Trends in Biotechnology

Engineering the Plant Secretory Pathway for the Production of Next-Generation Pharmaceuticals

--Manuscript Draft--

Manuscript Number:	TIBTECH-D-20-00008R1
Article Type:	Review
Keywords:	Biologics, folding, glycosylation, cleavage, chaperones, proteases
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Abstract:	Production of biologics in plants, or plant molecular pharming, is a promising protein expression technology that is receiving increasing attention from the pharmaceutical industry. In the past, low expression yields of recombinant proteins and the realization that certain post-translational modifications may not occur optimally limited the widespread acceptance of the technology. However, molecular engineering of the plant secretory pathway is now enabling the production of increasingly complex biomolecules using tailored protein-specific approaches to ensure their maturation. These involve the elimination of undesired processing events, and the introduction of heterologous biosynthetic machinery to support the production of specific target proteins. Here we discuss recent advances for the production of pharmaceutical proteins in plants, which leverage the unique advantages of the technology.

- Plants are an alternative pharmaceutical manufacturing platform with unique advantages compared to conventional technologies.
- Engineering the secretory pathway in plants enables the production of biologics that would otherwise accumulate at low levels or in an improperly processed form.
- Host proteases can be inactivated by co-expressing broad-spectrum protease inhibitors or by manipulating the pH along the secretory pathway.
- Glycoengineering strategies enable the production of human-like glycoforms that can be tailored to improve their biological activity.
- Introducing heterologous chaperone machinery improves the production of target proteins where the endogenous machinery does not efficiently mediate folding.
- Furin processing and tyrosine sulfation can be achieved *in planta* by introducing the required biosynthetic machinery.

Engineering the Plant Secretory Pathway for the Production of Next-Generation Pharmaceuticals

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Abstract

Production of biologics in plants, or plant molecular pharming, is a promising protein expression technology that is receiving increasing attention from the pharmaceutical industry. In the past, low expression yields of recombinant proteins and the realization that certain post-translational modifications may not occur optimally limited the widespread acceptance of the technology. However, molecular engineering of the plant secretory pathway is now enabling the production of increasingly complex biomolecules using tailored protein-specific approaches to ensure their maturation. These involve the elimination of undesired processing events, and the introduction of heterologous biosynthetic machinery to support the production of specific target proteins. Here we discuss recent advances for the production of pharmaceutical proteins in plants, which leverage the unique advantages of the technology.

Plant molecular pharming of pharmaceuticals

Producing complex biologics in plants, or plant molecular pharming, has been proffered as a cheap protein production technology with lower infrastructure requirements compared to conventional mammalian cell expression systems, an improved safety profile and the potential for rapidly scaling up production. These features make this technology highly attractive for manufacturing in resource-limited regions and for rapid vaccine production in response to pandemic disease outbreaks, or even bioterrorism threats. However, in practice the technology has had limited impact in these areas, and instead has been largely confined to niches where the accepted paradigm of pharmaceutical production has failed to satisfy market demands [1]. Retrospectively, use of this technology has been hindered by a myriad of factors including concerns regarding the safety of plant-specific glycoforms, an unclear regulatory pathway and limited capacity for scale up which have now been addressed [2].

The idealistic notion of cheap, oral vaccines that was once the major driving force of the technology has largely been replaced by the acceptance that plant-made pharmaceuticals (PMPs) will require some level of formulation to ensure consistency of dose and activity. Another paradigm shift is the favouring of transient expression systems such as "agroinfiltration"-mediated expression (see Glossary) over the use of transgenic plants, which was the major driving force of the technology during its infancy [3]. This is largely due to the speed and convenience of transient expression systems, which allow large-scale production in

weeks rather than years, and product yield is also generally better via transient expression [4]. Given the lack of a clear regulatory pathway for PMPs, it is unsurprising that the first US Food and Drug Administration (FDA)-approval for a plant product was for a veterinary vaccine against Newcastle disease virus in 2006, produced in transgenic tobacco suspension cells [3]. It was only in 2012 that the first PMP was approved for human use; this was a recombinant human enzyme (glucocerebrosidase) for treatment of Gaucher's disease that was produced in carrot cell suspension cultures [5]. More recently, there has been an increasing acceptance of *Nicotiana benthamiana* as the preferred expression host due to its ease of transformation, the development of versatile expression vectors for transient expression, rapid generation of biomass and a defective posttranscriptional gene silencing system [6].

Subsequently, many other PMPs have been entered into clinical testing, including antibodies for the therapeutic treatment of cancer and infectious diseases, recombinant enzymes for use as replacement therapies, and vaccines for both seasonal and pandemic influenza. The production of influenza vaccines is an especially promising application for plant molecular pharming due to the slow production time and limited manufacturing capacity for traditional egg-derived influenza vaccines [7]. The pioneering work of Medicago Inc. in this area has demonstrated the almost unprecedented capacity of the platform for rapid manufacturing and production scale-up, by producing 10 million doses of a candidate H1N1 vaccine within a month of receiving the sequence information [8, 9]. Most importantly, the vaccines – composed of heavily glycosylated viral hemagglutinin – were reported to be safe even in individuals with pre-existing allergies to plant-specific glycoepitopes [10]. Following these promising results, it was announced that Health Canada had accepted a New Drug Submission for a quadrivalent influenza vaccine from Medicago Inc, and that it was anticipated that the vaccine would be commercially available before the start of the 2020 flu season in Canada. This represents an unprecedented success in the field, and yet it still received considerably less attention than the emergency authorization of a cocktail of plant-produced monoclonal antibodies to treat Ebola virus disease. Although this treatment did not meet the threshold for efficacy, it highlighted the potential of PMPs on a global stage for the first time [11].

