Cover illustration

Tissue engineering is an integrative field that combines knowledge from several disciplines such as cell biology, biochemistry, medicine, and material sciences. The cover of this Special Issue on Strategies in Tissue Engineering – showing, an organ, a tissue sample, a bioreactor and a DNA strand, represents the holistic nature of this discipline and the multitude of expertise necessary to drive it forward. Forming the background to the image is the Würzburg Residence, a UNESCO World Heritage Site in Bavaria, Germany, and the location of the 3rd International Conferences “Strategies in Tissue Engineering”. © Katja Schenke-Layland.

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Introduction

Erythropoietin (EPO) is a glycoprotein mainly produced in the adult kidney, and was initially highlighted for its action on the hematopoietic system [1, 2]. EPO is also expressed in several non-hematopoietic tissues, where it protects from apoptosis and inflammation due to hypoxia, toxicity or injury [3, 4].

Recombinant human erythropoietin (rhEPO), a glycohormone, is one of the leading biopharmaceutical products. The production of rhEPO is currently restricted to mammalian cell expression systems because of rhEPO’s highly complex glycosylation pattern, which is a major determinant for drug-efficacy. Here we evaluate the ability of plants to produce different glycoforms of rhEPO. cDNA constructs were delivered to Nicotiana benthamiana (N. benthamiana) and transiently expressed by a viral based expression system. Expression levels up to 85 mg rhEPO/kg fresh leaf material were achieved. Moreover, co-expression of rhEPO with six mammalian genes required for in planta protein sialylation resulted in the synthesis of rhEPO decorated mainly with bisialylated N-glycans (NaNa), the most abundant glycoform of circulating hEPO in patients with anemia. A newly established peptide tag (ELDKWA) fused to hEPO was particularly well-suited for purification of the recombinant hormone based on immunoaffinity. Subsequent lectin chromatography allowed enrichment of exclusively sialylated rhEPO. All plant-derived glycoforms exhibited high biological activity as determined by a cell-based receptor-binding assay. The generation of rhEPO carrying largely homogeneous glycosylation profiles (GnGnXF, GnGn, and NaNa) will facilitate further investigation of functionalities with potential implications for medical applications.

Keywords: Erythropoietin · Glycoengineering · Plants · Purification · Sialylation
drug efficacy has been reported [11, 12]. Consequently, the oligosaccharide structures of native and rhEPO have been studied extensively. The hormone has been shown to have a large N-glycan microheterogeneity but an otherwise homogeneous protein backbone. Native and rhEPOs, isolated from urine and Chinese hamster ovary (CHO) cells, respectively, carry large amounts of elongated and branched structures (i.e. sialylated bi-, tri-, and tetra-antennary oligosaccharides). Native hEPO isolated from serum, which presents the physiologically active form of the hormone, exhibits in general the same type of N-linked oligosaccharides in terms of branching patterns as CHO-derived rhEPO but the relative amounts of the individual structures are different. A major difference is the presence of relatively large amounts of non-sialylated GnGnF structures and the absence of tetra-antennary oligosaccharides in circulatory hEPO [13]. Notably large glycans variations can occur between different individuals’ urinary EPO [14]. These observations indicate that N-glycans actively contribute to the modulation of hEPO activities in vivo.

The importance of terminal sialic acid for the circulatory half-life of rhEPO is well documented [11, 15, 16], and many investigations have concentrated on enhancing the presence of this carbohydrate formation [17, 18]. Indeed, hyper-sialylated rhEPO with a prolonged half-life and subsequently enhanced drug efficacy has been reported [12]. Relatively little is known about the impact of other glycoforms. However, increased understanding is desirable in view of the large N-glycan heterogeneity of hEPO and the recent findings of the non-erythropoietic activities of the hormone. Accordingly, it would be extremely valuable to establish an rhEPO with a defined N-glycosylation pattern for future research.

Plants are a suitable alternative platform for the expression of complex human recombinant proteins. In recent years the ability to modulate the plant N-glycosylation profile toward human-like structures and thus alter the in vivo activities of therapeutic proteins has attracted great attention [19, 20]. Previous attempts to produce rhEPO in plants have resulted in the generation of a recombinant hormone exhibiting in vitro activity [21–24]. Unfortunately, most of these studies largely neglected the N-glycosylation status of the recombinant hormone, or reported the presence of unusual oligosaccharide structures [25]. Moreover, rhEPO expressed in tobacco cells failed to show in vivo activity, most probably due to the lack of sialic acid residues [26]. In addition, success in purifying the plant-derived recombinant hormone was also limited.

