Chlamydomonas reinhardtii: Protein Glycosylation and Production of Biopharmaceuticals

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Abstract Recently, microalgae species such as *Chlamydomonas reinhardtii* have been investigated as potential biofactories for the production of biopharmaceuticals (Mathieu-Rivet et al., Front Plant Sci 5:359, 2014; Rasala and Mayfield, Photosynth Res 123:227–239, 2015). Biopharmaceuticals are protein-based pharmaceuticals which are produced recombinantly in living cells used as biofactories (Walsh, Nat Biotechnol 28:917–924, 2010; Walsh, Nat Biotechnol 32:992–1000, 2014). The pharmaceutical market encompasses more than 200 biopharmaceutical products (Walsh, Nat Biotechnol 32:992–1000, 2014). Most of these biopharmaceuticals are glycosylated proteins, and it is currently well established that their glycosylation is primordial for their stability, half-life, and biological activity (Walsh, Nat Biotechnol 28:917–924, 2010; Lingg et al., Biotechnol J, 7:1462–1472, 2012). Since enzymes involved in the glycosylation processing are localized in the endoplasmic reticulum and the Golgi apparatus, biopharmaceuticals produced in *C. reinhardtii* must travel through these organelles, which are components of the secretory pathway, to acquire an appropriate glycosylation. In this chapter, the *C. reinhardtii* protein glycosylation pathways as well as its capacity to synthesize and transport nucleotide sugars will be described and discussed.

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1 Introduction

Since the mid-twentieth century, microalgae have been used for the production of food and high-value added compounds like carotenoids (Sasso et al. 2012; Spolaore et al. 2006). Recently, microalgae have emerged as an alternative production system for new biotechnological applications. As photosynthetic organisms, microalgae are very efficient in converting sunlight into chemical energy, which feature makes them attractive for the production of carbohydrates, lipids, and hydrogen. Therefore, algal biomass represents a great potential for generating new sources of sustainable biofuels (Beer et al. 2009; Lam and Lee 2012; Merchant et al. 2012). Some microalgae species including *Chlamydomonas reinhardtii* have been also investigated as solar-powered protein biofactories for the production of biopharmaceuticals (Barrera and Mayfield 2013; Hempel and Maier 2016).

Biopharmaceuticals are protein-based pharmaceuticals which are produced recombinantly in living cells used as biofactories. Currently, there are more than 200 products on the biomedical market reaching a total cumulative sales value of $140 billion for 2013 (Walsh 2014). These products include antibodies, vaccines, human blood products, enzymes, hormones, and growth factors. Among the different categories, monoclonal antibodies (mAbs) and their derivatives form the largest group and the most lucrative product class with total sales reaching $63 billion in 2013 (Walsh 2014). MAbs are used to treat a wide range of diseases, with oncology and autoimmunity/inflammation covering more than 80% of the market. In 2010, the combined sale value of 25 actively marketed mAbs in these areas was $43 billion of which 75% were generated solely from the top five mAbs producers including Avastin®, Rituxan®, Humira®, Remicade®, and Herceptin (Elvin et al. 2013).

A substantial portion of the biopharmaceuticals on the market is represented by glycosylated proteins (Walsh 2010). Since it has been well established that glycan moieties attached to recombinant proteins are essential for the protein stability and bioactivity and mediate the efficacy of the glycosylated biopharmaceuticals (Lingg et al. 2012), the development of an expression system allowing an efficient manufacturing of the protein glycosylation is essential. Additionally, over the past decade, at least four nonhuman carbohydrate epitopes have been able to induce an immune response in humans (van Beers and Bardor 2012). Therefore, glycan variants of a biopharmaceutical must be adequately analyzed and controlled to ensure product quality and safety (Lingg et al. 2012; Zhang et al. 2016).

Biopharmaceuticals are currently produced in various heterologous expression systems ranging from bacteria to mammalian cell cultures (Demain and Vaishnav 2009; Huang et al. 2012; Wong 2005). Among those, mammalian cells such as Chinese hamster ovary cells (CHO) represent the industrial workhouse cell lines (Hossler et al. 2009), covering more than 50% of the market. For example, among the 18 recombinant biopharmaceuticals which have been approved between January 2008 and June 2011 by the US Food and Drug Administration (FDA), 12 were produced using mammalian cell expression systems, three were manufactured in *Escherichia coli*, and the remaining three were produced by using baculovirus,

yeast, or transgenic goats (Zhu 2012). Despite the industrial success of mammalian cell lines, a constantly increasing need for large amount of biopharmaceuticals, their high production cost, and potential virus contamination have driven scientists to explore new alternative for biopharmaceuticals production.

