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Fine-tuning the *N*-glycosylation of recombinant human erythropoietin using *Chlamydomonas reinhardtii* mutants

S. Leprovost^{1,2,†}, C. Plasson^{1,†}, J. Balieu¹, M-L. Walet-Balieu³, P. Lerouge¹, M. Bardor¹ and E. Mathieu-Rivet^{1,*} 

¹Université de Rouen Normandie, Normandie Univ, GlycoMEV UR 4358, SFR Normandie Végétal FED 4277, Innovation Chimie Carnot, IRIB, GDR CNRS Chemobiologie, Rouen, France

²Institute for Plant Biology and Biotechnology (IBBP), University of Münster, Münster, Germany

³Infrastructure de Recherche HeRaLeS, Plate-forme protéomique PISSARO, Université de Rouen Normandie, Rouen, France

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*Correspondence (Tel 00 (33)2 35 14 67 24;

email elodie.rivet@univ-rouen.fr)

[†]Equal contribution of the co-authors.

Summary

Microalgae are considered as attractive expression systems for the production of biologics. As photosynthetic unicellular organisms, they do not require costly and complex media for growing and are able to secrete proteins and perform protein glycosylation. Some biologics have been successfully produced in the green microalgae *Chlamydomonas reinhardtii*. However, post-translational modifications like glycosylation of these *Chlamydomonas*-made biologics have poorly been investigated so far. Therefore, in this study, we report on the first structural investigation of glycans linked to human erythropoietin (hEPO) expressed in a wild-type *C. reinhardtii* strain and mutants impaired in key Golgi glycosyltransferases. The glycoproteomic analysis of recombinant hEPO (rhEPO) expressed in the wild-type strain demonstrated that the three *N*-glycosylation sites are 100% glycosylated with mature *N*-glycans containing four to five mannose residues and carrying core xylose, core fucose and *O*-methyl groups. Moreover, expression in *C. reinhardtii* insertional mutants defective in *xylosyltransferases A* and *B* and *fucosyltransferase* resulted in drastic decreases of core xylosylation and core fucosylation of glycans *N*-linked to the rhEPOs, thus demonstrating that this strategy offers perspectives for humanizing the *N*-glycosylation of the *Chlamydomonas*-made biologics.

Keywords: *Chlamydomonas reinhardtii*, biologics, erythropoietin, glycosylation, glycoengineering.

Introduction

The biopharmaceutical market size was about US \$343 billion in 2021 (Walsh and Walsh, 2022). In this market, sales of recombinant proteins like antibodies or hormones represent US \$271 billion with an increase of 44% between 2017 and 2021. Currently, recombinant proteins are mainly produced in Chinese HAMSTER OVARY (CHO) cells but this type of production is expensive and provide variable outcomes. As a consequence, there is a growing interest in the development of new, cheaper and effective expression systems. In this context, microalgae represent an attractive alternative. As photosynthetic unicellular organisms, they do not require costly and complex media for growing and most of them are classified by the USA Food and Drug Administration as 'Generally Recognized As Safe'. Moreover, as other eukaryotes, they are able to secrete proteins in the culture medium and perform protein *N*-glycosylation. A few studies have reported the expression of glycosylated therapeutic proteins in microalgae, highlighting the potential of these organisms as innovative cell biofactories. For example, a fully assembled recombinant IgG antibody directed against hepatitis B surface antigen has been produced in the diatom *Phaeodactylum tricoratum* (Hempel *et al.*, 2011) and has been demonstrated to be of good quality, glycosylated and able to efficiently bind human Fcγ receptors (Vanier *et al.*, 2015, 2017).

With regards to green microalgae, about 10 proteins, including human erythropoietin (hEPO), growth factors, interleukin 2 and

the receptor binding domain of the viral protein Spike, have been produced in *Chlamydomonas reinhardtii* (Berndt *et al.*, 2021; Deghani *et al.*, 2020; Eichler-Stahlberg *et al.*, 2009; Jarquín-Cordero *et al.*, 2020; Kiefer *et al.*, 2022). However, post-translational modifications including glycosylation of these therapeutic proteins expressed in *C. reinhardtii* are poorly documented (Smyth *et al.*, 2021). This is a major concern as the *N*-glycan biosynthesis pathways differ between *C. reinhardtii* and mammals and give rise to structurally different final glycan structures. For example, some of the biologics produced in CHO cells can exhibit sialylated polyantennary *N*-glycans that may be required for their *in vivo* half-time and bioactivity. In contrast, mature glycans *N*-linked to *C. reinhardtii* endogenous proteins exhibit α(1,3)-fucose (core-Fuc) linked to the proximal *N*-acetylglucosamine (GlcNAc) of the core chitobiose of non-canonical Man₄GlcNAc₂ (M4) and Man₅GlcNAc₂ (M5), in which the arrangement of mannose residues differs from the canonical structures described in plants or mammals (Table S1). Moreover, two xylose (Xyl) residues are linked to M4 and M5, one of them being β(1,2) linked to the core mannose (core-Xyl) and the other one on the trimannosyl linear branch. It should be noted that another pentose, arabinose, was recently identified on protein *N*-glycans from another microalga species belonging to the Chlorophyta (Mócsai *et al.*, 2020), and as a consequence, we cannot definitively rule out that arabinose residues are not present in *Chlamydomonas* *N*-glycans. In addition, mannose residues are partially *O*-methylated (Mathieu-Rivet *et al.*, 2013;

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Vanier *et al.*, 2017). Core-Xyl and core-Fuc have been shown to potentially induce immune reactions in mammals (Bardor *et al.*, 2003). Thus, the production of biologics in *C. reinhardtii* raises the question of the engineering of the *N*-glycosylation of Chlamydomonas-made biologics in order to remove putative immunogenic glycoepitopes and to make them suitable for future human therapy. Recently, the genes encoding some of the transferases responsible for the transfer of core-Xyl and core-Fuc epitopes, *xylosyltransferases A* and *B* (Cre09.g391282, *XTA* and Cre16.g678997, *XTB*) and *fucosyltransferase* (Cre18.g749697, *FUT*), have been identified. Moreover, the investigation of the insertional double-mutant $IM_{XTA} \times IM_{XTB}$ and triple-mutant $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ demonstrated that the inactivation of these genes resulted in drastic decreases in core xylosylation and core fucosylation of glycans *N*-linked to their endogenous proteins and as a consequence, reduces the putative immunogenicity of *C. reinhardtii* proteins (Lucas *et al.*, 2020; Oltmanns *et al.*, 2020; Schulze *et al.*, 2018).

