دانشگاه تبریز

تولید پروتئین‌های داروئی نوترکیب در گیاهان:

مزایا و چالش‌ها

بهرام باغبان

دکتری بیولوژی مولکولی و بیوتکنولوژی

عضو هیئت مدیره انجمن ایمنی زیستی جمهوری اسلامی ایران

و عضو کمیته ایمنی زیستی وزارت علوم، تحقیقات و آموزش عالی کشور

مریم احساسات‌وطن

دانشجوی دکتری بیوتکنولوژی گیاهی

Chronology of Plant Molecular Pharming/ Plant Made Pharmaceuticals

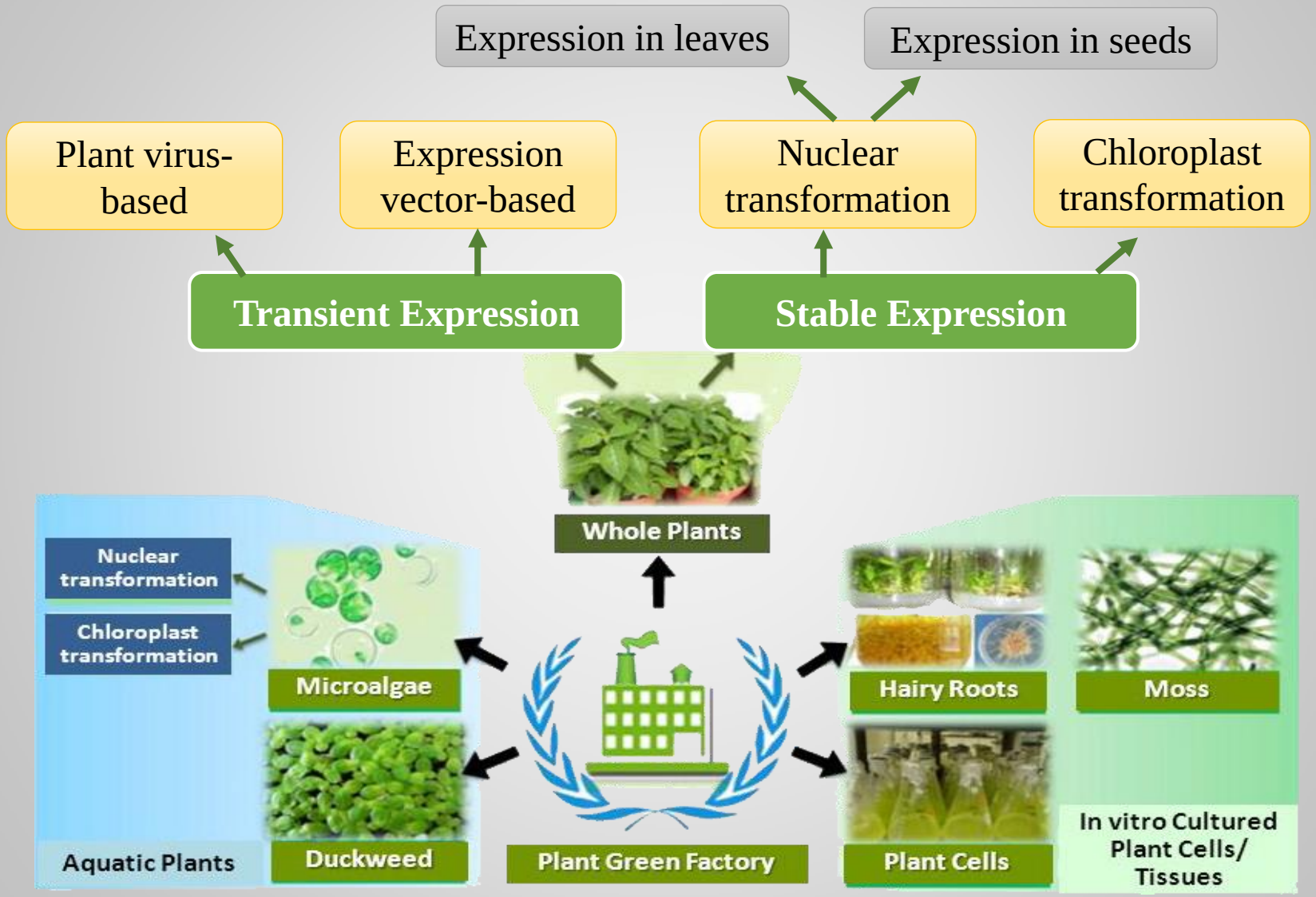
- rDNA technology (1971)
- Recombinant protein expression (1972-3)
- Recombinant biomedicine (1976)
- First commercial human insulin in E coli (1982)
- First report on the producing Human Growth Hormone in tobacco and sunflower callus tissue(1986)
- Emerging the plant molecular pharming/farming, by the “authenticity” of plant-derived recombinant proteins with the production of the first human protein (serum albumin) with confirmed native structure in transgenic tobacco and potato (1990)
- The field of molecular pharming gained support and interest from the plant biotechnology community (during the 1990s)