Several good manufacturing practice (GMP)-compliant factories have also now been established for the production of PMPs, including contract manufacturing organizations. This is a major advance given that much of the pioneering work in the field was conducted in academia and the lack of suitable manufacturing facilities impeded the clinical testing of these products. The development of improved expression technology has also resulted in drastic

improvements in the yields of many recombinant proteins, enabling the clinical testing of pharmaceutical products which otherwise would not be feasible for further development. A major advance in this regard is the exploitation of *A. tumefaciens* to deliver virus-derived expression vectors for recombinant protein expression in plants [12]. These "deconstructed" viral expression vectors support the carefully regulated co-expression of multiple proteins—by varying the bacterial inoculum used for infiltration—and rapid production scale up that is a major selling point for molecular farming [13].

Over the last decade, however, it has become apparent that low accumulation or inadequate biological activity of some proteins produced in plants may be related to the inefficiency of specific processing events during their synthesis. This observation has resulted in another paradigm shift: this time instead of empirical optimization of expression parameters, researchers are focussing on developing new host engineering approaches to facilitate the production of specific target proteins. The low accumulation of certain proteins *in planta* due to differences along the secretory pathway, compared to their usual expression hosts, is an important contributory factor in the reluctance of mainstream "pharma" companies to adopt molecular pharming technology [2]. Accordingly, this review will discuss the potential for engineering the processing pathways to alleviate these bottlenecks and improve the production of complex pharmaceuticals.

Preventing proteolysis of heterologous proteins along the secretory pathway

The extensive plant protease repertoire poses a challenge for the recovery of high yields of recombinant proteins that are susceptible to proteolysis. The *N. benthamiana* proteome contains 1243 putative proteases, although it is acknowledged that not all of these will impact molecular pharming as many are organelle-specific and do not have activity in leaves [14, 15]. Papain-like Cys proteases, subtilases, and pepsin-like Asp proteases are believed to be the main proteases that are responsible for proteolysis of recombinant proteins in plants, as they are abundant in plant leaves and display broad substrate recognition [14]. The impact of host proteases has been well documented for monoclonal antibodies which often display clear evidence of proteolysis in plants, but they are also assumed to contribute to low yields reported for other recombinant proteins [16, 17]. A recent investigation into the *N. benthamiana* response to agroinfiltration suggests that an increase in proteolysis may actually occur as part of the host immune response to *A. tumefaciens* [15]. Several strategies have been proposed to limit proteolytic degradation *in planta:* these include targeting recombinant

proteins to organelles where they are sequestered from host proteases, engineering of the protein sequence to remove susceptible sites, targeted knockdown of specific host proteases and the co-expression of broad-spectrum protease inhibitors (PIs). The latter approach is the fastest and may be the most feasible as it enables the temporal depletion of target proteases without compromising the development of the host, and could be applied to the production of diverse classes of recombinant proteins.

This strategy yielded limited success until recently; however, 3 new PIs (NbPR4 and NbPot1 from *N. benthamiana* and human HsTIMP) were reported to dramatically increase the accumulation of α-galactosidase, erythropoietin and the broadly neutralizing HIV antibody VRC01 in *N. benthamiana*. Interestingly, despite targeting unrelated classes of proteases, no synergistic affect was observed when all 3 PIs were co-expressed. More importantly, these new PIs had a far greater impact than any of the PIs tested previously, and although the effect was very much protein-dependent, the authors observed as much as 27-fold improvement in protein yield under the conditions tested [18].

Perhaps unsurprisingly, proteolytic activity along the secretory pathway is pH-dependent, suggesting that modifying the pH could be used to mitigate proteolytic degradation [19]. The expression of the influenza virus M2 proton channel protein in plants enables a localized pH increase in the Golgi apparatus, which has been reported to improve the accumulation of acid-labile proteins, including certain influenza virus hemagglutinins [19, 20]. It has not yet been established if this has an impact on protein glycosylation or other processing events in the Golgi, which are enzyme-mediated processes and therefore influenced by the pH. The potential synergy of this approach with PI co-expression has been also proposed, but is not yet reported in the literature [19].

Production of human-like glycosylation *in planta*

The majority of PMPs - including antibodies, hormones and many vaccine antigens - are glycosylated, and the attached glycans play diverse roles. N-glycosylation, a major type of protein glycosylation, links glycans to asparagine residues [21]. The N-glycans influence processes such as protein folding, quality control, protein trafficking and the interaction with other proteins. For example, specific types of human IgG1 N-glycans impact the binding to different Fcγ receptors and thus modulate the immune response [22]. While the first steps of N-glycosylation and N-glycan processing in the ER are in principle conserved between mammals and plants, N-glycan processing steps differ considerably in the Golgi apparatus,

