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A review

Plant Molecular Farming: Evolution of Genetic Engineering Strategies to Overcome Glycosylation Challenges in Therapeutic Protein Production

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Abstract:

Plant Molecular Farming (PMF) represents an innovative and promising approach for producing therapeutic and commercial proteins using genetically modified plants as bio-factories. Despite the economic and safety advantages offered by this technique compared to traditional production systems, the disparity in glycosylation patterns between plants and mammals constitutes a major obstacle limiting the clinical application of plant-produced proteins. This article reviews the development of plant molecular farming, highlights the competitive advantages of this production platform, a with focus on challenges associated with post-translational modifications, particularly glycosylation. The article also discusses modern genetic engineering strategies, including employment of CRISPR/Cas9 technology for multiplex gene targeting, aimed at producing Cas9-free, homozygous plant lines capable of synthesizing recombinant proteins with fully human-like glycan compositions. This review provides a comprehensive analysis of current developments in this field, and highlights future prospects for plant molecular farming as a sustainable, regulatory-compliant platform for producing the next generation of biopharmaceuticals.

Keywords: plant molecular farming, recombinant proteins, glycosylation, CRISPR/Cas9, biopharmaceuticals

1.Introduction

Plant Molecular Farming represents one of the most prominent contemporary applications of plant biotechnology, where genetically modified plants are employed as green bio-factories to produce therapeutic proteins, antibodies, vaccines, and industrial enzymes of high medical and commercial value [1]. The concept dates back to the late 1980s and has witnessed accelerated development thanks to advances in genetic engineering and understanding of the molecular mechanisms regulating gene expression in plants. Plant molecular farming involves introducing foreign genes into plants to produce specific proteins, capitalizing on their ability to perform photosynthesis and self-growth at low cost compared to traditional production systems, such as bacterial bioreactors or mammalian cell cultures [2]. This field is gaining increasing importance in light of the global need to enhance the efficiency of biological drug production and reduce their costs, particularly in developing countries.

Therapeutic proteins have constituted a qualitative leap in the history of modern medicine; since recombinant insulin provided treatment for millions of diabetic patients, through clotting factors that restored hope for hemophilia patients, to monoclonal antibodies that revolutionized the treatment of cancer and autoimmune diseases, these biological molecules are no longer a scientific luxury but have become a

fundamental pillar of contemporary healthcare [3]. However, this exceptional therapeutic success faces a major economic challenge; production costs using traditional systems remain exorbitant, making them

unattainable for the vast majority patients in developing countries, and impose a heavy financial burden even on advanced healthcare systems [4]. This stark disparity between therapeutic efficacy and economic cost places researchers before an urgent ethical and scientific responsibility: the search for alternative production platforms that combine efficiency and economic viability.

Here, Plant molecular farming emerges as one of the most promising solutions, as plants offer unique advantages not found in any other production system; they rely on sunlight and cheap natural resources instead of expensive culture media, production scale can be simply expanded by increasing cultivated areas without massive infrastructure investments, and they provide a safe environment for producing complex proteins due to their inability to transmit human pathogens [5]. These combined advantages make Plant molecular farming an ideal candidate for reducing production costs compared to traditional systems, which could contribute to transforming life-saving drugs from an elite commodity into an accessible health service for all [6].

However, the path to achieving this promise was not entirely paved; researchers soon discovered a subtle yet critically important molecular challenge: the difference in glycosylation patterns between plants and mammals. Glycosylation, the process of adding sugar chains to proteins after their synthesis critically affects protein stability, therapeutic efficacy, circulatory half-life, and most importantly, its immunogenicity [7]. When human proteins are produced in plants, they acquire specific plant sugar decorations (β-1,2-xylose and core α-1,3-fucose) that may be recognized by antibodies in the human body, neutralizing their therapeutic efficacy and inducing unwanted immune responses [7]. This problem formed the core challenge that preoccupied researchers for three decades and drove the development of advanced genetic engineering strategies to "humanize" post-translational modification pathways in plants .With the advent of the era of precise gene editing, specifically the CRISPR/Cas9 technique, Plant molecular farming entered an entirely new phase .This technology enabled targeting and modifying multiple genes simultaneously, allowing researchers to disable genes responsible for undesirable plant glycosylation and introduce human genes to synthesize biologically compatible glycan patterns [8]. These efforts yielded successive achievements, culminating in 2025 with the successful simultaneous editing of seven genes responsible for plant glycosylation (five FucT genes and two XylT genes) in *Nicotiana benthamiana* tobacco plants, and the production of 12 independent, Cas9-free, homozygous lines, with confirmation of enzymatic activity loss and absence of negative morphological effects compared to wild-type plants [9]. These engineered lines represent the first *N. benthamiana* plants completely free of Cas9 and modified in all seven glycosyltransferase enzyme sites, providing a stable and versatile genetic platform for future production of recombinant therapeutic proteins. In parallel, complementary strategies were developed targeting the ALG3 and GNTI genes, enabling the production of highly uniform tri-mannose glycans, and two pharmaceutical models (Varlilumab and β-glucocere-brosidase) were successfully produced in these modified plants [10]. Recent studies have also demonstrated the possibility of using DNA-free editing systems such as ErCas12a RNP to achieve editing efficiency of up to 95.3% in *N. benthamiana* plants without the need to introduce foreign genes [11].