Comparison of different expression platforms for the production of pharmaceuticals

Comparisons	Transgenic Plant	Plant Cell Culture	Bacteria (e.g. Ecoli)	Yeast (*Pichia pastoris)*	Mammalian Cell Culture (eg CHO)	Transgenic Animals
Overall cost	Very low	Medium	Low	Medium	High	High
Scale-up capacity	Very high	Medium	High	High		
Production scale	Worldwide	Limited	Limited	Limited	Limited	Limited
Protein yield	High	High	Medium	High		High
Protein folding accuracy	High	High	Low	Medium	High	High
Glycosylation	Minor differences	Minor differences	None	Incorrect	Correct	Correct
Product quality	High	High	Low	Medium	High	High
Contamination risks	Low	Low	Endotoxins	Low	Virus, Prions, oncogenic DNA	Virus, Prions, oncogenic DNA
Safety	High	Non-specific	Low	Unknown	Medium	High
Storage cost	Inexpensive	Moderate	Moderate	Moderate	Expensive	Expensive

Further, Transient expression system and tobacco cell free lysate

Platforms based on transgenic plants



Recombinant protein Expression Platforms in plants

(1) Nuclear genome by developing transgenic plants either biolistics or *Agrobacterium* mediated transformation
(2) Plastid genome by developing transplastomic plants using the biolistics or PEG approach
(3) Plant tissues transiently expressing recombinant proteins by inoculating plant viral vectors.
In the third case, the sequences are, usually leaves.

Recombinant Protein Production in Plants: Challengesand Solutions
Elizabeth E. Hood and Deborah V. Vicuna Requesens
Argelia Lorence (ed.), *Recombinant Gene Expression: Reviews and Protocols, Third Edition*, Methods in Molecular Biology, vol. 824, DOI 10.1007/978-1-61779-433-9_25.

Strategies *to* Increase Expression of Recombinant Proteins in plant system by:

1- developing efficient expression cassette,

2- Genetics approach considering transgene copy number and host vigorous germplasm

3- Protein Accumulation and Stability

1- developing efficient expression cassette

1-1. Transcription

Promoters;

Use of the CaMV 35S promoter from the cauliflower mosaic virus as a strong constitutive one and the most popular choice for dicotyledonous plants

use of the maize ubiquitin-1 promoter or the *Agrobacterium tumefaciens* nopaline synthase (*nos*) promoter, for higher expression in monocots.

Use of tissue and developmental stage specific promoters like the maize zein, rice and wheat glutenin, and pea legumin gene promoters.

1-2. Transcript Stability

-Polyadenylation sites directly affect message stability and Use of alternate polyadenylation sites in expression cassette.

-Use of efficient terminators specific sequences from plants and plant viruses, such as 35S terminator and the potato proteinase inhibitor II (Pin II) terminator,

-Use of single intron in a proper place of gene construct increases stability and accumulation of mRNA in plants, particularly grasses. In monocots, the introns known to stimulate expression are the maize *Adh1*, *Ubi1*, *Sh1*, and actin and the rice *sal*T, *act1*, and *tpi* genes, among others. In dicots, examples of introns known to elevate expression include the potato *ST-LS1* gene and the *Arabidopsis UBQ3*, *PAT1*, and *EF-1α* genes. In plants, introns affect mRNA accumulation post transcriptionally. They facilitate maturation and enhance the stability of new transcripts.

-Remove cryptic destabilizing regions from messengers of the gene of interest.

1-3. Replication

high-level expression with a suitable promoter is susceptible to posttranscriptional gene silencing, reducing the accumulation of the mRNAs.

Use of alternative method of plant virus-based vectors achieves high expression of foreign proteins in transient transformations systems. The recombinant proteins are encoded from an engineered vector that offers high-level expression by RNA-directed RNA amplification using viral replicases.

Viral genomes are small and easy to manipulate, and the infection of plants with this type of vector is simpler and faster than stable transformation methods.

This system has some difficulties and presents some challenges. There is a size limitation on the foreign protein expressed, the foreign gene is not heritable, the genes inserted into viral genomes can sometimes be spliced out, and there are concerns regarding the potential spread of the modified viruses in the environment.