which is the cellular site for complex N-glycan formation. Compared to humans, the structural diversity of N-glycans in plants is drastically reduced, which is related to the fact that they have fewer glycosyltransferases that elongate complex N-glycans. Nonetheless, plants produce some non-human N-glycans that may have adverse effects on recombinant biopharmaceuticals, and limit the broad use of plants as an alternative to mammalian cell lines for pharmaceutical production. Plant-produced recombinant glycoproteins carry α1,3-fucose and β1,2-xylose residues attached to the common core N-glycan (GnGnXF - **Figure 1A**). These two sugar residues are not found on human glycoproteins and are therefore potentially immunogenic. Consequently, the initial glyco-engineering approaches in plants focused on the elimination of these plant-specific N-glycan modifications [23], which recently resulted in CRISPR-Cas9 engineered *N. benthamiana* completely devoid of α1,3-fucose and β1,2-xylose residues (GnGn - **Figure 1B**) [24]. In addition, plants can elongate the core N-glycan on specific proteins with β1,3-galactose and α1,4-fucose, giving rise to the Lewis A epitope (**Figure 1A**). Although these structures are found in human tissues, for example in glycosphingolipids, a negative impact of Lewis A structures on the efficacy, stability or antigenicity of recombinant glycoproteins cannot be excluded. For example, when present on recombinant glycoproteins, Lewis A-containing N-glycans could interact with carbohydrate binding receptors resulting in enhanced turnover [25]. It is therfore desirable to eliminate these modifications in order to prevent unwanted cellular processes, simplify the N-glycan processing pathway and prevent competing enzymatic reactions. Knockout of the responsible β1,3-galactosyltransferase and the α1,4-fucosyltransferase in *Physcomitrella patens* resulted in the production of erythropoietin lacking the Lewis A epitope [26]. Another limitation of plants is the occurrence of truncated N-glycans (paucimannosidic) that are generated by the removal of terminal GlcNAc residues from the core N-glycan (MMXF - **Figure 1A**). Transient knockdown of a β-hexosaminidase considerably increased the amounts of complex N-glycans on human α1-antitrypsin produced in *N. benthamiana* [27]. Combining these three approaches for the elimination of unwanted enzymes acting on complex N-glycans will result in a plant-based platform for the production of different recombinant glycoproteins with homogenous complex N-glycans.

To produce complex N-glycans that carry modifications absent in plants, glycosyltransferases and whole pathways for the biosynthesis, transport and transfer of missing nucleotide-sugars have to be introduced. Plants lack common mammalian-type modifications including the formation of additional antennae e.g. tri- or tetra-antennary N-glycans – (bisected and branched), β1,4-linked galactose (galactosylated) and capping with sialic acid (N-

acetylneuraminic acid – sialylated) residues (**Figure 1B**). Transient or stable expression of the corresponding enzymes and pathways in *N. benthamiana* resulted in the production of numerous glycoproteins carrying human-type N-glycans with sialic acid [28-30]. Importantly, these tailored proteins are functional and display similar characteristics to mammalian cell culture-derived variants. Moreover, the coordinated expression of the mammalian sialic acid pathway and human polysialyltransferases resulted in the formation of polysialic acid-containing glycoproteins [21]. These types of glycans are currently the most elaborate human-type structures produced in plants.

As outlined above, remarkable progress has been made in humanization of plant N-glycans in plants. In contrast, until recently, differences in N-glycosylation occupancy have been widely neglected in glyco-engineering approaches. While the single N-glycan site present in the human IgG Fc region is almost 100% glycosylated when expressed in mammalian cells, when produced in plants more than 10% of the recombinant protein is underglycosylated at this site [23]. This phenomenon is dependent on the expression host and the extent of sequon occupancy has been observed to differ between different plant species as has been described for the anti-HIV antibody 2G12 [31-33].These differences in N-glycosylation efficiency are caused by yet unknown features of the plant oligosaccharyltransferase (OST) complex that catalyses the initial step of protein N-glycosylation, the transfer of the preassembled oligosaccharide to asparagine residues present in the consensus sequence Asn-X-Ser/Thr (X any amino acid except proline). The plant OST complex consists of multiple subunits whose precise composition and individual function is currently poorly understood [34, 35]. Transient expression of a single-subunit oligosaccharyltransferase from *Leishmania major* increased the N-glycan occupancy on different recombinant glycoproteins produced in *N. benthamiana* [23]. However, the *L. major* OST did not improve the occupancy on all tested sites, and there are indications that its expression can interfere with other processes in the secretory pathway, resulting in reduced overall yield of the produced protein. Consequently, the expression of the enzyme needs to be better controlled; other single-subunit OSTs should also be explored for their activity in plants, and efforts should be undertaken to understand the current limitation of the plant OST complex.

In contrast to engineering of N-glycans, comparatively little attention has been paid to the generation of tailored O-glycans. Linkage of O-glycans to Ser/Thr residues is the second major type of protein glycosylation in the secretory pathway. O-glycans are present on

biopharmaceuticals like etanercept [36] and on viral envelope glycoproteins that are potential vaccine candidates [37]. In humans, the most common type of O-glycosylation is initiated by the transfer of a single N-acetylgalactosamine (GalNAc) residue catalysed by a family of 20 N-acetylgalactosaminyltransferases. Plants are attractive hosts for the engineering of this type of O-glycosylation as they completely lack N-acetylgalactosaminyltransferases and glycosyltransferases for elongation and branching of O-glycans. Hence, mammalian-type O-glycans can be generated *de novo* without the interference from competing endogenous glycosyltransferases [38]. However, proline residues next to O-glycosylation sites on mammalian proteins like IgA1 are frequently converted to hydroxyproline residues that can be further extended with pentoses (**Figure 1A**) [39]. These protein modifications increase the heterogeneity of plant-produced proteins, impede a detailed site-specific analysis of engineered O-glycans, and may have adverse properties that affect the functionality or immunogenicity of PMPs. A single prolyl-4-hydroxylase responsible for the conversion to hydroxyproline has been eliminated from *P. patens,* and erythropoietin produced in the knockout plant completely lacked the modification [40]. In *N. benthamiana* this engineering approach is more challenging as the number and function of the involved prolyl-4-hydroxylases are currently unknown. The recently completed sequencing of the glyco-engineered *N. benthamiana* ΔXT/FT line now allows for thorough mining of the *N. benthamiana* genome and transcriptome for candidates to set up genome editing approaches for their elimination [41], in order to ultimately combine N- and O-glycan engineered platforms.

