Engineering of complex protein sialylation in plants

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Sialic acids (Sias) are abundant terminal modifications of protein-linked glycans. A unique feature of Sia, compared with other monosaccharides, is the formation of linear homo-polymers, with its most complex form polysialic acid (polySia). Sia and polySia mediate diverse biological functions and have great potential for therapeutic use. However, technological hurdles in producing defined protein sialylation due to the enormous structural diversity render their precise investigation a challenge. Here, we describe a plant-based expression platform that enables the controlled in vivo synthesis of sialylated structures with different interlinkages and degree of polymerization (DP). The approach relies on a combination of stably transformed plants with transient expression modules. By the introduction of multigene vectors carrying the human sialylation pathway into glycosylation-destructed mutants, transgenic plants that sialylate glycoproteins in α2,6- or α2,3-linkage were generated. Moreover, by the transient coexpression of human α2,8-polysialyltransferases, polySia structures with a DP >40 were synthesized in these plants. Importantly, plant-derived polySia are functionally active, as demonstrated by a cell-based cytotoxicity assay and inhibition of microglia activation. This pathway engineering approach enables experimental investigations of defined sialylation and facilitates a rational design of glycan structures with optimized biotechnological functions.

protein polysialylation | *Nicotiana benthamiana* | recombinant proteins | glycoengineering | in planta sialylation

In mammals, glycoproteins and glycolipids are frequently terminated with sialic acid (Sia) residues. These negatively charged sugar residues (i.e., *N*-acetylneuraminic acid) play essential roles in many aspects of life: e.g., in cell–cell interactions, in cell signaling, and for protein stability (1, 2). A unique feature of Sia is that, unlike other sugars, it often forms homo-oligomers or polymers, with its most complex form polysialic acid (polySia) reaching a degree of polymerization up to 400 (3). This sugar polymer is remarkable in that it plays multiple roles across different species from bacteria to humans. Its exceptional physicochemical properties provide the polySia with a large exclusion volume that physically inhibits interactions mediated by polySia-containing molecules, as shown for the major polySia carrier in humans, the neural cell adhesion molecule, NCAM (1, 2, 4). Recent studies demonstrated the effect of polySia in a variety of basic biological processes, like neural cell regeneration and antiinflammatory processes (5). Furthermore, the role of polySia in extraneural tissues and cells has also been described, revealing novel roles of polySia in protection and repair/regeneration and in immunological processes (recently reviewed by refs. 1, 2, 5, and 6). Notably, chemical or in vitro polySia engineering has made a substantial impact on pharmacokinetic properties of recombinant glycoprotein therapeutics (7, 8). These examples demonstrate a manifold functional impact of polySia and highlight the great potential for the development of novel polySia-dependent therapeutics.

The enormous structural diversity that is immanent in the mammalian sialom complicates understanding the biological function of individual sialylated glycan structures. Advanced technologies to generate and probe diverse sialylated glycoproteins are necessary not only to further elucidate the biological significance of sialic acid-containing molecules but also to pursue medicinal and industrial applications. For the recombinant expression of sialylated proteins, preferentially mammalian/human cells are used because they are able to cap N-glycans (e.g., β1,4-linked galactose) with α2,3-linked and α2,6-linked Sia, the latter representing the prevalent variant in human cells (1). Although such cells produce properly sialylated glycoproteins, they have several limitations, like glycan heterogeneity and the incapability to produce distinct glycoforms on demand, that hamper further investigation on the biological impact of sialylation and the development of optimized therapeutic proteins. Substantial efforts have been devoted to genetic engineering of mammalian cells and other organisms to expand their glycosylation capacity and to improve or alter glycosylation, including sialylation (9, 10). Despite impressive recent achievements, designed sialylation strategies are largely elusive.

Plants are increasingly being recognized as an interesting expression system for human glycoproteins, not least due to their ability to produce glycoproteins with homogenous N-glycan structures. Although significant parts of the N-glycosylation machinery

Significance

Sialic acid (Sia) residues are essential monosaccharides in mammals and confer multiple biological functions. Their precise generation is important for both structure–function studies and biotechnological applications. We describe a unique technology that enables the controlled generation of protein sialylation in *Nicotiana benthamiana*. The plant engineering approach relies on a combination of endogenous glycan deconstruction and the introduction of human sialylation capabilities. An arrangement of transgenic and transient expression modules resulted in the targeted synthesis of Sia structures in three different linkage types, reaching a polymerization degree exceeding 40 residues (polySia). Importantly, the obtained functional activities of polySia point to novel biotherapeutic applications. Our results highlight the exceptional flexibility of the plant-based expression platform for engineering complex posttranslational protein modifications.