Here, we conducted a comprehensive study to evaluate the ability of plants to produce different glycoforms of rhEPO. Respective cDNA constructs were delivered to N. benthamiana and transiently expressed by a viral-based magnICON® expression system [27]. LC-electrospray ionization-MS (LC-ESI-MS) used for N-glycosylation profiling, revealed that the recombinant hormone carries complex N-glycans with and without plant-specific epitopes (GnGnXF and GnGn), depending on whether the expression host is the wild type (WT) or the glycosylation mutant AXTF (a mutant lacking the plant-specific N-glycan residues xylose and core α1,3 fucose, [28]). Synthesis of bisialylated rhEPO (NaNa) was achieved by co-expressing the protein with the mammalian genes necessary for in planta sialylation [29]. By fusing the short peptide tag ELDKWA to rhEPO, the recombinant hormone was purified via immunofinity chromatography and finally sialylated rhEPO was enriched with Sambucus nigra agglutinin (SNA) lectin chromatography. Cell-based receptor binding assay demonstrated the expression of a highly active hormone.

2 Material and methods

2.1 Construction of erythropoietin expression vectors

A 585-bp fragment containing the cDNA from hEPO (amino acids 28-194), codon optimized for dicot plants and with a C-terminal Strep II tag (WSHPQFEK) was purchased from Mr. Gene GmbH, Regensburg (Germany) (http://www.bionity.com). This sequence was used as a template in a set of PCR reactions using different primer combinations to amplify Bsal-Bsal hEPO fragments with a C- or N-terminal Strep II tag and fragments where the Strep II tag was replaced by the ELDKWA sequence (Table S1 of Supporting information). All four PCR products were digested with Bsal and cloned into a magnICON® tobacco mosaic virus-based 3′-module vector (pICH21595, Bayer BioScience NV Research, Gent, Belgium) containing two Bsal sites designed for directional cloning of the target gene [27]. The resulting vectors were named StrephEPO, rhEPOStrep, ELDrhEPO, and rhEPOELD (Fig. 1A). The 5′-module (pICH20999) contains the signal peptide from the barley α-amylose sequence to direct proteins to the secretory pathway. A binary vector (pICH14011) expressing the recombinase was used to allow in planta assembly of the two viral modules.

2.2 Plant material and transient protein expression

WT N. benthamiana plants, the glycosylation mutants AXTF (lacking plant-specific N-glycosylation, i.e. β1,2-xylose and core α1,3-fucose, [28]) and Gal′ (synthesizing β1,4-galactosylated structures, [30]), were grown at 22°C with a 16 h light: 8-h dark photoperiod. Four to five-week-old plants were used for agroinfiltration experiments as described previously [30]. To express the rhEPO, the 3′-magnICON® vector containing rhEPO cDNA was co-infiltrated with the corresponding 5′-vector carrying the signal peptide in combination with the binary vector con-
taining the recombinase [27]. To modulate the N-glycosylation profiles, binary vectors containing the cDNA of the mammalian genes necessary for protein sialylation were co-infiltrated with the viral based vectors: namely, UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE), N-acetylneuraminic acid phosphate synthase (NANS), CMP-N-acetylneuraminic acid synthase (CMAS), CMP-sialic acid transporter (CST), and α2,6-sialyltransferase (ST) [29]. Agrobacteria carrying the magnICON® constructs were infiltrated using OD 600 0.05–0.2 and those carrying the other binary constructs were infiltrated using OD600 0.05 (1.0 OD600 corresponds to 5 × 10^8 cells/mL).

2.3 Purification of SiarhEPO

2.3.1 2F5 immunoaffinity chromatography

Affinity chromatography was performed on a 2F5 antibody affinity column using the ÄKTAPurifier-10 FPLC system (GE Healthcare). 5 mg of purified 2F5 antibody (kindly supplied by Dr. Florian Rücker, Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria) were coupled to a 1 mL pre-packed NHS-activated high performance Sepharose column (GE Healthcare) according to the manufacturer’s instructions. Infiltreated leaf material (20 g) was ground in liquid nitrogen and thawed in an equal volume v/w of ice-cold extraction buffer (100 mM Tris-HCl pH 7.4, 30 mM ascorbic acid, 0.9% NaCl). The slurry was centrifuged (35 000 g for 30 min at 4°C) and the supernatant was vacuum filtered (Macherey-Nagel filter circles MN619eh, 2–4 µm). The filtrate was further clarified by isoelectric precipitation at pH 4.5 (precipitation of ∼25% of total soluble proteins (TSPs) including RuBisCO), centrifuged as above and after adjustment to pH 7.4 incubated on ice for 15 min. Prior to loading on the chromatography column, the extract was passed through a 0.45 µm filter (Millipore Stericup Durapore low binding membrane PVDF, 73 mm/0.45 µm). The column was washed with 10 column volumes of binding buffer (PBS, pH 7.4) and the sample was applied onto the column at a flow rate of 0.9 mL/min. After washing with binding buffer, proteins were eluted with 50 mM glycine/HCl pH 2.8 and immediately neutralized with 0.5 M Tris. Column washing and elution were carried out at a flow rate of 1 mL/min. Prior to SDS-PAGE analysis the samples were precipitated in ice-cold acetone (4× sample volume) for 1 h at −20°C and centrifuged for 10 min at 16 000 g. The precipitate was air-dried, resuspended in 3× Laemmli buffer and heated at 96°C for 8 min.