Herein, we report on the structural investigation of a therapeutic human glycoprotein hormone, hEPO, recombinantly expressed in a wild-type *C. reinhardtii* strain and in $IM_{XTA} \times IM_{XTB}$ and $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ mutants for which the *N*-glycosylation pathway has been impaired. hEPO was selected as a model biopharmaceutical molecule because it exhibits three *N*-glycosylation sites on N24, N38 and N83, together with one *O*-glycosylation site on S126. Moreover, glycans account for 40% of the protein molecular weight and are essential for its stability and *in vivo* biological activity (Wasley *et al.*, 1991). The main objective of this study is to investigate whether knock-out strategies developed for the deletion of *N*-glycan immunogenic epitopes in *C. reinhardtii* are suitable for the glycoengineering of a therapeutic protein since this aspect is crucial for further exploitation of microalgae for the production of human-compatible biologics.

Results

rhEPO expressed in various genetic backgrounds is *N*-glycosylated

The sequence encoding hEPO was synthesized in fusion with the fluorescent protein mClover at the 3'-end of *hEPO* to help the selection of the best transformants exhibiting highest expression levels of the fusion protein rhEPO::mClover (Berndt *et al.*, 2021; Einhaus *et al.*, 2021, 2022; Freudenberg *et al.*, 2021, 2022; Lauersen *et al.*, 2016, 2018; Perozeni *et al.*, 2020; Wichmann *et al.*, 2018). The sequence coding for the signal peptide of the carbonic anhydrase CAH1 was added upstream *rhEPO::mClover* sequence to allow the secretion of the protein in the culture medium (Molino *et al.*, 2018). Finally, two copies of *RBCS2* intron 1 as well as one copy of *RBCS2* intron 2 were added inside the *rhEPO::mClover* sequence, as this strategy led to significant improvements of the expression levels of nuclear transgenes (Baier *et al.*, 2018, 2020). The expression of this optimized *hEPO* sequence was then driven *via* the combination of the PSAD promoter with FDX1 terminator (Figure 1a). CC5325 wild-type (WT) *C. reinhardtii* strain and mutants impaired for *XTA* and *XTB* activities (double-mutant $IM_{XTA} \times IM_{XTB}$) and for both xylosyltransferases and *FUT* activities (triple-mutant $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$) were transformed with this construct. Positive clones expressing the highest levels of rhEPO were then selected according to the fluorescence level of mClover for further analysis by Western blot (Figure 1b). In all transformants, a signal at the

molecular weight of ~65 kDa was immunodetected using an anti-hEPO antibody in proteins isolated from the culture medium, whereas only a very weak band was immunodetected in the total protein extracts isolated from the cell pellets (Figure 1b). This suggests that the major part of expressed rhEPOs is secreted in the culture medium. The amount of secreted rhEPO in the culture medium was then quantified by an ELISA assay. rhEPO was found to be expressed at levels ranging from 0.02 in the triple mutant to 4.46 ng/mL in the reference strain. Despite the efforts made for optimizing the construction allowing the expression of rhEPO, these expression yields remained low compared to data published by Eichler-Stahlberg *et al.* (2009). This is likely due to the genetic background of the strain used for this study in which epigenetic mechanisms probably limit the expression of nuclear transgenes (Neupert *et al.*, 2020).

Recombinant human EPO fused to mClover was purified from the culture media by affinity chromatography. The electrophoretic mobility of purified rhEPOs was then investigated after treatment with deglycosylating enzymes to get insights into their *N*-glycosylation profiles. It is to note that the mClover does not contain any *N*-glycosylation site. The analysis was performed on rhEPOs expressed in WT as well as in the double-mutant $IM_{XTA} \times IM_{XTB}$ and the triple-mutant $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ (Figure 1c). Whereas Endo H is able to only release oligomannosidic *N*-glycans ranging from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$, PNGase F is able to cleave all glycans *N*-linked to Asn of glycosylation sites, except those having a core-Fuc (Tretter *et al.*, 1991). As depicted in Figure 1c, the electrophoretic mobilities of rhEPOs were not affected after deglycosylation with Endo H, suggesting that rhEPOs do not carry oligomannosidic *N*-glycans. In contrast, after PNGase F treatment, additional signals at lower molecular weights than rhEPOs were immunodetected for rhEPOs expressed in WT strain, $IM_{XTA} \times IM_{XTB}$ and $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ mutants (Figure 1c). These results suggested that rhEPOs expressed in *C. reinhardtii* are *N*-glycosylated with mature *N*-glycans rather than oligomannoside structures. Moreover, the occurrence of additional electrophoretic bands after PNGase F treatment demonstrated that the three glycosylation sites are occupied in rhEPOs with either core-Fuc containing *N*-glycans (resistant to PNGase F) and mature *N*-glycans devoid of this specific glycoepitope (sensitive to PNGase F), each electrophoretic bands of lower mobility corresponding to rhEPOs lacking core-Fuc on one to three *N*-glycosylation sites of rhEPO.

