Chapter

Next Generation of Transgenic Plants: From Farming to Pharming

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Abstract

The number of approaches related to recombinant protein production in plants is increasing rapidly day by day. Plant-based expression offers a safe, cost-effective, scalable, and potentially limitless way to rapidly produce recombinant proteins. Plant systems, which have significant advantages over animal and yeast recombinant protein production systems, are particularly promising for the large-scale production of antibodies and therapeutic proteins. Molecular pharming with transgenic plant systems become prominent among other production systems with its low cost, absence of human or animal pathogen contaminants, and the ability to use post-translational modifications such as glycosylation. The ability to produce recombinant pharmaceutical proteins in plant seeds, plant cells and various plant tissues such as hairy roots and leaves, through the stable transformation of the nuclear genome or transient expression, allows for the establishment of different production strategies. In particular, the rapid production of candidate proteins by transient expression, which eliminates the need for lengthy transformation and regeneration procedures, has made plants an attractive bioreactor for the production of pharmaceutical components. This chapter aimsto exhibit the current plant biotechnology applications and transgenic strategies used for the production of recombinant antibodies, antigens, therapeutic proteins and enzymes, which are used especially in the treatment of various diseases.

Keywords: molecular pharming, plant-derived pharmaceutical, therapeutic proteins, transient expression, recombinant proteins

1. Introduction

In the last couple of decades, many initiatives have been carried out in which different disciplines came together for sustainable farming in respect to the increasing food demands of the world population which is expected to reach 9 billion thresholds in 2050. Conventional breeding, mutation breeding and especially transgenic technology have been frequently used to prevent yield losses which are caused by the decreasing amount of arable land due to various reasons such as urbanization, desertification and salinity and drought, various diseases, weeds and insects. With advances in modern biotechnology, many approaches have been used to improve crop varieties, from marker-assisted selection (MAS) to recently developed new plant breeding techniques (NPBTs). In particular, genetic modifications have significantly expanded the genetic pool that has been used by plant breeders since the mid-90s. Thus, the development of new plants with many different agricultural traits has gained great momentum.

After the acceleration of commercialization of transgenic crops, which were initially developed for agronomic purposes such as insect and herbicide tolerance for only producers, especially stacked GM events, in which two or more characteristics are introduced together, emerged. Approximately 30 different transgenic plants with many different characteristics for both the producer and the consumer as disease resistance, abiotic stress tolerance, increase in nutrition and food quality, including fruits and vegetables as *Phaseolus vulgaris* (bean), *Solanum melongen* (eggplant), *Cucumis melo* (melon), *Carica papaya* (papaya), *Prunus domestica* (plum), *Beta vulgaris* (sugar beet), had been approved [1, 2]. Stacked GM crops have a combination of several traits and even four different traits (genetic modification) are possible to be located in one GM event, as in the Widestrike™ Roundup Ready Flex™ cotton and Herculex™ RW Roundup Ready™-2 maize samples [3]. Moreover, in the last decade, new genome editing techniques such as zinc finger nuclease (ZFN) technology, clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease systems and transcription activator-like effector nucleases (TALENs), which provides precise genome modifications, enabled the reproduction of plants which does not contain recombinant DNA. The recently adopted cisgenesis/intragenesis, RNA interference and new genome editing techniques have enabled the development of many genetically edited organisms (GEOs) as well.

Along with all these developments, it has been understood after the 2000s that GMOs, which have become increasingly dominant in the agricultural field, can also be utilized effectively in medicine and industrial fields. Especially, the increase in chronic and infectious diseases as a result of overpopulation, and the outbreak of epidemics and pandemics cause demands that exceed the production capacity of molecules used for diagnosis, treatment and prevention. To satisfy these demands, cost-effective systems that can produce high-quality products, and allow to take action quickly are developed. In addition, it is a fact that models that support small-scale production will be needed in recent years, as is the case with personalized medicines. Especially in the last two decades, it has been possible to produce plant-based pharmaceutical proteins that can be used in the diagnosis and treatment of many diseases with the developments in gene transfer and production strategies of recombinant proteins in plants.

Thanks to improvements in basic points such as new transformation strategies (stable or transient), transformation methods, appropriate promoter selection and codon optimization, high-scale and lower cost recombinant proteins in plants can be produced rapidly [4, 5]. Thus, the interest in producing recombinant proteins for direct use as a product without aiming for a specific change in phenotype or metabolism and obtaining them in purified form or crude plant extract has increased. This potential provided by the production of recombinant pharmaceutical proteins in plants for therapeutic applications was realized much later than in bacterial, yeast and animal systems. On the other hand, in the early periods, interest in the production of non-pharmaceutical products such as industrial enzymes, cosmetic ingredients, biosensors or biocatalysts, feed, biofuel in plants due to their short development times, low purification costs and less regulatory burdens was much higher than pharmaceutical products [6].

The first applications of molecular farming were come to fruition in the late 1980s by producing various antibodies and human-specific proteins in transgenic plants. Among all expression systems, plant-based systems have started to be preferred as an attractive alternative with their advantages in low production cost, high level of transgene expression, rapid scalability, the riskless transmission of human and animal pathogens and production of proteins with secondary modification (**Table 1**). It is also possible to produce recombinant proteins in dry tissues such as seeds or grains, which reduces storage and transportation costs and puts plant

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	Production cost	High scale production	Development times	Product quality	Glycosylation	Purification-storage cost and diffuculty	Safety
Bacteria	Low	High	Low	Low	None	Low	Medium (Endotoxi)
Yeast	Medium	High	Medium	Medium	Incorrect	Low	Medium
Insect	Medium	Medium	Medium	Medium	Correct	Medium	Low
Mammalian cel culture	High	Low	High	Very High	Correct	Medium	Very Low (Virus, prion and oncogens)
Transgenic Animals	Very high	Medium	Very high	Very High	Correct	Medium	Very Low (Virus, prion and oncogens)
In vitro plant culture (Hairy roots, cell suspension culture)	Medium	High	High	High	Minor differences	Medium	Very High
Stable transgenic plants	Medium	Medium	Very High	High	Minor differences	High*	Very High
Transient plants	Low	High	Low	High	Minor differences	High*	Very High

Edible forms don't have any purification-storage cost and difficulty.

Table 1.
Comparison of different production systems according to their efficiency.

systems one step ahead. While plants can produce complex proteins similar to other eukaryotic systems, they can modify these proteins post-translationally. However, plants can present enhanced post-translational glycosylation modifications, unlike yeast and insects, which have prokaryotic expression systems or very simple glycosylation patterns. Plant cells have slight differences in their glycosylation patterns compared to mammalian cells, and these differences can be rearranged by genetic engineering [7]. Since different glycan structures affect the stability of glycoproteins, subcellular targeting, immunogenicity, pharmacokinetic behavior and biological activity, it is essential to produce the recombinant protein with the appropriate glycosylation pattern [8, 9]. One of the most significant strategies which are developed to control the glycosylation of recombinant proteins in plants is subcellular targeting that prevent the addition of undesired sugar residues. Another important strategy is glycoengineering that avoids the addition of plant glycans and even replaces them with human proteins [9]. In the following years, studies have accelerated and the term of molecular pharming has become prominent with the increasing demand for the production of recombinant pharmaceutical proteins in many different plant systems [9].

