Chapter

Prospects for the Production of Recombinant Therapeutic Proteins and Peptides in Plants: Special Focus on Angiotensin I-Converting Enzyme Inhibitory (ACEI) Peptides

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Abstract

Molecular pharming is a cost-effective, scalable, and safe system to produce high-quality and biologically active recombinant therapeutic proteins. Thus, plants are emerging alternative platform for the production of pharmaceutically relevant proteins such as vaccines, antibodies, antibody derivatives, and some serum-derived proteins. Additionally, plants have also been used to produce bioactive and immunogenic peptides. The efficacy, selectivity, specificity, and low toxicity make them particularly well-suited therapeutic agents for various indications, for instance, cardiovascular and infectious diseases, immunological disorders, and cancer. In the broad range of known bioactive peptides, angiotensin I-converting enzyme inhibitory (ACEI) peptides derived from food proteins have attracted particular attention for their ability to prevent hypertension. So far, several ACEI peptides have been identified in food proteins, mainly in milk, eggs, and plants. The industrial production of ACEI peptides is based on enzymatic proteolysis of whole food proteins, which leads to the release of small bioactive peptides with ACE-inhibitory activity. The problems associated to such procedures, namely, cost and loss of functional properties, have demonstrated the need to develop more straightforward methods to produce ACEI peptides. One viable hypothesis, discussed in this chapter, is to genetically engineer crop plants to produce and deliver antihypertensive peptides.

Keywords: plant engineering, recombinant protein, bioactive peptides, angiotensin I-converting enzyme, ACEI peptides

1. Introduction

The term "molecular pharming," blend of pharmaceutical and farming, surfaced in the literature in the 1980s to refer to the production of high-value compounds in

transgenic animals. Nowadays, the expression is mainly employed to the production of recombinant pharmaceutically relevant proteins or secondary products in plants [1–3].

The roots of molecular pharming can be traced back to the mid-1980s when plants started to be genetically engineered to act as bioreactors that produced pharmaceutically relevant proteins. Barta et al. [4] demonstrated that tobacco and sunflower callus tissues were capable of expressing transcripts of a human growth hormone fusion gene. Although no protein was detected, this was the first report of plants expressing human genes and established plants as a potential production system for recombinant therapeutic proteins. Later on, the expression of a full-sized IgG in tobacco [5] was a major breakthrough since it revealed the ability of plants to produce complex functional mammalian proteins of pharmaceutical relevance. In 1990, the "authenticity" of plant-derived recombinant proteins was proved even further with the production of the first human protein (serum albumin) with confirmed native structure in tobacco and potato [6].

After several studies that demonstrated the capacity of various plant species and systems to produce recombinant pharmaceutical proteins and peptides, during the 1990s, the field of molecular pharming gained support and interest from the plant biotechnology community. The scientific attention was followed by commercial interest, with many start-up companies being created to capitalize the advantages of plants in relation to the established platforms. These advantages include being a more cost-effective, scalable, and safer means of producing pharmaceutically relevant proteins and peptides. In opposition to the fermentation-based traditional platforms that require a massive investment in bioreactors, plant-based production systems can be established with minimal investment and offer a myriad of different hosts and platforms [7]. However, the expectation that plants could easily compete for the market share of some well-established biopharmaceutical platforms, such as Chinese hamster ovary (CHO) cells, and that they could motivate the mainstream pharmaceutical industry to switch platforms was overinflated. The CHO epithelial cell lines are the most commonly used mammalian hosts for industrial production of therapeutic recombinant proteins. The technical limitations of plants, especially their lower yields compared to mammalian cell lines, allied to the colossal existing investment in fermentation infrastructures, the unfavorable public opinion on OGMs, and regulatory uncertainty, lead the mainstream pharmaceutical industry to be cautious and to a consequent stagnation of the molecular pharming field in the 2000s [8, 9]. This situation induced a change of paradigm concerning molecular pharming: the initial vision of a highly scalable and low-cost production system, while still valid, was replaced by the idea of a production system for certain niche products that are not easily manufactured by conventional systems [8, 9].

Molecular pharming embraces several platforms and technologies with different advantages and limitations, related by their use of plant tissues. Conversely to conventional biopharmaceutical production systems that are based on few selected platforms, particularly the bacterium *Escherichia coli*, yeasts such as *Pichia pastoris*, and mammalian cell lines such as Chinese hamster ovary (CHO) cells [3], pharming platforms range from plant cells or unicellular plants growing in bioreactors to whole plants growing in soil or hydroponic environments. Further, the technologies include stable integration of DNA into the nuclear genome or plastid genome and transient expression by infiltrating leaves with expression vectors based on *Agrobacterium tumefaciens*, plant viruses, or hybrids [3, 8]. This great diversity of molecular pharming confers adaptability and flexibility, allowing the selection of the most suitable platform for each product, but has also conduced to fragmentation. This fragmentation meant that in the early days of molecular pharming there was no driving force to establish molecular pharming as a single competitive platform. Consequently, no actions were made to match the industry requirements for high yields, standardized procedures, and good manufacturing practices (GMP) [7, 9]. More recently, efforts have been made to mimic the mainstream

biopharmaceutical industry and place a focus only on a small number of platforms, namely, plant cell cultures, nuclear transgenic plants, and leafy plants transiently transformed [3, 10]. Since 2010 the attention of the biopharmaceutical industry to molecular pharming has been renewed as result of its consolidation on a small number of platforms and some target products that meet industry demands [8, 9].

In 2012, the FDA approval of the first recombinant plant-derived therapeutic for human use, Protalix Biotherapeutics' taliglucerase alfa (Elelyso™), was an important breakthrough for molecular pharming. The enzyme taliglucerase alfa is a carrot cell-expressed human recombinant β-glucocerebrosidase and is prescribed for the treatment of Gaucher's disease, a lysosomal storage disorder [11]. Imiglucerase, a recombinant form of glucocerebrosidase commercialized under the name Cerezyme®, was already produced in CHO cells. In this production platform, the enzyme required subsequent in vitro exposure to mannose residues in order to have biological activity, resulting in a time-consuming and expensive manufacturing process. Besides, this platform also has potential safety problems, namely, the risk of viral contamination, allergies, and other adverse reactions. In comparison, the plant-based platform is safer and less time-consuming and has reduced production costs, since the mannose units are posttranslationally added in vivo [11]. Glucocerebrosidase is a clear example of a target product in which safety, cost, and downstream processing issues were solved by switching from a traditional platform to molecular pharming. Another example that gathered mediatic exposure was ZMapp, a cocktail of three chimeric monoclonal antibodies targeting the Ebola virus surface glycoprotein produced in *Nicotiana benthamiana* using a hybrid transient expression system, the magnICON system. ZMapp was developed during the Ebola outbreak of 2014 by Mapp Biopharmaceutical Inc. (San Diego, USA), following initial studies on nonhuman primates [12]. ZMapp has since been used in humans under emergency compassionate protocols [13] and randomized controlled trials [14].

