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Molecular farming – The slope of enlightenment

Rainer Fischer[a,b,c,*], Johannes F. Buyel[b,d]

[a] Indiana Biosciences Research Institute (IBRI), 1345 W. 16th St., Suite 300, Indianapolis, IN 46202, USA
[b] Institute for Molecular Biotechnology, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany
[c] Purdue Institute of Inflammation, Immunology and Infectious Disease, Purdue University, 207 S. Martin Jischke Drive, West Lafayette, IN 47907, USA
[d] Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Forckenbeckstrasse 6, 52074 Aachen, Germany

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ABSTRACT

Molecular farming can be defined as the use of plants to produce recombinant protein products. The technology is now >30 years old. The early promise of molecular farming was based on three perceived advantages: the low costs of growing plants, the immense scalability of agricultural production, and the inherent safety of plants as hosts for the production of pharmaceuticals. This resulted in a glut of research publications in which diverse proteins were expressed in equally diverse plant-based systems, and numerous companies were founded hoping to commercialize the new technology. There was a moderate degree of success for companies producing non-pharmaceutical proteins, but in the pharmaceutical sector the anticipation raised by promising early research was soon met by the cold hard reality of industrial pragmatism. Plants did not have a track record of success in pharmaceutical protein manufacturing, lacked a regulatory framework, and did not perform as well as established industry platforms. Negative attitudes towards genetically modified plants added to the mix. By the early 2000s, major industry players started to lose interest and pharmaceutical molecular farming fell from a peak of expectation into a trough of disillusionment, just as predicted by the Gartner hype cycle. But many of the pioneers of molecular farming have refocused their activities and have worked to address the limitations that hampered the first generation of technologies. The field has now consolidated around a smaller number of better-characterized platforms and has started to develop standardized methods and best practices, mirroring the evolution of more mature industry sectors. Likewise, attention has turned from proof-of-principle studies to realistic techno-economic modeling to capture significant niche markets, replicating the success of the industrial molecular farming sector. Here we argue that these recent developments signify that pharmaceutical molecular farming is now climbing the slope of enlightenment and will soon emerge as a mature technology.

1. Introduction

Molecular farming can be defined as the production of recombinant proteins[1] in plants, where the aim is to recover and utilize the protein product rather than the plant itself (Ma et al., 2003; Stoger et al., 2014; Tschofen et al., 2016). The target protein is either extracted and purified or used as part of a crude extract, whereas the plant is merely a host and is either discarded and destroyed at the end of the process or utilized as a separate side-stream (Buyel, 2019). Molecular farming often involves the use of whole terrestrial plants such as tobacco and cereals, but the technology also encompasses other plant-based systems, including plant cell and tissue cultures (Santos et al., 2016), aquatic plants (Everett et al., 2012), moss (Decker and Reski, 2012), algae (Rosales-Mendoza et al., 2012), and even in vitro transcription and translation systems derived from plant cells (Buntru et al., 2014). The diversity of these plant-based systems means that molecular farming comprises a range of different platforms that have the potential to compete in many different markets, ranging from technical enzymes and research reagents that are typically produced in bacteria and yeast, to biopharmaceutical proteins that are usually produced in mammalian cells, particularly Chinese hamster ovary (CHO) cell lines (Schillberg et al., 2019).

The ability to compete across different markets reflects the specific advantages of the individual plant-based systems. For example, transgenic plants are inexpensive and massively scalable compared to CHO cells (Buyel et al., 2017), transient expression systems allow production to be scaled up much more quickly than any fermenter-based platform (Hiatt et al., 2015; Holtz et al., 2015), and plant cells offer the ability to

* Corresponding author at: Indiana Biosciences Research Institute (IBRI), 1345 W. 16th St., Suite 300, Indianapolis, IN 46202, USA.
E-mail address: rfischer@indianabiosciences.org (R. Fischer).

[1] The term may sometimes include small molecules in addition to proteins, but only proteins are considered in this article.

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produce uniquely tailored glycan structures (Schillberg et al., 2017; Fischer et al., 2018). All plant-based systems can be considered intrinsically safer than mammalian cells for pharmaceutical products because they do not support the replication of mammalian viruses (Hundleby et al., 2018). They also address consumer demands for products that are 'certified animal free' (Spiegel et al., 2018). Despite these advantages, molecular farming in plants has not supplanted the current generation of industrial recombinant protein manufacturing technologies and only a handful of products have reached the market (Fischer et al., 2014; Schillberg et al., 2019). In this review article, we consider the reasons for this slow progress, evaluate the latest generation of molecular farming platforms from an economic perspective, and predict how the technology may evolve in the future.

2. First-generation molecular farming: hope or hype?

Molecular farming was born following the publication of an article in *Nature* describing the production of a functional recombinant antibody in tobacco plants (Hiatt et al., 1989). This was soon followed by an article in *Bio/technology* (later rebranded *Nature Biotechnology*) in which functional human serum albumin was produced in tobacco and potato plants as well as tobacco suspension cells derived from the transgenic tobacco line (Sijmons et al., 1990). These pioneering studies can be considered as the technology trigger which led to an explosion of proof-of-principle studies in which a vast range of different plant species and platforms were used as production hosts (Twyman et al., 2003; Spiegel et al., 2018). Soon it became apparent that these studies fell into two major although not entirely separate camps, one involving the use of many different platforms to produce candidate pharmaceutical proteins – mainly antibodies and vaccines, but also replacement blood products and enzymes (Fischer and Emans, 2000) – and the other involving the use of primarily cereal seeds for the production of technical enzymes and protein-based research reagents (Hood, 2002). From the beginning, these two camps, hereafter described as pharma and non-pharma, focused on different priorities. The pharma camp was interested in proof of principle and the broad capabilities of plants to produce functional proteins for medical applications, whereas the non-pharma camp immediately seized upon the commercial potential of technical products and focused on process economics, including the development of efficient downstream processing (DSP) strategies (Buyel et al., 2015; Fischer et al., 2012). A survey of the industry landscape in 2005 revealed that at least 50 companies had been founded in an attempt to capitalize on molecular farming, most focusing on the pharma sector (Twyman et al., 2005).

The rush to launch startup companies to capitalize on new technology is one of the key features of the Gartner hype cycle, which is depicted in Fig. 1. The first part of the cycle (which is not actually a cycle but more a technology evolution timeline) involves a *technology trigger* which leads to early pre-commercial activity, leading in turn to a gradient of expectations, often boosted by media speculation. At the *peak of inflated expectations*, a dash of cold reality exposes certain limitations of the technology which precipitates a slide into a *trough of disillusionment*. Certainly the pharma camp fits this profile well. Molecular farming for the production of pharmaceutical proteins (sometimes described as *molecular pharming*) was in hindsight promoted as a panacea before the technology was sufficiently established and mature. For example, the diversity of molecular farming platforms was presented as an advantage, allowing the platform to be tailored to the product rather than forcing the product on one of the small number of favored platforms used by industry. However, the reliance of industry on a small number of platforms (predominantly the bacterium *Escherichia coli*, certain yeast species, and a selection of mammalian cell lines, especially CHO cells) reflected the natural maturation of recombinant protein production technology and the consolidation of expertise into platforms around which the regulatory framework had evolved. Rather than offering industry a disruptive technology based on greater choice, greater scalability, lower costs and enhanced safety, molecular farming instead raised the alarming prospect of an entrenched industry having to accommodate a different way of thinking about recombinant protein production by adopting a new set of platforms without a track record and without any regulatory framework. Understandably, industry largely abandoned pharmaceutical molecular farming after some initial curiosity and fell back on the trusty microbial and mammalian cells that had served well for decades, despite their limitations. The early pharmaceutical molecular farming bubble collapsed, many of the startups ceased trading or revised their business strategies, and the world went on much as before. Or did it?

Although the molecular farming of pharmaceutical products reached a peak of inflated expectations in the early 2000s, the non-pharma camp was enjoying quiet success. The key player was the company Prodigene, Inc. (College Station, TX, USA), which worked on the development of maize lines producing technical reagents and industrial enzymes (Hood et al., 1997; Kusnadi et al., 1998; Witcher et al., 1998). They initially selected avidin, which is normally extracted from hens' eggs. Because there was an existing market for the product, the company focused on the economics of their new production process, specifically the product yield (as a proportion of plant biomass) and stability during processing. Indeed, Prodigene was the first company to consider the DSP aspects (and costs) of molecular farming in detail. The yield of the avidin product was 230 mg per kg of seed, it was structurally indistinguishable from egg avidin, and remained completely stable under the maize processing conditions used by the company, making it directly competitive with the existing avidin from eggs (Hood et al., 1997). Prodigene also produced β-glucuronidase, which likewise was shown to be structurally near identical to its natural counterpart and stable during processing, with a yield of ~80 mg per kg of seed (Witcher et al., 1998). The company also developed a plant-based version of recombinant trypsin, and even branched into the development of pharmaceutical products such as the *E. coli* heat-labile toxin as a vaccine candidate, having established an economic process for product recovery (Lamphear et al., 2002).