Following the enhanced knowledge of gene transfer to plants and finely-tuned gene transfer methods specific to many species such as *Nicotiana tabacum* and *N. benthamiana* (tobacco plant), *Lactuca sativa* (lettuce), *Glycine max* (soybean), *Oryza sativa* (rice), *Solanum lycopersicum* (tomato), *Medicago sativa* (alfalfa), *Zea mays* (maize), it is possible to produce many recombinant proteins including monoclonal antibodies, enzymes, growth factors, therapeutic proteins and vaccines in various plant tissues or cell. Recombinant protein production can be accomplished in the whole plant, as well as in certain parts such as seeds, leaves and fruits [10]. The selected production strategy must express the protein with high efficiency and fully come up with the requirements of the regulations in terms of safety and production quality. To produce a recombinant protein, optimization of the coding sequences of a gene that is desired to transfer, determination of the gene expression strategy as stable or transient, selection of convenient plants and cost-efficient methods for isolation and purification of target proteins are required. Stable gene transfer which is performed by introducing the target gene into the nuclear or plastid genome by *Agrobacterium* and particle bombardment respectively results in stable expression in plant tissues. Although the methods for plant transformation vary according to target species, the target genome (nuclear or plastid), the structure of the gene to be transferred, there are two common approaches: direct (e.g. biolistic or microparticle bombardment) or indirect (*Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*). It is possible to obtain a whole transgenic plant by using plant tissue culture methods together with mentioned transformation methods [11]. Moreover, high-scale production of recombinant proteins becomes possible for many different plant species by establishing various cultures (e.g., callus culture, hairy root culture or suspension culture) from transformed plant tissues or cells [12].

It is crucial to state that developing transgenic plants by stable transformation is more time-consuming by comparing to transient expression systems. In addition, stable transformation strategies need improvement to increase the level of transgene expression. Especially, due to the random insertion of the transgene into the nuclear genome, the different profiles that emerge as a result of the positional effect should be followed meticulously. Unstable gene expression, as well as transgene-induced gene silencing, may occur together with multiple insertions [4]. Unlike stable expression, when we focus on recombinant protein production strategies based on transient expression of transgenes carried by bacteria (*A. tumefaciens*) or viral vectors (tobacco mosaic virus (TMV), cauliflower mosaic virus (CMV), alfalfa mosaic virus (AVM)), it is seen obviously that stable integration of transgene

is not necessary. Thus, it becomes possible to produce recombinant protein rapidly by avoiding time-consuming transformation and regeneration procedures. With transient expression by *Agrobacterium* infiltration or viral vectors, it is possible to ensure rapid expression of the transgene even 3–4 hours after transformation and to produce recombinant protein by reaching the maximum expression level in the range of 18–48 hours [4]. Other important advantages of transient expression are that they can reach higher expression levels compared to stable transformation and maintain gene expression in the range of 10–14 days.

One of the important handicaps in recombinant protein production in plants is the lack of suitable, reliable and inexpensive purification methods for each plant. This deadlock becomes even more difficult when the purification of pharmaceuticals must fulfill the stringent criteria mandated by the "good manufacturing practice (GMP)" standards. It is known that purification practices performed in accordance with legal standards comprise approximately 80% of the total costs [13]. Apart from purification by chromatography, membrane filtration and fusion of various polypeptides, there is a need to develop more efficient and cost-effective purification strategies. On the other hand, with the redesign of plant parts containing the target recombinant protein in a form suitable for an oral route such as edible vaccines, it is possible to get rid of the heavy financial burdens of purification strategies. One of the issues that need to be carefully considered regarding the production of pharmaceutical proteins in plants is regulation and biosafety issues. Especially, economic damages to farmers and the food industry as a result of the co-mingling of food and feed crops with plants designed for pharmaceutical production, the spread of transgenes through pollen or seeds, undesirable exposure of non-target organisms such as insects, birds and horizontal gene transfer are among the emerging risks [14].

2. Transgenic plants in farming

Since the discovery of genetic mechanisms of reproduction and biodiversity, plant breeders methodically try to exploit agronomically desired traits for more profitable crop production in many aspects. In the last quarter of the twentieth century, genome manipulation techniques known as genetic engineering were introduced into various organisms. In the early years of this novel technology in plant science, desired traits were related to higher yield, resistance to various biotic and abiotic stress factors. Considerable success has been obtained in enhancing photosynthetic capacity, increasing root and leaf size, stimulating vegetative growth, improving biomass and more. Following transgenic approaches aimed nutritional quality through enhancing various biomolecule production and increased shelf life as well [15]. In recent days, transgenic technology may even offer some solutions to the global energy crisis through improvements in biofuel production. A total of 17 million farmers from 29 different countries surged in biotech crops in the period from 1996 to 2019 after the first successful commercialized release. During these 24 years, global biotech crop planting increased 112- fold from 1.7 million to 190.4 hectares. In the second third of this period, trends in leading biotech crop-producing countries started to change as well. Since 2011, 24 developing countries produced 56% of total biotech crops while 5 leading industrial countries share the rest of the 44%. This trend tends to accelerate as long as other developing countries realize the potential. A total of 19 countries among 29 are considered as "biotech mega" with at least 50,000 hectares planting. United States, Brazil, Argentina, Canada and India are the leading planters. Soybean, maize, cotton and canola are the most planted biotech crops worldwide (**Figure 1**). Plants as alfalfa, sugar beet,

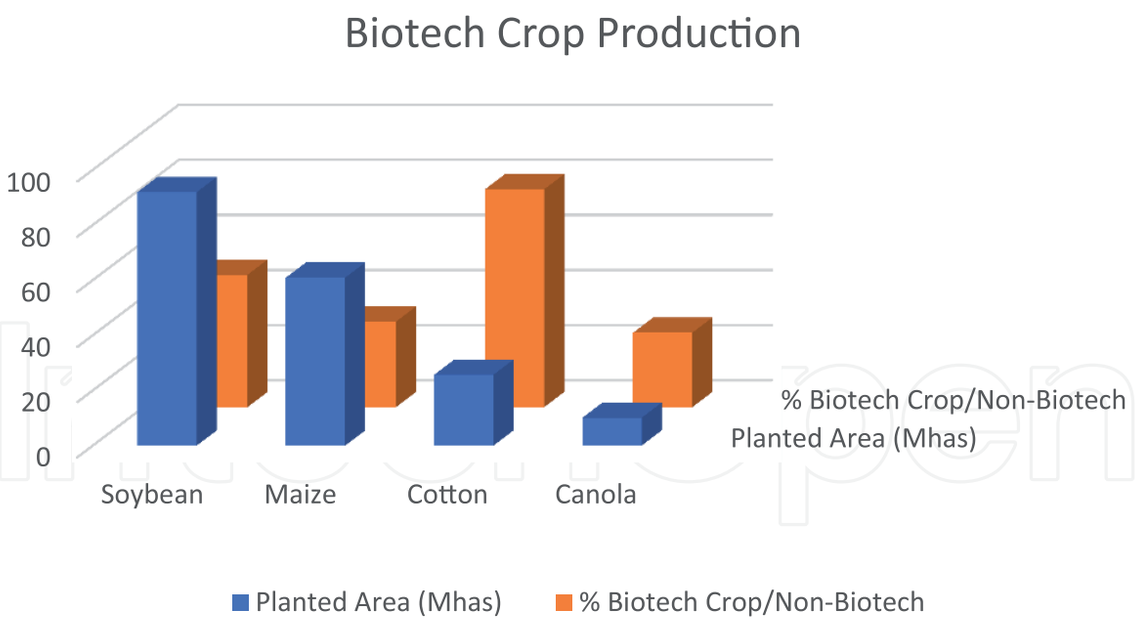

Figure 1.
Worldwide biotech crop production [16].

potato, apples, squash, papaya and eggplant share only 1.8% of total production besides these four plants.

Biotech crops, which have reached 224.9 billion US dollar global value have also contributed to conserving biodiversity by preventing deforestation, reducing pesticide requirement, decreasing CO_2 emissions in agricultural applications and alleviating socio-economic conditions of small farmers.