Following these examples of success, there has been a continuous increase in clinical trial applications and manufacturing capacity, which has also been correlated with the conception of more tangible regulations concerning plant-derived pharmaceuticals.

Although plants are still unlikely to substitute the established platforms [8], the recent promising developments in the field of molecular pharming demonstrate that glucocerebrosidase was not a lone case of success and that plant-based platforms could provide countless opportunities for the biopharmaceutical market. Plants combine the advantage of a full posttranslational modification potential with simple growth requirements and theoretically unlimited scalability in the case of field-grown whole plants. Plant-based platforms are versatile and allow the targeting of recombinant proteins and peptides produced to different organs or subcellular compartments, which provides an additional protection against proteolysis. Finally, plants are a safe host for therapeutic protein and peptide production since they do not harbor human or animal pathogens [15]. Therefore, instead of facing the red ocean of established pharmaceutical industries [16], molecular pharming is now evolving as a disruptive technology that creates its own marketplace by offering rapid drug development and production, unparalleled scalability, unique quality attributes such as tailored glycan structures, individualized therapies, and oral or topical applications of minimally processed plant tissues, thus reducing downstream costs [17].

2. Plant platforms for the production of therapeutic proteins and peptides

The continuous development of genetic engineering technologies for plants has resulted in an expansion of well-established plant-based platforms [18]. Molecular

pharming encompasses platforms based on stably transformed whole-plants transgene insertion in the nuclear or plastid genome, transient expression using agroinfiltration, viral and hybrid vectors; microalgae and aquatic plants (e.g., duck-weed) stably transformed; and in vitro culture systems (e.g., cell suspensions, hairy roots, and moss protonema) [19]. Each platform has particular advantages and limitations; therefore its selection is done on a case-by-case basis, depending on economic considerations as well as on the product characteristics and intended use [20].

2.1 Platforms based on transgenic plants

Transgenic plants have been the most widely used platforms for recombinant protein production. To obtain stable transgenic lines, the gene encoding the desired protein is cloned into an expression construct, which generally includes a promoter and regulatory elements that ensure efficient RNA processing and protein synthesis [21]. This expression construct is then stably integrated into the plant nuclear genome, resulting in the stable inheritance of the transgene and expression of stable pharmaceutical proteins over generations [22]. Two major transformation strategies have been employed to insert expression constructs into the nuclear genome: *Agrobacterium*-mediated transformation in dicotyledonous species (dicots) and particle bombardment of DNA-coated gold or tungsten beads in monocotyledonous species (monocots) [3]. Transgenic plant lines offer several advantages as platforms for molecular pharming: they are suitable for long-term production of recombinant pharmaceutical proteins and are highly scalable, as each line can be used to produce seeds, which increase the number of plants in every generation. Ultimately, the production capacity of recombinant pharmaceutical proteins in transgenic plants is practically unlimited, as it only depends on the number of hectares available for the plant culture. The major drawbacks of transgenic plants are the long development and scale-up timescales, the unreliable production yields, and the potential spread of pharmaceutical crops in the environment and into the food chain by outcrossing and seed dispersal [3].

The development of simple transformation technologies has expanded the number of host plants available for molecular pharming. Currently, the major molecular pharming transgenic platforms are based on leafy crops, seeds, fruits, and vegetable crops. Leafy crops are benefic in terms of biomass yield and high soluble protein levels. Additionally, leaf harvesting does not need flowering and thus considerably reduces contamination through pollen or seed dispersal [23]. One disadvantage of leafy crops is that proteins are synthesized in an aqueous environment, which is more prone to protein degradation, resulting in lower production yields [24]. In fact, the mature leaves possess very large extra cytoplasmic vacuolar compartments containing various active proteolytic enzymes that are involved in the degradation of native and foreign proteins. This is particularly problematic in the case of therapeutic peptide production because short heterologous peptides have an inherent instability in plant cells [25]. In addition to the protein instability, the harvested material has limited shelf life and needs to be processed immediately after harvest.

Tobacco has been the most widely used leafy crop for molecular pharming. The major advantages of using tobacco to express pharmaceutical proteins are its high biomass yield, well-established technology for gene transfer and expression, year-round growth and harvesting, and the existence of large-scale infrastructure for processing [23]. However, the natural production of nicotine and other alkaloids in tobacco poses some safety issues in its use as a host system for heterologous protein production. Therefore, tobacco varieties with low nicotine and alkaloid levels have been produced to diminish the toxicity and overcome those safety issues. Recent studies have led to the approval of the first monoclonal antibody produced in

transgenic tobacco plants, in phase I clinical trial [26]. Additionally, a 2018 publication reported the stable expression of adalimumab (a monoclonal antibody against *tumor necrosis factor-alpha (*TNF-α)) in tobacco plants [27]. Other leafy crops commonly used in molecular pharming include alfalfa and clover [19].

As an alternative to leafy crops, plant seeds have proven to be versatile hosts for recombinant proteins of all types, including peptides or short and long polypeptides as well as complex, noncontiguous proteins like antibodies and other immunoglobulins [28]. The expression of proteins in seeds can overcome the shortcomings of leafy crops in terms of protein stability and storage. Seeds possess specialized storage compartments, such as protein bodies and vacuoles, which provide the appropriate biochemical environment for protein accumulation, thus protecting the proteins expressed in seeds from proteolytic degradation [29]. Reports have demonstrated that antibodies expressed in seeds remain stable for at least 3 years at room temperature without detectable loss of activity [30]. Furthermore, the small size of most seeds permits to achieve a high recombinant protein concentration in a small volume, which facilitates extraction and downstream processing and reduces the costs of the overall manufacturing process [31]. One essential property of seeds is dormancy, which not only permits the stability of recombinant proteins but also allows a complete decoupling of the cycle of cultivation from the processing and purification of the protein [28]. Finally, proteins expressed in the seed do not normally interfere with vegetative plant growth, and this strategy also reduces exposure to herbivores and other nontarget organisms such as microbes in the biosphere [21]. Several crops have been studied for seed-based production, including cereals, such as maize, rice, barley, and wheat; legumes, such as pea and soybean; and oilseeds such as safflower and rapeseed. Maize has several advantages for seed-based expression of proteins; it has the highest biomass yield among food crops, and it is easy to transform, in vitro manipulate, and scale up [24]. These potentialities were explored by Prodigene Inc. for the production of the first commercially available plant-made protein, avidin (a protein with affinity for biotin used in biochemical assays). Other maize-derived protein products developed by this company include β-glucuronidase, aprotinin, laccase, and trypsin [32]. Prodigene was the first company to demonstrate the commercial benefits of plant-based platforms and was also a forerunner in the study of the economic impact of downstream processing in molecular pharming, having developed several successful approaches to recover intact and functional recombinant seeds from maize [3].

