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In Planta Protein Sialylation through Overexpression of the Respective Mammalian Pathway*[S]

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Many therapeutic proteins are glycosylated and require terminal sialylation to attain full biological activity. Current manufacturing methods based on mammalian cell culture allow only limited control of this important posttranslational modification, which may lead to the generation of products with low efficacy. Here we report *in vivo* protein sialylation in plants, which have been shown to be well suited for the efficient generation of complex mammalian glycoproteins. This was achieved by the introduction of an entire mammalian biosynthetic pathway in *Nicotiana benthamiana*, comprising the coordinated expression of the genes for (i) biosynthesis, (ii) activation, (iii) transport, and (iv) transfer of Neu5Ac to terminal galactose. We show the transient overexpression and functional integrity of six mammalian proteins that act at various stages of the biosynthetic pathway and demonstrate their correct subcellular localization. Co-expression of these genes with a therapeutic glycoprotein, a human monoclonal antibody, resulted in quantitative sialylation of the Fc domain. Sialylation was at great uniformity when glycosylation mutants that lack plant-specific *N*-glycan residues were used as expression hosts. Finally, we demonstrate efficient neutralization activity of the sialylated monoclonal antibody, indicating full functional integrity of the reporter protein. We report for the first time the incorporation of the entire biosynthetic pathway for protein sialylation in a multicellular organism naturally lacking sialylated glycoconjugates. Besides the biotechnological impact of the achievement, this work may serve as a general model for the manipulation of complex traits into plants.

The outstanding specificity of therapeutic glycoproteins places them among the fastest growing class of pharmaceutical products. Many of these drugs need terminal sialylation, the final and most complex step of human *N*-glycosylation, for optimal therapeutic potency. Therefore manufacturing is currently restricted to mammalian cell-based systems that are able to perform this important posttranslational modification, although with major limitations. The naturally present glycosylation repertoire of mammalian host cells promotes the generation of a number of different terminal structures and thus leads to heterogeneous glycosylation. Moreover, these modifications may differ from the authentic human glycosylation. For example, CHO[4] cells, widely used for the expression of recombinant glycoproteins, do not naturally produce human type α2,6-sialylation. These shortcomings in many cases lead to drugs with reduced biological potency, of which recombinant hormones such as human erythropoietin and interferons are prominent examples (1). In addition, about 10% of the *N*-glycans in the Fc region of human serum IgG is sialylated (2). Although the anti-inflammatory effects of sialylated IgG have recently been demonstrated (3), very little information on the impact of this common modification at the molecular level is available (4). This explains the general interest in novel expression hosts and strategies to engineer glycosylation not only to increase the value of therapeutic proteins but also to better understand the role of glycosylation in general and sialylation in particular in fundamental biological processes.

Plants are considered an attractive alternative expression platform for therapeutic human glycoproteins because they are cost-effective, highly scalable, free from human pathogens, and, importantly, can carry out complex *N*-glycosylation (5, 6). In addition, plants glycosylate proteins with considerable uniformity, which may provide an advantage over mammalian-based expression systems. However, plants lack sialylated *N*-glycans, which restrict their utility as a versatile production platform. Recent studies have shown that the plant *N*-glycosylation pathway can be modified toward human-type glycosylation (7–9). Elimination of plant-specific glycosylation in our laboratory and by others has resulted in the generation of plant

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2 and Table 1.

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[4] The abbreviations used are: CHO, Chinese hamster ovary; GalT, β1,4-galactosyltransferase; ST-GalT, modified β1,4-galactosyltransferase; ST, α2,6-sialyltransferase; CMAS, CMP-*N*-acetylneuraminic acid synthetase; NANS, *N*-acetylneuraminic acid phosphate synthase; GNE, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase; CST, CMP-Neu5Ac transporter; mAb, monoclonal antibody; HIV, human immunodeficiency virus; GFP, green fluorescent protein; Rubisco, ribulose-bisphosphate carboxylase/oxygenase; WT, wild type; MES, 4-morpholineethanesulfonic acid; MS, mass spectrometry; MS/MS, tandem MS; LC, liquid chromatography; ESI, electrospray mass ionization; TOF, time-of-flight.

lines (*e.g.* ΔXT/FT) that produce therapeutically relevant proteins carrying a human-like glycosylation profile (GnGn structures). Importantly, monoclonal antibodies produced in such lines exhibited enhanced effector functions (7–9). In addition, we demonstrated recently that by overexpressing a modified version of the human β1,4-galactosyltransferase (GalT), plant *N*-glycans can be extended with β1,4-linked galactose at great uniformity (10). Furthermore, these structures serve as acceptor substrates for subsequent terminal sialylation. However, sialylation is particularly difficult to accomplish even in the presence of β1,4-galactosylated structures because plants lack some further essential prerequisites: (i) the biosynthetic capability to produce the sugar nucleotide precursor CMP-sialic acid, specifically CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac); (ii) a transporter that delivers CMP-sialic acid into the Golgi in sufficient amounts; and (iii) a sialyltransferase (ST) to transfer sialic acid from CMP-Neu5Ac to terminal galactose on the nascent glycoprotein. Moreover, these proteins and their substrates must work in a highly coordinated fashion at different stages of the pathway, and thus organelle-specific targeting of several components is required to enable proper protein sialylation (see Fig. 1). Initial attempts to introduce Neu5Ac residues into plant *N*-glycans have resulted in the expression of some of these proteins in plants (11, 12), and recent progress in our laboratory allowed the synthesis of the sugar nucleotide CMP-Neu5Ac from endogenous metabolites by the simultaneous overexpression of three mammalian enzymes in *Arabidopsis thaliana* (13). However, *in planta* protein sialylation has not yet been achieved.

In this study, we aimed to achieve *in planta* sialylation in *Nicotiana benthamiana*, a tobacco-related plant species particularly well suited for recombinant protein expression (14). Six mammalian proteins comprising the sialic acid pathway were co-expressed together with a monoclonal antibody (mAb) in *N. benthamiana* wild type (WT) and glycosylation mutant plants thereof. Purified mAbs were subjected to glycosylation analyses and tested for functional integrity.