Transgenic plants are generally classified into three generations. First-generation GM crops were developed against various biotic and abiotic stress factors, while the second generation targeted better nutritional quality [15]. The remarkable amount of progress has been achieved in countless laboratory practices with many different plant species. On the other hand, commercial GM varieties are considerably limited compared to these laboratory practices. As it can be seen in **Table 2**, all commercial GM traits belong to the first and second generations of transgenic approaches. In laboratory practices, abiotic stress tolerance is one of the key aspects of desired crops. However, abiotic stress tolerance mechanisms and pathways are extremely intricate. Defining a particular gene target for an abiotic stress factor and introducing it to a susceptible variety is usually an insufficient strategy due to these complex responses within and between species. Plants have developed stress signal perception and transduction pathways that regulate stress-inducible genes through transcription factors (NAC, WRKY, MYB, bZIP, DREB/CBF), kinases and phosphatases. Main stress-inducible genes are kinases, molecular chaperones, osmoprotectants, transcription factors [17–20].

Many biomolecules are identified with their known protective roles against abiotic stresses. Therefore, these molecules are potential gene targets for transgenic abiotic stress tolerance approaches. Transgenic regulations of solutes such as glycine betaine, mannitol, trehalose and proline which acts as an osmoprotectant, metal chelator, antioxidative defense molecule and signal molecule have been used to enhance stress tolerance in many plants. For an instance, codA expressing GM indica rice plant which has enhanced glycine betaine production through increased choline oxidase activity, present induced water stress tolerance [21]. Abiotic stress factors usually cause misfolding and precipitation of crucial proteins. Heat shock proteins (HSPs) act as molecular chaperones and mediate folding, assembly, translocation and degradation of misfolded proteins. As a molecular chaperon,

Plant / Commercia GM Trait	Abiotic Stress Tolerance	Altered Growth/Yield	Disease Resistance	Herbicide Tolerance	Insect Resistance	Modified Product Quality	Pollination control system	TOTAL
Alfalfa – *Medicago sativa*				4		2		6
Apple – *Malus* x *Domestica*						9		9
Argentine Canola – *Brassica napus*				35			22	57
Bean – *Phaseolus vulgaris*			1					1
Carnation – *Dianthus caryophyllus*				4		1		5
Chicory – *Cichorium intybus*				3			3	6
Cotton – *Gossypium hirsutum* L.				45	50			95
Cowpea – *Vigna unguiculata*					1			1
Creeping Bentgrass – *Agrostis stolonifera*				1				1
Eggplant – *Solanum melongena*					1			1
Eucalyptus – *Eucalyptus sp*.		1				14		15
Flax – *Linum usitatissimum* L.				1				1
Maize – *Zea mays* L.	7	2		215	210		6	440
Melon – *Cucumis melo*								0
Papaya – *Carica papaya*			4					4
Petunia – *Petunia hybrida*						18		18

Plant / Commercia GM Trait	Abiotic Stress Tolerance	Altered Growth/Yield	Disease Resistance	Herbicide Tolerance	Insect Resistance	Modified Product Quality	Pollination control system	TOTAL
Pineapple – *Ananas comosus*						2		2
Plum – *Prunus domestica*			1			12		13
Polish canola – *Brassica rapa*				4				4
Poplar – *Populus sp*.					2			2
Potato – *Solanum tuberosum* L.			19	4	30			53
Rice – *Oryza sativa* L.				3	3			6
Rose – *Rosa hybrida*						1		1
Safflower – *Carthamus tinctorius* L.						9		9
Soybean – *Glycine max L*.	2	1		35	6			44
Squash – *Cucurbita pepo*			2			3		5
Sugar Beet – *Beta vulgaris*				3		19		22
Sugarcane – *Saccharum spp*.	3				3	2		8
Sweet pepper – *Capsicum annuum*			1			2		3
Tobacco – *Nicotiana tabacum* L.				1		1		2
Tomato – *Lycopersicon esculentum*			1		1	2		4
Wheat – *Triticum aestivum*				1		2		3
Total	12	4	29	359	307	99	31	841

Table 2.
Registered commercial GM traits and related crops [16].

transgenic *Trichoderma harzianum* hsp70 is shown to increase heat and other abiotic stress resistance in *Arabidopsis thaliana* plants [22]. Also, late embryogenesis abundant (LEA) proteins, aquaporins and calcineurin B-like proteins with antioxidant, membrane protection and ion binding functions are widely preferred targets [1]. Rab28 LEA gene over-expressing maize plants are reported to have improved desiccation tolerance under a constitutive maize promoter [23]. Likewise, transgenic expression of halophilic fungus *Aspergillus glaucus* AgGlpF gene encoding an aquaporin protein confers extreme salt tolerance in soybean [24].

Other commercially registered GM traits are altered insect and disease resistance, growth/yield, herbicide tolerance, modified product quality and pollination control system. Insect and disease resistance is mainly obtained through introducing natural pest genes from insects, fungi and bacteria to target plants. The most known example of the application is δ-endotoxin insecticidal protein-expressing cry gene transfer from *Bacillus thuringiensis* (Bt) which is extremely effective against lepidopterans, dipterans and coleopterans. In recent days, there are several alternatives to Bt toxins including lectins, protease inhibitors, antibodies, peptide hormones [15]. In particular, protease inhibitors (PIs) have devastating effects on insect digestive systems since most insects facilitate serine-type proteinase enzymes in digestion. Serine-type mustard trypsin inhibitor −2 (MTI-2) expressing *Brassica napus (L.)* plant are proven to present insecticidal properties on *Pterostichus madidus* beetle. It was also found effective on *Plutella diamondback* moth xylostella as the intermediary pest species [25]. As an alternative to Bt toxin alone, lectin genes were also introduced for insecticidal properties. Codon-optimized synthetic Bt Vip3Aa gene under CaMV35S promoter and *Allium sativum* leaf agglutinin gene under phloem-specific promoter transformation significantly improved *Helicoverpa armigera* resistance. As mentioned in environmental stress factors earlier, transcription factors are known to play important roles in plant resistance to environmental stress factors. MYB4L transcription factors were shown to induce ethylene pathway and enhance tobacco mosaic virus (TMV) resistance in *Nicotiana benthamiana*. On the other hand, silencing the transcription factor intensified the susceptibility [26]. Similarly, the WRKY17 transcription factor was proven to both enhance artemisinin biosynthesis in a traditional Chinese medicinal plant *Artemisia annua,* and provide resistance against *Pseudomonas syringae*. Two defense marker genes, pathogenesis-related 5 (PR5) and NDR1/HIN1-LIKE 10 (NHL10), were significantly increased in AaWRKY17-overexpressing transgenic *A. annua* plants as well as AaWRKY17 directly bound to the promoter region of the artemisinin biosynthetic pathway gene amorpha-4,11-diene synthase (ADS) and promoted its expression.

Herbicide resistance can be maintained through two transgenic approaches. The first involves the modification of the target enzyme to overcome herbicide sensitivity. In the second approach herbicide, detoxifying pathways are introduced to the susceptible target plants. Glyphosate (N-phosphonomethylglycine) is a highly efficient, low-toxicity, broad-spectrum and nonselective herbicide that has been widely applied. Glyphosate specifically inhibits the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimate pathway and prevents aromatic amino acid synthesis unselectively in any plants. Therefore, fortification of target enzymes in intended plants is crucial. Recently, co-transformation of a codon-optimized glyphosate oxidase gene WBceGO-B3S1 from a variant BceGO-B3S1 and a glyphosate-tolerant gene I. variabilis-EPSPS from the bacterium *Isoptericola variabilis* into an *O. sativa* variety by *Agrobacterium*-mediated genetic transformation resulted in high glyphosate tolerance [27]. In the second approach, the herbicidal effects of glufosinate, which is a nonselective, glutamine synthetase (GS) inhibitor, are targeted. The GS enzyme produces glutamine amino acids from ammonia and glutamate. Glufosinate causes glutamine deficiency in

susceptible plants through ammonia and glyoxylate accumulation, inhibition of photosynthesis due to defected chloroplast structure. Transformation of the pat gene from *Streptomyces viridochromogenes* to susceptible plants leads to expression of phosphinothricin acetyltransferase (PAT) enzyme in plants which metabolize glufosinate into N-acetyl-L-glufosinate (NAG). This non-toxic compound does not inhibit GS enzymes [28].