Maize has also been used to produce recombinant pharmaceutical proteins, including enzymes, vaccines, and antibodies [32, 33]. One of the most notable therapeutic proteins produced in maize is Meristem Therapeutics' gastric lipase, an enzyme intended for the treatment of exocrine pancreatic insufficiency—a disease significantly affecting cystic fibrosis sufferers—that has completed phase II clinical trial. In addition to this enzyme, Meristem Therapeutics has developed two other maize-derived products, human lactoferrin (phase I clinical trial), whose intellectual property was later acquired by Ventria Bioscience (http://www.ventria.com/), and collagen (pre-clinical stage).

Rice is another leading platform for recombinant protein and peptide production. Similar to maize, rice is easy to transform and scale up, but unlike maize, rice is self-pollinating, which reduces the risk of horizontal gene flow. Ventria Bioscience, in its ExpressTec platform, has used rice to produce recombinant pharmaceutical proteins, including human albumin, transferrin, lactoferrin, lysozyme, and vaccines against human rabies and Lyme disease. Its lead therapeutic candidate VEN100, whose active ingredient is lactoferrin, has been shown to reduce significantly antibiotic-associated diarrhea in high-risk patients and recently completed phase

II clinical trial [34]. Rice has also been widely used as host for peptide expression, especially for the production of allergen peptides (e.g., pollen and mite allergies) [35, 36]. Recent studies report that rice has the potential to offer an oral delivery system for vaccine antigens and therapeutic proteins and peptides [25, 35, 37].

Barley seeds have also been developed as commercial platforms. In comparison to other cereal crops, barley is less widely grown. However, this fact added to the self-pollinating nature of barley can be viewed as an advantage since the risk of contamination and outcrossing with non-transgenic crops is minimized. Considering this benefit, an Iceland-based company, ORF Genetics (https://orfgenetics.com/), has targeted barley grain as the expression host for several human cytokines and growth factors [19]. Other molecular pharming companies, such as Ventria Bioscience and Maltagen, have also been developing barley-based production platforms. Although barley is still recognized for its recalcitrance to transformation, over the last decade some progress has been made in the development of reliable transformation procedures [38].

The use of legume seeds, such as soybean and pea, for the production of recombinant pharmaceutical proteins, has been less explored than cereal-based platforms, with platforms based on legume seeds having yet to achieve commercial success. However, the fact that legume seeds have exceptionally high protein content (20–40%) can be exploited to achieve high yields of recombinant protein [39]. Soybean seeds have been used to express recombinant growth factors [40, 41], coagulation factors [42], and vaccine peptides [43]. Transgenic pea seeds have been previously used to produce a single-chain Fv fragment (scFV) antibody used in cancer diagnosis and therapy [44]. In another study, pea seeds were used to produce a vaccine that showed high immunogenicity and protection against rabbit hemorrhagic disease virus [45].

Safflower and rapeseed seeds are rich in oil and are, thus, referred as oilseeds. Oilseeds can provide useful recombinant pharmaceutical protein production systems. SemBioSys (http://www.sembiosys.ca/), with its oleosin-fusion platform, has been a pioneer in that field. Oleosins are the principal membrane proteins of oil bodies; oleosins confer peculiar structural properties to the oil bodies that offer simple extraction and purification procedures [46]. In the oleosin-fusion platform the recombinant protein is fused with oleosin and consequently targeted to the oil bodies. The fusion protein is then recovered through simple purification of the oil bodies and separated from oleosin by endoprotease digestion. Commercial production of hirudin in safflower by SemBioSys constituted the first report of an oilseed-derived protein [47]. The company has been focusing on safflower as its primary host ever since, with safflower-derived insulin being in phase I clinical trial [32].

Finally, fruit and vegetable crops can also be employed for molecular pharming. A major advantage of protein expression in fruit and vegetable crops is that edible organs can be consumed uncooked, unprocessed, or partially processed, making them particularly suitable for the production of recombinant subunit vaccines, nutraceuticals, and antibodies designed for topical application [29]. The oral delivery of recombinant therapeutics is one of the differentiating factor of molecular pharming in comparison to mainstream biopharmaceutical production systems, with several pharmaceutical products being produced in tomato fruits, potato tubers, and lettuce leaves for this purpose [3]. Tomato fruits are particularly useful for protein expression because the fruits are palatable as raw tissue but can also be lyophilized and stored for a long time [25]. Recently, human coagulation factor IX (hFIX) was expressed specifically in tomato fruits, constituting the first report on the expression of hFIX in plant [48]. Another study described the expression in tomato fruits of a thymosin α1 concatemer [49], an immune booster that plays an important role in the maturation, differentiation, and function of T cells.

The thymosin α1 concatemer derived from transgenic tomatoes exhibited biological activity and was proven to stimulate the proliferation of mice splenic lymphocytes in vitro. Moreover, thymosin α1 specific activity was higher when produced in tomato than in *Escherichia coli*, demonstrating the authenticity of the plant-made product. Other examples of tomato fruit expression include F1-V [50], a candidate subunit vaccine against pneumonic and bubonic plague, and β-secretase [51], to serve as a vaccine antigen against Alzheimer's disease.

In conclusion, platforms based on transgenic plants are a promising alternative to the conventional biopharmaceutical production platforms since they provide a stable source of pharmaceutical proteins and are also the most scalable of all molecular pharming platforms. This scalability of transgenic plants ensures the production of recombinant pharmaceutical proteins at levels previously inaccessible, namely, the commodity bulk production of monoclonal antibodies. In the current scenario of growing pharmaceutical demand, especially in developing countries, the use of transgenic plants can be game changing since they provide a highly scalable and low-cost means of producing medicines.

2.2 Platforms based on transplastomic plants

Transplastomic plants are a valuable alternative to transgenic plants for the production of recombinant pharmaceutical proteins. Transplastomic plants are obtained by the insertion of expression constructs into the plastid genome by particle bombardment. Since the *Agrobacterium* T-DNA (transfer DNA) complex is targeted to the nucleus, it is unsuitable for gene transfer to chloroplasts [24, 52]. Following the transformation procedure, the bombarded leaf explants are regenerated, and transplastomic plants with homoplastomic transformation (in which every chloroplast carries the transgene) are finally selected, recurring to a selection medium containing spectinomycin or in combination with streptomycin [53].

Plastid transformation can result in high yields of heterologous proteins because multiple copies of the genome are present in each plastid, and photosynthetic cells may contain hundreds or thousands of plastids [54]. As an example, the expression of a proteinaceous antibiotic in tobacco chloroplasts has achieved up to 70% of the total soluble proteins, which is the highest recombinant protein accumulation accomplished so far in plants [55]. Furthermore, chloroplasts provide a natural biocontainment of transgene flow since genes in chloroplast genomes are maternally inherited and consequently not transmitted through pollen, thereby avoiding unwanted escape into the environment. Other advantages of chloroplast engineering include the ability to express several genes as operons, and the accumulation of recombinant proteins in the chloroplast, thus reducing toxicity to the host plant [24].