EXPERIMENTAL PROCEDURES

Construction of Plant Expression Vectors—The binary vectors used for the expression of mammalian UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (p19GNE), *N*-acetylneuraminic acid phosphate synthase (p19NANS), modified β1,4-galactosyltransferase (ST-GalT), α2,6-sialyltransferase (ST), as well as 2G12 (pTRAp-Ds; anti-HIV antibody) have been described previously (10, 11, 13, 15).

For the expression of other mammalian proteins, we used three binary vectors (p18, p19, and p20), derivatives of pPT2 (16) only differing in their inserted tags (hemagglutinin, *myc*, and GFP, respectively, see Fig. 2). A truncated form of the human CMP-*N*-acetylneuraminic acid synthetase (CMAS) lacking 40 N-terminal amino acids (13) was amplified by PCR with the primers CMAS11F/CMAS5R (Table 1) and cloned into the XbaI/BamHI sites of p18, resulting in N-terminal hemagglutinin-tagged CMAS binary vector (p18CMAS). cDNA of the mouse CMP-Neu5Ac transporter (CST, German Resource Center for Genome Research accession number IRAVp968C0627D) was amplified by PCR with primers CST2F/CST3R (Table 1) and cloned into the XbaI/BamHI site of p19, resulting in a C-terminal Myc-tagged CST vector (p19CST). For subcellular localization experiments, the cDNAs from GNE, NANS, CMAS, and CST were cloned into the p20 vector XbaI/BamHI site (17), which resulted in C-terminal GFP fusion vectors (p20 series, see Fig. 2). For PCR amplification of cDNAs, gene-specific primers were used (GNE3F/4R, NANS3F/4R, CMAS12F/6R, and CST2F/3R, Table 1).

TABLE 1
Primers used in this study
Underlined letters indicate restriction sites.

Primer	Restriction enzyme	Sequence (5′–3′)
GNE3F	XbaI	tataTCTAGAatggagaagaacgggaacaac
GNE4R	BglII	tataAGATCTgtggatcctgcgcgttgtgta
NANS3F	XbaI	tataTCTAGAatgccgctggagctggagctg
NANS4R	BamHI	tataGGATCCagacttgatttttttgccatg
CMAS11F	XbaI	tataTCTAGAaagcccccgcacctggcagcccta
CMAS12F	XbaI	tataTCTAGAatgaagcccccgcacctggcagcccta
CMAS5R	BamHI	tataGGATCCctatttttggcatgaattattaac
CMAS6R	BamHI	tataGGATCCttttggcatgaattattaac
CST2F	XbaI	tataTCTAGAatggctccggcgagagaaaatg
CST3R	BamHI	tataGGATCCcacaccaatgattctctcttt

All binary vectors were transformed into the *Agrobacterium tumefaciens* strain UIA 143. The origin of the mammalian protein used in this study is summarized in supplemental Table 1.

Transient Protein Expression in N. benthamiana—5–6-week old *N. benthamiana* plants (4–6 leaf stage) were used for the transient expression of heterologous proteins by agroinfiltration as described previously (9, 17). For confocal laser scanning microscopy studies, agrobacteria carrying the respective binary vector (p20GNE, p20NANS, p20CMAS, and p20CST) were grown overnight in LB medium supplemented with kanamycin (50 μg/ml) and gentamycin (25 μg/ml) at 29 °C. 1 ml of bacteria culture was washed twice in infiltration buffer (50 mM MES, pH 5.6, 2 mM sodium phosphate, 0.5% w/v D-glucose, and 300 μM acetosyringone) and resuspended to a final A_{600} of 0.3 (~2,5 × 10^8 cells/ml). For the *in vivo* synthesis of CMP-Neu5Ac, bacterial suspensions containing p19GNE, p19NANS, and p18CMAS were diluted to A_{600} of 0.3, mixed in a 1:1 ratio, and co-infiltrated into epidermal cells of *N. benthamiana* leaves.

In co-expression experiments of ST-GalT and 2G12, the respective bacterial suspensions were diluted to an A_{600} of 0.3–0.5 and mixed 1:1 prior to infiltration. For co-expression of all seven binary vectors, bacterial suspensions of p19GNE, p19NANS, p18CMAS, p19CST, ST-GalT, ST, and 2G12 were diluted to A_{600} of 0.3 and mixed in a 1:1 ratio.

Confocal Laser Scanning Microscopy—3 days after infiltration of agrobacteria containing the p20GNE, p20NANS, p20CMAS and p20CST, the GFP expression was monitored in *N. benthamiana* leaves using a Leica TCS SP2 confocal laser scanning microscope as described before (17).

In Vivo CMP-Neu5Ac Analysis—*N. benthamiana* leaves (0.1 g) co-infiltrated with p19GNE, p19NANS, and p18CMAS were used to analyze the *in vivo* synthesis of CMP-Neu5Ac as described previously (13). Briefly, the supernatant from the homogenized samples was passed through a C18-RP SPE cartridge, and the flow-through was applied to a 10-mg HyperSep Hypercarb SPE cartridge (Thermo Scientific). This was washed with 1 ml of H_2O, and CMP-Neu5Ac was eluted with 0.3 ml of 60% AcCN in 65 mM ammonium formate buffer. The eluate was

freeze-dried. The samples were analyzed on a Hypercarb column (0.32 × 50 mm, Thermo Scientific) using a 65 mM ammonium formate buffer of pH 3.0 as the aqueous solvent. Analytes were detected with an ESI-Q-TOF Ultima Global (Waters) in the MS/MS mode with MS1 set on m/z = 613.1 Da and the mass of the $[M\text{-}H]^-$ ion of CMP-Neu5Ac, and simulated selected ion monitoring of m/z = 322.0 Da ($[M\text{-}H]^-$ of CMP) was performed with MS2.