1-4. Translation

Codon optimization should be a priority.
Along with optimizing the codons of transgenes, certain plant and plant viral 5’ untranslated sequences, such as the tobacco mosaic virus and the potato virus leader sequences, have been used to boost translation initiation.
It is necessary to modify any sequence located immediately next to the translation start site to fit the consensus initiation sequence, which can vary from species to species.
Furthermore, we should try to avoid predicted mRNA secondary structures, since they might impede translation or result in premature termination of transcripts.
Use of translational fusion between the recombinant protein of interest and a second proteinfor stabilizing the fused protein or improving the solubility and folding of the protein.

2- Genetics approach in transgene copy number and …

Increasing the transgene copy number

One way to achieve this is to self-pollinate single-transgenic homozygous plants or crossing high-expressing transgenic events with each other. However, transgenes present in multiple copies are more likely to be silenced at a transcriptional or posttranscriptional level.

Silencing is strongly correlated with the number of transgenes in vegetative tissue .

Several strategies have been utilized to reduce this problem.

Transplastomic plants, are not subject to silencing.

Additionally, the formation of antisense transcripts can be prevented by flanking the transgenes with matrix attachment region sequences .

Expressing a protein in seed rather than vegetative tissue could reduce this phenomenon.

A second genetic approach to boost expression and generate high protein accumulation is to make use Unique germplasm, such as high oil in maize, which can be used to increase yield, influence germination, and maintain or improve transgene expression. Other germplasm includes transgenic lines expressing a cytokinin-synthesizing enzyme under the control of a senescence inducible promoter, creating two normal-size embryos.

3. Protein Accumulation and Stability

In fact, high mRNA levels do not guarantee high levels of protein accumulation. One of the most critical factors influencing the accumulation of heterologous proteins is the presence of proteases in the host plant.

Expressing proteins in specific organelles, directing expression to a tissue or at a particular time, coexpression with protease inhibitors, or use of protein fusions.

Use of proper signal peptide to target in the mitochondria, the chloroplast, and the secretory pathway.

Also, the presence of chaperone proteins in the ER provides help to fold and assemble foreign proteins. An additional benefit of targeting foreign proteins to the ER is the ability to obtain posttranslational modifications of recombinant proteins, especially glycosylation.

Alternatively, when seeds are the target organ, protein storage vacuoles (PSVs) are a suitable subcellular compartment.

Time specific and tissue specific and inducible promoters are involved in this part.

Developing plastidial platform for recombinant protein expression system by Transplastomic plants

Plastids/Chloroplasts

- Plastids are plant cellular organelles with a ~120–150 kb genomes size
- 1 to 100 chloroplasts per plant cell
- The chloroplast genome most commonly includes around 100-250 genes
- Genome present in 1,000–10,000 copies per cell (Bendich,1987)
- Maternally inherited in most angiosperm plant species (Hagemann, 2004) Plastid genomes resemble bacterial genomes in many aspects and also contain some features of multicellular organisms, such as RNA editing and split genes (Exon-Intron)

Advantages of chloroplast transformation

- High level of expression
- Multigene engineering
- Maternal inheritance
- No gene containment
- No gene silencing
- No positional effect
- No pleiotropic effect
- No epigenetic effects
- No vector sequence
- Disulfide bound formation