In this context, this scientific review aims to survey the historical development of Plant molecular farming and analyze the competitive advantages that make it a promising platform for therapeutic protein production, while dissecting the glycosylation problem at the molecular level and explaining the impact of structural differences between plant and animal glycans on the immunogenicity and therapeutic efficacy of produced proteins. It also reviews the genetic engineering strategies used to overcome these challenges, particularly the latest achievements in multiplex gene editing using CRISPR/Cas9, discusses the existing technical and regulatory challenges and the impact of recent legislation on the future of this field, and finally envisions the future prospects of plant molecular farming by analyzing emerging research trendsartificial intelligence and synthetic biology technologies, and evaluating the potential of integrating them into developing more efficient and flexible production platforms.

The ultimate goal of this review is to draw a comprehensive picture of the reality and prospects of this promising technology, which possesses the real potential to bring about a radical change in the way biopharmaceuticals are produced. With the increasing demand for rapid responses, to health emergencies is escalating, and the gap between medical progress and equitable distribution of its benefits is widening, plant molecular farming emerges as one of the solutions capable of achieving the desired balance between quality and cost, between innovation and sustainability, and between scientific progress and human justice.

Methodology

This study is a narrative review that aims to sumarize and critically discuss the current literature on glycol-engineering in plant-based expression systems. Unlike a systematic review, this work does not follow PRISMA

guidelines but provides a comprehensive syn-thesis of published evidence from peer-reviewed studies.

Search Strategy

A literature search was conducted using scopus, PubMed, Web of Science, and Google Scholar. Articles published between 2002 and 2026 have been considered. The search rent combinations of the Keywords: plant molecular farming, recombinant proteins, glycosylation, CRISPR/Cas9, biopharmaceuticals. Only peer-reviewed articles published in English were included in this review, encompassing both original research articles and review papers related to plant glycol-engineering and the production of therapeutic proteins. Conference abstracts, duplicate publications, non-English articles, and studies that were not relevant to the scope of this review were excluded.

Scientific basis and techniques used

Molecular agriculture is based on the principles of plant genetic engineering, whereby the gene responsible for producing the desired protein is isolated from its original source, whether human, animal or microbial, and then integrated into suitable plant expression vectors (Figure 1). The main techniques used include genetic transformation using Agrobacterium tumefaciens bacteria, gene bombardment with micro-particles, and protoplast-mediated transformation [12].

Successful gene expression in plants requires the selection of strong and appropriate promoters, intra-cellular targeting signals to direct the protein to the appropriate organelles such as the endoplasmic reticulum or chloroplasts, and regulatory sequences to ensure the stability of the mRNA and the protein product[13]. Expression can occur in the whole plant or in specific organs such as leaves, seeds, roots, or even in suspended plant cell cultures.