Finally, transplastomic production platforms offer the possibility of oral delivery [54, 56]. In fact, it has been demonstrated that chloroplast-derived therapeutic proteins, delivered orally via plant cells, are protected from degradation in the stomach, probably due to the bioencapsulation of the therapeutic protein by the plant cell wall. They are subsequently released into the gut lumen by microbes that digest the plant cell wall, where the large mucosal intestine area offers an ideal system for oral drug delivery [57].

A shortcoming of expressing proteins via the chloroplast genome is that routine plastid engineering is still limited to tobacco, a crop that is not edible and thus unsuitable for oral delivery of therapeutic proteins. In addition, the synthesis of glycoproteins is not possible in the chloroplast system, as plastids do not carry out glycosylation [24]. Nevertheless, the expression of human somatotropin [58] in tobacco established that chloroplasts are capable of properly folding human proteins with disulfide bonds. In another study, the production of native cholera toxin B subunit

[59] demonstrated the capacity of chloroplasts to fold and assemble oligomeric proteins correctly. Other therapeutic proteins expressed in tobacco chloroplasts include interferons alpha-2a and alpha-2b [60, 61] and anti-cancer therapeutic agents such as human soluble tumor necrosis factor (TNF) [62] and azurin [63]. Recently, chloroplast transformation of lettuce has also been developed [64, 65] to provide oral delivery transplastomic systems [66, 67]. Several therapeutic proteins were produced in lettuce chloroplast, namely, proinsulin [66, 67], tuberculosis vaccine antigens [68], and human thioredoxin 1 protein [69]. The chloroplast production platform has yet to achieve commercial success, though the referred developments in this field augur a promising future for therapeutic protein production in chloroplasts.

2.3 Transient expression platforms

Transient expression is a phenomenon that occurs when genes are introduced into plant tissues and are expressed for a short period without stable DNA integration into the genome [3]. Traditionally, transient expression was used to verify expression construct activity and to test recombinant protein stability. This strategy allowed the identification and elimination of initial transformation problems, and thus the prospect of regenerating the desired transgenic lines was significantly improved. Recently, there has been an emergence of transient expression for the commercial production of recombinant pharmaceutical proteins. The advantages of transient expression platforms include the ease of manipulation, speed, low cost, and high yield of proteins. In comparison to transgenic plants, transient expression permits to achieve higher recombinant protein yields because there are no position effects (suppression of transgene expression by the surrounding genomic DNA following integration) [70].

Transient expression systems utilize the beneficial properties of plant pathogens to infect plants, spread systemically, and express transgenes at high levels, causing the rapid accumulation of recombinant proteins [8]. Currently, the major transient expression platforms are based on *Agrobacterium tumefaciens*, plant viruses, or hybrid vectors that utilize components of both (magnICON® technology).

The agroinfiltration method involves the vacuum infiltration of a suspension of recombinant *A. tumefaciens* into the plant leaf tissue, with the transgenes being then expressed from the uninterrupted T-DNA [8, 71]. Using this method, milligram amounts of recombinant protein are produced within a few weeks without the need to select transgenic plants, a process that takes months to years to be completed. This system has been commercially developed in tobacco [72] and alfalfa [73] but is also applicable to other crops such as lettuce [74], potato [75], and *Arabidopsis* [76]. An advantage of *Agrobacterium*-mediated transient expression is the fact that it allows to produce in plants complex proteins assembled from subunits [70].

Another transient expression technology is based on the use of plant viruses. In this technology, the gene of interest is inserted among viral replicating elements, episomically amplified and subsequently translated in the plant cell cytosol [77]. To date, the most efficient and high-yielding platforms have been developed using RNA viruses [78]. These plant viruses include *Tobacco mosaic virus* (TMV), *potato virus X* (PVX), and *Cowpea mosaic virus* (CPMV) (reviewed in [8]). The advantages of virus-based production include the rapid recombinant protein expression, the systemic spread of the virus, and the fact that multimeric proteins such as antibodies can also be produced by coinfecting plants with noncompeting vectors derived from different viruses [79, 80]. Transient expression vectors based on virus have been used to express peptides and long polypeptides (at least 140 amino acids long) as fusions to the coat protein, resulting in the assembly of chimeric virus particles (CVPs) displaying multiple copies of the peptide or polypeptide on its surface [77, 81].

Transient expression based in plant viruses has been commercially adopted by the now-closed Large Scale Biology Corporation (Vacaville, USA) that used a TMV-based vector for the production of patient-specific idiotype vaccines for the treatment of B-cell non-Hodgkin's lymphoma, which had successfully passed the phase I clinical trials [82].

Finally, the third transient expression strategy is based on hybrid systems that incorporate components of the T-DNA transfer and virus replication systems [3]. These hybrid systems use deconstructed viruses obtained by removing the coat protein (responsible for systemic movement) of the noncompeting virus strains and use *Agrobacterium* as the vehicle for the systemic delivery of the resulting viral vectors to the entire plant. These systems effectively address most of the major shortcomings of earlier plant-based technologies by providing the overall best combination of the following features: high expression level, high relative yield, low up- and downstream costs, very fast and low-cost R&D, and low biosafety concerns [83]. Consequently, there has been a commercial development based on several hybrid systems. One of most notable examples is the magnICON® system developed by Icon Genetics (https://www.icongenetics.com/) (formerly owned by Bayer Innovation, Dusseldorf, Germany; now a subsidiary of Nomad Bioscience, Halle, Germany), which features a deconstructed *Tobacco mosaic virus* (TMV) genome and *A. tumefaciens* as a delivery vehicle [83]. Another example is the iBioLaunch platform developed by the Fraunhofer Center for Molecular Biotechnology, which also features a deconstructed TMV genome [3]. Finally, the CPMV-HT platform is based on a deleted version of *Cowpea mosaic virus* RNA-2 and also allows the "hypertranslation" of recombinant proteins without virus spreading [8].

Examples of therapeutic recombinant proteins produced in these platforms have been generally reviewed in [3]. Recombinant protein production using transient expression is now being mobilized to a large scale with several companies developing scalable, automated plant-based GMP biomanufacturing facilities to efficiently produce large amounts of pharmaceuticals within weeks. Such facilities include the ones of the Fraunhofer Center for Molecular Biotechnology (Newark, DE) (https://www.fraunhofer.org/), Medicago Inc. (Quebec, Canada) (http://www.medicago.com/), Icon Genetics (Bayer; Halle, Germany) (http://www.icongenetics.com/), Texas A & M (College Station, TX), and Kentucky BioProcessing LLC (Owensboro, KY) (http://www.kbpllc.com/) [19].

In conclusion, the ability of transient plant expression systems to produce large quantities of recombinant protein, coupled to the use of current technology to increase yields, and the many promising technical solutions seems to be favorable compared with mammalian- or insect cell-based systems in quality, cost, and scale [19]. In case of emerging threats, transient platforms are advantageous since they produce large amounts of recombinant proteins rapidly (milligram quantities per plant within a few days) and can be scaled up quickly, currently providing the only reliable platform for rapid response situations [9]. During the H1N1 pandemic, the first batches of H1N1 virus-like particles (VLPs) could be produced by Medicago Inc. as soon as 3 weeks after the Centers for Disease Control and Prevention released the new influenza hemagglutinin sequence [73]. Similar lead times were reported for the H5N1 VLP vaccine [84]. Recently, the application of tobacco plant-based transient production systems, at Kentucky BioProcessing (KBP), to produce antibody lots against Ebola, was shown to significantly decrease the amount of time required for production over traditional methods, increase the quantity of antibody produced, and reduce the cost of manufacturing. Finally, at the other end of the market scale, transient expression platforms are economical for the production of pharmaceuticals for very small markets, such as orphan diseases and individualized therapies.