The first two generations of transgenic plants in farming applications consistently enhanced food supply and essential traits. Following the improvements within this technology, it became more friendly to the environment, less risky for human health, more profitable to small scale farmers and more regulated around the world. The third generation will be the era of recombinant pharmaceuticals as plant-based vaccines against pathogens in human health and beneficial health products as therapeutic proteins, monoclonal antibodies, hormones, enzymes along with transgenic bioenergy plants which are not going to be discussed in this chapter.

3. Transgenic plants in pharming

Excluding transgenic farming applications which were summarized in the previous section, the term of molecular farming through transgenic plants refers utilization of plants for recombinant protein production instead of microorganisms and animal cell in fermenter systems. When we narrow this definition from recombinant proteins to pharmaceutical products still in plants basis, we define the alternative term "molecular pharming". Before the revolutionary contribution of Stieger et al. [29] in which they presented the capability of plant cells to produce multimeric assembled mammalian antibodies in *Acetabularia mediterranea*, the general opinion was that functional full-length mammalian antibodies would not assemble in plants without mammalian chaperones. Before this accomplishment, there were various well-established production systems as *Escherichia coli* in prokaryotes, *Saccharomyces cerevisiae* in single-cell eukaryotes, Chinese Hamster Ovary (CHO) cells, non-secreting murine myeloma (NS0) cells, Sp2/0 HEK293 mammalian cells for recombinant protein production in which there were boundaries of the expensive fermenter and good manufacturing practice (GMP) required for pharmaceutical production. After successful production practices of mammalian IgG1 antibody in tobacco [30] and human serum albumin (HSA) in transgenic potato and tobacco plants [31], the molecular pharming approach rapidly accelerated and production systems like tobacco, potato, tomato, alfalfa, safflower, carrot, lettuce, strawberry, moss, duckweed, maize, wheat and rice were emerged. Besides the plant species, there are various approaches differing in plant tissues (whole plant, hairy roots, cell suspension etc.), expression type (stable, transient, transplastomic, tissue-specific, inducible) and product targeting (post-translational modifications and accumulation targets) [4]. Today, there are thousands of different recombinant proteins produced in plant systems in which we can also include antibody, vaccine, hormone or enzyme type pharmaceuticals. There is also an increasing number of companies producing commercial plant-based therapeutics (**Table 3**). Along with the obvious advantage of pharming in scale-up production, this approach also has a downside on downstream processing (including maintaining product quality, extraction and purification) due to the wide range of plant metabolites.

3.1 Monoclonal antibodies, viral antigens and vaccines

Recombinant monoclonal antibody (mAbs) production in pharming applications has grown rapidly since the first reported IgG1 antibody in transgenic tobacco by

Company Name	Product			References
iBio, Inc	IBIO-201 Prophylaxis of SARS-CoV-2 Spike Protein Fused Lichenase Protein / *Nicotiana benthamiana*	ACE2-FC Prophylaxis of SARS-CoV-2 Human Angiotensin Converting Enzyme 2 (ACE2) Fused To A Human Immunoglobulin G Fc fragment / *Nicotiana benthamiana*	IBIO-400 Prophylaxis of Classical Swine Fever (CSF) CSFV E2 Glycoprotein / *Nicotiana benthamiana*	[32]
Medicago Inc.	MT-7529 Prophylaxis of H7N9 Influenza / *Nicotiana benthamiana*	MT-2355 Prophylaxis Of Pertussis, Diphtheria, Tetanus, Poliomyelitis and Prophylaxis of Hib Infection in Infants / *Nicotiana benthamiana*	MT-2271 Prophylaxis of Seasonal Influenza / *Nicotiana benthamiana*	[33]
	MT-5625 Prophylaxis of Rotavirus Gastroenteritis / *Nicotiana benthamiana*	MT-2766 Prophylaxis of SARS-CoV-2 / *Nicotiana benthamiana*	MT-8972 Prophylaxis of H5N1 Influenza / *Nicotiana benthamiana*	
Icon Genetics	ZMapp Prophylaxis of Ebola virüs / *Nicotiana benthamiana*	Denka Prophylaxis of Norovirus / *Nicotiana benthamiana*	-	[34]
SemBioSys Genetics Inc.	Milano Production of apolipoprotein AI / *Carthamus tinctorius*	SBS-1000 Plant-Produced Insülin / *Carthamus tinctorius*	-	
Protailx	Elelyso Prophylaxis of type 1 Gaucher's disease / *Daucus carota*	PRX 102 Prophylaxis of Fabry disease / *Daucus carota*	PRX-105 Use in treatment of Organophosphorus poisoning / *Daucus carota*	[35]
Wuhan Healthgen Biotechnology Corp.	OsrhLF Recombinant Human Lactoferrin / *Oryza Sativa*	OsrHSA Human Serum Albumin / *Oryza Sativa*	OsrhEGF Human Epidermal Growth Factor / *Oryza Sativa*	[36]
	OsrhbFGF Human Basic Fibroblast Growth Factor / *Oryza Sativa*	rhIGF-1 LR3 Human Insulin-like Growth Factor-1 LR3 / *Oryza Sativa*	OsrhVEGF Human Vascular Endothelial Growth Factor / *Oryza Sativa*	
	OsrhKGF Keratinocyte Growth Factor / *Oryza Sativa*	OsrhLF Human Lactoferrin / *Oryza Sativa*	OsrhFN Human Fibronectin / *Oryza Sativa*	
	OsrhLYZ Human Lysozyme / *Oryza Sativa*	OsrhAAT Human α-1 Antitrypsin / *Oryza Sativa*	-	
Planet Biotechnology Inc.	PBI-220 Immunoadhesin of anthrax / *Nicotiana benthamiana*	(DPP4-Fc) Immunoadhesin of Middle East Respiratory Syndrome (MERS) / *Nicotiana benthamiana*	-	[37]

Company Name	Product			References
Merck KGaA	L1294 Lactoferrin / *Oryza Sativa*	A9731 Albumin / *Oryza Sativa*	L9545 Leukemia Inhibitory Factor / *Oryza Sativa*	[38]
	T3705 Transferrin / *Oryza Sativa*	L1667 Lysozyme / *Oryza Sativa*	616371 Aprotinin / *Nicotiana tabacum*	
	B0939 B Lymphocyte Activating Factor / *Nicotiana tabacum*	T3449 TrypZean® / *Zea Mays*	B0814 Bone Morphogenetic Protein 7 (BMP-7) / *Nicotiana tabacum*	
Angıo-Proteomıe	rAP-0487 Interleukin-12 p40 / *Nicotiana benthamiana*	rAP-2263 Growth Hormone / *Nicotiana benthamiana*	rAP-2375 Myostatin / *Nicotiana benthamiana*	[39]
Abbexa Ltd	abx263080 Bone Morphogenetic protein-7	abx263465 Fibroblast Growth Factor	abx260381 B-Cell-Activating Factor	[40]
SoyMeds, Inc.	soy-mSEB Prophylaxis of Staphylococcal Enterotoxin B / *Glycine max*	-	-	[41]
G+FLAS Life Sciences	The RBD Prophylaxis of SARS-CoV-2 / *Nicotiana benthamiana*	-	-	[42]
Kentucky BioProcessing, Inc.	V-101 Prophylaxis of Seasonal Flu / *Nicotiana benthamiana*	V-201 Prophylaxis of SARS-CoV-2 / *Nicotiana benthamiana*	-	[43]
Ventria Bioscience	VEN100 Prophylaxis of Clostridium Difficile	VEN BETA Prophylaxis of Enterotoxigenic *E. coli* (ETEC) / On Sale	-	[44]
Thermo Fisher Scientific Inc.	A35934 Leukemia Inhibitory Factor / *Hordeum vulgare*	-	-	[45]
ORF Genetics	ISOkine Production of Human Growth Factors & Cytokınes / *Hordeum vulgare*	MESOkine Production of Anima-Like Growth Factors & Cytokınes / *Hordeum vulgare*	DERMOkine EGF (Epidermal Growth Factor) / *Hordeum vulgare*	[46]

Table 3.
Plant-based therapeutic producing companies and commercial products.