2.3.2 SNA lectin affinity chromatography

Affinity chromatography was performed using the ÄKTAPurifier-10 FPLC system on a 1.6 mL SNA lectin affinity column (GALAB Technologies GmbH). The SiarhEPOELD containing eluates from the 2F5 immunoaffinity chromatography were identified by dot blot using antibodies against hEPO, WTrehEPOELD:rehEPOELD expressed in WT plants, SiarhEPOELD:rehEPOELD co-expressed with the genes for the mammalian sialic acid pathway [29] in N. benthamiana Gal+ (mutant lacking β1,2-xylose and core α1,3-fucose, and carrying terminal β1,4-galactose, [30]). C1 and C2: CHO-derived hEPO (50 and 100 ng, respectively). Data are representative of three independent experimental repeats. The sizes of molecular weight marker proteins are shown in kiloDalton.
NaCl, 2 mM MnCl$_2$, 2 mM CaCl$_2$, 2 mM MgCl$_2$, pH 7.0) and slowly (0.4 mL/min) applied to the column using a P1 peristaltic pump (GE Healthcare). Sample loading and column washing steps were performed at 4°C. The binding fraction was eluted with 0.3 M lactose in 50 mM glycine/HCl pH 3.2 buffer at a flow rate of 0.8 mL/min at room temperature. Subsequently the eluates were analyzed by dot blot with anti-hEPO antibodies and the positive fractions were pooled. The buffer was exchanged by passing the sample through an LC-4 solid phase extraction (SPE) column (Supelclean – Supelco), washing it with water and eluting it in 2 mL of 60% ACN + 0.1% TFA. After drying in the speed-vac the samples were resuspended in water.

2.4 SDS-PAGE and immune-based analyses

Recombinant proteins fractionated by 12.5% SDS-PAGE were either stained with CBB G-250 [31] or used for western blot analysis. Those used for immunoblot analysis were blotted to Hybond-ECL$^\text{TM}$ nitrocellulose membranes (Amersham), blocked for 1 h in 1×PBS supplemented with 3% w/v BSA and 1% v/v Tween 20 and detected using Streptavidin-peroxidase (Boehringer Mannheim, Art. Nr. AB-286-NA; 1:800 diluted) as a second antibody. The dot blot was prepared by spotting 10 µL of sample onto the nitrocellulose membrane and blocking for 1 h, and was detected with anti-hEPO antibodies as above.

Quantification of rhEPO was carried out by a double sandwich ELISA using mouse monoclonal hEPO antibodies (Genzyme, Art. Nr. AE-7A5) for coating (dilution 1:10,000) and biotinylated (according to GE healthcare, RPN2202) polyclonal rabbit hEPO Abs (R&D Systems; Art. Nr. AE-286-NA; 1:800 diluted) as a second antibody. Streptavidin-peroxidase (Boehringer Mannheim, Art. Nr. 1 096 044) was used for detection. The color reaction was measured by an ELISA Reader at 492/620 nm. The CHO-derived rhEPO standard was provided by Polymun Scientific GmbH (Vienna).

2.5 Profiling of N-glycopeptides

N-glycan analysis of rhEPO was carried out by LC-ESI-MS of tryptic glycopeptides as described [32]. Briefly, the purified samples were submitted to reducing SDS-PAGE and the immunoreactive bands were cut from the gel, S-alkylated, double-digested with trypsin and endoproteinase Glu-C, eluted from the gel fragment with 50% acetonitrile and separated on a Biobasic C18 column (150 mm × 0.32 mm, Thermo Electron) with a gradient of 1–80% acetonitrile containing 65 mM ammonium formate, pH 3.0. This double digestion allows the discrimination of all three rhEPO glycopeptides:glycopeptide 1:ENITGCAE$^{31}$; glycopeptide 2:HCSLNENITVDP-TK$^{46}$; and glycopeptide 3:GQALLVNSSQPWEFLG-VDK$^{97}$. Positive ions were detected with a quadrupole-time of flight (Q-TOF) Ultima Global mass spectrometer (Waters, Milford, MA, USA). Summed and deconvoluted spectra of the glycopeptides elution range were used for identification of glycoforms.