The techno-economic focus of Prodigene and other companies in the same space, such as SemBioSys Genetics (Calgary, Canada) working with safflower, Ventria Bioscience (Fort Collins, CO, USA) working with rice, and ORF Genetics (Kópavogur, Iceland) working with barley, led to the first commercial successes in the late 1990s. Prodigene's initial technical products were picked up and marketed by Sigma-Aldrich Fine Chemicals (St Louis, MO, USA) whereas SemBioSys Genetics, Ventria Bioscience and ORF Genetics developed strategies to market their cosmetic ingredients and research reagents. Ventria created a department for this purpose (InVitria) while also working independently on pharmaceutical products, ORF Genetics came to an agreement with Sif Cosmetics to market products containing ORF ingredients, and SemBioSys formed a subsidiary (Botaneco Specialty Ingredients) to commercialize cosmetic, personal care and dermatology products under the brand name Hydresia. In other words, the hype cycle that affected pharmaceutical molecular farming did not taint the camp working principally on technical and cosmetic products because they had done their homework and looked at the commercial potential of their platform from the outset. But trouble was nevertheless looming on the horizon…

3. Negative press

Although the use of cereals to produce technical enzymes and reagents made commercial sense, the early pioneers of this technology were about to be pushed into the trough of disillusionment by a perfect storm of unexpected events. In 2002, ProdiGene found itself at the center of a highly-publicized debate about protocols to contain pharmaceutical crops produced in the field (Hundleby et al., 2018). The widely reported case in Nebraska involved volunteer transgenic maize plants expressing recombining avidin, which grew among soybean

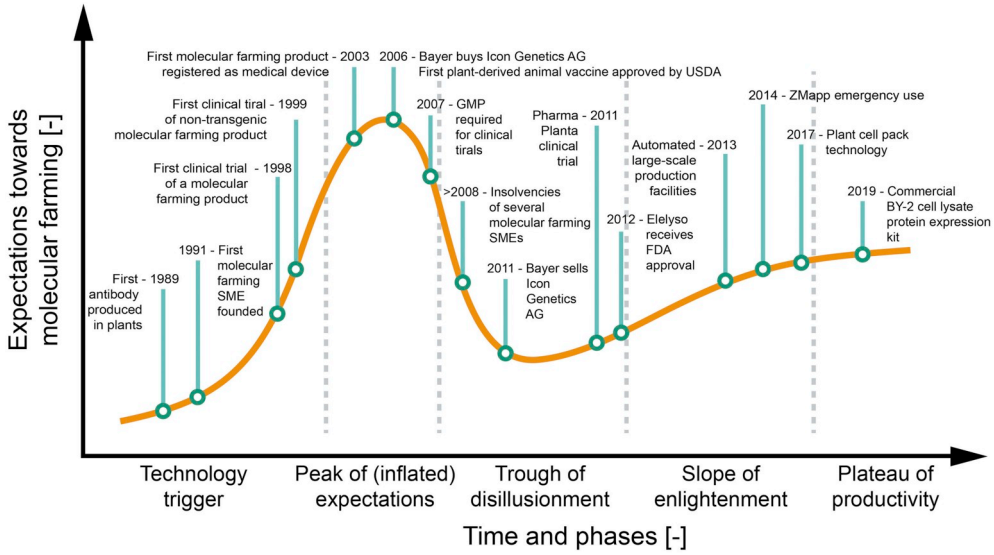

Fig. 1. The Gartner hype cycle applied to pharmaceutical molecular farming, with key events in the recent history of this field highlighted and dated.

plants cultivated the following year. When maize plant material was identified in the soybean crop, soybean grain in the storage silos was impounded and destroyed. In the resulting settlement, Prodigene accepted a $250,000 civil penalty, and agreed to pay a further $3 million for the quarantine and ultimate destruction of 500,000 bushels of soybean,[2] plus the cost of cleaning all the facilities and equipment. In a second incident in Iowa, the avidin maize line cross-pollinated with maize in an adjacent field, requiring 150 acres of potentially contaminated maize to be destroyed. These cumulative costs and fines caused the company to go into liquidation, and precipitated a tightening of the regulations compared to those applied to conventional genetically modified (GM) crops (Spök et al., 2008; Hundleby et al., 2018).

The fall of Prodigene in the USA coincided with a rising tide of public opinion against GM crops in Europe, with global implications as countries trading with Europe started to adopt similar practices. The GM industry ultimately stabilized into zones of broad consumer acceptance (the USA, South America, China and India) and zones where the cultivation of GM crops is effectively banned (Europe), with other countries adjusting their policies according to their political and trading alignments. Despite the efforts of researchers and companies to draw a boundary between conventional GM food/feed and the special status of molecular farming, the field of molecular farming was nevertheless drawn into the escalating conflict between the food industry, regulators, environmental activists, media, politicians and public. Researchers and business owners who until the mid-2000s were eager to promote the economic benefits of plants were now scrabbling to clarify the measures taken to protect the environment and the food/feed chain. Pharmaceutical molecular farming became an indoor pursuit and even the molecular farming of technical reagents was largely restricted to greenhouses and other containment facilities, with only Ventria Bioscience still growing its production crops outdoors. This company grows rice, which is self-pollinating and has no wild relatives in Colorado, providing a geographical form of isolation which fulfils the need for containment. SemBioSys Genetics was another casualty of the first wave of molecular farming, despite its success in the non-pharma field and its development of several pharma products, particularly a biosimilar insulin that reached phase I/II trials, and Apo AI (Milano).

A fascinating insight into the strategic decision making by some of the key players in the early development of pharma and non-pharma molecular farming (including Ventria, ORF Genetics and SemBioSys) is provided in the series of interviews with executives from 16 companies conducted byPaul et al. (2015). This sets out the insider view of the reasons for commercial success and failure, and offers a perspective not only from the viewpoint of the small startups offering molecular farming technologies, but also some of the large industry players that were initially tempted by this disruptive technology, or ultimately left with its legacy and the decision to continue investment or abandon it all together.

4. Beyond the trough of disillusionment

After the setbacks of the 2000s, the surviving molecular farming companies began to regroup. Again, there was a difference between the pharma and non-pharma camps, with the latter having an easier route to recovery because they had already penetrated significant markets. The regulatory burden and DSP costs of non-pharma product development were also much lower, a factor which persuaded ORF Genetics to remain firmly in the non-pharma camp (Paul et al., 2015). Several new companies have been founded or significantly expanded in the last 15 years to focus on the production of non-pharma growth factors and cytokines for non-medical/cosmetic use, including Agrenvec (Madrid, Spain) which uses plant viruses in tobacco, and Natural Bio-Materials (Jeollabuk-do, Korea) which uses rice cell suspension cultures. Other companies produce industrial enzymes, such as Infinite Enzymes (Jonesboro, AR, USA) and Origin Agritech (Beijing, China), in both cases using maize. As before, these newer companies are enjoying quiet success and have established themselves in key markets, in some cases because they offer 'certified animal free' research-grade reagents and cosmetic ingredients.

The molecular farming of pharmaceutical products has taken longer to recover and has faced a steeper learning curve. There are several reasons for this, which we will discuss in turn: the stricter regulation of pharma products and the changing regulatory environment; the corresponding inertia of industry which relies on fermentation infrastructure; the performance of plants compared to established platforms leading to the pursuit of best practices; and the techno-economic realities of pharmaceutical molecular farming. The distribution of molecular farming companies today and 15 years ago is compared in Fig. 2 and a 'then and now' snapshot of the industry is provided in Table 1.

[2] One bushel of soybean grain is equivalent to 27.22 kg (https://grains.org/markets-tools-data/tools/converting-grain-units/).

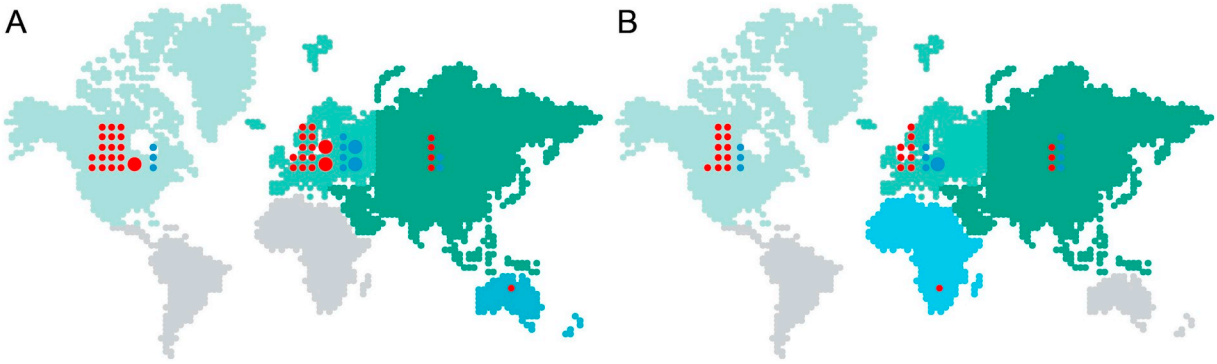

Fig. 2. World map showing the evolution of molecular farming companies from 2005 (A) to 2020 (B). On each map, large dots represent major industry players and small dots represent small/medium enterprises, stacked in the continent where principal operations take place. Red dots represent companies focusing on pharmaceutical products and blue dots are companies restricting their pipeline to non-pharma products, although the difference is not based on the product per se but rather on the intended use. The same product can be used for pharma and non-pharma applications, with pharma applications requiring a much more stringent production process that complies with pharmaceutical good manufacturing practice. For companies with subsidiaries in different continents, only the main company is counted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. The slope of enlightenment – a regulatory framework

The traditional biopharmaceutical industry has consolidated around a small number of platform technologies for multiple reasons, but one of the main ones is that this provides a simpler regulatory environment. Although the biopharmaceutical industry has several core production platforms (*E. coli*, yeast and mammalian cells), and a handful of others used more rarely, in almost every case the platform is based on the same principles: the cultivation of a genetically-defined cell line under precisely-specified physical and chemical conditions, and a defined process for product extraction and purification. Each step during upstream production and downstream processing can therefore be carefully controlled and regulated.

Bringing plants into this comfortable relationship was a disruptive innovation, and the important role played by the regulators in the maturation of pharmaceutical molecular farming cannot be overstated. Without a regulatory framework there is no industry confidence in the technology, but of course without some industrial take-up of the technology there is no impetus to develop a regulatory framework. The consequences of this Catch 22 situation were clearly laid out in the EU-funded framework project Pharma-Planta, which was launched in 2004 and took on the ambitious goal of developing a pharmaceutical product candidate from first principles all the way to phase I clinical trials, in this case the HIV-neutralizing antibody 2G12 produced in transgenic tobacco plants. The regulatory challenges facing the consortium were substantial and changed during the project, requiring compensatory changes in strategy:

(1) The challenges facing the consortium concerned the regulation of pharmaceutical products and the manufacturing process: specifically, the project aimed to develop a pharmaceutical product using a new production platform for which there was no existing regulatory framework. It is important to emphasize that there was never any intention to grow the tobacco plants in the open field, only in a contained greenhouse environment, so there was no need to consider the regulations governing the field cultivation of GM crops. However, the consortium needed to adapt the existing regulations for biopharmaceutical manufacturing to work with non-clonal plants as a production platform.
(2) When the project launched it was still possible to develop pharmaceuticals all the way to phase I clinical trials without using a process that complied with good manufacturing practice (GMP). These rules changed during the project and the consortium was suddenly faced with the need to develop a GMP-compliant production process from first principles, given that a new platform was involved.