2.4 Callus and plant cell suspension cultures

Plant cell suspension cultures grow as individual cells or small aggregates and are usually derived from *callus* tissue by the disaggregation of friable callus pieces in shake bottles and are later scaled up for bioreactor-based production. Recombinant pharmaceutical protein production is achieved using transgenic explants to derive the cultures or by transforming the cells after disaggregation, usually by co-cultivation with *A. tumefaciens*. The co-cultivation of plant cell suspensions and recombinant *A. tumefaciens* has also been used for the transient expression of proteins [85]. Since these plant cell suspension cultures are grown in sterile contained environments, they provide a cGMP-compatible production environment that is more acceptable to the established pharmaceutical industry and regulatory authorities [3, 86]. These systems have added benefits of complex protein processing compared to bacteria and yeasts and increased safety compared to mammalian cell systems, which can harbor human pathogens. Another advantage of plant suspension cultures is the very low maintenance cost in comparison to other fermenter-based eukaryotic systems such as mammalian or insect cells. Moreover, the possible secretion of the target protein into the culture medium simplifies downstream processing and purification procedures [87, 88]. Nevertheless, plant cell cultures also have some limitations such as poor growth rates, somaclonal variation (particularly due to chromosomal rearrangements, common in plant cell cultures generated by *calli*), and gene silencing, together with the inhibition of product formation at high cell densities, formation of aggregates, cell wall growth, as well as shear-sensitivity for some species [89]. However, high levels of functional recombinant protein in plant cell suspension cultures were already obtained [87]. Besides, the previously mentioned first licensed recombinant pharmaceutical protein, Elelyso™, was produced in plant cell suspension cultures (reviewed in [88]). Tobacco has been the most popular source of suspension cells for recombinant protein production. Tobacco plants proliferate rapidly and are easy to transform, but other plant species have also been used to generate suspension cells, including rice and *Arabidopsis thaliana,* alfalfa, soybean, tomato, *Medicago truncatula*, and carrot [85, 88, 90]. Carrot suspension cells have been used by the aforementioned Protalix Biotherapeutics to produce a recombinant glucocerebrosidase. This case of commercial success shows that suspension cell cultures have potential as a viable system for large-scale protein production. Recently, carrot callus cultures, expressing epitopes from the cholesteryl ester transfer protein, were accessed for the potential of becoming an atherosclerosis oral vaccine [91].

3. Optimization of plant expression levels

The lower expression levels in comparison to the established biopharmaceutical platforms were one of the major obstacles for the commercialization of molecular pharming [9]. Therefore, numerous techniques have been developed to enhance protein expression, including codon optimization of protein sequences, to match the preferences of the host plant, targeting subcellular compartments that allow proteins to accumulate in a stable form; the use of strong, tissue-specific promoters; and the testing of different plant species and systems [25].

Protein synthesis can be increased by optimizing the components of the expression construct to maximize transcription, mRNA stability, and translation or by diminishing the impact of epigenetic phenomena that inhibit gene expression [92]. In this field, the general strategy is to use strong and constitutive promoters, such as the cauliflower mosaic virus 35S RNA promoter (CaMV 35S) and maize ubiquitin-1 promoter (ubi-1), for dicots and monocots, respectively. However, organ- and

tissue-specific promoters are also being used to drive expression of the transgenes to a specific tissue or organ such as the tuber, the seed, and the fruit. Additionally, inducible promoters, whose activities are regulated by either chemical or external stimulus, may equally be used to prevent the lethality problem. Furthermore, transcription factors can also be used as boosters for the promoters to further enhance the expression level of the transgenes [53].

Protein stability can be increased by targeting proteins to cell compartments that reduce degradation. Protein targeting also affects the glycan structures added to proteins and the type of extraction and purification steps required to isolate the protein from the plant matrix. Proteins can be targeted to the secretory pathway by an N-terminal signal peptide, which is cleaved off for the release of the protein into the endoplasmic reticulum (ER). Proteins that do not require posttranslational modification, e.g., glycosylation, for their activity, can be targeted to the chloroplast using N-terminal transit peptides [93]. In addition, the target gene can be used to transform chloroplast directly, with highly enhanced protein accumulation. Moreover, posttranslational modifications of the ER lumen can also be avoided by expressing the protein as translational fusion with oleosin protein, which target the expression of the foreign protein to oil bodies of the seeds [28]. Other subcellular compartments like the protein-storing vacuoles are now being explored for recombinant protein accumulation, as it has been observed in rice seed endosperm [94].

4. Downstream processing

In the early years of molecular pharming, scientific studies were focused on demonstrating that plants could produce adequate quantities of recombinant pharmaceutical proteins and confer an oral delivery means. This led to downstream processing and the costs associated to it being basically overlooked. Downstream processing is now known to be an economically critical part of biomanufacturing processes (it can account for up to 80% of the total cost in a therapeutic protein production line) and also to be a key component of the regulatory process for evaluating the safety of pharmaceutical products [7]. The goal and the general steps for downstream processing are similar between plant and other expression systems: to recover the maximal amount of highly purified target protein with the minimal number of steps and at the lowest cost. The basic steps for downstream processes include tissue harvesting, protein extraction, purification, and formulation [22]. However, since in molecular pharming the costs of downstream processing are product-specific rather than platform-specific, the evaluation of downstream processing strategies and costs associated to it has to be done on a case-by-case basis. Nevertheless, even if unit operations have to be developed based on the properties of the product, others have to be developed based on the properties of the expression host. Plants produce process-related contaminants that require specific processing steps to ensure removal of fibers, oils, superabundant plant proteins such as RuBisCO, and potentially toxic metabolites such as the alkaloid nicotine in tobacco [8]. These secondary metabolites can be recovered from plant cells or tissues using methods such as adsorption, precipitation, and chromatography, often requiring phase partitioning and the use of mixtures of organic solvents. Several approaches have been used to facilitate downstream processing, including secretion of recombinant proteins, eliminating the plant cell disruption step; targeting of proteins into the protein bodies, oil bodies, or plastoglobules; and the use of affinity tags such as poly-histidine tags with the target protein, allowing protein purification by affinity chromatography [25]. In addition, oral delivery of whole plants or crude extracts containing the pharmaceutical relevant proteins can also be a way to simplify downstream processing and to

easily distribute medicines to those in need. Furthermore, the optimization of plant's expression level can also ease downstream processing, with higher protein concentrations conducting to higher protein volumes [7].