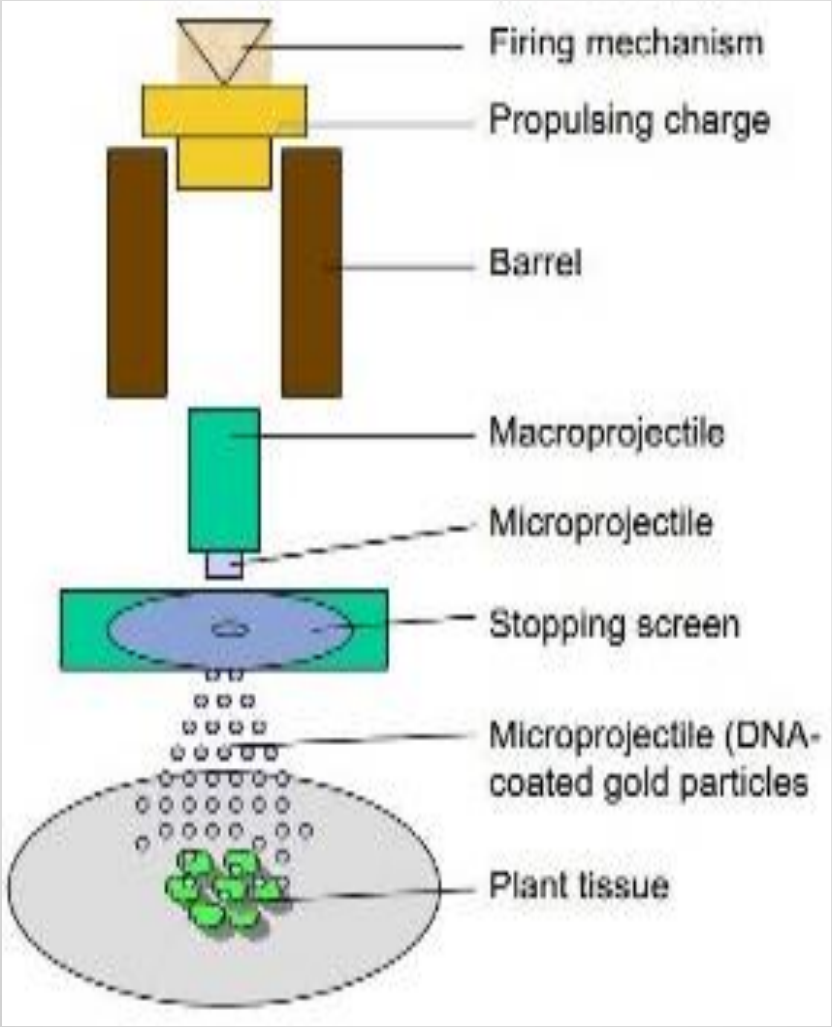
Factors involved in protein expression in plastid

- **Homologous recombination sequence**
- **Marker genes**
- **Promoter**
- **5’ and 3’ UTR sequences**
- **Shine-Dalgarno sequences**

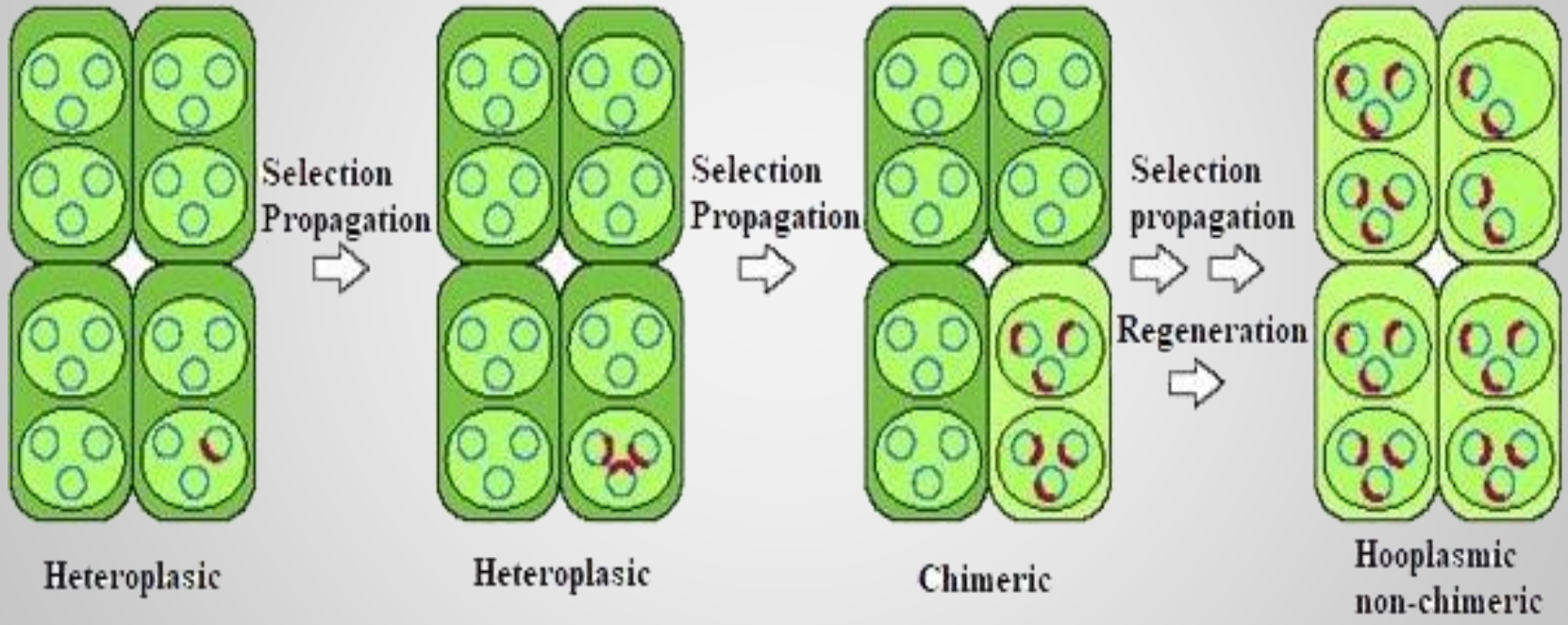
Chloroplast transformation requires

- **A chloroplast specific expression vector.**
- **A method for DNA delivery through a double membrane of the chloroplast.**
- **An efficient selection for the transplastome.**

