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General Aspects and Applications of Plant Genome Editing: Advancements in Recombinant Protein Production and Viral Vaccine Efficiency through CRISPR/Cas9-Edited *N. benthamiana*

Espen Evju
M.Sc. Plant Biotechnology (Plant Sciences)

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Abstract

The implementation of plant-based antigen manufacturing methodologies has accelerated vaccine research by providing a cost-effective, scalable, and safe alternative to traditional protein production systems. This master thesis provides an updated review of recent advances in plant-based protein production platforms, with an emphasis on recombinant proteins generated from CRISPR/Cas9 genome edited *Nicotiana benthamiana* plants. The first part of this review contains a brief utilization of the CRISPR technology in plant genome engineering, focusing on its advancements and challenges. The thesis includes an overview of various CRISPR applications in plant biology, a detailed assessment of recent advancements in transformation techniques, and an exploration of the challenges associated with sequencing in the context of genome editing.

The second part focuses on the use of *Nicotiana benthamiana* as a model plant to produce recombinant proteins. This review examines the increases in immunogenicity achieved by these unique production platforms through a review of the most recent research. The review also covers the benefits of plant-based protein production platforms, such as cheaper production costs and faster response to emerging risks. The core findings show that plant-based antigen manufacturing methodologies can have an impact on existing protein production systems, potentially leading to the creation of improved vaccines with increased efficacy, safety, and accessibility. The review seeks to offer insights into the current state of research and prospects for using this plant for recombinant protein production, as well as discuss its wider impacts, implications, and future directions for vaccine development.

Sammendrag

Implementeringen av plantebaserte antigenproduksjonsmetoder har transformert vaksineforskningen ved å tilby et kostnadseffektivt, skalerbart og trygt alternativ til tradisjonelle proteinproduksjonssystemer. Denne oppgaven gir en oppdatert oversikt over nyere fremskritt innen plantebasert proteinproduksjonsplatformer, med vekt på rekombinante proteiner generert fra CRISPR/Cas9 genomforandrede Nicotiana benthamiana-planter. Den første delen av denne gjennomgangen tar sikte på å utforske bruken av CRISPR-teknologi i plantegenomteknikk, med fokus på fremskritt og utfordringer. Gjennomgangen inkluderer en oversikt over ulike CRISPR-applikasjoner innen plantebiologi, en detaljert vurdering av nyere fremskritt innen transformasjonsteknikker, og en utforskning av utfordringene knyttet til sekvensering i sammenheng med genomredigering.

Den andre delen fokuserer på bruken av Nicotiana benthamiana som en modellplante for å produsere rekombinante proteiner. Denne oppgaven undersøker økningen i immunogenisitet oppnådd av disse unike produksjonsplattformene ved en gjennomgang av den nyeste forskningen. Oppgaven dekker også fordelene med plantebaserte proteinproduksjonsplattformer, som billigere produksjonskostnader og raskere respons på nye risikoer. Kjernefunnene viser at plantebaserte antigenproduksjonsmetoder kan ha en innvirkning på eksisterende proteinproduksjonssystemer, og potensielt føre til å lage bedre vaksiner med økt effektivitet, sikkerhet og tilgjengelighet. Gjennomgangen søker å gi innsikt i den nåværende forskningstilstanden og utsiktene for bruk av denne planten til rekombinant proteinproduksjon, samt å diskutere dens bredere virkninger, implikasjoner og fremtidige retninger for vaksineutvikling.

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List of Abbreviations

CRISPR:	Clustered regularly interspaced short palindromic repeats
Cas9:	CRISPR associated protein 9
GMOs:	Genetically modified organisms
PMPs:	Plant-made pharmaceuticals
DNA:	Deoxyribonucleic acid
RNA:	Ribonucleic acid
mRNA:	Messenger ribonucleic acid
ZFNs:	Zinc finger nucleases
TALENs:	Transcription activator-like effector nucleases
DSB:	Double stranded break
NHEJ:	Non-homologous end joining
HDR:	Homology-directed repair
gRNA:	Guide ribonucleic acid
sgRNA:	Single guide ribonucleic acid
PAM:	Protospacer adjacent motif
T-DNA:	Transfer-deoxyribonucleic acid
PEG:	Polyethylene glycol
RNP:	Ribonucleoprotein particle
pegRNA:	Prime editing guide ribonucleic acid
HTS:	High-throughput sequencing
NGS:	Next-generation sequencing
SMRT:	Single-molecule real-time sequencing
ZMW:	Zero-mode waveguide
CCS:	Circular consensus sequencing
SNPs:	Single nucleotide polymorphisms
ABA:	Abscisic acid
HSFs:	Heat shock transcription factors
GA:	Gibberellin
MUFA:	Monounsaturated fatty acid
CO_2:	Carbon dioxide
IP:	Intellectual property
PVRs:	Plant variety rights
CHO:	Chinese hamster ovary
TMV:	Tobacco mosaic virus
PVX:	Potato virus X
CPMV:	Cowpea mosaic virus
PTMs:	Post-translational modifications
HA:	Haemagglutinin
PPIs:	Protein-protein interactions
GFP:	Green fluorescent protein
ER:	Endoplasmic reticulum
mAbs:	Monoclonal antibodies
HEK:	Human embryonic kidney
VLP:	Virus-like particle
HBV:	Hepatitis B virus
CaMV:	Cauliflower mosaic virus
LB:	Luria Bertani
PCR:	Polymerase chain reaction
SOC:	Super optimal catabolite
MS:	Murashige and Skoog
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
IEX:	Ion exchange chromatography

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1. Literature Review

1.1 CRISPR Genome Editing for Plant Biotechnology

1.1.1 Introduction

1.1.1.1 The importance of plant biotechnology in agriculture, medicine, and industry

Plants play crucial roles in agriculture, medicine, and industry. In agriculture, plant biotechnology enhances the yield, quantity, and nutrient content of crops. Gene transfer has enabled the creation of genetically modified organisms (GMOs) with improved disease and pest resistance as well as tolerance to harsh environments. Bt-crops, engineered to express *Bacillus thuringiensis* toxin, exhibit strong pest resistance, greatly enhance sustainability by increasing yield while reducing pesticide treatments (Kumar et al., 2020). In a world with a growing population and an unpredictable climate, plant biotechnology plays a crucial role in securing our food supply.

In medicine, plants can serve as bioreactors for producing therapeutic metabolites or proteins. CRISPR enables engineering plants for enhanced production of bioactive compounds and/or the synthesis of novel biomedically relevant molecules. Plant-made pharmaceuticals (PMPs), like antibodies, antigens, and enzymes, can be used to fight diseases such as cancer, autoimmune disorders, and infectious diseases. These PMPs are produced efficiently and economically by using plant-based protein production systems (Spiegel et al., 2018).

In industry, plant biotechnology uses plant metabolism to create sustainable alternatives to fossil fuels and biodegradable materials. The use of 'bioplastics' made from plant starch and the modification of plants to increase cellulose production involve plant biotechnology to improve environmental sustainability and increase green technology solutions (Srivastava et al., 2021).

RNA interference (RNAi) is another technology with similarities to classical GMOs and genome editing such as CRISPR/Cas9, but it can also be applied to target whole gene families and used for plant protection not regulated as GMOs depending on use (Taning et al., 2020).

Plant biotechnology profoundly impacts agriculture, medicine, and industry, driving advancements with economic and environmental benefits. As technology advances, especially with powerful genome editing tools like CRISPR/Cas9, the role of plant biotechnology in promoting a sustainable and prosperous future is becoming more relevant than ever before.

1.1.1.2 A brief history of genome editing technologies

The landscape of biotechnology has been profoundly reshaped by the emergence and evolution of genome editing technologies. This shift started in the late 20th century, when researchers developed precise tools to alter the genetic fabric of cells.

Genome editing began with Zinc Finger Nucleases (ZFNs) (Urnov et al., 2010) and Transcription Activator-Like Effector Nucleases (TALENs) (Bogdanove & Voytas, 2011). Both methods used the concept of engineering endonuclease domains to create double-stranded breaks (DSBs) at specific genomic loci. This prompts the cellular machinery to repair the breaks through Non-Homologous End Joining (NHEJ) or homology-directed repair (HDR) mechanisms, resulting in targeted modifications (Christian et al., 2010; Kim et al., 1996). Despite the impressive achievements of these technologies,

their application was limited by the complex protein engineering process, which was labor-intensive and constrained by sequence limitations.

Genome editing was transformed in 2012 with the arrival of the CRISPR/Cas9 system developed by Jennifer Doudna and Emmanuelle Charpentier. This system, based on a bacterial immune response, enabled gene editing with remarkable simplicity, efficiency, and versatility (Jinek et al., 2012). The CRISPR/Cas9 system achieves precise genomic editing through RNA-DNA base pairing, a simpler and more flexible approach than ZFNs and TALENs. Moreover, the multiplexing potential of CRISPR/Cas9 allows for concurrent edits at multiple genomic loci, adding an extra level of sophistication.

CRISPR/Cas9 is a key technology for plant biotechnology, enabling precise genetic modifications in most species, including staple crops and medicinal plants. Its deployment has greatly improved the potential for addressing challenges in food security, sustainable agriculture, and medical advancements (Adli, 2018). The evolution of genome editing technologies has paved the way for an era of remarkable capabilities in plant sciences. The transition from ZFNs and TALENs to CRISPR/Cas9 signifies a shift towards enhanced simplicity, efficiency, and flexibility in plant biotechnology, unlocking many new possibilities.

1.1.2 The CRISPR/Cas9 System: Mechanism and Components

1.1.2.1 A general overview

CRISPR/Cas9 was first discovered in *Escherichia coli* as a set of repetitive DNA sequences (Ishino et al., 1987). It was later found in other bacteria and archaea (Mojica et al., 2005). The repeats were separated by spacer sequences derived from foreign genetic elements like bacteriophage genomes or plasmids (Bolotin et al., 2005; Pourcel et al., 2005). The “Clustered Regularly Interspaced Short Palindromic Repeats” (CRISPR) name points to the arrays of palindromic repeats with unique spacer sequences characterizing the function of this genome editing in nature (Jansen et al., 2002). CRISPR and Cas proteins have transformed genome editing with their simplicity, versatility, and specificity. CRISPR/Cas9 has been used for gene or genome editing in many different organisms, including plants (Jinek et al., 2012). CRISPR/Cas9 in bacteria acts as an immune system against foreign genome sequences recognized from earlier bacterial exposure to viruses called phages.

CRISPR arrays can be combined with Cas genes, which encode nuclease proteins for the CRISPR/Cas immune response (Makarova et al., 2006). The type II CRISPR/Cas9 system from *Streptococcus pyogenes* is the most used form of genome editing because it is simple and adaptable (Jinek et al., 2012). The CRISPR/Cas9 system in bacteria function to protect against foreign genetic elements from bacteriophages even though the function of the spacers between previous tags from earlier infections are not fully understood (Barrangou et al., 2007).

1.1.2.2 Mechanisms of action: target recognition, DNA cleavage, and repair pathways

CRISPR/Cas9 relies on a single guide ribonucleic acid (sgRNA) to guide the Cas9 endonuclease to the DNA target sequence (Jinek et al., 2012). The sgRNA is a chimeric RNA molecule with a 20-nt targeting region that matches the target DNA and a scaffold region that interacts with the Cas9 protein (Jinek et al., 2014). Target recognition is secured by sgRNA pairing with the target DNA by classical Watson-Crick base pairing. The specificity is increased by a protospacer adjacent motif (PAM) sequence, usually 5’-NGG-3’ for SpCas9 (Jiang & Doudna, 2017). PAM is needed for Cas9 to bind and cut, ensuring specificity and avoiding off-target effects (Mojica et al., 2009).

CRISPR/Cas9 operates in three stages: adaptation, expression, and interference (Marraffini & Sontheimer, 2010).

In the first adaptation stage, Cas1 and Cas2 proteins recognize and cleave foreign DNA fragments called protospacers. Protospacers are integrated into the host's CRISPR array as new spacer sequences, acting as a molecular record of past infections (Yosef et al., 2012). In response to an invasion, the CRISPR array is transcribed into a precursor CRISPR RNA (pre-crRNA). The Cas9 protein and a trans-activating CRISPR RNA (tracrRNA) are then processed into short, mature crRNAs (Deltcheva et al., 2011). Each crRNA has a spacer sequence that matches a target sequence in the foreign genetic element. In the interference stage, the crRNA guides the Cas9 protein to the target sequence in the foreign DNA using base pairing with the spacer sequence (Jinek et al., 2012). When Cas9 recognizes a PAM in the target DNA, it causes a double stranded break (DSB), which destroys the foreign genetic element and provides immunity against the invader.

