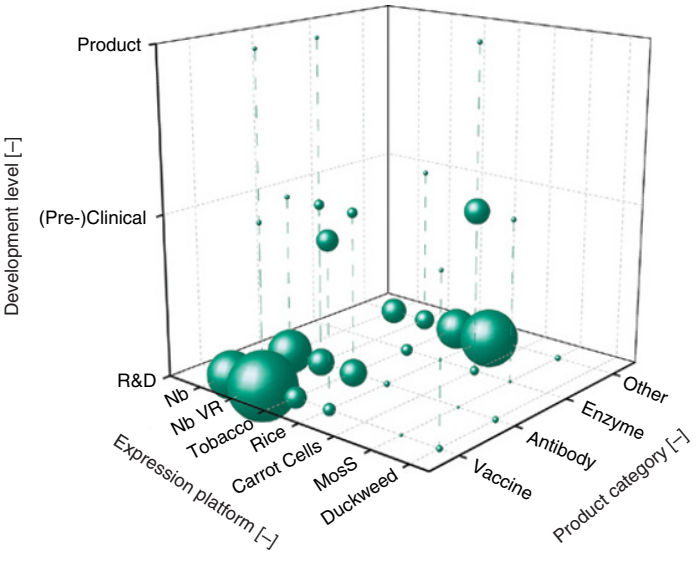


**Figure 1.2** Frequency of molecular farming publications according to the expression platform, product type, and development level. The spot radius is proportional to the number of publications from January 2011 to June 2016. Nb, *N. benthamiana*; Nb VR, *N. benthamiana* viral replicon.

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

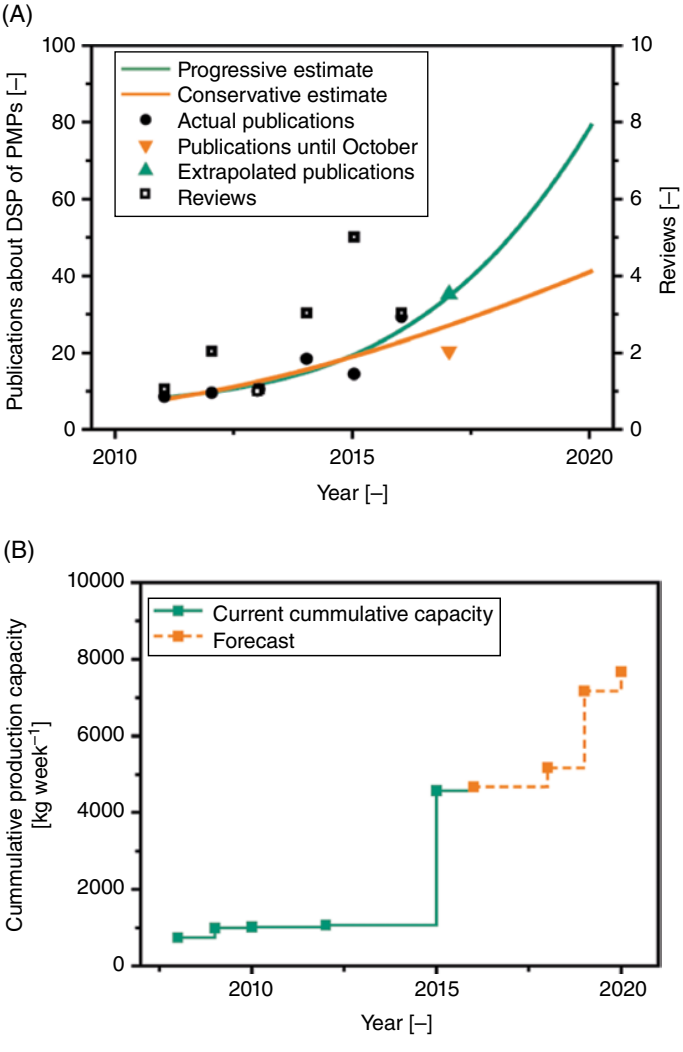


**Figure 1.3** Increase in the number of publications concerning the downstream processing of PMPs and the corresponding increase in manufacturing capacity. (A) The number of total publications (circles) and review articles (open squares) focusing on the downstream processing of PMPs has increased since 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