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Optimisation strategies for sustainable molecular farming

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Abstract. Molecular farming is a rapidly developing field of modern plant biotechnology that can provide industrial-scale support of biologically active therapeutics for medicine and veterinary. The review aimed to identify the main strategies for improvement of the efficiency of therapeutic protein production in plants. To achieve the aim, analyses of Scopus and PubMed databases, generalisation, and critical evaluation of data regarding to strategies enhancing the efficiency of therapeutic protein production in plants were conducted. Key criteria that should be covered in selection of a vector for transformation were identified. Super-binary or tertiary vectors are recommended to optimise the genetic transformation of recalcitrant species. For transient transformation, magnICON systems and geminivirus-based vectors were proposed. The ways to overcome plant recalcitrance to the *in vitro* culture and to the genetic transformation were reviewed. Methods such as overexpression of morphogenic genes (WOX, BBM) that increase the efficiency of regeneration were mentioned. The newest gene delivery systems for precise and accurate gene insertion were reviewed. Tissue-specific, inducible, or active at certain stages of plant development, promoters are recommended to increase the expression of target proteins. The study determined that protein stability can be increased by its targeting to cell compartments or co-expression with protease inhibitors. The study also established that correction of protein post-translational modifications can improve its biological activity. Reduction in toxic secondary metabolites of the host is also among the goals. The conclusions can be used to develop new strategies for optimisation of plant transformation, increasing the yield of recombinant protein, and improving its therapeutic efficacy

Keywords: plant biotechnology; genetic engineering; plant-based expression systems; pharmaceutical proteins

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INTRODUCTION

Molecular farming is a modern technology for obtaining pharmaceutically valuable proteins in transgenic plants for medical and veterinary purposes. In contrast to other systems for expressing recombinant proteins (e.g., bacteria, yeasts, animal and insect cell cultures) plants are characterised by their ability to express physiologically active heterologous proteins, accumulate large amounts of biomass in a relatively short period of time, without the risk of contamination with pathogens to which humans and animals are susceptible. This significantly reduces the safety concerns associated with such products and the cost of their purification. However, the commercialisation of plant-derived therapeutic proteins is limited by low efficiency of existing genetic transformation protocols for an economically relevant plants, low protein yield and glycosylation pattern differences that influence the efficacy of the plant-derived protein *in vivo*.

The review aimed to improve the yield and quality of recombinant therapeutic proteins by highlighting several strategies that can be applied at different stages of the technology development. J.K. Bharathi *et al*. (2024) summarised these strategies, including optimised gene-delivery methods, appropriate vector design, use of codon-optimised sequences of the genes of interest, and the peculiarities of plant cell culture approaches. Several specific methods used in experimental studies to increase the efficiency of transformation were also investigated. The gene integration efficiency can be increased by using transposon systems, as was reported by V. Kumar *et al.* (2024). The overview by S. Kambampati *et al*. (2025) stated that plant transformation using gene editing systems (e.g., CRISPR-Cas9, zinc fingers) delivered via nanoparticles could also achieve higher gene integration accuracy. The following studies highlighted strategies aimed to increase transgene expression. M.O. Pydiura & Ya.B. Blume (2023) reported that transposons enhance the expression of transgene. The expression also increased with integration of genome elements of plant viruses into vectors (e.g., polymerases). This was demonstrated in research conducted by Ya.R. Sindarovska & M.V. Kuchuk (2024) for transient expression in *Nicotiana benthamiana*.

The improvement of transformation protocols for the recalcitrant species is also noteworthy. M. Mirzaee *et al*. (2022) discussed the strategies applied for efficient transformation of monocots, which are considered as one of the most recalcitrant species. The study mentioned that the selection of suitable promoters, codon optimisation, incorporation of 5` and 3` untranslated regions (UTRs) into mRNA to increase its stability, and subcellular targeting of the recombinant protein expression are the main strategies that increase the efficiency of the transformation. Methods for improved regeneration were also developed for recalcitrant species. Some more strategies to overcome the recalcitrance of these species were discussed. M.B. Belaffif *et al*. (2025) discussed that introduction of morphogenic regulator genes such as Babyboom (BBM), Wuschel-related homeobox (WUS), and other growth-regulating factors enhanced regeneration rates in several recalcitrant plants (e.g., apple – *Malus domestica*, rice – *Oryza sativa*). X. Han *et al.* (2025) demonstrated that various in-planta methods can be applied for recalcitrant species.