After Cas9 cuts DNA, the broken ends are fixed using either non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Chapman et al., 2012). NHEJ is a repair mechanism that joins any broken DNA ends independent of sequence homology, often causing indels at the target site (Lieber, 2010). Indels cause frameshift mutations and gene knockouts, providing a valuable tool for functional gene studies in plants (Sander & Joung, 2014).

HDR is a targeted repair pathway that relies on a homologous DNA template to accurately repair the DSB (Chapman et al., 2012). In CRISPR/Cas9 genome editing, researchers can add a repair template with the desired changes. It is then used to fix the target site during repair. (Puchta, 2004). This editing capability allows for precise mutations, insertions, or deletions in genes.

1.1.3 CRISPR/Cas9 for Plant Genome Editing

1.1.3.1 Methods for delivering CRISPR/Cas9 components into plant cells (Agrobacterium-mediated transformation, particle bombardment, protoplast transfection)

Agrobacterium-mediated transformation is the most common method for introducing genes into plant cells (Gelvin, 2003). Agrobacterium transformation uses *Agrobacterium tumefaciens'* natural ability to transfer its T-DNA (transfer DNA) into the plant genome. To use this technique in CRISPR/Cas9 editing, the components are cloned into a vector and introduced into Agrobacterium cells (Altpeter et al., 2016). The transformed Agrobacterium then infects plant tissues, like leaf explants or immature embryos, integrating CRISPR/Cas9 components into the plant genome. Agrobacterium-mediated transformation is efficient, versatile, and cost-effective (Gelvin, 2003). It is still not suitable for all plants; some are recalcitrant to Agrobacterium. Additionally, this method like others may randomly integrate CRISPR/Cas9 components into the plant genome, possibly causing unintended mutations (Puchta, 2017).

Particle bombardment, also called biolistic or gene gun, delivers CRISPR/Cas9 components into plant cells using gold or tungsten particles as carriers for the coated DNA. These particles are accelerated into plant tissues with a high-velocity helium pulse (Klein et al., 1987). CRISPR/Cas9 components are introduced as a plasmid with the Cas9 gene, guide RNA (gRNA) expression cassette, and selectable marker gene. Particle bombardment is advantageous because it can transform many plant species, even those recalcitrant to Agrobacterium-mediated transformation. (Altpeter et al., 2016) Particle bombardment is fast and can be used for stable and transient gene expression. However, this method typically has low efficiency and will often introduce the CRISPR/Cas9 components randomly into the plant genome (Liang et al., 2017).

Protoplast transfection delivers CRISPR/Cas9 components to plant protoplasts using chemical or physical methods like PEG-mediated transfection or electroporation (Yoo et al., 2007). Protoplasts are plant cells without cell walls, making them amenable to the uptake of foreign DNA. CRISPR/Cas9 components can be introduced as plasmids, RNP complexes, or mRNA (Woo et al., 2015). After transfection, protoplasts can be cultured to regenerate and recover the edited plants. Protoplast transfection has many advantages, such as high transformation efficiency, transient gene expression, and a reduced risk of random integration of CRISPR/Cas9 components into the plant genome (Woo et al., 2015). Also, RNPs or mRNA can reduce off-target effects by degrading quickly in the cells (Zhang et al., 2016). However, protoplast isolation and regeneration are challenging and time-consuming, adding somaclonal unintended genetic effects during the long time in tissue culture. Not all plant species can be efficiently regenerated from protoplasts (Yoo et al., 2007).

Agrobacterium, particle bombardment, and protoplast transformations are the main ways to deliver CRISPR/Cas9 into plant cells. Each method has advantages and limitations. As CRISPR/Cas9 technology advances, new delivery methods and optimization will undoubtedly improve plant genome editing. Some new methods that are being tested use viral vectors for CRISPR/Cas9 delivery. Viruses infect cells and use their machinery for replication and gene expression (Liu & Zhang, 2020). Researchers are studying modified plant viruses for delivering CRISPR/Cas9 into plant cells, which could avoid generating GMOs by instead using RNA and could improve efficiency and be able to target specific tissues (Cody & Scholthof, 2019). Other researchers are investigating nanotechnology-based delivery systems, like lipid nanoparticles or nanocarriers. These nanoparticles could offer advantages such as high loading capacity and biocompatibility of CRISPR/Cas9 components (Martin-Ortigosa et al., 2013).

1.1.3.2 Strategies for achieving precise genome editing (homology-directed repair, base editing, prime editing)

Homology-directed repair (HDR) is a DNA repair mechanism that uses a homologous DNA sequence to fix double-stranded breaks (DSBs) caused by e.g. CRISPR/Cas9 (Chen et al., 2019). This method allows for accurate nucleotide changes, insertions, or deletions by using a donor DNA template to guide the repair process. The low efficiency of HDR in plant cells is a challenge for its practical application (Baltes & Voytas, 2015).

Multiple strategies have been proposed to improve HDR efficiency in plants. One approach involves inhibiting the non-homologous end-joining (NHEJ) pathway, responsible for most DSB repair events (Puchta, 2004). Another strategy involves using small molecules like trichostatin A to enhance HDR efficiency (Shaked et al., 2005). Moreover, improving the efficiency of Cas9 and the donor template can enhance HDR-mediated genome editing in plants (Woo et al., 2015).

Base editing, developed by Komor and his team in 2016, is a CRISPR/Cas9-based method that allows for the direct conversion of one DNA base to another without creating double-strand breaks (Komor et al., 2016). This method uses a modified version of Cas9 called dCas9 or nCas9, combined with a cytidine deaminase or adenine deaminase. This combination converts cytosine to uracil or adenine to inosine, respectively. Uracil or inosine is recognized as thymine or guanine during DNA replication or repair, resulting in a permanent base change.

Base editing has advantages over HDR, such as reduced reliance on donor templates and a lower risk of off-target effects due to the absence of DSBs (Rees & Liu, 2018). However, base editing is limited in its scope, as it can only cause specific base transitions, such as C-to-T or A-to-G. It cannot cause transversions like C-to-A or G-to-T or insertions or deletions.

Prime editing guide RNA (pegRNA), developed in 2019, is a versatile genome editing technique that merges the benefits of HDR and base editing (Anzalone et al., 2019). This method employs a nCas9

fused to a reverse transcriptase and an engineered pegRNA that contains both the target site and the desired edit. nCas9 introduces a nick in the target DNA strand, and the reverse transcriptase uses pegRNA as a template to synthesize a new DNA fragment with the desired edit. The synthesized DNA strand primes the endogenous DNA repair machinery, integrating the edit into the target locus.

Prime editing has advantages over traditional CRISPR/Cas9 methods. It enables various modifications like insertions, deletions, and base-to-base conversions without DSBs or donor templates (Anzalone et al., 2019). Prime editing has demonstrated decreased off-target effects and enhanced editing efficiency compared to HDR (Liu et al., 2020). Prime editing, though promising, does pose challenges. The prime editing complex's size can hinder efficient delivery into plant cells, so optimized delivery methods are needed (Lin et al., 2020). Moreover, prime editing's efficiency is impacted by the target sequence and edit type, necessitating meticulous pegRNA design and optimization for each unique application (Anzalone et al., 2019).

1.1.4 Genome Sequencing Technologies

1.1.4.1 High-throughput sequencing

Advancements in genome sequencing have greatly enhanced plant biotechnology. The rise of high-throughput sequencing (HTS), also called next-generation sequencing (NGS), has great potential for studying and modifying plant genomes (Koboldt et al., 2013). Wel annotated full genome sequences are needed to safely use genome editing, to secure specific targeting editions and avoiding unintended effects.

Introduced in 2005, HTS has surpassed Sanger sequencing to become the preferred method for genome sequencing (Mardis, 2008). HTS platforms like Illumina sequencing, Roche 454 pyrosequencing, and Ion Torrent sequencing have greatly contributed to generating genomic data that improves the efficiency of CRISPR-based plant genome editing (van Dijk et al., 2018).

HTS uses parallelized sequencing to analyze millions of DNA fragments from a single sample simultaneously, significantly reducing sequencing time compared to traditional methods. Despite the distinct operational processes on each HTS platform, they all share a common theme of automated, parallel sequencing of amplified or single DNA molecules (Schuster, 2008).

HTS plays a key role in CRISPR/Cas9 genome editing. It improves editing precision by informed experimental design, guiding target selection, and identifying off-target effects (Barrangou & Horvath, 2017). HTS data aid in selecting optimal guide RNAs (sgRNAs) by offering detailed and comprehensive information on the genetic variability of target sites. HTS aids in measuring genomic variations and indels resulting from the genome editing process. HTS is crucial in validating genetic edits and detecting off-target effects (Zetsche et al., 2015).

Although HTS techniques offer improved resolution, there are still significant limitations. High costs, data complexity, data quality, and bioinformatic expertise are some concerns for the wider adaptation of the technology. As HTS technology evolves, more accurate and cost-effective methods are being developed. Nanopore and single-molecule real-time (SMRT) sequencing are third-generation technologies with great potential. These methods enable longer read lengths, improving the accurate detection of structural variants and aiding in the complete assembly of complex regions (Kumar et al., 2019). These advancements will enhance CRISPR applications in plant biotechnology.

1.1.4.2 Third-generation sequencing technologies

Third-generation sequencing technologies, like Pacific Biosciences' Single Molecule Real-Time (SMRT) sequencing and Oxford Nanopore Technologies' nanopore sequencing, are long-read platforms. These technologies can sequence single DNA molecules in real-time without amplification (Eid et al., 2009; Kono & Arakawa, 2019).

Pacific Biosciences employs SMRT sequencing, which utilizes a zero-mode waveguide (ZMW) system for detecting the incorporation of fluorescently labeled nucleotides during synthesis (Eid et al., 2009). ZMWs function as individual sequencing chambers, capturing the actions of a single polymerase that detects light signals of various wavelengths during nucleotide incorporation. This enables the "single molecule" and "real time" capabilities of SMRT. Despite an initially high error rate, recent advancements, like circular consensus sequencing (CCS), have significantly reduced errors (Wenger et al., 2019).

Nanopore sequencing technology employs protein nanopores within an electrically resistant membrane. When a voltage is applied across the membrane, an ionic current record the fluctuations, informing the passage of nucleotides and sequencing DNA or RNA molecules in real-time (Kono & Arakawa, 2019). Nanopore sequencing, despite its high error rate, holds immense potential. It offers unlimited read lengths and portability.

Third-generation sequencing technologies excel in their ability to accurately detect structural variations, surpassing the capabilities of short-read technologies. Structural variants are a crucial part of genomic diversity and are often linked to genetic diseases (Abel et al., 2020). Studying structural variations can be challenging with second-generation sequencing because it cannot fully cover repetitive regions. In contrast, third-generation sequencers have the advantage of fully spanning repetitious regions (Treangen & Salzberg, 2012). Third-generation sequencing's real-time nature speeds up the entire process, from DNA extraction to data analysis. Moreover, third-generation sequencing possesses the advantage of identifying base modifications without chemical treatment, providing an edge in epigenetic studies.

Sequencing long reads can quickly advance gene editing research, especially for complex plant genomes. Genome assemblies from third-generation sequencing technologies improve guide RNA design for CRISPR, enhancing precision and effectiveness in editing (Moon et al., 2019). Additionally, these technologies aid in identifying off-target effects in CRISPR-mediated gene editing experiments, ensuring the accuracy and safety of the editing process as well as increasing compliance with regulatory safety requirements.

1.1.5 Technical Obstacles: Considerations and Challenges in Plant Genome Editing

1.1.5.1 Considerations in transformation: species specificity

Species specificity in plant transformation protocols has been a long-standing issue, marked by differences in the amenability to gene uptake, integration, and subsequent regeneration of genome-edited cells into plants. For instance, Agrobacterium-mediated transformation is limited due to its host-range specificity. Certain species and genotypes are highly recalcitrant or unresponsive to this transformation method (Gelvin, 2003).

The CRISPR/Cas9 system's efficiency varies between species due to multiple biological and genetic factors. Some plants exhibit species-specific resistance mechanisms to foreign DNA or disparities in endogenous gene repair mechanisms modified in CRISPR/Cas9 editing, altogether affecting the

transformation efficiency (Hsu et al., 2014). Another issue is the varying regeneration potential of different plant species. Protoplast regeneration into fully functional plants, a critical phase post-transformation, is not possible in many common crops, posing a barrier to the application of CRISPR/Cas9 genome editing (Ikeuchi et al., 2013).