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Data deposition: The sequence for the NCAM Ig5FN1 domain codon optimized for *Nicotiana benthamiana* reported in this paper has been deposited in the GenBank database (accession no. KU052570).

See Commentary on page 9404.

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are conserved between plants and human cells, plants lack essential parts of the glycosylation repertoire that contribute to the glycan diversity typically observed in mammalian cells. For example, plants lack a number of glycosyltransferases that are mainly responsible for the diversification and elongation of glycans, including branching, galactosylation, and sialylation. Also, plants lack the machinery for biosynthesis of the nucleotide sugar CMP-sialic acid and its transport from the cytoplasm to the Golgi apparatus, where it serves as the donor substrate for different sialyltransferases. Nevertheless, various knock-in and knock-out/knock-down studies have demonstrated the enormous flexibility of plants to synthesize complex human-type glycosylation on demand (11). A prominent example is the generation of a *Nicotiana benthamiana* (*N. benthamiana*) glycosylation mutant (ΔXTFT) that synthesizes human type N-glycans, namely GnGn structures ($GlcNAc_2Man_3GlcNAc_2$) at high homogeneity (12). The superior efficiency of glycoengineered monoclonal antibodies (mAbs) produced in these plants has recently been highlighted by ZMapp, the mAb mixture developed for the treatment of Ebola virus-infected patients (13). In addition, the introduction of entire biosynthetic pathways in a transient manner allowed the in planta production of mono- and biantennary α2,6-sialylated N-glycans on therapeutically interesting proteins (14, 15). The approach required the coordinated expression of six foreign glycosylation proteins in different subcellular compartments of *N. benthamiana* plants. Despite this remarkable achievement, the transient expression methodology is prone to batch-to-batch inconsistencies. A transgenic approach that confers stable pathway engineering would be a reliable alternative to overcome these shortcomings and would lay the basis for elusive engineering approaches like polysialylation.

Here, we focused on the modulation of the plant N-glycosylation pathway for the synthesis of complex sialylated structures by a targeted combination of transgenic and transient expression modules. We used the glycosylation mutant ΔXTFT for the transgenic introduction of the mammalian sialylation pathway (comprising six mammalian proteins), which resulted in $\Delta XTFT^{Sia}$ plants synthesizing mono- and bisialylated structures in either α2,6- or α2,3-linkage. Subsequently two human polysialyltransferases (ST8Sia-II and ST8Sia-IV) were transiently delivered to $\Delta XTFT^{Sia}$ by agroinfiltration. The two polysialyltransferases transferred Sia residues in planta to a recombinantly coexpressed Ig5FN1 module of the human neural cell adhesion molecule (NCAM). High performance liquid chromatography (HPLC) of fluorescently labeled polySia chains liberated from Ig5FN1 demonstrated the synthesis of polySia chains that in length extended 40 residues. Functional activities of polySia were determined by cell-based assays. Plant-derived polySia inhibits cytotoxicity of free histones and exhibits attenuation of nitric oxide production of LPS-stimulated microglia cells.

Results

Stable Engineering of an N-Glycan Processing Pathway Toward the Formation of Defined Sialylated Structures ($\Delta XTFT^{Sia}$). To test the capability of *N. benthamiana* ΔXTFT for tolerance of N-glycan augmentation/diversification toward sialylated structures, we pursued the generation of transgenic ΔXTFT plants that stably express six mammalian proteins involved in sialylation (14). A library of various promoter–terminator constructs was generated and evaluated by a transient expression approach. To this end, two binary vectors were generated (16): one for the expression of the proteins necessary for the synthesis of the activated nucleotide sugar precursor CMP-Sia (pCe144: GNE, NANS, and CMAS) and one for the expression of proteins necessary for synthesis of the N-glycan acceptor substrate (β1,4-galactosylated N-glycans), Golgi transport, and transfer of sialic acid to nascent glycoproteins (pGb371: STGalT, CST, and ST) (Fig. S1 and Table S1). A mutated version of UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (GNE), a key enzyme for the biosynthesis of CMP-sialic acid (17), was generated. The mutated GNE variant (GNE^{R263L}) lacks the binding site for the feedback inhibition and resulted in the synthesis of four times higher amounts of CMP-Sia (1 μmol·g^{-1} vs. 0.25 μmol·g^{-1} fresh weight when native GNE was used). Both vectors (pCe144, pGb371) were cotransformed into the ΔXTFT genome, and different primary transformants that were genomic PCR-positive for all six foreign genes were used to assess their sialylation capacity. For this purpose, recombinant glycoproteins were transiently expressed and subsequently subjected to N-glycan analysis by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). Plants that produced recombinant proteins with large amounts of sialylated structures were brought to a homozygous stage, resulting in a previously unidentified plant-based expression platform ($\Delta XTFT^{Sia}$).

