Plant made pharmaceuticals (PMPs) as medicinal products for human use – a review of current regulatory issues and challenges to achieve a marketing authorisation in the EU

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List of abbreviations

APHIS	Animal and Plant Health Inspectorate
BSE	Bovine Spongiform Encephalopathy
CHO	Chinese Hamster Ovary cells
CHMP	Committee for Medicinal Products for Human use
COGS	Cost of Goods
COPD	Chronic Obstructive Pulmonary Disease
DNA	Deoxyribonucleic Acid
DSP	Down Stream Processing
EMA	European Medicines Agency
EFSA	European Food and Safety Agency
EPA	Environmental Protection Agency
ER	Endoplasmic Reticulum
ERA	Environmental Risk Assessment
EU	European Union
FDA	Food and Drug Administration
GCD	Human Glucocerebrosidase
GACP	Good Agricultural and Collection Practice
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
GM	Genetic Modification / Genetically Modified
GMO	Gene Modified Organism
GMP	Good Manufacturing Practice
HIV	Human Immunodeficiency Virus
MA	Marketing authorisation
mAB	Monoclonal Antibody

NPBT	New Plant Breeding Techniques
NTWG	New Techniques Working Group
PBS	Plant-based (production) System
PMF	Plant Molecular Farming
PMP	Plant Made Pharmaceutical
QMS	Quality Management System
mRNA	messenger Ribonucleic Acid
RMP	Risk Management Plan
SME	Small/Medium-sized Enterprise
US	United States
USDA	United States Department of Agriculture
USP	Upstream Processing
WHO	World Health Organisation

1 Introduction

1.1 Context and problem formulation

The population of Europe and the rest of the world is growing and getting more and more elderly. For 10 years now there is an unbroken trend, where there are more people of 65 years than children less than 15 years old, and combined with that there is an observable increase in the number and spectra of different types of diseases (Kaplan, W. et al., 2013). Due to the above-mentioned trend, the importance for biologicals or large biomolecule drugs like vaccines, blood and blood components, gene therapy agents, recombinant therapeutic proteins, living cells and tissues is vastly growing worldwide.

The major problem with the conventional production platforms for therapeutic proteins is that they cannot accomplish the challenge of providing sufficient quantities of recombinant proteins in order to enable a fast reaction to emerging disease outbreaks. The second major problem is that the production costs of the proteins are enormous. Especially where biologicals are most needed, namely in underdeveloped countries where the number of infectious diseases is high, these expensive therapeutical proteins are not affordable and therefore the population in these countries are underserved with life-saving therapeutics. The World Health Organisation (WHO) and the European Union (EU) funded several long-term research programmes and the pharmaceutical industry has also taken interest to resolve these global problems in the long run.

Recently, plants have become an important platform for recombinant protein production (Penney, C. A. et al., 2011). The concept of plants producing pharmaceutical proteins, a research area called plant molecular farming (PMF), is to utilise plants and plant tissues as ‘biofactories’ for therapeutic proteins that have primarily been derived from bacterial or animal origin. The development of different plant genetic transformation methods gave plants the possibility to express ‘foreign’ genes via stable or transient expression. Furthermore, plants are capable of accumulating non-native proteins in the cells and plant organs like plastids (Sil et al., 2014). Upfront costs in the manufacturing of plant made pharmaceuticals (PMP) including the investment for the facilities and COGS are much lower compared to conventional production platforms and nowadays the scalability of products has improved significantly (Phoolcharoen et al., 2014), (Sack et al., 2015b; Ahmad, P. et al., 2012), (Stoger, E. et al., 2014).

Although there has been continuous research for almost 30 years and the biotechnology boom in the early 90's, the development of PMPs is still at an early stage. PMPs have suffered in the past from several technical and regulatory setbacks. This is due to the fact that there is no defined sort of plant-based production system (PBS). Each platform consists of a puzzle of variables and therefore needs to be authorised on a case-by-case basis. In order to produce a reasonable amount of a target recombinant protein, many factors in the manufacturing process must be taken into account. The PBS can vary from whole plants to cell-based suspensions. In the EU, the regulatory approach is very precautionary and follows a complex and step-wise approach in terms of the approval of genetically modified (GM) host plants. This leads to the situation that at present in Europe no therapeutic protein for human use has yet been approved or is still blocked in between the product idea and feasibility phase (Sparrow et al., 2013).

Another aspect that contributes to a stringent EU legislation concerning GM products in the EU, is a mixed acceptance within society due to the fear of comingling of GM plants with the food/feed-chain. Due to this negative attitude to GM plants, many countries will not permit experimental field trials in their territories.

1.2 Objectives

The aim of this master thesis, which is based on a literature review, is to gain knowledge, which regulatory aspects have an influence on the development and market authorisation of PMPs.

The following questions are in focus:

- Which regulatory directives and guidelines are under which circumstances applicable?
- What are the hurdles in the current regulatory framework in the EU?
- What lessons can be learned from first PMF experiences (Elelyso and from the Pharma-Planta project) regarding the approval process of PMPs?
- Is there a need to adapt the current regulatory framework in the EU in relation to the technical progress of PMPs and if so, how should the regulations regarding PMPs be adapted?

It will be discussed if the regulatory burden for PMF should be reduced, in light of emerging new technologies that modify the genetics of plants.

Note: Focus will be placed on PMPs for the therapeutic human use such as vaccines, antibodies or enzymes. Industrial PMPs or PMPs for the medicinal use in animals will not be not covered in this thesis.

1.3 Methodology

This study is based on literature reviews, document analysis and Internet database browsing. The information was sourced through Internet searching and cross-referencing guidelines.

The following search terms were used: plant-made pharmaceuticals, regulatory, plant derived pharmaceuticals, plant molecular farming, recombinant protein and GMO.

2 General aspects concerning PMPs

2.1 What are PMPs?

PMPs are the result of a breakthrough application in biotechnology. Plants can produce therapeutic proteins that could be used by physicians to fight life-threatening diseases. In this process, plants themselves become 'biofactories' that produce therapeutic recombinant proteins. These proteins are then extracted and purified in the pharmaceutical production.

As plants are eukaryotes they are capable of storing the desired recombinant proteins in subcellular compartments like chloroplasts and performing adequate folding and post-translational modification, which is important in order to produce also complex and multimeric proteins. With these characteristics, useful proteins like vaccines, monoclonal antibodies (mABs), therapeutic enzymes, growth factors and growth hormones have already been produced in plants in order to efficiently combat major health problems like chronic and infectious diseases (Jin, T. et al., 2015) (Stoger, E. et al., 2014).

For as long as mankind has been in existence, plants have always been suppliers of therapeutic substances. Medicinal plants containing healing metabolites such as essential oils, glycosides, saponins etc. are traditionally used as herbal medicinal products. However, the concept of PMPs is that plants are no longer used as medicinal plants in the traditional sense, but function as bioreactors for the expression of recombinant proteins against chronic and infectious diseases.

2. General aspects concerning PMPs

In contrast to the conventional production systems of recombinant proteins, plants-based production systems can vary from whole plants or plant tissues to suspension cells, hairy roots, moss, duck-weed, microalgae etc. (Xu et al., 2011).

The use of PBSs enables companies to develop new recombinant proteins within a short period. This can contribute to the realisation of personalised medicines and fulfilling the global needs for higher scales of vaccines, mABs or therapeutic enzymes in the near future (Xu et al., 2011a).

The original idea behind the development of PMPs was to vaccinate people by eating antigen-producing fruit or vegetables, that would deliver vaccines in a safe and cost-effective manner and to increase the number of childhood vaccinations globally. The concept of edible vaccines was born. In a highly competitive medicinal products market with costly established production methods the prospect of using edible vaccines was innovative and appealing. It was proposed that plants could be grown close to the consumers using local farming techniques. The ambitions were to cut down the overall costs of vaccination on grounds of transport expenses, production, purification and other down stream processes (DSPs) necessary for vaccines, that were manufactured in conventional production platforms (Thomas, 2011), (Ahmad, P. et al., 2012; Arntzen et al., 2005), (Moustafa et al., 2015).

In the 90's many small/medium-sized enterprises (SMEs) were established with the goal of raising capital with molecular farming, respectively PMF, (Fischer et al., 2014). The research area of PMF has evolved towards the controversies regarding GM food and plant breeding biotechnology. However, these developments were not mainly driven by industry, but via international funded research programs. Especially in Europe, where the acceptance regarding genetic engineering is not big in society, the development of PMPs stagnated and major commercial plant research organisations moved their GM operations abroad and settled, for example, in the United States (US) or Canada. Therefore, the settings of the local regulatory prerequisites and public acceptance play a key role in the business strategy of molecular farming start-ups (Paul et al., 2015). Continuous and successful research of biotechnical start-up companies will additionally serve to stimulate renewed interest in PMPs from large pharmaceutical companies, due to the high relevance and importance of more proven and approved products.

2.2 Drawbacks, current limits and advantages of PMPs

Conventional production platforms that produce recombinant therapeutic proteins like bacteria, yeast or mammalian cells have their own strengths and weaknesses. Mammalian cells such as Chinese hamster ovary cells (CHO cells) are generally able to produce identical, human-like proteins but the disadvantage of this production platform is that the yields of recombinant proteins are low. Furthermore, these proteins bear the risk of harbouring dangerous agents for example viruses, prions like bovine spongiform encephalopathy (BSE), pathogens or oncogenes. Therefore, specific purification steps need to be conducted during DSP, which increases the manufacturing costs.

Prokaryotic production platforms like bacteria or yeasts can produce recombinant proteins in high rates, but a major disadvantage is that they cannot perform the complex post-translational processes, which are typical for eukaryotes, such as glycosylation and correct protein folding. Hence, the number of different recombinant proteins that prokaryotic production platforms are able to produce is limited. Another disadvantage is that they store proteins in inclusion bodies and can produce endotoxins, which requires specific purification steps during DSP (Alvarez et al., 2014).

Nevertheless, in the continuous research over the past 30 years, some of the technical disadvantages of PBS have been addressed and many shortcomings have been improved. This fact gives a hint that plants are more flexible and capable compared to conventional production platforms in terms of the production of recombinant proteins.

However, a main drawback that remains in the EU is the current regulatory framework concerning the development and commercialisation of PMPs – which is the main subject of this thesis.

In the following, general disadvantages, limits and advantages of PMF will be outlined.

2.2.1 Technical / manufacturing related issues

Problems due to long development times and low yield – The early research stages of PMFs were characterised by an extensive development time and low yields of expression levels compared to conventional production platforms of recombinant proteins. Low yields of PMPs either result from low-level transcript expression or from instability of the recombinant protein (Obembe et al., 2011). By the invention of new expression methods, which will be in-

troduced in chapter 3.3.3, the mean development time of two years was successfully reduced to two or three weeks in order to produce gram-level quantities. Furthermore, solutions were found how to address the problem with the instability of proteins during manufacturing (Wilken, L. R. et al., 2012).

High Scalability – Today, the high scalability of the production of recombinant proteins in plants should be emphasised. They are relatively easy to scale-up, as they offer a rapid gene to protein turnaround time. Conventional protein-manufacturing practices are facing a major global capacity shortage for the production of biologics (Sil et al., 2014). The traditional methods cannot produce a sufficient amount of therapeutic proteins in order to meet patient needs. Once there are more authorised PMPs available, plants can help to reduce the shortage of biologics (Fischer et al., 2014), (Xu et al., 2011b).

Low upstream costs – As the technology is based on natural and renewable resources the production costs are significantly lower (Sil et al., 2014). Additionally, investment costs of plant facilities are cheaper compared to equivalent bioreactors. Hence, the production and cost advantages of PMPs can allow more capital to be invested in the development of new therapeutics (Sil et al., 2014). This outlook can offer patients a greater and faster access to new medicines in the long run (Phoolcharoen et al., 2014), (Sack et al., 2015b), (Stoger, E. et al., 2014).

DSP and purification – It has been estimated that over 70 % of the total production costs are related to the extraction and purification of the proteins. The processing steps are expensive, as purification is generally product specific and relies on the chromatography media, which isolate particular proteins (Fischer et al., 2015). Therefore, DSP is a critical step, concerning the commercialisation of a potential protein candidate. One approach to reduce DSP costs was the development of PMPs in plant cells as in vitro cultures, where the protein of interest would be directly secreted into the culture medium (Wilken, L. R. et al., 2012).

GxP issues - GxP collectively refers to all guidelines for ‘Good Practice’, which has an important meaning, particularly in medicine, pharmacy and in pharmaceutical chemistry. Establishing a PBS that meets current good manufacturing practice (GMP) requirements and good agricultural and collection practice (GACP) is a great challenge especially in terms of the cultivation of whole plants (see chapter 3.1). One problem with whole plants is, that they are susceptible to pests and diseases, or can be contaminated by agrochemicals or fertilizers (Alvarez et al., 2014). As the aim is to guarantee batch-to-batch consistency of the target protein, it is an overall requirement that the manufacturing facility should be as controllable as

possible; this could be achieved in a contained/confined environment like bioreactor tanks or glasshouses. At present, there are globally only a few dedicated production facilities, which can manufacture PMPs under GMP-compliant conditions.

Risk of gene transfer – There is a potential risk of gene transfer considering the food or feed chain. Therefore the cultivation of whole plants requires working under specific regulations for GMOs in the field and only after an approved risk assessment (see chapter 3.2.1 and chapter 3.2.2), (Xu et al., 2011a), (Obembe et al., 2011).