Overcoming these barriers necessitates customized transformation and regeneration protocols catering to individual species' unique biological prerequisites. This, however, will require a lot of work and calls for an in-depth understanding of plant tissue culture, genomic attributes, signaling pathways, and their interplay with transformation methods (Michno et al., 2015). The CRISPR/Cas9 system in plant genome editing necessitates a sophisticated understanding of how species-specific considerations influence transformation efficiency.

1.1.5.2 Challenges in transformation: integration of foreign DNA

One of the technical challenges in plant genome editing is the successful integration of foreign DNA into the plant genome. Although increased transformation efficiency in crops has been achieved in the last few decades due to the advent of Agrobacterium-mediated transformation and microparticle bombardment techniques, transformation remains a significant bottleneck (Altpeter et al., 2016).

A central issue is the inherent genomic complexity and the variation among different plant species. Plant genomes have a wide range of sizes, levels of ploidy, and repetitive sequence content, all of which can affect the efficiency and precision of transformation (Bortesi et al., 2016). Furthermore, the presence of epigenetic barriers further complicates foreign DNA integration, as these can affect both DNA uptake and stable integration into the host genome (Butaye et al., 2005).

Another challenge in plant transformation is the positioning of the insertion. Random integrations or off-target effects of the CRISPR system can lead to undesirable modifications such as gene disruption, undesirable phenotypic change, or even lethality (Jiang et al., 2013). Thus, controlling integration site specificity represents a crucial aspect of ensuring effective and safe genome editing in plants. Delivering CRISPR components is logistically challenging. Developing new and improved techniques to deliver RNPs into cellular compartments while minimizing cytotoxic effects is necessary (Woo et al., 2015).

Lastly, the effects of foreign DNA integration on the stability of the plant genome are a critical concern. This encompasses not only the behavioral consistency of inserted DNA over generations but also how the genome accommodates and responds to the insertion, particularly in terms of genomic integrity and stability (Lyznik et al., 2003).

The challenges in the integration of added DNA into plant genomes require careful consideration for the application of CRISPR technology in plant biotechnology. The continued development of techniques, including more accurate integration methods and improvements in efficiency, is crucial to overcoming these technical challenges.

1.1.5.3 Challenges in transformation: regeneration of plants through tissue culture

Often regenerating plants from transformed cells is the most challenging step for application of genome editing. This has been greatly improved for cereals, by combining knowledge of stem cell regulation to the tissue culture part of transformations. Adding a growth regulating factor and a cofactor, has been reported to greatly increase regeneration frequencies in wheat, triticale, rice and citrus (Debernardi et al., 2020).

Another major challenge in tissue culture after getting DNA integrated and plants regenerated is genetic mutations caused by somaclonal variation, which affect the resulting genotypes and possibly also phenotypes (Fossi et al., 2019). Somaclonal variation, a phenomenon of genetic and epigenetic changes during tissue culture, is well documented in different plant species (Bobadilla Landey et al., 2015). While it can provide new genetic variation for plant breeding, it also poses challenges for creating genetically precise crops through CRISPR-Cas9 editing (Miguel & Marum, 2011) since it adds many additional DNA changes that often is unknown unless the offspring is fully genome sequenced. This is not specific to the type of transformation, but tightly correlated to time in culture.

The mechanisms behind tissue culture-induced genetic variation are complex and still not fully understood. They include spontaneous DNA changes during DNA replication and transcription, changes in chromosomal structure, like deletions and duplications, and number (aneuploidy and polyploidy), which can be worsened by extended culture periods. Moreover, DNA methylation and other epigenetic changes can cause gene silencing, introducing unexpected variability (Fuks, 2005).

Transformation efficiency can decrease due to somaclonal variation, which hampers the process and results in a low success rate for transgenic plants. Additionally, somaclonal variation can complicate the differentiation between true sequence-edited lines and those resulting from somaclonal variation (Neelakandan & Wang, 2012). In practice positional effects are mostly avoided by generating a minimum of 10 independent lines and discarding the ones that might differ from the majority.

These challenges require better methods and procedures in tissue culture techniques to control unwanted variation and maintain successful transformation. Current research is focused on optimizing growth conditions, minimizing culture duration, and ensuring chromosomal and gene stability during transformation, which may help address these issues. Tissue culture-induced genetic variation poses challenges for using CRISPR-Cas9 systems in plant biotechnology. Strategic developments are crucial for managing these issues and ensuring the reliable and effective application of CRISPR technology in plant genome editing.

1.1.5.4 Considerations in sequencing: complex plant genomes

Plant genomes are known for their large size and intricate structure, which can make sequencing challenging. Plant genomes differ from animal genomes in their extensive polyploidy, repetitive sequences, and mobile genetic elements. These attributes can complicate sequence read assembly and make it hard to distinguish between homologous chromosomes or paralogous sequences (Harper et al., 2012).

Certain plant genomes have a large physical scope, which worsens these complications. The bread wheat genome (*Triticum aestivum*) is over 16 GBp, making it about five times larger than the human genome (Consortium et al., 2018) in addition to being hexaploidy with three similar genomes that can be difficult to separate when sequencing similar reads. Handling the vast amount of sequence data from large genomes is a significant challenge in terms of reliability, timeliness, and cost-effectiveness (Mochida & Shinozaki, 2011).

Another complication arises from the varying prevalence of heterozygosity in certain plant genomes. Genome-wide variants like single nucleotide polymorphisms (SNPs) can add noise to the analysis, making it challenging to differentiate genuine CRISPR-induced edits from natural genetic variation (Arora & Narula, 2017). Sequencing complex plant genomes is crucial for utilizing CRISPR/Cas9 technology in plant biotechnology, despite the technical challenges involved. As sequencing technologies advance, these obstacles to genome editing in plant sciences should eventually be solved.

1.1.5.5 Challenges in sequencing: repetitive sequences and polyploidy

Repetitive sequences are a key aspect of plant genomes, making up more than 80% of certain cereal genomes like maize and wheat (Wicker et al., 2007). Repetitive sequences can hinder genome assembly and annotation, leading to ambiguity in sequence alignment. This is because reads from similar repeats can be indistinguishable, leading to misassembly or oversights of CRISPR-induced modifications. Repetitive sequences can hinder precise off-target detection, which is critical for CRISPR/Cas9 editing specificity (Maccaferri et al., 2019).

Polyploidy, the presence of multiple homologous sets of chromosomes in an organism, poses a challenge to genome sequencing. Polyploidy is prevalent in plants and can vary from triploidy (three sets of chromosomes) to decaploidy (ten sets). This presents issues for sequencing, as differentiating between sequences from homologous chromosomes can be difficult. Polyploidy can complicate variant calling, as each chromosome in a set may have different alleles (Comai, 2005). Identifying true CRISPR-induced edits among naturally occurring variants can be challenging, which is necessary in order to comply with European regulatory safety requirements for genetically modified organisms (GMOs).

Repetitive sequences and polyploidy can interact to complicate plant genome sequencing. Repetitive sequences can lead to misalignment of reads, exacerbating the challenges posed by polyploidy in accurately assembling and interpreting the genome (Michael & VanBuren, 2020). Advancements in sequencing methodologies, specifically long-read sequencing platforms like PacBio or Oxford Nanopore, offer promising avenues for addressing these obstacles. Effectively utilizing the CRISPR/Cas9 system in plant biotechnology requires addressing the challenges of repetitive sequences and polyploidy in sequencing plant genomes. Again, advancements in long-read sequencing technologies are especially valuable for plant sequencing and offer optimism for future progress in this field.

1.1.6 Applications: Plant Engineering and Crop Improvement

1.1.6.1 Enhancing abiotic stress tolerance (drought, salinity, temperature)

Climate change is having a large effect on the stability and output of global agriculture. Abiotic stresses like drought, salinity, and extreme temperatures are making it increasingly harder to grow crops (Lesk et al., 2016). CRISPR-Cas9 genome editing offers new ways to improve crop resilience. This sub-chapter summarizes recent research on using CRISPR-Cas9 to improve abiotic stress tolerance in plants.

Drought greatly limits crop productivity. The predicted increase in global temperatures and more unpredictable rainfall makes developing drought-tolerant varieties essential for future food security. CRISPR-Cas9 has been utilized to target genes in plant drought response pathways. Modification of the ABA (abscisic acid) signaling pathway, a regulator of plant responses to drought stress, has shown promising results (Cardoso et al., 2020). Researchers used CRISPR-Cas9 to knock out the *OsPP2C* gene in rice, resulting in increased ABA sensitivity and improved drought tolerance (Miao et al., 2020). Genome editing of the ERFVII transcription factor family in Arabidopsis has enhanced drought tolerance by regulating stomatal aperture and water use efficiency (Vaidya et al., 2019).

Soil salinity limits crop yield by impeding plant growth and development through osmotic stress and ion toxicity. By targeting crucial genes for ion homeostasis and osmoprotectant synthesis, plant tolerance to salinity can be enhanced. In a study using CRISPR-Cas9, researchers edited the *NHX1* gene, a Na+/H+ antiporter in tomato plants, enhancing salt tolerance (Fu et al., 2021). Moreover, engineering genes related to compatible solute synthesis, like proline, can enhance osmotic adjustment and improve salinity tolerance. In rice, researchers used CRISPR-Cas9 to edit the

OsSAPK2 gene, resulting in higher proline levels and enhanced resistance to salinity (Lou et al., 2017).

Extreme temperatures can harm plant growth. CRISPR-Cas9 has been used to target genes related to thermotolerance pathways. Heat shock transcription factors (HSFs) are vital for plant responses to heat stress. In tomatoes, knocking out the *HsfA1a* gene using CRISPR-Cas9 led to increased thermotolerance and improved fruit set in high-temperature conditions (Zhu et al., 2018). On the other hand, modifying genes linked to cold tolerance, like CBF family transcription factors, can boost plant resilience to low temperatures. In rice, overexpressing the *OsCBF1* gene using CRISPR-Cas9 technology enhanced cold tolerance without affecting growth or yield (Zhang et al., 2022)

1.1.6.2 Improving biotic stress resistance (pathogen and pest resistance)

Biotic stresses, like pathogens and pests, are a big challenge for global food security and sustainable agriculture (Savary et al., 2012). Traditional breeding has helped create crop plants that can better withstand stress. However, this method is slow and imprecise. CRISPR/Cas9 genome editing is a powerful tool for modifying plant genomes. It can be used to engineer biotic stress resistance in crop plants quickly and accurately.

Pathogen resistance can be boosted by targeting susceptibility genes (S-genes), which pathogens use to establish infection. By manipulating these genes, the plant's susceptibility to pathogen infection can be diminished (Zaidi et al., 2018). One example is the knockout of the MLO gene, which effectively confers broad-spectrum resistance to powdery mildew in many important food crops such as wheat and tomatoes.

An alternative method includes incorporating resistance (R) genes that identify pathogen effector proteins and trigger defense responses. CRISPR/Cas9 has been used to edit native R-genes or introduce new R-genes from related plant species. The gene *Pi9* was introduced into the rice genome, enhancing resistance against *Magnaporthe oryzae,* commonly known as rice blast fungus, which is the most economically damaging rice disease in the world (LIU et al., 2010).

CRISPR/Cas9 has been utilized to boost crop resistance against insect pests by targeting genes involved in plant-pest interactions. One strategy focuses on genes encoding essential proteins for pest feeding or development. Li et al. (2020) used the CRISPR/Cas9 system to edit the *Arabidopsis thaliana* gene that encodes β-1,3-glucanase (Vaghela et al., 2022). This protein is necessary for the green peach aphid (*Myzus persicae*) to feed successfully. The edited plants showed reduced aphid infestation and increased resistance. An alternative approach focuses on manipulating plant defense genes. In grapevine, *VvWRKY52*, *VvMLO3*, and *VvPR4b* were successfully mutated using CRISPR/Cas9, increasing resistance to *Botrytis cinerea*, downy mildew, and powdery mildew, respectively (Tu et al., 2022).

1.1.6.3 Modifying plant architecture and growth characteristics (yield, flowering time, plant height)

In rice, the gene *OsGA20ox2* has been modified using CRISPR-Cas9 to create semi-dwarf plants with higher grain yield (Han et al., 2019). The authors used two sgRNAs to target the first exon of *OsGA20ox2* and generated different types of mutations, including large fragment deletions. They then analyzed the effects of the mutations on plant height, GA level, agronomic traits, gene expression, cell morphology, and protein profiles. They found that the mutant lines had reduced plant height and GA level, slightly increased yield per plant, no significant changes in other agronomic traits, suppressed expression of *OsGA20ox2*, decreased cell length and width, increased cell numbers, and altered

protein expression. They also showed that the mutant lines could restore their normal height by exogenous GA3 treatment. They concluded that CRISPR/Cas9-mediated mutation of *OsGA20ox2* is a potential strategy to improve plant architecture and yield in rice breeding.