In this context, plant molecular farming (PMF), which refers to biotechnological production of plant-based biopharmaceuticals, is gaining more and more attention. The growing interest in PMF research encourages development of active research and projects, which could be identified in more than 120 companies, universities, and research institutes worldwide (Obembe et al. 2011). Meanwhile, PMF field is becoming a lucrative biotechnology industry that attracts an increasing number of specialized startup and biotechnology companies. This rapid development observed in PMF area is certainly due to the successful production of several plant-made biopharmaceuticals (De Muynck et al. 2010). Moreover, an important breakthrough was achieved in 2012 when the first plant molecular farming product was approved by the FDA for use in humans (Maxmen 2012). This successful story concerns taliglucerase alfa, a recombinant form of human glucocerebrosidase, an enzyme developed in carrot cells by Protalix BioTherapeutics for the treatment of the lysosomal storage disorder Gaucher's disease (Shaaltiel et al. 2007). In addition, two clinical trial applications for plant-derived pharmaceuticals were also approved in the European Union (EU), one for insulin produced in safflower and another for an HIV-neutralizing monoclonal antibody produced in transgenic tobacco plants (Ma et al. 2015; Stoger et al. 2014). In both cases, new manufacturing processes based on transgenic plants were developed to ensure compliance with pharmaceutical good manufacturing practice (GMP; Ma et al. 2015).

Since 25 years, there is an increasing interest to use microalgae for biopharmaceutical production (Rasala and Mayfield 2015). Microalgae are cheap and easy to grow, classified in generally recognized as safe (GRAS) organisms, which feature makes them potentially attractive cell factories for the large-scale production of biopharmaceuticals. In addition, microalgae can be cultured in closed bioreactor systems to overcome gene dissemination often encountered with transgenic plant breedings (Hempel and Maier 2016). So far, several microalgae species have been evaluated for their potential to express biopharmaceuticals. Among them, *C. reinhardtii* is currently the most deeply investigated (Mathieu-Rivet et al. 2014; Rasala and Mayfield 2015).

2 Production of Biopharmaceuticals in *Chlamydomonas reinhardtii*

Several studies attempted to demonstrate that *C. reinhardtii* is an efficient cellular factory for the production of recombinant proteins, especially biopharmaceuticals (Barahimipour et al. 2016; Dauvillée et al. 2010; Eichler-Stahlberg et al. 2009; Gregory et al. 2013, 2016; Mayfield and Franklin 2005; Mayfield et al. 2003; Rasala

et al. 2010; Sun et al. 2003; Surzycki et al. 2009; Tran et al. 2009, 2013). Most of the efforts in this microalga have been focused on the chloroplastic expression of a transgene of interest.

Chloroplastic expression of a transgene has been favored over expression in the nucleus since this strategy allows accumulation of relatively higher yields of recombinant proteins within the chloroplast (Mayfield et al. 2007; Surzycki et al. 2009). For example, Manuell and collaborators managed to express a mammalian protein, the bovine mammary-associated serum amyloid (M-SAA), up to 5% of the total soluble proteins (Manuell et al. 2007). In *C. reinhardtii,* the chloroplastic compartment represents more than 40% of the cellular volume providing a favorable environment and preventing proteolysis of a recombinant protein. Thus, about 30 biopharmaceuticals have been produced through chloroplastic expression (for a complete review, refer to Almaraz-Delgado et al. 2014; Pérez Espana et al. 2016). These works include studies on several antibodies such as the large single-chain antibody directed against glycoprotein D protein from Herpes simplex virus (lcs-HSV8) (Mayfield et al. 2003), the single-chain fragment HSV8-scFv (Mayfield and Franklin 2005), the heavy chain of human monoclonal antibody directed against anthrax protective antigen 83 (PA83) (Tran et al. 2009), as well as immunotoxin such as the *sc*Fv (single-chain antibody) directed against a B-cell surface molecule called CD22, fused to domains II and III of exotoxin A (PE40) from *Pseudomonas aeruginosa* (Tran et al. 2013). These examples illustrate the high potential of this strategy for the production of complex proteins, as the chloroplast contains the enzymatic arsenal and chaperones required for disulfide bond formation and proper folding of proteins (Mayfield et al. 2007; Tran et al. 2013). Compared to bacterial expression system, this feature renders *C. reinhardtii* attractive for such production of recombinant proteins. Despite these advantages, the transformation of chloroplast appears not to be the best strategy for the expression of biopharmaceuticals. As it has been already mentioned, the majority of biopharmaceuticals are glycosylated proteins for which chloroplastic expression is not appropriate. In fact the chloroplast organelle lacks the glycosylation machinery which is localized in the endoplasmic reticulum (ER) and Golgi apparatus (Dance 2010). However, two works carried out in rice (Chen et al. 2004) and in Arabidopsis (Villarejo et al. 2005) reported the existence of an alternative route for secreted proteins in the chloroplast, suggesting the possibility for some proteins to be glycosylated in the ER and the Golgi apparatus prior to their import into the chloroplast. Therefore, we can hypothesize that such a pathway may exist in *C. reinhardtii*, which would open the door to new strategies for optimizing the expression of glycosylated proteins within its chloroplast.