The review aimed to explore the key strategies explored to improve the efficiency of therapeutic protein production in plants. For this review, the studies published in journals indexed in Scopus were used. The systematic literature search by keywords was performed in Scopus and PubMed databases. The search was filtered by year (2000-2025). Strategies used by authors among selected articles to increase the expression or stability of recombinant proteins and improve their biological availability were analysed. The data obtained were critically evaluated, compared, and grouped into semantic sections.

VECTOR DESIGN

The transfer of the target gene into the plant genome (stable nuclear and chloroplast transformation) or into the cell nucleus for its transient expression is conducted using vectors that are transferred into the plant cell by various metods (Zhi *et al.*, 2022). For plant genetic transformation and transient expression, vectors based on *Agrobacterium tumefaciens* Ti-plasmids, *Agrobacterium rhizogenes* Ri-plasmids, and plant viruses of the genera *Bunyavirus, Potexvirus, Tobamovirus, Mastrevirus, Tobravirus, Hordeivirus, Cytorhabdovirus,* and *Nucleorhabdovirus* are used (Mahmood *et*

al., 2023). The vector choice is fundamental in the efficiency of plant transformation and target protein yield. For stable nuclear transformation, Ti- or Ri-plasmids are used; specific binary and ternary vectors were created on their basis. A binary vector was obtained by removing the genes responsible for crown gall formation found in the wild-type *A. tumefaciens* plasmid. This resulted in a system consisting of a T-DNA binary vector with the gene of interest and a helper Ti-plasmid, containing virulence genes (*vir*) necessary for the insertion of T-DNA into the nuclear genome. Such binary vectors as pBIN19, pBI121, and pPZP are widely used, as reported by T. Komori *et al.* (2007). However, the use of binary vectors demonstrates rather low efficiency for species that are difficult to transform (e.g., monocots). This led to the emergence of updated vectors with increased number of *vir* genes. In particular, these are super-binary systems, in which the T-DNA binary vector contains additional *vir* genes, and ternary vector systems containing an additional third plasmid with a *vir* region. J. Jeong *et al*. (2025) demonstrated that the use of ternary vectors for the transformation of corn, sorghum, and soybeans increases the efficiency of transformation by 1.5-21.5 times. Thus, the choice of vector for stable transformation is determined by the sensitivity of the producer to *Agrobacterium*-mediated transformation.

Chloroplast transformation and transient expression result in relatively higher protein yield compared to stable nuclear genetic transformation, but the chloroplast transformation is rather complicated, while transient expression may cause some ecological concerns. To perform chloroplast stable transformation, vectors containing flanking sequences homologous to plastid DNA regions are developed. M. Narra *et al*. (2025) demonstrated that addition of flanking sequences enabled the insertion of the target fragment to the selected site via homologous recombination. In addition, the developed vectors usually contain regulatory elements, binding sites for proteins that link RNA with expression regulators (PPR – pentatricopeptide repeat), and a selective marker (usually, spectinomycin/streptomycin resistance gene). Thus, a substantial aspect in the vector design for plastid transformation is the selection of the insertion site and the isolation of the necessary sequence for the synthesis of flanking regions.

Transient protein expression is primarily achieved by using viral vectors. The main biological limitation of virus-based systems is the sensitivity of the selected host to the virus on which the vector is based. Q. Chen *et al*. (2013) reported that an effective strategy for transient expression is the use of vectors based on deconstructed plant RNA viruses, such as the magnICON system. As reported by Y. Gleba *et al*. (2013), there are many modifications to the magnICON system, and they are based on tobacco mosaic virus (TMV), potato virus X (PVX), cowpea mosaic virus (CPMV), and bean yellow dwarf virus (BYDV) DNA. With magnICON system, several monoclonal antibodies (mAb), antigens, and other therapeutics proteins have been expressed transiently. J. Hurtado *et al*. (2020) highlighted expression of anti-Chikungunya virus mAb with TMV-based vectors (pICH21595 and pICH11599), K.T. Hamorsky *et al*. (2013) reported about anti-HIV mAb expression with single-component vector based on TMV/TVCV – pICH38099. H. Karauzum *et al*. (2012) reported that the TMV- and PVX-based vectors were used to express staphylococcal enterotoxin B, meanwhile in H. Nausch *et al*. (2012) used TMV-based pICH29912 vector and PVX-based pICH27727, pICH31160 vectors for interleukin-6 expression.