Finally, several purification strategies have been investigated to separate target transgenic proteins from host plant proteins, which are tailored for each individual protein based on its solubility, size, pI, charge, hydrophobicity, or affinity to specific ligands, and the parallel characteristics of plant host proteins. Chromatographic methods, such as affinity chromatography, have been the most extensively used. However, recently increasing attention is being paid to non-chromatographic methods to provide alternatives for large-scale production [22].

5. Heterologous production of bioactive angiotensin I-converting enzyme inhibitory (ACEI) peptides

In the broad range of known bioactive peptides, angiotensin I-converting enzyme inhibitory (ACEI) peptides derived from food proteins have attracted particular attention and have been studied the most comprehensively for their ability to prevent hypertension [95]. In this chapter we will further focus on the possibility to genetically engineer crop plants to produce and deliver antihypertensive ACEI peptides, therefore creating alternative sources to fight hypertension and prevent cardiovascular disease.

5.1 Cardiovascular disease and the renin-angiotensin system

Cardiovascular disease (CVD) has been recognized as the leading cause of death in developed countries. Hypertension or high blood pressure is one of the major independent risk factors for CVD [96]. States of CVD include conditions such as coronary heart disease, peripheral artery disease, and stroke. Hypertension is a condition defined by a blood pressure measurement of 140/90 mmHg or above and is thought to affect up to 30% of the worldwide adult population [95]. The kinin-nitric oxide (KNO) system and the renin-angiotensin system (RAS), **Figure 1**, play a crucial role in the control of hypertension by the action of angiotensin I-converting key enzyme (EC 3.4.15.1; ACE) [96–99].

Several synthetic ACE inhibitors such as captopril, enalapril, and lisinopril have been prescribed for the treatment of hypertension, congestive heart failure, and diabetic neuropathy [100]. However, their consumption is associated with various side effects including cough, skin rashes, hypotension, loss of taste, angioedema, reduced renal function, and fetal abnormalities [95]. The side effects associated to synthetic ACE inhibitors and the high prevalence of hypertension have led scientists to search for natural and safer therapies. Interestingly, the study of ACEI peptides has revealed that they do not have significant effects on blood pressure in normotensive subjects, suggesting a convenient mechanism that avoids acute hypotensive effects. Based on this finding, it is hypothesized that ACEI peptides could be used in initial treatment of mildly hypertensive individuals or even as supplemental treatments [101].

5.2 Antihypertensive ACEI peptides

So far, several ACEI peptides have been identified in food proteins, mainly in milk, eggs, and plants, currently constituting the most well-known class of bioactive peptides [102–104]. These peptides are inactive within the sequence of parent proteins, but they can be released by enzymatic proteolysis in vivo or

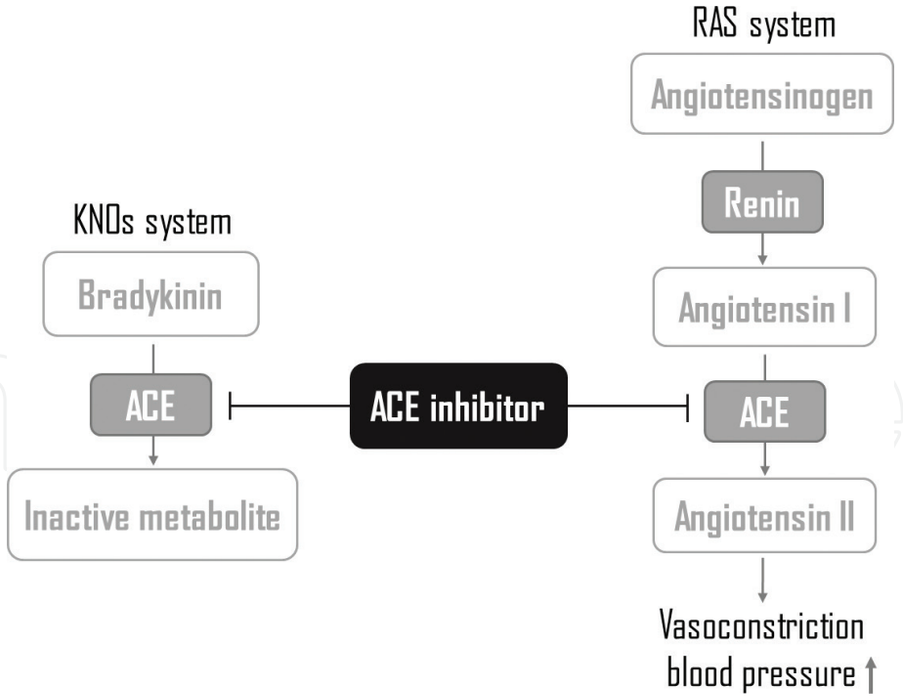

Figure 1.
The kinin-nitric oxide (KNO) system and the renin-angiotensin system (RAS). The left side (KNO system) shows the mechanism of the action of ACEI on ACE that cleaves bradykinin, a nonapeptide acting as vasodilatory hormone, and causes the formation of an inactive heptapeptide. In the right side (RAS system), the inhibition of ACE activity plays an important physiological role in regulation of blood pressure by inhibiting the conversion of the hormone angiotensin I to angiotensin II, a potent vasoconstrictor (figure adapted from Erdmann et al. [96]).

in vitro, for example, during gastrointestinal digestion or during food processing. A common feature shared by the majority of ACEI peptides is the generally short sequence, i.e., 2–12 amino acids in length. However, some larger inhibitory sequences have been identified in milk fermented with *Enterococcus faecalis* [105] and *Lactobacillus casei* Shirota [106], in koumiss [107], tuna [108], bonito [109], and rotifer [110]. Studies have also indicated that binding to ACE is strongly influenced by the substrate's C-terminal tripeptide sequence. Hydrophobic amino acid residues with aromatic or branched side chains at each of the C-terminal tripeptide positions are common features among potent inhibitors. The presence of hydrophobic Pro residues at one or more positions in the C-terminal tripeptide region seems to positively influence a peptide's ACE-inhibitory activity [95]. In general, the peptides showing higher activity against ACE have Tyr, Phe, Trp, or Pro at their C-terminus [95]. The peptides TQVY from rice [111], MRW from spinach [112], and YKYY from wakame [113] are some examples of this principle. **Table 1** reviews some examples of ACEI activities of plant origin, whose peptides responsible for such activity may be potential sources for the heterologous production of ACEI peptides.

The most common method to produce and identify ACEI peptides is through enzymatic hydrolysis of food proteins with gastrointestinal enzymes such as pepsin and trypsin or with commercial proteases such as Alcalase™ [127]. ACEI peptides have also been produced with *Lactobacillus, Lactococcus lactis*, and *E. faecalis* strains during milk fermentation [105, 106]. Nevertheless, there are problems associated to this type of industrial production of ACEI peptides, including the difficulty to isolate the peptide of interest from the complex mixture of compounds produced by enzymatic hydrolysis, the high cost, low recovery, and the low bioavailability. These disadvantages denote the need to develop new and alternative approaches for their production.