Efficient production of glycosylated biopharmaceuticals requires nuclear expression of the transgene encoding for the protein of interest and its targeting to the secretion pathway to acquire either *O*- or/and *N*-glycosylation. In contrast to the numerous recombinant proteins produced in the chloroplast (Almaraz-Delgado et al. 2014;

Table 1 Secreted biopharmaceuticals produced in *C. reinhardtii*

Biopharmaceuticals	Function	Protein titer	Reference
Erythropoietin	Treatment for anemia	100 μg L^{-1} of culture medium	Eichler-Stahlberg et al. (2009)
GBSS-AMA1	Malaria vaccine	0.2–1 μg mg^{-1} of purified starch	Dauvillée et al. (2010)
GBSS-MSP1	Malaria vaccine	0.2–1 μg mg^{-1} of purified starch	Dauvillée et al. (2010)
HIV antigen P24	Putative HIV vaccine	0.25% of total cellular proteins	Barahimipour et al. (2016)

Pérez Espana et al. 2016), only a few examples of nucleus-expressed proteins have been reported in *C. reinhardtii* (Lauersen et al. 2013b; Rasala et al. 2012), and among them, only four are biopharmaceuticals (Table 1; Barahimipour et al. 2016; Dauvillée et al. 2010; Eichler-Stahlberg et al. 2009). Strategies used for the production of these four biopharmaceuticals were very distinct. The recombinant erythropoietin (EPO) was secreted in the medium thanks to the insertion of the secretion signal of the arylsulfatase ARS2 in its *N*-terminal part (Eichler-Stahlberg et al. 2009). The two malaria vaccine antigens AMA1 and PSP1 were fused to a truncated granule-bound starch synthase (GBSS) which allow their accumulation in the chloroplast (Dauvillée et al. 2010). Recently, the HIV antigen P24 was accumulated in the cytosol and was shown to represent up to 0.25% of the total cellular proteins (Barahimipour et al. 2016). Despite these efforts, the yields reached in these attempts were very low (Table 1). So far, nuclear expression appeared to be a trickier strategy which does not allow reaching high protein levels. Molecular mechanisms suspected to cause the low expression of nuclear transgenes in *C. reinhardtii* and strategies used to circumvent this limitation are reviewed in another chapter of this book. However, a number of studies conducted recently reported the development of new tools which improve the yield of nuclear-expressed proteins. Major recent advances concern the selection of strains used for the production (Lauersen et al. 2013a, b; Neupert et al. 2009), vectors, and promotors (Lauersen et al. 2015b; Rasala et al. 2012; Scranton et al. 2016), as well as growth conditions (Lauersen et al. 2015a)**.** As an illustration, the comparison of several cultivation systems showed that cells grown in mixotrophic conditions, using both acetate and carbon dioxide as carbon sources, are able to produce relatively high amounts of recombinant protein (10 mg L^{-1} of culture medium) (Lauersen et al. 2015a). This result demonstrates that *C. reinhardtii* can be a competitive biofactory for the production of therapeutic proteins.

Besides the numerous attempts made in order to increase the yields of recombinant protein, another strategy involves maximizing the economic value of *C. reinhardtii*, in order to reduce the high costs of biopharmaceutical manufacturing processes. A concomitant production of other valuable product or use of industrial waste waters to grow the microalgae expressing the protein of interest could be a way to optimize the production costs (Gong et al. 2011).

3 *N*-Glycosylation Pathway in *Chlamydomonas reinhardtii*

In Eukaryotes, *N*-linked glycosylation of secreted proteins is an extensive and universal posttranslational modification which results in the covalent attachment of an oligosaccharide onto asparagine residues belonging to the consensus sequence Asn-X-Ser/Thr/Cys, in which X represents any amino acid except proline.