Q. Chen *et al*. (2013) noted that the use of vectors based on geminiviruses (in particular, BYDV) can be more preferable, in comparison to the magnICON system, due to a wider range of susceptible species and a shorter replication period. Furthermore, N. Pisuttinusart *et al*. (2024) used the pBYR2eK2Md (pBYR2e) vector, which demonstrated high efficiency in the expression of a anti-respiratory syncytial virus mAb. The pBYR2fp vector used by J.-H. Ha *et al*. (2017) enabled efficient expression of human fibroblast growth factor. Additionally, the effectiveness of the geminivirus-based vectors can be increased when the gene of interest is co-deliverd with a Rep/RepA-supplying vector, coding Rep and RepA proteins necessary for replication. The successful super-transformation of *N. benthamiana* plants stably producing Rep/RepA proteins under control of an ethanol-inducible promoter with a geminivirus-based vector, encoding Rep/RepA, p19 suppressor, and vitronectin under control of the same promoter (INPACT platform) was also reported by B. Dugdale *et al*. (2018).

The use of other vectors has also been reported. In particular, D.A. Morris *et al*. (2021) determined that agroinfiltration of *N. benthamiana* with a TMV-based vector (GENEWARE system) yielded significantly higher amounts of cholera toxin subunit B compared to previous studies. Similar observations were made by A. Varsani *et al*. (2006) in the case of human papillomavirus (type 16) L1 protein expression. The PVX-based vectors are used for the efficient expression of human fibroblast growth factor (vector pgR107) in J. Liu *et al*. (2007) research, human granulocyte-macrophage colony-stimulating factor (vector pPVXG1) in F. Zhou *et al*. (2006), and keratinocyte growth factor 1 (pgR107) in Z.-G. Feng *et al*. (2014) researches. Thus, the choice of vector type for plant transformation should be based on the selected transformation method, the plant's sensitivity to *A. tumefaciens* (in the case of stable nuclear transformation) and viruses (in the case of virus-based transient expression). For chloroplast transformation, it is necessary to determine correctly the site of gene integration to use a suitable flanking sequence.

TRANSFORMATION METHOD OPTIMISATION

The choice of delivery method for various genetic constructs determines the overall effectiveness of plant transformation. Physical, chemical, and biological methods of genetic information transfer are utilised depending on the type of transformation (stable, transient) and the characteristics of the host. Stable transformation is applied to obtain producers that can inherit and transmit the target gene for the protein of interest to subsequent generations. Such expression results in comparatively low protein yield. Therefore, this method is used for plants with high biomass production. In contrast, transient expression demonstrates higher protein yield during a short period of time, but there is a need for repetitive rounds of plant treatment to obtain the target product.

Optimisation of transformation methods can relate to both the selection of optimal conditions for DNA transfer and to the improvement of plant regeneration during stable transformation. For example, callus cultivation in pneumatic bioreactors has been proposed by S.S. Barretto *et al*. (2017) to increase the regeneration frequency of tobacco callus expressing the C subunit of tetanus toxin. Another strategy for improvement of plant regeneration is the induction of overexpression of transcription factors. The WUSCHEL-related homeobox (WOX) transcription factors are essential plant growth and development regulators. In a study on *N. tabacum* overexpressing the *MaWOX* gene isolated from mulberry, conducted by H. Zhou *et al*. (2025), an increase in regeneration rates and the *Agrobacterium*-mediated transformation efficiency was demonstrated. J.A. Teixeira da Silva (2005) reported that *Agrobacterium*-mediated treatment with sonication promotes shoot regeneration from transverse thin cell layers as explants. J. Zhang *et al*. (2025) reported that the regeneration rate increased while overexpressing BBM morphogenic genes under constitutive and somatic embryogenesis-specific promoters. For genetic transformation, the expression of BBM morphogenic genes under inducible promoters is preferential, as their constitutive expression results in developmental abnormalities and regenerant mortality was highighted by S.D. Bakulin *et al*. (2025). The study by D.U. Santos-Ballardo *et al*. (2019) on cell pre-treatment with 0.5 M sorbitol prior to particle bombardment demonstrated optimised transformation efficiency rate at 60%. Improved transformation rate in chloroplast transformation with biolistic could be achieved through increasing the size of plastids. Research with overexpression of the *FtsZ1* gene (regulating chloroplast division) in transgenic *Solanum tuberosum* increased organelle size (generating macro-chloroplasts), as was previously reported by A. Occhialini *et al*. (2020). Such modified plants can be a suitable hosts that produce pharmaceutical proteins.