A. thaliana Transformation and in Vitro Activity Assay of the CMP-Neu5Ac Transporter—A. thaliana wild-type plants were transformed with p19CST (see Fig. 2) by floral dipping (16). Kanamycin-resistant plants were screened by PCR with gene-specific primers to confirm the presence of the coding sequences. Microsomal fractions were prepared according to Fleischer and Kervina (18) in the extraction buffer containing 1 mM EDTA and protease inhibitors (Complete Mini, EDTA-free, Sigma). Microsomes were suspended in 100 μl of Solution A (10 mM Tris-HCl, pH 7.0) containing 250 mM sucrose, 1 mM $MgCl_2$, 0.5 mM β-mercaptopropanol, and protease inhibitors per gram of initial material. The CMP-Neu5Ac transporter assay was started by the addition of 50 μl of solution A containing 0.05 μCi of ^{14}C-labeled CMP-NeuAc to 50 μl of microsomes. The reaction was performed in a 30 °C water bath for 5 and 10 min and stopped by the addition of 1 ml of ice-cold Solution A supplemented with 1 μM non-radioactive CMP-NeuAc (stop reaction mix). The reaction mixture was poured on an nitrocellulose filter (Advantec Toyo, A045A025A) and thereafter washed three times with 1 ml of ice-cold stop reaction mix. The radioactivity remaining on the filters was counted using a scintillation counter.

IgG Purification—2G12 infiltrated leaves were ground in liquid nitrogen, resuspended in ice-cold extraction buffer (100 mM Tris-HCl pH 6.8, 40 mM ascorbic acid, 500 mM NaCl, 1 mM EDTA), and centrifuged (35,000 × *g*/30 min/4 °C). Supernatant was vacuum-filtrated (Macherey-Nagel filter disks MN619eh, 2–4 μm), and the filtrate was further clarified by isoelectric precipitation at pH 4.8 (precipitation of ~25% of total soluble protein including Rubisco) and centrifuged. Clarified supernatant was adjusted to pH 6.8 and incubated on ice for 20 min. After an additional centrifugation step, the extract was passed through a 0.45-μm filter (Millipore Stericup Durapore, low binding polyvinylidene difluoride membrane, 73 mm/0.45 μm) before loading on the chromatography column. Protein A affinity chromatography was performed using the ÄKTAPurifier-10 fast protein liquid chromatography system (GE Healthcare) and a prepacked protein A-Sepharose high performance column (HiTrap ProtA HP 5 × 5 ml; GE Healthcare) according to the manufacturer's instructions.

Analysis of Glycopeptides—N-Glycan analysis of 2G12 was carried out by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) of tryptic glycopeptides (2, 9). Briefly, the heavy chain of SDS-PAGE separated IgGs was excised from the gel, *S*-alkylated, digested with trypsin, and subsequently analyzed by LC-ESI-MS. Note: During this procedure, two glycopeptides are generated that differ in 482 Da (2).

Structural Identification of Sialylated N-Glycans—The verification of the linkages of Neu5Ac and galactose as α2,6- and β1,4-, respectively, on diantennary *N*-glycans was carried out by chromatography on porous graphitic carbon with detection by ESI-MS (19). The retention times of free, reduced *N*-glycans were compared with that of a panel of all possible diantennary mono- and disialylated *N*-glycans of the composition $Neu5Ac_{1-2}Gal_2GlcNAc_4Man_5$ (19). Structural assignment of the glycan with a mass corresponding to the composition $Neu5Ac_1Gal_1GlcNAc_3Man_3$, which could be either the $MNa^{6\text{-}4}$ or the $Na^{6\text{-}4}M$ isomer, was accomplished as follows. The anchor point of all isomer assignments is the synthesis of the MGn glycan using GlcNAc transferase I (19) and the generation of the pair MGn/GnM by enzymatic degradation of GnGn. The isomer pair $A^4Na^{6\text{-}4}/Na^{6\text{-}4}A^4$ was originally identified by separation and digestion down to MGn or GnM. Here again, the two peaks were separated and individually digested with β-galactosidase and β-*N*-acetylhexosaminidase. The retention times of the resulting isomers $MNa^{6\text{-}4}$ or $Na^{6\text{-}4}M$ were thus determined to be 35.9 and 44.7 min, respectively.

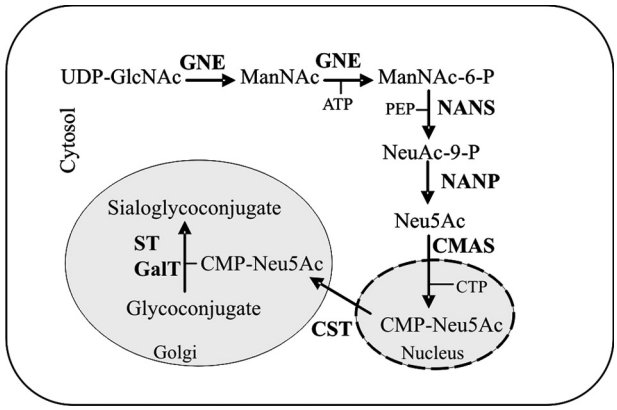


FIGURE 1. **Schematic representation of the mammalian pathway for sialylation of glycoconjugates.** The enzymes involved in the process are: GNE, NANS, Neu5Ac-9-phosphate phosphatase (*NANP*), CMAS, CST, GalT, and ST. *ManNAc-6-P*, ManNAc-6-phosphate; *NeuAc-9-P*, NeuAc-9-phosphate; *PEP*, phosphoenolpyruvate.

2G12 Antigen Binding and HIV Neutralization Assay—2G12 antigen binding specificity was carried out by an enzyme-linked immunosorbent assay-based assay using recombinant gp160 as antigen (9, 15). Neutralization assays were performed using an AA-2 cell-based syncytium inhibition assay as described previously (9, 20). Briefly, 2-fold dilution series of antibodies were preincubated with virus at 10^2-10^3 50% tissue culture infective dose ($TCID_{50}$)/ml for 1 h at 37 °C. CD4-positive human AA-2 cells were then added at a cell count of 4×10^5 cells/ml and further incubated for 5 days. The read-out was performed according to the method of Reed and Muench (21), and the presence of at least one syncytium was scored positive. All assays were performed as duplicates.