The consortium learned quickly that the development of new regulations for plant-made pharmaceuticals was not going to be straightforward, and that orthodoxy was favored over innovation, resulting in various situations in which the regulators attempted to insert the square peg of tobacco plants into the round hole of the regulations developed for genetically consistent cell lines. One example was the intense negotiations required to avoid the need for master and working cell banks – a concept which makes sense for clonally propagated cells but not for sexually reproducing plants. This was eventually addressed by developing the concept of master and working seed banks. Ultimately, the Pharma-Planta project was able to obtain enough regulatory advice to guide the development of a GMP-compliant production process (Ma et al., 2015; Sack et al., 2015). Companies working on disruptive technologies generally do not discuss their interactions with regulators, partly to protect their intellectual property and partly because regulatory consultation is chargeable and there is an understandable reluctance to spend money on advice only to then pass it on for free. Pharma-Planta was a game-changer in this regard because it was a publicly-funded project, so the regulatory path it developed could be made available to all. The irony of a publicly-funded consortium paying a publicly-funded regulatory authority for advice was not lost on either the consortium or its EU project officers, and although the consortium was not exempt for the costs it was at least granted a significant discount. The consortium also expected to be advised on how to proceed and was somewhat taken aback to be 'treated like a company' and therefore required to bring proposals to the table which were given yes/no answers. In turn, the regulators were rather bemused by the consortium's request for instructive guidance rather than permission. The take home message was that the current regulatory system is designed for industry and not publicly-funded research, and more could be done to make the regulatory system more research friendly, including informal and instructive advice rather than yes/no decisions.

There are significant regional differences in current regulatory guidelines covering the production of proteins by molecular farming in whole plants. The original FDA recommendations were already flexible, accommodating all whole plant systems and those based on plant organs (FDA/USDA, 2002). In contrast, the original EMEA (now EMA) guidelines were largely unworkable, and even the post-consultation updates still exclude transient expression (EMEA, 2009). Several companies in the USA and Canada now base their business model on GMP manufacturing by transient expression in tobacco, but this is not possible in Europe (Tremblay et al., 2010; Whaley et al., 2011). The bumpy evolution of the regulatory landscape also goes some way to explaining

Table 1
Survey of the molecular farming commercial landscape, 2005 vs 2020.

Major industrial players								
Company	**Country**	**Continent[1]**	**Link[2]**	**Major product(s)**	**Major platform(s)**	**Comments**	**2005[3]**	**2020[4]**
BASF Plant Science	Germany	Europe		Cosmetic products	Maize and rice	Wide plant biotechnology portfolio, but acquired Crop Design (Belgium) as a subsidiary in 2006 along with its molecular farming platforms, focusing on personal care products marketed by Henkel (see separate entry). Currently does not have a molecular farming program.	Y	N
Bayer CropScience	Germany	Europe		Antibodies	Transient expression	Formed in 2002 when Bayer AG acquired Aventis CropScience (and by extension, Plant Genetic Systems). Focused on crop protection, but diversified into molecular farming by agreements with several SMEs (AltaGen, Biolex, LemnaGene) and its acquisition in 2006 of Icon Genetics (see separate entry). Relinquished molecular farming in 2011.	Y	N
Dow AgroSciences	USA	North America		Animal vaccines	Tobacco cell cultures	Developed a portfolio of plant-derived antibodies and vaccines, in partnership with Epicyte and Centrocor, co-developed the CONCERT plant cell platform in collaboration with Fraunhofer IME. Responsible for the first plant-derived (veterinary) vaccine to receive regulatory approval from the USDA although not marketed. Now Corteva Agriscience. No longer has molecular farming portfolio.	Y	N
Henkel	Germany	Europe		Cosmetic products	Maize and rice	Cooperation with Crop Design and BASF to produce personal care products.	Y	N
Hokusan	Japan	Asia		Canine α-interferon	Transgenic strawberry	Partnership with AIST. Product launched 2014 but not sold after late 2015.	N	N
Monsanto Protein Technology	USA	North America		Avicidin (antibody)	Transgenic maize	Division of Monsanto concerned specifically with biopharmaceuticals with a focus on the development of large-scale processes for the production of therapeutic proteins in maize. Collaborated with NeoRX to produce the Avicidin anti-EpCAM antibody for the treatment of colorectal cancer (withdrawn during phase II trials). Monsanto decided to withdraw from molecular farming in 2004.	N	N
Syngenta International	Switzerland	Europe	www.syngenta.com	Biopharmaceuticals;	Transgenic safflower	Formed in 2000 through the merger of Novartis agribusiness and Zeneca agrochemicals. Among many plant biotechnology products, Syngenta was involved in the development of at least six biopharma products with partners including SemBioSys Genetics. Ceased pharmaceutical molecular farming in 2012.	Y	N
				Amylase in biomass for ethanol production	Transgenic maize	Subsequently began to develop non-pharma molecular farming products.	N	Y
Synthon	Netherlands	Europe		Biopharmaceuticals	*Lemna minor*	Formed in 1991 as a conventional pharmaceutical company, began developing biopharmaceuticals in 2008. Acquired the Biolex LEX System in 2012. Sale included two antibodies. Ceased molecular farming activities in 2014.	N	N
SMEs								
Company	**Country**	**Continent**	**Link**	**Major product(s)**	**Major platform(s)**	**Comments**	**2005**	**2020**
Agragen	USA/ Finland	North America/ Europe		Biopharmaceuticals	Flax	Founded in 1995, focusing on the genetic improvement of flax, including the use of flax for molecular farming and other biotechnology purposes (e.g. biofuels).	Y	N
Agrenvec	Spain	Europe	www.agrenvec.com	Diagnostic antibodies and cytokines/growth factors as cell culture reagents	Transient expression using TMV vectors in *N. benthamiana*	Founded in 2001 as a spin-off from INIA (Agriculture and Food Research National Institute).	Y	Y
AltaGen/ Serologicals	USA	North America		Biopharmaceuticals	Transgenic plants (various species) and cell cultures	Formed in 2002 as a merger between Phytagenics and Sierra Biosource. Licensed protein expression technology to Bayer. Acquired in 2004 by Serologicals, and in 2006 by Millipore, which ceased molecular farming.	Y	N

(*continued on next page*)

Table 1 (*continued*)

Angany	Canada	North America	www.angany.com	Allergens as bioparticles	Transient expression in tobacco	Has French subsidiary for innovation.	N	Y
AntoXa Corp	Canada	North America	www.antoxacorp.com	Anti-ricin antibody	Transient expression in tobacco	A subsidiary of PlantForm.	N	Y
Applied Phytologics, Inc.	See entry under Ventria Bioscience							
Axis Genetics	UK	Europe		Vaccines	Transgenic potato, CPMV system	Formed in the 1980s after a management buyout. Focused on the production of vaccines in transgenic potatoes and in plants infected with recombinant CPMV. Liquidated in 1999 after failing to secure adequate funding.	N	N
BioApp	Korea	Asia	http://bioapp.co.kr/	Porcine virus marker vaccine	Transient expression in *N. benthamiana*	Founded in 2011, first product approved 2019.	N	Y
Biocem	France	Europe		Gastric lipase	Transgenic tobacco and maize	IP transferred to Meristem Therapeutics.	Y	N
Biolex	USA	North America		Locteron (α-interferon)	*Lemna minor* (LEX) platform	IP on LEX platform acquired by Synthon in 2012. Synthon ceased molecular farming work in 2014.	Y	N
Biosource Technologies Inc.	See entry under Large Scale Biology Corp.							
Cape Bio Pharms	South Africa	Africa	www.capebiopharms.com	Antibodies and enzymes	Transient expression in *N. benthamiana*	Established in 2014.	N	Y
Ceres	USA	North America		Biopharmaceuticals	Various	Major developer in all areas of plant biotechnology, including pharmaceutical production. Switched focus to biofuels and alternative energy crops. Acquired by Forage Genetics International.	Y	N
Chlorogen	USA	North America		HSA, vaccines, interferon, ILGF	Tobacco chloroplasts	Established 2002; dissolved 2007.	Y	N
Cobento	Denmark	Europe		Human intrinsic factor, transcobalamin	Arabidopsis	Website shut down 2009.	Y	N
CollPlant	Israel	Asia	www.collplant.com	Collagen	Transgenic tobacco	Established in 2004.	Y	Y
Crop Design	Belgium	Europe		Platform technology	Maize and rice	Founded in 1998 as a spin-off from the Flanders Interuniversity Institute for Biotechnology, focusing on protein expression technology in rice grains. Acquired by BASF in 2006, and operates as a subsidiary. Appears focused on trait development rather than molecular farming.	Y	N
CropTech Development	USA	North America		Uronidase, irunosidase, glucocerebrosidase and vaccines	Transgenic tobacco leaves. MeGA PharM system allows post-harvest expression.	Established in 1992, dissolved in 2003. IP transferred to Tobacco Ventures.	N	N
Diamante SRL	Italy	Europe	www.diamante.tech	Vaccines	Plant virus nanoparticles expressed in tobacco	A spinoff from the University of Verona.	N	Y
Epicyte Pharmaceutical	USA	North America		Antibodies	Technology development for any plant species	Founded in 1996. Acquired by Biolex in 2004.	N	N
ERA Plantech ERA biotech ZIP Solutions	Spain	Europe	www.zipsolutions.cat	Enabling technology	ZERA fusion, Splittera purification	ZERA tech developed for molecular farming in plants, now marketed for all production hosts.	Y	Y
Evogene	Israel	Asia		Enabling technology	Tomato trichome	Founded in 2002 as spin-off from Compugen. Appears to have abandoned molecular farming and focused entirely on traits after 2006.	Y	N
Farmacule	Australia	Australasia		INPACT expression technology	Tobacco, banana and sugarcane	Founded in 2001 as a spin-off from Queensland University of Technology. Focused on precision gene expression in transgenic plants using the INPACT regulated expression technology. Now Leaf Energy/Leaf Resources suggesting acquisition and change of strategy.	Y	N
GenoMine	Korea	Asia		Vaccines	Various	Founded in 2001. Main focus is genomic and proteomic technology. No longer developing molecular farming products.	Y	N
Greenovation	Germany	Europe	www.greenovation.com	α-galactosidase for Fabry disease, antibodies	Moss	Founded in 1999.	Y	Y