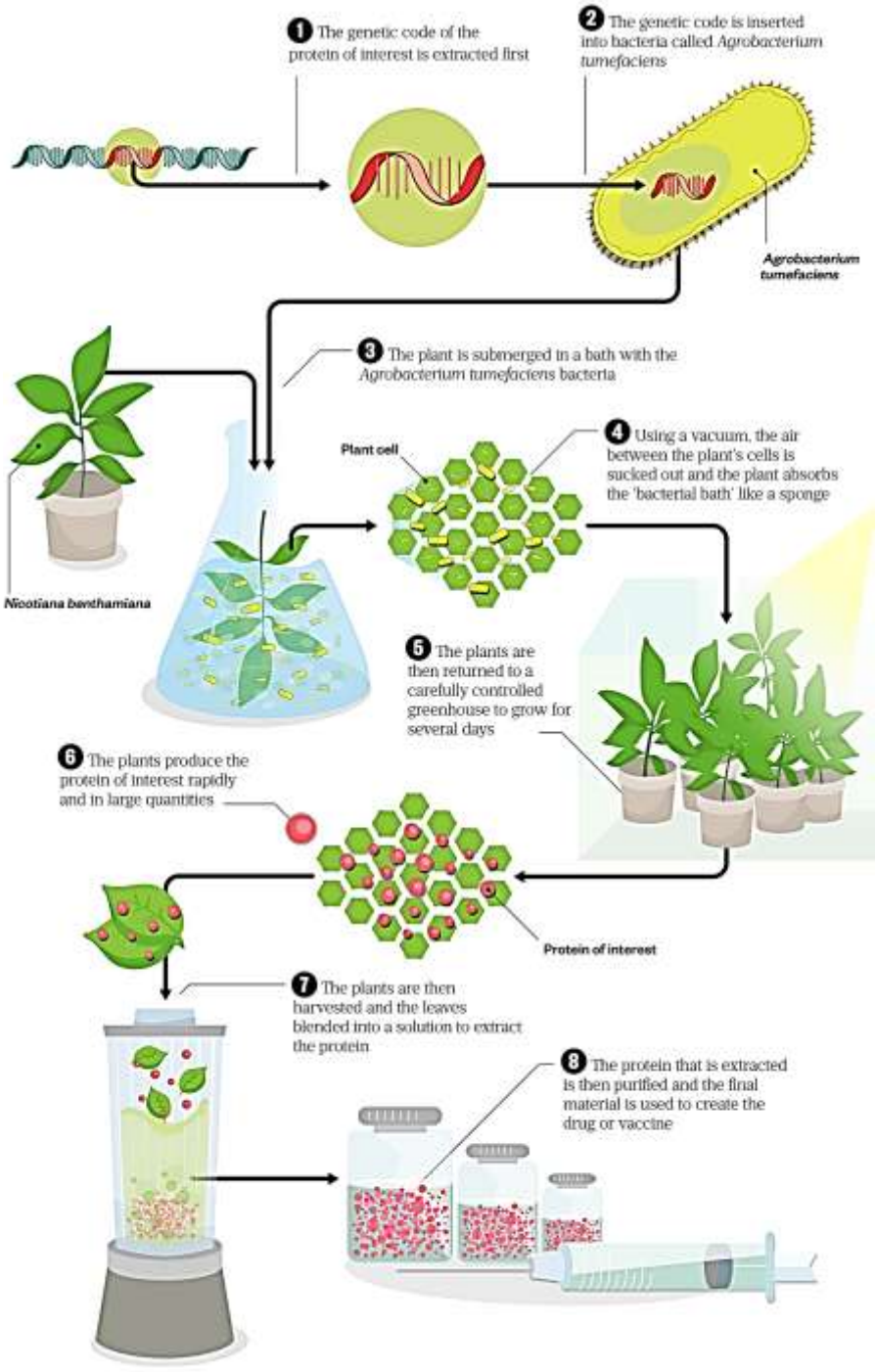

Figure 1. Plant-based production of recombinant proteins.

Competitive Advantages of Plant Molecular Farming

Plant molecular farming is characterized by a set of competitive advantages that make it an attractive alternative to traditional production systems (bacterial, yeast, and animal) [14]. These advantages can be summarized as follows:

Economic Efficiency

Low cost represents one of the most important drivers for adopting plant molecular farming. Plants rely on photosynthesis to generate energy required for their growth, eliminating the need for expensive culture media required by bacterial or cell fermentation systems [14].

are produced. As the need for rapid responses continues to grow, to health emergencies is escalating, and the gap between medical progress and equitable distribution of its benefits is widening, plant molecular farming emerges as one of the solutions capable of achieving the desired balance between quality and cost, between innovation and sustainability, and between scientific progress and human justice.

Moreover, the infrastructure required for plant cultivation is significantly less expensive compared to sophisticated bioreactors, whose construction cost may reach hundreds of millions of dollars. This economic dimension is particularly important for producing biopharmaceuticals for developing country markets, where high prices constitute a major barrier to patient access to treatments [15]. Additionally, whole plants or their parts can be used as natural storage media, reducing storage and transportation costs. Studies have shown that genetically modified seeds can store recombinant proteins for years at room temperature without significant loss of activity [16].

Biosafety

Plant systems exhibit lower risks of contamination with human pathogens compared to animal systems, as these pathogens-such as prions and animal viruses cannot replicate efficiently within plant cells [17]. This grants plants an inherent biosafety advantage and reduces purification costs and safety testing required for pharmaceutical products[18].

Furthermore, plants do not produce endotoxins like Gram-negative bacteria, simplifying purification procedures and reducing costs [19]. Additionally, produced proteins can be directed to specific plant parts (such as seeds or fruits) to facilitate their isolation and reduce contamination with other plant compounds.