To test the sialylation capability of $\Delta XTFT^{Sia}$ in detail, we analyzed three human glycoproteins that are naturally sialylated at different intensities: i.e., erythropoietin (EPO), α1-antitrypsin (A1AT), and IgG. Although purified IgG and EPO exhibit GnGn structures when expressed in ΔXTFT, A1AT carries mainly $Man_3GlcNAc_2$ N-glycans due to an endogenous β-hexosaminidase activity in plants (18) (Fig. 1). All three glycoproteins were efficiently sialylated when expressed in $\Delta XTFT^{Sia}$ (Fig. 1). Note that, for IgG sialylation, *Arabidopsis thaliana* α1,3-fucosyltransferase (FUT11) was transiently coexpressed because core α1,3-fucosylation enhances sialylation of the Fc glycans, which are naturally inefficiently sialylated (19). Transgenic $\Delta XTFT^{Sia}$ plants stably express rat α2,6-sialyltransferase (ST6) and synthesize α2,6-linked Sia as previously shown using a transient expression approach (14). To test whether α2,3-linked Sia can be synthesized in planta on a recombinant glycoprotein, ST6 was replaced by a construct that expresses human α2,3-sialyltransferase (ST3). Indeed, transient

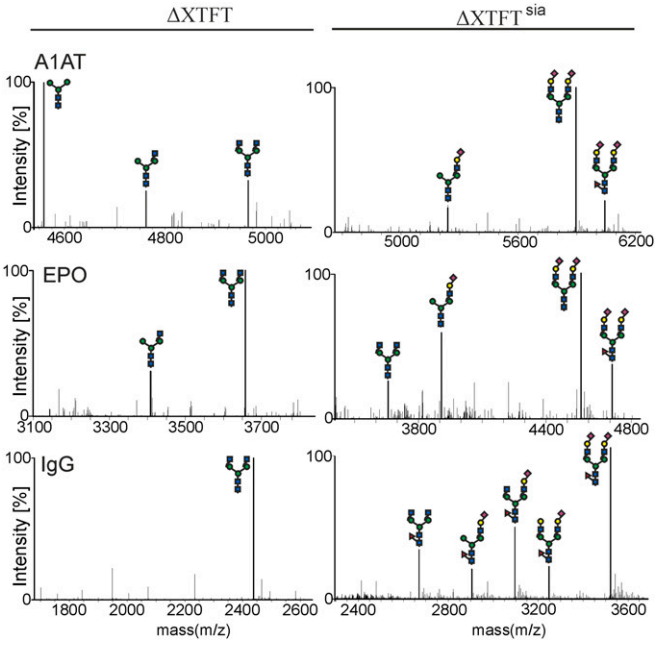

Fig. 1. Recombinant human glycoproteins are capped with terminal sialic acid in transgenic $\Delta XTFT^{sia}$ plants. Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) of selected tryptic glycopeptides from A1AT (Asn^{83}, K/ A^{70}ADTHDEILEGLNFNLTEIPEAQIHEGFQELLR^{101}), EPO (Asn^{83}, R/G^{77}QALLVNSSQPWEPLQLHVDK^{97}), and IgG (Asn^{297}, R/E^{293}EQYNSTYR^{301}) expressed in *N. benthamiana* ΔXTFT and $\Delta XTFT^{sia}$. Glycan diagrams were drawn using the Consortium for Functional Glycomics symbols (www.functionalglycomics.org). A1AT, human α1-antitrypsin; EPO, human erythropoietin; IgG, human Ig 1 (monoclonal antibody).

coexpression of ST3, with pCe144, β1,4-galactosyltransferase, and the CMP-sialic acid transporter, resulted in the synthesis of sialylated N-glycan structures on EPO (Fig. S2). The sialylation efficiencies of ST6 and ST3 are comparable; both enzymes transiently sialylate up to 90% of the acceptor substrate. Importantly, besides a considerable decrease in fertilization with reduced seed yield, transgenic ΔXTFT^{Sia} plants do not exhibit any obvious morphological or developmental changes compared with ΔXTFT or *N. benthamiana* WT. Likewise, recombinant glycoprotein production was not hampered by extensive genetic engineering and glycosylation pathway modifications, indicating the generation of a robust plant-based platform for the production of glycoproteins with defined terminal sialic acid residues.