RESULTS

In Planta Synthesis of CMP-Neu5Ac—The initial step in the mammalian sialylation pathway is the biosynthesis of the activated sugar nucleotide precursor cytidine monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac). To achieve the generation of this precursor from the endogenously present metabolite UDP-GlcNAc, the action of at least four enzymes is needed (Fig. 1): (i) GNE, a bifunctional enzyme, which catalyzes the conversion of UDP-GlcNAc to ManNAc and the phosphoryla-

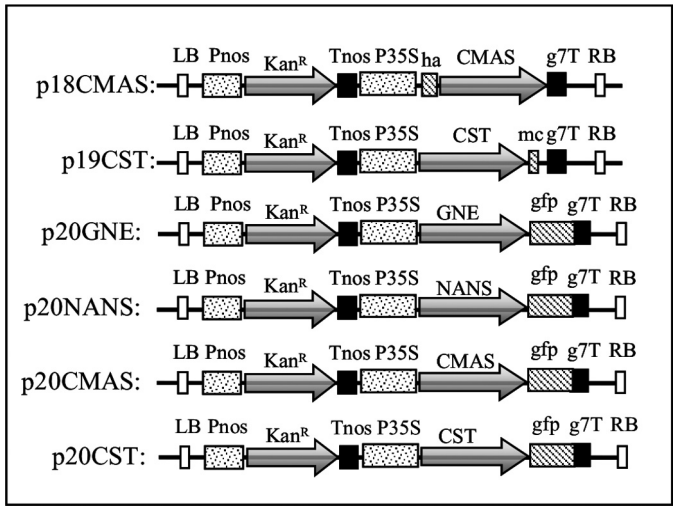


FIGURE 2. **Schematic representation of the different plant expression cassettes of the binary vectors generated in this study.** *Pnos*, nopaline synthase gene promoter; *Tnos*, nopaline synthase gene terminator; *Kan*^{R}, neomycin phosphotransferase II gene; *P35S*, cauliflower mosaic virus promoter; *ha*, hemagglutinin epitope tag; *g7T*, *Agrobacterium* gene 7 terminator; *mc*, c-Myc epitope tag; *LB*, left border; *RB*, right border.

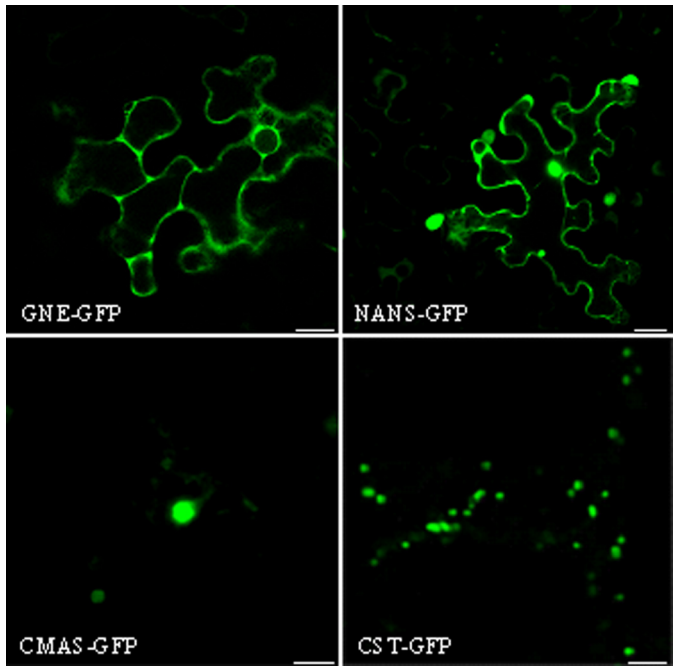


FIGURE 3. **Subcellular localization of fluorescently tagged GNE, NANS, CMAS, and CST proteins.** The corresponding constructs were transiently expressed in *N. benthamiana* leaf epidermal cells and analyzed by confocal laser scanning microscopy 3 days after inoculation. GNE- and NANS-GFP exhibited a typical cytoplasmic labeling, whereas CMAS-GFP showed exclusive staining of the nucleus. CST-GFP displayed a punctuate labeling throughout the cell, which indicates Golgi localization. *Scale bar* for p20CST = 5 μM; for others, *scale bar* = 20 μM.

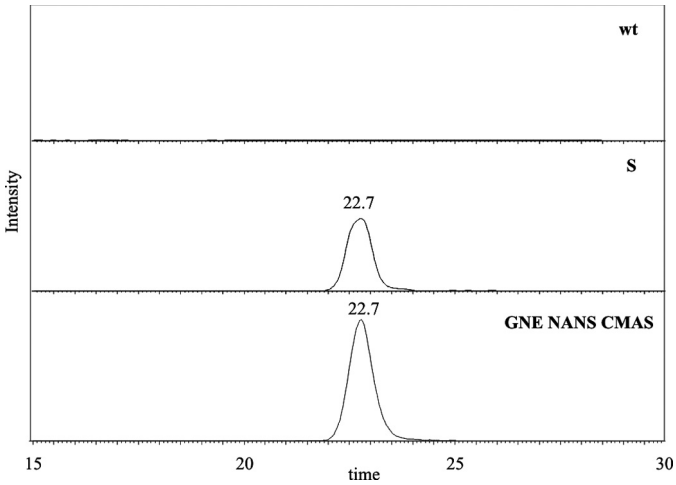


FIGURE 4. ***In planta* synthesis of CMP-Neu5Ac.** LC-ESI-MS/MS analysis of molecular mass 613.1 Da from extracts of *N. benthamiana* WT, from CMP-Neu5Ac standard (*S*), and from extracts of *N. benthamiana* co-expressing the GNE, NANS, and CMAS mammalian proteins (*GNE NANS CMAS*) was performed. The peak at 22.7 min in the standard and the GNE+NANS+CMAS sample corresponds to the CMP fragment obtained by fragmentation of CMP-Neu5Ac ($[M\text{-}H]^-$ = 613.1 Da).