Scalability and Operational Flexibility

Plant production scale can be easily expanded by increasing cultivated areas, without need for massive investments in fermentation infrastructure [20]. The diversity of available plant systems allows great flexibility in designing production processes:

·**Field crops:** Such as cereals (maize, rice, wheat) and tobacco, suitable for large-scale production

·**Aquatic plants:** Such as algae, providing rapid production in controlled environments

·**Plant cell cultures:** Enable precise control over production conditions and comply with Good Manufacturing Practice (GMP) regulations [21].

·**Transient expression systems**: Allow rapid protein production within days or weeks, making them ideal for responding to health emergencies like pandemics [22].

Post-Translational Modifications

Plants possess the ability to perform post-translational modifications that ensure proper folding and maintain structural and functional integrity of recombinant proteins [23]. This advantage makes plants superior to bacterial systems that lack these capabilities, and safer than animal systems that may transmit pathogens.

Scope of Therapeutic Products

Biological drug production tops the list of Plant molecular farming applications, with successful trials in the production of human hormones such as insulin and growth hormone, blood clotting factors for the treatment of haemophilia, and therapeutic enzymes for lysosomal storage diseases. Monoclonal antibodies produced in plants, known as "plantibodies," represent a promising area for the treatment of cancer, autoimmune diseases, and infectious diseases [12].

In the field of vaccines, Plant molecular farming has proven effective in producing edible vaccines against diseases such as viral hepatitis, cholera, and influenza. These vaccines have the significant advantage of being easy to store and transport without the need for refrigeration, and can be administered orally without injection, making them suitable for developing countries with limited health infrastructure [24].

Challenges in Plant Production:
The Glycosylation Issue

Despite these competitive advantages, plant expression systems face technical challenges limiting their widespread use in therapeutic protein production. Foremost among these is the difference in glycosylation processes between plants and mammals [25].

Concept of Glycosylation and Its Functional Importance

Glycosylation is a post-translational modification involving covalent addition of sugar chains (glycans) to specific amino acid residues in proteins [26]. These covalent additions affect critical aspects of protein function:

·**Protein folding:** Sugar chains help guide proper protein folding and stabilize its three-dimensional structure [27].

·**Stability:** Increase thermal stability and protect against proteolytic degradation [28].

·**Half-life:** Extend functional lifespan of protein in circulation by preventing renal clearance [29].

·**Biological activity:** Directly affect therapeutic protein efficacy and receptor binding ability [30].

·**Cellular signaling**: Participate in cellular recognition, intercellular interactions, and immune responses [31].This modification is classified into two main types [32].

N-glycosylation: Where glycan binds covalently to nitrogen atom of asparagine (Asn) residues within a specific sequence (Asn-X-Ser/Thr). This process follows a complex biochemical pathway beginning in the endoplasmic reticulum and ending in the Golgi apparatus.

O-glycosylation: Where sugar molecule is added to the hydroxyl group of serine (Ser) or threonine (Thr) residues. This type differs from the first in that it occurs entirely in the Golgi apparatus and produces more diverse patterns.

Structural Differences Between Plant and Animal Glycosylation

Plants and mammals share an evolutionarily conserved pathway for N-glycosylation, which begins in the endoplasmic reticulum with the synthesis of a common core structure ($Glc_3Man_9GlcNAc_2$) [33].

However, the later stages of N-glycan maturation differ fundamentally between the two kingdoms **(Table1)**, as they are catalyzed by distinct sets of glycosy-ltransferase enzymes [34]. Plant N-glycan patterns are characterized by the presence of specific sugar residues, such as the addition of β-1,2-xylose by xylosyltransferase and core α-1,3-fucose by fucosyl-transferase [7], while their mammalian counter-parts contain β-1,4-mannose, core α-1,6-fucose, β-1,4-galactose, in addition to sialic acids at the termini of the chains.

These structural differences pose a significant challenge in therapeutic protein production, as they may induce immune responses in humans [35]. Studies have shown that plant xylose and fucose residues can be recognized by pre-existing antibodies in human serum, leading to [36]:

Neutralization of therapeutic protein efficacy Accelerated protein clearance from circulation via the reticul-oendothelial system Potential hypersensitivity reactions upon repeated exposure Formation of immune complexes that may deposit in tissues.

This phenomenon limits use of plant systems for producing therapeutic glycoproteins intended for human use, necessitating development of effective strategies for engineering plant glycosylation pathways [34].

Table 1: Comparison of glycosylation patterns in plant and animal systems

Feature	Plant System	Animal System (Mammals)
Added sugar residues	β-1,2-xylose, α-1,3-fucose	β-1,4-galactose, sialic acid
Fucose linkage	α-1,3 to core	α-1,6 to core
Sialylation	Usually absent	Present
Immunogenicity	May induce immune responses	Immunologically compatible
Structural complexity	Limited	High and diverse

Nicotiana benthamiana the most widely used model in Plant molecular farming due to its high efficiency in transient gene expression and susceptibility to Agrobacterium-mediated transformation contains five XylT isoforms and two FucT isoforms [37]. This multiplicity results from the plant's polyploid genome nature, and poses a significant challenge to complete inactivation of these enzymes, especially when using single-gene RNA interference (RNAi) techniques that may not succeed in simultaneously inactivating all isoforms [38].