Expression of rhEPOs in glycosylation mutants results in the secretion of glyco-engineered rhEPO lacking immunogenic core-Xyl and core-Fuc epitopes

The peptide sequence and the *N*-glycosylation profile of rhEPOs expressed either in WT or in the double $IM_{XTA} \times IM_{XTB}$ and triple $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ mutants were then determined through a glycoproteomic approach (Balieu *et al.*, 2022). The purified proteins were separated by SDS-PAGE and digested by trypsin and Glu-C. The resulting peptides and glycopeptides were analysed by nano-liquid chromatography coupled to electrospray mass spectrometry (LC-ESI MS/MS). The overall protein sequence coverages were determined to be about 60% (Figure S1). Peptides containing N24, N38 or N83 were not detected in any sample, thus suggesting that they are fully *N*-glycosylated. In contrast, the peptide E117-R131 devoid of any *O*-glycan modification was detected in the LC-ESI MS/MS analyses of all samples, suggesting that the *O*-glycosylation site at S126 is not

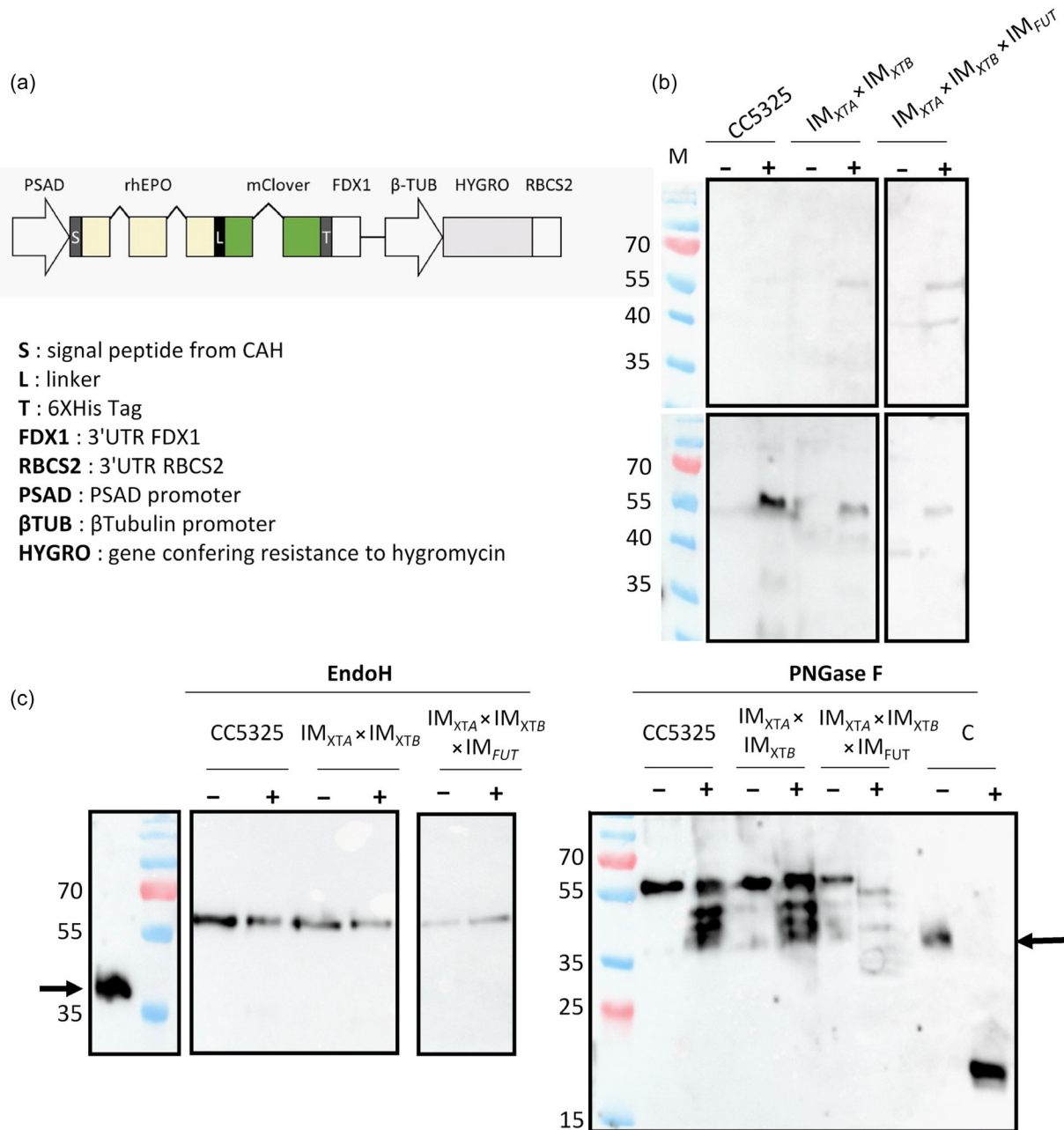


Figure 1 rhEPO is secreted in the culture medium in *C. reinhardtii* transformants and is N-glycosylated. (a) Construct used for the expression rhEPO in *C. reinhardtii*. (b) Western blot analyses using an anti-hEPO antibody performed on total proteins from the cell pellets (upper panel) and TCA precipitated proteins from the culture medium (bottom panel) of CC5325, $IM_{XTA} \times IM_{XTB}$ and $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ mutants. (–) Proteins from non-transformed strains used as negative controls. (c) rhEPOs were purified from the culture media, submitted to a deglycosylation with Endo H or PNGase F and then analysed by Western blot using an anti-hEPO antibody. (–) and (+): Samples non-treated or treated by Endo H or PNGase F. C: positive control, corresponding to commercial rhEPO. The black arrows point out on intact commercial rhEPO.

occupied in rhEPOs produced in *C. reinhardtii* (Figure S1). In order to determine the N-glycan structures and their distribution on the three N-glycosylation sites, the mixtures of peptides and glycopeptides released by the endoprotease digestions were submitted to a targeted LC-ESI MS/MS analysis (Balieu *et al.*, 2022). To this end, peptides giving MS/MS spectra exhibiting N-glycan diagnostic fragment ions at m/z 204 (GlcNAc) and 366 (Man-GlcNAc) were selected as being glycopeptides. Whatever the strain in which rhEPO was expressed, the three

glycosylation sites of rhEPOs were found to be occupied in rhEPOs by a mixture of mature glycans with relative abundances depending on the protein N-glycosylation site (Figure 2 and Table S2).