2.2.2 Protein-related issues

Instability of recombinant protein and bio containment – Proteins bear the difficulty that they are very fragile and can be attacked by proteases, which leads to low yields of PMPs (Obembe et al., 2011). However, when administered for therapeutic use, they must be able to withstand the harsh conditions in the gastrointestinal tract to reach the effector sites, which are the mucosal surface and gut-associated lymphoid tissue. Plants offer the great advantage to protect the proteins from degradation due to bio-encapsulation. The plant cell wall provides an initial barrier, but proteins can be additionally protected through accumulation in subcellular compartments such as plastids or seed storage organelles (Hefferon, 2013). Therefore, they can withstand chemical, thermal and enzymatic degradation. As an example, the use of lyophilised carrot cells for the oral delivery of a therapeutic enzyme is currently being tested in clinical trial phase II and furthermore freeze-dried tobacco, Arabidopsis and lettuce cells also showed effective results (Sack et al., 2015a).

Post-translational modifications in order to produce safe, complex and 'hard-to-make' therapeutic proteins – Plants are very efficient protein producers. As eukaryotes, plants are more similar to mammalian cells and therefore have a clear advantage over bacteria for the performance of post-translational modifications like disulphide bond formation, subunit assembly or proteolysis. The post-translational modifications in plants can be controlled and as a result help to fold functional proteins (Hefferon, 2013), (Phoolcharoen et al., 2014). Additionally, they can assemble multimeric proteins and excel at producing complex therapeutic proteins (Thomas, 2011). By this clear advantage, the production of diverse tailor-made therapeutic proteins for the personalised medication of individual patients is possible (Yusibov et al., 2011), (Xu et al., 2011b).

Safe biologics – A clear advantage over mammalian and microbial cells is that plant cells lack harmful human or animal pathogens or endotoxins (see also chapter 5.2). Although plants can contain potentially harmful metabolites or plant viruses, there are no reports that plant viruses cause diseases in humans or other mammals, as humans are exposed to a large number of plant viruses daily in food (Pelosi, A. et al., 2012).

Non-mammalian glycosylation – Many therapeutic proteins are glycoproteins, and a human-like glycosylation pattern can be necessary for full biological activity and therapeutic efficacy. For this reason, there should only be little difference between the plant and human glycosylation patterns of proteins, in order to prevent immunogenic or allergic reactions. The research area of glyco-engineering has evolved 'humanised' plant lines that produce PMPs with mammalian glycosylation. This is achieved by knocking out enzyme genes in order to include plant specific glycans and/or mammalian glycosylation genes into the plant cell (Chen, 2011), (Obembe et al., 2011).

Variety of recombinant proteins – Because of a choice of different expression methods (see chapter 3.3.3) in whole plants or plant cell cultures, various recombinant subunit vaccines, therapeutic proteins and mABs can be produced. Some of these products have been tested in early phase clinical trials and show good safety and efficacy (Fischer et al., 2014).

2.3 The current status of PMPs

The pros and cons of PMPs, as described in the previous chapter, implies that a range of technical and regulatory factors has to be considered during the research and development, to enable a successful and commercial production. PMPs have suffered in the past from several technology and regulatory setbacks. One of the key features of PMF that distinguishes it from other manufacturing concepts of recombinant proteins is that there is no standardised platform or single technological basis. Diverse platforms and methods have been developed with distinct and overlapping benefits. The only common factor is that they utilise plants and plant organs. The platforms can vary from plant cells or simple plants like duckweed or mosses growing in bioreactors containing fully defined synthetic substrate to whole plants growing in soil or soil-free environments. The expression methods differ from stable transgene integration to transient expression using bacterial, viral, or chimeric vectors (Stoger, E. et al., 2014). Currently, PMPs are being developed for the treatment of disorders such as Alzheimer's disease, cancer, chronic obstructive pulmonary disease (COPD), Crohn's disease, cystic fibrosis,

diabetes, geriatric and child diarrhoea, heart disease, Hepatitis C, human immunodeficiency virus (HIV), iron deficiency, kidney disease, multiple sclerosis, obesity, rheumatoid arthritis and spinal cord injuries etc. (Sil et al., 2014).
The development of PMPs is a major challenge for small biotechnology start-up companies, because on one hand they often lack the regulatory know-how, so that when a promising target protein has been found, cooperation with a big pharmaceutical company is very likely as they help to provide the regulatory, technical and marketing know-how. However, the regulations for PMPs in the EU are more stringent than in any other part of the world and therefore a positive outcome of a marketing approval process is unpredictable and long-termed.
This leads to the situation, that many biotech companies switched to the development of plant-derived active ingredients for the cosmetic industry, that require a less complex, costly and time-consuming approval process (Paul, et al., 2015).
Due to the uncertainties in the approval process of PMPs in the EU, the interest of major pharmaceutical companies has decreased in recent years and start-up companies settled their research facilities out of the EU (Sparrow et al., 2013), (COGEM, 2009). In order to revitalise the research area of PMPs, multiple global research programs have been started, to pave the way from development of a PMP to the first clinical trial phase. As an example the EU funded project Planta Pharma successfully established a PBS of whole plants, see section 5.1.
Table 1 gives a status overview of the different clinical phases of current PMP projects.
To date, only one individual PMP has been approved for human application worldwide. Elelyso was approved by the US Food and Drug Administration (FDA) in 2012, a medicine developed by the biotech firm Protalix, which is indicated for the rare Gaucher disease, see chapter 5.2.
It does not seem that PMPs will fully replace conventional production platforms of recombinant proteins, since these platforms have evolved during the last years and continue to improve. Rather, PMF can be seen as a niche technology for the production of PMPs (Sparrow et al., 2013). PMF can be advantageous particularly for products, which cannot be easily manufactured by conventional production platforms, for instance if a target protein contains glycan structures, which are difficult to generate (Stoger, E., et al., 2014).

What are the prerequisites in the research and development of PMPs? What specific documents are required in order to apply for a PMP in the EU? These questions will be addressed in the next chapter.

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

Table 1 – Examples of current PMPs assessed in clinical trials (Sack et al., 2015b)

3 Dossier requirements for an MA application in the EU

Regulation (EC) 726/2004 is the European pharmaceutical legislation. An applicant for an MA for a biotechnological medicinal product should submit to the European Medicines Agency (EMA) an application dossier with all necessary administrative information, quality, non-clinical and clinical data of the medicinal product. These data would then be assessed in accordance with the centralised procedure.

3.1 Prerequisites: GACP, GMP, GLP and GCP

The research and development phase as well as the complete manufacturing of PMPs needs to adhere to the 'Good Practice'-principles as follows:

Good agricultural and collection practice

The guideline on good agricultural and collection practice (GACP) for starting materials of herbal origins is not considered as a GMP guideline per se, but serves as a basis for the establishment of an appropriate quality assurance system (EMA, GACP, 2006). In order to obtain a reproducible quality of an herbal starting material, it is important to set an adequate quality assurance system for the wild collection and cultivation, harvesting and first processing steps of host plants. The GACP guideline closes the gap between the activities in the field and the industrial processing of medicinal products, which takes place in accordance with the principles of the GMP guideline. As GACP is primarily targeted at non-transgenic medicinal plant cultivation, it is not alone adequate to define good transgenic crop production in terms of GM plants. Additionally, a system with inspections and certifications needs to be established in order to fill the gap sufficiently.

Good Manufacturing Practice

When recombinant proteins are intended for medical use they are covered by the same regulatory guidelines for manufacturing as all other pharmaceuticals, and when these proteins enter clinical development it is a prerequisite according to the new Clinical Trials Regulation that the protein must have been produced according to GMP (Fischer et al., 2012). Therefore, the importance of GMP compliance becomes relevant from the very beginning of clinical development (Pelosi, A. et al., 2012).

The GMP requirements are laid down in Directive 2003/94/EC, in Volume 4 of the Rules governing medicinal products (EC SANCO, 2012). Special needs for the manufacturing of

biological medicinal products are contained in Annex 2 of the EU GMP guideline covering products like vaccines, immune sera, antigens, hormones, cytokines, enzymes, products of fermentation including mABs and products derived from recombinant Deoxyribonucleic Acid (DNA).

In principle, the well-characterised GMP regulations that are applicable to therapeutic proteins produced in bacteria and mammalian cells are directly transferrable to plants (Fischer et al., 2012). In practice, only plant cells in a bioreactor can fully meet the GMP criteria concerning the manufacturing of recombinant proteins in a contained and sterile environment (Fischer et al., 2012). This implies that only a production method based on controlled conditions can guarantee batch-to-batch consistency, which is a crucial aspect in a GMP-compliant production platform (Tekoah, Y. et al., 2015).

Adherence to the current GMP regulations can safeguard the identity, strength, quality and purity of PMPs, when manufacturing steps are properly validated and controlled (Tekoah, Y. et al., 2015). To build a GMP-compliant manufacturing facility, the establishment of a stringent quality management system (QMS) is required, to settle robust operating procedures, detect and investigate product quality deviations, and collaborate with reliable testing laboratories (Tekoah, Y. et al., 2015). This controlled production system helps to prevent events of contamination, mix-ups, deviations and errors and guarantees that the PMP can meet strict quality standards. At present, only a few plant-based production facilities can meet these GMP standards and are certified (Tekoah, Y. et al., 2015).

Good Laboratory Practice

Good laboratory practice (GLP) is a quality system concerning the organisational process and conditions, under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded, achieved and reported (OECD Principles of GLP and comliance monitoring, 1998).

As a prerequisite for a clinical development program and for a marketing authorisation (MA) procedure it is necessary to generate high quality and reliable test data concerning the safety of pharmaceutical products.

In general the primary goals of preclinical safety evaluation are:

- to find an initial safe dose followed by dose escalation schemes in humans;
- to detect potential target organs for toxicity and further toxicity studies; and
- to detect safety parameters in clinical trials.

As the development of biopharmaceuticals is a rapidly evolving scientific area, a common understanding and continuing exchange of scientific opinions is in this case inevitable.

Good Clinical Practice

The ultimate test of a PBS is whether a developed product can be utilised in clinical trials. As a requirement to move on from research laboratory to the clinical trial phases, the product must gain approval from competent authorities (Thomas, 2011).

Good clinical practice (GCP) forms rules for conducting clinical trials that are internationally recognised and prepared in accordance with ethical and scientific aspects. In the EU the Clinical Trial Regulation is valid for all medicinal products (Regulation (EU) No 536/2014, 2014). The focus is to protect study participants and their informed consent, as well as to ensure the quality of study results. As a pharmaceutical product derived from any expression system used must be extensively characterised before the conduction of a clinical trial, this leads to the fact that many products do not reach a clinical trial phase (Thomas, 2011), (see also chapter 3.5).

3.2 Specific aspects regarding administrative information (Module 1)

3.2.1 Potential hazards / risks of GMOs

Concerns about the risks from transgenic organisms initially emerged when the first GMOs were developed in the 1970s. Particularly in the EU, the anti-GM lobby began to grow by the end of the 1990s in parallel to a growing number of organic food supporters (Paul et al., 2015). Consequently, almost all the major agricultural companies moved their biotechnology activities away from the EU (Paul et al., 2015).

The main concerns associated with transgenic plants are:

- the threat to biodiversity by transgenes spread due to cross-pollination and pollen dispersal (Moustafa et al., 2015);
- seed or fruit dispersal;
- gene flow and unintended exposures to non-target organisms like insects, soil microorganisms due to the biological activity of PMPs on living cells (Obembe et al., 2011);
- higher expression levels of target proteins due to the optimisation of expression methods (Spok, 2007).

3.2.2 Risk analysis of PMPs

In many countries across the world, transgenic plants and their products need to be evaluated in a risk analysis before they can be used for experimental or commercial use. The risk analysis process for transgenic plant activities is the same as for any activity with GMOs (Sparrow et al., 2013). A risk analysis consists of the three key elements:

- risk assessment;
- risk management;
- risk communication.

Risk assessment always refers to a basis of known and accepted risks (COGEM, 2009). Any risk must exceed a certain threshold value or baseline before the plant in question receives a negative safety assessment (Ramessar, K. et al., 2008). The level of risk is estimated based on the seriousness and possibilities of harm. For an overall risk assessment a range of issues
(areas of risk) needs to be taken into consideration to ensure that human and animal health as well as the environment are appropriately protected (Sparrow et al., 2013).

Examples for possible areas of risk, that need to be assessed in terms of PMF, are:

- molecular characterisation of the GM plant (information on the structure and stability of the favoured traits);
- a comparative analysis of compositional, phenotypic and agronomic characteristics to detect differences between the GM plant and their derived products and non-GM comparators;
- the toxicological assessment of GM, for example, the impact on human and animal health of potentially biological relevant changes in the GM plant and/or derived food and feed resulting from GM;
- interactions between the GM plant and target organisms respectively non-target organisms;
- the assessment of the allergenic and immunogenic potential of the new protein (this assessment would determine whether a transgenic plant would be approved for a field release);
- the nutritional assessment to demonstrate that the food and feed derived from a GM plant is nutritionally not harmful to humans and/or animals;
- potential for gene transfer;

- potential interactions with biogeochemical processes and with the abiotic environment;
- impacts of altered farming practices due to the cultivation of GM plants;
- the scientific quality of the proposed post-market (environmental) monitoring plan (Sparrow et al., 2013).