Additional Challenges in Plant Production

Beyond the glycosylation issue, plant molecular farming faces other challenges including [39]:

- Low expression levels: Especially for complex or large proteins
- Variability among transformed lines: Complicating reproducibility and scale-up efforts
- Proteolytic degradation: By endogenous plant proteases .
- High purification costs: Due to presence of plant compounds such as alkaloids and phenolics .
- Regulatory uncertainty: Regarding approval of genetically modified plant products.
- Environmental safety concerns: About gene flow to related wild plants

Strategies for Improving Productivity and Quality

To overcome the mentioned challenges, a set of technical strategies have been developed to improve quantity and quality of proteins produced in plants [39].

Codon Optimization

Codon usage bias can profoundly affect heterologous protein expression in host organisms [40]. Most recombinant proteins produced in plants are of human origin, thus their sequences contain codons matching human rather than plant cellular frequencies. Codon mismatch leads to translation stalling or abortion [41]. Studies have shown that codon optimization favoring host plant codons can significantly increase translation efficiency and protein yield. In a recent study, codon optimization increased stem cell factor (SCF) yield by 25-30 fold in BY-2 tobacco cells [42]. However, there are conflicting reports regarding codon optimization effects. For instance, codon optimization of human erythropoietin (EPO) or interferon gamma (IFNγ) for plant expression showed no appreciable advantage in protein yield. Studies on codon optimization of human papillomavirus (HPV-16 L1) coat protein in *N. benthamiana*. showed that native genes with human codons performed better than those optimized to reflect plant codons [43].

Promoter and Regulatory Element Selection

Proper transcription initiation, termination, and polyadenylation are key components of gene expression [44]. Having the appropriate combination of promoters and terminators is crucial in constructing gene expression vectors. Effective terminators significantly affect gene transcription and mRNA processing for nuclear export to cytoplasm [45]. Untranslated regions (5'-UTR and 3'-UTR) also play important roles in determining mRNA stability and translation efficiency [46].

Types of promoters used:

- Strong constitutive promoters: Such as Cauliflower Mosaic Virus 35S promoter (CaMV35S), the most common, and plant ubiquitin promoters [47].
- Tissue-specific promoters: Such as seed-specific promoters for protein storage [48].
- Inducible promoters: Responding to specific chemical or physical signals

Several new effective terminators have been identified, such as the Arabidopsis heat shock protein terminator (HSP) and tobacco EU terminator, which showed significantly higher efficiency than traditional NOS terminator in target protein production [49].

Subcellular Targeting

Subcellular targeting of recombinant proteins is an effective strategy for improving their yield and quality [50, 51]. Proteins can be directed to

.Endoplasmic reticulum (ER): Using retention signals (such as KDEL or HDEL), protecting protein from proteases and providing favorable environment for folding and disulfide bond formation .

Apoplast: Facilitates protein assembly and simplifies extraction .

Vacuoles: Provide stable storage environment

Oil bodies: Facilitate protein purification through flotation.

Chloroplasts: Allow high-level production while isolated from rest of cell .

Studies have shown that directing IgG3 antibodies to endoplasmic reticulum or apoplast achieved highest expression levels in plants (over 130 mg/kg fresh weight) [52].

Gene Silencing Suppression

Plants possess defense mechanisms that silence introduced genes via RNA interference (RNAi) [53]. To overcome this obstacle, one can:

- Use viral silencing suppressors such as P19 protein [54].
- Use genetically modified plants with silenced-suppressed background
- Design vectors that reduce recognition of introduced sequence as foreign [54].

Protease Inhibition

Plant proteases pose a major challenge to recombinant protein stability. Strategies to overcome this problem include [13]:

- Subcellular targeting: Directing protein to cellular compartments poor in proteases
- Production in storage organs: Such as seeds containing natural protease inhibitors
- Using genetically engineered plants with low protease activity
- Co-addition of protease inhibitors: During extraction and purification
- Protein modification: Removing sites susceptible to protease cleavage

Deconstructed Viral Vectors

Deconstructed viral vectors represent a significant advancement in transient expression systems in plants.These vectors utilize elements from plant viruses (such as Tobacco Mosaic Virus TMV and Potato Virus X PVX) after removing harmful genes and adding target protein genes [57, 58]. Their advantages include:

ΔXF System: Fundamental Achievement in Plant Glycosylation Inactivation

The ΔXF system (XylT and FucT knockout) was developed through inactivation of XylT and FUT11/12 genes responsible for α-1,3-fucose addition in Arabid-opsis, then in *N. benthamiana* [70]. In this system, plants produce glycoproteins with simplified glycan composition (GnGn-type) free from immunogenic plant residues. This system has proven effective in producing monoclonal antibodies with improved properties [34].