Human Polysialyltransferases Are Functional When Transiently Expressed in ΔXTFT^{Sia}. Sialic acid residues are usually not extended further with other monosaccharides, except with another sialic acid. Sialyltransferases of the mammalian ST8Sia family catalyze oligo- and polysialylation of glycoproteins through transfer of α2,8-sialic acids from CMP-sialic acid to the nonreducing ends of α2,6- or α2,3-Sia acceptors (20, 21). If the degree of polymerization (DP) exceeds 8 (DP >8), such structures are regarded as polySia chains and can be as long as DP >400 (3). PolySia is a prominent structural feature on the termini of N- or O-glycans of a very small number of mammalian proteins (3). In mammals, ST8Sia polysialyltransferases (polySTs) are type II membrane proteins that reside in the Golgi. Two human polyST members have been characterized, ST8Sia-II and ST8Sia-IV (21, 22), and are responsible for the polysialylation of NCAM. PolySTs are themselves modified by autopolysialylation with α2,8-linked polysialic acid chains (23). Although autopolysialylation is not a prerequisite for polyST enzymatic activity, it enhances NCAM polysialylation (24).

Here, we examined the capability of polySTs to polysialylate glycoproteins in planta when transiently expressed in ΔXTFT^{Sia}. Appropriate plant expression vectors coding for Strep-tagged human ST8Sia-II and ST8Sia-IV were generated (Fig. S3 and Table S1) and transiently expressed in *N. benthamiana* leaves by agroinfiltration. PolyST expression was monitored in total protein extracts by immunoblotting using Strep-specific antibodies. A specific signal was obtained at the expected size (i.e., 58 kDa) for both enzymes expressed in ΔXTFT^{Sia} (Fig. 2*A*). Interestingly the recombinant polySTs could not be detected in ΔXTFT (of WT) plants that lacked sialylation capacity, indicating the requirement of sialylation for protein stability. Because the autopolysialylation capacity of the enzymes is well documented (23–25), we examined the presence of polySia structures on ST8Sia-II and -IV expressed in ΔXTFT^{Sia} by Western blotting using a polySia-specific antibody. Although strong signals were obtained on total protein extracts at the high molecular weight range in plants expressing polySTs, none were detected when the respective expression constructs were absent (Fig. 2*B*). Endoneuraminidase-N (EndoN) digestion, which specifically cleaves α2,8-linked polySia, abolishes the signal indicating the presence of polySia structures in ΔXTFT^{Sia} infiltrated with polySTs (Fig. 2*C*). No polySia-specific signals were obtained when endogenous plant total soluble proteins were analyzed (Fig. S4*A*), indicating that they are not substrates for polysialylation. Subcellular localization of the recombinant enzymes was determined by live-cell confocal microscopy of ΔXTFT^{Sia} leaf epidermal cells expressing ST8Sia-II and ST8Sia-IV fused to GFP (ST8Sia-II-GFP and ST8Sia-IV-GFP) (Fig. S3). Fluorescent protein fusions were transiently coexpressed with the Golgi marker ST6-mRFP (26) in ΔXTFT^{Sia} and analyzed 3 d postinfiltration. The punctate fluorescence of both ST8Sia-II-GFP/ST8Sia-IV-GFP (Fig. 2*D*) and of ST6-mRFP (Fig. 2*D*) shows significant colocalization (Fig. 2*D*) (Fig. 2*D* and Fig. S4*B*), indicating localization of polyST fusions in the Golgi, the expected subcellular site for their action.