Moreover, it is necessary to investigate direct and indirect, as well as immediate and delayed long-term effects. Although crops used for PMF are not intended to enter the food/feed chain, a safety assessment as an integral part of the precautionary approach in the EU would still be required. The European Food and Safety Agency (EFSA), (see also chapter 4.2) released guidance notes on the risk assessment for GM crops for non-food/non-feed uses (Sparrow et al., 2013).

Risk management evaluates, selects, and implements plans or actions to ensure that potential risks are appropriately handled. Therefore, identified risks are assessed concerning their acceptance, minimisation or reduction and, if necessary appropriate prevention and controls are established. As regulators rely on risk assessments to make a reasonable decision whether the use of a GM plant is favourable, it should be comprehensible what assumptions have been made during the risk assessment, and what uncertainties associated with each identified risk are still present. Risk management needs to be separated from risk assessment both functionally and temporally in order to avoid any conflict of interest and to protect the scientific integrity of the risk analysis (Sparrow et al., 2007).

Risk communication is defined as an active exchange of information and opinions between risk managers, risk assessors and other parties dealing with potential risks throughout the risk analysis. The risk assessment findings are discussed and justification is given why decisions were made concerning risk management (Sparrow et al., 2007).

3.2.3 Pharmacovigilance aspects

The necessity of a Risk Management Plan (RMP), that represents a detailed description of the risk management system, for all newly authorised medicinal products was introduced in the new pharmacovigilance legislation in 2010 (Regulation (EU) No 1235/2010 and Directive 2010/84/EU – amending Regulation (EC) No 726/2004 and Directive 2001/83/EC – accompanied by the Commission Implementing Regulation (EU) No 520/2012). In the EU, an RMP in accordance with current EU legislation and pharmacovigilance guidelines should be presented, although this is not r specified respectively for GMOs. The summary of main identi-

fied and potential risks of the medicinal product and missing information serves as the basis for an action plan for pharmacovigilance and risk minimisation activities. Appropriate measurements should be set in order to identify biologicals that would be prescribed or sold in a country. The directive covers that all medicinal products with a new active substance and biological medicinal products, including biosimilars, are priorities for pharmacovigilance. Furthermore, reference is given to International Conference on Harmonisation (ICH) or EU/US guidelines on the post marketing safety monitoring.

3.2.4 Environmental Risk Assessment

Potential risks to the environment due to the use and/or disposal of the medicinal product should be addressed in the Environmental Risk Assessment (ERA) and proposals for appropriate labelling associated with the environmental risks should be made.

As a requirement, the ERA should be carried out in accordance with the principles set out in Annex II of Directive 2001/18/EC (see chapter 4.1) and in connection with the guideline on the ERA of medicinal products consisting of, or containing, GMOs (Directive 2001/18/EC). The ERA applies without prejudice to other relevant safety requirements as regards risk assessment, risk management, labelling, monitoring, public information and safety clauses provided by the community.

3.3 Specific aspects regarding quality (Module 3)

In the following chapters the aspects, which play a key role in the manufacturing of PMPs, will be introduced.

3.3.1 Peculiarities in the drug development – state of the art of science and technology

In the past, the biopharmaceutical industry traditionally focused only on a few conventional production platforms. The focus was to improve the production platforms gradually in order to increase efficiency and performance and therefore keep the up-front costs as low as possible. Here the regulatory framework evolved on established production techniques.

Plants are, in contrast to the conventional production platforms, more flexible and meanwhile diverse PBS were successfully established (see Figure 1). Because of a multiple choice of production methods of PMF, the research and development can focus on the product rather

than on the production platform, thus representing an innovative and challenging approach in terms of recombinant protein production.

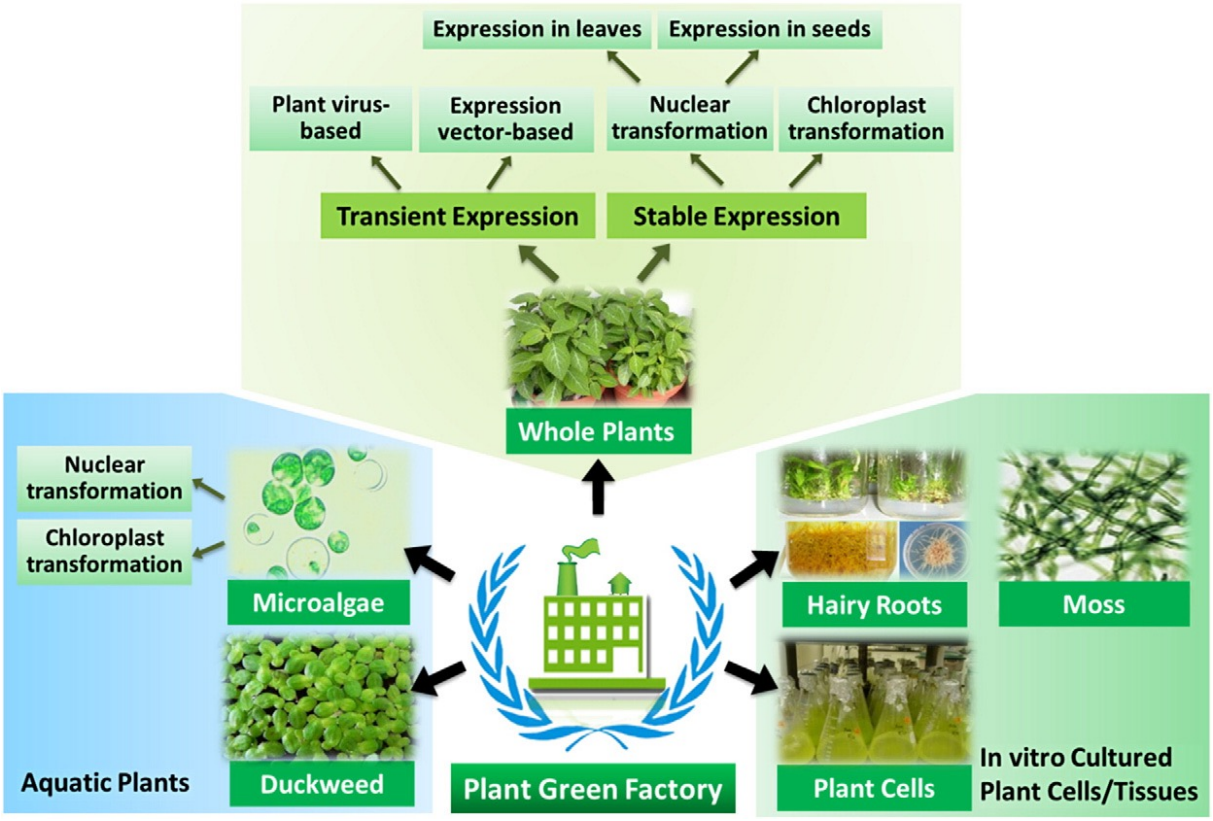

Figure 1 – Various plant expression platforms for the production of recombinant proteins (Xu et al., 2011a)

In general the biopharmaceutical production pathway is characterised by the following production steps: growing, harvesting, transport, storage, is considered as the upstream processing (USP) and the second manufacturing phase, the DSP is characterised by the extraction and purification of the protein (Obembe et al., 2011).

One of the main outstanding issues in the development of PMPs is to achieve sufficient yields of the protein of interest (Tekoah, Y. et al., 2015). A strategy to improve the yields of recombinant proteins needs to be assessed on a case-by-case basis, as it may not work for the production of another protein (Penney, C. A. et al., 2011). In terms of genetic engineering, there are adjustable parameters, which can potentially optimise a PBS; there are on the one hand inside features like messenger Ribonucleic Acid (mRNA) and protein primary structure and on the other hand outside features like the choice of host system, vector system or culture conditions. Both groups, inside and outside features are closely connected and influence each other (Ullrich, 2015). Due to the consistent impulse to improve protein yields, various techniques

like stable or transient expression in plants, nuclear or the production in chloroplasts, subcellular targeting, optimisation of the codons, choice of promoters, choice of host plants and specific plants tissues were developed and will be introduced in the following chapters (Pelosi, A. et al., 2012). The issue for the feasibility of scale-up in order to achieve high protein yields in accordance to GMP requirements is a key issue that needs to be considered in the early development phase of a PMP. Although only a small scale may be sufficient to show that a protein candidate is safe and efficacious in proof-of-concept trials, it is crucial to demonstrate that the therapeutical protein can be produced in a batch-to-batch consistent manner, plus in an adequate quality.

Another main issue to place PMPs on the market successfully and make them competitive to their counterparts derived from conventional production platforms is to keep the production costs as low as possible. For instance, the cost of goods (COGS) plays an important role, as it must be consistent and favourable to ensure profitability for the manufacturer and cost-effectiveness for patients. The main cost factor concerning the manufacturing process of PMPs is related to DSP expenses and can account for up to 80% (Obembe et al., 2011), (Xu et al., 2011b). This is because biopharmaceuticals for GMP manufacturing may require protein purities as high as 99%. (Wilken, L. R. et al., 2012). In this light, an oral delivery of mABs and vaccines appears economically advantageous as no or only a reduced purification would then be necessary.

3.3.2 Choice of host crop – cells and whole plants

The selection of an appropriate host plant to implement molecular farming technology is important for its general success, as an efficient production of recombinant proteins needs to be assured.

In order to gain increased yields of recombinant proteins, an ideal PBS should exhibit the following features:

- ease of genetic manipulation by either stable transformation or transient expression;
- high protein expression level;
- low overall costs;
- low proteolytic activity in the metabolism of the plant;
- high product stability in the expression environment;

- low concentration of secondary metabolites, that may influence structural changes of the protein or biological properties or which may complicate purification processes;
- capability of post-translational modifications, uniformity in the glycosylation pattern and proper protein folding;
- homogeneous dispersion in a bioreactor (Huang, 2012), (Ruiz et al., 2015), (Moustafa et al., 2015).

In Table 2, the different plant expression systems are introduced in light of the features: production time, scalability and regulatory feasibility.

Expression system	Commercially viable species	Time for production	Scalability	Regulatory compliance
Whole plants:				
• Stable transgenic plant	Corn, soy, safflower, rice	3–6 months	Unlimited field culture	Difficult
• Transient plants	Nicotiana sp., lettuce	2–7 days	Greenhouse limited	Moderate
In vitro cultured plants:				
• Hairy roots	Nicotina sp.	14–30 days	20.000 L	Easy
• Cell suspension cultures	Tobacco BY-2, carrot, rice	7–20 days	100.000 L	Easy
• Moss	Physomitrella patens	14–30 days	200 L	Easy
Aquatic plants:				
• Duckweed (closed system)	Lemna sp., Spirodela sp.	20–40 days	10.000 L	Moderate
• Microalgae	Chlamydomonas reinhardtii			
➢ Open system		20–40 days	Ltd. by water surface area	Difficult
➢ Photobioreactor		14–30 days	10.000 L	Moderate
➢ Conventional bioreactor		7–20 days	200 L	Easy

Table 2 – Comparison of different expression systems (Xu et al., 2011)

In vitro plant cell-based systems

In vitro PBSs are used for the production of PMPs and combine the benefits of whole-plants with those of microbial or mammalian cell cultures in bioreactors (Tekoah, Y. et al., 2015). The concept of in-vitro PBS is to be as similar as possible to well-known microbial and mammalian cells, which are meeting the current regulatory requirements set by the main health agencies like the FDA and EMA (Huang, 2012). Characterised as in-vitro PBS are aquatic plants, plant cell cultures, hairy root cultures, moss and green algae (Sil et al., 2014), (Wilken, L. R. et al., 2012).

Plant cell suspension cultures are usually derived from stably transformed plant tissues by Agrobacterium-mediated transformation (see chapter 3.3.3). Therefore callus cells derived from explants (harvested directly from target tissue) of transgenic plants can be grown in a chemically defined media to establish transgenic cell suspension cultures. Similar to conventional production platforms, it is possible to establish a cell banking system for plant suspension cultures. The cell banking system consists of a master cell bank derived from an appropriate cell line identified as the future production line, as well as a working cell bank used for continuous manufacturing (Tekoah, Y. et al., 2015). This cell bank is the cell culture source that will eventually be taken for producing the protein for clinical trials and later for commercial purposes (Tekoah, Y. et al., 2015). This cell banking system and the cell line will be completely characterised according to applicable guidelines, in order to meet the requirements of the competent authorities. For now, there is no specific guideline for plant cell culture cell banks, but the requirements and reasoning are similar to those of mammalian cell cultures, where applicable (Tekoah, Y. et al., 2015).

Normally the supplement of plant growth regulators is required in the medium to enhance rapid cell growth and maintain cell morphology (Huang, 2012).

In comparison to whole-plant cultivation, plant cell culture platforms provide several advantages:

Shorter production cycles – The timescale for the protein production in plant cell culture requires days or weeks compared to months in whole plant expression systems.

Rapid growth – With cell doubling time as short as one day, proteins can be synthesised at high levels and can either be stored in intracellular compartments or secreted into the culture medium, that facilitates the purification of the target proteins (see chapter 3.3.5) (Xu et al., 2011b).

Simple DSP – A simplified procedure for secreted protein isolation/purification from an inexpensive, well-defined medium lacking exogenous proteins, which would complicate the purification process.