However, new methods of DNA delivery into plastids have been proposed. For instance, A. Matsuoka & P. Maliga (2021) highlighted an approach to edit the *VirD2* and *VirE2* genes of *A. tumefaciens* to use female gametocytes as explants in the chloroplast transformation with the floral dip protocol. Genetically engineered *A. tumefaciens* integrates T-DNA into the plastid because of the removal of nuclear localisation signals, and fusion of the C-terminus of the target protein with VirF Type IV secretion system signal. Another method proposed by C. Thagun *et al*. (2019) is based on the self-assembled complex, containing DNA, cell penetrating peptide (CPP), and chloroplast targeting peptide (CTP), delivered via syringe/vacuum

infiltration, microinjection into leaves, roots, fruit parts, and tubers. One of the methods for the genetic transformation of recalcitrant plants developed by A. Jakubiec *et al*. (2021) is the creation of "minichromosomes". They are based on the beet curly top geminivirus (BCTV) and contain the Rep protein required for vector replication, which recognises specific VOR sequences (viral origin of replication), flanking the transgene. For chloroplast introduction, cognate VORs flanking the chloroplast cassette were used. This vector was delivered biolistically and amplified independently of the chloroplast genes. The study also reported the case of episomal plasmid usage, which can be used for recombinant protein expression in chloroplasts without integration into plastid DNA proposed by A. Occhialini *et al*. (2024). Similarly to classic chloroplast transformation, this approach can be used for stable expression of multiple genes. Previous research noted that carbon nanotubes (CNT) could be used as novel carriers for recombinant DNA for plant transformation. As discussed by O.M. Burlaka *et al*. (2015), CNTs have higher cell-penetrating properties, enabling the delivery process tracking and non-covalent loading of the DNA. Thus, the biological properties of recombinant DNA provide no changes.

The various strategies can be used to increase the efficiency of transient expression. J.F. Buyel & R. Fischer (2012) demonstrated that protein yield depends on concentration of *Agrobacterium* cells, temperature, plant, leaf age and its position. The optimal cell density for *N. tabacum* transformation is 0.13-2 at OD_{600}, plant age – 6-7 weeks after seeding. This was supported by Y.V. Sheludko *et al*. (2007). The study determined that for *Nicotiana* species optimal leaf age for treatment is 1-6 days, and the plant age – 8-10-week-old. To increase the rate of transient expression, many more approaches can be applied. K. Norkunas *et al*. (2018) summarised that addition of the virus-derived elements into the vector (e.g., CMV 2b, TBSV p19) decreased post-translational gene silencing effects; addition of molecules that enhance transformation rate (acetosyringone, vanillin, cinnamic acid), reduce of oxidation stress (e.g., PVP, lipoic, ascorbic acid) and also addition of surfactants (e.g., Silwet-L77, Pluronic F-68, Tween 20) into a co-cultivation or infiltration medium, and heat shock treatment of the plants after transformation enhanced transient expression rate. Thus, treatment of 6-week-old leaf plants with Silwet L-77 (optimal concentration – 0.001%, as higher concentration caused leaf damages), resulted in 60-80% transient expression ratio as has reported by Y. Zhang *et al*. (2020). H. Zhao *et al*. (2017) additionally mentioned that agents which reduce DNA methylation (e.g., 5-azacytidine), also increased the efficiency of expression.

Several optimised transient expression systems such as AGROBEST (*Agrobacterium*-mediated enhanced seedling transformation), FAST (Fast Agro-mediated Seedling Transformation), and magnICON were proposed by Y. Gleba *et al*. (2005). The AGROBEST method is based on the use of a disarmed *Agrobacterium* strain with pre-induced vir-genes and an optimised culture medium, resulting in a 100% transformation rate of seedlings. It is necessary to maintain a stable pH of 5.5, as it suppresses calcium ion uptake, thus inhibiting immunity against *Agrobacterium* infection. The FAST method is based on the co-cultivation of seedlings in the medium with surfactants. The absence of specialised equipment renders this method simple and low-cost, and it can also be applied to some plants recalcitrant to *Agrobacterium* infection, such as rice. The magnifection technology (magnICON) is based on *Agrobacterium*-mediated delivery of viral DNA vector encoding RNA viral replicons, which are later spread cell-to-cell within the plant. This method is fast and does not require specialised equipment, while providing high transformation efficiency and expression level (Gleba *et al*., 2005).