Source	ACEI activity (IC50; μM)	Antihypertensive activity (mmHg)	Dose (mg/kg)	Reference
Chlorella vulgaris	29.6	Not determined	—	[114]
Chebulic myrobalan	100	Not determined	—	[115]
Bitter melon	8.64	−31.5 to −36.3	2–10	[116]
Mung bean	13.4	Not determined	—	[117]
Pea	64	Not determined	—	[118]
Peanut	72	Not determined	—	[100]
Potato	18–86*	Not determined	—	[119]
Rapeseed	28	−11.3	7.5	[120]
Rice	18.2	−40	30	[111]
Soybean	14–39*	−17.5	2	[121]
Soybean	21	Not determined	—	[122]
Soybean	1.69	Not determined	—	[123]
Soybean	17.2	Not determined	—	[124]
Spinach	0.6–4.2*	−13.5 to −20*	20–100	[112]
Wakame	21–213*	−50	50	[113]
Walnut	25.7	Not determined	—	[125]
Wheat	20	Not determined	—	[126]

Different values for the same plant product related to the ACEI peptide sequence.

Table 1.
Examples of ACEI peptide activity from different plant origin.

5.3 Heterologous production of ACEI peptides in plants

In recent years, the application of recombinant DNA technologies for the production of ACEI peptides at a large scale and low cost has gathered attention in the biotechnology community. Investigation has been focused on the development of expression methods for antihypertensive peptide production in different plant crops [128]; and here, we tried to provide some promising examples.

Thus far, the main strategies that have been adopted are as follows: the overexpression of ACEI peptide precursor proteins and the production of particular peptides as heterologous components [101], the modification of some storage proteins to produce chimeric proteins carrying ACEI peptides [101], and also the generation of multimer proteins containing tandem repeats of ACEI peptides, flanked by protease recognition sequences that allow the peptide release during gastrointestinal digestion.

5.3.1 Rice

Transgenic rice plants that accumulate novokinin (RPLKPW), a potent antihypertensive peptide designed according to the structure of ovokinin (2–7) (RADHPF), as a fusion with the rice storage protein glutelin, have been generated. The engineered peptide is expressed under the control of endosperm-specific glutelin promoters and specifically accumulates in seeds. Oral administration of either the RPLKPW-glutelin fraction or transgenic rice seeds to spontaneously hypertensive rats (SHRs)—the main model for assessing the in vivo activity of ACEI peptides (e.g., [108, 111, 122])—significantly reduced systolic blood pressures, suggesting

the possible application of transgenic rice seed as a nutraceutical delivery system and particularly for administration of antihypertensive peptides [129].

Wakasa et al. [130] attempted the generation of transgenic rice seeds that would accumulate higher amounts of novokinin peptide by expressing 10 or 18 tandemly repeated novokinin sequences, with the KDEL endoplasmic reticulum retention signal at the C-terminus, and using the glutelin promoter along with its signal peptide. Although the chimeric protein was unexpectedly located in the nucleolus and the accumulation was low, a significant antihypertensive activity was detected after a single oral dose to SHRs. More importantly, this effect was observed over a relatively longer duration time, with intervals of 5 weeks between doses as low as 0.0625 g transgenic seeds per kg.

5.3.2 Soybean

Soybean [*Glycine max* (L.) Merr.] is an attractive option for the production of ACEI peptides given that soybean seeds contain a large amount of total protein. Therefore, there has been an effort to generate soybean lines with improved ACEI properties foreseeing the creation of novel functional foods.

Matoba et al. [128], introduced novokinin (RPLKPW) into homologous sequences of a soybean β-conglycinin α' subunit by site-directed mutagenesis. Founded on first achievements from an *E. coli* expressed protein, the muted β-conglycinin α' subunit carrying novokinin repeats were also expressed in soybean. This chimeric protein accumulated at levels of up to 0.2% of extracted protein from transgenic soybean seeds [131]. Still, the levels of expression were too low, and it was not possible to assess the in vivo effects of these soybean seeds.

Novokinin has also been expressed in transgenic soybean seeds in a fusion form along with a β-conglycinin α' subunit. Interestingly, a reduced systolic blood pressure was observed in SHRs after administering a dose of 0.15 g kg^{-1} of protein extracts. A similar effect was attained following administration of a 0.25 g kg^{-1} dose of defatted flour. Thus, it was concluded that this chimeric protein produced in soybean possessed an antihypertensive activity [132].

Additionally, a synthetic gene of His-His-Leu (HHL), an ACEI peptide derived from a Korean soybean paste, was tandemly multimerized to a 40-mer, ligated with ubiquitin as a fusion gene (UH40), and subsequently expressed in *E. coli*. Following digestion with leucine aminopeptidase, the 405-Da HHL monomer was recovered by reverse-phase high-performance liquid chromatography (HPLC). MALDITOF mass spectrometry, glutamine-TOF mass spectrometry, N-terminal sequencing, and measurement of ACE-inhibiting activity confirmed that the resulting peptide was the HHL [133]. The potential use of this antihypertensive chimeric protein in soybean has yet to be assessed.

5.3.3 Tomato and tobacco

A modified version of amarantin, the main seed storage protein of *Amaranthus hypochondriacus*, carrying four tandem repeats of the ACEI dipeptide Val-Tyr into the acidic subunit of amarantin, was expressed in cell suspension cultures of *Nicotiana tabacum* L. NT1. Protein hydrolysates obtained from transgenic *calli* showed high levels of inhibition of the angiotensin-converting enzyme, with an IC50 value of 3.5 μg ml^{-1}, and 10-fold lower levels than that of protein extracts of wild-type cells (IC50 of 29.0 μg ml^{-1}) [134]. This was the first time that a chimeric protein comprising an ACEI peptide was produced in plant cell suspension cultures.

This modified version of amarantin was also expressed in the fruit of transgenic tomato plants. Protein hydrolysates from transgenic tomato fruits showed in vitro ACE inhibition, with IC50 values ranging from 0.376 to 3.241 μg ml^{-1};

this represented an increase of up to 13-fold in the inhibitory activity when compared with the protein hydrolysates of non-transformed fruits [135]. These two results suggest the possible application of tobacco plant cell suspension cultures and transgenic tomato fruits for massive production of this engineered version of amarantin, which could be especially used as an alternative hypertension therapy [134, 135].

5.3.4 Amaranth

Although amaranth has not been genetically modified to produce ACEI peptides, the feasibility of developing a modified amarantin acidic subunit has been widely assessed [129, 134–139]. Recently, the in vivo effect of an *E. coli*-modified amarantin protein, four units of Val-Tyr dipeptides (VY) in tandem, and one of Ile-Pro-Pro tripeptides (IPP) incorporated in the amarantin acidic subunit (AMC3) was evaluated in SHRs in a one-time oral administration experiment. This study showed that enzymatic hydrolysates of AMC3-containing ACEI peptide (4xVY and IPP) sequences had significant in vivo antihypertensive action [138]. The positive reports of amarantin expression in *E. coli* [136, 138, 139] along with the sustained expression of amarantin-modified proteins in tobacco [134] and tomato [135] prospect the successful production of ACEI peptide fusion proteins in amaranth.