Improved consistency of the protein product – Due to controlled growth conditions with complete containment, batch-to-batch consistency in product yield, quality and homogeneity is ensured and GMP requirements can be met (Wilken, L. R. et al., 2012).

Low culture costs – Like microbial systems, plant cell-based systems bear low culture costs, because simple synthetic media are used and allow rapid scale-up of the protein product (Tekoah, Y. et al., 2015). In order to achieve the desired production process the growing media composition, including the aeration, mixing rate and nutrients is controlled (Tekoah, Y. et al., 2015).

Safety – The best advantage that plant cell systems have over mammalian cell cultures is that plant cells do not harbour any known human pathogens. Furthermore, a closed bioreactor system prevents gene flow in the environment and contamination of the food chains (Huang, 2012).

Fewer regulatory burdens and environmental compliance – Because the quality and safety of the recombinant protein is ensured, concerns regarding the release of GMO can be limited or fully eliminated.

Even if the above-mentioned advantages are obvious, the overall yield and usability of in-vitro PBS is limited. As the system is more similar to microbial production systems, the protein size and complexity is restricted and the yield of recombinant protein reduces significantly during the late stationary phase due to an increased proteolytic activity. In addition, the system is limited to small numbers of well-characterised plant cell lines only, such as tobacco, rice, carrot, Arabidopsis (Obembe et al., 2011).

Whole plants:

One advantage of using whole plants as a production platform is the fact, that production scalability is nearly unlimited (Xu et al., 2011).
Other economic factors that play a key issue on the selection of an appropriate host plant are:

- biomass yield;
- facilities for storage;
- transportation;
- value of the PMP itself;
- preproduction costs;
- scaling and maintenance costs;
- containment costs;
- availability of labour and land area;
- length of production cycle;
- costs of DSP and edibility (Obembe et al., 2011).

The drawbacks of whole plants are:

- long development times for stable transgenes;
- instability of product yield and quality;
- issues with possible fertiliser contamination;
- impact of pests;
- diseases and weather conditions in the 'production factory';
- the challenge of meeting current GMP standards (Xu et al., 2011b).

Non-food/non-feed crops - An appropriate example for a non-food crop often used in PMF is tobacco. Tobacco can gain high biomass yields due to high scale-up capacity, has a non-food/non-feed status, therefore the co-mingling with the food and feed chain is prevented and transgenic techniques are well-established. A supplemental advantage of tobacco is that a year-round growth and harvesting is possible. By these features, tobacco is the most favoured whole plant host for molecular farming. However, the disadvantage of tobacco is, due to the large numbers of toxic substances like nicotine and other alkaloids, special DSP is required, which increases the manufacturing costs (Obembe et al., 2011), (Moustafa et al., 2015).

Leafy crops and fruits – Green leaf crops like lettuce, alfalfa or soybean or fruits like banana, papaya etc. offer high biomass and large numbers of soluble proteins. The major advantage with these plants is that their edible parts can be consumed without being cooked or (partially) processed. The disadvantage is that they do need to be processed directly after harvest in order to avoid a great loss of PMPs due to proteolysis (Moustafa et al., 2015), (Obembe et al., 2011). Hence, it is necessary to desiccate the leaves, freeze or treat them directly after harvest. At present, it is unlikely that PMPs will be administered in this way, as it is difficult to ensure batch-to-batch consistency and determine the amount of correct dosage (Sparrow et al., 2007).

Seed crops – Cereals like rice and maize are gaining popularity as they have natural biochemical processes that help to accumulate stable proteins and enable dehydration and preservation and produce high levels of protein (Sparrow et al., 2007). Therefore, seeds can minimise the requirement for fermenters as well as a cold chain for storage, thus reducing production costs. However, they also bear a high risk of contamination due to cross-pollination and pollen dispersal (Sparrow et al., 2013); in this case the use of barley and legumes like peas or beans can lower the dispersal risks. In Figure 2 the production schemes of PMPs in expressed seed crops and leafy crops are compared.

When choosing a suitable host plant, the consequences of the chosen plant on the surrounding environment, human health and food safety should all be taken into account (Moustafa et al., 2015). There are recommendations available from regulatory bodies in the US or Canada, that some plants are not suitable to be used for field production (Sparrow et al., 2013). Plants like oilseed rape (canola) and alfalfa are considered not suitable, because they require insect pollination and are reproductively compatible with local weed species, plus they have multiple year seed dormancy. Therefore, they have an increased risk of transgene spread. On the other hand, these crops could be beneficial hosts if grown in containment (Sparrow et al., 2013).

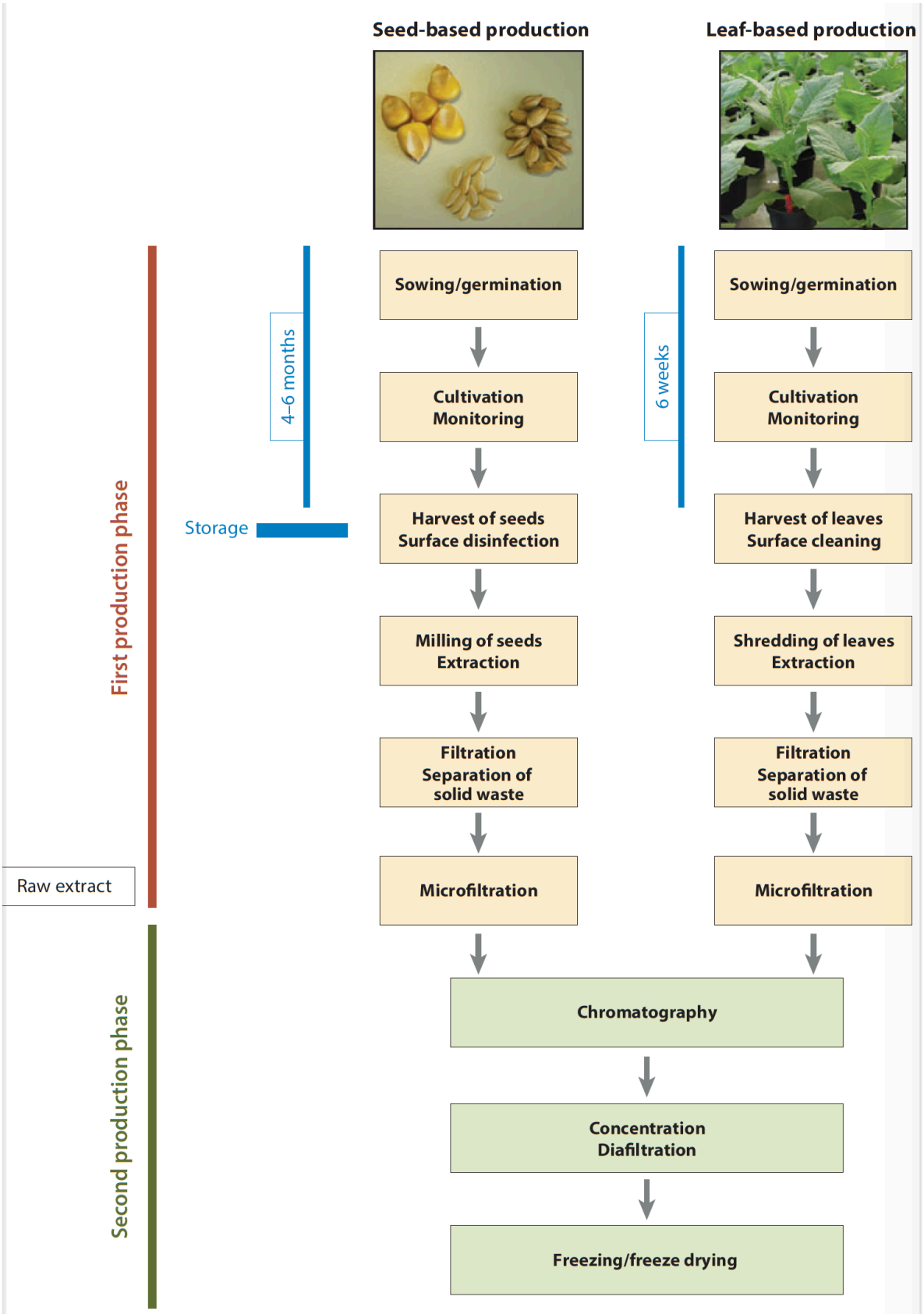

Figure 2 – Example of seed- and leaf-based production schemes (Stoger, E. et al., 2014)

3.3.3 Plant transformation methods

There are two main categories of plant transformation to produce PMPs: stable expression and transient expression. In stable production systems the insertion of foreign genes into the genome of a host plant is involved. Therefore the foreign genes in the host's genetic lineage over multiple generations are maintained (Penney, C. A. et al., 2011).

In transient expression systems bacterial or viral vectors are utilised to insert transgenes into a host cell (Penney, C. A. et al., 2011). The foreign gene or transgene is expressed only for a brief timespan (Penney, C. A. et al., 2011). The characteristics of both expression systems are described in the following paragraphs, while the pros and cons are summarised in figure 3.

Stable Expression:

From a regulatory point of view, the application of stable transgenic plants is more favourable to transient expression, because they are recognised under EMA guidelines (EMA, 2008), and only one organism needs to be regulated (Fischer et al., 2014). Additionally, stable transgenic plants are permanent genetic resources where the reintroduction of bacteria in every production generation is not required, hence the batch-to-batch consistency is easier to maintain (Fischer et al., 2012). It is possible to target the proteins to different subcellular compartments like the cytoplasm, the nucleus, the plastids and the vacuole. Furthermore they can be secreted into the apoplast or retained in the ER, thus providing various choices for posttranslational modifications (Gleba, Y. et al., 2005). High protein yields and post-translational stability can be achieved by stable insertion of transgenes into the nuclear or plastidal genome and by optimising the gene expression systems.

The choice of plant tissue, organ and cellular compartments to express and accumulate the proteins of interest has a strong influence on the integrity and the functionality of the final product (Moustafa et al., 2015). Due to this reason, plastids transformation (or transplastomic transformation) has been developed recently, where for example, chloroplasts carry the transgenes; protein expression from plastids can be boosted due to the high copy number of chloroplast genomes in each cell. Plant viruses, which have small genomes, make them relatively easy to manipulate. The benefit is that chloroplast transformation offers bio-encapsulation, which prevents the newly acquired genes from spreading and contributes to the stability of the fragile proteins. The disadvantage is that the recombinant proteins expressed in the plastids are more bacteria-like in terms of post-translational modifications (Hefferon, 2013).

Transient Expression:

Transient expression is characterised by the use of a specific bacterium *Agrobacterium tumefaciens*, plant viruses, or hybrid vectors with components of both (Magnifection method, (Gleba, Y. et al., 2005; Hayden, E. et al., 2014)). Unlike in stable transformation systems, foreign proteins that are expressed in plants by transient expression using virus-based vectors are not heritable by following generations (Jin, T. et al., 2015). These plant pathogens can infect plant tissues in shortest and most convenient time in terms of PMF (Obembe et al., 2011), (Sack et al., 2015b). The method is called **Agro-infiltration or Agro-inoculation:** liquid Agrobacterium sp. suspension infiltrates a plant or plant tissue. The Agrobacterium delivers transfer DNA from which genetic material is expressed locally and transiently in the plant to produce a specific protein (Sparrow et al., 2013). After the experiment the plant is usually destroyed in order to extract the target proteins, hence progeny of the inoculated plant tissue are not possible. However, progeny derived from an agroinoculated plant material in which absence of stably integrated genes is provided may not be considered a GMO and therefore will not fall under the scope of the GMO legislation (Sparrow et al., 2007), (Sparrow et al., 2013). The infiltrated plant material may not be considered as being GMO per se, but in this case, the transformed Agrobacterium cultures may be considered as GMO. Consequently, the infiltrated material will still fall under GM legislation and therefore a large-scale use of GM bacteria and/or viruses requires strict containment (Fischer et al., 2012).
As GMOs are included in the manufacturing process, this system will not necessarily reduce the level of regulatory requirements to produce PMPs, but can diminish the impact on the risks associated with the PBS system (Rybicki, E. et al., 2012).

As there are some variables that influence the production process like the strain of the agrobacteria, the growth medium, the cell and bacterial concentrations, the co-incubation period and the temperature, an optimisation for each plant species or cell type is likely and needs to be performed (Tekoah, Y. et al., 2015). Furthermore, transformation-accelerating metabolites e.g. acetosyringone can boost transformation efficiency. All these factors play an important role in the selection of certain plant cells (Tekoah, Y. et al., 2015). In the past, transient expression methods were mainly used for a quick validation of expression constructs, today they are used routinely for the production of considerable amounts of proteins (mg to g level) within a few weeks as research material for preclinical studies (Obembe et al., 2011). Commonly, protein yields between 4 to 20-fold higher than by stable transformation can be achieved, and viral vectors can produce even higher yields than Agrobacterium-induced expression systems (Alvarez et al., 2014).

On the one hand, transient expression is good for high gene expression within a short timeframe, and on the other hand best suited for low-volume protein production such as personalised therapeutics, seasonal vaccines, and other specialised markets (Jin, T. et al., 2015), (Wilken, L. R. et al., 2012).