Therefore, the choice of transformation method is one of the steps that determines the efficiency of obtaining a producer with the desired characteristics. Optimisation of stable transformation methods mainly includes strategies that improve gene integration into the genome and improve regeneration frequency. Optimisation of transient expression involves creation of conditions that increase the permeability of the explant to *Agrobacterium*, thereby increasing the entry of vectors into the plant.

STRATEGIES FOR OPTIMISATION OF GENE EXPRESSION AND PROTEIN TRANSLATION

Translation is a subtantial step for the expression of a recombinant protein. M. Beihaghi *et al*. (2018)

in with a study on transient expression of CCL21 protein highlighted the Kozak sequence and ER retention signal (SEKDEL) to the upstream region of codon-optimised target gene sequence. The strategy enhanced translational efficiency and protein accumulation in plants. M.O. Pydiura & Ya.B. Blume (2023) reported that the inclusion of introns is central in the enhancing of the target protein expression. The study suggested that introns may contribute to the stabilisation of mRNA or improve its processing, thereby increasing the overall efficiency of protein synthesis.

In some cases, the use of constitutive promoters in transplastomic plants negatively affects the expression of the protein of interest and the host's physiology. Such observations were made by J.I. Arevalo-Villalobos *et al*. (2020) when expressing vaccine candidates against multiple sclerosis (BV5S2, BV6S5, and BV13S1) under the constitutive promoter. The constitutive expression of these proteins was toxic for host plants, and successful expression was achieved by the Algevir expression system (geminivirus-based vector with inducible AlcA promoter from *Aspergillus nidulans*). The last one is based on the geminivirus elements with an ethanol-inducible promoter, which leads to regeneration and stable expression of the peptides. K. Saba *et al*. (2019) demonstrated that tuberculosis antigen ESAT-6 expressed under T7 ethanol-inducible promoter in plastids caused no pleiotropic effects. Therefore, the use of inducible promoters is recommended for the expression of pharmaceutical proteins in transplastomic hosts.

The choice of promoters can influence the success of transient expression. Strong promoters accelerate efficient protein synthesis, like those isolated from cotton GhEF1A8 with a 5'UTR region. B. Sun *et al*. (2016) demonstrated that GhEF1A8 provides higher expression rates of the target gene compared to the CaMV 35S promoter. For edible plants (e.g., tomato – *Solanum lycopersicum*), an effective solution is the use of promoters involved at certain stages of plant development. N. Kurokawa *et al*. (2013) proposed to use the E8 promoter, which becomes active during fruit ripening and results in higher protein accumulation. The study showed that protein yield in fruits was at least 4-times higher with E8 promoter, compared to CaMV 35S promoter. This can be explained by the fact that CaMV 35S promoter reduces its activity during fruit ripening, thus resulting in lower yields that was demonstrated by S.H. Park *et al*. (2022) in with a study on colorectal antigen expression in tomato fruits. The analysis of expression revealed that protein expression was higher in leaves, compared to fruits, therefore the researches also suggest using promoters with high activity when fruit ripening. Another approach to increase the efficiency of the biosynthesis is the editing of regulatory elements. J. Limkul *et al*. (2015) reported that usage of 5`UTR from *Arabidopsis* alcohol dehydrogenase, along with HSP (heat shock protein) terminator increased the yield of β-glucocerebrosidase up to 2 times. S. Kanagarajan *et al*. (2021) demonstrated that addition of the Kozak sequence into the 5`UTR region and 12-amino acid linker could improve expression of heterodimeric proteins (e.g., hemoglobin), providing additionally correct folding and stability of the protein. E. Frangedakis *et al*. (2021) reported that gene expression could be greatly increased due to the presence of an mRNA site for pentatricopeptide repeat (PPR), which stabilises mRNA. Such stabilisation occurred due to protection against exoribonuclease degradation, which can increase protein yield up to 15% of the TSP.

There are several studies in which increased efficiency of translation was achieved when plant viral elements and RNA silencing suppressors were inserted into the vector. P. Habibi *et al*. (2018) demonstrated that insertion to the vector of the p19, p1, p0 genes, which prevent gene silencing, resulted in higher levels of griffithsin (used as a potential HIV entry inhibitor) expression. S. Kanagarajan *et al*. (2021) demonstrated that p19 suppressor was effective for transient expression of human fetal hemoglobin with the p19 gene solely. M.-C. Jiang *et al*. (2019) highlighted the efficiency of p19 suppressor and ER retention signal co-expression with human interferon gamma. Transient co-expression of human iduronate-2-sulfatase with p38 suppressor and folder enhancer (human caltreticulin fused with GB1 – streptococcal protein G, domain B1), also demonstrated high protein yield production in *N. benthamiana*. K. Matsuo & T. Matsumura (2017) proved that transgenic *N. benthamiana* plants with repressed DCL2 and DCL4 genes, which mediate foreign RNA cleavage, produce larger amounts of acidic human fibroblast growth factor transiently, compared to wild-type