2.6 In vitro activity assay of rhEPO

The in vitro biological activity of rhEPO$^{\text{ELD}}$ was measured in an UT-7 cell-based proliferation assay. Briefly, the UT-7 cell line [33] was maintained in RPMI 1640 (Biochrome AG) supplemented with 10% fetal calf serum (PAN Biotech.), 4 mM L-glutamine and 5 ng/mL EPO. The cells were washed with EPO-free culture medium and incubated for 4 h at 37°C and 7% CO$_2$. Increasing amounts of CHO-derived rhEPO and plant-derived rhEPO$^{\text{ELD}}$ (ranging from 0.03 to 30 000 ng/mL) were added to 100 µL of medium containing about 10$^4$ cells in a 96-well culture plate, resulting in a final rhEPO concentration of 0.01–10 000 ng/mL per well. After 4 days at 37°C and 7% CO$_2$, 10 µL of an MTT (Thiazolyl Blue Tetrazolium Bromide; Sigma) solution (5 mg/mL) were supplied to each well and the plate was incubated for 4 h as before. Finally, 100 µL of 10% SDS (in 0.01 M HCl) were added to each well and mixed thoroughly at 37°C to dissolve blue crystals before absorbency was read at 570 nm (reference wavelength 690 nm). The results of the fivefold determination were evaluated using MS Excel Solver. The half-maximal effective UT-7 cell proliferation dose (ED50) was used to compare the activities of plant- and CHO-derived rhEPO.

3 Results

3.1 Vectors for rhEPO expression

Transient expression of rhEPO was achieved with magnICON$^\text{TM}$ viral based vectors [27] designed to contain the respective cDNAs with a Strep II tag either at the N- or C-terminus (Strep-rhEPO, rhEPO$^{\text{Strep}}$, Fig. 1A). Agrobacterium tumefaciens suspension cultures carrying the recombinant plasmids were delivered to the expression host N. benthamiana by agroinfiltration. Infiltrated leaves were harvested 5 days post-infiltration (dpi) and expression of the recombinant hormone was monitored by western blot analysis of TSPs. Antibodies against hEPO exhibited a strong signal at the expected size of 30-kDa for both constructs; however, no signal was detected when antibodies against the Strep II tag were used (data not shown). These results indicate that either the tag is cleaved off or it is not accessible at either of the termini. In search for an alternative purification strategy we used an immunoaffinity-based purification with the epitope ELDKWA (derived from HIV-1-gp41) in combination with the corresponding mAb 2F5 [34]. hEPO constructs with a
N- or C-terminal tag (ELDrhEPO, rhEPOELD, Fig. 1A) were delivered to *N. benthamiana* and western blot analysis of TSP at 5 dpi showed strong signals at the expected size with both anti-hEPO and 2F5 antibodies (Supporting information Fig. S1). Consequently, we used the C-terminal-tagged rhEPO construct (rhEPOELD) in subsequent experiments.

### 3.2 Transient expression of rhEPOELD in *N. benthamiana*

Various infiltration experiments were carried out to explore the maximum expression levels of rhEPOELD in *N. benthamiana*. The OD<sub>600</sub> of agrobacterial suspension was varied (0.5–0.05 OD<sub>600</sub>) and leaves were infiltrated at different developmental stages. In addition, timecourses were implemented. Western blot analyses revealed that the optimum rhEPOELD expression could be achieved upon infiltration of middle-aged leaves (from plants that carry six to eight leaves) using agrobacteria at a concentration of OD<sub>600</sub> 0.2. A timecourse from 2 to 11 dpi showed an expression maximum between 4 and 5 dpi (Fig. 1B). Western blot analysis with anti-hEPO antibodies showed a strong signal at the expected size of 30-kDa. In addition, two other bands were detected at positions 26- and 22-kDa (Fig. 1B). CHO-derived rhEPO was detected as an ~40-kDa band (Fig. 1B, right panel C1, C2). This size discrepancy between plant- and CHO-derived rhEPO is most probably due to differences in N-glycosylation. The expression level of rhEPO, as determined by ELISA, varied from 50 to 85 mg rhEPO/kg fresh leaf material depending on the OD<sub>600</sub> and dpi. With an OD<sub>600</sub> of 0.2, a maximum expression level of 85 mg rhEPO/kg fresh leaf material was detected at 4 dpi, corresponding to ~2% of TSP.