As each plant needs to be inoculated individually, this makes a large-scale production particularly labour-intensive. This drawback was recently addressed by the development of fully-automated agroinfiltration systems to infiltrate plants like tobacco within short time. With the vacuum or airbrushing method, it takes only minutes to spray hundreds of plants with an Agrobacterium suspension containing the vector (Taicheng, J. et al., 2015). This enables a bulk vaccine production within 3-10 days of transgene vector becoming available. In contrast, stable plant transformation systems have a minimum lead-time of several months (Jin, T. et al., 2015). As transient expression systems allow a rapid testing of different genetic constructs. As plants do not have to be stably transformed over months these features offer clear advantages over current production platforms for influenza vaccines production (Penney, C. A. et al., 2011). Additionally, the safety and efficacy conducted in animals was confirmed (Fischer et al., 2014). The rapid nature of the expression method offers the opportunity that a PBS facility, normally used for the production of other recombinant proteins, could be utilised to rapidly design a vaccine and switch manufacture in the case of an emerging pandemic (Fischer et al., 2014). For now, influenza vaccines are mainly produced in fertile hen eggs using the prevalent influenza strains, as determined biannually by the WHO Global Influenza Surveillance Network (Penney, C. A. et al., 2011).
A recombinant version of the virus infects and grows in the egg. In the DSP, the egg proteins are separated from the viral particles. Although the egg-based production method is well established and cost-effective, a clear drawback of the production system is the lengthy process, which takes here 5 to 6 months from the determination of vaccine strains to vaccine release (Penney, C. A. et al., 2011). In case of a pandemic, there is then a significant gap between the outbreak and the availability of a vaccine in high yields (Fischer et al., 2014). This clearly demonstrates one advantage of transient expression systems over conventional methods.

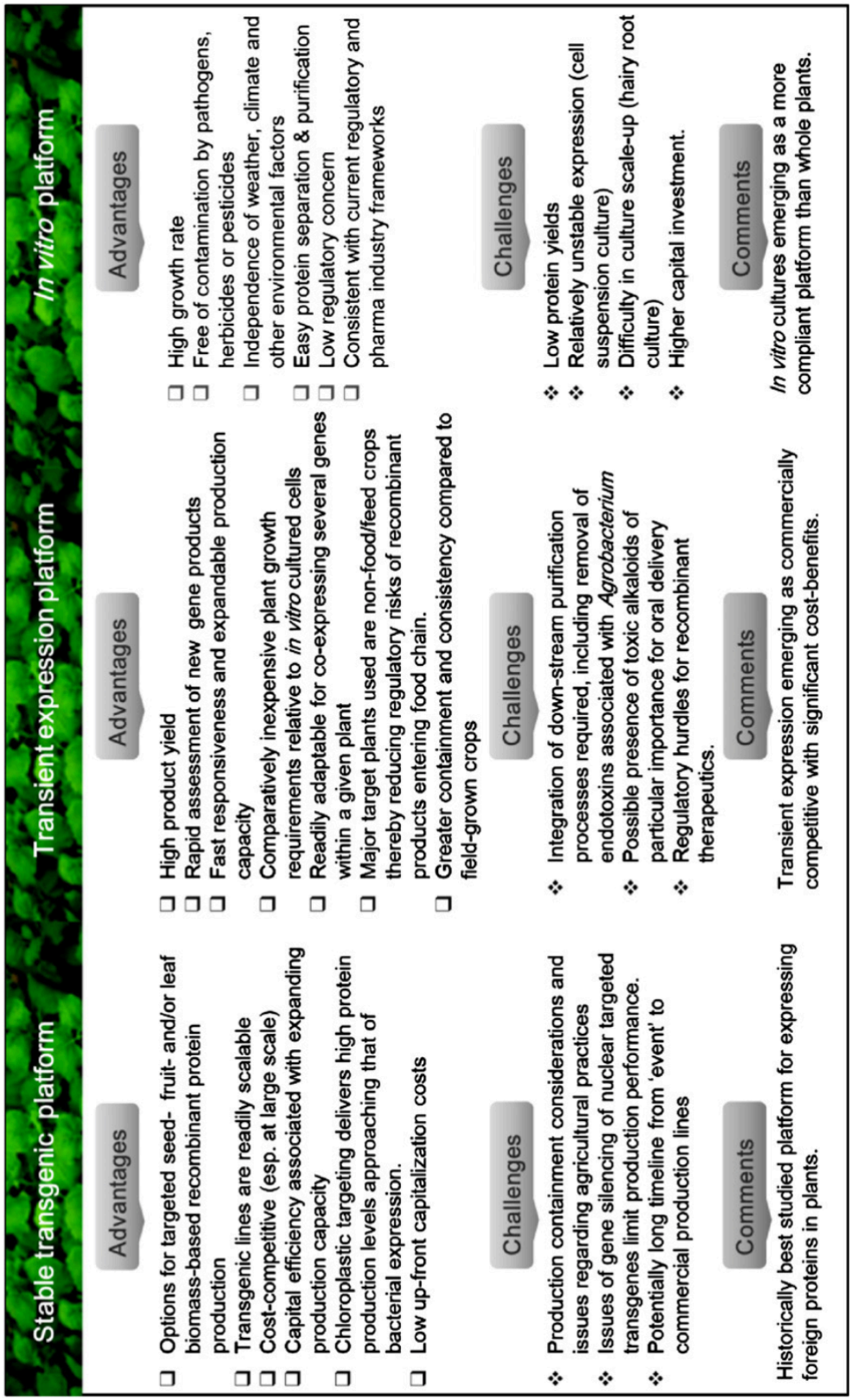

Figure 3 – Comparison of benefits and challenges associated with the three major types of plant production platforms (Xu et al., 2011)

3.3.4 Target protein delivery

Each PMP is developed in a case-by-case approach. While some PMPs are secreted and purified from the medium, others are targeted to accumulate within different cell compartments (Tekoah, Y. et al., 2015). The sub-cellular location, in which the protein acquires post-translational modifications, has a significant impact on the yield and stability of the protein and enables control of the glycosylation pattern, which has a great impact on the biological activity of the protein (Tekoah, Y. et al., 2015). The physiological conditions in cellular compartments like pH and salt concentrations can accelerate or inhibit protease activity (Moustafa et al., 2015). Given these considerations, the vacuole, the Endoplasmic Reticulum (ER), the cytoplasm and seeds have proven to be effective target locations, which enable a preservation of long-term stable pharmaceuticals (Moustafa et al., 2015). Proteins that do not require post-translational modification like glycosylation for their activity can be targeted by the chloroplasts, or if the protein is expressed directly by plastids transformation, which is for example possible in tobacco (see chapter 3.3.3), (Obembe et al., 2011).

An important aspect that should not be disregarded and which was the original idea in terms of PMPs is the accumulation of proteins in edible plant organs (particularly leaves, seeds, and fruits). An important advantage of the delivery of PMPs in edible plant organs reduces or even eliminates the costs of DSP as bio-encapsulation have a positive influence on the stability. This give advantages specifically for developing countries because recombinant proteins can be preserved in desiccated seeds for years (Fischer et al., 2014), (Tekoah, Y. et al., 2015). Although the idea of targeting recombinant proteins in edible crops seems to offer cost-effective and convenient prospects, edible plants directly used as oral vaccines, need to be handled with caution (Moustafa et al., 2015).
The difficulties are dose standardisation and the low efficacy of the oral route for immunisation. Furthermore there is the issue regarding the regulation of transgenic plants with fears of contaminating the food/feed chain (Xu et al., 2011a). Thus, this approach will probably be restricted to specific drugs and host systems, or alternatively non-food/non-feed crops should be considered in such cases (Moustafa et al., 2015). When they become feasible, edible vaccines should be administered only under the supervision of doctors or health care professionals (Sil et al., 2014).

3.3.5 Down stream processing (Purification)

DSP refers to the isolation and purification of recombinant proteins in every bio-manufacturing process. The targeted, purified protein should be free from compounds like potential allergens, toxins, teratogens and carcinogens and so on, to ensure that any remaining impurities in the final product are within a safe range. Key examples are nicotine from tobacco leaves and oxalic acid from alfalfa, or more generally, cellulose from plant tissues. As DSP can take 80% of overall production costs, the purification of proteins represents the most expensive production step in the manufacturing process (Obembe et al., 2011), (Xu et al., 2011b). Therefore, an improvement of DSP methods can significantly reduce the overall costs associated with plant cell-based production systems (Xu et al., 2011b).

Concerning regulatory aspects, DSP is crucial to assess the safety of pharmaceutical products. The highest purity is required in order to meet the regulatory standards. The final processed product will be held to the same standards of quality and efficacy as those imposed for established bacterial, yeast and mammalian cell-based production platforms (Fischer et al., 2015). The efficiency of DSP is influenced by the recombinant protein concentration, the complexity of the plant extracts, cell-free culture media, and the required final product purity (Wilken, L. R. et al., 2012).

The EMA and respectively the FDA have established strict guidelines to ensure safety and efficacy of the final product; especially when the product is intended for clinical trials, DSP must meet current GMP standards (FDA, USDA, 2002), (EMA, 2008). Therefore, a pure and homogeneous product, that reduced contaminants to an acceptable level, is requested, and that enables a uniform administration in order to ensure accurate dosage (Pelosi, A. et al., 2012). In the case of plants, it is an initial problem to characterise the nature of those contaminants and to ensure that they have been reduced to a safe level (Fischer et al., 2012). Safety levels are well established and the characterisation of contaminants is known in bacteria and animal cells. The processes have developed over a long time to remove endotoxins from bacterial cultures and viruses derived from mammalian cells. However, this is not applicable to plants. Plants can contain potentially harmful secondary metabolites or plant viruses. To date, there have been no incidences where plant viruses induce diseases in humans or other mammals, as humans are exposed to a large number of plant viruses daily in their food. Nevertheless, the regulatory guidelines do not accept unquantified risks and one risk can be sufficient to hold up further development (Pelosi, A. et al., 2012).

Protein extraction may be required in some cases to avoid toxic alkaloids present in some plant species or tissues. Depending on the production systems being used (whole plant, plant tissue, or cell suspensions) protein purification will be more or less extensive (see figure 4). In general the protein purification requires following steps:

- harvest of plant materials;
- extraction of total proteins;
- purification of the target recombinant protein.

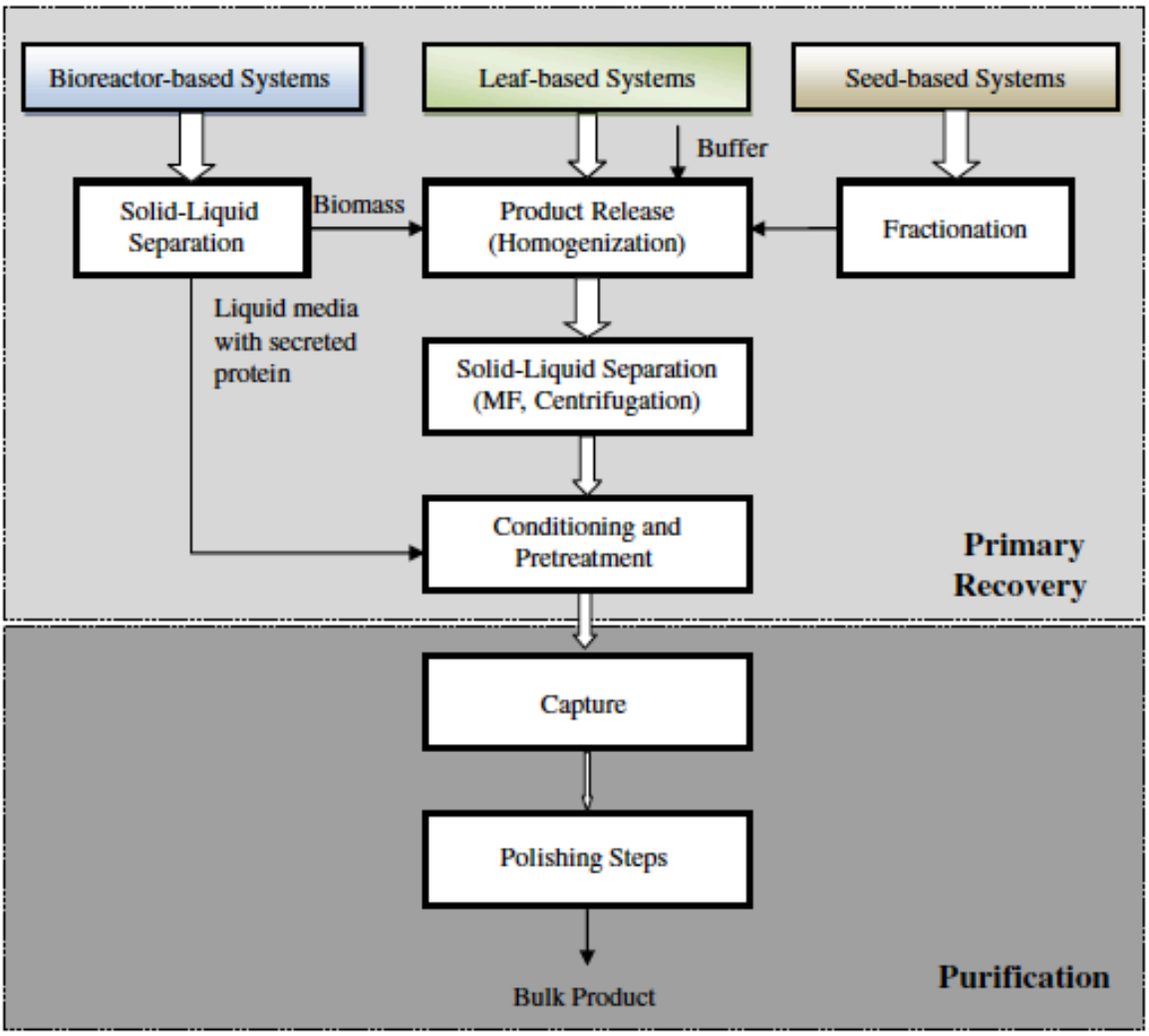

Figure 4 – Overview of DSP of recombinant proteins from bioreactor-based, leaf-based and seed-based systems (Wilken, L. R., et al., 2012).