plants. Targeting protein expression to cellular compartments is also an effective strategy to increase yield. A. Sorrentino *et al*. (2005) noted that for human tissue transglutaminase, higher yields (up to 2.5 times) were observed when the protein was fused with the apoplast targeting signal. Similar observations were made by S. Singhabahu *et al*. (2015) for adenosine deaminase expressed in tobacco BY-2 suspension cells. In this case, the protein yield increased up to 336 times compared to the cytosol-directed proteins.

Apoplast targeting in S. Wirth *et al*. (2004) was noted to provide a fourfold increase in expression for the epidermal growth factor in *N. benthamiana*. However, for some proteins, the apoplast targeting was inefficient. As reported by N.R. Nair *et al*. (2014) for human granulocyte colony stimulating factor stably expressed in BY-2 cells and targeted to ER, on the contrary, increased protein yields up to 4 times compared to apoplast targeting. Moreover, in the case of β-glucuronidase, addition to the E7 protein and targeting of expression into thylakoid lumen, M. Morgenfeld *et al*. (2014) reported up to 30-40 times higher protein expression. As was previosly reported by J. Patel *et al*. (2007), fusion of elastin-like polypeptides (ELP) with interleukin-10 not only enhanced the purification efficiency but also increased transient or stable expression of the target proteins in tobacco leaves.

Thus, the efficiency of expression and translation can be improved by various methods. To improve translation, it is recommended to optimise the codons of the target gene and use a co-expression strategy with genes that regulate mRNA degradation. Expression can be improved by selecting a promoter that considers the characteristics of the transformation method (for stable transformation, inducible promoters are recommended), the site of protein accumulation, and the stage of plant development at which synthesis will occur. Protein expression can also be improved by fusion with various proteins that direct protein expression to the desired cellular compartment.

IMPROVEMENT OF PROTEIN STABILITY AND ENHANCING THE BIOAVAILABILITY OF RECOMBINANT PROTEINS

Final protein yield is greatly influenced by stability in plants. Increased protein stability is frequently achieved by co-expression of protease inhibitors or auxiliary proteins, which stabilise the target protein and protect it from proteolysis. It is known that if the protein is expressed in the cytosol, it can be degraded by proteases. M. Niemer *et al*. (2014) showed that co-expression of the protein with protease inhibitors (e.g., tomato cystatin 9, tomato cathepsin D), or, in the case of transient expression, by using young leaves, which tend to have lower protease content, the protein yield could be increased up to 40%. Fusion of interleukin-2 with different protease inhibitor proteins (e.g., CMTI – *Cucurbita maxima* trypsin inhibitor 1) resulted in a protective effect against trypsin activity. Protease level decreased when suspension culture of tobacco cells was immobilised in the alginate, resulting in the rise of protein yield up to 33-fold. This was demonstrated by K. Nakashima *et al*. (2013) on NT1 cells expressing human alkaline phosphatase.

Another strategy to protect proteins from proteolysis is directing protein translation into the endoplasmic reticulum (ER). Hence, the addition of ER retention signal at the C-terminus (C-KDEL signal) could enhance protein expression in *N. tabacum*. This was shown in A. Bidarigh Fard *et al*. (2019) research with etanercept (a recombinant fusion protein of TNFR2 receptor with the Fc portion of human IgG1 used for autoimmune diseases treatment) expression. Similar observations were made by N. Gutierrez-Valdes *et al*. (2024) for anti-HIV Fc fusion proteins, and by K. Deepa *et al*. (2013) for human platelet-derived growth factor BB. B. Dugdale *et al*. (2018) in the study on vitronectin transient expression in *N. benthamiana* also showed that ER localisation of the protein enhances its accumulation. Similar observations were made by M. Mazalovska & J.C. Kouokam (2020). The study demonstrated that addition of ER retention signal to lectin III, enhances protein expression up to 3 times, when transiently expressed in *N. benthamiana*. However, some recombinant proteins may be unstable in ER due to folding defects in this compartment (e.g., Nef protein of the HIV). M. de Virgilio *et al*. (2008) proposed fusing of the target protein (Nef) with zeolin that forms protein bodies in which protein is more stable. Some proteins when their expression is targeted in ER, may lose their biological activity. This was reported by A.P. Soni *et al*. (2022)

when expressing tumor growth factor β1 and fusing ER retention signal with C-terminus. Thus, they suggest that the optimal strategy for such proteins is chloroplast transformation.