Andrew Hiatt [30]. In the beginning, it was not an easy task due to the complicated nature of antibodies on basis of post-translational modifications, folding patterns and structural assembly. IgG is the simplest immunoglobulin structure, therefore only two plant genes are required to produce fully functional IgG in plant systems. On the other hand, IgA which has four heavy chains and four light chains requires the expression of four genes at the same time. Early antibody studies also considered producing antibody fragments, mini bodies, large single chains, single-chain variable fragments (scFvs), bispecific scFvs, diabodies and fusion proteins as well [47]. Some

achievements on targeting the mAbs into different plant cell parts for accumulation followed in a short time. The first plant-derived IgM was produced in *N. tabacum* and targeted into chloroplasts. Constructed chimeric genes and the barley aleurone α-amylase signal peptide coding sequence which had already been used successfully to transport bacteriophage T4 lysozyme from transgenic tobacco cells to the intercellular spaces was introduced to *N. tabacum* to initiate the secretory pathway of chimeric IgM in transgenic plants. Subcellular localization of IgM, presented the assembly of the antibody in the endoplasmic reticulum and the targeted accumulation in chloroplasts. Assembly and targeting of complex foreign protein in the transgenic plant were shown through fusing the individual chains to a plant signal peptide [48]. Secretory IgA was also expressed recombinantly. Four transgenic *N. tabacum* plants were generated that expressed a murine monoclonal antibody kappa chain, a hybrid immunoglobulin A-G heavy chain, a murine joining chain and a rabbit secretory component, respectively. Sexual crosses were achieved among the transgenic tobaccos resulting expression of all four protein chains simultaneously. These chains were assembled into a functional, high molecular weight secretory IgA which recognized the native streptococcal antigen I/II cell surface adhesion molecule. Transgenic plants were suggested as suitable systems for large-scale production of assembled recombinant secretory IgA for passive mucosal immunotherapy since plants require a single cell to assemble secretory antibodies while mammalian cells require two different cell types [49]. Transgenic plant-derived antibodies (plantibodies) are thought to be particularly effective in topical immunotherapies which are based on the antigenic competition by using immunomodulators to induce hypersensitivity. In recent days, mABs are designed for various purposes as chemotherapeutics for cancer, antibody-mediated passive immunization against highly contagious infectious diseases as SARS and COVID-19, curing or slowing down disease progression, active immunization through antigens (**Table 4**).

mABs may aim cancer cells in different mechanisms. They can directly bind and flag cancer cells for immune cells prevent angiogenic properties, stimulate disruption of the cell membrane, block immune system inhibitors, retard cancer growth, act as chemotherapy or radiotherapy agent carrier. Targeted antigens are generally related to growth and differentiation including epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), human epidermal growth factor receptor 2 (HER2), vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR), fibroblast activation protein (FAP). mAbs are not native products of plant tissues. However, the transgenic plant approach provides the capacity to induce production and modification of mAbs through posttranslational modifications as glycosylation to enhance therapeutic efficacy. Pembrolizumab which is an anti-human PD-1 monoclonal mAb has been produced in wild-type *Nicotiana benthamiana* through transient expression. 344.12 ± 98.23 μg/g fresh leaf weight Pembrolizumab accumulation was obtained after 4 days of agroinfiltration. Molecular characterization of plant-based Pembrolizumab was compared to mammalian cell-based commercial counterpart Keytruda®. Physicochemical properties of plant-based Pembrolizumab were found comparable to Keytruda® with similar secondary and tertiary structures. Both products presented no aggregation differences and binding efficacy to PD-1 protein and inhibitory activity between programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) interaction. In this respect, researchers concluded that plant-produced Pembrolizumab could induce IL-2 and IFN-γ production and plant-based production of functional Pembrolizumab can be utilized for immunotherapy purposes [50]. In another anticancer approach, heavy and light chains of mAb BR55-2 were expressed separately and assembled in plant cells of transgenic tobacco plants (*N. tabacum* cv. LAMD609). Production was as high as 30 mg/kg of fresh leaves in the first generation of plants. Like in mammalian counterpart, the Fc

Plant	Target	Protein	Expression system	Transformation method	Efficiency	Purification	References
Nicotiana benthamiana	Cancer	Pembrolizumab (PD-1) monoclonal antibody	Transient Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	344.12 ± 98.23 µg/g FLW	Size Exclusion Chromatographyband protein A Affinity Chromatography, The ÄKTA Pure Fast Protein Liquid Chromatography (FPLC)	[50]
Glycoengineered (ΔXFT) Nicotiana benthamiana	Chikungunya virus (CHIKV)	The Monoclonal Antibody anti-CHIKV	MagnICON-based Transient Expression	*Agrobacterium tumefaciens*-mediated	130 µg/g FLW	Low pH Precipitation and Protein A Chromatography	[62]
Nicotiana benthamiana	Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)	The Monoclonal Antibody CR3022	Transient Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	130 µg/g FLW	Ni affinity Chromatography	[58]
Nicotiana benthamiana	Zika virus	The Monoclonal Antibody 2A10G6	BeYDV-Based Transient Expression	CaMV 35S promoter / -	3–5 g/kg FLW	G column Chromatography	[63]
Nicotiana benthamiana	Hand-Foot-Mouth Disease (HFMD)	The Monoclonal Antibody D5	Transient Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	50 µg/g FLW	0.45 µm Membrane Filter and protein A Chromatography	[64]
Oryza sativa	Porphyromonas gingivalis	MAb specific for FimA (Fimbrial protein fimbrillin)	Nucleer / Stable Expression	Rice Amylase 3D (Ramy3D) promoter / Particle Bombardment	3.44 µg/g FLW	G-affinity Chromatography	[65]
Nicotiana benthamiana	Dengue Virus (DENV)	The Monoclonal Antibody E60	Transient Expression	magnICON® / *Agrobacterium tumefaciens* mediated	0.8–4.8 mg/g FLW	Ammonium Sulfate precipitation and Protein A Affinity Chromatography.	[66]
Nicotiana benthamiana	AIDS (acquired immunodeficiency syndrome)	The Monoclonal Antibody 2G12	Nucleer / Stable Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	7-10 µl/ml Leaf Extract	PDF4 Filter Purification	[67]
Nicotiana tabacum	AIDS (acquired immunodeficiency syndrome)	The Monoclonal Antibody 2G12	Transient Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	-	Protein A Chromatography	[68]