3.4 Specific non-clinical aspects (Module 4)

The ICH guideline S6 (R1) – preclinical safety evaluation of biotechnology-derived pharmaceuticals – gives support to improve the quality and consistency of preclinical safety data in the development of biopharmaceuticals (ICH guideline S6 (R1), 2011). The objectives of the preclinical safety studies are to define pharmacological and toxicological effects prior to initiation of human studies and the clinical trial program. In-vitro and in-vivo studies are necessary tests in order to clarify the characteristics of a therapeutic compound. Biopharmaceuticals that are comparable in the structure and pharmacological properties to an already well-known product, for which there is wide clinical experience, toxicity testing may be reduced.

Toxicity studies should be performed in compliance with GLP (see chapter 3.1); however, conventional toxicity testing may not always be appropriate for biopharmaceuticals due to the unique and various biological and structural properties. As some studies require the settlement of specialised test systems, which are often required for biopharmaceuticals, they may not always meet GLP. Therefore, areas of non-compliance need to be clarified and their impact evaluated in relation to an overall safety assessment. Some cases may lack of full GLP compliance, but that does not necessarily mean that the data obtained from preclinical trials cannot be used for the support of clinical trials and the application for MAs.

3.5 Specific clinical aspects (Module 5)

The development of new regulatory guidelines for the production of biopharmaceuticals in plants and the Clinical Trial Regulation (Regulation (EU) No 536/2014, 2014) overlapped in the course that biopharmaceutical products must be manufactured in line with GMP in order to be used in a clinical trial phase I (Alvarez et al., 2014). Therefore, GMP becomes a necessity right from the start of clinical development (Fischer et al., 2012). In the clinical development, it is a general regulatory requirement in terms of GM plants, to document and explain their origins, purpose and safety regarding the expression vector. Furthermore, also the safety of the plant species and the impact of the transgene insertion need to be documented (Fischer et al., 2012). The focus on transgene insertion as a regulatory issue has driven researchers to look for less stringent approaches in order to improve yields of recombinant proteins that do not involve a stable nuclear transformation (Fischer et al., 2012) (see chapter 3.3.3).

The goal of most clinical studies has been to generate recombinant proteins that resemble the native protein as closely as possible, thereby enabling regulatory bodies to assess potential PMPs as ‘bio similar’ rather than novel agents (Webster, D. E. et al., 2012).

At present, the engineering of recombinant proteins with improved physico-chemical and/or pharmacological properties has the aim of generating a protein with better pharmaceutical characteristics, termed ‘biobetter’ (Webster, D. E. et al., 2012). Both protein engineering and host plant system engineering are under focus of current research and although all is still at an early stage, the results show significant promise (Webster, D. E. et al., 2012).
In order to meet these aims there should be as little difference between plant-derived proteins and human proteins as possible. The comparison of the glycosylation pattern is in this case one important aspect and although mammals and plants attach both N- and O-linked glycans, the systems are not identical (Tekoah, Y. et al., 2015). Many therapeutic proteins are in fact glycoproteins, and the glycans can be necessary for full biological activity, immunogenicity, appropriate pharmacokinetic properties, correct targeting and stability (Fischer et al., 2012). N-glycosylation occurs in several stages, the first of which involves the attachment and modification of an oligosaccharide precursor in the ER. This is similar in all eukaryotes. Enzymes, which are distinct in plants, mammals, insects and yeast, process the N-glycan resulting in species-specific oligosaccharide structures (Fischer et al., 2012). Plant-derived glycoproteins differ from those of mammals at the point that they carry specific

β1,2-xylose and core α1,3-fucose residues that are not found in mammals, but lack β1,4-galactose, α1,6-fucose and terminal sialic acid residues that are present in mammals (Xu et al., 2011b), (Fischer et al., 2012).

For now, there has been no strong evidence that plant glycans cause immunogenicity or allergenicity in the administered human subjects (Moustafa et al., 2015), (Tekoah, Y. et al., 2015), (Bosch, D. et al., 2013b). Moreover, plant-derived proteins often exhibit glycan structures that can be superior to proteins derived from mammalian cell cultures in terms of the homogeneity of glycan composition and bioactivity (Webster, D. E. et al., 2012). For example, saponins, a diverse group of metabolic glycosides and present in higher plants, have been shown to enhance systemic and oral delivery of antigens, therefore can function as an adjuvant during oral delivery (Pelosi, A. et al., 2012). Nevertheless, the potential impact of plant glycans on protein structure, function and safety remains an important regulatory issue and the main regulatory authorities are giving special attention in terms of safety and precaution (Fischer et al., 2012).

How are PMPs regulated in the EU and which laws are applicable? These questions will be addressed in the following chapter.

4 Marketing authorisation for a PMP under the regulation of (EC) 726/2004

4.1 The regulation of plants as GMOs in the EU

Genetic modification can be used to create organisms with new characteristics that have never before existed in the environment, and can therefore bear unconsidered risks (COGEM, 2009). In the Cartagena Protocol, an international agreement on trans-boundary movement of GMOs, signed by 153 countries including the EU and in force since 2003 (implemented by Regulation 1946/2003), a GMO is defined as 'any living organism that possesses a **novel combination of genetic material** obtained through the use of modern biotechnology' (COGEM, 2009). This statement cannot be simply transferred to plants, as nowadays it is not easy and clear to classify which product can be considered as a GM plant. Due to the recent development of transient expression techniques (see chapter 3.3.3) in plant biotechnology, the final products like recombinant proteins no longer contain foreign genes in their genome. This makes them difficult to distinguish from conventional plant breeding products. In the

EU, they fall under the GMO legislation and therefore need to be clearly labelled as GMOs; whereas in other countries, for example in the US, they are considered as conventional plant breeding products (COGEM, 2009), (Bundesamt für Umwelt (CH), 2012).
The current legislation on GMOs in the EU no longer seems suitable to cope with recent scientific innovations in plant biotechnology and therefore a revision is postulated in several publications, (Bundesamt für Umwelt (CH), 2012), (COGEM, 2009).

In the EU the GMO regulation is set down in various directives and regulations see Table 3. There are directives on activities with GMOs in laboratories (contained use) and for open field trials (deliberate release), on labelling and traceability, on food safety, and so on.

In terms of the authorisation of GM plants, there are two categories:

1. Cultivation in contained use (grown in laboratories, glasshouses, or contained facilities such as cell culture systems grown in bioreactors);
2. Cultivation for 'deliberate release into the environment' (for experimental purposes or for commercial cultivation) (Sparrow et al., 2013).

EU Directives 90/219/EC (amended by Directive 98/81/EC and lately by 2009/41/EC) deals with the **contained use of GMOs** and EU Directive 2001/18/EC (amended by 2008/27/EC) and Council Directive 90/220/EC are subject 'on the **deliberate release into the environment of genetically modified organisms**'. This means that within the EU, any field grown pharmaceutical crop, either food or non-food crop, requires an application under 2001/18/EC to the competent authority in the country where seeding is intended. Part B of the directive is dedicated for research trials and Part C for the commercial use of GM plants as in plant-derived products (Sparrow et al., 2007), (COGEM, 2009). The regulation does not differentiate field trials concerning pharmaceutical crops from food/non-food crops (Sparrow et al., 2007). By the end of the 1990s, Dir. 90/220/EC had to be replaced because various EU countries like France, Denmark, Italy, etc. announced a decision to block the authorisation of GM crops (COGEM, 2009).

However, MAs of pharmaceutical products derived either from plant cells grown in containment or from whole plants are granted on regulation 726/2004/EC (formerly 2309/93/EU). The relevant national authority oversees these regulations during the early clinical trial phase and by the EMA (Sparrow et al., 2013).

If the final product is intended for food and/or feed use, its potential use is regulated under the

scope of GM food and feed Regulation 1829/2003/EC and watched by the European Food and Safety Agency (EFSA) (Sparrow et al., 2013).

Directive/Regulation	Covers	Relevance to Pharma - Covers	Authority
Directive 2009/41/EC	Contained use of GMOs	• Early laboratory R&D phase • GMOs grown in enclosed cell culture systems (bioreactors) • GM plants grown in containment, i.e. glasshouses or controlled environment rooms	Member States National competent authorities
Directive 2001/18/EC	Deliberate release to the environment	• Part B of the directive covering experimental field release • Part C covering cultivation at the commercial level	Member States National competent authorities Member States National competent authorities, European Commission, EFSA
Directive 2001/10/EC	Safety and efficacy testing of drugs	Clinical trials	Overseen by the Member States National competent authorities, at the R&D stage and by EMEA at the marketing stage
Regulation (EC) 726/2004	Laying down community procedures for the authorization and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency	Would follow the existing regulations developed for mammalian/ bacterial systems and draw guidance from EMEA guidance notes published on the 'quality of biological active substances produced by stable transgene expression in higher plants'	EMEA (assessment only), European Commission, Member States National competent authorities (market authorization)

Table 3 – GMO regulation in the EU modified from (Sparrow et al., 2013)

4.1.1 Process-based approach of GMOs in the EU for whole transgenic plants

The EU legislation follows a **process-based legislation** in terms of the deliberate release of GMOs to the environment (Directive 2001/18/EC). Process-based legislation means that **not an altered organism with new traits is of relevance, but the way the organism was obtained**.

New techniques in plant biotechnology using genetic modification in the development process, for receiving final products without having foreign genes in their genome, make it hard to distinguish these from conventional plant breeding products. A clear obstacle of a process-based GMO regulation is that it will always fail to be in accordance with the latest scientific developments, as biotechnology is a rapidly evolving research area. It is therefore essential to state clearly in the regulations how GM is to be defined and what techniques or processes lead to GM plants. The problem is that the legal description of the techniques that lead to the production of GMOs in the EU (Dir. 2001/18/EC) no longer reflects the scientific state of the art (COGEM, 2009). In the past, the use of stable expression techniques in GM plants always led to an identifiable GM product. Foreign DNA inserted into the genome of the host plant led to a plant with new characteristics. The current techniques use GM as an intermediate step without causing a change of the genome of the host plant (see also section 3.3.3), (COGEM, 2009).

Hence, the issue of the process-based approach is that for each new technique, it must be decided whether it falls under the GMO legislation or not.

Therefore the EU appointed a working group (national techniques working group, NTWG) that reviewed several techniques and determined whether the GMO legislation is applicable for the products derived from these techniques (see also chapter 6.2), (Sparrow et al., 2007).

The determination of the different techniques is challenging and there is a likely risk that there will be a lack of consistency in decision-making whenever a new technique is developed. For companies, especially for biotechnical start-up firms, this means a high-risk approach to research on new inventive techniques due to legislative/regulatory uncertainty. Therefore, the interest from different stakeholders is to adopt the EU legislation from the currently process-approach to a more product-based approach.

4.1.2 Product-based approach of GMO like in the US for whole transgenic plants

Unlike the EU, the US has no specific legislation regarding GM crops. The US, Canada and some other countries decided to place GMOs under their general legislation (COGEM, 2009). The decision was already made in 1986 and to date no specific GMO regulations were needed, as the existing general legislation was deemed sufficient and still suitable.

Here, a strict **product-based approach** is followed**, where the characteristics of the desired protein is important** regardless the techniques used and the way it is produced.
The advantage of a legislation based on a product approach appears to be more flexible to scientific advances and more suitable for safety assessment, because the host plant is assessed only by its characteristics and not by the way it was produced. This kind of regulation does not hinder scientific inventions, as each new technique will not need to be evaluated to determine if it falls under the scope of GMO legislation or not (Sparrow et al., 2013), Bundesamt für Umwelt (CH), (2012).
Due to the lack of specific GMO regulations, several different official bodies assess GM plants in the US: the United States Department of Agriculture (USDA), the Environmental Protection Agency (EPA) and the FDA. Their responsibilities can overlap. The USDA deals with the cultivation of GM crops, the EPA administers the regulation of pesticides and the FDA is pendant to the EMA, responsible for the MA of GMs. The Animal and Plants Health Inspection Service (APHIS) and the USDA regulate safety assessment and the authorisation of field trials, the transport and import of GMOs. The APHIS assesses environmental safety issues of pharmaceutical plants and decides on permissions for the release of a plant into the environment, for field-testing (Sparrow et al., 2007), (FDA, USDA, 2002).

Seen from a global perspective, the FDA in the US has been very proactive and committed to the development of the regulatory framework of PMPs and this has contributed significantly to the promotion of the research field of PMF (Paul et al., 2015).

The first draft guideline for plant-derived pharmaceutical proteins (‘Guidance for Industry - Drugs, Biologics, and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals’, (FDA, USDA, 2002)) was developed jointly by the USDA, which regulates veterinary biologics through the Centre for Veterinary Biologics and the FDA. The guidelines covered all imaginable platforms from whole transgenic plants, aquatic plants, moss and algae to transient expression systems. The guideline is flexible in terms of GMP considerations, requiring information about plant characteristics, the manufacturing process and pre-clinical testing (Fischer et al., 2012). Although this draft guideline was never imple-

mented, it gives an insight of USDA and FDA regulatory opinions concerning PMPs. Aside from this draft guidance and the revised APHIS field-testing requirements, no specific regulations have yet been adopted regarding PMPs in the US (Fischer, R., et al., 2012), (Sparrow et al., 2013).