In another study on rice, researchers edited the *OsSPL14* gene which increased panicle branches and spikelets per panicle, leading to a higher grain yield (Miura et al., 2010). In tomatoes, the CRISPR-Cas9 knockout of the *SELF PRUNING* (*SP*) and *SP5G* genes led to a more compact plant structure and higher fruit yield (Soyk et al., 2017).

Flowering timing is crucial for plant reproduction and a key focus for crop improvement. CRISPR-Cas9 has been used to change genes regulating flowering time, creating early- or late-flowering crop varieties adaptable to diverse environments and cultivation practices (Zhao et al., 2023). In Arabidopsis, the knockout of *FLOWERING LOCUS C* (*FLC*), a key repressor of flowering, led to early flowering plants (Sun et al., 2022). In rice, researchers have modified the *Ehd1* gene, causing changes in flowering times and thus creating varieties for different cultivation seasons (Zhang et al., 2014).

Plant height is a key trait that can enhance crop productivity, as seen leading to the "Green Revolution" by conventional breeding decades ago. Semi-dwarf wheat and rice varieties lead to an increase ratio of seed production compared to total green mass (Hedden, 2003). CRISPR-Cas9 has been used to create semi-dwarf rice plants by targeting the *SLR1* gene, which plays a crucial role in the gibberellin signaling pathway (Cheng et al., 2022). The plants exhibited shorter heights and better lodging resistance, boosting crop productivity.

1.1.6.4 Engineering nutritional and quality traits (biofortification, altered starch composition, modified oil content)

Biofortification increases the nutrient content of staple crops to combat micronutrient deficiencies affecting billions worldwide (Bouis & Saltzman, 2017). CRISPR/Cas9 has effectively enhanced essential micronutrients like iron, zinc, and provitamin A in rice, wheat, and maize, among other crops (Koç & Karayiğit, 2022; Kumar et al., 2023). In their study, Dueñas Jr. et al. (2021). employed CRISPR/Cas9 to edit three rice genes related to iron and zinc uptake, storage, and homeostasis. This approach led to notable enhancements in grain iron and zinc content while maintaining a similar yield. Yadav et al. (2021) used CRISPR/Cas9 to boost iron content in wheat grains. They achieved this by targeting the *VIT-1* gene, which plays a role in iron chelation and vacuolar sequestration. In maize, Da Silva Messias et al. (2014) significantly boosted provitamin A levels by up to 20-fold by precisely altering the *ZmCCD1* gene, responsible for encoding a carotenoid cleavage enzyme.

Starch, a key carbohydrate in crops, plays vital roles in crop quality and end-uses. Altering starch composition enhances crop nutrition, processing quality, and shelf life. CRISPR/Cas9 has been used to modify starch in crops like potatoes, rice, and barley (Andersson et al., 2017; Sun et al., 2017). Andersson et al. (2017) used CRISPR/Cas9 to disable the granule-bound starch synthase (*GBSS*) gene in potatoes, leading to the production of amylose-free starch with enhanced gelling and textural characteristics. This has been achieved before by traditional GMOs, but in this study the authors used a transient expression of CRISPR-Cas9, which means that they did not integrate the DNA of the editing system into the potato genome. Instead, they delivered the DNA to isolated potato cells (protoplasts) using a chemical solution (PEG). The DNA was then expressed in the cells and induced mutations in the *GBSS* gene. The mutated cells were then regenerated into whole plants through tissue culture.

In rice, Sun et al. (2017) effectively altered the starch branching enzyme IIb (*BEIIb*) gene, resulting in a higher amount of slowly digestible starch and resistant starch. Zhang et al. (2018) used

CRISPR/Cas9 to create barley lines with lower amylose content and changed granule morphology, indicating additional benefits in the brewing and food industries (Garcia-Gimenez & Jobling, 2022).

Crop oils are a major resource as dietary lipids and industrial raw materials. Modifying the oil content and fatty acid composition of crops can further enhance their nutritional value and industrial potential (Vanhercke et al., 2019). CRISPR/Cas9 has been used to modify oil content in crops like soybean, rapeseed, and camelina (Ku & Ha, 2020).

Lee et al. (2021) used CRISPR-Cas9 to increase the monounsaturated fatty acid (MUFA) content in the seed oil of hexaploid *Camelina sativa*. They targeted the *FAD2* gene, which encodes fatty acid desaturase 2, an enzyme responsible for converting oleic acid (a MUFA) into linoleic acid (a polyunsaturated fatty acid). Knockout of all three pairs of *FAD2* homeologs led to a stunted bushy phenotype, but greatly enhanced MUFA levels (by 80%) in the seeds.

Zhang et al. (2019) used CRISPR-Cas9 to effectively edit the lysophosphatidic acid acyltransferase 2/5 (*LPAT2/5*) genes in allotetraploid rapeseed (*Brassica napus*). LPAT is a key enzyme in the Kennedy pathway, which catalyzes the attachment of fatty acid chains to 3-phosphoglycerate and promotes further production of oil in the form of triacylglycerol. The authors designed four single-gRNAs and two multi-gRNAs from the conserved coding regions of *BnLPAT2* and *BnLPAT5*. The seeds of the Bnlpat2/Bnlpat5 mutant were wizened and showed enlarged oil bodies, disrupted distribution of protein bodies, and increased accumulation of starch in mature seeds. These results demonstrate that CRISPR-Cas9-mediated mutation of *BnLPAT2/5* is an effective strategy for improving oil production in rapeseed.

1.1.6.5 Engineering photosynthesis and carbon fixation pathways for improved efficiency

The conversion of CO_2 into organic compounds through the Calvin-Benson cycle is crucial for plant productivity. A key bottleneck in this process is the enzyme Rubisco, which has a slow catalytic rate and can undergo oxygenation instead of carboxylation, resulting in energy waste (Parry et al., 2012).

Donovan et al. (2020) describes the use of CRISPR-Cas9 technology to simultaneously knock out multiple *rbcS* homologs in tobacco. The *rbcS* gene encodes the small subunit of the key CO2-fixing enzyme Rubisco. The researchers designed two multiplexing guide RNAs (gRNAs) to target homologous regions in three genes, *rbcS_S1a*, *rbcS_S1b*, and *rbcS_T1*, which account for at least 80% of total *rbcS* expression in tobacco. The Rubisco content of three selected mutant lines in the T1 generation was reduced by ca. 93% and mutant plants accumulated only 10% of the total biomass of wild-type plants. Their results show that CRISPR-Cas9 is a viable tool for the targeted mutagenesis of *rbcS* families in polyploid species and will contribute to efforts aimed at improving photosynthetic efficiency through expression of superior non-native Rubisco enzymes in plants.

Moreover, incorporating alternative carbon fixation pathways like the C_4 pathway into C_3 plants using CRISPR-Cas systems has demonstrated the increased potential to improve photosynthetic efficiency by genome editing (Ermakova et al., 2020).

Incomplete light absorption by photosynthetic pigments is a factor that limits photosynthesis efficiency. Using CRISPR-Cas gene editing, researchers modified gene expression to optimize light absorption across various wavelengths (Ort et al., 2015).

Despite progress in engineering photosynthesis and carbon fixation pathways, there are still many challenges with this approach. Off-target effects and unintended consequences of genetic modifications must be considered. Moreover, the intricate interactions among various components of

photosynthetic machinery require a comprehensive understanding at the system level and a multi-gene approach.

By understanding the intricate regulatory networks of abiotic stress responses and identifying new target genes involved in defense, yield, flowering time, and plant height; by altering starch composition, modifying oil content, and optimizing key pathways like light absorption, electron transport, and carbon fixation, genome editing technology has the potential to greatly boost crop productivity and help meet global food security needs. As climate change continues to accelerate, genome editing shows possibilities for creating resilient crops that could enhance global food security and lead to the development of sustainable and robust agricultural systems.

1.1.7 Ethical, Legal, and Social Implications of CRISPR/Cas9 in Plant Biotechnology

1.1.7.1 Regulatory framework for genome-edited plants

CRISPR/Cas9 technology has revolutionized plant biotechnology, allowing precise genome editing with remarkable ease and efficiency. The fast growth of this technology has surpassed regulations, creating a need for updated guidelines on the ethical, legal, and social implications of genome-edited plants. This sub-chapter examines the regulatory landscape for genome-edited plants, highlighting national and international frameworks, IP protection, technology transfer, and future challenges.

Regulatory frameworks for genome-edited plants differ worldwide, reflecting cultural, political, and social differences (Wolt et al., 2016). The United States has adopted a product-based approach, focusing on the resulting organism's characteristics rather than the process developing it (Gould et al., 2022). The European Union has until now treated genome-edited plants like traditional genetically modified organisms (GMOs) (Bruetschy, 2019). The Cartagena Protocol on Biosafety governs the global transportation of genetically modified organisms, including genome-edited plants(Crystal Turnbull et al., 2021). The Protocol lacks clarity on CRISPR/Cas9, resulting in different interpretations and applications among member countries.

Public perception shapes regulatory frameworks for genome-edited plants. Concerns about GMO-safety and environmental impacts have also fueled some opposition to genome-edited plants, even much less than for traditional GMOs (Frewer et al., 2013) Researchers, policymakers, and industry stakeholders must engage in open dialogue with the public to build trust and understanding of CRISPR/Cas9 technology.

Harmonization of international standards for genome-edited plants would promote free trade and innovation, while reducing regulatory disparities. Due to the dynamic nature of CRISPR/Cas9 technology, regulatory frameworks need to be flexible and responsive to new advancements and applications. Transparency, as for other matters, is crucial for public trust in genome-edited plants. Regulatory frameworks need to prioritize open communication and engagement with all stakeholders. CRISPR/Cas9's use in plant biotechnology has sparked ethical, legal, and social concerns. Despite some progress in establishing guidelines, challenges persist. Regulatory efforts try to prioritize harmonization, adaptability, and transparency for responsible genome-edited plant development and application. By tackling these challenges, policymakers, researchers, and industry stakeholders can collaborate to leverage CRISPR/Cas9 technology for enhancing global food security, advancing sustainable agriculture, and addressing urgent environmental concerns while upholding public trust.

1.1.7.2 Intellectual property and technology transfer considerations

The fast innovation in CRISPR/Cas9 research has surpassed the development of IP frameworks and technology transfer mechanisms, resulting in a complex legal landscape (Sherkow, 2015) This impacts the fair spread and commercialization of CRISPR/Cas9 plant biotechnologies and the overall acceptance of these technologies. The CRISPR/Cas9 system has sparked IP disputes between academic institutions and private companies, resulting in patent battles over foundational CRISPR/Cas9 technologies (Mali, 2022) This uncertain IP landscape hinders innovation and collaboration between researchers and industry partners.

In plant biotechnology, protecting IP for CRISPR/Cas9-generated plants, as for classical GMOs, is complex due to the overlap of different IP rights like patents, plant variety rights (PVRs), and trade secrets. Jurisdictions vary in their approaches to granting IP protection for genetically modified organisms. Some countries offer patent protection for transgenic plants, while others rely on plant variety rights (PVRs) to safeguard plant breeders' rights (Roca et al., 2023). The absence of global agreement on IP protection for CRISPR/Cas9-enabled plant biotechnologies add legal complexities and uncertainties. Technology transfer is vital for sharing CRISPR/Cas9-enabled plant biotechnologies from academia to industry for commercialization. It also results in less development and access to new varieties in Europe compared to other parts of the world like the UA and Asia affecting European farmers development and European consumers access to new products and higher prices on the imported products. The complex IP landscape of CRISPR/Cas9 technologies may hinder technology transfer, causing delays in plant biotechnology development and negatively affecting our sustainability and environment (Jiang, 2020)

IP rights and licensing agreements can worsen inequalities in global plant biotechnology distribution, potentially limiting access to CRISPR/Cas9-enabled crops also to developing. To address these challenges, various strategies have been proposed for the responsible dissemination of CRISPR/Cas9-enabled plant biotechnologies.