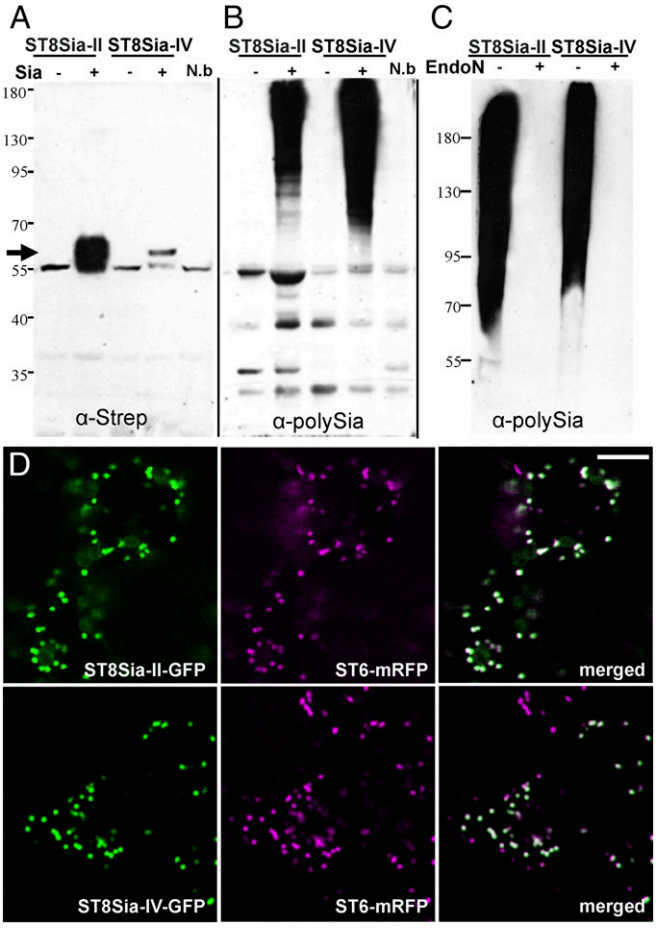

Fig. 2. Polysialyltransferases are active in *N. benthamiana* leaves and located in the Golgi. (*A*) Western blot analysis of total proteins extracted from ΔXTFT (Sia –) and ΔXTFT^{Sia} (Sia +) infiltrated with ST8Sia-II and ST8Sia-IV constructs (Fig. S3). Approximately 50–100 μg of proteins were loaded per lane. Apparent protein molecular masses are indicated in kilodaltons. Specific signals at ~58 kDa were detected using anti-Strep antibodies (arrow) in ΔXTFT^{Sia}. Total proteins extracted from noninfiltrated leaves (N.b) served as negative control. In all samples a 55-kDa protein was visible, which most probably corresponds to rubisco, the most abundant protein in plant leaves. (*B*) Immunoblotting using the anti-polySia antibody mAb735. (*C*) Immunoblotting using mAb735; samples were treated with (+) or without (–) endoneuraminidase N (EndoN). (*D*) Subcellular localization of the recombinant enzymes was determined by live-cell imaging of ΔXTFT^{Sia} leaf epidermal cells expressing ST8Sia-II and ST8Sia-IV fused to GFP (ST8Sia-II-GFP and ST8Sia-IV-GFP) (Fig. S3). Fluorescent protein fusions were transiently coexpressed with the Golgi marker ST6-mRFP in ΔXTFT^{Sia} and analyzed 3 d postinfiltration. The punctate fluorescence of both ST8Sia-II-GFP and ST8Sia-IV-GFP (green) and of ST6-mRFP (magenta) shows significant colocalization (white, merged). (Scale bar: 6.5 μm.) The integrity of the GFP fusions was determined by immunoblotting with anti-GFP antibodies (Fig. S4*B*).

The Recombinantly Expressed Ig5FN1 Module of NCAM Is Polysialylated in Vivo in Glycoengineered Plants. The Ig5FN1 module of NCAM is a well-characterized target for polysialylation (27). Thus, this module was used as a reporter for in planta polysialylation. The Ig5 domain carries three N-glycosylation sites (ASN4–ASN6), with sites 5 and 6 being decorated with polySia structures (28) (Fig. S5*A*). A secreted version of Ig5FN1 (Fig. S5*B*) was expressed in ΔXTFT^{Sia} and ΔXTFT leaves. Upon inspection of the intercellular fluid (IF) (representing the secretome of a plant cell) on SDS/PAGE and subsequent Coomassie Brilliant Blue staining, a strong signal was visible at position 35 kDa, corresponding to the glycosylated Ig5FN1 polypeptide (Fig. 3*A*). The band was excised