tion of ManNAc to ManNAc-6-phosphate; (ii) NANS, which condenses ManNAc-6-phosphate and phosphoenolpyruvate, resulting in Neu5Ac-9-phosphate; (iii) a specific phosphatase acting on Neu5Ac-9-phosphate; and (iv) a CMAS that activates the resulting primary sialic acid (*N*-acetylneuraminic acid) in the nucleus to CMP-Neu5Ac. Here we set out to simultaneously express cDNAs from the respective mammalian enzymes. In previous experiments, we showed that *A. thaliana* transformed with mammalian GNE and NANS accumulated Neu5Ac rather than Neu5Ac-9-phosphate (13), indicating the presence of a Neu5Ac-9-phosphate homologue that catalyzes the dephosphorylation step in this plant species (22). Thus this protein was not further considered in the present study. For a better understanding, all constructs used in this study are illustrated in Fig. 2. As the correct subcellular targeting of these proteins is an essential prerequisite, live cell imaging was used to determine the localization of transiently expressed GFP fusion proteins. Confocal laser scanning microscopy studies exhibited that GNE and NANS were located in the cytoplasm and that CMAS was found exclusively in the nucleus (Fig. 3). These findings are consistent with the intracellular localizations of these proteins in mammalian cells. Subsequently, the mammalian cDNAs of these three enzymes were simultaneously expressed in *N. benthamiana* leaves, and it was found that these plants contained substantial amounts of CMP-Neu5Ac (5.7 nmol g^{-1} fresh weight) as determined by ESI-TOF-MS/MS analysis (Fig. 4). These results are in good agreement with our data derived from *A. thaliana* transformed with the three mammalian genes (13).

Transfer of CMP-Neu5Ac into the Golgi—The transfer of the activated donor substrate into the Golgi is accomplished by CST. Thus we overexpressed the respective mammalian cDNA in *N. benthamiana*. In a first step, correct localization of CST was determined by CSLM, and the transiently expressed GFP fusion exhibited Golgi labeling, the predicted compartment for this protein (Fig. 3). Transgenic *A. thaliana* plants stably expressing CST were used to carry out *in vitro* transporter assays. An accumulation of CMP-Neu5Ac in microsomes was observed in transformed plants (Fig. 5), demonstrating the expression of a functional protein.

In Planta Protein Sialylation—Finally, specific sialyltransferases (ST) transfer sialic acid to the acceptor substrate A^4A^4, a β1,4-galactosylated, diantennary *N*-glycan. In humans, Neu5Ac transfer is preferentially in α2,6- but also α2,3-linkage.

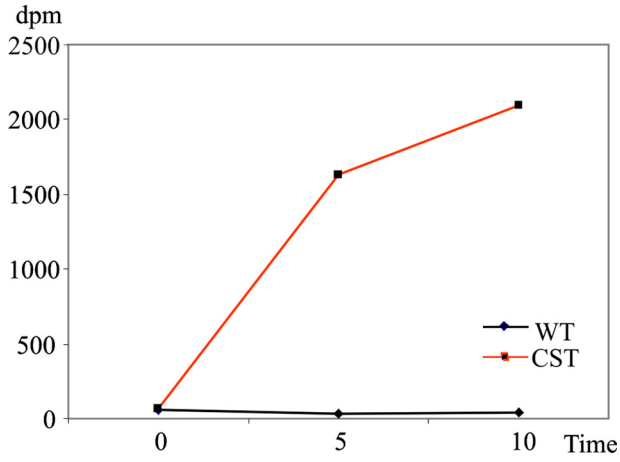


FIGURE 5. ***In vitro* CMP-Neu5Ac transporter activity.** Microsomal membrane vesicles derived from *A. thaliana* WT and transgenic CST-expressing line (*CST*) were incubated with [^{14}C]CMP-Neu5Ac for 0, 5, and 10 min. The vesicles were bound to nitrocellulose filter, and their incorporated radioactivity was measured using a scintillation counter. The activity of CMP-Neu5Ac transporter is assessed by the incorporation of more than 2000 dpm after 10 min in CST plants.

Here we set out to generate Neu5Acα2,6-Gal linkage, the major glycoform of important therapeutic proteins, such as erythropoietin, interferons, or IgG. The mammalian α2,6-sialyltransferase has been expressed in plants previously (11). As β1,4-galactosylated *N*-glycans are not present in plants, we first converted endogenous structures to highly galactosylated forms by overexpressing a modified version of the human GalT that acts in a late stage of the pathway (ST-GalT (10) in *N. benthamiana* WT). The enzyme was co-expressed together with a therapeutically relevant glycoprotein, *i.e.* 2G12, a human anti-HIV mAb (23). Subsequently, the *N*-glycosylation profile of purified 2G12 was determined by ESI-TOF/MS analyses as described previously (2). The glycosylation profile exhibited the generation of quantitative 2G12 galactosylation with three major glycoforms, namely MAX_i, $MAXF_i$ and AAXF (Fig. 6*a*). (The subscript "i" indicates that other isoforms of the same mass, *e.g.* AMX and MAX, may theoretically exist). In addition, smaller peaks representing galactosylated and non-galactosylated glycoforms were detected (*e.g.* MGnX, MA_i, $MGnXF_i$, Man5Gn, GnGnXF, and Man8).

To finally accomplish the transfer of CMP-Neu5Ac to galactosylated *N*-glycans, all six proteins (GNE, NANS, CMAS, CST, ST-GalT, and ST) were co-expressed with 2G12 in *N. benthamiana* WT. The *N*-glycans of purified 2G12 exhibited efficient sialylation. The three major galactosylated variants synthesized in the presence of ST-GalT were quantitatively converted to $MNaX_i$, $MNaXF_i$, and NaNaXF (Fig. 6*b*). To further fathom the potential of this engineering approach, glycosylation mutants lacking plant-specific glycan residues, *i.e.* lacking only the β1,2-xylose (ΔXT) and also lacking the core α1,3-fucose (ΔXT/FT) (9), were used as expression hosts. *N*-Glycans of 2G12 produced in ΔXT and ΔXT/FT were also efficiently sialylated and exhibited a highly uniform glycosylation pattern with two dominant glycan species in ΔXT, namely $MNaF_i$ and NaNaF (Fig. 6*c*), and three major sialylated glycan species in ΔXT/FT, namely MNa_i, ANa_i, and NaNa (Fig. 6*d*). In the two glycosylation mutants, over 80% of the glycoforms were sialylated, which demonstrates the coordinated expression and functional integrity of the mammalian proteins in plants. As the presence of plant homologues for enzymes of the sialylation pathway has been suggested (24, 25), we wanted to determine whether the recombinant expression of all six proteins is required to accomplish *in vivo* sialylation. A series of expression studies where single proteins were omitted was carried out; however, no sialylated glycoforms were detected in any of these experiments (data not shown).