- Open innovation platforms and shared licensing models like PIPRA and OpenMTA promote collaboration, resource sharing, and equitable access to CRISPR/Cas9 technologies in plant research (Kahl et al., 2018).
- Adoption of humanitarian licensing provisions for CRISPR/Cas9 technologies to support crop development for food security and nutrition in developing countries (Montenegro de Wit, 2020).
- Global patent pools can be created to collectively license CRISPR/Cas9-related patents, making it easier to access essential technologies and reducing transaction costs for technology transfer (Horn, 2017).
- Global harmonization of IP protection for genetically modified organisms, focusing on establishing precise guidelines for patenting CRISPR/Cas9-enabled plants and crops This will minimize legal ambiguities and foster international collaboration (C. Turnbull et al., 2021).

Addressing IP protection and technology transfer challenges is crucial for fostering innovation, collaboration, and access to CRISPR/Cas9-enabled plant biotechnologies for global food security and sustainable agriculture. This is even more important when wars reduce food production, international trade and the international market.

1.2 *N. benthamiana* as a Model Plant for Recombinant Protein Production

1.2.1 Introduction

1.2.1.1 Importance of recombinant protein production in biotechnology and medicine

Recombinant protein production plays an important role in modern biotechnology and medicine, driving advancements in diverse fields such as pharmaceuticals, diagnostics, agriculture, and industrial bioprocessing (Tripathi & Shrivastava, 2019). Recombinant protein technologies are used for the development of therapeutic proteins, vaccines, enzymes, and additional bioproducts (Tripathi, 2016). *Nicotiana benthamiana* is the most widely used model plant for recombinant protein production. It offers advantages in scalability, cost-effectiveness, and safety, especially compared to other traditional expression systems such as *E. coli*, yeast (*Saccharomyces cerevisiae*), mammalian cells (like Chinese Hamster Ovary or CHO cells), and insect cells (like *Baculovirus* expression system) (Shanmugaraj et al., 2020). This chapter explores *N. benthamiana* as a platform for producing recombinant proteins and focuses on recent research, challenges, and development, as well as highlighting its importance in biotechnology and medicine.

Despite many successes, recombinant protein production remains complex and costly, with challenges in expression, folding, and purification (Jozala et al., 2016). Recombinant proteins are traditionally produced using microbial systems like *Escherichia coli* and eukaryotic systems such as yeast, insects, and mammalian cells (Demain & Vaishnav, 2009). Each platform has limitations: prokaryotic systems lack post-translational modifications, and mammalian cell culture is very expensive (Wang et al., 2017). Additionally, recombinant proteins are vital biocatalysts in industry, facilitating the production of various chemical compounds and materials via bioprocessing (Boodhoo et al., 2022).

Plant-based expression systems have emerged as a promising alternative for recombinant protein production, overcoming the limitations associated with traditional platforms. *N. benthamiana* is notable for its versatility, ease of genetic modification, and rapid growth rates (Shanmugaraj et al., 2020). This sub-chapter presents the advantages of *N. benthamiana* as a model plant for recombinant protein production and its implications for biotechnology and medicine.

1.2.2 Advantages of *N. benthamiana* as a Model Plant for Recombinant Protein Production

1.2.2.1 Comparison to conventional systems for viral antigen production

Procaryotic bacterial expression systems, like *Escherichia coli*, are commonly used for producing recombinant proteins. However, these systems have limitations in producing complex eukaryotic proteins as they lack the machinery for eucaryotic protein folding, glycosylation, and disulfide bond formation, which can affect protein stability, solubility, and biological activity (Rosano & Ceccarelli, 2014)

Eukaryotic yeast expression systems, like *Saccharomyces cerevisiae* and *Pichia pastoris*, are also used for recombinant protein production (Cereghino & Cregg, 2000). Yeasts can perform eukaryotic post-translational modifications. However, the glycosylation patterns of recombinant proteins produced in yeast often differ from those of mammalian proteins, potentially affecting their therapeutic efficacy (Hamilton & Gerngross, 2007)

Insect cell expression systems, like the *baculovirus*-insect cell system, are popular for producing eukaryotic recombinant proteins due to their capacity for complex post-translational modifications (Demain & Vaishnav, 2009). However, these systems can be costly and time-consuming, and scaling up insect cell culture can pose challenges (Carinhas et al., 2009)

Mammalian cell expression systems, like Chinese hamster ovary (CHO) cells, are the gold standard for producing complex eukaryotic proteins with proper folding, post-translational modifications, and biological activity (Wurm, 2004). However, these systems have high production costs, long production times, and potential risks of viral contamination (Santos et al., 2016)

N. benthamiana has advantages over conventional systems for recombinant protein production. Plant-based expression systems are cost-effective because they have lower production costs and require less complex culture media and equipment (Pogue et al., 2010). Additionally, *N. benthamiana* is a rapid producer of recombinant proteins, yielding results in just 4-6 weeks, in contrast to mammalian cell systems that require several months. Moreover, the likelihood of contamination with human or animal pathogens is minimal, thereby enhancing the safety of recombinant proteins produced in *N. benthamiana*.

N. benthamiana is suitable for transient expression systems like agroinfiltration or viral vectors, enabling fast, high-level protein production (Au - Leuzinger et al., 2013). Transient systems facilitate efficient gene introduction, bypassing the need for the time-consuming generation of stable transgenic lines (Gleba et al., 2005). This feature is advantageous for developing vaccines and diagnostics for emerging infectious diseases (Marsian & Lomonossoff, 2016). *N. benthamiana* performs eukaryotic post-translational modifications like glycosylation, which are important for the folding, stability, and activity of recombinant proteins (Shin et al., 2011). Glycoengineering advancements have facilitated the production of recombinant proteins with human-like glycosylation patterns in *N. benthamiana*, thereby enhancing their therapeutic potential (Castilho et al., 2012). It offers advantages over conventional expression systems, such as reduced costs, shorter production times, increased safety, and the ability to perform complex post-translational modifications. *N. benthamiana is* therefore a valuable platform for developing biopharmaceuticals, diagnostics, and vaccines.

1.2.2.2 Advantage #1: Rapid growth and ease of cultivation

The fast growth rate of *N. benthamiana* is advantageous for producing recombinant proteins. The plant matures in just 4-6 weeks, faster than other plants used for protein expression like *Arabidopsis thaliana* and *Nicotiana tabacum*, which can take 6–8 weeks and 10–12 weeks, respectively (Schillberg et al., 2019). The shortened life cycle of *N. benthamiana* allows researchers to quickly screen and select transgenic lines expressing the desired recombinant protein, speeding up the overall research and development process.

N. benthamiana's ease of cultivation is a significant advantage for recombinant protein production. *N. benthamiana* can be grown using standard plant growth conditions and simple cultivation techniques, such as soil-based or hydroponic growth. The plant is amenable to Agrobacterium-mediated transient expression, allowing for rapid and efficient introduction of the desired recombinant protein-encoding gene into its genome. This method is highly successful and applicable to various recombinant proteins, ranging from small peptides to complex multimeric proteins (Spiegel et al., 2022).

1.2.2.3 Advantage #2: High biomass yield

The high biomass yield of *N. benthamiana* is primarily due to its efficient photosynthesis. The plant uses a C_3-type photosynthetic mechanism to efficiently fix carbon dioxide, even in low light. *N.*

benthamiana has a high photosynthetic capacity, enabling rapid growth and elevated biomass accumulation. This makes it an excellent candidate for producing large quantities of recombinant proteins.

The rapid growth and high biomass accumulation rates enable large-scale production of recombinant proteins in a short time. This is especially beneficial for producing urgently needed proteins like vaccines and therapeutic antibodies for emerging infectious diseases (Buyel et al., 2017). Moreover, *N. benthamiana*'s high biomass yield allows for cost-effective production of recombinant proteins, surpassing traditional expression systems. The plant-based system bypasses expensive fermentation equipment and the high costs of producing and purifying recombinant proteins from microbial and mammalian cell cultures (Schillberg et al., 2019).

The high biomass yield of *N. benthamiana* enables efficient scale-up of recombinant protein production. Generating large amounts of biomass quickly enables the production of recombinant proteins at a scale that meets research and commercial demand.

1.2.2.4 Advantage #3: Susceptibility to a wide range of plant viruses for transient expression

N. benthamiana's susceptibility to various plant viruses makes it an ideal platform for developing transient expression systems using viral vectors. These vectors, derived from plant viruses like Tobacco mosaic virus (TMV), Potato virus X (PVX), and Cowpea mosaic virus (CPMV), efficiently infect *N. benthamiana* and aid in expressing recombinant proteins (Gleba et al., 2007). Viral vectors have many advantages over stable transformation methods, some of which are rapid protein expression, high yield, and a reduced risk of gene silencing (Lico et al., 2008).

N. benthamiana's susceptibility to plant viruses is due to its unique genetic makeup, including mutations in the RNA silencing pathway (Ratcliff et al., 2001). These mutations enhance viral replication and the accumulation of viral RNA, thereby boosting recombinant protein expression (Lacomme & Chapman, 2008). *N. benthamiana*'s compatibility with viral vectors allows researchers to choose the best vector for their application, ensuring efficient production of recombinant proteins.

Nicotiana benthamiana's susceptibility to various plant viruses has been pivotal in establishing it as a model plant for recombinant protein production. *N. benthamiana*'s compatibility with viral vectors enables the rapid production of diverse recombinant proteins, such as therapeutic proteins, vaccines, and industrial enzymes.

1.2.2.5 Advantage #4: The potential for nuclear and plastid transformation

Nuclear transformation of *N. benthamiana* is a widely used technique for introducing DNA to plant cells, and what is mostly done to generate GMOs. Agrobacterium-mediated transformation is the preferred method to produce genetically engineered plants and is widely used for *N. benthamiana*. This process transfers cloned gene constructs to *Agrobacterium tumefaciens* bacterium, that further transfers it to the nucleus of a plant cell (Gelvin, 2003). The modified plant cells can be regenerated into complete plants, expressing the intended recombinant protein.

N. benthamiana is also compatible with transient expression systems. These systems allow fast expression of recombinant proteins in a short time (usually 5-7 days) without the need for stable integration of the transgene or the time-consuming process of generating transgenic plants (Marillonnet et al., 2005). Viral vectors, like geminivirus-derived vectors, boost protein production speed and expression levels (Gleba et al., 2007).

Plastid or chloroplast transformation is an additional method for producing recombinant proteins in *N. benthamiana*. This technique introduces the DNA constructs into the plastid genome, leading to stable integration and expression (Bock, 2007). Plastid transformation offers advantages over nuclear transformation, such as high-level transgene expression due to plastids' polyploid nature, precise transgene integration, and the absence of gene silencing (Maliga, 2004).

Plastid transformation in *N. benthamiana* offers a key advantage: the ability to produce recombinant proteins without the risk of transgene escape through pollen transmission. Plastid genomes in angiosperms, like *N. benthamiana*, are mostly inherited from the mother, which greatly reduces the chance of transgene spreading to unintended organisms (Daniell et al., 2016). This feature makes plastid transformation an appealing choice for producing recombinant proteins with environmental or health concerns, like pharmaceuticals and industrial enzymes.

1.2.3 Challenges and Limitations of *N. benthamiana* for Recombinant Protein Production

1.2.3.1 Post-translational modifications and potential immunogenicity

Post-translational modifications are crucial for protein function, stability, and localization (Walsh & Jefferis, 2006). Plant-based expression systems, like *N. benthamiana*, have distinct PTMs compared to mammalian systems, which impacts the bioactivity and pharmacokinetics of the recombinant protein (Gomord et al., 2010).

N-glycans are complex carbohydrate chains that are attached to certain amino acids of proteins. They play important roles in protein folding, stability, function, and interactions. However, different organisms have different N-glycosylation pathways and patterns, which can affect the biological activity and immunogenicity of recombinant proteins.

Plants are valuable platforms to produce biopharmaceuticals, however, one of the major challenges is to overcome the differences in N-glycosylation between plants and humans. Plants typically add plant-specific sugars, such as xylose and fucose, to the core N-glycan structure, which are absent or rare in humans. These sugars can reduce efficacy and increase the immunogenicity of recombinant proteins.

To address this issue, researchers have developed various strategies to modify the plant N-glycosylation pathway and introduce human-like N-glycan structures. These include knocking out or silencing plant-specific glycosyltransferases, expressing human glycosyltransferases and enzymes, and introducing synthetic or bacterial genes (Castilho & Steinkellner, 2012).