Addressing folding constraints along the secretory pathway

The folding of proteins is carefully regulated in the ER to ensure that only properly folded proteins progress through the secretory pathway. This process is mediated by chaperones, both to assist with protein folding, and to impose quality control on the nascent proteins [42]. The two main chaperone folding pathways are mediated by BiP and calnexin/calreticulin (CNX/CRT), although the systems are not mutually exclusive [43, 44]. In the case of glycoproteins, chaperone-mediated folding is co-ordinated by the processing of host-derived glycans which control entry and exit from the CNX/CRT folding pathway [45, 46]. Although the plant chaperone machinery is less well characterized than in other eukaryotes, CNX, CRT and BiP exist in several forms [42].

Given that glycans co-ordinate protein interactions with ER-resident chaperones, the observation that some proteins may be underglycosylated in plants has important implications

for protein folding in the system [23]. It is conceivable that underglycosylation could compromise targeting into the CNX/CRT folding pathway and could contribute to the inefficient production of certain proteins in plants. To illustrate this, the removal of a single glycan sequon from cholera toxin B was reported to result in severe leaf necrosis and the upregulation of representative ER-stress markers when the mutant protein was expressed in *N. benthamiana* [47]. This pathology following recombinant protein expression in plants is indicative of ER stress arising from the accumulation of misfolded proteins.

Recently it has also been suggested that the inefficient production of certain viral glycoproteins in plants may be related to differences in the endogenous chaperone machinery compared to the native host where these proteins are usually expressed, although other processing events are also required for their maturation (Box 1) [48, 49]. In order to address this a suite of human chaperones were recently co-expressed with several prototype viral glycoproteins in *Nicotiana benthamiana*. The co-expression of CRT resulted in a dramatic improvement in the accumulation of many of these glycoproteins and enabled the production of antigens which could not previously be produced at detectable levels in plants. The observation that CRT co-expression relieved the host ER-stress response following HIV Env gp140 expression further suggests that inefficient folding of the protein is related to the endogenous plant chaperones [50]. Similarly, the co-expression of the type 3 secretion chaperone CesT from *Escherichia coli* was reported to improve the expression of the bacterial receptor protein Tir in *N. benthamiana* where the chaperone does not occur naturally [51]. This approach may also be useful for the assembly of virus-like particles in plants where this is a chaperone-mediated process, although this has yet to be explored.

Targeted maturation of heterologous proteins along the secretory pathway

Many proteins that are of interest as pharmaceutical targets require additional post translational modifications (PTMs) for their biological activity, some of which may be absent or occur inefficiently in plant cells [52, 53]. Cleavage by furin proteases is a common PTM required for functioning of many enzymes, growth factors and hormones, and for the maturation of viral fusion glycoproteins [54, 55]. These maturation requirements are an important consideration for the production of viral glycoprotein-based vaccines which may be aberrantly folded, or where the glycoprotein may not be properly incorporated into the viral particle, in the absence of proper processing [56, 57].

Until recently, furin processing of heterologous proteins in plants had not been documented due to the absence of the enzyme in the secretory pathway [58]. Even the prototypic plant produced viral glycoprotein, influenza virus HA, has been reported to be predominantly unprocessed when transiently expressed in *N. benthamiana* [59]. Lately, several studies have reported the ectopic expression of the protease to achieve maturation of target proteins. This approach has enabled the production of appropriately processed transforming growth factor beta (TGF-β) isoform 1, *Bacillus anthracis* protective antigen 83 (PA83), blood clotting factor IX, and more recently a cleaved HIV Env "SOSIP"-type glycoprotein trimer [58, 60]. Interestingly, a soluble version of furin was reported to enable more efficient processing of P83A *in planta* than the full length protein, presumably due to the improved accumulation of the protease [60]. We have achieved efficient processing of an HIV Env glycoprotein in *N. benthamiana* by replacing the native cleavage motif with a hexa-arginine motif (RRRRRR) which is more efficiently recognized. This was the first successful production of a furin-cleaved viral glycoprotein in a plant system. In addition to furin, other proteases may also be involved in the proteolytic processing of viral glycoproteins, such as the subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) in the case of arenaviruses, or endosomal cathepsins for paramyxovirus glycoproteins [57, 61-63]. It is presently unclear if these enzymes are present in plants, and if they are, whether the endogenous levels are sufficient to accommodate processing of an overexpressed glycoprotein. A consideration for the production of proteolytically processed viral glycoproteins is whether this approach would be compatible with the co-expression of broad spectrum PIs to improve accumulation *in planta*.