Identification of α-2,6-Linked Sialic Acid—The transfer of Neu5Ac to 2G12 galactosylated structures causes a mass shift of 291.1 Da of the respective peaks in MS spectra. To confirm that this shift actually corresponds to a α2,6-linkage of Neu5Ac to β1,4-linked galactose, LC-ESI-MS analysis was carried out as described previously (19) using ΔXT/FT-derived 2G12 (Fig. 7*A*). This method allows identification of individual glycoforms by the comparison with elution positions of well defined standards. Notably, α2,3- and α2,6-sialylation lead to different retention times (2, 19). The peaks with retention times of 39.9 and 42.7 min co-eluted with the standards A^4Na^{6-4} and $Na^{4-6}Na^{4-6}$, respectively (Fig. 7, *B–E*), confirming the presence of mono- and disialylated glycans with Neu5Ac in α2,6-linkage on plant-produced 2G12. To further identify the isoform of the incompletely processed structure assigned as MNa, the peak was analyzed against the corresponding standards and identified as MNa^{6-4} (Fig. 7, *F* and *G*).

Functional Integrity of 2G12 Glycoforms—To test the functional properties of sialylated mAb, antigen binding and HIV neutralization assays were carried out as described recently (9, 10) using 2G12 derided from ΔXT/FT. No differences in binding capacities were found between CHO cell-derived 2G12, *N. benthamiana* WT 2G12 with mainly GnGnXF glycans, and sialylated 2G12 (Table 2). Finally, the ability of highly sialylated 2G12 to neutralize HIV was examined using a syncytium inhibition assay (Tab. 2). As for antigen binding, no significant differences between the different glycoforms were observed, which demonstrates the full integrity of plant-derived sialylated 2G12 glycoforms.