from the gel and analyzed by LC-ESI-MS. Glycopeptide analysis revealed the presence of peaks corresponding to GnGn or bisialylated structures (NaNa: $Neu5Ac_2Gal_2GlcNAc_2Man_3GlcNAc_2$) depending on the expression host (ΔXTFT or $\Delta XTFT^{Sia}$) (Fig. 3*A*). For the in planta synthesis of polySia structures, Ig5FN1 was coexpressed with polySTs in $\Delta XTFT^{Sia}$. In addition, mouse core α1,6-fucosyltransferase (FUT8) was coexpressed to achieve core α1,6-fucosylation (29) because this glycan residue positively influences the synthesis of polySia structures on Ig5FN1 (30). The presence of polySia structures was first monitored by Western blotting of IF resident proteins using the polySia-specific antibody. Strong signals at high molecular weight were visible in samples coexpressing polySTs, Ig5FN1, and FUT8 whereas none were visible when one of these proteins was not coexpressed, indicating specific addition of polySia to Ig5FN1 but not to native plant proteins (Fig. 3*B*). The signal was abolished after EndoN digestion, pointing to the presence of polySia structures on Ig5FN1 (Fig. 3*B*). In a next step, the degree of polymerization was analyzed. High performance liquid chromatography (HPLC) of fluorescently labeled polySia chains liberated from IF-derived Ig5FN1 demonstrated the synthesis of polySia chains exceeding a length of 40 Sia residues (Fig. 3*C*). Notably, no differences were observed in Ig5FN1 expression levels whether expressed in ΔXTFT or $\Delta XTFT^{Sia}$ with or without polySTs, demonstrating that the generated transgenic expression platform, in combination with the transient expression modules, is highly suitable for the in vivo generation of this carbohydrate polymer on recombinant proteins.

PolySia Generated in Plants Is Functional in Cell-Based in Vitro Assays. Previous findings have demonstrated that α2,8-linked Sia-polymers can abolish the cytotoxic effect of extracellular histones as seen for both bacterial colominic acid and soluble polySia from NCAM (31, 32). Here, a cytotoxicity inhibition assay against extracellular histone-mediated cytotoxicity of neutrophil extracellular traps (NETs) was performed to determine plant-derived polySia-mediated biological activity. Human umbilical vein endothelial cells (HUVECs) (with or without histone treatment) were cultured in the presence of IF-containing bisialylated Ig5FN1 or polySia-Ig5FN1, and activities were determined by a lactate dehydrogenase (LDH) cytotoxicity assay. Indeed, cytotoxicity induced by extracellular histones could be significantly decreased upon polySia-Ig5FN1 treatments whereas no significant effect was detected with bisialylated Ig5FN1 (Fig. 4*A*). In a second approach, affinity-purified polySia-Ig5FN1 was tested for its capability to inhibit microglia activation, another activity mediated by polySia structures (33). When present in BV-2 microglial cells, plant-produced polySia-Ig5FN1 was at least as potent as free soluble polySia in reducing the lipopolysaccharide (LPS)-induced production of nitric oxide (Fig. 4*B*), a pharmacological target and key regulator of the inflammatory response (34).

Discussion

Given their exposed position on protein-bound glycans, sialic acids are important mediators of cellular interactions, including cell signaling and specific immune responses (35). Moreover, for recombinant glycoprotein therapeutics, the positive effect of increased sialylation on pharmacokinetic properties is well-known (36).

In this study, we introduce a flexible approach for the generation of targeted sialylation. We demonstrate that whole plants well tolerate not only glycan deconstruction (ΔXTFT) but also stable genomic integration of an entire glycosylation pathway. Importantly, both engineering approaches could be combined ($\Delta XTFT^{Sia}$), and the resultant glycosylation capacity remained stable at least for three generations, the time period evaluated thus far. Future studies will need to address potential challenges during extensive upscaling of glycoprotein production, including