Plant	Target	Protein	Expression system	Transformation method	Efficiency	Purification	References
Zea mays	AIDS (acquired immunodeficiency syndrome)	The Monoclonal Antibody 2F5	Transient Expression	CaMV 35S promoter/ Particle Bombardment	0.61 ± 0.28 μg/ ml Seed extract	-	[69]
Nicotiana tabacum	AIDS (acquired immunodeficiency syndrome)	The Monoclonal Antibody 4E10	Nucleer / Stable Expression	CaMV 35S promoter / *Agrobacterium rhizogenes*-mediated	10.43 RDW	-	[70]
Nicotiana benthamiana	West Nile virus (WNV)	The Monoclonal Antibody pHu-E16	(TMV)-based Transient Expression	*Agrobacterium tumefaciens*-mediated	0.8 mg/g FLW	Ammonium Sulfate Precipitation and Protein A Chromatography	[71]
Nicotiana tabacum	Hepatitis B Virus	Anti-HBsAg Monoclonal Antibody	Nucleer / Stable Expression	Phaseolin promoter	6.5 mg/g of Seed	Protein A Affinity Chromatography, Size-exclusion chromatography (SEC) and High-performance liquid chromatography (HPLC)	[72]
Nicotiana tabacum	Rabies Virus (RABV)	The Monoclonal Antibody E559	Nucleer / Stable Expression	-	1.8 mg/kg FLW	Protein A Chromatography	[73]
Lactuca sativa	Ebola and West Nile viruses	The Monoclonal Antibodies 6D8 and hE16	MagnICON-based Transient Expression	*Agrobacterium tumefaciens*-mediated	0.27 mg/g	DEAE anion-exchange chromatography with DEAE Sepharose FF 26/20 resin	[74]
Nicotiana tabacum	Streptococcus-mediated dental caries	The Monoclonal Antibody Guy's 13	Nucleer / Stable Expression	CaMV 35S promoter / *Agrobacterium rhizogenes*-mediated	58 μg/g RDW	Affinity Chromatography	[70]
Nicotiana tabacum	Cancer	The Monoclonal Antibody BR55-2	Nucleer / Stable Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	30 mg/kg FLW	Protein A Chromatography	[51]

Table 4.
Monoclonal antibodies produced from transgenic plants.

domain of the plant mAbP presented a similar binding to FcγRI receptor (CD64) and bound to both SK-BR3 breast cancer cells and SW948 colorectal cancer cells, specifically. This plant-derived BR55-2 also inhibited SW948 tumor growth in nude mice, efficiently and was suggested as a possible immunotherapy option [51]. Both samples represent IgG-type plant-derived antibodies. However, more complex IgA-type plant-derived products are also presented with better efficacy, recently. The secretory component (SC) of immunoglobulin A (SIgA), which is an efficient therapeutic antibody against mucosal pathogens, was successfully expressed in *A. thaliana*. The expression level of SC was increased in the plant system through the insertion of endoplasmic reticulum retention signal peptide, KDEL (Lys-Asp-Glu-Leu), into a binary vector with translational enhancer and an efficient terminator [52]. This approach was also reported as useful against food poisoning causing *E. coli* virulence factor Shiga toxin through recombinant IgA (S-hyIgA) produced in transgenic *A. thaliana* plants [53].

Plant-derived antibodies and viral antigens were also targeted for highly contagious infectious diseases as SARS, Ebola, Zica, Hepatitis B, AIDS and even the most recent COVID-19 (**Tables 5** and **6**). SARS-CoV-2 is a single-stranded RNA-enveloped virus, which has 29,881 bp genome encoding 9860 amino acids belonging to structural proteins, spike (S), envelope (E), membrane (M) and nucleocapsid (N) and 16 non-structural proteins (such as 3-chymotrypsin-like protease, papain-like protease and RNA-dependent RNA polymerase) and 9 accessory proteins. N protein which is the most abundant viral protein shown to be highly conserved in CoV families. It is highly immunogenic during CoV infections. It is a major target for antibody responses and also contains T cell epitopes [54, 55]. Post-transcriptional gene silencing suppressor p19 protein from tomato bushy stunt virus substantially demonstrated the transient expression of recombinant SARS-CoV nucleocapsid (rN) protein in *Nicotiana benthamiana*. The rN protein accumulated up to a concentration of 79 μg per g fresh leaf weight in the agrobacteria-infiltrated plant leaf after the third day of infiltration. BALB/c mice were intraperitoneally vaccinated with pre-treated plant extract emulsified in Freund's adjuvant and plant-expressed recombinant SARS-CoV N protein-induced strong humoral and cellular responses in mice [56]. Also, iBio company developed a plant-derived vaccine targeting the N protein in their IBIO-202 program which is under pre-clinical trials. M and E proteins contribute very low on protection owing to their small ectodomains for immune cell recognition and small molecular sizes and poorly immunogenic for humoral responses. Glycosylated S proteins cover the SARS-CoV-2 surface and bind to the host cell receptor angiotensin-converting enzyme 2 (ACE2) during viral cell entry. Hence, S protein is the main viable vaccine target against the ongoing pandemic for the time being [57]. Rapid production of SARS-CoV-2 receptor-binding domain (RBD) and spike-specific monoclonal antibody CR3022 were achieved in *Nicotiana benthamiana*. Both RBD and mAb CR3022 were transiently produced with the highest expression level of 8 μg/g and 130 μg/g leaf fresh weight respectively at 3 days post-infiltration. The plant-produced RBD exhibited specific binding to the SARS-CoV-2 receptor, angiotensin-converting enzyme 2 (ACE2) [58]. In July 2020, phase 1 study was initiated for a plant-derived virus-like particle (VLP) vaccine candidate called CoVLP which expresses the SARS-CoV-2 spike glycoprotein (CoVLP: NCT04450004). It has been evaluated through 21 days apart from two doses of 3.75 μg, 7.5 μg or 15 μg vaccine alone or with AS03 or CpG1018 adjuvants in healthy adults 18–55 years of age. Based on the available data two-dose schedule of CoVLP at 3.75 μg per dose adjuvanted with AS03 has been carried forward into ongoing phase 2/3 studies in Canada and the United States, with planned expansion to additional countries in Latin America and Europe [59]. In December 2020, the Kentucky BioProcessing company announced its phase 1 plant-derived

Plant	Disease	Antigent	Expression system	Transformation method	Efficiency	immunization	İmmun responce	References
Nicotiana Tabacum / Lactuca sativa	type 1 diabetes	human proinsulin (hpINS) fusion protein with CTB	Chloroplast / Stable Expression	psbA promoter / biolistics	3.33–15.3 mg/g CTB-hpINS (12.9–24.4 of TSP)	Oral / 250-500 μg	Decrease IL-10	[75]
Spinacia oleracea /	Type-2 diabetes (T1D)	glutamic acid decarboxylase (GAD65)	Nucleer / magnICON® Based Transient Expression	BAK/ *Agrobacterium tumefaciens* mediated	SO: 544 μg/g FW	-	-	[76]
Beta vulgaris	Type-2 diabetes (T1D)	glutamic acid decarboxylase (GAD65)	Nucleer / magnICON® Based Transient Expression	BAK/ *Agrobacterium tumefaciens* mediated	BV: 113 μg/g FW	-	-	[76]
Nicotiana tabacum	Parkinson's disease (PD)	LTB-Syn chimeric protein	Nucleer/ Stable Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	0.27 μg/g	İntraperitoneal / 10 μg	Induced IgG	[77]
Oryza sativa	Allergic Asthma	*Dermatophagoides pteronyssinus* allergen (Der p 1)	Nucleer/ Stable Expression	GluB-1 promoter / *Agrobacterium tumefaciens* mediated	7.5% TSP	Oral / 0.5 or 5 mg, intraperitoneal / 6.8 mg	Decrease IgE IgG2a and IgG2b, inhibition of IL-4, IL-5, and IL-13	[78]
Lycopersicon esculentum	Alzheimer's disease,	Human β-amyloid Aβ	Nucleer/ Stable Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	0.7% TSP	Oral / 5 g	NA	[79]
Oryza sativa	Pollen Allergy	The T cell epitope peptides of Cry j I and Cry j II	Nucleer/ Stable Expression	GluB-1 promoter / *Agrobacterium tumefaciens* mediated	0.5% TSP	Oral / 70 μg	Decrease IgE IgG2a and IgG2b, inhibition of IL-4, IL-5, and IL-13	[80]

Table 5.
Plant-based vaccines for non-infectious diseases.