3.1 N-*Glycosylation Pathway in Plants and Mammals*

In eukaryotic cells, the process of *N*-linked glycosylation is initiated within the endomembrane system of the ER and is continued within the Golgi apparatus for the latest maturation steps. The *N*-glycosylation is initiated in the ER with the biosynthesis of a lipid-linked oligosaccharide (LLO) precursor. The LLO synthesis starts on the cytosolic face of the ER membrane by the stepwise addition of monosaccharides *N*-acetylglucosamine (GlcNAc) and mannose (Man) on a dolichol pyrophosphate (Dol-P-P) to form a $Man_5GlcNAc_2$-P-P-Dol which is then flipped into the lumen of the ER (Helenius and Aebi 2002; Helenius et al. 2002). Here, the precursor undergoes elongation by the addition of several Man and glucose (Glc) residues to form the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$-P-P-Dol (Aebi 2013). This product is subsequently transferred by the oligosaccharyltransferase (OST) complex onto the asparagine of the *N*-glycosylation consensus sequences of the neo-synthesized polypeptides. The precursor is afterward trimmed by the action of α-glucosidases I and II, ER-mannosidase into a high-mannose type *N*-glycan or oligomannoside ($Man_8GlcNAc_2$). In parallel to these events, the nascent protein is submitted to the calnexin-calreticulin control quality cycle to ensure its proper folding. When the glycoprotein is correctly folded, it can then leave the ER to enter the Golgi apparatus where its *N*-glycans will be further subjected to modifications: α-mannosidase I, *N*-acetylglucosaminyltransferase I (GnT I), α-mannosidase II, and finally *N*-acetylglucosaminyltransferase II give rise to an intermediate $GlcNAc_2Man_3GlcNAc_2$ which is common to mammals and land plants (Lerouge et al. 1998; Strasser 2016). Thereafter, this intermediate undergoes further maturation into complex-type *N*-glycans. Even if the *N*-glycosylation processing is highly conserved, the final processed *N*-glycan structures present a high variability when comparing different species together (Brooks 2011; Bardor et al. 2011). The resulting organism-specific complex-type *N*-glycans reflect the difference in the Golgi enzyme repertoires, for example, mature *N*-glycans from plant bore core β1,2-xylose, core α1,3-fucose (Fig. 1), and to a less extent $Lewis^a$ antennae (Lerouge et al. 1998; Bardor et al. 2011; Brooks 2011). In contrast, major maturated *N*-glycans in humans are bearing core α1,6-fucose and Neu5Ac-sialylated lactosamine antennae (Fig. 2; Brooks 2011). In addition to this interspecies differences, *N*-glycan structures variability can be found even in different cell types of the same species or within one cell (Varki et al. 2009b).

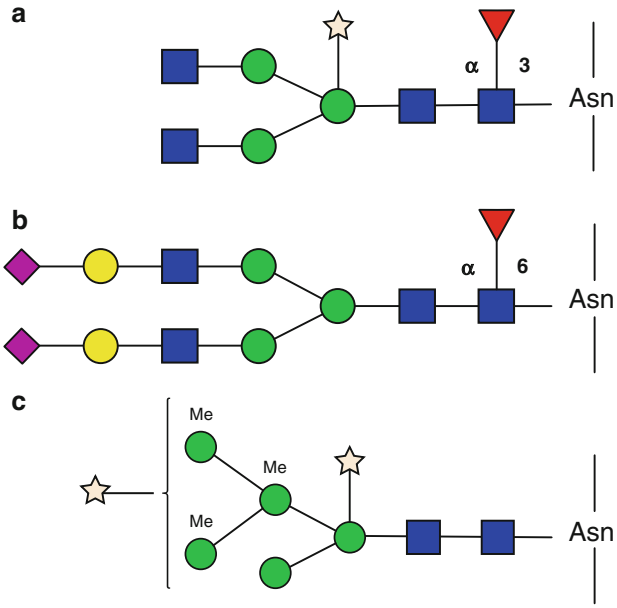

Fig. 1 Major *N*-glycan structures identified in land plants (**a**), in humans (**b**), and in *C. reinhardtii* (**c**). The *symbols* used for representing the *N*-glycan structures are the ones from the Consortium for Functional Glycomics (Varki et al. 2009a). The specific linkage of the core fucose has been indicated to highlight the difference between land plants and mammals. *Me* methyl group, *blue-filled squares* N-acetylglucosamine, *green-filled circles* mannose, *yellow-filled circles* galactose, *filled stars* xylose, *red-filled inverted triangles* fucose, *violet filled diamonds* N-acetylneuraminic acid