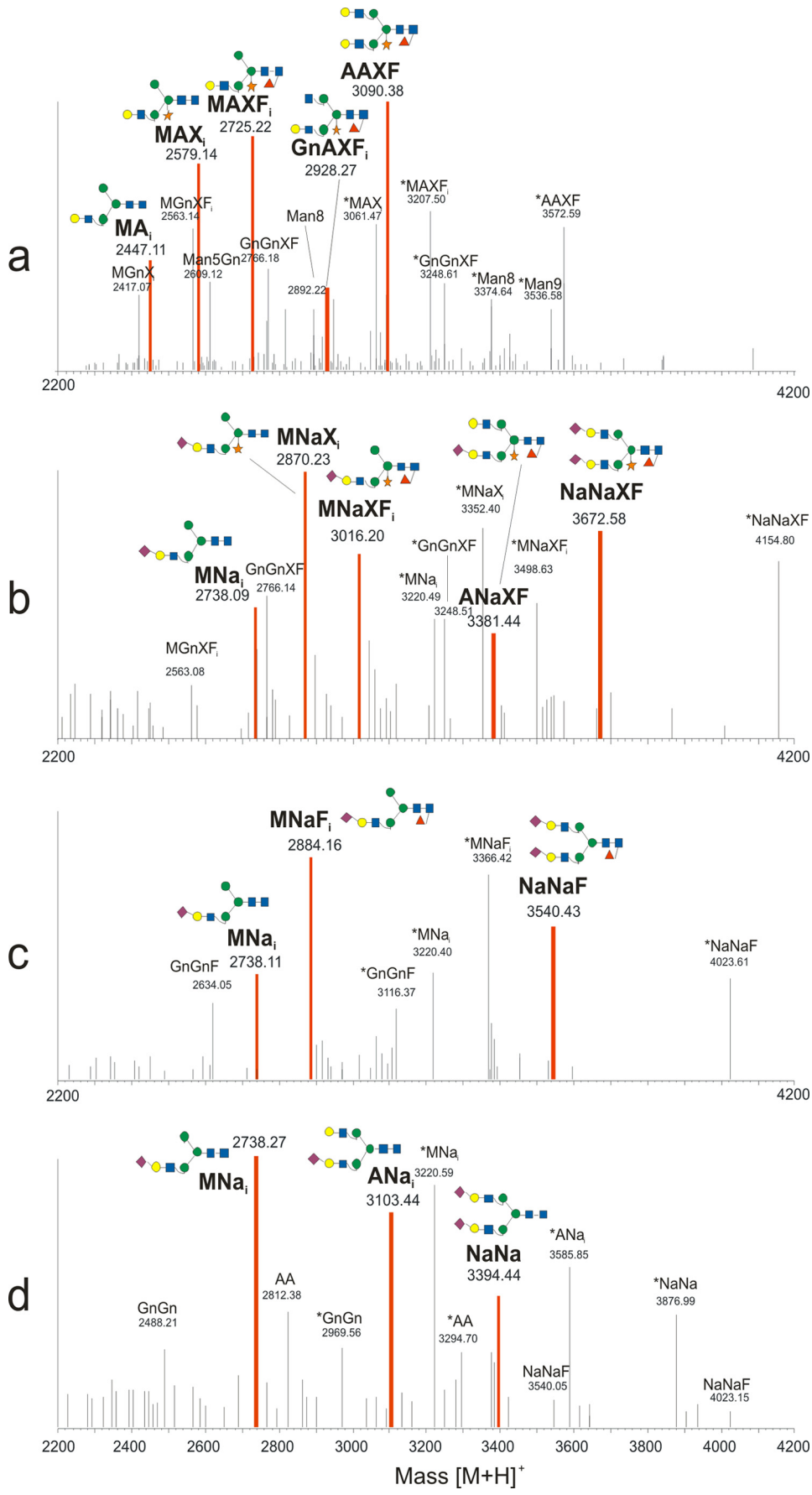
DISCUSSION

Here we report the efficient *in planta* sialylation of a therapeutic protein as exemplified by a recombinant mAb. This was achieved by the coordinated overexpression of six mammalian proteins that act at various stages of the biosynthetic pathway in different subcellular compartments. Quantitative sialylation was facilitated by the specific modification of some of these proteins, *i.e.* elimination of activity-destroying amino acid sequences (in the case of CMAS) (13) and by enabling proper subcellular localization (in the case of ST-GalT) (10). Notably, nearly all of the available acceptor substrates (*i.e.* galactosylated glycans) present in WT and in the glycosylation mutants were sialylated. The synthesis of incompletely processed structures such as MNa_i, $MNaF_i$, and $MNaX_i$ is most likely due to inappropriate overexpression of ST-GalT interfering with the action of GlcNAc-T II. Incompletely processed glycans with one antenna terminating in mannose ($MAXF_i$ and MA_i, Fig. 6*a* and data not shown, respectively) were present in WT and ΔXT/FT plants expressing only ST-GalT, an observation

already made previously (10). This shortcoming may be overcome by optimizing the expression system as demonstrated for stably transformed GalT$^+$ plants (10). Note that GalT$^+$ plants were not considered for sialylation experiments because they are not yet in a homozygous stage.

The high amount of sialylated glycoforms of 2G12 upon expressing proteins of the mammalian biosynthetic pathway points at a considerable degree of functional conservation between plant and mammalian cells. Moreover, the entire procedure did not interfere with IgG expression, which is remarkable in the light of the complexity of the process.

As reported for *A. thaliana* transformed with *GNE*, *NANS*, and *CMAS* (13), transient expression of these genes in *N. benthamiana* efficiently converted ManNAc-6-phosphate to Neu5Ac rather than to Neu5Ac-9-phosphate, suggesting the presence of a so far uncharacterized endogenous phosphatase acting on sialic acid-9-phosphate. On the other hand, although the presence of plant homologues for the CMP-Neu5Ac transporter and sialyltransferases has been suggested (24, 25), our experiments indicate that all required mammalian genes need to be expressed to achieve *in planta* protein sialylation. Notably, transient sialylation did not result in any obvious phenotype in the infiltrated leaves, indicating that at least certain plant tissues/organs can tolerate the incorporation of highly charged sialic acid into proteins. This is not surprising because *N*-glycans of total plant proteins derived from plants expressing all six genes of the sialylation pathway exhibited a relatively low amount of sialic acid (supplemental Fig. 1), in contrast to the efficient sialylation of the secreted mAb. The formation of disialylated mAb is remarkable because in human B-lymphocytes, α2,6-ST preferentially generates monosialylated Fc glycans (26). In this light, the *in planta* sialylation of a recombinant protein is extremely