Chloroplast transformation by biolistic method



Selection of homoplasmic non-chimeric plants



Vaccine antigens and biopharmaceuticals engineered via plastid genome of higher plants

Traits	Gene	Expression	Host plant	References
HIV/AIDS	gp120, gp41	16 µg g−1 FW	Tobacco	(Morgenfeld et al., 2014)
Human papiloma virus	GUS-E7	3–4% TSB	Tobacco	(Chan et al., 2016)
Polio virus	CTB-VP1	4–5% TSP	Tobacco	(Lakshmi et al., 2013)
Tuberculosis antigens	CTB-SAT6CTB-Mtb72F	7.5% TSP	Tobacco	(Gorantala et al., 2014)
Tuberculosis antigens	CTB-ESAT6	0.75% TSP	Lettuce	(Gorantala et al., 2014)
Bacterial	Pa	2.5–4% TSP	Tobacco	(Gottschamel et al., 2016)
Dengue virus	EDIII	0.8–1.6 TSP	Tobacco	(Oey et al., 2009a)
Bacterial phage lytic protein	plyGBS	>70% TSP	Tobacco	(Su et al., 2015)
Pompe disease	CTB-GAA	0.1–0.2% TLP	Tobacco	(Lim et al., 2011)
Thioredoxin 1	hTx1	15% TSP	Lettuce	(Ruiz, 2002)
Insulin liken growth factors	IGF-1n	32% TSP	Tobacco	(Oey et al., 2009b)
Endolysin Cpl-1	Cpl-1	10% TSP	Tobacco	(Arlen et al., 2007)
Interferon-α2b(IFN-α2b)	IFN-a2b	21% TSP	Tobacco	(Wang et al., 2011)
Interferon-α2b(IFN-α2b)	bFGF	0.1% TSP	Tobacco	(Morgenfeld et al., 2009)

Platforms based on transgenic plants

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 post translationally 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].

Platforms 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 disadvantage of leafy crops is that proteins are synthesized in an aqueous environment, which is more prone to protein degradation, resulting in lower production yield. 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. 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. 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 . Other leafy crops commonly used in molecular pharming include alfalfa, clover and lettuce.

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. 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. 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.

Rice has also been widely used as host for peptide expression, especially for the production of allergen peptides (e.g., pollen and mite allergies).

Recent studies report that rice has the potential to offer an oral delivery system for vaccine antigens and therapeutic proteins and peptides.

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**. 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.

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. In another study, pea seeds were used to produce a vaccine that showed high immunogenicity and protection against rabbit hemorrhagic disease virus.

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. 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 oilseedderived protein . The company has been focusing on safflower as its primaryhost ever since, with safflower-derived insulin being in phase I clinical trial.

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. 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.

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. 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, 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, a candidate subunit vaccine against pneumonic and bubonic plague, and β-secretase, to serve as a vaccine antigen against Alzheimer's disease.

What is needed after 30 years on PMPs :

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).

The market is still dominated by prokaryotic and mammalian expression systems, the former offering high production capacity at a low cost, and the latter favored for the production of complex biopharmaceutical products. Although plant systems are now gaining widespread acceptance as a platform for the larger-scale production of recombinant proteins, there is still resistance to commercial uptake. This partly reflects the relatively low yields achieved in plants, as well as inconsistent product quality and difficulties with larger-scale downstream processing. Furthermore, there are only a few cases in which plants have demonstrated economic advantages compared to established and approved commercial processes, so industry is reluctant to switch to plant-based production. some plant-derived proteins for research or cosmetic/pharmaceutical applications have reached the market, showing that plants can excel as a competitive production platform in some areas.

Suggestions:

Although transient expression by viral and hybrid system in plants and cell free system in BY2 tobacco cell lines have been started in recent years but still we need to develop some novel and synthetic, semi synthetic biological or sub biological circuits and production system or we do not know any…….
which is certainly accessible in your growing minds,

Thanks for your interest and attention

We all of us
wait impatiently your
creature and
contribution
See you at coming
days.