Sequences of N-linked glycans of rhEPO glycopeptides from WT were determined on the basis of the MS/MS fragmentation patterns of glycopeptides as illustrated for $[M + 3H]^{3+}$ ions assigned to HCSSLNENITVPDTK (Figure 3a). Major ions were assigned to M4- and M5-based structures depicted in Table S1.

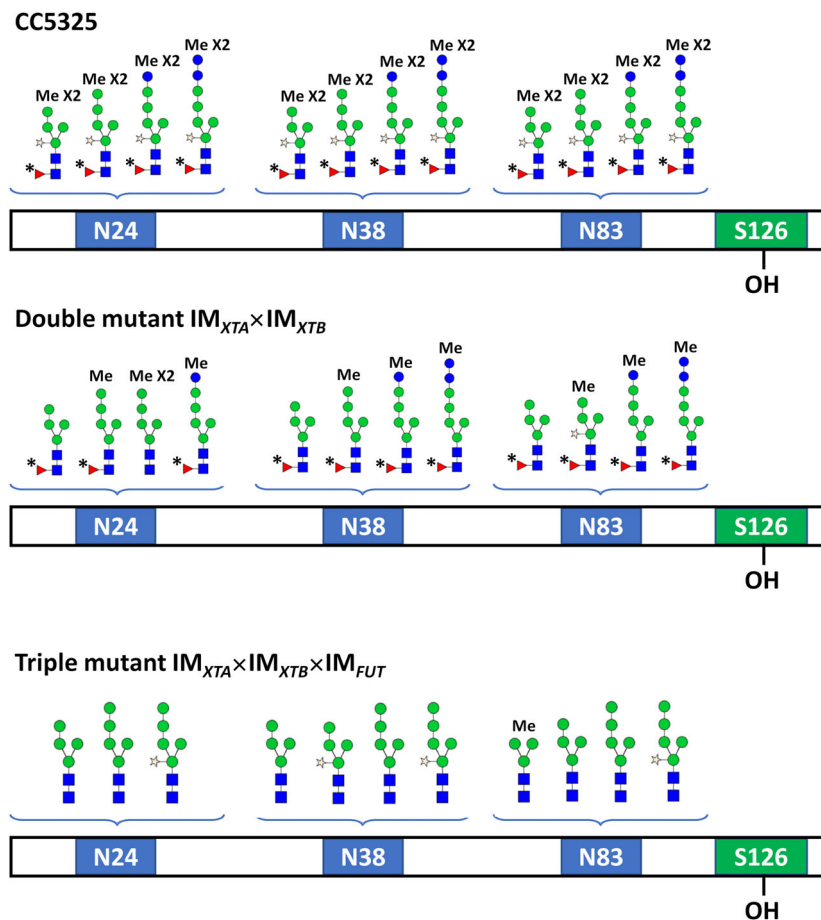


Figure 2 Distribution of *N*-glycans on the three *N*-glycosylation sites of rhEPOs produced in *C. reinhardtii* CC5325, $IM_{XTA} \times IM_{XTB}$ and $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ mutants on rhEPOs. For structures containing one xylose residue, this xylose residue is represented as $\beta(1,2)$ linked to the core mannose but it may also be linked to the trimannosyl linear branch (Lucas *et al.*, 2020). Other *N*-glycans were identified either lacking Fuc on the proximal GlcNAc or with additional *O*-methylations. Blue square: GlcNAc; green circle: Man; blue circle: Glc; yellow star: xylose, red triangle: $\alpha(1,3)$ -fucose, red triangle with a star: *O*-methyl-fucose, Me: methyl group on Man.

Based on *m/z* values of molecular ions and fragmentation patterns, these structures were found to be M4 and M5 substituted by Xyl and Fuc residues, and *O*-methyl groups in accordance with previous reports on the *N*-glycosylation of endogenous proteins in *C. reinhardtii* (Lucas *et al.*, 2020; Mathieu-Rivet *et al.*, 2013; Oltmanns *et al.*, 2020; Schulze *et al.*, 2018) (Figure 2 and Table S2). Although we cannot definitively conclude on the location of the Xyl residues on *N*-glycans, we can assume that at least one of them is attached to the core mannose *via* a $\beta(1,2)$ linkage as the purified rhEPO is recognized by the anti- $\beta(1,2)$ -Xyl antibodies that specifically recognize the core Xyl (Figure S2; Faye *et al.*, 1993). Surprisingly, MS/MS fragmentation of rhEPO glycopeptides from WT also revealed the presence of one *O*-methyl fucose residue linked to the proximal GlcNAc. Fragment ions assigned to the peptide attached to a GlcNAc are usually observed in the fragmentation patterns of *N*-glycopeptides together with ions differing by 146 Da for *N*-glycans having a Fuc residue on the proximal GlcNAc. However, we observed ions differing by 160 Da from peptide carrying a single GlcNAc residue which suggests a substitution of the proximal GlcNAc by an *O*-methylated Fuc instead of a Fuc (Figure 3a). Core fucosylation was previously reported in *C. reinhardtii* (Mathieu-Rivet *et al.*, 2013; Oltmanns

et al., 2020) but not *O*-methyl fucosylation. This suggests that *O*-methylation of proteins *N*-glycans in *C. reinhardtii* is not restricted to mannose residues but also occurs on the core-Fuc.

Compared to structures identified on rhEPO expressed in WT, rhEPO from the double $IM_{XTA} \times IM_{XTB}$ mutant impaired for XTA and XTB activities displayed structures containing also four to five mannose residues as well as *O*-methyl-Fuc, but lacking most Xyl substitutions (Figure 3b). Likewise, mainly M4 and M5 oligomannosides were found on the three *N*-glycosylation sites of rhEPO expressed in the $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ triple mutant (Figure 2 and Table S2). A few xylosylated structures were detected in this triple mutant which is consistent with the results of the Western blot analysis using an anti- $\beta(1,2)$ -Xyl antibody that has been performed on purified rhEPO (Figure S2). It is worth noting that fragment assigned to an *O*-methyl Fuc residue linked to the proximal GlcNAc was not observed in MS/MS spectra of glycopeptides from the $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ triple mutant that is defective in core *N*-glycan fucosylation (Figure 3c).