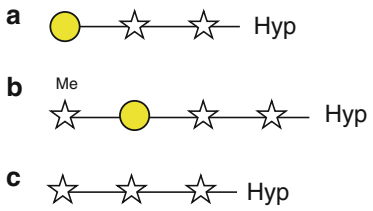

Fig. 2 Linear *O*-glycans structures linked to hydroxyproline residues identified in *C. reinhardtii* (**a**) and (**b**) as compared to those in land plants (**c**). Structures (**a**) and (**b**) have been isolated from *C. reinhardtii* outer cell wall proteins (Ferris et al. 2001; Bollig et al. 2007), and structure (**c**) represents extensins characterized in *A. thaliana* and *N. tabacum* (Bollig et al. 2007). The *symbols* used for representing the *O*-glycan structures are the ones from the Consortium for Functional Glycomics (Varki et al. 2009a). *Me* methyl group, *yellow-filled circles* galactose, *stars* arabinose, *Hyp* hydroxyproline. Both sugars (galactose and arabinose) present a furanose ring

3.2 N-*Glycan Structures in* Chlamydomonas reinhardtii

The mature *N*-glycan structures borne by the endogenous proteins of *C. reinhardtii* were recently identified and showed substantial differences in comparison to those described in plants and humans (Fig. 1; Mathieu-Rivet et al. 2013). In Mathieu-Rivet et al. (2013), the *N*-glycosylation has been studied in three distinct *C. reinhardtii* strains: CC-503 cw92, CC-400 cw15, and CC-1036 pf18. The two first strains are cell wall-deficient strains used as references (Merchant et al. 2007). The latter one possesses a cell wall but is completely impaired in its motility (Witman et al. 1972). The soluble and membrane-bound proteins were extracted from those strains and studied through either glycomic and/or glycoproteomic approaches combined with mass spectrometry analysis. Such studies allowed the identification of two types of *N*-glycans: oligomannosides and maturated *N*-glycans, which account, respectively, for 70% and 30% of the *N*-glycan population. Within the population of oligomannosides, the $Man_5GlcNAc_2$, $Man_4GlcNAc_2$, $Man_3GlcNAc_2$, and $Man_2GlcNAc_2$ were shown to be the main *N*-glycans (Mathieu-Rivet et al. 2013). The complex-type *N*-glycans in *C. reinhardtii* were identified as oligomannosides bearing one or two xylose residues and methyl group substitutions on the C6 on the three outer mannose residues (Mathieu-Rivet et al. 2013; Fig. 1c). The two xylose residues have been determined to be either β(1,2) linked to the core β-mannose as previously demonstrated in land plants (Brooks 2011; Bardor et al. 2011; Strasser 2016) or (1,4) linked on a terminal mannose residue (Fig. 1c; Mathieu-Rivet et al. 2013). Such *N*-glycan structures are unique when compared to plant and mammalian *N*-glycan structures (Fig. 1). The absence of additional GlcNAc residues on these mature *N*-linked glycans also suggested that they may derive from GnT I-independent Golgi events.

3.3 *Synthesis of the Oligosaccharide Precursor Within* Chlamydomonas reinhardtii *Endoplasmic Reticulum*

In silico analysis of the *C. reinhardtii* genome allowed the identification of a number of putative ortholog genes encoding for enzymes potentially involved in the LLO biosynthesis and its transfer onto the nascent glycoprotein (Mathieu-Rivet et al. 2013, 2014). Three monosaccharides serve as building blocks for the synthesis of LLO, namely, GlcNAc, Man, and Glc. They emerge from the cytosolic primary metabolism as nucleotide-activated sugar forms: UDP-GlcNAc, GDP-Man, and UDP-Glc, respectively. These activated forms are then used by the glycoenzymes involved in the synthesis of the LLO precursor. CrALG5 (Cre16.g652850) is a putative dolichol-phosphate glucosyltransferase able to transfer a Glc residue onto dolichol-phosphate. The newly synthesized Glc-P-Dol is then translocated within the ER membrane to