More Advanced Systems: ΔXTFT and Introduction of Human Pathways

More advanced systems such as ΔXTFT have been developed that additionally inactivate other genes in glycosylation pathway, producing simpler glycans (Man5-type) resembling early stages of human glycosylation [71]. Human genes have also been introduced such as:

·β-1,4-galactosyltransferase (B4GALT1): For galactose addition

·α-2,6-sialyltransferase (ST6GAL1): For sialic acid addition [25].

This dual modification (plant inactivation + human introduction) produced plants capable of synthesizing proteins with fully human-like glycan compositions [72].

Importance of Producing Cas9-Free Lines

Production of Cas9-free plant lines is essential for compliance with biosafety and regulatory standards in molecular farming. The reasons for this importance can be summarized as follows:

- **Biosafety:** Persistence of Cas9 gene may lead to off-target editing at other genomic sites, causing unexpected genetic changes [73].
- **Genetic stability**: Cas9 elimination ensures stability of genetic modifications and prevents any subsequent nuclease activity that might cause unwanted changes
- **Regulatory acceptance:** Bringing modified plants closer to traditional breeding concepts facilitates regulatory assessment and increases public acceptance [74].
- **Regulatory compliance:** Most legislation in European Union, Japan, and Korea requires segregation of introduced genes after desired modification is achieved [75].
- **Practical applicability:** Cas9-free lines are closer to commercial application due to absence of continuous editing concerns

Glycan Analysis Techniques

To evaluate success of glycoengineering strategies, advanced analytical techniques have been developed including [76].

- **High-Performance Liquid Chromatography (HPLC):** For separation and identification of glycan patterns [77].
- **Mass Spectrometry:** For detailed structural determination of glycans [78].
- **Lectin arrays:** For rapid detection of specific patterns
- **Capillary Electrophoresis (CE):** For quantitative glycan analysis

These techniques are essential for verifying success of gene editing strategies and ensuring quality of final products [79].

Toward a Regulatory-Compliant Production Plat-form : Recent Results

Multiplex Editing Methodology

In recent studies, an integrated approach was adopted to address previous limitations by targeting all seven glycosyltransferase isoforms in *N. benthamiana*, including FucT5, using multiplex CRISPR/Cas9 editing [9](**Table 3**). Multiple sgRNAs were designed to target conserved regions in these genes, increasing editing efficiency and reducing likelihood of escape mutants

Table2: The Role of Genetic Engineering and CRISPR/ Cas9 Technology in the Production of Therapeutic Proteins in Plants

Objective/ Technology	Target Protein/ Gene	Plant organism Used	Main Result/Observation	Ref.
Production of Therapeutic	Protein GA733-FcK	*Nicotiana tabacum)*	Studying the gene expression and glycosylation pattern of the protein in tobacco seedlings.	[62]
Production of Therapeutic Protein	Prostate cancer antigen (PAP-IgA Fc and PAP-IgA FcK)	*Nicotiana tabacum*	Achieving high expression levels of the protein with a high proportion of dimerized proteins in the leaves.	[63]
Production of Vaccine Candidate	GA733 (colorectal cancer vaccine) and GA733-Fc (complex protein)	*Nicotiana tabacum*	Demonstrating the feasibility of producing the vaccine. The strategy of fusing the Fc fragment of human immunoglobulin with the KDEL signal aids in purification and increases accumulation	[64]
Production of Therapeutic Protein	Recombinant human erythropoietin	*Nicotiana benthamiana*	Reaching high expression levels of up to 85 mg/kg in fresh leaves.	[65]
Production of Growth Factor	Human epidermal growth factor (hEGF)	*Nicotiana benthamiana*	Producing large amounts of recombinant protein using transient expression,especially when combined with a "P19" inhibitor and using codon-optimized sequences	[66]
Production of Antiviral Protein	Human interferon alpha-2b	*Daucus carota*	Higher protein activity in young leaves compared to mature leaves. The potential of using the taproot expression system to produce sufficient amounts of the protein.	[67]
Gene Editing (CRISPR/Cas9)	Knockout of genes responsible for adding specific plant glycans (β(1,2)-xylosyl ransferase and α(1,3)-fucosyl-transferase)	*Nicotiana tabacum*	Reducing the presence of plat-specific proteins (β(1,2)-xylose and α(1,3)-fucose) responsible for potential mmuno-genicity, leading to the production of glycoproteins without plant-specific glycan chains (more compatible with humans).	[68]
Gene Editing (CRISPR/Cas9)	Double knockout of "dicer-like protein 2 and 4"genes	*Nicotiana benthamiana*	Potential increase in the production of "human fibroblast growth factor 1" by inhibiting plant defense mechanisms that might interfere with foreign protein production	[69]
Protein Production in Bioreactors (Additional Examples)	Various human proteins(e.g.,BChE, hCTLA4Ig, hGH1, t-PA) and antigens/ antibodies	Rice,cabbage, tobacco (Physcomitrel la patens), melon	Demonstrating that plants are promising models for producing complex therapeutic proteins insufficient quantities.	[70]