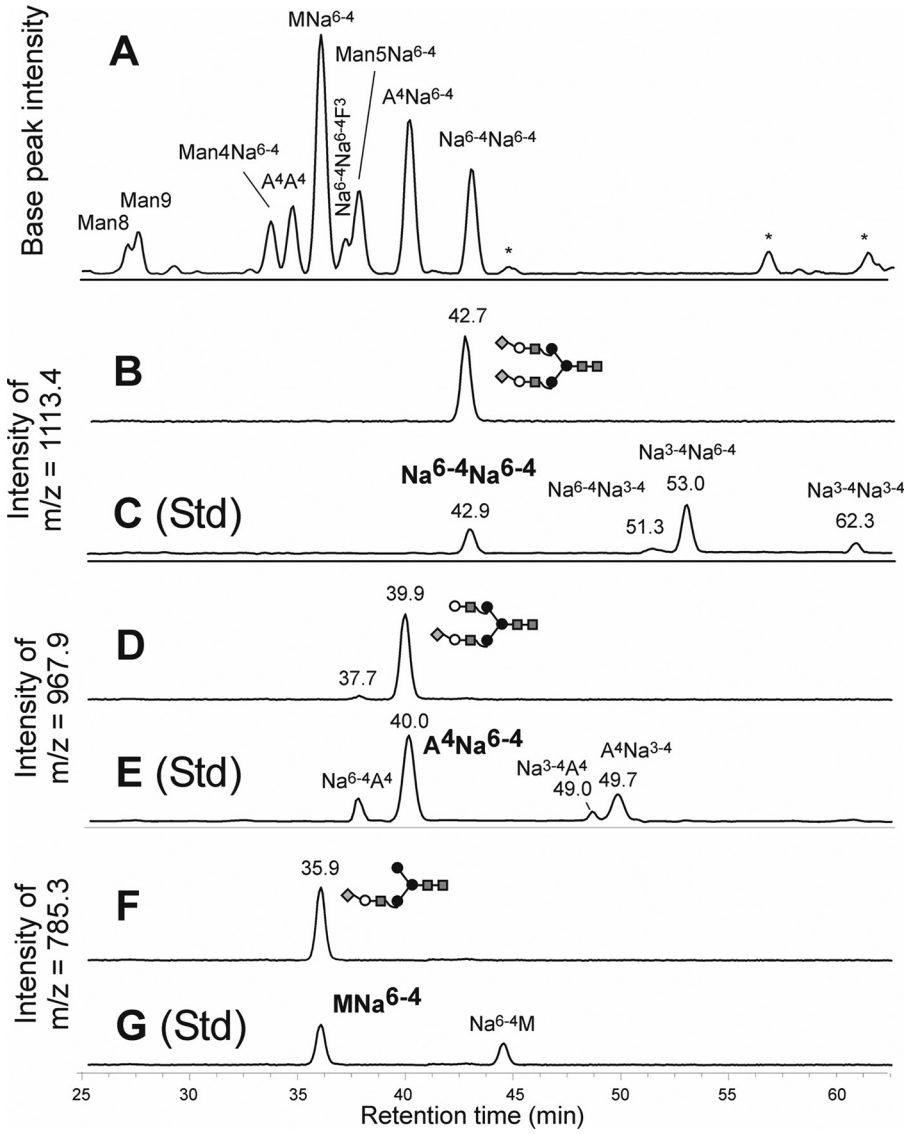


FIGURE 7. **Isomer assignment of mono- and disialylated *N*-glycans.** Glycans of the ΔXT/FT-derived 2G12 were enzymatically released, reduced, and subjected to LC-ESI-MS with a carbon column. *Trace A* shows the base peak intensity of this chromatogram. *Trace B* depicts the extracted ion intensity of the $[M+2H]^{2+}$ ions of disialylated glycans ($m/z = 1113.4$ Da) in the 2G12 sample, whereas *trace C* represents a standard mixture (*Std*) containing the four possible isomers with two sialic acid residues in either α2,3-linkage or α2,6-linkage to β1,4-galactose. 2G12-Derived *N*-glycans co-elute with $Na^{6-4}Na^{6-4}$, which has both sialic acids in α2,6-linkage. *Trace D* shows the extracted ion chromatogram of monosialylated *N*-glycans with two galactose residues ($m/z = 967.9$ Da), and *trace E* depicts the corresponding reference run. Here the standard A^4Na^{6-4} with a 6-linked Neu5Ac on the lower arm co-elutes with the peak from 2G12. *Traces F* and *G* identify the major peak from the base peak chromatogram as MNa^{6-4} by co-elution with the respective standard.

high and significantly outperforms the sialylation ability of commonly used mammalian cell-based expression lines that generate low amounts of sialylated *N*-glycans (2). The rarely used Nawalma cell line is an exception, generating mAbs with an increased sialylated Fc domain (27). A substrate with more exposed *N*-glycans than Fcs would be expected to be furnished with higher amounts of disialylated *N*-glycans. This glycoform has indeed been generated for erythropoietin and lactotransferrin upon expression in glyco-engineered *Pichia pastoris* (28). The sialylation of lactotransferrin was performed *in vitro* for reasons that were not disclosed (29).

Here we show that sialylated 2G12 exhibits similar *in vitro* HIV neutralization potency to other glycoforms derived from plants and CHO cells, demonstrating full integrity of the protein. Notably, this neutralization experiment entirely relies on antigen-antibody binding and does not involve effector functions of the antibodies. Several studies have demonstrated the impact of proper Fc glycosylation for the biological activities of mAbs with the general agreement that mAbs lacking core α1,6-fucose exhibit a dramatically increased antibody-dependent cell mediated cytotoxicity activity (30, 31). In contrast, increased sialylation of IgGs results in reduced antibody-dependent cell mediated cytotoxicity activity because of a decreased binding to the FcγRIIIa receptor (4, 32). On the other hand, Fc sialylation of serum IgG is a major determinant in anti-inflammatory processes in autoimmune disorders (3). The rapid generation of highly α2,6-sialylated Fc as described in this work may substantially contribute to further elucidation of the underlying molecular mechanisms and may facilitate the generation of hypersialylated IgGs for the use as potent drugs to treat autoimmune diseases.

This proof of concept study completes many years of intensive efforts to fully humanize plant glycosylation. Moreover, it is the first report demonstrating that a multicellular organism naturally lacking sialylated glycoconjugates can incorporate the entire biosynthetic pathway for protein sialylation. The lack of any phenotype in infiltrated leaves indicates the feasibility of generating plants stably expressing the entire pathway. Recent advances in multigene delivery (33)

FIGURE 6. **Mass spectra of tryptic glycopeptides of 2G12 transiently expressed in *N. benthamiana* WT and glycosylation mutants.** *a*, 2G12 co-expressed with ST-GalT in *N. benthamiana* WT. *b–d*, 2G12 co-expressed with six mammalian proteins involved in the sialic acid pathway (GNE, NANS, CMAS, CST, ST-GalT, and ST) in *N. benthamiana* WT (*b*) and in two glycosylation mutants ΔXT (*c*) and ΔXT/FT (*d*) lacking plant-specific glycosylation. *i* refers to two isoforms of the same mass that cannot be distinguished by mass spectrometry. Note that two tryptic glycopeptides (GP1 and the incompletely cleaved GP2) are generated, which differ by 482 Da. For better readability, GP1 glycoforms are *highlighted*, and GP2 forms are marked with an *asterisk*. Peak labels were made according to the ProGlycAn system (supplemental Fig. 2).

TABLE 2

Functional properties of 2G12 glycoforms

In vitro HIV-1 neutralization activity was determined by a syncytium inhibition assay. 2G12 antigen-binding tests were carried out by enzyme-linked immunosorbent assay using recombinant gp160 as antigen. GnGnXF, major glycoform obtained from 2G12 produced in *N. benthamiana* WT; Neu5Ac glycoforms, sialylated 2G12 glycoforms as shown in Fig. 6*d*; CHO, CHO-cell derived 2G12, generates a mixture of glycoforms (9,10).

2G12	Antigen binding	IC_{50}
	%	*μg/ml*
GnGnXF	117	3.71
Neu5Ac glycoforms	121	2.09
CHO	100	7.40

should allow the efficient generation of such plants to accomplish *in planta* sialylation in the future. The availability of such lines, together with recently generated glyco-engineered mutants displaying human-type glycosylation of great uniformity (9, 10), pave the way not only for generating therapeutic glycoproteins with optimized biological activities obtained by a customized *N*-glycosylation profile but also for studying the impact of different glycoforms in biological processes. In case whole plants would not tolerate protein sialylation during their complete developmental process, one of the six genes could be transiently expressed together with the protein of interest. Another alternative to this hurdle would be the use of tissue-specific or inducible promoters to specifically turn on protein sialylation in leaves.

Glycoengineered plants in combination with newly developed plant virus-based transient expression systems, allowing the generation of a virtually unlimited number of different proteins at high amounts within 1 week after DNA construct delivery (6, 34), provide a considerable advantage over existing glyco-modified expression platforms, including yeast and mammalian cells. Besides the importance of this work in biotechnological applications, it may serve as a model for the manipulation of complex metabolic pathways into plants for the generation of varieties with new traits.

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