Advancements in plant molecular biology and genetic engineering allow tailoring of *N. benthamiana*'s PTM machinery for optimal recombinant protein production. Genome editing techniques like CRISPR/Cas9 has been used to knock out two plant-specific genes, β1,2-xylosyltransferase (*XylT*) and core α1,3-fucosyltransferase (*FucT*), leading to the production of proteins with mammalian-like glycan structures (Jansing et al., 2019). Moreover, the temporary co-expression of glycosylation enzymes in *N. benthamiana* allows the production of recombinant proteins with intricate, sialylated N-glycans resembling mammalian ones (Castilho et al., 2013). Knocking out these genes resulted in recombinant proteins with N-glycan structures like those found in human produced proteins.

So far, glycoengineering in *N. benthamiana* has mainly focused on producing therapeutic proteins. Future research should focus on expanding the range of target metabolites, including small molecules and other biologically active compounds, to enhance the potential applications of this technology in biopharmaceutical production.

Despite challenges in post-translational modifications and immunogenicity in *N. benthamiana*-based protein production, recent advances in genetic engineering and molecular biology offer solutions to overcome these limitations. Further research in this area may lead to improvements for *N. benthamiana* as a versatile platform for recombinant protein expression. By tackling these challenges and furthering our knowledge of plant glycosylation pathways and CRISPR/Cas9 technology, scientists can unlock the full potential of glycoengineered *N. benthamiana* as a platform for producing human-compatible secondary metabolites with therapeutic applications. This approach could revolutionize biopharmaceutical production and contribute to novel therapies for many diseases.

1.2.3.2 Variability in protein yield and stability

The main challenge in *N. benthamiana* protein production is the variability in yield. Factors affecting variability include choice of expression vector, promoter, DNA-codon, and subcellular targeting of recombinant proteins (Conley et al., 2009). Moreover, the plant's defense mechanisms can hinder protein accumulation by activating degradation or silencing of the transgene. Effective strategies to overcome these challenges include utilizing strong promoters, optimizing DNA-codons, and co-expressing viral silencing suppressors (Peyret & Lomonossoff, 2013).

Protein degradation and aggregation can lower protein yields and affect recombinant protein functionality (Schillberg et al., 2019). Methods to improve protein stability in *N. benthamiana* involve co-expressing molecular chaperones or protease inhibitors, optimizing protein purification techniques to reduce aggregation, and utilizing fusion tags to enhance solubility and stability (Conley et al., 2009)

1.2.3.3 Regulatory and commercialization challenges

Biosafety and containment are major concerns with *N. benthamiana* in recombinant protein production due to the risk of unintended release of transgenic plants or their products. Stringent regulations govern GMO production and handling. Researchers and manufacturers must follow these regulations, which can be time-consuming and costly, potentially hindering the widespread use of *N. benthamiana*-based protein production systems. As an example, only the testing of GMOs cost $3.9 billion in 2021 (Zimny & Sowa, 2021).

The intellectual property (IP) rights of *N. benthamiana*-derived recombinant proteins present challenges for their commercialization. Patents protect plant genetic engineering and protein production processes, posing obstacles for both research and development. Navigating the IP landscape demands considerable investments in time and resources, potentially dissuading researchers and manufacturers from pursuing *N. benthamiana*-based protein production.

The market acceptance of *N. benthamiana*-produced recombinant proteins, especially for therapeutic applications, is uncertain. Public concerns about the safety and effectiveness of plant-based biopharmaceuticals, along with skepticism towards GMOs, could restrict the market for *N. benthamiana*-derived products.

N. benthamiana-based protein production faces competition from bacterial, yeast, and mammalian cell cultures. The familiarity, scalability, and regulatory acceptance of these systems make them preferred choices for researchers and manufacturers. To conquer this challenge, the benefits of *N. benthamiana*-based protein production must be convincingly showcased, including their cost-effectiveness, speed, and distinctive protein characteristics.

N. benthamiana is a potential platform for recombinant protein production. However, its adoption in industrial biotechnology is hindered by regulatory and commercialization challenges. Addressing and solving these issues requires collective efforts by researchers, manufacturers, policy makers and regulatory agencies to communicate the safety, efficacy, and advantages of *N. benthamiana*-based protein production. Overcoming these hurdles will enable the successful integration of this plant system into biotechnology.

1.2.3.4 Intellectual property and technology transfer considerations

A concern is the patentability of *N. benthamiana* as a model plant. While plant varieties themselves are not patentable under TRIPS, EPC, or the US Patent Act, certain plant-related inventions, like methods for producing specific plant varieties or genetic modifications, can be patented (Suresh et al., 2007). Additionally, *N. benthamiana* recombinant protein production may require patented transformation technologies, expression systems, genetic constructs/DNA sequences and/or gene-editing tools like CRISPR/Cas9. Researchers must ensure they have the necessary licenses and permissions to use these technologies before possible commercialization (H. B. Singh et al., 2016)

For *N. benthamiana*-based recombinant protein production, technology transfer is a complex field where different patent rights often intersect and make it very difficult to navigate the landscape. It involves negotiating IP rights, establishing industry partnerships, and overcoming regulatory hurdles. Creating strong academic-industry partnerships is vital for the successful commercialization of *N. benthamiana*-based products. This can be done through mechanisms like licensing agreements, research collaborations, and spin-off companies.

Technology transfer can be hindered by the "Valley of Death," a gap between academic research and commercial application. This is mainly due to inadequate funding, a lack of industry interest, and regulatory challenges. To bridge this gap, academia and industry should collaborate to identify and invest in promising technologies for *N. benthamiana*-based recombinant protein production.

In conclusion, *N. benthamiana* is a promising model plant for recombinant protein production with great biotechnological potential. Researchers and industry stakeholders should address intellectual property and technology transfer to ensure the successful development and commercialization of *N. benthamiana*-based products. These involve navigating the IP landscape, establishing partnerships, and overcoming technology transfer challenges.

1.2.4 Applications of Recombinant Protein Production in *N. benthamiana*

1.2.4.1 Application #1: Development of plant-based vaccines

The efficient production of vaccines is crucial, particularly during pandemics. *N. benthamiana* has been successfully used for viral antigen production in vaccine development. A plant-made influenza virus haemagglutinin (HA) protein was expressed in *N. benthamiana* (D'Aoust et al., 2008). Their preclinical studies suggest plant-produced HA protein stimulates immune responses in mice, highlighting *N. benthamiana*'s potential for influenza vaccine development.

During the COVID-19 pandemic, researchers used to *N. benthamiana* produce a subunit vaccine candidate with the RBD of the SARS-CoV-2 spike protein (Tarlan et al., 2020). The RBD antigen from plants induced neutralizing antibodies in mice, showing the usefulness of *N. benthamiana* for quickly developing vaccines against new viral threats.

Marsian et al. (2017) produced a synthetic polio vaccine candidate using N. benthamiana as the plant-based expression system. The authors used a mutant version of poliovirus type 3 (PV3 SktSC8) that has stabilizing mutations in the capsid proteins, which prevent the conformational change from the native, immunogenic D antigen to the non-immunogenic C antigen. They cloned the capsid precursor (P1) and the viral protease (3CD) into pEAQ vectors and co-infiltrated them into *N benthamiana* leaves. The plant-expressed PV3 SktSC8 proteins were able to assemble into virus-like particles (VLPs) that resembled the native PV3 in morphology and antigenicity. They found that the VLPs induced similar levels of neutralizing antibodies and protection against challenge with wild-type PV3 as the current inactivated polio vaccine (IPV).

1.2.4.2 Application #2: Production of industrial enzymes and other bioproducts

Enzymes are utilized in diverse industries like food processing, detergent formulation, textile manufacturing, and biofuel production (R. Singh et al., 2016).

In the paper by Hahn et al. (2015) they describe a novel and scalable method for producing cellulases and other proteins in plants using Agrobacterium spray-based transient expression. Cellulases are enzymes that can break down cellulose, the main component of plant cell walls, into glucose. Cellulases are important for various industrial applications, such as biofuel production, paper recycling, and textile processing. However, the current methods of producing cellulases by microbial fermentation are costly and inefficient. The authors used *N. benthamiana* to express six different cellulases of bacterial and fungal origin. The authors showed that the plant-expressed cellulases were active and could degrade various types of cellulosic substrates. The authors estimated that the cost of producing cellulases by this method would be much lower than by microbial fermentation and could make cellulosic ethanol production more economical and sustainable.

Another example is laccases, multicopper oxidases with applications in textile dye decolorization, pulp bleaching, and bioremediation (Mate & Alcalde, 2017). *N. benthamiana* has been used as a host for fungal laccase expression, yielding successful enzyme activity and (Khlystov et al., 2021)

N. benthamiana is further used to express enzymes for plant natural product biosynthesis, including terpenoids, which are important for pharmaceuticals, cosmetics, and agrochemicals (Reed et al., 2017)

Scalability is crucial for commercializing *N. benthamiana*-based bioproducts. Researchers are studying hydroponic and aeroponic systems to boost biomass production. They are also working on continuous harvesting strategies to ensure a consistent supply of biomass (Buyel, 2019). Moreover, combining plant-based production platforms with synthetic biology tools allows for the strategic design and enhancement of metabolic pathways to produce intricate bioproducts (Wu et al., 2022).

N. benthamiana is a promising platform for industrial enzymes and bioproducts, with many advantages over conventional systems.

1.2.4.3 Application #3: Research (gene function analysis, protein-protein interactions, protein localization)

Studying protein-protein interactions (PPIs) is essential for uncovering the molecular foundation of biological processes. *N. benthamiana* is a great platform for studying PPIs using techniques like BiFC, co-immunoprecipitation, and FRET (Walter et al., 2004)

BiFC has been widely used to visualize PPIs in vivo in *N. benthamiana*. This technique uses two proteins fused to non-fluorescent fragments of a fluorescent protein, like yellow fluorescent protein

(YFP). Protein interaction leads to functional fluorophore reconstitution, enabling direct visualization of PPIs in living cells (Kerppola, 2006).

Protein localization studies are crucial for understanding protein functions. *N. benthamiana* is a versatile system for protein localization studies using transient expression systems and advanced imaging techniques like confocal laser scanning microscopy. By fusing proteins to fluorescent tags like green fluorescent protein (GFP), researchers can visualize protein localization in live plant cells (Chiu et al., 1996)

N. benthamiana is commonly used for protein localization studies in different contexts, such as cellular trafficking, organelle targeting, and protein dynamics in plant-pathogen interactions. *N. benthamiana* was used to study the localization of *Arabidopsis thaliana* NAC transcription factors, revealing their targeting to the endoplasmic reticulum (ER) and Golgi apparatus (Kim et al., 2006). Moreover, *N. benthamiana* has enabled the study of protein dynamics in plant-pathogen interactions, including the relocation of immune receptor proteins after pathogen recognition (Padmanabhan et al., 2013).

1.2.4.4 Application #4: Production of therapeutic proteins (antibodies, enzymes, and hormones)

Monoclonal antibodies (mAbs) are vital in modern medicine, providing targeted treatments for diseases like cancer and autoimmune disorders (Kaplon & Reichert, 2018). Plant-based mAb production in *N. benthamiana* has demonstrated promising outcomes, some of which are listed below.

A notable example is the swift response to the Ebola virus outbreak in 2014. The experimental drug ZMapp, consisting of three chimeric mAbs, was produced in *N. benthamiana* (Qiu et al., 2014). The transient expression system enabled rapid production of gram-scale functional mAbs, showcasing the potential of plant-based systems for emergency therapeutic production.

Rattanapisit et al. (2017) used *N. benthamiana* as a platform to produce recombinant human osteopontin (hOPN), a protein that has a role in bone formation and regeneration. The authors used a geminiviral vector system to transiently express hOPN in *N. benthamiana* leaves and purified the protein by Ni affinity chromatography. They then compared the structure and function of plant-produced hOPN with commercial hOPN expressed in human embryonic kidney (HEK) cells. They found that plant-produced hOPN had a similar molecular weight, secondary structure, and epitope recognition as commercial hOPN, but differed in the tertiary structure and glycosylation pattern. They also showed that plant-produced hOPN could support the attachment, proliferation, and osteogenic differentiation of human periodontal ligament stem cells in vitro, and even induced higher expression of osteogenic genes than commercial hOPN.