Table 3: Evolution of glycoengineering systems in plants

System	Genetic Modifications	Characteristics of Produced	Ref.
ΔXF system	Inactivation of XylT + FUT11/12	Xylose and Fucose Free, GnGn Type	[70]
ΔF system	Inactivation of FuctT only	Absence of α-1,3-Fucose only	[82]
ΔX system	Inactivation of XylT only	Absence of β-1,2-Xylose only	[82]
ΔXTFT system	Additional inactivation of othe enzymes	Man5 type (simpler)	[71]
Galactosylation system	ΔXF + B4GALT1 Input	Addition of Human Galactose	[25]
Sialylation system	ΔXF + ST6GAL1 insertion	Addition of human sialic acid	[83]
Integrated System	Inactivation of 7 genes+ Cas9 segregation	Complete absence of plant-specific patterns, Cas9-free lines	[9]

Key Results

Through rigorous genetic screening of successive generations, the following results were achieved [80]:

1 .Comprehensive elimination: Plants carrying homozygous mutations in all seven target sites were isolated, confirming complete inactivation of XylT and FucT enzymes

2. Genetic segregation: Complete elimination of introduced Cas9 gene was confirmed by the second generation (T2) through Mendelian segregation, producing completely Cas9-free lines

.3Functional verification: Biochemical analyses of proteins expressed in these lines showed absence of plant xylose and fucose residues, with appearance of glycan patterns approaching human patterns

Additional Achievements in Glycoengineering

Recent years have witnessed important achievements in plant glycoengineering:

Production of Complex IgM Antibodies: In 2023, researchers succeeded in producing SARS-CoV-2 neutralizing IgM antibodies in glycoengineered plants [80]. IgM represents the largest antibodies (approximately 21 human protein subunits correctly assembled into pentameric structures). These plant-produced antibodies showed human-like glycan patterns consistent with predominance of a single glycan type, and up to

390-fold increase in virus neutralization capacity compared to parent IgG1 antibodies. This achievement confirms enormous potential of plants for producing complex human proteins with targeted post-translational modifications [80].

Glycoengineering of Therapeutic Antibodies:

The cetuximab antibody an IgG1 therapeutic antibody carrying additional glycosylation sites in Fab region was produced in engineered plants using different transient expression systems (pEAQ, magnICON, pTra) [60]. All systems showed ability to produce the antibody with targeted glycan patterns in similar quantities, confirming universal applicability of plant glycoengineering.

Production of Complex Human Proteins: Studies have succeeded in producing a variety of complex human proteins in engineered plants, including coagulation factors, lysosomal enzymes, and cytokines [81].

Scientific and Applied Significance

The importance of these results is not limited to demonstrating feasibility of comprehensive elimination of plant-specific glycosylation, but also establishes an integrated production platform with unique specifications:

- Regulatory compliance: Produced lines are free from introduced genes, facilitating licensing and marketing processes

- Genetic stability: Homozygous mutations ensure trait stability across generations
- Application readiness: These lines represent a ready platform for producing recombinant proteins with fully human-like glycan compositions
- Flexibility: Can be used with different expression systems (stable or transient).

Future Prospects and Potential Applications
New Classes of Therapeutic Proteins

Genetically engineered plant lines free from plant glycosylation patterns open the door to producing a wide range of therapeutic proteins requiring precise glycosylation for their biological activity [84]. Prominent potential applications include:

- **Monoclonal antibodies:** Where glycosylation patterns critically affect their efficacy in killing cancer cells via Antibody-Dependent Cellular Cytotoxicity (ADCC). Fucose removal significantly increases this activity [85].
- **Protein hormones:** Such as erythropoietin (EPO) and growth hormone, where glycosylation patterns determine blood half-life [86].
- **Cytokines:** Such as interferons and interleukins
- **Coagulation factors:** For hemophilia [87].