(*continued on next page*)

Table 1 (*continued*)

Guardian Biosciences	Subsidiary of Korean company Nexgen (see separate entry)							
Hayashibara	Japan	Asia		Edible vaccines	Various	At the forefront of Japanese biotechnology and marketing recombinant interferon since 1988. Diversified into plant-derived edible vaccines and related products but research program discontinued.	Y	N
iBio Inc/Caliber	USA	North America	www.ibioinc.com	Vaccines, antibodies, plasma products	Transient expression in tobacco	Formed 2009 after name change from iBiopharma, major collaboration with Fraunhofer CMB, CDMO from 2018.	N	Y
Icon Genetics / Denka	Germany	Europe	www.icongenetics.com	Vaccines	Transient expression in tobacco (Magnicon platform)	Formed in 1999, focusing on production platforms based on transgenic foliage and virus infected plants. Major innovation was the MagnICON system (magnifection) for rapid, high-level expression of proteins using virus vectors. This technology prompted acquisition by Bayer CropScience in 2006, and it is operated as a subsidiary before several reorganizations and eventual acquisition by Nomad Bioscience in 2012, then Denka in 2015 (see separate entry for Nomad).	Y	Y
Infinite Enzymes	USA	North America	www.infiniteenzymes.com	Enzymes	Transgenic maize seeds	Established in 2008.	N	Y
Intrucept Biomedicine	USA	North America	www.intrucept.com	Antimicrobial and antiviral proteins	Transient expression, tobacco	Formed in 2006. Development in collaboration with Kentucky Bioprocessing.	N	Y
Kentucky Bioprocessing	USA	North America	www.kentuckybioprocessing.com	Aprotinin	Transient expression, tobacco	Formed in 2006 using technology assets, infrastructure and key personnel from Large Scale Biology Corp., focusing on the production of proteins in plants infected with plant viruses (GENEWARE platform). Recombinant aprotinin for research use is the company's sole product, but has IP for pharma products. From 2014, part of Reynolds American. Contracted to produce ZMapp by Mapp Biopharmaceutical (see separate entry).	N	Y
Large Scale Biology	USA	North America		Aprotinin, antibodies, interferons	Transgenic tobacco	Formed in 1987 as Biosource Technologies Inc. Developed and built the world's first bioprocessing facility for plant-derived proteins, focusing on the production of proteins in transgenic plants and plants infected with plant viruses (GENEWARE platform). Ceased trading in 2006. Antibody products subsequently developed by Icon Genetics Interferon marketed by Sigma-Aldrich. Facilities taken over by Kentucky Bioprocessing.	Y	N
Leaf Expression Systems	UK	Europe	www.leafexpressionsystems.com	Contract protein manufacturing	Tobacco leaves with Hypertrans transient expression system	Based on expression technology from John Innes Centre.	N	Y
LemnaGene	France	Europe		Enabling technology	*Lemna minor*	Acquired by Biolex in 2005.	Y	N
LenioBio	Germany	Europe	www.leniobio.com	Enabling technology	Cell-free expression system	Combines technologies from Fraunhofer IME and DowDuPont (now Corteva AgriSciences).	N	Y
Maltagen	Germany	Europe		HSA, lactoferrin, lysozyme	Transgenic barley seeds	Website no longer online after 2010.	Y	N
Mapp Biopharmaceutical	USA	North America	www.mappbio.com	ZMapp	Transient expression in tobacco	Commercialization arm is called LeafBio.	Y	Y
MBP Cologne GmbH	Germany	Europe		Enabling technology	Potato and rapeseed	Formed in the 1999 as a spin-out from the Max Planck Institute, with a focus on the production of recombinant protein in potato tubers and rapeseeds. Liquidated in 2002 due to lack of funding.	N	N
Medicago	Canada	North America	www.medicago.com	Seasonal flu vaccine	Alfalfa until ~2005 then switch to transient expression in *N. benthamiana*	Formed in 1999. Strategic alliance with Mitsubishi Tanabe from 2013.	Y	Y
Meristem Therapeutics	France	Europe		Lipase, antibodies,, lactoferrin, collagen antitrypsin, lactoferrin	Transgenic tobacco and maize	Formed in 1997 and focused on large-scale processes for the production of therapeutic proteins in tobacco, maize, rapeseed, potato and other crops. Liquidated in 2008. IP portfolio sold to Ventria Bioscience in 2012.	Y	N
Natural Bio-Materials	Korea	Asia	www.nbms.co.kr	Trypsin, growth factors, cytokines (cosmetics)	Rice cell suspension cultures	Established in 2008.	N	Y

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Table 1 (*continued*)

NexGen	Korea	Asia	www.nexgenbiotech .com	Growth factors	Transient expression, tobacco	Formed in 1999, has North American subsidiary Guardian Biotechnologies Inc.	Y	Y
Nomad Bioscience/ Nambawan Biotech/ UAB Nomads	Germany/ Lithuania	Europe	www.nomadbioscience.com/	Vaccines	Transient expression in tobacco (Magnicon platform)	Acquired Icon Genetics from Bayer in 2012 and NHL product candidate in 2013. Sold 51% of shares in Icon to Denka along with IP for vaccines and diagnostics in 2015 and remaining shares in 2017 (see entry for Icon Genetics). Created Nambawan Biotech as wholly owned subsidiary in 2015 to commercialize healthcare product candidates other than vaccines.	N	Y
Novoplant	Germany	Europe		Veterinary antibodies	Potato tubers, peas, rapeseed, flax seed	Formed in 1998, no longer trading in 2006.	Y	N
ORF Genetics	Iceland	Europe	www.orfgenetics.com	Growth factors and cytokines (cell culture reagents) and cosmetics	Transgenic barley seeds	Formed in 2002. Distribution agreement with Sif Cosmetics for cosmetic products.	Y	Y
Origin Agritech	China	Asia	www.originseed.com.cn	Phytase (biomass)	Transgenic maize seeds	Founded in 1997.	N	Y
Pharmaplant	Germany	Europe	www.pharmaplant.com	Medicinal plant breeding	Medicinal plants	Metabolites only, no protein expression.	Y	Y
PhycoTransgenics/ PhycoBiologics	USA	North America		Enabling technology	Algal platform	Website closed 2007.	Y	N
Phytomedics	USA	North America		Alkaline phosphatase	REPOST platform (rhizosecretion) in tobacco	Website closed 2011.	Y	N
Phyton Biotech	USA	North America	www.phytonbiotech.com	Taxanes	*Taxus* spp	Not proteins only taxanes: 80,000 L plant cell cultivation.	Y	Y
Phytoprotein Biotech	Singapore	Asia		Vaccines and antibodies	Plant cell suspension cultures	Formed in 2000; website closed 2004.	N	N
Planet Biotechnology	USA	North America	www.planetbiotechnology.com	Antibodies and immunoadhesins	Transgenic tobacco leaves	Still active, but website not updated since 2015.	Y	Y
Plant Advanced Technologies (PAT)	France	Europe	www.plantadvanced.com	Bioactives and recombinant proteins	Milking plant roots and exudates	Formed in 2005 as University of Lorraine spinoff. Major platform is milking plant roots for bioactives, but PAT Friday technology involves producing proteins in the exudates of carnivorous plants.	Y	Y
Plantechno	Italy	Europe		Glucocerebrosidase, Apo-A1	Transgenic rice and tobacco seeds	Formed in 1995 as a spinout from Universitá Cattolica S. Cuore di Agraria Istituto di Botanica e Genetica. Website up till 2017 but news not updated since 2007.	Y	N
PlantForm	Canada	North America	www.plantformcorp.com	Trastuzumab	Transient expression in tobacco	Established in 2008.	N	Y
Plantigen	Canada	North America		GAD, cytokines	Transgenic tobacco leaves	Formed in 1999 by the London Health Sciences Centre in Canada. Website closed 2011.	Y	N
Planton	Germany	Europe		AMPs in potato	Potato tubers	Formed in 2001. No longer seems to be focusing on plants.	Y	N
PlantVax	USA	North America	www.plantvax.net	Enzymes, vaccines, antibodies	Unknown	Founded in 2007, produces recombinant enzymes commercially and collaborates for the production of other proteins.	N	Y
Prodigene	USA	North America		Avidin, trypsin, GUS, aprotinin	Transgenic maize seeds	Formed in 1996, and was the first company to commercialize proteins from transgenic plants (1998). Distribution agreement with Sigma-Aldrich. Dissolved in 2004 following fines and penalties for environmental breaches.	Y	N
Protalix Biotherapeutics	Israel	Asia	www.protalix.com	Glucocerebrosidase (replacement enzyme)	Tobacco cells (carrot cells used for Elelyso)	First FDA-approved pharmaceutical from plants, 2012. Partnered with Pfizer in USA.	Y	Y
Root Lines Technology / SamaBriva	France	Europe	www.samabriva.com	Lysosomal storage disease enzymes	Rhizosecretion and root cultures	RLT is a spin-off at the University of Picardy Jules-Vernes, formed in 2011. SamaBriva uses their RhizoBriva root culture platform.	N	Y
Quantum Tubers	USA	North America		Oral vaccines	Potato tubers	Website offline after 2014.	Y	N
SemBioSys	Canada	North America		Insulin, ApoA1	Safflower	Founded in 1994 as a spinout from the University of Calgary. Focused on the expression of pharmaceuticals and technical proteins in oilseeds (safflower) using proprietary oleosin fusion technology. Collaborated with Syngenta (see separate entry) to produce their front-line biological products. Formed spin off in 2007 to market cosmetics (marketed as the brand Hydresia) which later merged with Advitech. Short-lived agreement with Tasly Pharmaceuticals. Terminated operations in 2012.	Y	N

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Table 1 (*continued*)