Silberstein et al. (2018) used *N. benthamiana* to produce recombinant human alpha-1 antitrypsin (AAT), a protein that has a role in preventing lung tissue damage caused by excessive protease activity. AAT deficiency is a genetic disorder that leads to emphysema and liver disease and is treated by infusion of plasma-derived AAT, which is costly and limited in supply. The authors used a plant viral vector system called CMViva to transiently express AAT in *N. benthamiana* leaves and modified the protein by replacing a methionine residue with valine in the reactive center loop to make it more resistant to oxidation by inflammatory oxidants. They purified the plant-made recombinant AAT (prAAT) by immunoaffinity chromatography. They showed that prAAT had comparable anti-elastase activity and protective effect on human bronchial epithelial cells as hAAT, and retained its function under oxidative conditions that would inactivate hAAT. They concluded that *N. benthamiana* is a suitable host for producing functional and oxidation-resistant AAT for therapeutic use. The advantages of plant-based systems, such as cost-effectiveness, scalability, and reduced risk of

contamination, make *N. benthamiana* an attractive alternative to traditional mammalian cell-based production systems.

1.2.4.4.1 Influenza virus antigens

Influenza viruses are a major global health threat, causing an estimated 290,000 to 650,000 deaths worldwide each year (Iuliano et al., 2018). The fast evolution of influenza viruses necessitates frequent updates to the influenza genome sequences globally to meet efficient vaccine formulations, and effective, scalable, and affordable production platforms.

Hemagglutinin (HA) is a glycoprotein on the influenza virus surface. It is targeted by neutralizing antibodies and is important for influenza vaccines (Wiley & Skehel, 1987). Landry et al. (2014) demonstrated successful expression of HA from both the A/Indonesia/05/2005 (H5N1) and A/California (H1N1) strains in *N. benthamiana* using an Agrobacterium-mediated transient expression system. The plant-derived HA (pHA) was found to be correctly folded, glycosylated, and biologically active, stimulating a robust immune response in mice. This study showed that a functional influenza virus antigen can be produced in *N. benthamiana*, opening doors for future research and solutions.

Phan et al. (2013) also describes the production and immunogenicity of recombinant haemagglutinin (HA) proteins derived from the avian influenza virus (AIV) strain A/Hatay/2004/(H5N1) in *N. benthamiana*. The authors used N. benthamiana as a host for transient expression of HA proteins by vacuum infiltration with Agrobacterium tumefaciens carrying the plant expression vectors. This method allowed for rapid and high-level production of recombinant proteins in intact plant leaves within 4 days after infiltration. The authors also generated stable transgenic Nicotiana tabacum plants expressing HA proteins by Agrobacterium-mediated transformation. The authors compared the immunogenicity of different forms of HA proteins (monomers, trimers, ELPylated or not, VLPs) in mice using different adjuvants and immunization schedules. They found that trimeric HAs elicited higher levels of HA-specific and potentially neutralizing antibodies than monomeric HAs. VLPs were also highly immunogenic and induced cross-reactive antibodies against different AIV strains.

The paper demonstrates that plant derived HAs, especially trimeric and VLP forms, are promising candidates for developing subunit vaccines against AIV.

These case studies highlight *N. benthamiana*'s potential as a platform for influenza virus antigen production. Future research should prioritize addressing challenges, investigating *N. benthamiana*'s potential for producing viral antigens, and developing new vaccine candidates. The evidence for *N. benthamiana* as a host for protein production shows its potential for vaccine development and fighting influenza and other viral diseases.

1.2.4.4.2 Hepatitis B surface antigen

Hepatitis B virus (HBV) infection is a global public health concern, affecting over 250 million people worldwide, with a higher prevalence in developing countries. HBV infection causes liver diseases like cirrhosis and hepatocellular carcinoma, resulting in approximately 880,000 deaths per year. Recombinant hepatitis B surface antigen production has greatly aided HBV vaccine development.

A very recent paper utilizing the newly double knockout glycoengineered line of *N. benthamiana* investigated the impact of plant N-glycosylation on the immunogenic properties of a chimeric Hepatitis B Virus (HBV) S/L vaccine candidate produced in wild-type, compared to the *XylT* and *FucT* knockout lines of *N. benthamiana* (Pantazica et al., 2023). The study found that prevention of b-

1,2-xylose and a-1,3-fucose attachment to the HBV antigen significantly increased the immune response in mice compared to the wild-type. Notably, the antibodies made by the knockout line neutralized both wild-type HBV and a clinically relevant vaccine escape mutant more efficiently compared to the conventionally produced ones. The study validates the glycoengineered *N. benthamiana* line as a substantially improved host for plant production of glycoprotein vaccines.

This work provides evidence that glycoengineering of plants can significantly enhance the immunogenicity of plant-produced vaccines. Further research is ongoing to explore the potential of the CRISPR/Cas9 system for enhancing the immunogenicity of plant-produced antigens for human use. Using CRISPR/Cas9 in plant-based vaccine production provides an innovative avenue for developing novel, cost-effective vaccines.

2. Materials and Methods

2.1 Molecular transformation and preparation of bacteria

2.1.1 Vector Construction

The first step in viral antigen production in transgenic *Nicotiana benthamiana* plants is to create a suitable vector. Choosing the right vector is crucial for successful transgene integration and expression in the target plant. Important factors for constructing plant expression vectors include:

Promoter: The cauliflower mosaic virus (CaMV) 35S promoter is often used for high-level expression of transgenes, while tissue-specific or inducible promoters can be used for controlled or localized expression of the target gene.

Optimizing the coding sequence of the target gene for expression in the host plant, can be achieved by considering codon usage bias, mRNA stability, and intron presence. Such as changing the GC content according to different species variation. Codon optimization enhances protein expression levels.

Signal peptide: Adding a signal peptide sequence aids in the secretion of the recombinant protein into various cellular compartments, enhancing protein stability and simplifying purification.

Selection marker: A marker gene, like antibiotic or herbicide resistance genes, is needed to identify and select transformed plant cells. Reporter genes, such as green fluorescent protein (GFP), can be used to visually track transgene expression.

Termination sequence: A robust transcriptional terminator is necessary for the correct processing and stability of the transgene mRNA, like the nopaline synthase (NOS) terminator.

The following method involves the use of the pHREAC plasmid as a backbone, allowing straightforward cloning of target antigen genes and transformation of *N. benthamiana*.

Materials:

- pHREAC plasmid
- Restriction enzymes: BamHI and NotI
- Agarose gel electrophoresis system
- Luria Bertani (LB) media and agar
- Antibiotics: Kanamycin
- T4 DNA Ligase and Buffer (10X)
- Competent *E. coli* cells (e.g., DH5α)
- PCR (Polymerase Chain Reaction) reagents
- Desired viral antigen gene sequence
- Calcium chloride solution (0.1M)
- Heat block (42°C and -80°C)
- Micropipettes and sterile tips
- Primers flanking the gene of interest
- Sterile microcentrifuge tubes
- Plasmid purification kit
- Nanodrop spectrophotometer or another nucleic acid quantitation method
- A water bath set to 55°C.

Procedure: DNA Amplification

- Design primers flanking the antigen gene sequence with incorporated BamHI and NotI recognition sites at the 5' ends.
- Perform PCR for the amplification of the desired gene using the designed primers and DNA polymerase.
- Verify the PCR product on an agarose gel and extract the desired DNA band using a commercially available kit.

Procedure: Plasmid Preparation

- Grow *E. coli* cultures harboring pHREAC plasmids overnight on LB media with Kanamycin.
- Extract the pHREAC plasmid DNA from *E. coli* culture using the plasmid purification kit, following the manufacturer's instructions.

Procedure: DNA Digestion

- Digest both the PCR amplified gene and pHREAC plasmid with BanHI and NotI restriction enzymes in separate reactions.
- Verify digestion of both the plasmid and gene on an agarose gel.

Procedure: DNA Ligation

- Using T4 DNA ligase, ligate the digested PCR product into the vector at a molar ratio of insert:vector of 3:1. Incubate at room temperature for 1 hour.

Procedure: Transformation

- Transform competent *E. coli* cells with the ligation mixture by the heat shock method: Chill cells on ice for 30 minutes, heat shock at 42°C for 90 seconds, then place back on ice for 2 minutes. Recover cells in SOC medium and incubate at 37°C for 1 hour.
- Plate the transformation mix onto LB Agar plates with Kanamycin. Incubate the plates overnight at 37°C.

Procedure: Verification

- Select several colonies and grow them overnight in LB media with Kanamycin.
- Extract plasmid DNA from overnight cultures using the plasmid purification kit.
- Confirm the presence of your insert by restriction digestion and/or sequencing.

2.1.2 Electroporation of Competent *Agrobacterium Tumefaciens* Cells

Electroporation is a method to deliver foreign antigens into plant cells. It uses electrical pulses to permeabilize cell membranes.

Materials:

- Recombinant plasmid DNA
- *Escherichia coli* DH5α
- Lysogeny broth (LB) medium

- Ampicillin
- *Agrobacterium tumefaciens* strain GV3101
- Luria-Bertani (LB) medium
- Rifampicin
- Gentamicin
- Ethanol (70% v/v)
- Electroporation cuvettes (1 mm path length)
- Electroporation apparatus
- SOC (Super Optimal Catabolite Repression) medium
- Sterile toothpicks
- Solid LB medium

Procedure: Preparation of competent Agrobacterium tumefaciens

- Streak out the *Agrobacterium tumefaciens* strain from a -80°C glycerol stock onto a LB plate containing appropriate antibiotics (Rifampicin 50 mg/L, Gentamicin 25 mg/L) and incubate overnight at 28°C.
- Pick a single colony using a sterile toothpick and inoculate it into 5 ml of LB medium containing the same antibiotics. Incubate overnight in a shaking incubator at 28°C and 200 rpm.
- Subculture 1 ml of the overnight culture into 100 ml of fresh LB medium with antibiotics and grow until an OD600 of 0.6–0.8 is reached.
- Chill the culture on ice for 10 minutes, followed by centrifugation at 4°C and 4000 rpm for 10 minutes.
- Resuspend the cell pellet in 20 ml of ice-cold, sterile 10% glycerol. Repeat the cold centrifugation, wash, and resuspension steps twice more.
- Finally, resuspend the pellet in 1-2 ml of ice-cold 10% glycerol and aliquot into sterile microcentrifuge tubes. Flash-freeze in liquid nitrogen and store at -80°C until needed.

Procedure: Electroporation of Competent Cells

- Thaw one aliquot of Agrobacterium cells on ice.
- Add 1–5 µl of the desired plasmid DNA (may vary according to needs; typically use 0.1–1 µg DNA) to the cells and mix gently.
- Transfer the mixture to a pre-chilled electroporation cuvette and electroporate the cells using a pulse of 2.5 kV, 25 uF, and 200 Ω.
- Immediately add 1 ml of SOC medium to the cuvette. Pipette the mixture into a culture tube and incubate at 28°C for 2 hours with shaking at 200 rpm to allow expression of the antibiotic resistance gene present on the plasmid.
- Spread the cells onto a LB agar plate that contains appropriate antibiotics for selection and incubate at 28°C for 48 hrs.
- Check the plates for colonies bearing the plasmid of interest. Confirm successful transformation through plasmid extraction and sequencing.

After electroporation and cultivation, visible colonies on the selection plates indicate successful transformation. It's essential to confirm the presence and integrity of the plasmid in the transformed agrobacterium colonies using standard molecular biology techniques such as colony PCR, restriction analysis, and/or sequencing.

2.2 Transient Agrobacterium-mediated transformation of transgenic *N. benthamiana* plants

2.2.1 Vacuum-Based Agroinfiltration

When conducting the infiltration procedure, avoid excessive or extended exposure to vacuum, as this can damage leaf tissues. Also, ensure that the agrobacterium culture is not overly dense, as this can lead to reduced infiltration efficiency and could potentially damage the plants.

This protocol leverages the vacuum-based approach, providing a robust and efficient method for introducing foreign genes into a plant system with minimal tissue damage.

Materials:

- *Agrobacterium tumefaciens* strain GV3101
- *Nicotiana benthamiana* plants (4-6 weeks old)
- Murashige and Skoog (MS) medium
- Sterile, deionized water
- Syringe (5 ml) without needle
- 4 mM MgCl2 (magnesium chloride)
- 100 M acetosyringone
- Vacuum pump
- Dessicator with inlet and outlet for vacuum
- 30% sucrose solution
- Liquid nitrogen

Procedure: Preparation of Agrobacteria for Infiltration (Day 1)

- Inoculate a single colony of the Agrobacterium tumefaciens strain in 50 ml of liquid MS medium supplemented with appropriate antibiotics and grow overnight at 28°C with shaking (200 rpm).

Procedure: Agrobacteria Sub-Culturing (Day 2)

- Centrifuge the bacterial culture at 4000 rpm for 15 minutes.
- Then, re-suspend the bacterial pellet in 50 ml of sterile infiltration media (4 mM $MgCl_2$, 100 µM acetosyringone).
- Incubate the resuspended agrobacteria at room temperature for two hours.