Glycoproteomic analyses of rhEPO indicated some oligosaccharides attached to the three glycosylation sites are *N*-glycans containing 6 or 7 hexoses (Figure 2). These are M5 *N*-glycans with two additional terminal Glc residues that are transiently transferred on M5 in the ER to ensure the quality control of the

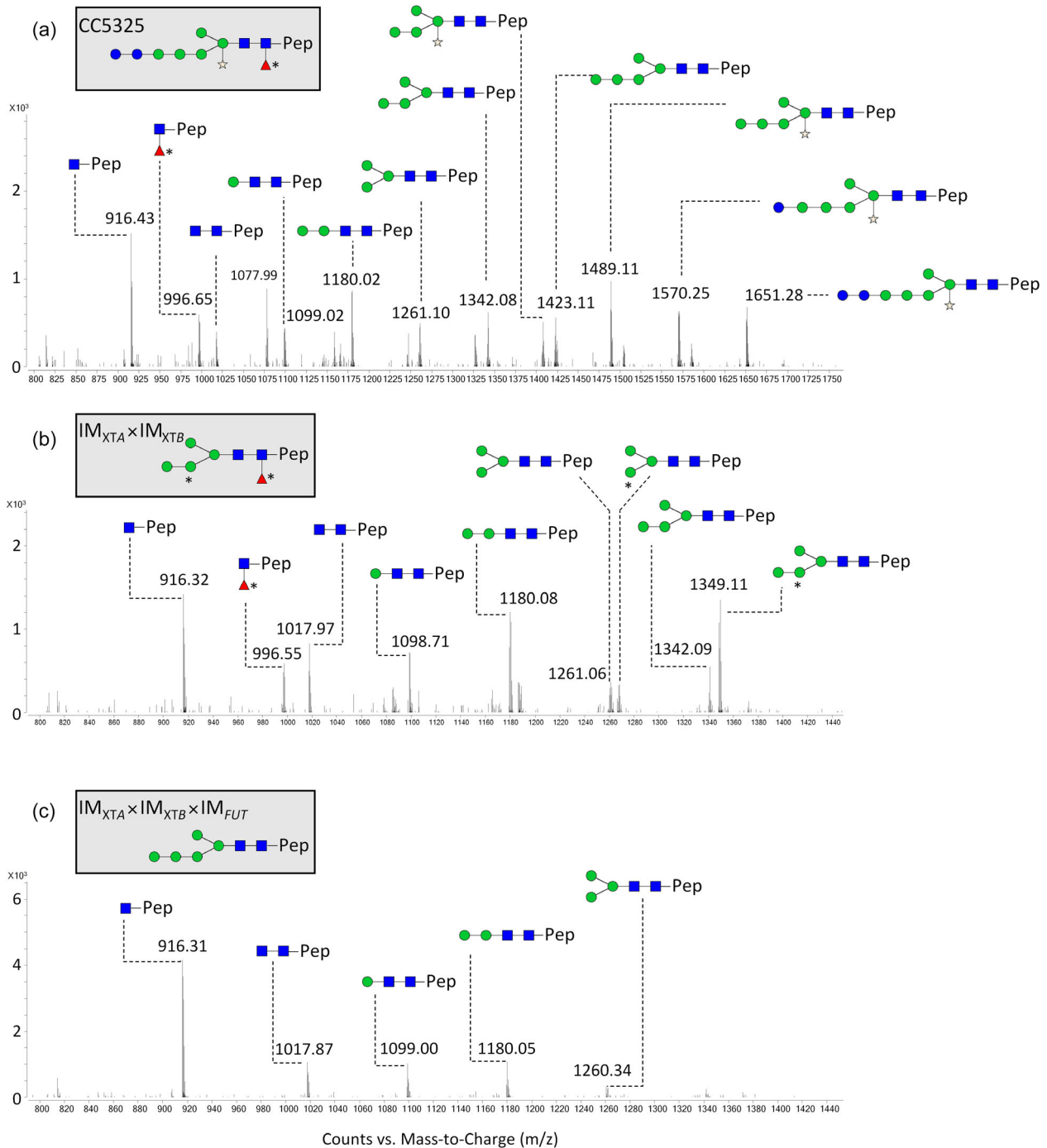


Figure 3 MS/MS spectra of $[M + 3H/3]^{3+}$ ion at m/z 1154.144 (a), 952.750 (b) and 948.738 (c) that were assigned to rEPO peptide HCSLNENITVPDTK (Pep) *N*-linked to G2M5XF* in CC5325, M4F** in $IM_{XTA} \times IM_{XTB}$ and M5 in $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$. Main doubly charged ions were assigned to the glycopeptide fragments. Blue square: GlcNAc; green circle: Man; blue circle: Glc, yellow star: $\beta(1,2)$ -xylose and red triangle: $\alpha(1,3)$ -fucose. *O-methylation.

glycoproteins (GM5 and G2M5; Table S1) (Lucas *et al.*, 2018). These minor mono- and diglycosylated *N*-glycans, together with their xylosylated and *O*-methylated derivatives, have been previously reported in *C. reinhardtii* *N*-glycan profiles (Lucas *et al.*, 2020; Oltmanns *et al.*, 2020; Schulze *et al.*, 2018). However, to check whether these glucosylations do not result from the expression of rEPO, a glycan analysis of endogenous proteins from WT and mutant lines was carried out. As shown in

Figure S3, GM5 and G2M5, as well as GM5X and G2M5X, were observed in MALDI TOF MS glycan profiles obtained from total proteins from the different preparations.

Discussion

The development of alternative systems for the expression of biologics combining reduced production cost and reduced

safety risks like adventitious contamination from virus represents a major challenge in the near future (Brady and Love, 2021). During the last 20 years, *C. reinhardtii* has been extensively studied as a microalga model for the production of recombinant proteins. However, despite recent progress allowing increasing the expression level of secreted recombinant proteins (Schroda and Remacle, 2022), the glycosylation of a glycoprotein recombinantly expressed in this model has never been reported so far.