4.2 Regulatory requirements for PMPs in Europe

One of the challenges in order to gain a MA for a PMP is the cooperation and communication with the different bodies that regulate human health, environment and agriculture. Furthermore, the inter-jurisdictional harmonisation in terms of achieving an MA in different countries is a difficulty (Arntzen et al., 2005).

Similar to the US and as mentioned in chapter 4.1, the regulation of PMPs in the EU falls to several regulatory bodies whose authorities overlap (Sparrow et al., 2007). There remains some overlap between the European Food Safety Authority (EFSA), which deals with the practicalities of growing GM plants and has a mandate to consider non-food plants as well as food/feed crops and the EMA, responsible for granting the MA of biopharmaceuticals (Masip, G. et al., 2013). It depends on the choice of the host plant (food or feed) and growing location (containment or field grown) to what extend the authorities are involved during the assessment (Sparrow et al., 2007), (Masip, G. et al., 2013).

Regulatory bodies have to deal with a number of challenges regarding the regulation of GM plants. Each PMP and each PBS is unique and can be produced in a versatile array of manufacturing techniques and plant-based systems, ranging from whole plants growing in the soil to plant suspension cells growing in a defined synthetic medium in a bioreactor (Ahmad, 2014).

Until 2002, regulatory guidance existed for the production of PMPs only as draft legislation based strongly on the existing regulations suitable for mammalian cells (Fischer et al., 2012). However, this legislation was not suitable for MA applications involving whole plants (Fischer et al., 2012).

There was a noticeable evolution of the regulatory framework in the EU in 2002. Until then, the EMA guidance was narrow in scope, as the regulations were only applicable for stable transgenic plants (see chapter 3.3.3) and were principally drawn from the regulations covering cells and fermenters from the conventional production platforms of recombinant proteins.

Some researchers struggled to meet the existing regulations and developed plant-cell-based platforms that had similar characteristics to conventional production platforms and therefore could benefit from prior experience with microbes and animal cells. Conceptually, the focus was on containment, consistency and similarity to mammalian cells (Ma, J. et al., 2005a), (Ma, J. et al., 2005b), (Xu et al., 2011b). Therefore, the products from plant-cell-based production systems were able to meet requirements under GMP. It is then no wonder, that due to this important aspect, the first approved PMP for human use was a plant cell derived product (Elelyso, see chapter 5.2).

The switch from experimental platforms to commercially optimised manufacturing processes arose quickly, and made plants increasingly competitive in the market for valuable metabolites and recombinant proteins. It should be noted, that proteins derived from cell suspension cultures based on medicinal plants are treated as 'natural products', whereas proteins gained form whole plant expression systems will fall under the GM legislation, which are introduced hereafter (Fischer et al., 2015).

New regulatory framework for field cultivation of whole (transgenic) plants – as the above described regulations were nearly impossible to implement with whole-plant systems, the EMA published draft guidance on 'the quality of biological active substances produced by stable transgene expression in higher plants' (Fischer et al., 2012). The current version has been in effect since February 2009 (EMA, 2008). In parallel, the FDA/USDA issued a comparable document 'Drugs, Biologics, and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals' (FDA, USDA, 2002), (see also chapter 4.1.2).

However, products developed from whole plant systems are still trailing behind those from plant cells, due to their complexity. At present, several protein candidates are now in clinical development (Fischer et al., 2012), (Fischer et. al, 2014).

Application for open field production trials – As a prerequisite for the cultivation of whole transgenic plants, it is necessary to submit a notification for the deliberate release of transgenic plants for open field. In accordance with Directive 2001/18/EC, Part B, the permission to conduct field trials is delegated to national authorities (see chapter 4.1) and this has emerged in the EU under divergent conditions (Nausch, H. et al., 2015). Today, in most EU countries, field trials with GM plants are completely blocked either by legislation or by systematic destruction of trials, hence possibilities for publicly funded research is presently hindered (Nausch, H. et al., 2015). For example, in 2014 no field trials were conducted in Germany. Based on this fact, the use of transgenic plants in Germany will only be possible after the

development of a plant that provides significant advantages for the consumer (Nausch, H. et al., 2015). At present research funding organisations seem to be reluctant to fund even research on the development of innovative plants. A change can only come with innovations and it takes an estimated 20 years from the first experiments to MA (Sparrow et al., 2013), (Nausch, H. et al., 2015).

4.3 Differences in the PMP authorisation between the EU and the US

The current regulatory guidelines for whole plants vary in the EU and USA. The original FDA guidelines were flexible to incorporate any system based on whole plants or plant organs (FDA, USDA, 2002), whereas EMA guidelines, published in 2008 after a lengthy consultation and enacted in 2009, only consider GM plants and therefore excluded transient expression systems (EMA, 2008). In the US it is possible to conduct the manufacturing based on transient expression in leafy crops like tobacco or alfalfa under GMP requirements, but is still hindered by the GM legislation in the EU (Fischer et al., 2014). In addition, the costs to cultivate a GM plant in the EU are about 25% higher compared to the costs in the US, with an average amount of 6.8 million euros for the admission of a transgenic crop. These costs derive from the larger amount of requested data by the EU authorities (COGEM, 2009). Aside from the licensing costs, the average time for assessment of the licensing procedure is in the EU more than twice as long. A licensing procedure in the US is estimated to be on average 18 months, whereas in the EU it takes about 47 months (COGEM, 2009). This explains why the entire commercial infrastructure for transient expression in whole plants has moved out of the EU (Stoger, E. et al., 2014), (Fischer et al., 2014).

On the other hand – and this is a contradiction in the EU policy – the purified biopharmaceuticals can be imported into the EU in precisely the same way as those produced in any other platform. A similar paradox is applicable for GM foodstuffs, which cannot be grown in the EU but are imported in enormous quantities. By reducing the regulatory framework only on the production process rather than the product itself, this is driving the technology and its economic benefits similarly away from the EU (Masip, G. et al., 2013), (Stoger, E. et al., 2014). These inharmonious regulations separated the molecular farming community into two groups, with some stakeholders focusing on the development of platforms based on plant cells, which were thought to be more compatible with the EMA regulations, while others demanded new guidelines suitable for whole plants (Fischer et al., 2014). Therefore, the Pharma-Planta project was founded.

5 Case studies: the Pharma-Planta project and MA Elelyso

5.1 The Pharma-Planta project: Initiative to establish the regulatory framework for plant–made pharmaceuticals

Pharma-Planta (http://www.pharma-planta.net) was an EU funded project (EU Sixth Framework Integrated project) that ran from 2004 until 2011. The main objectives of the project were:

- to establish an approved PBS for pharmaceuticals in whole plants and help to roadmap appropriate regulatory monitoring in the EU;
- to produce mABs in transgenic plants under GMP, passing pre-clinical toxicity testing and entering phase I human clinical trials in the EU (Sparrow et al., 2007);
- to demonstrate a practical commitment to the humanitarian use of PMPs in developing countries. A PMP licensing strategy for humanitarian intentions was worked out and 'open access' was given concerning the used production technology (Sparrow et al., 2007).

From an initial panel of eight target molecules, the HIV-neutralising human mAB 2G12 was selected for fast-track production, and transgenic tobacco was selected as the platform because of its scalability. In terms of tobacco, it was assumed that regulators would favour a non-food crop, as the potential risk to contaminate the food/feed chain would be reduced. Furthermore, the decision was made to grow whole tobacco plants in contained glasshouses, thus avoiding issues of 'a deliberate release into the environment' that would have been associated with a small-scale experimental field trial (Part B of Directive 2001/18/EC), (Sparrow et al., 2013). The desired finished product, an anti-HIV mAB derived from tobacco dissolved in a saline solution and intended to be administered as a topical cream (in the vagina or rectum), gained approval by the UK regulatory authority to enter human phase I clinical trials in 2011 (Sparrow et al., 2013). With the successful completion of the clinical trial phase I, one of the goals of the Planta-Pharma project was reached: the development of a novel biopharmaceutical manufacturing process from first principles laid out a manufacturing template for other organisations to follow (Stoger, E. et al., 2014), (Fischer et al., 2014).

The production of recombinant proteins in transgenic plants under non-sterile conditions in contained environments, like glasshouses, was connected with logistic, scientific and regula-

tory challenges throughout the process chain. Tasks like the generation and characterisation of stable plant lines, master and working seed banks, and the development of robust standard operating procedures (SOP), in order to make cultivation efficiently consistent and define the harvesting conditions, needed to be resolved (Sack et al., 2015a). Furthermore, DSP steps such as the efficient extraction of soluble proteins from plant biomass, extract clarification and host cell protein removal needed to be established (Sack et al., 2015a).

One of the goals in the Pharma-Planta project was a humanitarian use policy for PMF technology and this is currently pursued in the follow-up project 'Future-Pharma'. The Future-Pharma project has the benefit of the technical progress made in the Pharma-Planta project and is extending the work by focusing on phase II clinical studies, other product candidates, and new DSP and purification concepts (Stoger, E. et al., 2014).

5.2 First marketing authorisation for a PMP for the human use: Elelyso

An important break-through was achieved in 2012 when the first PMP was approved for human use: the enzyme taliglucerase alfa, called Elelyso. Elelyso, a recombinant type of human glucocerebrosidase (GCD) was developed by the Israeli biopharmaceutical company Protalix Biotherapeutics for the treatment of the lysosomal storage disorder, Gaucher disease (Stoger, E. et al., 2014).

Gaucher disease is a recessive hereditary lysosomal storage disorder and is considered as an 'orphan disease'. Among the population there are 1:60.000 live births worldwide with this disease; however the prevalence of Gaucher disease ovaries between differing populations (Cox, 2010).

GCD is administered intravenously, and the medical treatment brings, in most cases of Gaucher disease patients, a significant relief of symptoms. The treatment costs with this enzyme-replacement therapy based on recombinant CHO cell-derived GCD (marketed as Ceredase or Cerezyme by the biopharmaceutical company Genzyme) are enormous. The annual treatment costs are estimated to be nearly US $200,000 per patient. Cerezyme became the standard therapy of this hereditary, fatal disease (Cox, 2010). To reduce the production costs with plant cell-cultures as an alternative was an important drive to develop Elelyso and indeed Elelyso provides clear advantage with a 25% lower price per dose than Cerezyme (Mor, 2015). The production system of Elelyso is based on carrot cells and it is unique in that it is produced

in a closed, sterile culture system, contained in a multiuse, disposable bioreactor, termed ProCell System (Tekoah, Y. et al., 2015). GCD is a glycoprotein, and its function depends on terminal mannose residues that are preserved when produced in carrot cells (Fischer et al., 2012). Here plant-derived GCD offers a clear advantage over CHO cells-derived GCD: as plant-derived, GCD is retained in the ER during glycosylation modification and then targeted into the vacuole, the N-glycans are trimmed to expose mannose residues and the correct mannose glycosylation pattern can be made within the plant cells. Therefore, in vitro trimming of the glycans is eliminated in DSP, resulting in a significant cost reduction. In addition, the half-life of plant-derived GCD is longer than that of Cerezyme due to bio-encapsulation in the vacuole (Mor, 2015), (Huang, 2012), (Bosch, D. et al., 2013a), (Bosch, D. et al., 2013b).

The success story of Elelyso – in 2009, the FDA and Genzyme (manufacturer of Cerezyme) issued a notification to healthcare professionals regarding an incident of foreign particle contamination concerning Cerezyme (FDA Safety Alert, 2009). A calicivirus infected their production cell line. Due to this incident, the FDA gained awareness regarding a shortcoming of Cerezyme, which meant a serious safety concern for human patients. The immediate need to ensure a sustainable supply chain for this orphan drug and the lack of approved therapeutic alternatives to GCD gave Elelyso a push to go forward with the clinical development program (Paul et al., 2015), (Xu et al., 2011b). Hence it is no wonder that Elelyso was also the first plant-derived biologic to reach a phase III clinical trial and its successful completion. It is noticeable that Pfizer, the largest drug company worldwide, acquired licensing rights from Protalix based on a multi-million-commercialisation deal (Mor, 2015). Another important aspect that contributed to Elelyso's success story is the fact that Protalix chose to concentrate its efforts on a less radical plant-platform. Here, plant cells are grown in a reactor, which is comparable to conventional and well-established fermentation-based platforms (ProCellEx system). The production of GCD in full containment avoided a public debate respectively to GMOs and helped to achieve a product in accordance to current GMP requirements (Mor, 2015).

Protalix applied for an MA application not only in the US but also in the EU and nearly at the same time. The application was received by the EMA on 25 November 2010. Elelyso was granted an MA in the US on 1 May 2012.

In the EU, the Committee for Medicinal Products for Human Use (CHMP) considered the benefit-risk balance of Elelyso as favourable. However, the CHMP considered Elelyso too similar to another orphan medicinal product (called Vpriv) already authorised for the same

therapeutic indication in the EU, including a market exclusivity of 10 years due to the orphan drug designation. For Elelyso it was concluded that none of the derogations regarding an orphan market exclusivity under Regulation (EC) No. 141/2000 applied. Therefore, the refusal of granting the MA for Elelyso was recommended in June 2012.