reach the ER lumen where it will be used by glucosyltransferases to end up in the synthesis of the LLO precursor. Similarly, CrDPM1 (Cre03.g150950), a putative dolichol-phosphate mannosyltransferase, gives rise to Man-P-Dol which is then translocated within the ER lumen for further usage. LLO precursor synthesis is initiated on the cytosolic side of the ER membrane and starts with the formation of GlcNAc1-P-P-Dol through the action of an *N*-acetylglucosamine-phosphate transferase encoded by *CrALG7* gene (Cre16g.663100). The addition of a GlcNAc residue is then mediated by the β(1,4)-*N*-acetylglucosaminyltransferases CrALG13 and CrALG14 (Cre13.g585850 and Cre16.g669950, respectively) leading to the formation of a $GlcNAc_2$-P-P-Dol intermediate. To this point, it could be hypothesized that in *C. reinhardtii,* the three proteins CrALG7, CrALG13, and CrALG14 are organized within a protein complex as it has already been described for other organisms (Noffz et al. 2009; Lu et al. 2012). The mannosylation of the LLO starts with a first Man residue added to $GlcNAc_2$-P-P-Dol intermediate by the β(1,4)-mannosyltransferase CrALG1 (Cre12.g516550). The subsequent addition of four branched mannose residues is catalyzed by two putative α(1,3)- and α(1,2)-mannosyltransferases, CrALG2 (Cre11.g474450) and CrALG11 (Cre23.g767350), respectively. All these cytoplasmic steps result finally in the synthesis of a $Man_5GlcNAc_2$-P-P-Dol. Then, the putative CrRFT flippase (Cre22g.765100) is likely responsible for the flip-flop movement of this $Man_5GlcNAc_2$-P-P-Dol intermediate across the membrane bilayer into the ER lumen as previously described in yeast (Helenius and Aebi 2002; Rush et al. 2009). In yeast and mammals, the synthesis of the LLO continues within the ER lumen by the stepwise addition of four mannose residues through the action of ALG3, ALG9, and ALG12 prior to glucosylation of the $Man_9GlcNAc_2$-P-P-Dol by the ALG6, ALG8, and ALG10 glucosyltransferases (Aebi 2013). No ortholog genes for ALG3, ALG9, and ALG12 have been identified within the *C. reinhardtii* genome (Gomord et al. 2010; Levy-Ontman et al. 2014; Mathieu-Rivet et al. 2013) suggesting that the $Man_5GlcNAc_2$-P-P-Dol could be directly submitted to further glucosylation steps. Furthermore, orthologs for two α(1,3)-glucosyltransferases ALG6 (Cre16.g690150) and ALG8 (Cre09.g414250) are predicted in *C. reinhardtii* genome. In contrast, no ALG10 ortholog required for the transfer of the outer α(1,2)-glucose onto the LLO was found (Gomord et al. 2010; Levy-Ontman et al. 2014; Mathieu-Rivet et al. 2013). All together, these results suggest that the LLO precursor from *C. reinhardtii* might be restricted to the $Glc_2Man_5GlcNAc_2$ structure (Gomord et al. 2010). The lack of *ALG* genes (Samuelson et al. 2005) and the ER synthesis of truncated LLO structures have already been described for some parasitic unicellular organisms (Garénaux et al. 2008; Kelleher et al. 2007; Schiller et al. 2012). However, this bioinformatically predicted structure of LLO is in discrepancy with the identified *N*-glycans borne by the endogenous glycoproteins from *C. reinhardtii* (Fig. 1; Mathieu-Rivet et al. 2013). This discrepancy could reflect the existence of uncharacterized enzymes able to add supplementary hexose residues onto the oligosaccharide precursor. Therefore, more work needs to be done to confirm the LLO biosynthetic pathway and its structure within *C. reinhardtii*.

3.4 *Transfer and Maturation of the* N-*Glycans Within* Chlamydomonas reinhardtii *Golgi Apparatus*