Another PTM that does not occur naturally in plants is tyrosine sulfation [53]. Tyrosine sulfation is a common post-translational modification in secreted mammalian cell proteins and is often observed in broadly neutralizing antibodies targeting the HIV Envelope glycoprotein [64-67]. In the case of the broadly neutralizing anti-HIV antibody PG9, for example, the co-expression of human tyrosylprotein sulfotransferase in plants was required to achieve optimal neutralizing activity as the endogenous plant homologue does not mediate this function efficiently [68]. While this is the only reported example of engineered tyrosine sulfation in a plant-based expression system at this time, the approach is likely to become increasingly important to enable the production of other anti-HIV antibodies with therapeutic potential.

Similarly, γ-carboxylation has not been reported for plant-produced proteins, and homologues of human γ-carboxylase are not present in *Arabidopsis thaliana* [53]. γ-carboxylation is

important for the activity of coagulation factors, suggesting that in order to produce authentic versions of these proteins in plants, the host cell may need to be engineered to enable appropriate maturation [53]. Recently, blood clotting factor IX was transiently expressed in *N. benthamiana* plants, but the authors did not report whether recombinant factor IX was functional [60]. Whilst theoretically, γ-carboxylase could be expressed *in planta*, there appear to be additional co-factors required for this PTM as undercarboxylation is known to occur in mammalian cells - even when the enzyme is overexpressed [53].

Concluding remarks and future perspectives

New approaches to engineer the plant expression host now enable the production of novel pharmaceuticals that could not otherwise be produced at sufficient yields in plants, or which would not be appropriately processed (**Table 1**). This is enabling the production of increasingly complex biologics and the development of many broadly applicable engineering approaches. Critical focus areas going forwards include developing strategies to improve glycan occupancy of PMPs, and to produce "human-like" O-glycan structures and chaperone co-expression to overcome folding constraints (see Outstanding Questions). To date, the glycan occupancy and complexity has only been reported for a limited number of plant-produced proteins. Future work should look to expand the range of glycoproteins that are analysed in this way with particular emphasis on more heavily glycosylated viral glycoproteins. These analyses should be conducted as head-to-head comparisons with the cognate mammalian-cell produced proteins as they represent the accepted industry standard and therefore provide a rational starting point for glyco-engineering approaches to improve the glycosylation of these proteins. These experiments will also provide a more holistic insight into whether underglycosylation is a global problem for molecular farming or whether this phenomenon only occurs for a subset of proteins. Future work should also investigate how broadly applicable the co-expression of human chaperones is to improve protein folding in plants. So far, approaches have focused on ER-resident chaperones to improve the production of viral glycoproteins in plants. Future work should investigate if this approach would also improve the production of other glycoproteins which traffic through the ER, or if the co-expression of cytosolic chaperones would have similar benefits. The flexibility of the transient expression approach enables multiple engineering approaches to be combined for the tailor-made production of specific proteins (**Figure 2**). Whilst this enables high throughput optimization, an important consideration going forward into clinical testing is the capability of co-expressing multiple heterologous proteins in a single cell, for example where

an entire biosynthetic pathway may need to be introduced to accommodate the production of a particular protein. While this is feasible, a more realistic and reliable approach may involve a combination of transient and transgenic approaches, such as those described to achieve sialylation, or the development of specialized expression vectors for targeted applications [21].

While these engineering approaches all represent incremental advances in the field, considerable capital investment is still required to establish manufacturing capacity in low and middle income countries to fulfil the long unrealized promise of the technology in these regions [69]. We hope that the recent developments in the field will be sufficient to achieve a critical mass for "big pharma" to adopt plant molecular pharming on a global scale, rather than merely for niche applications. At this juncture we feel that the outlook is promising, and that the field is poised on the cusp of realization.

Acknowledgments

This work was supported in part by the Austrian Science Fund, FWF Project P31920-B32, at the University of Natural Resources and Life Sciences. Support at the University of Cape Town was provided by the South African Medical Research Council, the South African Research Chairs Initiative of the Department of Science and Technology and the Wellcome Trust Centre for Infectious Diseases Research in Africa.

Figure 1: N and O-linked glycoforms produced in A) wildtype and B) glyco-engineered plants. The predominant N-glycan species in plants comprises of complex glycans containing β1,2-xylose and α1,3-fucose residues (GnGnXF). Elongation of the core glycan gives rise to the Lewis A structure whereas processing of the terminal GlcNAc yields paucimannosidic structures (MMXF). Elimination of the enzymes responsible for imparting plant-specific glycan moieties (GnGn) has enabled the production of "human-like" glycan structures (bisected, branched) which have been further decorated with galactose and sialic acid residues. Plant O-glycan structures contain arabinose extensions of hydroxyproline which do not occur in mammals. Introduction of the appropriate biosynthetic machinery has enabled the production of mucin-type glycan structures in plants with sialic acid extensions. Sialic acid structures are depicted as N-acetylneuraminic acid (Neu5Ac).

Figure 2: Engineering strategies applied along the secretory pathway enable a tailor-made environment to support production of a recombinant target protein. A) Underglycosylation of recombinant proteins can be addressed by the co-expression of single subunit oligosaccharyltransferase enzymes. B) The co-expression of heterologous chaperone proteins can improve folding and production of specific proteins where the endogenous chaperone machinery differs from the native expression host of the protein. The figure depicts calreticulin associating with Erp57, a member of the protein disulphide isomerase family, to enable the chaperone to catalyse the formation of disulphide bonds. C) Ectopic expression of proteases and other enzymes can result in appropriate processing where certain post-translational modifications would not naturally occur in plants. D) The introduction of broad-spectrum protease inhibitors along the secretory pathway can mitigate proteolysis from host enzymes and improve production yields. Lastly, the glycosylation machinery can be engineered to produce human glycoforms *in planta*.

Box 1: maturation of envelope viral glycoproteins along the secretory pathway

Viral glycoproteins have complex maturation requirements which are dependent on sequential processing events that are mediated by the host biosynthetic machinery. Following translation, the nascent glycoprotein is glycosylated upon entry into the ER by the oligosaccharyltransferase complex. These host-derived glycans are recognized by the lectin chaperone machinery which co-ordinates the interaction of the protein with other chaperones and foldases to assist with protein folding. Correctly folded proteins translocate into the Golgi apparatus where they undergo proteolytic processing, by furin or other proteases, to assume their fusion-competent conformation. Further processing of the glycan structures also occurs in the Golgi apparatus to yield complex glycans. Subsequently the glycoproteins are trafficked to the plasma membrane where they associate with viral structural proteins to facilitate the budding of the virion.

Glossary

Agrobacterium: a Gram negative bacterium which hosts a bacterial tumour-inducing (Ti) plasmid. When infecting plants, a DNA segment encoding tumour morphology genes from the Ti plasmid (T-DNA) is conjugatively transferred into plant host cells and expressed causing galls on the plant. This process has been adapted to plant molecular farming enabling the transfer of a desired gene coding sequence into the nucleus of the host plant for protein expression.

Agroinfiltration: a method of physically forcing recombinant *Agrobacterium* cells into plant leaves. This is usually achieved under pressure using a hand syringe or by immersing leaves in Agrobacterium culture, drawing a vacuum and then releasing it upon which the bacteria are forced into the interstitial spaces of the leaves.

BiP: Binding immunoglobulin protein - an ATP-dependent ER chaperone belonging to the heat shock protein 70 (Hsp70) family.

Glycoprotein: A class of proteins containing host-derived sugar molecules (glycans) attached to their amino acid backbone. N-linked glycans are attached to the asparagine of the Asn-X-Ser/Thr sequon where X may be any amino acid except proline. In contrast, O-linked glycoproteins contain sugar molecules attached to Serine or Threonine residues.

Glycosylation: the enzymatic addition of sugar molecules (glycans) to proteins

Nicotiana benthamiana: a species of *Nicotiana* indigenous to Australia closely related to tobacco which is typically used in the laboratory as a model plant for research.

Oligosaccharyltransferase (OST) complex: a protein complex consisting of several subunits which resides in the endoplasmic reticulum membrane and which is central to the N-linked glycosylation pathway. It transfers a lipid-linked oligosaccharide at the ER membrane to selected asparagine (N) residues of nascent polypeptide chains on proteins as they enter the endoplasmic reticulum.

Plant-made pharmaceuticals (PMPs): proteins made in plants which are used for animal or human medical applications

Plant secretory pathway: Pathway along which newly translated proteins are trafficked through host cell organelles for their modification, processing and assembly. The pathway targets proteins into the endoplasmic reticulum through the trans-Golgi network and subsequently to the plasma membrane for extracellular secretion.

Post transcriptional gene silencing (PTGS): a mechanism utilised in plant cells to degrade endogenous mRNA in order to regulate gene expression.

Protease inhibitor: a substance that prevents proteins from being cleaved into smaller peptides which could result in their lack of function.

Sequon: a sequence of amino acids which serve as an attachment site for a glycan e.g. N-glycans are usually attached to the asparagine in the sequon Asn-X-Ser or Asn-X-Thr.

Sialylation: addition of sialic acid to the terminus of a oligosaccharide (sugar) chain in glycoproteins. Sialic acid refers to a family of derivatives of neuraminic acid, with the most common member being N-acetylneuraminic acid Neu5Ac.

Target protein(s)	Accessory protein(s)	origin	Application	Expression host	Reference
α-Gal, EPO, VRC01	NbPR4	*N. benthamiana*	Protease inhibition	*N. benthamiana*	[18]
α -Gal, EPO, VRC01	NbPot1	*N. benthamiana*	Protease inhibition	*N. benthamiana*	
α -Gal, EPO, VRC01	HsTIMP	*H. sapiens*	Protease inhibition	*N. benthamiana*	
HA, fusion proteins	M2 ion channel	Influenza	Increased pH	*N. benthamiana*	[20]
2G12	Cas9 – genome editing	*S. pyogenes*	Elimination of α1,3-fucose and β1,2-xylose	*N. benthamiana*	[24]
EPO	targeted knockout	NA	Elimination of α1,4-fucose and β1,3-galactose	*P. patens*	[26]
A1AT	RNA interference	NA	Reduced paucimannosidic N-glycans	*N. benthamiana*	**[27]**
2G12	Biosynthetic pathway	Mammalian	Sialic acid biosynthesis	*N. benthamiana*	[70]
PAT-SM6	Biosynthetic pathway	Mammalian	Sialic acid biosynthesis	*N. benthamiana*	[71]
EPO, A1T1, IgG, NCAM IG5FN1	Biosynthetic pathway	Mammalian	sialic acid synthesis and polysialylation	*N. benthamiana*	[21]
Variable,	Biosynthetic pathway	*P. aeruginosa*, *H. sapiens*	Mucin-type O-glycans	*N. benthamiana*	[72]
EPO-Fc fusion	Biosynthetic pathway	*H. sapiens*, *Y. enterocolitica*, *C. elegans*	Sialylated mucin-type O-glycans	*N. benthamiana*	[73]
EPO	targeted knockout	NA	Elimination of prolyl-4-hydroxylase	*P. patens*	[40]
Fc, IgG, IgE, IgA1, EPO-Fc, IFN-γ	LmSTT3D	*Leishmania major*	Modulation of N-glycan occupancy	*N. benthamiana*	[23]
HIV-1 Env gp140	Calreticulin	*H. sapiens*	Folding	*N. benthamiana*	[49]
RVFV Gn	Calreticulin	*H. sapiens*	Folding	*N. benthamiana*	
CHIKV E2ΔTM	Calreticulin	*H. sapiens*	Folding	*N. benthamiana*	
EBV $gp350_{Ecto}$	Calreticulin	*H. sapiens*	Folding	*N. benthamiana*	
Bacterial Tir	CesT	*E. coli*	Folding	*N. benthamiana*	[51]

TGF-β1	furin	*H. sapiens*	Cleavage	*N. benthamiana*	[58]
Factor IX	furin	*H. sapiens*	Cleavage	*N. benthamiana*	[60]
B. anthracis PA83	furin	*H. sapiens*	Cleavage	*N. benthamiana*	
HIV Env SOSIP	furin	*H. sapiens*	Cleavage	*N. benthamiana*	[49]
PG9, RSH	tyrosylprotein sulfotransferase	*H. sapiens*	Tyrosine sulfation	*N. benthamiana*	[68]

Table 1: Plant host engineering approaches applied for the production of pharmaceutical proteins.

α-Gal, α-galactosidase; EPO, erythropoietin; VRC01, anti-HIV IgG; HA, hemagluttinin; 2G12, anti-HIV IgG; PAT-SM6, anti-tumour IgM, A1AT, α1-antitrypsin; NCAM, neural cell adhesion molecule, PG9/RSH, anti-HIV IgG; IFN-γ, interferon-γ; TGF-β1, transforming growth factor-β1, RVFV, Rift Valley Fever virus; CHIKV, Chikungunya virus; EBV, Epstein-Barr virus; Tir, translocated intimin receptor.

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- Can synergistic improvements in heterologous protein production be achieved by combining host plant engineering approaches?
- Can the host plant biosynthetic machinery be engineered to impart completely humanized glycoforms?
- Will plant homologues of mammalian proteases support the efficient processing of recombinant viral glycoproteins?
- How can product homogeneity be achieved for proteins that require the co-expression of multiple accessory factors?
- How can we eliminate multiple prolyl-4-hydroxylases with overlapping substrate specificities without affecting the growth and biomass production of *N. benthamiana*?
- Can protease inhibitors be engineered in a way that they robustly prevent the degradation of different recombinant proteins?
- Does the efficient expression of mammalian or viral proteins require the co-expression of mammalian chaperones and folding factors?

Figure 1

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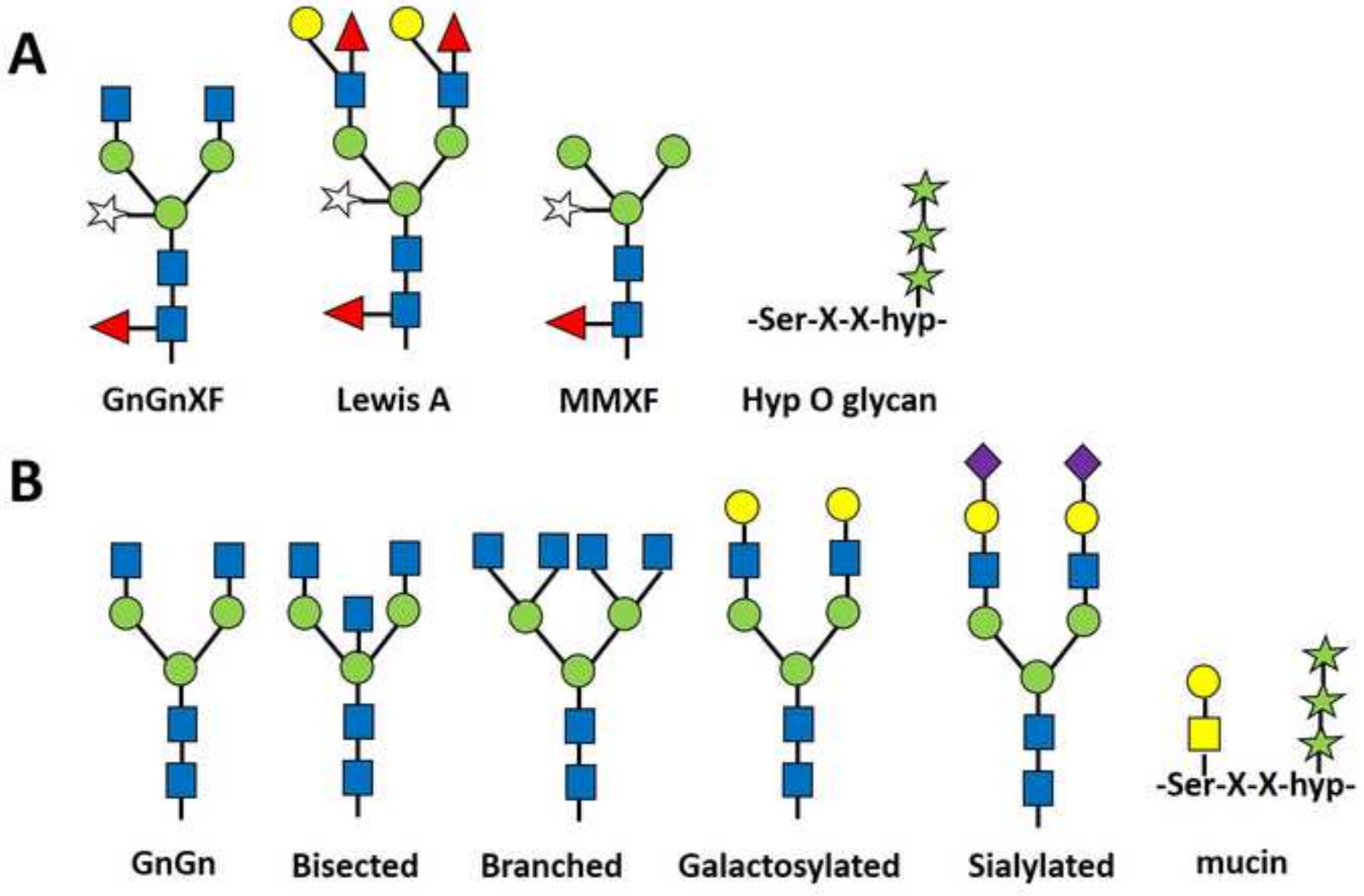

Figure 1 key

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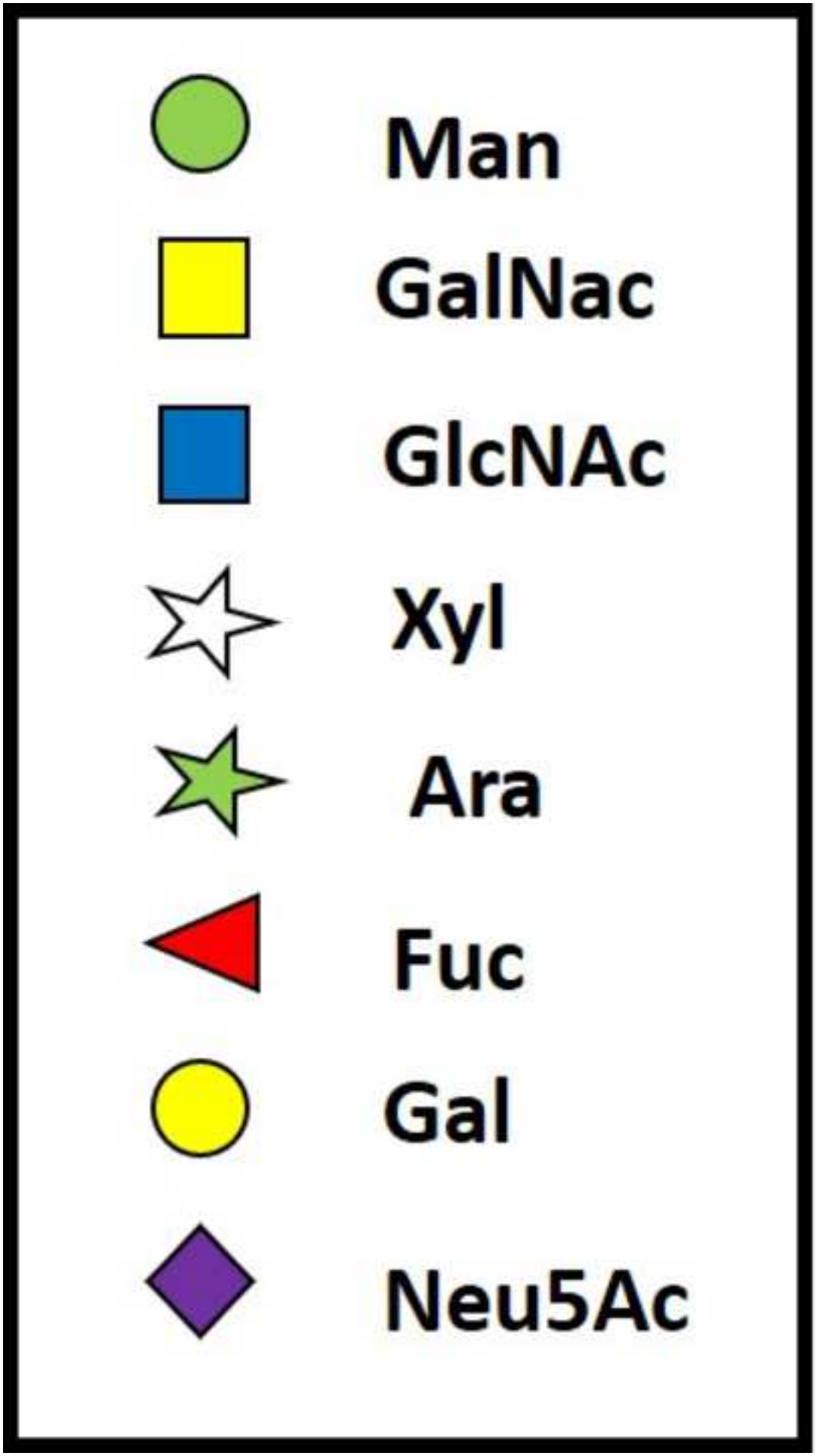

Figure 2