Engineering Additional Glycosylation Pathways

Beyond inactivating plant enzymes, human genes responsible for adding desirable human glycan patterns can be introduced [25]:

- β-1,4-galactosyltransferase (GalT): For galactose addition
- α-2,3- and α-2,6-sialyltransferase (SiaT): For sialic acid addition
- N-acetylgalactosamine (GalNAc) transferases: For producing additional human patterns
- Human fucosyltransferase (FUT8): For adding human α-1,6-fucose [88].

This would enable production of proteins with fully human glycan compositions, increasing their pharmaceutical compatibility and extending their circulatory half-life [89].

Applications in Vaccine Production

The COVID-19 pandemic demonstrated the importance of rapid and flexible production platforms for vaccines and therapeutic proteins [90]. Plant molecular farming can play a pivotal role in [91]:

- Rapid antigen protein production: Within weeks instead of months
- Developing recombinant vaccines: Against emerging and variant diseases
- Manufacturing rapid diagnostic proteins: For infection detection Producing Virus-Like Particles (VLPs): Using efficient plant technologies [92].
- Developing oral vaccines: Using edible plant tissues [93].

Successful plant-derived vaccines have already been produced against human papillomavirus, influenza viruses, and norovirus [93].

Future Technologies: Artificial Intelligence and Synthetic Biology

Research is moving toward integrating advanced technologies to improve plant production systems [94] Buyel:

- Artificial Intelligence and Machine Learning:
- Developing predictive models for optimizing gene sequences and promoters [95].
- Designing highly efficient sgRNAs with low off-target effects.
- Predicting protein structure and glycosylation patterns .
- Optimizing cultivation and purification conditions.

Synthetic Biology:

- Building complete synthetic glycosylation pathways in plants.
- Designing synchronized multigene expression systems [96].
- Developing "designed plant cells" (Chassis cells) as multipurpose production platforms [60]
- Building modular genetic parts libraries for rapid assembly

Advanced Gene Editing Technologies:

- Using CRISPR/Cas12 and CRISPR/Cas13 for more precise editing
- Developing DNA-free editing systems to avoid foreign DNA risks [97].
- Base editing and prime editing techniques for finer control [98].

Ongoing Challenges and Opportunities for Improvement

Despite significant progress, challenges remain requiring further research:

Technical Challenges: Batch-to-batch variability control: Remains challenging, especially in field systems

- Purification efficiency: Developing low-cost, high-throughput purification methods
- Glycan pattern control: Achieving complete homogeneity in glycosylation patterns
- Expression stability across generations: In stable transformation systems

Regulatory Challenges:

- Regulatory framework clarity: Especially for products modified by new gene editing methods
- Consumer acceptance: Overcoming societal concerns about genetically modified organisms
- Standardization: Establishing international standards for quality and safety of plant-derived products

Opportunities for Improvement:

- Developing specialized plant varieties for pharmaceutical production
- Improving vertical farming and controlled environment systems to reduce variability
- Standardizing protocols across laboratories and companies.

Current Limitations and Research Gaps

Despite the significant progress in plant glycoengineering for the production of recombinant glycoproteins, several limitations continue to restrict its broader application.

Low Productivity:

Plant-based expression systems generally produce lower yields of recombinant proteins than established mammalian cell expression systems.

Production challenges:

Enhancing manufacturing methods, downstream cleaning, and maintaining product consistency between production batches remain significant challenges for industrial applications .

Good manufacturing practice (GMP) requirements:

Commercial manufacturing must adhere to the requirements of Good Manufacturing Practice (GMP) to make a particular stable product , sustainable, and production reliable.

Regulatory Challenges:

Regulatory approval from organizations such as the FDA and EMA requires extensive evidence demonstrating product efficacy, safety, and stability of the glycan profile .

Future Research Directions

Future studies should be aware of increasing recombinant protein production, increasing post-technology glycoengineering technologies, using synthetic intelligence for protein optimization, implementing computerized molecular economics techniques, and introducing advanced genome augmentation techniques .

Conclusion

Plant molecular breeding has emerged as a promising platform for biopharmaceutical production by combining the financial benefits of agricultural infrastructure and the precision of genetic engineering . Over the past four decades, it has progressed from experimental studies to a viable platform for producing complex therapeutic proteins. Overcoming glycosylation challenges has been a major milestone in this development. Recent studies, including those discussed in this review, have demonstrated the successful elimination of plant-specific glycosylation patterns using multiplex CRISPR/Cas9 editing and the production of gene-free, regulatory-compliant plant lines. Engineered plants have also shown the ability to produce complex human proteins, such as IgM antibodies, with high efficiency.

These advances have strengthened the potential of plant molecular farming as a cost-effective and sustainable alternative to conventional production systems. Continued improvements in plant engineering and production technologies are expected to further enhance its application in the manufacture of safe and effective biopharmaceuticals.

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