SubTerra/Prairie Plant Systems	USA	North America		Underground cultivation	Legumes	SubTerra wholly owned by PPS Inc., with underground cavern in Michigan USA. PPS has two further sites in Canada. Rebranded in 2018 as CanniMed, now growing Medical Cannabis and no longer into molecular farming.	Y	N
Toxin Alert Inc	Canada	North America		Diagnostic antibodies	Unknown	Founded in 1998, focusing on food diagnostic products and the use of antibodies as diagnostic reagents. Investigated production capabilities in plants. Website no longer active in 2012.	Y	N
Transalgae	Israel	Asia	www.transalgae.com	Animal vaccines	Algae	Develops oral vaccines and insecticides expressed in transgenic algae and administered as biomass.	N	Y
UniCrop	Finland	Europe		Enabling tech	Camelina sativa	Website offline after 2007.	Y	N
Ventria Bioscience	USA	North America	www.ventria.com/	VEN120 (lactoferrin) for the treatment of inflammatory bowel disease	Transgenic rice seeds	Founded in 1993 as Applied Phytologics.	Y	Y
			www.invitria.com/	Growth factors and other cell culture reagents	Transgenic rice seeds	This is a division of Ventria for non-pharma products.	Y	Y
Vytrus Biotech/ Phyture Biotech	Spain	Europe	www.vytrus.com/	Cosmetic ingredients	Plant cells	Formed in 2009 as a spinoff from the University of Barcelona, renamed Vytrus in 2016. Initially focused on natural products, now includes molecular farming of proteins.	N	Y

[1]See continent map (Fig. 2).
[2]Links provided for companies still active in the molecular farming sphere in 2020.
[3]Data from Twyman et al. (2005).
[4]The colour coding refers to the major activities of each company (red = pharma, including veterinary; blue = non-pharma, including research reagents, diagnostics, cosmetics) used to assemble Fig. 2. Where companies are engaged in pharma *and* non-pharma activities, the prominent activity at the selected time point is indicated (except Ventria Bioscience, which has separate entities for pharma and non-pharma products and is therefore listed twice).

why the first pharmaceutical products of molecular farming were derived from plant cell suspension cultures, which are analogous in every way to CHO cells and could be accommodated under existing regulations (Tekoah et al., 2015). The orthodoxy was extended by the FDA to also include whole clonally propagated plants such as the duckweed *Lemna minor* (Everett et al., 2012) and likewise to moss and algae (Decker and Reski, 2012; Rosales-Mendoza et al., 2012).

6. The slope of enlightenment – consolidation

6.1. Overview

The consolidation of the biopharmaceutical industry around fermenter-based production and a very limited number of platforms (mainly *E. coli*, yeasts, and CHO cells, with a few products made in insect cells or transgenic animals) is not only a consequence of regulatory simplicity but also reflects the concentration of resources on compatible infrastructure and the accumulation of process knowledge. Put more simply, the biopharmaceutical industry has invested significantly in the same technology since the 1970s and it has served its purpose well. Incremental innovations that improve the performance of current technologies are welcome, but disruptive technologies that tear up the rule book and start again are greeted with extreme caution. Molecular farming falls into the latter category.

Even so, the molecular farming of pharmaceutical proteins is starting to follow the same pathway as the wider biopharmaceutical industry, and the process of consolidation and development of best practices has already begun. To some extent, this may also explain the earlier success of industrial molecular farming, which was initially restricted to cereal crops (Hood, 2002). Whereas the first wave of pharmaceutical molecular farming technologies was bewilderingly diverse, we now see a maturing portfolio that features three main platform types: plant cells and other clonal systems; transient expression platforms for rapid and scalable production campaigns; and transgenic plants for long-term production and scalability. Tobacco plants are the mainstay of the cellular and transient systems and are also a popular transgenic platform for pharmaceutical products, with cereals favored for non-pharma products (although this distinction is not strict, and is more to do with the way in which different companies have evolved to utilize particular platforms). For example, ORF Genetics and Ventria Bioscience use barley and rice, respectively, to produce a range of human proteins that are marketed as cosmetic ingredients and research reagents, but many of these same proteins (growth factors, cytokines and enzymes) could also be developed as pharmaceuticals and Ventria is developing various therapeutic products. Nevertheless, the focus on specific platforms has allowed the concentration of resources and further examples of innovation, which are discussed below.

6.2. Plant cell suspension cultures and other clonal systems

Plant cell cultures were at the forefront of pharmaceutical molecular farming because they bridged the gap between plants and existing cell-based production platforms, and could be accommodated with few changes to existing GMP regulations. It is therefore ironic that the two main advantages often attributed to molecular farming (the low cost of growing plants and the massive agricultural scalability) do not really apply to plant cells. Fermenter-based infrastructure is similarly expensive for microbes, plant cells and mammalian cells, so the up-front investment costs are of the same magnitude. The media for microbial fermentation is much less expensive than media for the growth of mammalian cells, with plant cells somewhere in the middle. Plant cells can also have certain disadvantages, such as slower growth compared to microbes, and the presence of a dominant vacuole which increases the size and volume of plant cells without increasing their productivity. Accordingly, the typical dry/wet cell mass of plant cells is ~20/400 g L^{-1} (Holland and Buyel, 2018) compared to ~200/400 g L^{-1} for bacteria (Bratbak and Dundas, 1984; Shiloach and Fass, 2005) and ~60/200 g L^{-1} for yeast (Kastilan et al., 2017).

Tobacco cells are the most widely-used platform, and Dow AgroSciences (Zionsville, IN, USA) used tobacco NT-1 cells (the CONCERT system) in 2006 to produce the first molecular farming product approved by the US Department of Agriculture (USDA) as a veterinary vaccine (Schillberg et al., 2013). The first molecular farming product approved by the FDA for use as a pharmaceutical in humans was also produced in plant cells, in 2012. This was taliglucerase alfa, manufactured in carrot cells (the ProCellEx system) by Protalix

Biotherapeutics (Karmiel, Israel), although the company uses tobacco cells for other products in their pipeline (Aviezer et al., 2009a, 2009b; Mor, 2015). Although not higher plant cells, other clonal systems have come to be regarded in the same context, including platforms based on the moss *Physcomitrella patens* (Greenovation Biotech GmbH, Heilbronn, Germany), the alga *Chlamydomonas reinhardtii* (Decker and Reski, 2012), and the duckweed *Lemna minor* (principally developed by the now-liquidated Biolex Therapeutics, Pittsboro, NC, USA).

6.3. Transient expression

Transient expression involves the introduction of genes into plants carried by viruses or the bacterium *Agrobacterium tumefaciens* (or some combination of the two). This approach is transient because the plant is not stably transformed and the target protein is expressed for only a few days before the plant succumbs or clears the vector (virus-based methods) or the expression construct is degraded (methods based on T-DNA transfer). But during the window of expression, large quantities of protein can accumulate, in some cases reaching >2 g kg^{-1} biomass in less than a week (Sainsbury and Lomonossoff, 2008; Sainsbury et al., 2010; Hiatt et al., 2015; Zischewski et al., 2016).

The most widely used transient expression system involves the infiltration of tobacco leaves with *A. tumefaciens*, as first applied to a molecular farming product by Vaquero et al. (1999). This differs from the process used to generate transgenic plants, where the bacterium is left in contact with plant tissue to allow time for stable T-DNA insertion followed by the regeneration of transformed cells into whole plants under selection. Instead, transient expression involves the injection or vacuum-mediated infiltration of millions of recombinant *A. tumefaciens* into the spaces between mesophyll cells in the leaf. This results in a large number of plant cells becoming infected and receiving T-DNA, which is translocated to the nucleus. Stable integration is not sought in this process. Instead, genes on the episomal T-DNA are expressed for a few days until it undergoes natural degradation (undetected stable integration may occur in some cells). Several companies have taken and adapted the agroinfiltration-based transient expression system described by Vaquero et al. (1999) and developed platforms in tobacco (*N. benthamiana* or *N. tabacum*) for rapid vaccine manufacturing, including influenza vaccines produced by Medicago (Québec, Canada/Durham, NC, USA) and iBio/Caliber Therapeutics (Bryan, TX, USA). These two companies, along with Kentucky Bioprocessing (Owensboro, KY, USA), have established large-scale automated facilities for the transient expression of vaccine products, marking a significant milestone along the slope of enlightenment (Holtz et al., 2015). The platform used by Medicago is based on the infiltration of *N. benthamiana* using recombinant *A. tumefaciens* and has proven particularly suitable for the production of virus-like particles (VLPs) as efficacious vaccine candidates (Rybicki, 2019). Their lead product is a human quadrivalent seasonal influenza vaccine candidate that has already completed phase II (Pillet et al., 2019) and phase III clinical trials (CT identifiers: NCT03301051, NCT03739112). The potential value of a rapidly scalable transient expression system was most clearly demonstrated by the production of ZMapp, a cocktail of three monoclonal antibodies for the treatment for Ebola hemorrhagic fever (Qiu et al., 2014) developed by Leaf Biopharmaceutical (the commercial arm of Mapp Biopharmaceutical, San Diego, CA, USA). ZMapp was produced by transient expression in tobacco using the Kentucky Bioprocessing facility, and was approved for compassionate use in 2014 (before the completion of clinical testing) during an outbreak of the viral disease in West Africa due to its life-saving potential and the lack of any alternatives. Although only seven patients could be treated with the available ZMapp stock, given the need for ~10 g of the antibody cocktail per patient, five of them ultimately recovered (Na et al., 2015). The subsequent phase II clinical trial enrolled 72 participants (short of the intended 200 enrollees due to the outbreak tailing off) thus reducing the statistical power of the study. Therefore, although the risk of death was reduced by 40% in ZMapp recipients, this did not reach the threshold for statistical significance and the drug was not considered efficacious (PREVAIL II Writing Group, et al., 2016).