In order to investigate whether this green microalga is a suitable expression system for the production of therapeutic glycoproteins, we report in this study on the first structural investigation of glycans linked to a biopharmaceutical glycoprotein hormone expressed in *C. reinhardtii*. hEPO was chosen because it is a small glycoprotein exhibiting both *N*- and *O*-glycosylation sites and it was already produced successfully in *C. reinhardtii* (Eichler-Stahlberg et al., 2009). hEPO was expressed in *C. reinhardtii*, a wild-type strain and mutants impaired for *N*-glycosylation. ESI-MS/MS analyses of rhEPO demonstrated that the three *N*-glycosylation sites are 100% glycosylated. Glycans *N*-linked to rhEPO expressed in WT strain correspond to mature oligosaccharides containing four or five mannose residues carrying core-Xyl and core-Fuc and *O*-methyl groups (Figure 2). Previous glycomic analyses performed in *C. reinhardtii* showed that the *N*-glycan population released from endogenous proteins was composed of a major part of non-canonical M5 arising from the *N*-glycan processing occurring in the endoplasmic reticulum (ER) and M3 to M5 substituted with core-Xyl, core-Fuc and *O*-methyl groups arising from maturation in the Golgi apparatus (Lucas et al., 2018; Mathieu-Rivet et al., 2013; Oltmanns et al., 2020). Thus, the fact that rhEPO harbours mainly mature structures rather than oligomannosides suggests that *N*-glycans are exposed to the surface of the glycoprotein in a such way that they are fully accessible to the Golgi enzymes. This feature has been also reported for hEPO or recombinant hEPO expressed in CHO cells (Yang et al., 2017). This makes hEPO a suitable glycoprotein model for studying glycoengineering strategies based on the inactivation of Golgi-resident glycosidases and glycosyltransferases. Then, the *N*-glycan structures of rhEPO produced in different glycosylation mutants of *C. reinhardtii* have been analysed. As observed on endogenous proteins of *C. reinhardtii*-mutant strains (Lucas et al., 2020; Oltmanns et al., 2020; Schulze et al., 2018), the inactivation of *XTA* and *XTB*, and *FUT* resulted in a drastic decrease in immunogenic core xylosylation and core fucosylation of glycans *N*-linked on rhEPO expressed in these mutants. However, core xylosylation was not fully suppressed (Figure S2) probably because of the presence of additional xylosyltransferases that are predicted in the *C. reinhardtii* genomes, likely contributing to the protein core xylosylation through a compensation phenomenon (Lucas et al., 2020; Oltmanns et al., 2020). Moreover, the inactivation of protein core xylosylation and core fucosylation in mutants gives rise to mainly short oligomannosides that are more suitable for further glycoengineering to human-like *N*-glycans by expression of human glycosyltransferases to build the missing sialylated lactosamine sequences that are required for *in vivo* half-life and activity of rhEPO (Takeuchi et al., 1989; Yuen et al., 2003).

It is worth noting that our analysis allowed the identification of an *O*-methyl Fuc on the proximal GlcNAc residue of *N*-glycans, whereas so far, only core-Fuc was detected in glycoproteomic analysis performed on endogenous proteins (Mathieu-Rivet et al., 2013; Oltmanns et al., 2020). This residue results probably

from the high accessibility of rhEPO *N*-glycans to Golgi *O*-methyltransferases. Unfortunately, although the construction allowing the expression of rhEPO was designed to optimize the yield of secreted rhEPO, this remained too low to perform the analysis of PNGase-released *N*-glycans from purified rhEPO by MALDI-TOF mass spectrometry. However, these *O*-methyl Fuc-containing structures were not observed in *N*-glycan profiles of total endogenous proteins from *C. reinhardtii*, suggesting that they are resistant to the deglycosylation by PNGase A. We presume that the *O*-methylation of Fuc residue linked to the proximal GlcNAc residue prevents the *N*-glycans cleavage by PNGase A. *O*-methyl Fuc has already been previously reported on *N*-glycans of proteins from *C. elegans* (Altmann et al., 2001), or more recently on terminal lactosamine epitopes of *N*-glycans of proteins from the oysters *Crassostrea gigas* and *Ostrea edulis* (Auger et al., 2023).

N-linked glycans containing terminal sialic acids were not found in the glycan profile of rhEPOs expressed in *C. reinhardtii*. This is consistent with previous reports (Lucas et al., 2020; Mathieu-Rivet et al., 2013; Oltmanns et al., 2020; Schulze et al., 2018). This aspect could constitute a limit to the use of such microalga model for the expression of therapeutic proteins like hEPO since its most active form harbours sialylated tetraantennary *N*-glycans. Moreover, it is well established that the presence of sialic acid residues protects EPO from its recognition by galactose-binding receptors located on erythrocytes, thus preventing its clearance and prolonging its half-life in serum (Elliott et al., 2014; Wasley et al., 1991). Therefore, knock-in strategies would be necessary in future studies to express heterologous glycosyltransferases and glycoenzymes that are required for the synthesis of sialylated *N*-glycans in *C. reinhardtii* as previously reported in moss and plants (Bohlender et al., 2020; Kallolimath et al., 2016).

hEPO also exhibits one *O*-glycosylation site on S126. hEPO *O*-glycan is a mucine-type oligosaccharide with a GalNAc *O*-linked to the serine residue (Sasaki et al., 1987). In our study, no *O*-glycan was observed on rhEPOs expressed in *C. reinhardtii*. Only few information is available on protein *O*-glycosylation in *C. reinhardtii*, except for hydroxyproline-rich glycoproteins (Mathieu-Rivet et al., 2020). To our knowledge, GalNAc has not been reported in *C. reinhardtii* and, as a consequence, gaining human glycans *O*-linked to a recombinant therapeutic protein produced in this green microalga would require both the implementation of the enzymatic machinery to supply the Golgi apparatus with the nucleotide-sugar UDP-GalNAc, and also the expression of appropriate glycosyltransferases such as a polypeptide: *N*-acetylgalactosaminyltransferase, as well as a core $\beta(1,3)$ -galactosyltransferase, that are required for the formation of the most common *O*-glycan extension (Castilho et al., 2012).