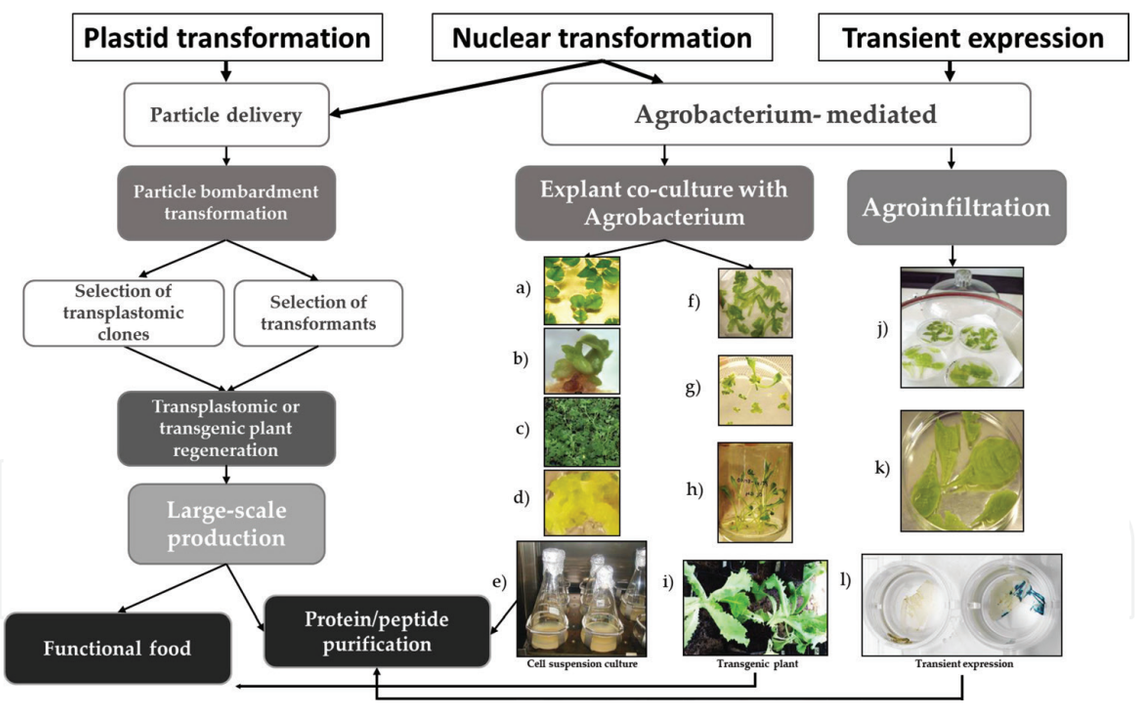

Figure 2.
Schematic representation of the technologies involved in different plant platforms for the production of therapeutically important proteins and peptides. ***Plastid transformation*** *by particle bombardment can result in regeneration of transplastomic plants, revealing high-yield heterologous production, with the possibility of protein/peptide oral delivery or purification.* ***Nuclear transformation*** *can be accomplished by particle bombardment or by Agrobacterium-mediated transformation, resulting in the regeneration of stable transgenic plants. Finally, the technology based on* ***transient expression****, here with the example of agroinfiltration. We present Medicago truncatula and lettuce as examples: (a) M. truncatula co-culture of leaf explants with Agrobacterium, (b) and (c) plant regeneration via somatic embryogenesis according to Araújo et al. [145], (d) and (e) establishment of a cell suspension culture from callus for protein/peptide production [146, 147], (f) lettuce leaf explant co-culture with Agrobacterium, (g) and (h) plant regeneration via shoot organogenesis at PCB lab, (i) lettuce transgenic plants which can be used for oral delivery, (j) and (k) agroinfiltration of lettuce leaf explants according to Negrouk et al. [74], and (l) example of a control explant (left) and transient expression of a 35S::GUS(int) cassette in lettuce leaves (right).*

5.3.5 Lettuce and Medicago truncatula

Lettuce (*Lactuca sativa*) is a commercially important crop belonging to the Asteraceae family. It is a diploid (2n = 18), autogamous species with a genome size of 2.7 Gb [140]. This crop is particularly suitable for oral delivery of therapeutics as its raw leaves are consumed by humans, and the time to obtain an edible product is only weeks, compared to the months needed for crops such as tomato or potato. Therefore, recently lettuce has been investigated as a production host for edible recombinant therapeutics [66, 67, 141]. Furthermore, the fact that stable transformation procedures for both nuclear [142] and plastid genomes [64], and transient expression [74], are widely available, is also an advantage. Lettuce has been used as production host for several recombinant therapeutics, virus-like particles (VLPs) and monoclonal antibodies [143], antigens [142, 144], and human therapeutic proteins [66, 69].

Medicago truncatula is a model plant from the legume family. It is a diploid (2n = 16), autogamous species, with a relatively small genome and short life cycle of 3–5 months. These characteristics enable this species to be used in molecular genetic studies and expression of foreign genes [145]. The phylogenetic distance to economically important crops is crucial in the choice of this plant by many researchers and funding agencies, since it allows comparative studies within the legume family. The methodologies for the establishment of long-term cell suspension culture are well recognized [146], and the potential of *M. truncatula* as expression host has also been established for the production of feed additives [20, 87], human hormones [90], and human enzymes [147].

The use of these two species in molecular pharming is at the center of a recent collaboration between the Plant Cell Biotechnology (PCB) Laboratory (ITQB UNL), the Cell Differentiation and Regeneration Laboratory (iBiMED UA), and the Institute of Plant Genetics (IPG PAS). This cooperation foresees the usage of these two species as exceptional hosts for the heterologous production and/or delivery of ACEI peptides, and a resume of this ongoing project is here schematically presented (**Figure 2**). This figure also provides an overview of the technologies involved in different plant platforms discussed in this chapter.

6. Conclusions

Molecular pharming has been recently and extensively reviewed, and the future of this technology has gathered some optimistic expectations. A myriad of studies have already demonstrated the capacity of various plant species and systems to produce recombinant pharmaceutical proteins and peptides. This technology has already been put to the test in case of emerging threats, where transient platforms proved to be strategic for rapid production of large amounts of recombinant proteins in response to pandemic situations. However, their usefulness for the production of functional foods still falls short of expectations, as well as the attainment of its full potential in bioactive peptide production. With the improvement of known plant platforms and development of new genetic engineering techniques and their exploration, it is forthcoming an evolution in the production of heterologous bioactive peptides, to which we hope to contribute with our ACEI pharming project. The advent of genome editing techniques (with the advantage of site- specific gene insertion), like the CRISPR/Cas9 methodology, will undoubtedly increase and democratize plant transformation events and will certainly contribute to the increase of genetically modified species for molecular pharming purposes.

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