Transient expression can also be mediated by plant viruses modified to carry additional genes for the target recombinant protein. Following the inoculation of host plants, the viruses cause a systemic infection which results in the production of recombinant protein throughout the plant until the plant clears the virus or succumbs to the infection. Because the use of infectious viruses is an environmental risk, most virus-based systems used for transient expression are deconstructed, which means vital parts of the virus machinery required for systemic infection or spreading to new hosts are removed. The virus is still capable of local spreading, leading to small infection foci each comprising a few hundred cells. Because this would limit the amount of recombinant protein recovered, the virus genome is delivered by agroinfiltration as above, such that many cells are infected independently, resulting in multiple infection foci, but the virus cannot spread systemically or to other plants. Several platforms have been developed based on deconstructed versions of Tobacco mosaic virus including the Launch Vector system (Fraunhofer CMB, Newark, DE, USA) and the Magnifection system (Icon Genetics, Halle/Saale, Germany). These systems can achieve high product yields, for example the Fraunhofer system was able to produce influenza A virus hemagglutinin subunits from strains H3N2, H5N1 and H1N1 at yields of 50–200 mg per kg fresh leaf biomass (Shoji et al., 2008, 2011). Another deconstructed virus system developed at the John Innes Centre (Norwich, UK) uses translational enhancers from Cowpea mosaic virus to generate hypertranslatable vectors, yielding up to 1.5 g recombinant protein per kg fresh leaf biomass without virus replication (Sainsbury and Lomonossoff, 2008; Sainsbury et al., 2010). This platform is now available for contract manufacturing via the spin-off company Leaf Expression Systems (Norwich, UK).

6.4. Transgenic plants

Transgenic plants are particularly suitable in the case of pharmaceutical and industrial products for which there is a large and continuous demand because the plants can be cultivated on an agricultural scale to yield 100–1000 kg of the pure protein per year. Here the molecular farming community has settled on two main platforms – leafy crops (principally tobacco, because of its extraordinary biomass yield and status as a non-food/feed crop) and cereal crops (principally maize, rice and barley) because the seeds are self-contained bioreactors that protect the product. Although the benefits of transgenic plants have been blunted somewhat in the case of pharmaceutical proteins by the slim likelihood that such crops would ever be grown in the open field due to product safety/reproducibility and environmental containment issues, we nevertheless consider the case of HIV-neutralizing antibodies, which have been produced in transgenic tobacco (Ma et al., 2015) and transgenic maize (Rademacher et al., 2008; Ramessar et al., 2008; Vamvaka et al., 2016c). In the Pharma-Planta project, the HIV-neutralizing human antibody 2G12 was produced in tobacco and envisaged as a component of a microbicidal cocktail to prevent HIV infections (Ma et al., 2015). However, several milligrams of antibody must be applied each time the microbicide is used to ensure a protective effect is achieved (Ramessar et al., 2010). This would be too expensive for production in CHO cells and would exceed global GMP manufacturing capacity. For example, the production of 1000 kg of an antibody in CHO cells per year would require 25 fermentation runs (14 days each) of 10,000 L, assuming a yield of at least 5 g L^{-1} and 80% recovery. This would be too expensive even for the industrialized world, but the primary target for HIV microbicides is sub-Saharan Africa, where the burden of disease is highest. The massive demand for inexpensive antibodies could only be met by growing pharmaceutical crops on an agricultural scale using local infrastructure, as envisaged by the humanitarian focus of the Pharma-Planta project (Ma et al., 2015).

The amount of land needed would depend on the yield per kg plant biomass, e.g. the typical yield of the 2G12 antibody produced by Pharma-Planta was 10 g pure product per tonne tobacco leaves (Ma et al., 2015), although other antibodies accumulate to >1.5 kg per tonne fresh mass (Zischewski et al., 2016). Assuming an intermediate scenario, the production of 1000 kg of antibody product at a yield of 1 kg per tonne biomass would require 1000 t of tobacco leaves (Buyel et al., 2017). Close cropped tobacco can be harvested several times per year and the overall biomass yield is ~100 t per hectare, equivalent to 10,000 t per km^2 (Stoger et al., 2002). The cost of growing and harvesting 0.1 km^2 of tobacco plants, even in containment, would be much lower than constructing a CHO facility and running 25 back-to-back fermentations every year.

7. The slope of enlightenment – development of best practices

7.1. Overview

The issues facing molecular farming have often been described in terms of the yield and purification challenges, reflecting the two perceived deficiencies of molecular farming in plants compared to established systems: lower yields and more complex DSP requirements (Schillberg et al., 2019). Plants generally achieve lower yields than microbes and animal cells during upstream production, partly due to the larger size of plant cells (meaning there are fewer productive bioreactors per unit biomass compared to microbial and animal systems), and partly because the yield is intrinsically lower given that plants are relatively new entrants in the biopharmaceutical manufacturing industry and there has been less time for process optimization. The purification challenge reflects the fact that animal cells and microbes generally secrete products into the medium, whereas plant cell suspension cultures and root cultures may do this but in most cases whole plants do not. The product must therefore be recovered by disrupting the plant tissue, releasing copious amounts of soluble and insoluble impurities that must be removed during DSP.

The yield problem has been addressed by incremental improvements in plant expression cassettes, the use of silencing suppressors, and the development of high-yielding transient expression systems as discussed above and in recent review articles (Twyman et al., 2013; Schillberg et al., 2019). The purification problem has been addressed by intensive work on the development of improved DSP steps focusing on clarification and early recovery. Approaches such as centrifugal extraction and rhizosecretion attempt to avoid the release of process-related impurities altogether at the cost of restricting the sub-cellular localization of the product to the apoplast, the space outside the plasma membrane, including the cell wall (Borisjuk et al., 1999). Others have compiled a set of conditioning steps, including acid precipitation and heat treatment, to remove host cell proteins early in the process, thereby simplifying purification and protecting the product from degradation (Buyel, 2015; Buyel et al., 2015). This work has been complemented by adapting solid–liquid separation techniques to the unique properties of plant-based manufacturing (compatibility with large quantities of dispersed solids) such as the introduction of screw-presses, optimized filter cascades, flocculants and filter additives. High-throughput screening is now used to identify suitable purification processes and can be supported and accelerated by the predictive (mechanistic) modeling of protein separation during chromatography (Buyel et al., 2013; Buyel and Fischer, 2012). In addition to these general aspects of process improvement, molecular farming has also developed certain best practices which focus strongly on the unique benefits of plants, and some of these recent innovations are discussed below.

7.2. Plant cell packs

One of the key advantages of microbial and mammalian cell systems is that tests carried out on a small scale generally translate well when the process is scaled up: for example, a high-producer CHO cell line in a shake flask generally behaves in a similar manner in a 20,000-L bioreactor. This link is missing in plants because there is no direct correlation between small-scale tests in cell/tissue culture and what happens to whole plants in a greenhouse or field due to the much longer growth time, the vastly different environmental conditions in the tissue culture and greenhouse/field scenarios, and aspects of plant growth such as photoperiod dependency which are not present in cell culture (Bendandi et al., 2010; Buyel and Fischer, 2012; Buyel et al., 2013; Goojani et al., 2013; Knödler et al., 2019). A new screening platform was recently described which is based on plant cell cultures that are exposed to a vacuum to remove the liquid medium, and cast into a porous mass known as a plant cell pack (PCP). The PCP is derived from plant cells but shares many features of the tissues in whole leaves, and likewise can be infiltrated with recombinant *A. tumefaciens* and used for transient expression (Rademacher et al., 2019). The PCPs can be cast at various scales, from microtiter plate wells to 150-mL columns, and they allow the high-throughput testing of different expression constructs, process conditions and product candidates before translating to transient expression in whole plants. Most importantly, in the initial tests, the PCPs were able to predict the relative expression levels of different constructs during transient expression in whole plants quite accurately, providing a means to circumvent the laborious and error-prone testing of multiple product variants in order to hit on an appropriate set of conditions.

7.3. BY-2 cell lysates

Cell-free protein synthesis is a powerful method for the high-throughput production of recombinant proteins, especially proteins that are difficult to express in living cells due to their toxic or inhibitory effects (e.g. mitotic inhibitors in eukaryotes, antimicrobial peptides in bacteria). Several systems based on cell lysates have been described, including at least one (wheat germ lysate) based on plants. However, a recent innovation in the field of molecular farming was the development of lysates based on tobacco BY-2 cells, which provide many of the benefits of the BY-2 system in terms of its potential for the production of diverse functional proteins, with the additional ability to produce proteins that are toxic in plants (Buntru et al., 2014). About 2 g of wet BY-2 cell biomass is required to generate 1 mL of the corresponding lysate, which can then produce recombinant proteins at concentrations of up to 270 mg L^{-1} using covalently closed plasmid templates, or up to 180 mg L^{-1} using a linear PCR product (~80 mg of DNA must be added per liter of lysate to trigger product formation). The BY-2 cell lysate is versatile, supporting the formation of disulfide bonds, glycans and allowing the co-translational integration of membrane proteins. This has allowed the synthesis of a functional full-size antibody, the enzyme glucose oxidase, and a transmembrane growth factor (Buntru et al., 2014).

7.4. Transplastomic plants

Transplastomic plants are generated by introducing DNA into the plastid genome, and are therefore a subset of transgenic plants (Bock, 2015). However, in contrast to nuclear transgenic plants, the transgene copy number is high because there are up to 20,000 plastids in a typical photosynthetic cell, there is no gene silencing, multiple genes can be expressed in operons, the recombinant proteins accumulate within the chloroplast thus limiting toxicity to the host plant, and the absence of functional chloroplast DNA in the pollen of most crops provides natural transgene containment (Svab and Maliga, 2007). The high transgene copy numbers and the absence of silencing have resulted in extraordinary expression levels, in the most extreme case reaching 70% of the total soluble protein in the plastid (Oey et al., 2009). However, plastids are evolutionarily derived from bacteria and therefore form

neither disulfide bonds nor glycans, making them unsuitable for the production of complex and/or glycosylated recombinant proteins. Reliable plastid transformation has been achieved in only a few crops – most plastid molecular farming studies concern tobacco or lettuce (Waheed et al., 2015) – but there is a large subgroup of studies in the literature dealing with plastid transformation and molecular farming in the alga *C. reinhardtii* (Rosales-Mendoza et al., 2012). Although molecular farming in plastids is practiced less widely than the standard nuclear transgenesis approach, there are some remarkable recent success stories for both pharmaceutical products (Hoelscher et al., 2018) and industrial enzymes (Schmidt et al., 2019). The latter case is particularly interesting from a commercial perspective because the authors expressed a soluble recombinant cellulase in tobacco plastids and tested the plants in the field, revealing that high-level recombinant protein expression had no effect on plant growth, photosynthesis and the accumulation of host proteins. However, they found that plants in growth chambers under constant conditions diverted their existing resources to produce the recombinant protein, resulting in a drop in the quantity of host cell proteins to compensate, whereas under the more variable field conditions the plants maintained the normal level of host cell proteins and accommodated the extra demand for recombinant cellulase by increasing the overall level of total soluble protein. These transplastomic tobacco plants were therefore able to grow normally in the laboratory and field even though up to 40% of the total soluble protein was the recombinant product because the plants were metabolically flexible, although they used different strategies to cope with the demand under constant and variable environmental conditions.