After completion, the LLO precursor is then transferred on the nascent protein onto the specific *N*-glycosylation consensus sites. This key reaction giving birth to glycoproteins is catalyzed by an oligosaccharyltransferase (OST; Aebi 2013). The OST has been described to be a hetero-oligomeric protein complex in yeast and mammals (Kelleher and Gilmore 2006). However, the OST from protists such as *Trypanosoma cruzi* and *T. brucei* are composed of a single catalytic subunit (Kelleher et al. 2007). In *C. reinhardtii*, several putative subunits have been identified through sequence homology search in the genome (Mathieu-Rivet et al. 2013). This includes the catalytic subunits CrSTT3A and CrSTT3B, respectively, encoded by the genes Cre02.g121650 and Cre07.g330100, as well as ribophorin orthologs (CrRPN1: Cre12.g523300 and CrRPN2: Cre08.g368450), CRDGL1 (Cre14.g614100), CrDAD1 (Cre02.g108400), and CrOST3 (Cre01.g063500), previously described for other eukaryotes such as yeast and mammals (Pfeffer et al. 2014). After its transfer on the protein, but before leaving the ER, the *N*-glycan is then trimmed through the removal of the glucose residues by the action of putative α-glucosidases (CrGSI: Cre13.g579750; CrGSIIA: Cre03.g190500; CrGSIIB: Cre05.g233250). The resulting oligomannoside structure of the glycoprotein will then be maturated within the Golgi apparatus via the contributions of putative mannosidases (Cre03.g189050 and Cre07.g336600) as well as xylosyltransferases and methyltransferases. Even if two xylose residues have been detected on the *N*-glycan structures, only one candidate gene encoding for the xylosyltransferase activity has been identified (Cre02.g126700). The amino acid sequence deduced from Cre02.g126700 displayed about 16.5% of identity with the β(1,2)-xylosyltransferase from Arabidopsis which catalyzes the transfer of a xylose onto the β-mannose of the core *N*-glycan (Strasser et al. 2000). However, considering the lack of information regarding conserved domains required for β(1,2)-xylosyltransferase activity, the assignment of such a sequence remains highly speculative without further functional evidence. Although two candidate genes encoding for putative fucosyltransferases have been identified in the genome (Cre31.g780450 and Cre18.g749047), only minute amount of a fucosylated glycan was detected by mass spectrometry in the glycan preparations from the strain CC-503 cw92 (Mathieu-Rivet et al. 2013). Therefore, further experiments would be required to functionally characterize these enzymes and demonstrate their capability to fucosylate *N*-glycans in *C. reinhardtii*. Contrary, no *GnT I* ortholog was found in *C. reinhardtii* (Mathieu-Rivet et al. 2013). Both these bioinformatics data and the structure of glycan *N*-linked to secreted proteins suggested that *C. reinhardtii N*-glycosylation pathway is a GnT I-independent processing (Fig. 1; Mathieu-Rivet et al. 2013) in contrast to the one described in land plants and mammals (Lerouge et al. 1998; Strasser 2016).

4 *O*-Glycosylation in *Chlamydomonas reinhardtii*

In additional to *N*-glycosylation, there is a second major type of glycosylation which is based on the attachment through an *O*-linkage of a glycan to the side chain of a serine or threonine residue of a mammalian protein (Corfield 2015) or to the side chain of serine or hydroxyproline residues of a plant protein (Nguema-Ona et al. 2014). In contrast to *N*-glycosylation, no consensus sequence has been identified for the *O*-glycosylation site. Moreover, the *O*-glycan structures are much more complex, highly heterogeneous, and divergent from one organism to another (Nguema-Ona et al. 2014; Solís et al. 2015). So far, less attention has been paid to the *O*-glycosylation of biopharmaceuticals because structural analysis of *O*-glycans is more challenging as compared to *N*-glycans and since there is no need yet for the pharmaceutical industries to provide detailed structural analyses of *O*-glycans to regulatory agencies (Zhang et al. 2013).

O-glycosylation can be found on few examples of biopharmaceuticals dedicated to therapeutic applications in humans. For example, recombinant human EPO, a tumor necrosis factor (TNF) receptor fused to an Fc part, coagulation blood factors IX and VII carry, respectively, *O*-GalNAc, *O*-Fuc, and *O*-Glc modifications (refer to Zhang et al. 2013; Walsh 2010). Functionally, similarly to *N*-glycosylation, *O*-glycosylation was found to impact on biopharmaceutical immunogenicity, secretion, and function (Zhang et al. 2013). Therefore, it is essential to understand the *O*-glycosylation capabilities of the expression system used for the production of biopharmaceuticals.

In this context, it is important to understand the capabilities of *C. reinhardtii* to synthesize *O*-glycans. So far, *O*-glycans have mainly been described in the cell wall of this organism. Indeed, *C. reinhardtii* cell wall does not contain cellulose but is made of crystalline glycoproteins which are essentially *O*-glycosylated proteins (Catt et al. 1978; Goodenough et al. 1986; Roberts et al. 1972). The amino acid sequences of these glycoproteins share a high degree of similarity with extensins, a class of plant hydroxyproline-rich glycoproteins (HRGPs) (Ferris et al. 2001; Woessner and Goodenough 1989). Especially, hydroxyproline residues have been identified as *O*-glycosylation sites in chaotrope-soluble glycoproteins which constitute the vegetative outer cell wall in *C. reinhardtii* (Bollig et al. 2007; Ferris et al. 2001; Miller et al. 1972). Using antibodies recognizing specifically the carbohydrate moiety (Smith et al. 1984) on endosomal fractions enriched either in ER or Golgi apparatus, Zhang and Robinson (1990) showed that glycoproteins could be detected in both compartments. This contrasts with the situation described in plants and mammals, where *O*-glycosylation only occurs in the Golgi apparatus (Gill et al. 2011; Matsuoka et al. 1995). The monosaccharide composition of these chaotrope-soluble glycoproteins highlighted the presence of arabinose and galactose as the most prominent monosaccharides, followed by glucose, xylose, and mannose as minor species (Bollig et al. 2007; Ferris et al. 2001;