Plant	Pathogen/Disease	Antigent	Expression system	Efficiency	Adjuvant	Immunization	Immun responce	References
Nicotiana benthamiana	SARS-CoV	SARS-CoV nucleocapsid (rN) protein	Nucleer / Transient Expression	0.8–1% of the TSP	With Complete Freund's Adjuvant/ Incomplete Freund's Adjuvant	İntraperitoneal / 500mg fresh leaves	Induced IgG1 and IgG2/ Increase IFN-_ and IL-10/ not chanced IL-2 and IL-4	[56]
Nicotiana tabacum	Ebola virus (EBOV)	Envelope-Associated Protein VP40	Nucleer / Stable Expression	2.6 µg/g FW	With Complete Freund's Adjuvant / Incomplete Freund's Adjuvant	Ebible (25 ng) / Subcutaneous (125 ng)	Induced IgM, IgG and intestinal IgA	[81]
Nicotiana benthamiana	Zika virus (ZIKV)	Envelope (E) Protein	Nucleer / Transient Expression	160 µg/g FW	With Aluminium Hydroxide Gel Adjjuvant	Subcutaneous / 50 µg x 24	Induced IgG1 and IgG2, Increase IFN-γ, IL-4 and IL-6	[82]
Oryza sativa	Vibrio cholerae	Cholera Toxin B-Subunit	Nucleer / Stable Expression	NA	Witout Adjuvant	Edible / 150 mg seed	induced IgG and mucosal IgA	[83]
Nicotiana benthamiana	Influenza A H1N1	Soluble Protein H1/ H1-VLP	Nucleer / Transient Expression	NA	-	-	Induced CD4+ and CD8+ T cells	[84]
Nicotiana benthamiana	Influenza A H5N1	The matrix protein 2 ectodomain (M2e) fused to N-terminal proline-rich domain (Zera®) of the γ-zein protein of maize	Nucleer / Transient Expression	125–205 mg/ kg FW	Without Adjuvant	İntramuscular / 4.5 µg	Induced IgG	[85]
Nicotiana tabacum / Lycopersicon esculentum	Yersinia pestis / Plague	The major capsular protein F1-V antigen fused	Nucleer NT:Transient Expression / LE:Stable Expression	NT: 1–4% FW LE: 4–10% mg DW	With Adjuvant NT: Aluminum Hydroxide T / LE: Cholera Toxin	NT: Subcutaneous (10µg purified) / LE: Edible (2 g fruit)	Induced Serum IgG1, IgG2a and mucosal IgA	[86]

Plant	Pathogen/Disease	Antigent	Expression system	Efficiency	Adjuvant	Immunization	Immun responce	References
Nicotiana benthamiana	Flavivirus / Yellow fever (YF)	YF virus envelope protein (YFE) fusion to the bacterial enzyme lichenase (YFE-LicKM)	Nucleer / Transient Expression	NA	With Alhydrogel Adjuvant	İntramuscular 5 µg x3/ 5 µg x 2 / 30 µg x 3	Induced IgG İncreased IFNγ	[87]

FW: Fresh Weight, TSP: Total Soluble Protein, NA: Not Available, DW: Dry Weight. NT: Nicotiana tabacum, LE: Lycopersicon esculentum. CaMV: Cauliflower Mosaic Virus

Table 6.
Plant-based vaccines for pandemics and epidemics infectious diseases.

cVLP vaccine. CPG adjuvant vaccine was administered in healthy adult subjects in two age groups, Part A (18–49 years) and Part B (50–85 years). The company also produces seasonal influenza vaccines through *Nicotiana benthamiana* plants [60]. BaiyaPharming™ produced a subunit-based plant-derived vaccine against SARS-CoV-2 in *N. benthamiana*. Baiya SARS-CoV-2 Vax 1 was chosen between six candidates which showed better immunogenicity in mice and monkeys. Baiya Phytopharm expects to initiate human trials by September 2021 [61]. Plant-derived vaccines for humans and animals stand out as a viable alternative that can be used to overcome the barriers of conventional vaccines. Within the scope of transgenic plants, it is possible to produce cost-effective, immunogenic and safer vaccines in plants with an enhanced amount, effective isolation and purification methods. Correct use of adjuvants along with the production of recombinant vaccine antigens also seems equally crucial for the future of this technology.

3.2 Replacement human proteins

Plants are usually referred to as molecular factories to provide humans with many useful molecules for many purposes. In the last decades, it has also become available to produce specific heterologous proteins as a replacement in humans. In this manner, the first plant-derived pharmaceutical was human growth hormone, which was expressed in transgenic tobacco as a fusion with *Agrobacterium* nopaline synthase enzyme in 1986 [88]. Today, recombinant human proteins are a considerable part of FDA-approved biotechnological drugs and recombinant plant-derived proteins are extended in many categories as industrial enzymes, research intended technical proteins, nutritional supplements and polymers as well as antibodies and vaccines which were mentioned in the previous section. Replacement human proteins include products as growth hormone, HSA, α-interferon, erythropoietin (EPO), human secreted alkaline phosphatase, aprotinin, collagen, α1-antitrypsin and more (**Table 7**).

Human growth hormone (hGH) has various biological functions on protein synthesis, cell proliferation and metabolism. After the first successful plant-derived production, many different strategies were achieved. In a recent approach, a synthetic hGH gene (shGH) has been synthesized in a plant expression vector under the control of the rice amylase 3D (Ramy3D) promoter. The plant expression vector was introduced into rice calli (*O. sativa* L.) via the particle bombardment transformation method. The shGH protein expression was verified and quantified as 57 mg/L in the transgenic rice cell suspension medium. Biological activities of the shGH were found similar to the conventional *E. coli*-derived recombinant hGH. Likewise, many different plant tissues and expression systems are suggested as effective hGH production replacements [89].

Human serum albumin (HSA) is the most abundant protein in human blood plasma. HSA is a soluble, globular, unglycosylated, monomeric multidomain protein. The single polypeptide of HSA consists of 585 amino acids with a range of structural configurations that fold into three helical domains [90]. It is also the first full-size native human protein expressed in plants and there is more than 500 tons annual demand. The transient expression level of the HSA gene in different genotypes was achieved in many plants. Recently, the A. tumefaciens strains LB4404 and GV3101 containing pBI121-HSA binary vector were infiltered in *Nicotiana benthamiana* and *N. tabacum* varieties. The bioactive HAS expression in tobacco leaves through the expression of the HSA gene in the plant system is suggested as the first transient expression success in literature [91].

α-interferon is the first human pharmaceutical protein produced in rice. The plasmid pIG3031 containing human α-interferon cDNA and the neomycin

Plant	Disease	Protein	Expression system	Transformation method	Efficiency	Purification	References
Nicotiana benthamiana	Osteogenic differentiation	Human Dentin Matrix Protein 1 (hDMP1)	Transient Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	0.3 μg/g FW	Ni affinity chromatography	[97]
Nicotiana benthamiana	In The Diagnosis Of Rift Valley Fever Virus (RVFV)	Nucleocapsid Protein (N-protein)	Nucleer/ Stable Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	500–558 mg/kg FLW	Ammonium Sulphate Precipitation, Nickel Affinity Column Chromatography and 6xhis-Tag Affinity Chromatography	[98]
Nicotiana tabacum	Anemia	Erythropoietin (rhEPO)	Nucleer/ Stable Expression	CaMV 35S promoter/ *Agrobacterium rhizogenes*-mediated	66.75 pg/mg medium TSP	Sephadex 25 columns, Ni-TED columns, HPLC	[99]
Nicotiana benthamiana	Dental Bone Regeneration	Osteopontin (hOPN)	Transient Expression	CaMV 35S promoter / *Agrobacterium tumefaciens*-mediated	100 ng/g FW	Ni affinity chromatography	[100]
Physcomitrella patens	Fabry disease	α-galactosidase A	Nucleer / Stable Expression	PEG-based	0.5 mg/ml TSP	Butyl-650 M, DEAE, S chromatography	[101]
Nicotiana benthamiana	Gaucher's disease (GCase)	Human Glucocerebrosidase	Nucleer/ Stable Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	68 μg/g FW	Concanavalin A (Con A) Agarose Column and Hydrophobic Interaction Chromatography (Phenyl-650C)	[102]
Nicotiana tabacum	Pompe disease	Acid Alpha Glucosidase (GAA)	Chloroplasts / Stable Expression	psbA promoter / Biolistic Mediated	190 μg/g of DW	-	[103]
Salvia miltiorrhiza	Angiogenesis, and Tissue Repair	Human acidic fibroblast growth factor 1 (FGF-1)	Nucleer/ Stable Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	272 ng/g FW	-	[104]