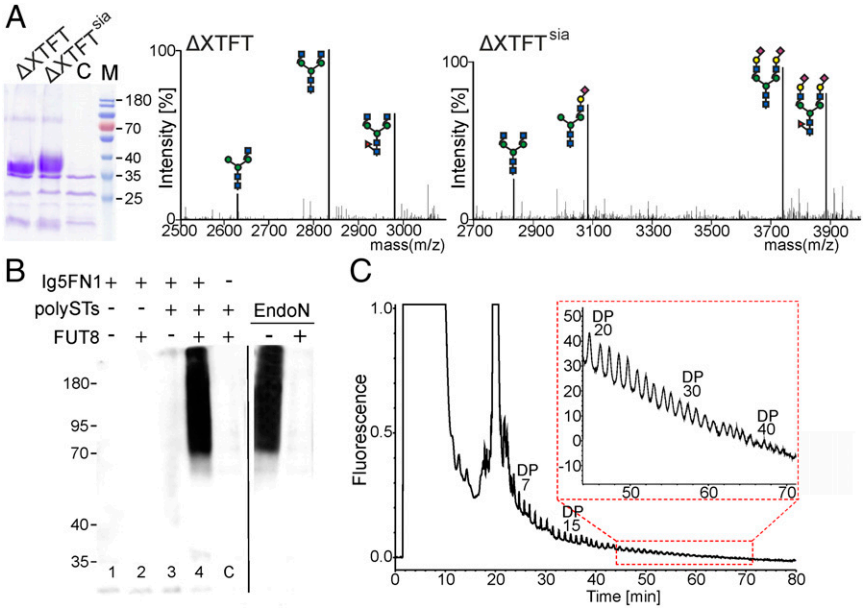

Fig. 3. Ig5FN1 is polysialylated when expressed in glycoengineered *N. benthamiana*. (*A*) Expression of Ig5FN1 was monitored by SDS/PAGE and Coomassie Brilliant Blue staining of intercellular fluid (IF) derived from ΔXTFT and $\Delta XTFT^{Sia}$. A strong band at ~35–40 kDa corresponds to the glycosylated Ig5FN1 polypeptide. Protein bands ≤35 kDa are associated with agrobacterial infection. IF from infiltrated leaves with *Agrobacteria* carrying an "empty" plasmid served as a negative control (*C*). Apparent protein molecular masses are indicated in kDa (M). Glycan analysis of IF-derived Ig5FN1 was performed by LC-ESI-MS of tryptic glycopeptides. The spectra for ASN5 ($R/D^{38}GQLLPSSNYSNIK^{51}$) are shown. (*B*) Western blot analysis using mAb735 of IF extracted from $\Delta XTFT^{Sia}$ infiltrated with (lane 1) Ig5FN1, (lane 2) Ig5FN1 coexpressed with human core αI,6-fucosyltransferase (FUT8), (lane 3) Ig5FN1 coexpressed with both ST8Sia-II and -IV (polySTs), and (lane 4) Ig5FN1 coexpressed with FUT8 and polySTs. As a control sample, (lane C) polySTs were coexpressed with FUT8 in the absence of Ig5FN1. Sample (4) was incubated without (–) and with (+) EndoN. (*C*) To determine the degree of polymerization (DP), purified polysialylated Ig5FN1 was labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) and separated by HPLC. The degree of polymerization is given for selected peaks.

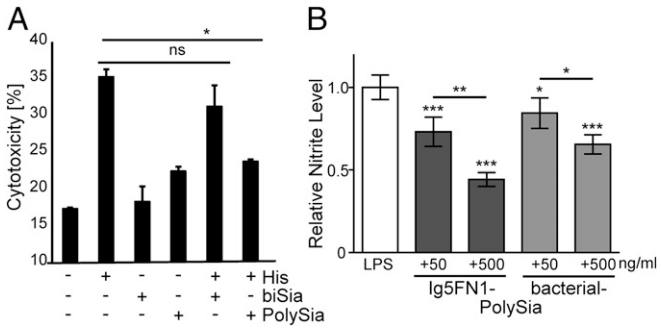

Fig. 4. Plant-produced polySia is functionally active in cell-based inhibition assays. (*A*) Histone-mediated cytotoxicity was determined by its exposure to HUVECs. In parallel, the cytotoxicity was analyzed in the presence of IF-containing bisialylated Ig5FN1 (biSia) or polysialylated Ig5FN1 (polySia). All values are means of four independent experiments. The statistical evaluation was performed by Student´s *t* test (unequal variances, two-tailed). Significance levels are indicated by n.s. (nonsignificant), $*P > 5\%$. (*B*) Nitric oxide production of BV-2 microglia cultured for 24 h in the presence of lipopolysaccharide (LPS, 1 $\mu g \cdot mL^{-1}$) and immunopurified polySia-Ig5FN1 or bacterial-polySia with corresponding concentrations of polySia, as indicated. Values are mean relative nitrite levels ± SD from ≥3 independent replicates for each condition. One-way ANOVA indicated significant effects ($P < 0.0001$), and Tukey's post hoc test was applied. ***, **, *, significant differences against controls or between selected groups with $P < 0.001$, 0.01, or 0.5.

gene stability and glycosylation consistency. Interestingly, neither vegetative growth behavior of engineered plants nor the expression of recombinant proteins was affected by the extensive glycan engineering approach. Note that a reduction of seed production was observed in $XTFT^{Sia}$ lines, for reasons that are not currently understood. It seems that sialylation of endogenous proteins interferes with the reproductive development of *N. benthamiana.* Certainly the sole presence of an engineered CMP-Sia pool in plants does not affect seed production, as demonstrated for *Arabidopsis* (37), and *N. benthamiana* transformed with only the three proteins needed for the synthesis of CMP-Sia. Nevertheless, in terms of the biotechnological applicability of $\Delta XTFT^{Sia}$, this phenomenon should not provide a major limitation.