7.5. Direct application of tissues and crude extracts

The leaves, seeds and fruits of many plants are edible, providing a route for the oral administration of pharmaceutical products such as protective antibodies (Zimmermann et al., 2009) and vaccine antigens (Merlin et al., 2017), several of which were produced in potato tubers, cereal seeds, salad leaves and tomato fruits for human clinical trials (Ma et al., 2005) before GMP manufacturing became mandatory (Fischer et al., 2012). Oral vaccines can induce mucosal immune responses via lymphoid tissues in the gut, particularly if multiple copies of the epitope are presented on the surface of a plant virus or VLP (Yusibov et al., 2011; Merlin et al., 2017). Systemic responses are also possible if there is prolonged contact with the immune system, and this can be achieved by encapsulating vaccine candidates in cereal or legume seeds, exploiting the presence of subcellular compartments that delay digestion (Hofbauer and Stoger, 2013). Although most studies have investigated the ability of oral antibodies and vaccines to prevent infectious diseases, the same principle has been used to demonstrate the efficacy of plant tissue containing autoantigens to prevent autoimmune diseases, including type 1 diabetes (Bertini et al., 2018). In addition to transgenic plants expressing antigens, transplastomic plants have also been used for this purpose, potentially offering epitope protection similar to that provided by seed storage compartments because of the enclosure of the recombinant protein in plastids (Kwon et al., 2018).

Seeds are also potentially a useful platform for the production of microbicides that are applied topically rather than ingested, such as the HIV-neutralizing antibodies 2G12 and 2F5 (Rademacher et al., 2008; Ramessar et al., 2008; Vamvaka et al., 2016c) and the antiviral lectins griffithsin and cyanovirin-N (Vamvaka et al., 2016a, 2016b). The transmission of HIV can be prevented by the application of neutralizing monoclonal antibodies and lectins, but the requirement for multiple microbicidal proteins to prevent virus 'escapes' makes traditional production platforms and potentially even transgenic plants too expensive for applications in developing countries. However, in a recent innovation, Vamvaka et al. (2018) described transgenic rice plants simultaneously expressing three different HIV-neutralizing proteins in the seeds (2G12, griffithsin and cyanovirin-N) and showed that the three components had synergistic activity in HIV neutralization assays, even in crude extracts, suggesting that transgenic plants producing appropriate absolute and relative doses could be used to produce pharmaceuticals required in 'cocktail' form directly. Previously, vaccine cocktails have been produced in separate plant lines followed by mixing the purified components, as shown for the cocktail of three ZMapp antibodies, and as various multivalent fusion constructs, in the case of a malaria vaccine cocktail (Boes et al., 2015). The production of such proteins in seeds would also allow the transport and storage of microbicides without a cold chain, and the preparation of microbial extracts at the point of care (Stoger et al., 2005).

7.6. Plant glycans

The *N*-glycosylation of proteins in humans and plants involves the passage of the nascent protein through the endoplasmic reticulum, where the addition and modification of glycans follows identical steps. Later, the partly-glycosylated protein enters the Golgi body, and here the process diversifies in a species-dependent manner: importantly, plant glycoproteins are modified with β1,2-xylose and core α1,3-fucose residues that are not present in mammals, whereas mammalian proteins are modified with β1,4-galactose and sialic acid residues that are not present in plants (Gomord et al., 2010). The presence of different glycan residues on recombinant plant-derived human glycoproteins and endogenous human counterparts can render the plant-derived proteins immunogenic, but the effects may be more extensive including the loss of stability or activity, different rates of clearance from the body, and different interactions with human cells. These differences can be either detrimental or beneficial. There has therefore been a twin effort to, on one hand, generate molecular farming platforms lacking plant glycans and in some cases incorporating human glycans (Strasser et al., 2009; Castilho et al., 2010) and, on the other hand, to develop 'biobetter' molecular farming products with glycan structures that improve the quality or efficacy of the protein due to the presence of plant glycans (Cox et al., 2006; Shaaltiel et al., 2007).

Two broad strategies have been used to prevent the formation of plant glycans, one involving the modification of the target protein to prevent its passage through Golgi body (usually achieved by adding a so-called retrieval sequence that ensures the protein is targeted to accumulate in the endoplasmic reticulum) and one involving the modification of the host, for example by the knockdown or knockout of genes encoding unwanted glycosyltransferases. The transient or stable expression of human-type glycosyltransferases and other components of associated metabolic pathways can complete the human glycan profile (Fischer et al., 2018). A more recent innovation is the use of genome editing to generate 'glycan friendly' hosts. This was initially achieved using TALENs to knock out both *XylT* genes and two of the five *FucT* genes in *N. benthamiana* (Li et al., 2016). This eliminated XylT activity but only reduced FucT activity, indicating that further *FucT* sequences remained active. The complete elimination of FucT activity was later achieved in BY-2 cells using the CRISPR/Cas9 system, involving a complex editing strategy which required simultaneous mutation at 12 or 14 targets (Hanania et al., 2017; Mercx et al., 2017). The most conserved regions of the genes were targeted so that one gRNA would introduce a break in several different alleles/homeoalleles. To confirm that the enzymes were no longer active, the mutated BY-2 cells in the first study were transformed with a construct encoding an antibody, and the resulting recombinant protein was devoid of plant complex-type glycans (Mercx et al., 2017). In the second study, the authors achieved the mutation of all 14 loci and transformed the mutated cells with a transgene encoding DNase I, which was also shown to lack plant complex-type glycans (Hanania et al., 2017). The simultaneous knockout of two *XylT* and four *FucT* genes using CRISPR/Cas9 has also been reported in *N. benthamiana* plants (Jansing et al., 2019). The sextuple knockout plants were devoid of XylT and FucT activity and produced a recombinant antibody that lacked plant complex-type glycans but retained its antigen-binding specificity and binding kinetics.