Another substantial factors in molecular farming are bioavailability and therapeutic efficacy of the recombinant plant-derived proteins. Proteins derived from plants may have reduced efficacy due to differences in post-translational processes, especially – glycosylation. To overcome this problem I. Andrasevic *et al*. (2025) proposed the strategy of recombinant protein expression in CRISPR-Cas9 edited *N. benthamiana* plants with knock-down genes of β-1.2-xylosyltransferase and α-1.3-fucosyltransferase, responsible for plant-specific protein N- glycosylation pattern. In case of Chikungunya virus antigen expression, J. Hurtado *et al*. (2020) reported that glycoengineered plants showed slightly higher productivity compared to wild-type plants. Another research in *N. tabacum* BY-2 suspension cells, performed by K. Fujiyama *et al*. (2007) addressed co-expression with β-1.4-galactosyltransferase gene, which resulted in "humanised" N-glycosylation pattern monoclonal antibody. Similar results were obtained by C. Navarre *et al*. (2017) for IgG antibody, "humanised" protein. The researchers expressed erythropoietin in tobacco plants.

For proteins that require an O-glycosylation pattern, the strategy of co-expression with genes responsible for *N*-acetylgalactosamine synthesis can be used, as was previously reported by I.A. Ramírez-Alanis *et al*. (2018). J. Jez *et al*. (2013) showed the successful case of sialylated human erythropoietin expression in plants. This was achieved by the addition of six mammalian genes responsible for sialylation into transformation vectors. To achieve improved efficiency of the plant-derived therapeutic proteins, the latter can be fused with specific peptides or other receptor-binding ligands, which increase their affinity to the target receptor and permeabilises the blood-brain barrier for the therapeutic drug delivery. This was demonstrated via fusion of myelin basic protein with cholera toxin B subunit (CTB) for Alzheimer's disease treatment, performed by N. Kohli *et al*. (2014). P.S. Lakshmi *et al*. (2013) noted that chloroplast-delivered *Mycobacterium tuberculosis* antigens (ESAT-6, Mtb72F) fused with CTB and cell wall protein LipY demonstrated that such fusion resulted in higher immunogenicity and the possibility of the development of low-cost oral-delivery vaccines.

The production of oral therapeutic agents in some plant species is limited due to the presence of toxic secondary metabolites. This is characteristic of the Solanaceae family species, which are often used for genetic transformation. Therefore, new strategies are being developed to reduce the expression levels of certain toxic secondary metabolites. The study by J. Jeong *et al*. (2024) highlighted the possibility ofnicotine reduction by induction of mutation in BBL and A622 genes by using the CRISPR-Cas9 gene editing system. K.D. DeBoer *et al*. (2011) reported that lowered nicotine content can be achieved via RNA interference method. D. Li *et al*. (2006) highlighted the expression of B-chain of insulin fused to CTB in tobacco plants with reduced nicotine content. Several metabolic engineering strategies were also applied to *S. tuberosum*. L.V.T. Shepherd *et al*. (2015) noted the engineered *S. tuberosum* plants with reduced content of glycoalkaloids, which was achieved via down-regulation of glycosyl transferases.

Increasie of protein stability in plants is a promising area of research, as the final product yield is highly dependent on this parameter. Even with the right choice of producer, transformation method, vector, and additional strategies that promote effective protein expression, protein degradation in the cell will significantly reduce the efficiency of the entire biosynthesis process. The study discussed that protein stability can be increased by targeting the endoplasmic reticulum and chloroplasts through the addition of signal sequences. Co-expression of target proteins with protease inhibitors is also possible. Bioavailability is also a critical parameter in the development of therapeutic protein biosynthesis technology. The main areas of work in this field are the "humanisation" of post-translational modification patterns and the minimisation of the expression of toxic plant secondary metabolites.