![Figure 2. N-glycan profiles of rhEPOELD derived from *N. benthamiana* WT plants harvested at 4 dpi. At this time point western blot analysis of TSPs with anti-hEPO antibody shows three reactive bands (22-, 26-, and 30-kDa, top right cartoon). The N-glycan analysis was carried out by LC-ESI-MS of glycopeptides obtained by double digestion as described in Section 2. This allows discrimination of the three EPO glycopeptides (Gp1-Gp3). The MS profile of glycopeptide 3 (Gp3) is shown (see Supporting information Fig. S2 for analysis of glycopeptide 1 and 2). Top: profile of the 30-kDa band; middle: profile of the 26-kDa band; bottom: MS profile from rhEPOELD isolated from the IF. Two independent repeats of the experiment were performed. Peak labels were made according to the ProGlycAn system (www.proglycan.com).](https://www.biotecvisions.com)
LC-ESI-MS analysis was performed to determine the N-glycosylation status of rhEPOELD expressed in WT N. benthamiana plants (WT-rhEPOELD). Corresponding bands (22-, 26-, and 30-kDa) were excised from denaturing gels loaded with TSP and double digested with trypsin and endoproteinase Glu-C to release three N-glycopeptides (Gp1-Gp3). Subsequent MS analysis revealed that all three N-glycosylation sites of WT-rhEPOELD carried a similar glycosylation pattern (Fig. 2 and Supporting information Fig. S2). The 26-kDa protein had a dominant single glycoform, i.e. GnGnXF, typical for plant-secreted proteins. This oligosaccharide formation was also present in the 30-kDa protein, however, the major fraction referred to ER-specific oligomannosidic structures (M8 and M9). Non-fucosylated complex glycans (GnGnX) and glycans containing galactose residues were detected in both bands. The galactosylated structures are most likely Lewis-a (Lea) carbohydrates (Gn(FA)XF), which have been previously reported on rhEPOFc expressed in N. benthamiana [35, 36] and are highly abundant on moss-derived rhEPO [24, 25]. MS analysis of the 22-kDa protein revealed non-glycosylated WT-rhEPOELD, which accounts for less than 5% of the recombinant hormone. The differences in size upon gel electrophoresis are most probably associated with different glycosylation of the recombinant protein. Signals in immuno-blot analysis with 2F5 mAbs, specific for the ELD-Tag, indicate intact N- and C-termini of the recombinant protein (Supporting information Fig. S1). rhEPOELD was abundantly present in intercellular fluid (IF), indicating efficient secretion of rhEPOELD to this compartment. Glycan analysis of secreted rhEPOELD displayed a homogenous profile of plant-specific complex N-glycans (GnGnXF) and no significant amounts of oligomannosidic structures were detected (Fig. 2).

### 3.3 Modulation of rhEPOELD N-glycosylation

We used N. benthamiana ΔXTFT as a host in a first set of experiments aimed at generating rhEPOELD with human-like N-glycosylation, i.e. lacking plant specific core α1,3-fucose and β1,2-xyllose residues. The recombinant EPO (AXXTFT-rhEPOELD) was produced in similar levels as WT-rhEPOELD. Also, AXXTFT-rhEPOELD was detected in three immunoreactive bands by western blot, exhibiting distinct glycosylation profiles: the 30-kDa protein band carried GnGn structures accompanied by high levels of oligomannosidic N-glycans (M8, M9), the 26-kDa band mainly carried complex GnGn structures and the 22-kDa band corresponded to non-glycosylated AXXTFT-rhEPOELD (Fig. 3). No N-glycans carrying core α1,3-fucose or β1,2-xyllose residues were detected. AXXTFT-rhEPOELD showed a largely homogeneous glycosylation profile.