6 Discussion

6.1 Lessons learned from the Pharma-Planta project and Elelyso

The Pharma-Planta project was important to roadmap a working regulatory system for the production of recombinant biopharmaceuticals in whole transgenic plants. The manufacturing process, developed in the project, can be used as a template for the production of further protein candidates, because the project was publicly funded and the intellectual property is 'open access' and not secured by patents. The USP in the manufacturing process, used in the Pharma-Planta project, involved the development of new concepts such as seed banking in whole plants, an equivalent of cell banking from the conventional production platforms and novel approaches to minimise the batch-to-batch variability and therefore meeting one important aspect considering GMP (Stoger, E. et al., 2014). It was shown that the production of recombinant proteins in whole plants is feasible according to GMP requirements.
This represented a milestone for this type of PBS.

In the case of Elelyso's success story, the research area of PMF benefited from the prior experiences with microbes and animal cells. The emphasis in the manufacturing process was laid on containment, consistency and similarity to mammalian cells, both conceptually and practically. By this method, the products from plant cell-based systems could be manufactured GMP-compliant and moved forward to commercialisation. It is outstanding, that the transfer from experimental scale to a commercially optimised manufacturing process could be realised in an in vitro PBS within a short time (Fischer et al., 2012), (Fischer et al., 2015).

The Pharma-Planta PBS and Elelyso's PBS are based on different production platforms, but both projects have in common the fact that they are using stable expression techniques, where foreign DNA is incorporated in the genome of the plant cells, in either the nucleus or plastids.

For stably expressed proteins, the regulatory guidelines are more certain as these guidelines have been established for a longer period (Fischer et. al, 2014). Currently there are a number of whole-plant produced biopharmaceuticals moving into the preclinical and clinical devel-

opment pipeline, though the plant cell culture system seem to be clearly emerging as a more immediate alternative PBS for producing complex pharmaceutical proteins (Xu et al., 2011b). It is expected, that based on the experience with stable expression systems like the plant-cell-based production platform used to produce Elelyso and with whole plant-based production platforms developed as in the Pharma-Planta project similar platforms will follow, which can be approved within a shorter timeframe.

Although the regulatory framework in the EU follows a process-based approach concerning host plants, and the regulatory guidelines for stably expressed PBS are more certain and have been longer established, there should also be a possibility to pave the way for more innovative expression techniques, like transient expression methods. At present, research is hindered by the current EU legislation due to reasons described in the following section (Fischer et. al, 2014).

6.2 Potential amendments in the regulation of PMPs in the EU

At present, GM plants must pass an extensive and costly GMO approval process in the EU. To approve a new transgenic plant, procedures exist that were established more than 10 years ago, when only a small volume of research data was available on the impact of transgenic plants on human and animal health, and the environment. Furthermore, the stepwise authorisation process of the host plant intended for PMF takes approximately 4-6 years and costs about 7-15 million Euros (COGEM, 2009). Even safety research on transgenic plants is restricted (see chapter 4.1.1). To summarise the situation: transgenic crops, which are needed as host plants in the manufacturing of PMPs, are more restricted in the EU than in other countries.

The process-based regulatory framework of GM plants in the EU is not science-based and the market approval process is disproportionally longer compared to other countries. For instance, the US and Canada implemented a product-based regulation, which appears more flexible to scientific advances (see chapter 4.1.2). Also the marketing approval time is less consuming and the application procedure less expensive (see chapter 4.3). Therefore, in order to avoid problems between trading countries, the EU needs to consider a revision of the existing regulatory framework in order to keep the system more flexible for agricultural biotechnology like in the US. The demand for a revision of the regulatory framework in the EU does not intend

to disregard the focus on risk assessment considering new traits and/or the product, but should not emphasize the technology used.

The following aspects show the weaknesses, that hinder the EU regulatory framework from being as flexible and cost efficient concerning the approval process as in the US.

Weakness in the EU regulatory framework

Since the adoption of Directive 2001/18/EC, an increasing number of techniques were developed to allow a precise genetic modification of plants for research and precision breeding (Hartung, F. et al., 2014). However, the major challenge for the new expression techniques is that it is not clarified, which or if any of these expression techniques adhere to current GM legislation.

As an example, point mutation or the small insertions/deletion of plant genes, induced by irradiation, chemicals or somaclonal variation (chromosomal rearrangements in plants occurring mainly in the callus), used in conventional breeding is a technique that is excluded from GMO legislation by Annex IB of Directive 2001/18/EC (Hartung, F. et al., 2014). The methods have been in use since 1950 and are excluded from GMO legislation as the mutations occur without the intervention of human mechanisms, although to a much lower frequency.
The appearance of mutations is considered a natural process, commonly used by breeders to exploit natural variation.

In 2007, eight new expression techniques were identified to be unclear and the NTWG (see chapter 4.1.1) completed the work in 2012, resulting in an assessment report, whether these techniques should be covered or not Bundesamt für Umwelt (CH), (2012). Two of the eight methods cause similar mutations to those that may occur in a natural process or by mutation breeding, only in a much more specific way. In principle, these two methods are similar to mutation breeding. Therefore, the general tendency would be to exclude these two methods from GMO legislation.

From a scientific point of view, new plant breeding techniques (NPBT) can often not be distinguished from conventionally bred plants and they do not seem to bear higher risks for human health and the environment.

Additionally, the increasing number of new plant genetic improvement methods enables various ways to specifically alter the genomic sequences of host plants resulting in plants that harbour little or no foreign DNA. In addition, there is no reason why a plant should be

categorised as a transgenic crop, if it does not contain stable and foreign DNA (Hartung, F. et al., 2014). If the host crop is intended for the production of PMPs it will be usually destroyed in order to extract the targeted proteins.

In order to avoid discussions about the possible offspring of transgenic plants, the implementation of transient expression methods or the integration of foreign DNA only in the rootstock part would be suggested, as there is no evidence of recombinant DNA transfer in the scion except for the callus region at the interface (Hartung, F. et al., 2014).

All the aforementioned weaknesses of the regulatory framework for PMPs in the EU lead to the conclusion, that a revision of the current GMO legislation should be considered, which excludes transient expression.

The NPBT allow highly precise genetic engineering in comparison to plants derived from conventional plant breeding, and it is therefore less likely that these plants bear more risks of side effects.

The current regulation of host plants should not be based solely on the technique used, as this is not an evidence-based approach and not appropriate for NPBTs. It is not justified that the transient expression method (without foreign DNA incorporated into a host plant) falls into a costly, time-consuming regulatory approval process, which is equated with the approval process of stable transgenic plants.

Suggestions for improvement of the regulatory framework in the EU

It is suggested that host plants, genetically modified by NPBTs or other future techniques that follow, should be evaluated according by the end product and its (new) traits and not to the technique, or how genetic modification was obtained in the plant. Therefore, the legislation in the EU should be harmonised with other regions of the world and be adapted to a more product-based assessment of GM plants.

The advantages of a product-based approach for the EU regulation are as follows:

- the product-based approach enables for more flexible and faster adaptation of new techniques (see chapter 4.1.2);
- harmonised market requirements with other trading countries (COGEM, 2009);
- clarification for R & D scientists considering the regulatory framework (COGEM, 2009);
- cost and time saving (see chapter 4.3).

To be able to fully exploit the potential of PMF, it is strongly necessary, that the regulatory authorities develop a policy framework concerning the field-grown production of PMPs (Sparrow et al., 2013). The placing on the market of a GM plant containing therapeutic proteins to be subsequently isolated, purified and formulated into a medicinal or industrial product, needs a separate authorisation from the European Commission under Part C of Directive 2001/18/EC (Penney, C. A. et al., 2011). During the field trial stage (Part B of Directive 2001/18/EC), it must be safeguarded that no GM material is placed on the market, unless in accordance with Part C of the Directive.
It is expected that the majority of GM plants used in the production for PMPs will be grown on a restricted acreage by contract farmers (Penney, C. A. et al., 2011). In this case the costly authorisation under Part C no longer appears appropriate (Sparrow et al., 2013). On the other hand, the commercialisation of medicinal plants produced under Part B conditions is not allowed in Europe. Therefore, amendments of Directive 2001/18/EC are necessary to allow the commercialisation of products from GM plants grown under conditions, which still need to be defined (e.g. limited acreage, contract cropping, and confinement) without the need of an authorisation under Part C (Sparrow et al., 2013).

7 Conclusion and Prospects

A promising future in the research of PMF is foreseen, as there are several unique areas of opportunity in the pharmaceutical market for PMF. Many shortcomings related to PBS were addressed in the continuous research over the past 30 years, by the improvement of different expression techniques.

In fact, plants seem to be more flexible and capable in the manufacturing of recombinant proteins compared to the conventional production platforms. Via emerging precise genetic engineering methods in plants, drug products can be manufactured much safer, more quickly, less expensive and/or on a larger production scale. The choice amongst different PBS enables the production of therapeutic proteins in a superior quality (e.g. biobetters) and additionally, complex proteins which cannot be produced in mammalian cells (CHO-cells) due to potential toxicity issues. Transient expression systems offer the potential to produce vaccines or virus-neutralising ABs, which can be manufactured within a shorter timescale compared to the current production platforms of for example influenza vaccines (Stoger, E. et al., 2014).

Although the prospects for PMF are promising and the benefits demonstrable, it is unlikely that PBS can compete and replace conventional manufacturing platforms in the near future in terms of blockbuster products like insulin.

At present, industry investments are still focusing in the conventional fermentation infrastructure and after years of refinements this has evolved a high product quality, process robustness, and regulatory certainty. PMF industry-sponsored research is strongly needed to optimise PBS in order to explore to its full potentials, and in order to meet the rising market demands of valuable therapeutic proteins and vaccines globally and also individually as personalized medicinal products. However, industrial funding can be attracted only by showing that more products can be marketed successfully (Stoger, E. et al., 2014). Therefore the participation of SMEs should be encouraged by incentives to establish more GMP-compliant PBS facilities.

Biotechnological start-ups will only gain new impetus to build research and development facilities in the EU when the regulatory framework for GM plants and PMPs is more certain and open for genetic engineering as in the US. Yet research in the EU is impeded by the current GM legislation and innovation cannot take place. The EU must act and be proactive in terms of streamlining and rationalising the regulatory pathway for PMF, if it wants to encourage PMF businesses and benefit directly from the technology. For the further development and widespread success of the technology, it is critical to find the correct balance between standardisation/containment/control in the first phase of production, including

plant cultivation. This must be sensible in terms of safety, but not so unreasonable that it would offset the main economic advantages offered by PMF. (Stoger, E. et al., 2014)

Therefore, the GMO legislation should be harmonised with other international regulatory approaches, to make regulatory processes more predictable and form a basis for the submission of multiple MA applications for valuable PMP candidates. It is no longer tolerable that potentially good, cost-effective medicines, that are needed for chronic or infectious diseases, which occur especially in underdeveloped countries, are not available due to the rigid and outdated GM legislations in the EU.

8 Summary

The number of patients with chronic diseases like diabetes, Alzheimer's disease or chronic obstructive pulmonary disease (COPD) is increasing globally and various epidemic threats like Ebola, human immunodeficiency virus (HIV) or influenza result, particularly in the underdeveloped countries, in a high mortality. In correlation to this matter, the market demand for vaccines, therapeutic proteins and monoclonal antibodies is growing. On the other hand, only a few approved medicinal products against rare diseases are available.

The production of recombinant, therapeutic proteins in conventional production systems such as genetically modified bacteria, yeast or mammalian cell cultures bears high costs and demonstrates other drawbacks, such as low yields or shortages and the occurrence of potential pathogens. Due to the high price of these medicinal products, many infectious diseases like Ebola, rabies, malaria mainly appearing in poor countries, cannot be reduced or eradicated, although medicinal treatment could be made available.

On the long run, transgene plants have the potential to serve as an alternative production platform for recombinant proteins. The research area plant molecular farming (PMF) is about the viable production of large biomolecule drugs; plants as biopharmaceutical factories can contribute to resolve the above mentioned problems as they offer potential advantages such as low cost of investment and low cost of goods, manufacturing scalability, speed of production and diversity of target products, coupled with related molecular engineering technologies including glyco-engineering.

Although there has been continuous research for almost 30 years, the development of plant medicinal products is still at an early stage. However, there are already a few success stories in this research area: in 2012 the first plant made pharmaceutical (PMP) for the human use was approved by the Food and Drug Administration (FDA) in the US and other candidates are currently entering clinical trial Phase II and Phase III, close to marketing approval. This thesis reviews the development status of PMPs to date. The current challenges in the drug development and issues regarding the manufacturing process of PMPs in order to operate to current regulatory guidelines are examined. Then the current regulatory framework, applicable for PMPs in the EU, and the differences between the EU and US are introduced; both regions have well-sourced regulatory systems settled. Based on the experience gained from the research and development of the EU funded Pharma-Planta project and of the first approved PMP for the human use (Elelyso) conclusions are drawn, considering the necessity to revise the regulatory guidelines specifically for PMPs. Furthermore, ambiguities

or gaps in the regulatory legislation are outlined. In the last chapter it is discussed, how these issues can be addressed in order to expedite the market availability of various PMPs in the near future.

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Declaration of Authenticity

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person where due reference is not made in the text.

Bonn, 05.11.2015

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