Materials and methods

Strains and growth media

The insertional mutants $IM_{XTA} \times IM_{XTB}$ (Lucas et al., 2020) and $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ (Oltmanns et al., 2020; Schulze et al., 2018) were used for these experiments. The double-mutant $IM_{XTA \times XTB}$ has been obtained by crossing the insertional strains LMJ.RY0402.087519 (Cre09.g391282, XP_042921158.1) and LMJ.RY0402.118417 (Cre16.g678997, XP_042916231.1) provided by the ClIP library (Li et al., 2016; Zhang et al., 2014). The triple-mutant $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ has been obtained by crossing an insertional mutant IM_{XTA} from the library described in Cheng et al., 2017, with the double-mutant $IM_{XTB} \times IM_{FUT}$

that was generated from strains LMJ.RY0402.049160 (Cre18.g749697, XP_001695259.2) and LMJ.RY0402.118417 (Cre16.g678997, XP_042916231.1) from the CLIP library. The strain CC5325 was chosen as a wild-type strain (WT) since it is the genetic background that was used to generate the mutants in the CLIP library (Li *et al.*, 2016; Zhang *et al.*, 2014). All the strains were grown on Tris-acetate phosphate (TAP) medium (Gorman and Levine, 1965) at a constant illumination of 400 $\mu\text{mol photons/m}^2/\text{s}$, at 25 °C.

Constructs and transformation

The 5'-part of the sequence encoding hEPO was fused to the 3' part of the sequence encoding for mClover, and a sequence coding for the SP from the carbonic anhydrase (SP-CA, Molino *et al.*, 2018) was added downstream to this construct, leading the expression of the fusion protein SP-CA::rhEPO::mClover. This sequence was then optimized using Intronsorter (Jaeger *et al.*, 2019) as follows: two copies of the first RBCS2 intron as well as one copy of the second RBCS2 intron were added, and the sequence was synthesized according to the nuclear codon bias in *C. reinhardtii*. The expression of this optimized sequence was then driven via the combination of the PSAD promoter with FDX1 terminator (López-Paz *et al.*, 2017). A second transcriptional unit was added to these constructions allowing the resistance to hygromycin.

After *in silico* design, all plasmids were synthesized *de novo* by polyplus transfection SA (<https://www.polyplus-sartorius.com>, Loos, France). Plasmids were delivered as lyophilized DNA, transformed and maintained in *Escherichia coli* DH5 α grown in lysogeny broth medium (LB)-containing ampicillin. All plasmid vectors were prepared by midi prep using the NucleoBond Xtra Midi kit from Macherey-Nagel (Düren, Germany) and then linearized by digest with EcoRV restriction enzyme at 37 °C. For each transformation, a starter culture of *C. reinhardtii* was grown in TAP media to near saturation ($\sim 5 \times 10^6$ cells/mL) and then diluted back into 0.5×10^6 cells/mL in 500 mL of TAP media 24 h before transformation. The next day, cells were collected by centrifugation at 1000 *g* for 5 min at 16 °C. The cells were then resuspended in 4 mL of TAP containing 40 mM sucrose. Depending on the cell pellet size, the final volume is about 5 mL. For one transformation, 250 μL is transferred in a 4 mm gap distance electroporation cuvette (Fisherbrand; FisherScientific), with 1–2 μg of linearized vector. The mixture is then incubated for 5 min at 16 °C before electroporation using a Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, Hercules, CA, USA) set to 800 V and 25 μF . Immediately after electroporation, the mixture is resuspended in 8 mL TAP 40 mM sucrose. After overnight recovery in 40 mM sucrose in TAP media with low light ($\sim 5 \mu\text{mol photons/m}^2/\text{s}$), cells were pelleted, resuspended in 5 mL of fresh TAP media and plated on solid TAP containing 15 $\mu\text{g/mL}$ zeocin or 15 $\mu\text{g/mL}$ hygromycin B. The positive clones expressing rhEPO::mClover were screened thanks to the fluorescence level of mClover. Individual colonies grown on TAP medium supplemented with selection antibiotic were picked and resuspended in 160 μL of TAP without antibiotic into a 96-well plate. After 5 days, the mClover fluorescence was analysed using the FlexStation[®] 3 Multi-Mode Microplate Reader (Molecular Devices LLC, San Jose, CA, USA) according to the following parameters: excitation: 480 nm; emission: 530 nm. mClover fluorescence was normalized to DO₇₂₀ to take into account differences in cell density.

Protein extraction

Total proteins were extracted from a 5 mL culture grown until near saturation (DO₇₂₀ \approx 1). After centrifugation and removing of the culture medium, the cell pellet was broken in 1 mL of Agrisera buffer (0.138 M Tris-HCl pH 8.5, 0.5 M LDS, 10% (v/v) glycerol, 0.5 mM EDTA) in a microtube containing lysis matrix D (MP Biomedicals™) with a Fastprep system (MP Biomedicals™) according to the following parameters: 4 cycles (40 s; 6.5 m/s). After centrifugation at 4000 *g* 10 min, 20 μL of supernatant was used for Western blot analysis. Proteins of the culture medium were precipitated with 10% TCA (v/v) at 4 °C overnight. After 3 washes with cold acetone 80% (v/v), the pellet is resuspended in 60 μL Agrisera buffer and 30 μL was used for Western blot analyses.