In a next step, we set out to generate rhEPOELD carrying sialylated N-glycans. Recently we showed in planta sialylation of recombinant mAbs through the overexpression of six genes involved in the mammalian sialic acid pathway [29]. We used N. benthamiana GalT⁺ as the expression host to accomplish sialylation in plant-derived rhEPOELD. This glycosylation mutant synthesizes mammalian-type terminal β1,4-galactosylated structures, providing the acceptor substrate for subsequent sialylation [30]. Thus, rhEPOELD was co-expressed in N. benthamiana Gal⁺ plants with mammalian genes required for the synthesis of sialic acid (GNE, NANS, and CMAS), its transport into the Gogi (CST) and its transfer to terminally β1,4-galactosylated N-glycans (ST) (see Section 2). Subsequently, western blot analysis with antibodies against hEPO was used to monitor the expression of the recombinant hormone (SiarihEPOELD, Fig. 1B, right panel). The expression maximum was reached at 4 dpi, however, it seems that SiarihEPOELD is more stable than WT-rhEPOELD over time (Fig. 1B, left panel). Moreover, immunoreactive bands shifted toward increased size over time (Fig. 1B), indicating accumulation of the sialylated recombinant hormone. Apart from the three reactive bands (22-, 26-, and 30-kDa) an additional 37-kDa band appeared at 6 dpi. A detailed N-glycan analysis of the proteins from individual bands revealed that the 22-kDa protein, which accounts for less than 5% of the recombinant hormone, was non-glycosylated. The three larger bands (26-, 30-, and 37-kDa) exhibited substantial fractions of sialylated N-glycans, with some variation in bisialylated (NaNa) and incompletely processed structures (NaM) (Fig. 4 and Supporting information, Fig. S3). In addition, significant amounts of GnGn and minor fractions of oligomannosidic structures (M8, M9) were also present. Notably, SiarihEPOELD derived from the apoplastic fluid was decorated almost exclusively with sialylated N-glycans (IF, Fig. 4).

### 3.4 Purification of SiarihEPOELD

Although the expression of rhEPO has been reported to be successful in various expression hosts, purification of the recombinant hormone remains a challenge. Previous attempts using a C-terminal His or Strep II tag to purify rhEPO proved unsuccessful. Although the expression levels of the hormone were similar to those obtained in this study, we were not able to purify the recombinant protein due to unspecific binding (His-tag) or loss of tag (Strep II tag) (our laboratory, unpublished data). In this study we explored immunoaffinity of the ELDKWA epitope to the mAb 2F5 [34] in view of establishing a new purification method. Five milligram of purified CHO-derived 2F5 were coupled to an NHS-Sepharose pre-packed chromatography column. Clarified leaf protein extracts from SiarihEPOELD were loaded onto the column and eluted at different pH conditions. A sharp elution peak was visible in eluates E5 and E6. These two eluates, with a total volume of 1 mL, contained about 90% of the purified product. We used CBB G-250 to detect the purified hormone as
rhEPO is more sensitive to Brilliant Blue R-250 [31]. As already seen in immunoblots (Fig. 1B), the Coomassie-stained gels of purified recombinant hormone shows three protein bands (Fig. 5A) that react with anti-hEPO antibodies (Fig. 5B). Coomassie-stained gels upon loading 0.3 mL out of 0.5 mL eluate showed alongside the three major rhEPO specific bands, minor contamination with plant proteins (Supporting information, Fig. S4). Moreover significant amounts of the recombinant protein were detected in the flow through (FT, Fig. 5B), indicating that there is still room for optimization of the purification procedure. The $N$-glycosylation profile of the purified products largely resembled those observed in TSP extracts, where $\text{SiarhEPO}^{\text{ELD}}$ is produced as a mixture of glycoforms including sialylated and high manniosidic structures. In our attempts to enrich the fraction of $\text{rEPO}^{\text{ELD}}$ carrying sialylated glycoforms, we performed SNA lectin chromatography as a second purification step.

A schematic representation of the experimental procedure is outlined in Supporting information Fig. S5. Briefly, samples from $\text{SiarhEPO}^{\text{ELD}}$ purified by 2F5-based immunoaffinity were applied to the SNA lectin column, and eluates containing the recombinant protein were pooled and passed through an LC4 SPE reversed phase column to remove lactose. Subsequent $N$-glycosylation analysis of the product obtained revealed the presence of virtually exclusively sialylated $N$-glycan structures. An effective enrichment of bi-sialylated fractions accompanied by minor fractions of monoantennary sialylated structures was obtained (NaA, NaM, Fig. 5C, EL). As expected, rhEPO$^{\text{ELD}}$ present in the FT carried exclusively oligomannosidic structures (Fig. 5C, FT), demonstrating the efficiency of the procedure. Note that although the SNA chromatography allowed isolation of fully sialylated rhEPO$^{\text{ELD}}$, this step caused a significant reduction in product yield, and thus may to be optimized.
Figure 4. N-glycan profiles of rhEPOELD co-expressed with genes necessary for in planta sialylation in N. benthamiana Gal+, harvested at 7 dpi (SiarhEPOELD). Western blot analysis of TSP extracts with anti-hEPO antibody shows several reactive bands (22-, 26-, 30-, and 37-kDa, top right cartoon). The glycosylation profile (Gp3) of 37-, 30-, and 26-kDa immunoreactive bands are shown; bottom: MS profile of SiarhEPOELD isolated from the IF. (see Supporting information Fig. S3 for analysis of glycopeptide 1 and 2). Two independent repeats of the experience were performed. Peak labels were made according to the ProGlycAn system (www.proglycan.com).
3.5 In vitro activity assay