CONCLUSIONS

Molecular farming is a promising area of modern plant biotechnology. However, active development is limited by the recalcitrance of many plant

species to existing genetic transformation protocols. Therefore, the analysis of strategies that can increase the efficiency of transformation and the yield of the target protein from the producer is a substantial and relevant research area. The efficiency of therapeutic protein expression in transgenic plants depends on several factors: vector selection and its design, transformation method, translation rate, mRNA and protein stability in the host cell, and the presence of correct post-translational modifications. For the correct selection of the vector, it is necessary to choose the proper transformation method for the biological characteristics and demands of the selected host. It is recommended to use super-binary or ternary vectors with Ti-plasmid-based ones for species recalcitrant to *Agrobacterium*-genetic transformation. In other cases (for example, transformation of dicotyledonous plants), effective transformation can be achieved using binary vectors. The choice of viral vector should be based on the sensitivity of the selected host to the applied virus.

The choice of transgene delivery method also depends on the biological characteristics of the host. As a result, plants with high biomass yield are rationally used for stable transformation, while plants with a short life cycle, relatively low biomass yield, and high sensitivity to plant viruses are used for transient transformation. The choice of optimisation strategy is aimed at improving regeneration, reducing the negative effects of possible interference between target protein synthesis and basic metabolism (stable transformation), and creating favorable conditions for the penetration of a sufficient number of vectors into the plant cell (transient transformation). Optimisation of transcription, translation, and post-translational modification levels should be considered at the vector design stage. To increase the translation rate, a strategy of codon optimisation, co-expression of the target protein with RNA silencing suppressors, and editing of gene regulatory elements is used. At the stage of vector design, it is possible to increase future protein stability by adding signal molecules directing protein expression to the proper cellular compartments. It is necessary to consider the post-translational modifications of the target protein that are essential for its activity *in vivo*.

For proteins with activity determined by certain patterns of glycosylation, sialylation, and other modifications, the need for additional genes or silencing of certain plant genes is considered at the vector development stage. The immunogenicity of therapeutic proteins can be enhanced by fusion of the target protein with peptides that have adjuvant properties or increase the affinity of the protein to the receptor. In addition, the bioavailability and safety of the protein can be increased by altering the secondary metabolism of the plant hosts to reduce the expression of toxic secondary compounds. Maybe soon specially edited plants amenable to genetic transformation producing high yields of correctly glycosylated pharmaceutical proteins without the necessity of complicated protein purification will appear.

Further development of more effective protocols for the genetic transformation and gene editing approaches of cereals, legumes, etc, is required. The stability of recombinant protein storage due to their low moisture and protease content and high protein yield in seeds of these hosts can increase the recombinant protein production. Therefore, molecular strategies to increase plant host regeneration or attempts to increase the bioavailability of obtained proteins are relevant areas of future research. The search for new hosts without harmful secondary metabolites for transient expression of therapeutic proteins and novel vector design is relevant.

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CONFLICT OF INTEREST

None.

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Стратегії оптимізації сталого молекулярного фермерства

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Анотація. Молекулярне фермерство – це галузь сучасної рослинної біотехнології, яка швидко розвивається та дозволяє експресувати біологічно активні терапевтичні засоби для медицини та ветеринарії у промислових масштабах. Метою цього огляду був пошук основних стратегій, що застосовуються для підвищення ефективності виробництва терапевтичних білків в рослинах. Для досягнення мети було застосовано аналіз баз даних Scopus та PubMed, узагальнення та критичну оцінку даних щодо стратегій, що підвищують ефективність виробництва терапевтичного білка в рослинах. Було визначено ключові критерії, які слід враховувати під час вибору вектора для трансформації. Для оптимізації генетичної трансформації стійких видів рекомендуються суперподвійні або третинні вектори. Для транзієнтної трансформації запропоновано системи magnICON та вектори на основі гемінівірусів. Розглянуто способи подолання стійкості рослин до культури *in vitro* та генетичної трансформації. Згадано такі методи, як надекспресія морфогенних генів (WOX, BBM), що підвищують ефективність регенерації. Розглянуто новітні системи доставки генів для точного та правильного введення генів. Для збільшення експресії цільових білків рекомендуються тканинно-специфічні, індуцибельні або активні на певних стадіях розвитку рослин промотори. Визначено, що стабільність білка можна підвищити шляхом його таргетування в клітинні компартменти або коекспресії з інгібіторами протеаз. Також в ході дослідження визначено, що корекція посттрансляційних модифікацій білка може покращити його біологічну активність; зменшення токсичних вторинних метаболітів хазяїна також є однією з цілей. Висновки можуть бути використані для розробки нових стратегій оптимізації трансформації рослин та збільшення виходу рекомбінантного білка

Ключові слова: біотехнологія рослин; генетична інженерія; рослинні системи експресії; фармацевтичні білки