Plant	Disease	Protein	Expression system	Transformation method	Efficiency	Purification	References
Helianthus annuus	Cardiovascular and Cerebrovascular Thrombus Diseases	Lumbrokinase (LK)	Nucleer/ Stable Expression	napA promoter / *Agrobacterium tumefaciens*-mediated	5.1 g/kg of Seed	Nothing	[105]
Nicotiana tabacum	Drug Carrier	Hydrophobin	Nucleer/ Stable Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	16.5% of TSP	Surfactant-Based Aqueous Two-Phase Separation	[106]
Nicotiana benthamiana	Anthrax	Anthrax Receptor Decoy Protein (immunoadhesin), CMG2-Fc	Transient Expression	CaMV 35S promoter / *Agrobacterium tumefaciens*-mediated	1.5% of TSP	-	[107]
Nicotiana benthamiana	Cardiopulmonary Bypass	r-aprotinin	GENEWARE® mediated Transient Expression	CP promoter / *Agrobacterium tumefaciens*-mediated	25 mg/kg FW	Size-exclusion chromatography (SEC) and High-performance liquid chromatography (HPLC)	[108]
Oryza sativa	Dwarfism, bone fractures, skin burns, and bleeding ulcers	Human growth hormone (hGH)	Nucleer/ Stable Expression	Rice Amylase 3D (Ramy3D) promoter / Particle Bombardment Transformation	57 mg/l medium TSP	-	[89]
Glycine max	Cardiovascular and Neurodegenerative Diseases	Fibroblast Growth Factor (Bfgf)	Nucleer/ Stable Expression	G1 (glycinin) promoter / *Agrobacterium tumefaciens*-mediated	2.3% of TSP	heparin-Sepharose CL-6B affinity chromatography	[109]
Nicotiana tabacum	Marker Used In Diagnosis	Placental Alkaline Phosphatase (SEAP)	Nucleer/ Stable Expression	CaMV 35S promoter/ *Agrobacterium tumefaciens*-mediated	3% of TSP	-	[110]

Table 7.
Replacement human proteins produced from transgenic plants.

phosphotransferase II coding sequence was introduced to Indica rice protoplasts via lipofection-mediated transformation. Transgenic plants were regenerated from transformed calli. Extracts of transgenic cell cultures and plants presented apparent interferon activity proven by the resistance of human amniotic cell lines to viral infection in the presence of plant extracts. This production encouraged many other strategies as human α-interferon cDNA was correctly expressed in rice cells [92].

Erythropoietin (EPO) is the first human replacement protein produced in tobacco suspension cells. EPO is a cytokine that regulates and maintains the physiological level of circulating erythrocytes. The survival of erythroid precursor cells is also achieved through EPO. It stimulates the proliferation and differentiation of the precursor cells by plasma membrane EPO-receptor interactions. The first transgenic approach introduced human Epo cDNA via *A. tumefaciens*-mediated gene transfer to tobacco BY2 cells (*N. tabacum* L. cv. Bright Yellow 2). EPO is a heavily glycosylated protein, therefore glycosylation of tobacco-derived EPO by smaller oligosaccharides led the molecule to remain attached to the cell wall. However, it induced the differentiation and proliferation of erythroid cells in in vitro biological activity trials [93]. Recently, mammalian cell-derived recombinant human erythropoietin (rhuEPOM) is a multimodal neuroprotectant in experimental stroke models. However, the rhuEPOM clinical trials were terminated due to the increased risk of thrombosis, largely ascribed to its erythropoietic function. A rhuEPO derivative without sialic acid residues was produced in a plant-based expression system which is lacking sialylation capacity to produce asialo-rhuEPO^{P}. Repeated intravenous injection (44 μg/kg bw) in mice presented no increase in hemoglobin levels and red blood cells. Hence, Asialo-rhuEPO^{P} that lacks erythropoietic activity and immunogenicity suggested as a great multimodal neuroprotectant for stroke treatment [94].

Pathogen contamination risk of animal-derived collagen initiated the need for safe recombinant production of this complex molecule. Collagen is the first human structural-protein polymer produced in a plant-derived system. The use of the tobacco plant as a novel expression system for the production of human homotrimeric collagen I was achieved in 2000. cDNA encoding the human proalpha1(I) chain was introduced to tobacco. Expressed recombinant procollagen has been folded to stable homotrimeric triple helix-shaped collagen as in animal cells [95]. *A. tumefaciens*-mediated transient expression of the recombinant hydroxylated homotrimeric collagen in tobacco plants that are co-transformed with a human type I collagen and a chimeric proline-4-hydroxylase (P4H) improved the quality of collagen by enhancing thermostability to 37°C [96].

Human 1-antitrypsin (AAT) is a 394-amino-acid glycoprotein that inhibits the activity of the serine protease neutrophil elastase. Healthy individuals control elastase activity by producing sufficient quantities of AAT into the bloodstream. When the circulating concentration drops below 15%, various diseases as emphysema, hepatitis and skin disorders occur. Various expression systems, cell types and tissues of plants were used for molecular pharming earlier. However, rice suspension cells were used for the molecular farming of recombinant human 1-antitrypsin (rAAT) in biologically active form for the first time in 1999. Transformation of rice callus tissues with a p3D-AAT expression vector containing the cDNA for mature human AAT protein was achieved. The promoter, signal peptide and terminator of a rice-amylase gene Amy3D, which tightly controls simple sugars such as sucrose, regulated expression and secretion of rAAT. Expression of the rAAT was initiated by removing sucrose from the cultured media or by allowing the rice suspension cells to deplete sucrose catabolically. For that time being, the rice cell culture system clearly contributed to the molecular pharming field.

As a concluding summary, plants have various upsides against traditional microbial and animal cell culture systems in respect to molecular pharming. Some of

these include cost efficiency, easier up-scale production, absence of human pathogens and accurate maturation (folding and assembly) of proteins. The potential of molecular pharming in plants attracts more and more entrepreneurs following the numerous successful products and companies. Achieving secretory properties and targeting of plant products also surpass many other production systems. The use of plants as bioreactors is well known, and its applications are increasing for both recombinant protein expression and recombinant pharmaceutical production. This chapter showcases the various plant biotechnology application and strategies as applied in the production of recombinant antibodies, antigens, therapeutic proteins and enzymes, that are used in the treatment of various diseases. Now with the COVID-19 pandemic, more than ever this approach is taking center stage. This is so important as it will reveal the hidden treasure that transgenic plants already offered but mired in the genetically modified organism debate and therefore rejected before these other applications beyond food could be readily realized. Indeed, different systems exist within the system utilizing many different parts and tissues of the plant to produce products. Furthermore, this system is more advantageous in a eukaryotic system that performs post-translational modification as would animal and yeast cells thus yielding the final desired therapeutic product comparable to what is already produced by humans, for example.

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