The in vitro biological activity of plant-derived rhEPO^{ELD} was evaluated by a UT-7 cell-based proliferation assay and compared with CHO-derived sialylated rhEPO. The latter consisted mainly of tri- and tetrasialylated core fucosylated N-glycans (CHO-rhEPO, Supporting information Fig. S6). Growth of the human leukemia cell line UT-7 is strictly correlated to the concentration of EPO in the culture medium. Cell-based in vitro assay indicated that plant-derived rhEPO^{ELD} was active (Supporting information, Fig. S7). Sia-rhEPO^{ELD} and CHO-rhEPO showed similar half maximum effective doses (ED50 values 0.66 and 0.53 ng/mL, respectively). As expected, non-sialylated WTrhEPO^{ELD} exhibited increased activity (ED50 0.03 ng/mL) compared with its sialylated form.

Figure 5. Analysis of 2F5 immunoaffinity-purified Sia-rhEPO^{ELD}. (A) SDS-PAGE Coomassie-stained gel of eluates 4–9 (E4–E9; no protein quantification was made), (B) corresponding western blot using antibodies against hEPO, Ext: TSP extract, FT: FT after immunoaffinity chromatography (5 µg TSP was loaded). Several independent repeats of the experience were performed. (C) N-glycan profiles of Sia-rhEPO^{ELD} purified by sequential 2F5 immunoaffinity and SNA lectin affinity chromatography (see also Supporting information Fig. S4). The glycosylation profile for glycopeptide 1 in the eluate (EL) and FT is shown. Peak labels were made according to the ProGlycAn system (www.proglycan.com).
4 Discussion

Erythropoietin is a multifunctional molecule produced and used by many tissues. Apart from its erythropoiesis action, other roles involve the acute and sub-acute biological responses to tissue damage [3, 4]. The extensive glycosylation of the protein is well characterized, and attempts have been made to generate hyperglycosylated forms of the protein in the course of developing drugs for treating anemia-associated conditions. However, considering the large N-glycan heterogeneity and multifunction of the hormone other glycoforms may be needed for new therapeutic applications [3, 4]. In the current study, we demonstrate the efficient expression of sialylated rhEPOELD in *N. benthamiana*, already at 4 dpi. This production speed provides advantages over other potential expression systems, such as mammalian cells. Terminal sialylation of rhEPOELD seemed to have a positive impact on the stability compared with WTrhEPOELD. Expression levels of up to 85 mg recombinant hormone/kg fresh leaf, corresponding to ~2% of TSP, were achieved. Compared to previous studies where up to 0.02% expression levels of rhEPO in plants were reported [37] our results demonstrate a significant improvement. Nevertheless the expression of rhEPOELD was relatively low compared with expression levels of other recombinant proteins using similar transient production systems, e.g., monoclonal antibodies are produced up to 50% of TSP [38–41]. Similarly, the expression of a recombinant EPO fusion (rhEPOFc) using the same magICON® expression system resulted in the generation of a chimeric recombinant hormone accompanied by a five to ten times higher level of free Fc [35, 36]. The main goal of this study was to generate rhEPOELD carrying different N-glycosylation profiles. With this intent, WT *N. benthamiana* and the glycosylation mutants ΔXTFT and GaIT⁺ were used as expression hosts. rhEPOELD derived from WT (WTrhEPOELD) was decorated mainly with plant-specific GnGnXF³ structures. These are the typical N-glycan formations expected on secreted plant glycoproteins. However, in addition significant amounts (ca. 30%) of oligomannosidic structures and Leα epitopes (ca. 15%) were detected. The presence of oligomannosidic structures was unexpected, they had not been detected previously in either native EPO or rhEPO expressed in mammalian cells [9, 11]. The fact that these structures were absent in IF-derived WTrhEPOELD, indicates that fractions of the protein are not properly secreted. It seems that part of rhEPOELD is sorted away from the secretory pathway and deposited in ER-derived compartments, a phenomenon observed previously upon expression of recombinant single chain antibodies in Arabidopsis seeds and *N. benthamiana* leaves [42, 43]. Leα epitopes were another N-glycan formation detected on WTrhEPOELD but not on native hEPO or CHO-derived rhEPO. Usually this glycoform accounts for only minor fractions of endogeneous Nicotiana proteins [28] and it had not been previously detected on plant-produced recombinant proteins [19, 20]. An exception is rhEPO expressed in moss cells, where large amounts of such oligosaccharide structures are present [25]. Leα structures have also been reported on a rhEPOFc fusion expressed in *N. benthamiana* [35, 36]. The reason for plant-derived rhEPO carrying relatively large amounts of Leα structures is currently not known.