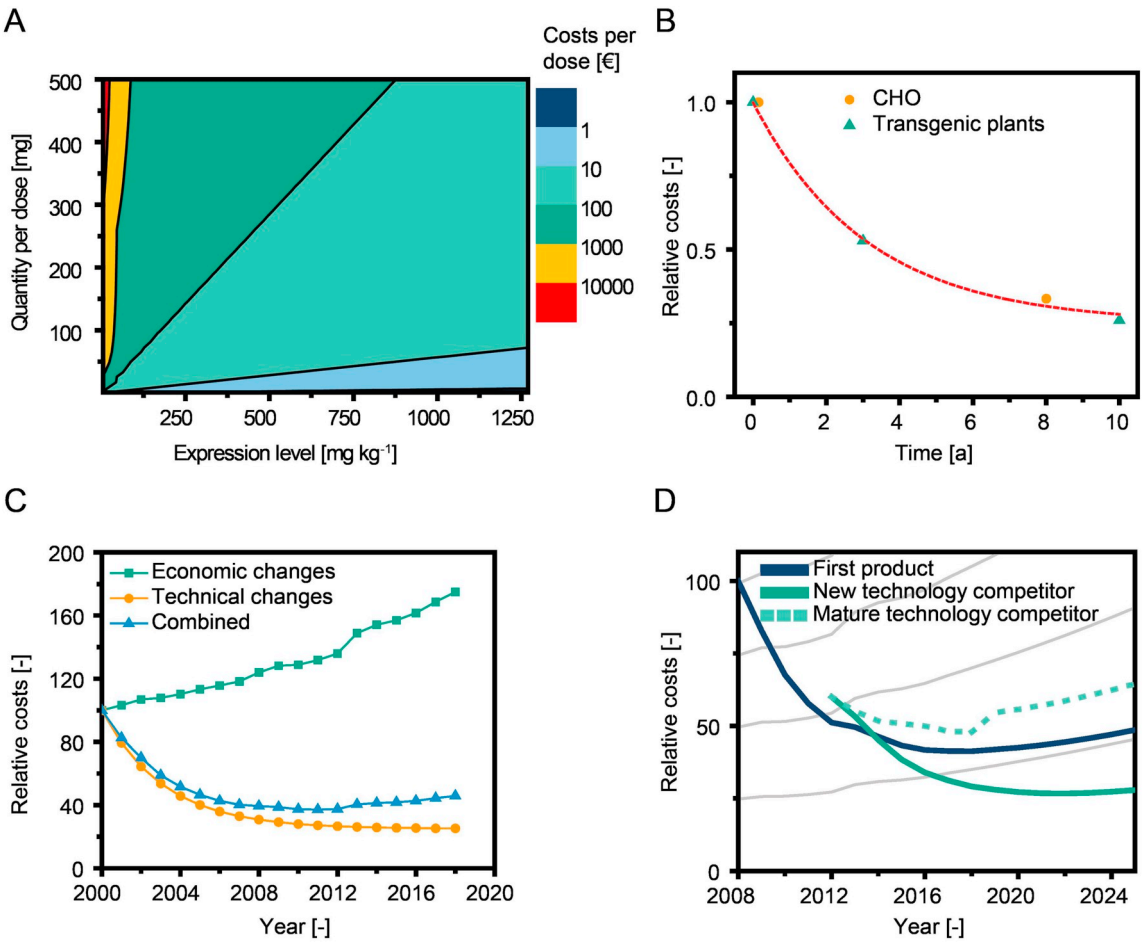

Fig. 3. Cost development for biotechnological processes. (A) Cost analysis for a plant-produced pharmaceutical antibody showing the dependence of costs on the expression level and dose requirements. The cost model is based on Buyel and Fischer et al. (2012). (B) Cost reduction in biotechnological manufacturing due to technology and process maturation (data from Kelley (2009) and our unpublished data). (C) Relative cost development based on economic changes (here, inflation and salary raises), technological improvements (as shown in B) and a combination of both effects. (D) Impact of economic changes and technology maturity on the competitiveness of a new product (green, starting 2012) compared to an established counterpart (blue, starting 2008). If the competing technology is new, cost savings can be substantial (solid green line), whereas for a new product using a mature technology, cost savings may be overcompensated by increasing costs due to inflation (dotted green line). Gray lines are 'isocost' lines based on the observed (up to 2018) and expected inflation and salary increase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The efforts to embrace the positive aspects of plant glycans are best exemplified by taliglucerase alfa (recombinant human glucocerebrosidase) marketed as Elelyso by Protalix BioTherapeutics for the treatment of Gaucher's disease. Unmodified glucocerebrosidase from human pancreas is unsuitable for enzyme replacement therapy because the terminal sialic acid, galactose and *N*-acetylglucosamine residues on the glycan chains inhibit endocytosis by macrophages, which is mediated by mannose receptors. Elelyso is manufactured in carrot cells, which in common with other plant cells do not synthesize glycoproteins containing sialic acid. Furthermore, targeting the protein to the vacuole prevents the extension of terminal mannose residues, thus promoting uptake of the enzyme by macrophages (Shaaltiel et al., 2007). The equivalent product manufactured in CHO cells, imiglucerase (marketed as Cerezyme by Sanofi-Genzyme, Cambridge, MA, USA), has terminal sialic acid residues, which are automatically added during the transit of mammalian glycoproteins through the secretory system. It is necessary to remove these residues *in vitro* to ensure that the enzyme functions correctly. Therefore, unlike the product manufactured in carrot cells, the CHO-derived enzyme requires an additional step which adds to the overall manufacturing and QA/QC costs. A third version of the enzyme, velaglucerase alfa (VPRIV, Shire Pharmaceuticals, now Takeda Pharmaceutical Company, Tokyo, Japan) is produced in human fibroblast carcinoma cell lines with a mannosidase I inhibitor included in the medium to prevent the extension of mannose residues. All three enzymes have been shown to be functionally equivalent (Tekoah et al., 2013).

8. The slope of enlightenment – techno-economic analysis

As stated above, the need for thorough techno-economic analysis was grasped immediately by the molecular farming companies developing non-pharma products and accordingly they suffered less than their pharma-focused siblings during the trough of disillusionment phase. In the last decade, the pharma camp has caught up in this respect, resulting in three major developments that encompass changes in the laboratory/production line through to strategic decisions on a corporate level.

First, techno-economic analysis has helped to identify major cost drivers in the production of plant-made pharmaceutical proteins at a shop-floor level. The foremost drivers are the DSP unit operations that were necessary to deal with the high burden of insoluble particles and host cell proteins in typical plant extracts. These steps were found to account for >80% of the total costs and the issue has been addressed, for example, by developing more efficient solid–liquid separation procedures (Menkhaus et al., 2004; Nikolov and Woodard, 2004; Wilken and Nikolov, 2012; Buyel, 2015; Buyel et al., 2015).

Second, and as a consequence of the first development, plant-based production has and still is in the process of converging towards a platform technology with a limited set of operations used for different products. Again, the convergence specifically applies to DSP, where most processes now use (a decreasing number of) filtration-based clarification steps followed by 2–3 chromatography-based purification steps with interspersed ultrafiltration/diafiltration to purify the product. An important realization is that the DSP for plant-derived pharmaceutical products now closely resembles that of any bacterial or CHO-derived recombinant protein. Therefore, the production of biopharmaceuticals may be regarded as a modular process in the future, where the host/expression system can be plugged into a standardized DSP that is most appropriate for the target protein. This has clearly been demonstrated in the large-scale transient expression facilities discussed above. For example, the iBio/Caliber production facility for influenza vaccines by transient expression in tobacco integrated the mobile and modular downstream processing suites provided by G-Con Manufacturing (College Station, TX, USA) to ensure process adaptability and rapid changeovers (Holtz et al., 2015).

The third development is the advent of reliable process and thus cost models for plant-based manufacturing (Mir-Artigues et al., 2019). These models have become increasingly sophisticated and often use the same professional software tools (e.g., SuperPro Designer; Intelligen Inc., Scotch Plains, NJ, USA) as the rest of the pharma industry to set up closed mass and cost balances (Nandi et al., 2016). Early examples were published by Ventria Bioscience for their transgenic rice platform (Nandi et al., 2005) and other key examples have been published for transgenic tobacco plants/cells (Wilken and Nikolov, 2012) and transient expression, in one case comparing the relative costs of pharma and non-pharma products (Tusé et al., 2014). This transition to professional cost modeling has accelerated the alignment of molecular farming with existing expression systems and will facilitate the future modularity of pharmaceutical production. The major benefit of these techno-economic models for molecular farming is that they allow the early assessment of economic viability for potential and new protein candidates. Therefore, they can facilitate informed decisions such as whether or not to pursue a novel candidate based on its potential revenue, for example as a function of the achieved or achievable expression levels and dose requirements (Fig. 3A). As well as providing an overview of the current factors affecting production costs, these models can be augmented in several ways to estimate future cost developments. For example, Kelley (2009) anticipated a reduction in monoclonal antibody manufacturing costs from \$300 g^{-1} in 2000 to \$100 g^{-1} in 2008 based on published estimates for the cost of goods sold (COGS) when using CHO cells due to the maturation of the technology and increasing process knowledge. When comparing this assumption for CHO cells with the tobacco-based production process for 2G12 first developed in 2009, we find (our unpublished data) that the costs gradually decreased from €10,000 g^{-1} in 2009 and €5500 g^{-1} in 2012 to less than €2500 g^{-1} in 2019 (Fig. 3B). However better-expressing recombinant antibodies such as M12 can yield ~50-fold more product from the same amount of biomass, thus reducing costs to below €100 g^{-1} (our unpublished data). Our data are thus in good agreement with the maturing technology assumption and are supported by other reports, which estimate a COGS of ~€170 g^{-1} for accumulation levels of 0.4 g kg^{-1} at a scale of 300 kg y^{-1} or accumulation levels of 1.0 g kg^{-1} at a scale of 110 kg y^{-1} (Nandi et al., 2016). However, a reliable comparison should also consider additional effects such as infrastructure costs and depreciation, personnel costs, operational expenses and inflation. We therefore suggest the use of models that consider both the cost reductions due to maturation and the completion of equipment depreciation as well as inflation and other rising trends like salary increases (Fig. 3C). This type of plot allows "what if" analysis to compare a given process with competitors that may enter the market at a later stage (Fig. 3D), thus predicting the window of profitability to avoid misguided investments such as Bayer's Factor VIII production facility. The manufacturing site was built in Wuppertal (Germany) between 2014 and 2018 at a cost of >500 million euros but closed down before the commissioning phase (Francisco, 2014; Hargreaves, 2018).

9. Conclusion – the future of molecular farming

As the Danish proverb goes, 'Predictions are difficult, especially about the future'. Nevertheless, we have highlighted some potential developments that appear plausible and/or interesting from our current perspective. Despite the potential advantages of molecular farming, the biopharmaceutical industry still favors their standardized cell-based platform technologies that have received heavy investment for many years. This has been rewarded in most cases by incremental improvements in product yield and quality. Following the early phase of the hype cycle, when molecular farming was promoted as a game-changing innovation on an industry-wide basis, the pioneers of this new industry eventually focused on the disruption of niche markets rather than the displacement of incumbent technologies such as CHO cells. And niche markets may have been the extent of the pharmaceutical molecular farming revolution were it not for the emergence of a new paradigm: the time-to-market benefits.

Ultimately, time-to-market factors are the strongest drivers in terms of host platform selection, including the duration of research and development (R&D), production scale-up and regulatory approval. Plants can gain a head start during R&D due to the speed of transient expression, but face more hurdles during scale-up because of a lack of suitable manufacturing sites. The latter might change as more niche products become available, along with the corresponding infrastructure, until at some point there will be a critical mass of production capacity sufficient to accommodate large-scale processes and thus attract the attention of big pharma once again, but this time on a paying basis. The seeds of this new revolution have already been sown, and the overview of the industry provided in Table 1 shows how. Whereas the industry landscape in 2005 was characterized by two largely separate camps focusing on pharma and non-pharma products, the situation today is much more integrated. There are still companies specializing in each area, but there are also mixed-model companies that have a foot in both camps, such as Ventria Biosciences with its pharma portfolio but separate department (InVitria) for non-pharma reagents and cosmetics. There are also companies that function effectively or explicitly as contract manufacturers alongside their own pipelines, such as Kentucky Bioprocessing with its non-pharma aprotinin product but the capacity to take on the manufacturing of pharma products under license, such as the ZMapp antibodies developed by Mapp Biopharmaceutical. This innovative use of technology and, more importantly, production capacity is something that the traditional biopharmaceutical manufacturing industry simply cannot replicate – because this would involve, for example, using expensive CHO cells to produce low-margin high-volume products such as technical enzymes. Plants have the advantage because they are economically as well as biologically versatile, and concepts such as versatility and adaptability may trump sheer productivity as a key platform selection criterion in the future as the time-to-market factor becomes the more desirable target.

Much of the adaptability of plants comes from the scalability of production and the more refined regulatory framework, which allows upstream production in bioreactors, greenhouses or fields to be 'plugged in' to pre-defined DSP modules, as exemplified by the iBio/Caliber + G-Con model described above. The attractiveness of plants has also increased due to the alignment of plant-based processes with ICH guidelines, accommodating principles of quality by design such as the use of design-of-experiments strategies to accelerate process development and improve process consistency (Buyel and Fischer, 2012; Buyel et al., 2013) and in-line process analytical technology to ensure robust quality assurance/quality control during the manufacturing process (ICH, 2012). The ability of molecular farming to exploit the unique advantages of plants, while also seeking to align with current

manufacturing paradigms, is a sign that this 30-year-old technology is at last moving along the slope of enlightenment to industrial maturity.

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