Efficient sialylation has been achieved in glycoengineered yeasts (38). This approach, which required the elimination of four yeast genes and introduction of 14 heterologous genes, provides certainly a suitably system for the expression of sialylated proteins. However, due to differences in protein folding and secretion, some complex, multimeric proteins might give higher yields in plants.

We demonstrate a rapid way to produce sialic acid in α2,3-linkage or α2,6-linkage, simply by the exchange of the two corresponding STs. The generation of "pure" Sia linkage-forms provides an advantage over mammalian cell systems because they either do not synthesize α2,6-linked Sia (e.g., CHO cells) or generate mixtures of both forms (most human cells). Although the impact of Sia linkage is often not clear, α2,6-sialylation is the preferred form in human serum IgG and shows increased effector functions compared with α2,3-linked variants (39). The ability to generate both linkages will allow a more precise investigation of their possible impacts.

Importantly, we demonstrate that $\Delta XTFT^{Sia}$ mutants are appropriate hosts for the generation of so far largely unexplored glycan structures: i.e., polySia. By the transient overexpression of human polySTs, the synthesis of polySia structures exceeding DP 40 was achieved. Interestingly, polySTs displayed autopolysialylation, and the presence of polyST proteins could only be detected when the entire sialic acid pathway is coexpressed. The impact of polySia on polyST activities is not entirely clear. One study claims that autopolysialylation is not essential for the enzymes' activity (23); however, other experiments point to its importance for efficient polysialylation of NCAM (24, 40). Our results indicate that (autopoly) sialylation positively affects the stability of the two polySTs in planta. Similar to plant expression, human polySTs are autopolysialylated when expressed in COS-1 cells but do not polysialylate any endogenous proteins, highlighting the protein specificity of polysialylation (24). By contrast, bacterial polySTs are more promiscuous than the protein-specific mammalian enzymes (41). Together with advances in creating mutated bacterial polyST versions that allow for the synthesis of oligo- and polysaccharides of defined size (42), the bacterial polySTs are promising candidates to further optimize the platform toward in planta site-specific synthesis of defined polySia on many different recombinant glycoproteins. In contrast to autopolysialylation of polySTs, Ig5FN1 required core fucose for polysialylation. It seems that this core glycan residue induces conformational modifications, thereby influencing the processing of glycan structures, an observation also noticed for the glycosylation modulation of the IgG-Fc N-glycan (19). Selective polysialylation determined by specific core residues has been reported for glycoproteins expressed in CHO cells (30). Nevertheless, others have shown that distinct types of glycans are not required for polyST activity (1).

We show, by two independent cell-based assays, the functional activity of plant-derived polySia structures. PolySia produced in planta blocked cytotoxicity of free histones and attenuated the nitric oxide production of LPS-stimulated microglia cells. These results are in line with findings on the impact of polySia structures recently reviewed (2). The physical and biological characteristics of polySia may confer novel functions to these molecules, such as protection of host cells during inflammatory processes (5) or promoting cell migration required for regeneration (43), to name two. These activities of protein-independent polySia have great potential for the development of novel carbohydrate-dependent therapeutics. Likewise, improving the efficacy of therapeutic proteins (e.g., erythropoietin and buturylcholinesterase) using polySia was demonstrated by chemical conjugation and in vitro approaches (7, 8, 44). Clinical trials of polySia-conjugated recombinant proteins are ongoing, indicating the pharmaceutical relevance of polySia (reviewed in ref. 44). The polySia-dependent approach is particularly interesting because a related method, namely the attachment of polyethylene glycol (PEGylation) to components, raised concerns in therapeutic applications, due to the lack of biodegradability and immunogenicity (44).

Collectively, our results underscore the importance of diverse sialic acid modifications and the demand for expression systems that can provide the generation of defined sialylated glycoproteins not only for structure–function studies but also for therapeutic applications. The plant-based expression platform reported here should enable a targeted design of sialylation for many mammalian glycoproteins, which will enable the experimental evaluation of the biological function of defined sialo-forms and will allow rational design of optimized glycan-dependent therapeutics.

Experimental Procedures

See *SI Experimental Procedures* for descriptions of procedures used for cloning of expression constructs, transient recombinant protein expression, glycosylation analysis by LC-ESI-MS, immunoblotting, subcellular localization by confocal laser scanning microscopy, and analysis of polysialylation by HPLC. In addition, protocols used for testing biological activity of plant-derived polySia are also provided.

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