N-glycans of rhEPOELD derived from ΔXTFT (AXTFTrhEPOELD) were essentially devoid of β1,2-xylase and core α1,3-fucose. Mainly two carbohydrate structures were present: complex GnGn and oligomannosidic structures. Surprisingly, no Leα epitopes were detected. Finally, using *N. benthamiana* GaIT⁺ as the expression host, an efficiently sialylated glycoform was produced (SiaΔrhEPOELD) by coexpressing hEPOELD with the genes necessary for in planta sialylation. Moreover, sialylation seemed to have a stabilization effect on the recombinant hormone as SiaΔrhEPOELD accumulated up to 9 days after infiltration, contrasting with the gradual reduction over time of WTrhEPOELD expression.

Mammalian cell produced rhEPO is usually purified by ultrafiltration followed by ion exchange chromatography, reversed phase chromatography and gel filtration. These techniques are hardly applicable to crude plant leaf extracts. We evaluated different immunoaffinity tags in our attempts to establish an efficient purification protocol for plant produced rhEPO. A C-terminal His-tagged rhEPO showed similar expression levels to rhEPOELD but we were not able to efficiently purify the recombinant protein due to unspecific binding of the abundant endogeneous protein RuBisCO to Ni-Sepharose (our unpublished data). Another widely used tag (Strep II tag) failed as well because the tag was not detectable in western blot analysis, even though the hormone was nicely expressed. While searching for alternatives, we came across the mAb 2F5, which strongly binds to the epitope ELDKWA localizing the HIV-1 envelope protein gp41 [34]. We established a new immunoaffinity protocol that allowed purification of the recombinant hormone at great homogeneity. The sequential immunoaffinity- and lectin affinity chromatography procedure allowed the enrichment of biantenary sialylated plant-derived rhEPOELD. Nevertheless, this procedure, particularly SNA treatment, caused a massive loss of the product, and finally only ca. 5% of the amount present in TSP could be recovered. The applicability of 2F5-ELDKWA-based immunoaffinity purification at industrial scale still needs to be evaluated and optimized. Previous studies reporting high level of 2F5 expression in CHO cells are an excellent basis for mass production of the mAb at reasonable costs [44, 45]. The fact that the immunoaffinity column could be reused several times indicates high stability of the 2F5-Sepharose. Finally, we demonstrated full activity of the plant-derived hormone. The degree of sialylation does not seem to influence receptor binding, as seen by similar binding affini-
ties of bisialylated $\text{Si}_2\text{hEPO}^{\text{RLD}}$ and $\text{CHO}_{\text{hEPO}}$, which mainly consists of tri- and tetrasialylated structures. On the other side, a higher receptor binding affinity of non-sialylated $\text{rhEPO}^{\text{RLD}}$ was observed, a phenomenon already described for $\text{hEPO}$ [46]. These results suggest a targeted manipulation of biological activities by alteration of the $N$-glycosylation profile. The results presented here together with the generation of branched $N$-glycosylated rhEPO in plants (tri and tetra-antennary structures, [35, 36]) serve thus as an excellent starting point for the synthesis of multisialylated rhEPO, the preferred glycoform currently used in anemia treatment (work is in progress). Bisialylated hEPO is an abundant glycoform of anemia-patients derived serum EPO. The function of this glycosylation form is not known. Our results allow structure-function studies on this (and other) individual glycoprofile(s), and thus may help to better understand the action of this multifunctional hormone.

In summary the ability to generate different rhEPO$^{\text{RLD}}$ with a targeted $N$-glycosylation profile paves the way to further investigate the impact of $N$-glycosylation on the function of the hormone, which in turn could lead to the discovery of new hEPO functions. Given the speed and function of the hormone, which in turn could lead to the discovery of new hEPO functions. Given the speed and function of the hormone, which in turn could lead to the discovery of new hEPO functions.

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