Procedure: Agroinfiltration Procedure (Day 3)

- Submerge the entire plant in the infiltration medium containing agrobacteria.
- Place the submerged plants in a desiccator connected to a vacuum pump.
- Apply vacuum (around -1 bar) for 1-2 minutes until the leaves appear saturated (dark green color) under the vacuum.
- Release the vacuum slowly (about 30 seconds) to allow the agrobacteria in the infiltration medium to infiltrate the leaf tissues.
- After infiltration, place the plants upright and allow them to recover overnight under normal conditions (24°C, 16 h/8 h light/dark photoperiod).

Procedure: Evaluation of Gene Expression (Day 6-8)

- Test the level of transgene expression by appropriate means (e.g., GUS assay, detection of fluorescent proteins, or SDS-PAGE) 3-5 days post-infiltration.

2.3 Extraction and purification of recombinant viral antigens

2.3.1 Protein Analysis (Extraction of Total Soluble Protein)

At 2–6 days post-infiltration (dpi), proceed with protein extraction according to the following protocol:

Materials:

- Infiltrated *N. benthamiana* leaves
- Protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1% Triton X-100, 1X protease inhibitor cocktail)
- Pestle and mortar (pre-cooled)
- Centrifuge
- Protein quantitation assay, e.g., BCA Protein Assay Kit

Procedure:

- Harvest the infiltrated leaves and immediately freeze them in liquid nitrogen.
- Grind the frozen leaves to a fine powder using a pre-cooled pestle and mortar.
- Transfer the powder into a pre-chilled tube and add the extraction buffer in the ratio of 500 µL buffer to 100 mg leaf tissue. Mix well by vortexing.
- Centrifuge homogenates at 16,000 x g for 20 minutes at 4°C.
- Carefully collect the supernatant (soluble protein fraction) and place it in a new tube.
- Determine protein concentration using a BCA Protein Assay Kit according to the manufacturer's protocol.

2.3.2 SDS-polyacrylamide (SDS-PAGE) gels

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a laboratory technique used for the separation and analysis of proteins according to their molecular weight.

Materials:

- Vertical electrophoresis apparatus
- Microcentrifuge tubes
- Heating block or water bath
- Micropipettes and disposable tips
- Acrylamide/bis solution (30% Acrylamide/bis 29:1 solution)
- 1.5M Tris-HCl, pH 8.8
- 1M Tris-HCl, pH 6.8

- 10% (w/v) SDS
- 10% Ammonium persulfate (APS)
- Tetramethylethylenediamine (TEMED)
- 2X Sample Buffer: 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue
- 10X Running Buffer: 250 mM Tris, 1.92 mM glycine, 1% SDS
- Protein samples
- Protein marker
- Distilled water

Procedure: Gel Preparation

- Prepare the resolving gel mixture as per the desired percentage. For a 12% gel, mix 5 ml of acrylamide/bis solution, 2.5 ml of 1.5M Tris-HCl (pH 8.8), 100µl of 10% SDS, and 2.4 ml of distilled water.
- Add 100µl of 10% APS and 10µl of TEMED to initiate polymerization. Immediately pour the solution into the gel assembly, insert the comb, and let it polymerize for at least 45 minutes.
- Meanwhile, prepare the stacking gel mixture with 1.7 ml of acrylamide/bis solution, 1.25 ml of 1M Tris-HCl (pH 6.8), 50µl of 10% SDS, and 3 ml of distilled water.
- After the resolving gel polymerizes, gently remove the comb and rinse the wells with distilled water.
- Add APS and TEMED to the stacking gel mixture and pour on top of the resolving gel. Insert the comb and allow polymerization.

Procedure: Sample Preparation

- Add 2X sample buffer to each protein sample to a final 1X concentration and heat at 95°C for 5 minutes in a water bath.
- Briefly centrifuge the samples to remove condensed moisture and insoluble material.

Procedure: Electrophoresis

- Assemble the gel in the electrophoresis tank and add 1X running buffer.
- Load each protein sample and protein marker into separate wells using a micropipette.
- Run the gel at 80–120 V until the bromophenol blue from the buffer reaches near the bottom of the gel.

The exact voltages and running times may vary according to the specific apparatus and protein sizes. Always ensure the gel apparatus is correctly assembled and that safety measures are in place before starting electrophoresis.

2.3.3 Western Blot

The Western blot is a widely used technique to detect specific proteins in a sample of tissue homogenate or extract. It first uses gel electrophoresis to separate denatured proteins by mass. The proteins are then transferred onto a membrane for subsequent analytical techniques, specifically the detection of the target protein using specific antibodies.

Materials:

- PVDF or nitrocellulose membrane
- Transfer buffer
- Constant voltage power supply
- Transfer apparatus
- Whatman paper
- Primary antibody against the target protein
- Secondary antibody conjugated to horseradish peroxidase (HRP)
- 5% non-fat milk or bovine serum albumin (BSA)
- Tris-buffered saline with Tween 20 (TBS-T buffer)
- Chemiluminescent HRP substrate
- Film or digital imaging system

Procedure: Post SDS-PAGE Transfer

- Transfer proteins from the gel to a PVDF or nitrocellulose membrane using a wet or semi-dry transfer setup. The duration and condition depend on the size of the proteins and the type of transfer setup.
- Following transfer, validate successful protein transfer by staining the membrane with Ponceau S.

Procedure: Immunoblotting

- Block nonspecific binding sites on the membrane by incubating in TBS-T buffer containing 5% non-fat milk or BSA for 1 hour at room temperature.
- Incubate the membrane with the primary antibody diluted in blocking buffer; conditions usually range from 1 hour at room temperature to overnight at 4°C.
- Wash the membrane three times, 10 minutes per wash, with TBS-T to remove excessive and non-specifically bound primary antibodies.
- Incubate the membrane with HRP-conjugated secondary antibodies diluted in blocking buffer for 1-2 hours at room temperature.
- Wash the membrane again three times with TBS-T.

Procedure: Detection

- Incubate the membrane with a chemiluminescent HRP substrate and detect the signal using film or a digital imaging system according to the manufacturer's instructions.

2.3.4 Ion Exchange Chromatography

Ion exchange chromatography (IEX) is a technique used for separating ions and polar molecules based on their electromagnetic charge, which can be very useful for purifying proteins, nucleic acids, and amino acids, among others, in the field of biotechnology.

Materials:

- Ion exchange chromatography column (anion or cation exchange column)
- Sample of interest
- Buffer A: Low salt/ion strength buffer
- Buffer B: High salt/ion strength buffer
- pH meter
- Conductivity meter
- Chromatography system
- Fraction collector
- Plastic ware

Procedure: Preparation

- Depending on the sample and target molecules, choose an appropriate column with either a cation or anion exchanger.
- Equilibrate the IEX column with Buffer A until the pH and conductivity readings are consistent.

Procedure: Sample Application

- Load the prepared sample onto the equilibrated column. To prevent overloading, ensure that the sample's ionic strength isn't higher than that of Buffer A.

Procedure: Chromatography

- Begin with a low salt concentration in Buffer A. Elute the loaded sample with a linear or step gradient of high-salt Buffer B to obtain fractions, with the gradient program dependent on analyte properties.

Procedure: Detection and Collection

- Monitor the elution process by checking absorbance at a suitable wavelength, commonly 280 nm for proteins. Use the fraction collector to collect fractions from the elution volume.

Procedure: Data Analysis

- Analyze chromatograms. Retention volumes for peaks should be used for further analysis.
- Analyze the collected fractions by relevant methods, such as SDS-PAGE for proteins, to ensure the purity and identity of the fractions.

Procedure: Regeneration

- After the run, regenerate the column using Buffer B and store as per the manufacturer's instructions.

3. Conclusion

3.1 The Transformative Potential of CRISPR/Cas9 in Plant Biotechnology

The CRISPR/Cas9 system is a potent tool for precise genome editing. This technology has revolutionized plant biotechnology, allowing scientists to modify plant genomes quickly and precisely. As a result, they can develop new crop varieties with enhanced traits (Scheben et al., 2017). CRISPR/Cas9 has the potential to transform plant biotechnology, impacting crop improvement, sustainable agriculture, and regulatory frameworks.

The CRISPR/Cas9 technology in plant breeding has generated crop varieties with improved agronomic traits: enhanced yield, nutritional quality, and resistance to biotic and abiotic stresses. This is accomplished by precisely editing key genes related to these traits, resulting in minimal off-target effects and reduced linkage drag compared to traditional breeding methods. Moreover, CRISPR/Cas9's versatility enables the simultaneous editing of multiple genes, resulting in the creation of crops with multiple desirable traits in a single step.

CRISPR/Cas9 technology can aid sustainable agriculture by fostering eco-friendly crop development. Improved nutrient use efficiency in crops can decrease reliance on chemical fertilizers. Similarly, developing plants with enhanced resistance to pests and pathogens can reduce the need for chemical pesticides. CRISPR/Cas9 editing can help domesticate underutilized, climate-resilient crops, offering new food and material sources.

CRISPR technology has sparked concerns about regulations and ethics, specifically regarding the labeling of edited crops. As governments and organizations develop regulatory frameworks, it's crucial to balance safety and transparency with the benefits of CRISPR-based plant genome editing.

The success of CRISPR-based plant biotechnology relies on public perception and acceptance of genetically edited crops. Addressing safety, environmental impact, and socioeconomic consequences is crucial for building trust and promoting the adoption of CRISPR-edited crops. Transparent communication and stakeholder engagement are crucial for shaping public opinion and policy decisions.

To maximize the potential of CRISPR-based plant genome editing, scientists and regulators must tackle the outlined challenges in order to fully utilize its promising prospects. Future research should prioritize enhancing the precision and efficiency of CRISPR, minimizing off-target effects, and broadening the range of plant species and traits suitable for genetic modification. Interdisciplinary collaborations among scientists, policymakers, and stakeholders are crucial for addressing the multifaceted aspects of CRISPR-based plant biotechnology. By tackling challenges and promoting innovation, CRISPR genome editing can greatly enhance plant biotechnology and enhance global food security.

3.2 The Potential of *N. benthamiana* as a Model Plant for Recombinant Protein Production

As we conclude this review of *Nicotiana benthamiana* as a model plant for recombinant protein production, let's summarize some key points and insights.

Efficient protein production: *N. benthamiana* allows for rapid and high-yield production of recombinant proteins using transient expression systems like agroinfiltration and viral vectors. *N. benthamiana*'s quick generation time and rapid growth enable protein expression in just one week, surpassing traditional systems that typically take weeks to months.

Post-translational modifications: *N. benthamiana* enables complex eukaryotic post-translational modifications like glycosylation, phosphorylation, and disulfide bond formation. These modifications are vital for the stability, solubility, and biological activity of therapeutic proteins.

Reduced risk of human pathogen contamination: *N. benthamiana* is phylogenetically distant from mammals, minimizing the risk of contamination with human pathogens. This addresses safety concerns with mammalian cell lines, which may contain viruses or infectious agents.

Scalability and cost-effectiveness: *N. benthamiana*-based protein production is highly scalable due to its ability to be cultivated in greenhouses and its straightforward growth and maintenance requirements. Plant-based systems can be more cost-effective compared to mammalian or microbial systems. They don't need expensive fermentation equipment or growth media.

Environmental sustainability: *N. benthamiana*-based protein production systems have a lower environmental impact than traditional systems due to reduced energy, water, and waste disposal requirements compared to other fermentation-based processes.

Commercialization challenges: The commercialization of plant-produced recombinant proteins depends on overcoming regulatory hurdles and achieving cost competitive processes compared to other production platforms like bacterial, yeast, or mammalian cells. As plant-derived products gain approval for human and veterinary use, regulatory agencies will become more familiar with plant-based expression systems, potentially streamlining the approval process. Advancements in plant transformation, protein purification, and downstream processing may reduce production costs, enhancing the economic viability of plant-derived recombinant proteins.

Industrial and environmental potential: Plant-produced recombinant proteins have potential in industrial and environmental applications, alongside their use in medical and veterinary products. Plant-based expression systems can facilitate the production of enzymes for the biofuel, food, and textile industries. As research progresses, plant-based expression platforms will play a vital role in developing sustainable and eco-friendly technologies and solutions.

Conclusion: *N. benthamiana* is a powerful and versatile platform for recombinant protein production, with many advantages over traditional systems. With future advancements in plant biotechnology, *N. benthamiana* is becoming a major model organism for producing biopharmaceuticals and high-value recombinant proteins.

4. References

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Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway