Glyco-modulation of therapeutic proteins produced in plants has shown great success. Plant-based expression platforms for tailored human-like N-glycosylation are based on the overexpression of foreign genes. However, drawbacks such as protein miss targeting, interference with endogenous glycosyltransferases, or with plant development hamper the widespread use of the technology. Here a technique that facilitates the generation of recombinant proteins with targeted N-glycosylation at high homogeneity is described. It is focused on the synthesis of human-type β1,4-galactosylation by the overexpression of the human β1,4-galactosyltransferase (GalT) in Nicotiana benthamiana. A GalT construct that targets the enzyme to the required late Golgi compartment (STGalT) is transiently co-expressed with two pharmacologically relevant glycoproteins. The impact of eight promoters driving the expression of STGalT is evaluated by mass spectrometry (MS)-based analyses. It is shown that five promoters (amongst them high expressors) induce aberrant non-human glycosylation. In contrast, three promoters, considered as moderately active, regulate gene expression to levels leading to an improved efficiency of di-galactosylation (and subsequent sialylation) on the reporter proteins. The results point to the importance of promoter choice for optimizing glycan engineering processes.

1. Introduction

Over the last decade, plants have emerged as a convenient, safe, and economical alternative system for the large-scale production of glycoproteins with therapeutic value.\(^1,2\)

The impact of different glycan modifications on the function of recombinant glycoproteins has been extensively reviewed highlighting the important advances in the field of glycobiology.\(^3\) Functional roles of glycoproteins are often directly related to specific N-glycan structures.\(^4\) Therefore, the efficacy of therapeutic glycoproteins can be maximized through modification of the N-glycan profiles.

In plants, numerous efforts have been undertaken to engineer therapeutic proteins with appropriate glycans to enhance their activity.\(^5,6\) Plants are particularly amenable to glyco-engineering and allow the reconstruction of entire human glycosylation pathways.\(^6\) Plant glyco-engineering encompasses the elimination of particular glycosidic linkages and the introduction of new ones. Attempts to establish plant-based expression platforms to tailor human-like N-glycosylation on recombinant proteins started with generation of mutants in different plant species that lack β1,2-xylosylation and core α1,3-fucosylation.\(^6\) The incorporation of new enzymatic reactions in plants may be accomplished by genomic insertion or transient expression of foreign genes coding for specific glycosylation proteins. However, such proteins sometimes need further modifications to act in a targeted way. For example, the correct subcellular localization of glycosyltransferases, directed by its cytoplasmic tail, transmembrane domain and stem (CTS) region,\(^7\) has profound implications on the final N-glycan profile. This was well illustrated by the efforts to achieve β1,4-galactosylation in plants where incorrectly located β1,4-galactosyltransferases (GalT) resulted in the generation of aberrant (hybrid) structures.\(^6\) Fully processed di-antennary β1,4-galactosylated structures were accomplished by targeting GalT to a late Golgi compartment using a chimeric version consisting of the CTS region of rat α2,6-sialyltransferase (ST) fused to the catalytic domain of GalT (STGalT).\(^8\)

Despite the enormous success, overexpression of proteins for glyco-modulation is not always straightforward. There are examples in the literature reporting drawbacks of gene overexpression including alterations on plant development and mislocalization of the target protein.\(^9–11\)

Previous data demonstrate that phenotypical modifications may occur upon stable in planta expression of foreign genes. A GalT construct that targets the enzyme to the required late Golgi compartment (STGalT) is transiently co-expressed with two pharmacologically relevant glycoproteins. The impact of eight promoters driving the expression of STGalT is evaluated by mass spectrometry (MS)-based analyses. It is shown that five promoters (amongst them high expressors) induce aberrant non-human glycosylation. In contrast, three promoters, considered as moderately active, regulate gene expression to levels leading to an improved efficiency of di-galactosylation (and subsequent sialylation) on the reporter proteins. The results point to the importance of promoter choice for optimizing glycan engineering processes.

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glycosyltransferases. Stable transgenic plant lines expressing STGalT, exhibit phenotypes associated with the enzyme expression levels. Galactosylated N-glycans, the acceptor substrate for protein sialylation, were generated either by transient or stable expression of STGalT under the control of the strong 35S promoter. In both cases, upon co-expression of the necessary enzymes to introduce a sialylation pathway (Sia), recombinant proteins were produced with a mixture of di- and monosialylated structures. Incomplete processed glycans were attributed to the interference of STGalT with endogenous proteins.

Such studies demonstrate that glyco-engineering is not a simple overexpression of a gene, but requires fine-tuning of various parameters such as correct subcellular localization and gene expression level. Promoter selection has become increasingly important to maintain appropriate level of transgene expression. The availability of a broad spectrum of promoters that differ in their ability to regulate gene expression can dramatically increase the success of the technology for glycoengineering. Common promoters used in transgenic plants are derived from Agrobacterium tumefaciens, plants and from plant viruses. The most widely used promoter is the cauliflower mosaic virus (CaMV) promoter of the 35S RNA. Promoters from A. tumefaciens nopaline synthase (nos), octopine synthase (ocs), and mannopine synthase (mas) genes are the most commonly used. Finally, plant constitutive promoters isolated from the highly expressing ubiquitin, actin, and rubisco genes provide valuable alternatives to viral and bacterial promoters.

Here we investigated the impact of different promoters driving STGalT expression on the generation of human-type galactosylated N-glycans on plant-derived glycoproteins. We used eight different promoters to transiently express STGalT in Nicotiana benthamiana and group them according to their capacity to modulate the glycosylation profile of two reporter proteins. We have established three groups of promoters that generate distinct galactosylation and sialylation profiles. Our findings clearly point to the importance of choosing the correct promoter to obtain targeted structures.

2. Experimental Section

2.1. Plant Material

Nicotiana benthamiana wild-type (WT) and the glycosylation mutant ΔXT/FT plants, with down-regulation of plant-specific N-glycan residues (core α1,3-fucose and β1,2-xylose), served as host for the expression of recombinant proteins. Plants were cultivated in a growth chamber at a constant temperature of 24 °C, 60% relative humidity, and a 16 h light/8 h dark photoperiod.

2.2. Expression Vectors

The binary vectors used for transient expression of the IgG1 monoclonal antibody (4E10) and alpha-1-antitrypsin (A1AT) were described previously. The two reporter glycoproteins were co-expressed with a chimeric human β1,4-galactosyltransferase that targets the enzyme to a late Golgi compartment (STGalT). For sialylation, the reporter glycoproteins were additionally co-expressed with the mammalian genes for biosynthesis of CMP-sialic acid, Golgi transport and transfer of sialic acids to the protein (Sia). To transiently express STGalT we generated a set of binary vectors where gene expression is driven by promoters from different origins including viral, agrobacterium and plants. Corresponding promoter fragments (Figure 1A) were PCR amplified and used to replace the 35S promoter of the original STGalT expression vector. In some constructs, the terminator sequence of the original vector was also substituted as depicted in Figure 1A.

Viral promoters include an 835 base pair (bp) fragment of CaMV35S promoter (35S), an enhanced variant with a tandem duplication of 231 bp of upstream sequences plus translational enhancer 5′-UTR from tobacco etch virus (2×35S) and a 346 bp long fragment from the cestrum yellow leaf curling virus (CmYLCV) promoter (CmpC). The promoter fragments were isolated by PCR from available binary plasmids. A. tumefaciens promoters used to drive STGalT expression were derived from the octopine (ocs, 360 bp) and mannopine (mas, 387 bp) synthase genes. The promoter fragments were isolated from commercially available cloning vectors using PCR.

Finally, plant promoters comprising constitutive promoters from Arabidopsis thaliana actin (Act, 1216 bp), ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) small subunit (RbcS1, 1002 bp), and ubiquitin 10 (Ubi10, 628 bp) were isolated by PCR either from A. thaliana genomic DNA or from commercially available vectors. All PCR fragments were amplified with primers with additional 5′-HindIII and 3′-XbaI restriction sites and inserted into the original STGalT expression vector digested in the same way.

2.3. Expression of 4E10 and A1AT

Four-to-five week old plants were used for expression of 4E10 and A1AT by agroinfiltration. Agrobacteria containing binary constructs for the reporter glycoprotein were infiltrated at an OD600 of 0.3 and glyco-modulating proteins at an OD600 of 0.05, unless stated otherwise (1.0 OD600 corresponds to 5×10⁶ cells mL⁻¹). Three days post-infiltration 4E10 was purified by protein A based affinity chromatography and A1AT was collected from the intercellular fluid (apoplast).

2.4. Glycan Analysis

The N-glycan composition of 4E10 and A1AT was determined using reversed-phase liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) of tryptic glycopeptides as described previously.

2.5. Subcellular Localization by Confocal Laser Scanning Microscopy

For subcellular localization experiments, STGalT was C-terminally tagged with green fluorescent protein (GFP) as described previously. The fusion construct was transiently
expressed in *N. benthamiana* leaf epidermal cells at different OD₆₀₀ (0.2, 0.05, and 0.005). GFP expression was monitored at two dpi using an upright Leica TCS SP5 confocal laser scanning microscope (CLSM). Post-acquisition image processing was performed in Adobe Photoshop CS6.

### 3. Results

Previous studies have provided evidence that different CTS domains fused to human β1,4-galactosyltransferase lead to distinct sub-Golgi location of the enzyme with a great impact on the generation of galactosylated N-glycans in plants.⁸,²² In addition to the CTS region, the expression level of GalT seems to correlate with aberrant galactosylation.⁹ We aimed to investigate the impact of promoter choice in controlling STGalT expression toward a homogenous galactosylation profile. To this end, we transiently co-expressed a monoclonal antibody 4E10 with STGalT under the control of eight different promoters. Well described constitutive promoters of different origin were chosen, encompassing 35S, 2x35S and CmpC (viral-origin), ocs and mas (bacterial-origin), Act, RbcS1, and...
Ubi10 (plant-origin, Figure 1A). 4E10 was immune affinity purified and glyco-profiling was performed by LC-ESI-MS. The results showed that STGalT driven by all promoters is able to galactosylate 4E10 (up to 82%). However, overall glycosylation profile of 4E10 depended on the promoter. According to the generated glycoforms, the promoters were clustered in three groups: Group 1 (2×35S and RbcS1) led to the most heterogeneous glycosylation profile of 4E10, harboring a mixture of at least seven glycoforms at similar levels. In addition to complex di- and mono-galactosylated (28% of AA and GnA) N-glycans, aberrant galactosylation (up to 50%, Man5A and Man4A, MA) were also detected (Figure 1B). Using group two promoters (35S, CnpC and Ubi10), 4E10 clearly showed a dominant glycoform, namely di-galactosylated glycans (AA) which, together with complex mono-galactosylated structures (GnA) account of up to 50% of the overall glycosylation. Nevertheless, significant amounts (up to 35%) of hybrid and incompletely processed galactosylated forms (Man4A, MA) are also detected. When STGalT is driven by group three promoters (mas, ocs, and Act), the galactosylation profile is more homogenous and 4E10 is decorated mainly with fully processed di-galactosylated (AA) structures (up to 46%). Together with complex mono-galactosylated glycans they account for 67% of all glycoforms. In addition, minor amounts of incompletely processed structures (17%, MA) but, no hybrid glycans were detected (Figure 1B). The galactosylation profile of 4E10 originated from STGalT driven by group three promoters (mas, ocs, and Act), the galactosylation profile is more homogenous and 4E10 is decorated mainly with fully processed di-galactosylated (AA) structures (up to 46%). Together with complex mono-galactosylated glycans they account for 67% of all glycoforms. In addition, minor amounts of incompletely processed structures (17%, MA) but, no hybrid glycans were detected (Figure 1B). The galactosylation profile of 4E10 originated from STGalT driven by group three promoters (mas, ocs, and Act), the galactosylation profile is more homogenous and 4E10 is decorated mainly with fully processed di-galactosylated (AA) structures (up to 46%). Together with complex mono-galactosylated glycans they account for 67% of all glycoforms. In addition, minor amounts of incompletely processed structures (17%, MA) but, no hybrid glycans were detected (Figure 1B). The galactosylation profile of 4E10 originated from STGalT driven by group three promoters (mas, ocs, and Act), the galactosylation profile is more homogenous and 4E10 is decorated mainly with fully processed di-galactosylated (AA) structures (up to 46%). Together with complex mono-galactosylated glycans they account for 67% of all glycoforms. In addition, minor amounts of incompletely processed structures (17%, MA) but, no hybrid glycans were detected (Figure 1B). The galactosylation profile of 4E10 originated from STGalT driven by group three promoters (mas, ocs, and Act), the galactosylation profile is more homogenous and 4E10 is decorated mainly with fully processed di-galactosylated (AA) structures (up to 46%). Together with complex mono-galactosylated glycans they account for 67% of all glycoforms. In addition, minor amounts of incompletely processed structures (17%, MA) but, no hybrid glycans were detected (Figure 1B).

4. Discussion

Methods to transiently express high levels of recombinant proteins and simultaneously modulate their glycosylation pattern in plants have been previously described[5] and a step-by-step protocol was recently established.[44] The approach allows generating recombinant proteins with a largely targeted glycosylation profile. It is, however, necessary to point out the importance of fine-tuning gene expression for effective glycengineering. For N. benthamiana, these steps include the correct subcellular localization of the foreign enzymes,[8,31] elimination of endogenous glycosylation enzymes,[17,25] and/or overexpression of exogenous glycosyltransferases.[14,23] Despite the great achievements, efficient N-glycan processing in vivo may sometimes need further “polishing”. Previously we reported on the phenotypic variation of transgenic plants stable expressing a chimeric human B1,4-galactosyltransferase (STb1Gal[8,9]), which was correlated to GaIT expression levels. It also became clear that STGalT expression over a certain threshold...
negatively impacts the generation of fully processed glycans and induce aberrant glycosylation. From the numerous factors that can influence gene expression, promoters are perhaps the most important player. Although strong promoters are beneficial to increase yields of recombinant proteins, they might not always be appropriate. Here we show that the ability of STGalT to decorate glycoproteins with galactose (and consequently with sialic acid) largely depends on the promoter used to drive its expression. This observation was valid for two tested glycoproteins (4E10 and A1AT). The relative high amounts of incompletely processed N-glycans (Man5A and Man4A) indicate that promoters from group 1 most probably induce high expression of STGalT, which, in turn competes with endogenous Golgi-α-mannosidase II (GMII) for the acceptor substrate. The same seems to be true for group 2 promoters, however, to a lesser extent. Interestingly, previous results have shown a comparable GFP expression when driven by CmpC, 35S, and ubiquitin promoters,[19] all placed in group 2 in this study. Also, fine tuning of the STGalT expression by replacing the 2xC235S for a weaker constitutive promoter (Gpa) was required to achieve the synthesis of glycans carrying mono- and bi-antennary Lewis x motifs.[11] Group 3 promoters appear to induce STGalT expression at levels most suitable for the synthesis of complex di-galactosylated (and as a consequence di-sialylated) glycans at large homogeneity. Collectively, we demonstrate that glyco-engineering through overexpression of foreign glycosylation enzymes requires optimized gene expression, at least for galactosylation, a fact that has not been addressed sufficiently previously. Many efforts have been applied to precisely target glyco-modulating proteins to correct subcellular compartments.
but, little attention has been paid on how to control their expression levels.

Transient expression systems have been developed for rapid analysis of promoter function, and although the system may not provide definitive information on gene expression patterns, it is powerful tool to gauge promoter strength. With this investigation we point to the importance of the choice of promoter to drive the expression of key enzymes on plant glycoengineering. In comparison to other glycosyltransferases, GalT has a broader acceptor substrate specificity and efficiently acts on all substrates carrying a terminal β-linked GlcNAc. This makes controlling the sub-cellular localization and expression of this enzyme a crucial step for successful glyco-engineering. With this optimized technology we contribute to the establishment of versatile plant expression systems as a platform for the production of a broad range of recombinant glycoproteins.

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Figure 3. A) Relative abundance of glycoforms detected on 4E10 co-expressed with STGalT constructs representing each group of promoters: RbcS1 (group 1), 35S (group 2), and Act (group 3). Representations of N-glycans detected by mass spectrometry are shown. STGalT constructs were co-expressed in N. benthamiana ΔXT/FT at different OD₆₀₀ (0.2, 0.01, and 0.005). Percentages are mean values of experiments carried out in two repetitions for each promoter. B) Top, schematic representation of the binary vector used for subcellular localization studies (35S::STGalT-GFP). Bottom, monitoring of the expression of 35S::STGalT-GFP at different OD₆₀₀ (0.2, 0.05, and 0.005) by CLSM. Life-imaging shows that the punctuate fluorescence of 35S::STGalT-GFP (green) typical for Golgi stacks decreased with the OD₆₀₀.
Conflict of Interest

The authors declare no commercial or financial conflict of interest.

Keywords

galactosylation, gene expression, plant glyco-engineering, promoters, recombinant expression

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