Purification of rhEPO-mClover

rhEPO-mClover was purified using the GFP-Trap™ Magnetic Agarose Kit (ChromoTek[®]), according to the manufacturer's instructions. Briefly, the medium was collected from a 200 mL culture grown until near saturation (DO₇₂₀ \approx 1) and then lyophilized before being re-suspended in 20 mL of phosphate buffer saline pH 6.7 containing a cocktail of protease inhibitor (Roche). After a centrifugation at 48 000 *g* for 2 h, the supernatant was loaded on an Amicon 50 KDa centrifugal filter unit 5000 *g* for 2 h, to get a final volume of 500 μL . The filter was rinsed with 500 μL of 2 \times dilution buffer provided in the kit, in such a way that secreted proteins were concentrated in final volume of 1 mL. Twenty-five microlitres of beads were then incubated in this volume overnight at 4 °C on a carousel rotator. After three washing using the provided buffer, 80 μL of LSB2X was added and the mixture was warmed at 95 °C for 5 min for further glycoproteomic analysis. For further deglycosylation treatment, rhEPO is eluted using 140 μL of acidic elution buffer provided in the kit and then neutralized with 20 μL of neutralization buffer.

PNGase F and Endo H treatment

For PNGase F deglycosylation, 110 μL of purified rhEPO was first denaturated in Tris-HCl 0.1 M pH8, 0.1% SDS (w/v) during 5 min at 100 °C. Then, deglycosylation is performed in Tris-HCl 0.1 M pH8, NP40 0.5% (v/v) using 1 U of PNGase F (Roche) during 24H at 37°C. Deglycosylation with 2000 U EndoH (Promega) was carried out on 50 μL of purified rhEPO according to the manufacturer's instructions.

Western blot analysis

Immunoblotting analyses were performed on total proteins from cells, the culture medium or on purified rhEPO. Proteins were separated on 12% SDS polyacrylamide gels and transferred to nitrocellulose membranes (0.2 μm ; Amersham™ Protran[®]) using the Pierce™ Power Blotter system (Thermo Scientific™) and semi-dry blotting method according to the manufacturer's instructions. rhEPO-mClover was detected using either anti-eGFP (1/6000e; Invitrogen AB_962098) or anti-hEPO (1/2000e; Abcam ab226956) as a primary antibody, and an HRP-conjugated secondary antibody (Invitrogen A16041 for anti-eGFP, and 65–6120 for anti-hEPO). For the analysis of the presence of xylose or fucose residue, rhEPO-mClover was detected using either anti-Xyl (1/3000e; Agrisera AS07 267) or anti-Fuc (1/2000e; Agrisera AS07 268) as primary antibodies and a HRP-conjugated secondary antibody (Invitrogen G5 G120).

ELISA assay

The amount of rhEPO was quantified using the Human EPO ELISA Kit (Invitrogen BMS2035-2) according to the manufacturer's instructions. For each clone expressing rhEPO, three independent cultures were grown for 5 days. After centrifugation (4500 *g*, 10 min) to pellet the cells, ELISA assays were performed on 50 μ L of the culture medium. The reported value for each clone corresponds to the average of three technical repeats.

Glycoproteomic analysis

The protein sequence and *N*-glycan profile of purified rhEPOs were determined through the analysis by LC-ESI MS/MS of peptides and glycopeptides released by trypsin and Glu-C digestions as previously described in Balieu *et al.* (2022). Peptide analyses by nano-LC-ESI-MS/MS were performed using a liquid chromatography system coupled to a Q-TOF 6545 XT AdvanceBio mass spectrometer (Agilent Technologies, Les Ulis, France). The liquid chromatography system consisted of a nano-LC 1200 system and an HPLC-chip cube interface (Agilent Technologies). The samples were resuspended in an acetonitrile/formic acid mixture of 0.1% 3/97 v/v. The samples were enriched and desalted using a 40 nL RP-C18 column, then the compounds were separated using an Agilent Zorbax C18 column (length 150 mm, internal diameter 75 μ m, particle size 5 μ m and pore size 30 nm). A linear gradient of 3% to 80% acetonitrile in 0.1% formic acid was carried out over 26 min at a flow rate of 350 nL/min. The mass range was 290–2000 *m/z* in MS1 and 59–3200 *m/z* in MS2. During each cycle, a maximum of five precursors sorted by charge state (discharged priority, mono-charged excluded) are isolated and fragmented in the collision cell with N2. The collision energy is automatically adjusted according to the *m/z* value. Dynamic exclusion of these precursors was activated after 1 spectrum in 0.2 min and the absolute threshold was 1000 (relative threshold 0.001%). The data were recorded and processed using Agilent Data Acquisition B.09.00 and Mass Hunter Qualitative B.09.00 software. For the identification of putative *O*-linked glycans on S126, we searched in the LC-ESI MS/MS data for glycopeptide candidates exhibiting C- or N-terminal peptide fragments of the S126-containing tryptic peptide E117-R131. No glycopeptide candidate was detected by this analytical approach.

N-glycan profiling of *C. reinhardtii* lines

For the determination of *N*-glycan profiles of *C. reinhardtii* CC5325, IM_{XTA}IM_{XTB} and IM_{XTA}IM_{XTB}IM_{FUT} lines, *N*-glycans were released from proteins by PNGase A, coupled to 2-amidobenzamide (2-AB) and analysed by MALDI-TOF mass spectrometry as reported in Balieu *et al.*, 2022.

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IM_{XTA} × IM_{XTB} × IM_{FUT}, and Dr Gaëtan Vanier who initiated this work.

Conflict of interest

All the authors declare no competing interests.

Data availability statement

Data sharing is not applicable to this article. These data have never been shared before on any repository database.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Sequence coverage determined by LC-ESI MS/MS analysis of the rhEPO clover expressed in the *C. reinhardtii* CC5325 strain. Black arrows point out the glycosylation sites.

Figure S2 MALDI-TOF MS of 2AB-labelled *N*-glycans released by PNGase A from proteins of *C. reinhardtii* CC5325 (A), $IM_{XTA} \times IM_{XTB}$ (B) and $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ (C). See Table S1 for structures.

Table S1 Structure, name and *m/z* values of $[M + Na]^+$ ions of 2-AB-derivatives of *N*-glycans.

Table S2 Distribution of *N*-glycans on the three glycosylation sites of rhEPOs produced in *C. reinhardtii* CC5325 strain, $IM_{XTA} \times IM_{XTB}$ and $IM_{XTA} \times IM_{XTB} \times IM_{FUT}$ mutants.