Miller et al. 1972). In addition, methylated sugars (i.e., MeHex and MePent which correspond to MeGal and MeAra) have been detected (Bollig et al. 2007; Ferris et al. 2001). Further analysis carried out by mass spectrometry and NMR led to the identification of *O*-glycans bound to hydroxyproline, which in *C. reinhardtii* correspond to linear and branched structures, with the first two L-arabinoses linked to each other in β(1–2) (Fig. 2; Bollig et al. 2007; Ferris et al. 2001). Besides these structural data, information concerning the enzymes involved in this process remains currently scarce. A prolyl-4-hydroxylase has been characterized (Keskiaho et al. 2007). This enzyme expressed in insect cells or in *E. coli* has been shown to efficiently hydroxylate the proline residues of synthetic peptides. Moreover, the downregulation of its transcription in *C. reinhardtii* affects the assembly of a proper cell wall, which is consistent with the role of hydroxyproline residues in the attachment of the oligosaccharide moiety. As far as glycosyltransferases involved in *O*-glycans biosynthesis are concerned, only arabinosyltransferase and galactosyltransferase activities have been detected in vitro using endomembrane preparations from *C. reinhardtii* (Zhang and Robinson 1990; Zhang et al. 1989). To the best of our knowledge, none of these enzymes have been further characterized.

In plant and mammals, *O*-glycosylation is known to occur also on serine residues (Gill et al. 2011; Kieliszewski and Lamport 1994; Velasquez et al. 2011). Although no data on such posttranslational modification has yet been reported in *C. reinhardtii*, a peptidyl-serine α-galactosyltransferase has recently been characterized (Saito et al. 2014). Saito and collaborators (2014) showed that crude cells extracts from the CC-503 cw92 strain could modify synthetic peptides by adding a galactose residue onto serine. Therefore, the enzyme was purified from the endosomal fraction, and its galactosyltransferase activity has been confirmed by an in vitro assay. Altogether, these results suggest that *O*-glycosylation of serine residues can occur in *C. reinhardtii*, even if there is yet no evidence that such *O*-glycoprotein exists in this microalga.

5 Synthesis and Transport of Glycan Building Bocks

Glycans, either *N*- or *O*-linked to proteins, are built by sequential addition of monosaccharides thanks to glycosyltransferases which use activated sugar donors as substrates. These sugar donors are synthesized within the cytosol prior to their transfer into the ER and Golgi compartments. Once they reach the lumen of the organelles, they can be used as substrates for the resident glycosyltransferases.

5.1 Nucleotide Sugar Biosynthesis

Nucleotide sugars result from the conversion of a sugar-1-phosphate in its corresponding nucleotide-diphosphate activated form (Bar-Peled and O'Neill 2011). Sugar-specific kinases and pyrophosphorylases which ensure this conversion are

usually localized within the cytosol. Currently, 10 and 30 different nucleotide sugars have been identified in animals and plants, respectively (Bar-Peled and O'Neill 2011). This large diversity as compared to animals can be explained by the important roles that they play as precursors of the cell wall polysaccharides and glycoproteins. Whereas the biosynthetic pathways of nucleotide sugars are well studied in plants (Bar-Peled and O'Neill 2011), these processes are poorly detailed in microalgae and especially in *C. reinhardtii*. However, based on homologies with Arabidopsis genes encoding for putative enzymes involved in the nucleotide sugar biosynthesis, we report here a bioinformatics analysis of the *C. reinhardtii* genome which suggests that this metabolic process would be conserved in this microalga (Table 2; Fig. 3).

Most of the nucleotide sugars derive from UDP-Glc which is a crossroad in the interconversion pathways. For example, UDP-galactose (UDP-Gal) and UDP-glucuronic acid (UDP-GlcA) derive, respectively, from the epimerization (Barber et al. 2006; Rosti et al. 2007) and the dehydrogenation of UDP-Glc (Klinghammer and Tenhaken 2007; Reboul et al. 2011) (Fig. 3). UDP-Glc results from the activity of an UDP-Glc pyrophosphorylase which uses Glc-1-P as a substrate (Kleczkowski et al. 2004). One sequence encoding for a putative protein sharing 35% identity with the *Arabidopsis thaliana* UTP-Glc-1-phosphate uridylyltransferase is predicted in the *C. reinhardtii* genome (Table 2; Fig. 3). However, its biological function has not yet been demonstrated.

As described previously, the synthesis of *N*-glycan oligosaccharide precursor in eukaryotes starts with the addition of two GlcNAc residues onto the dolichol-phosphate. Since these first steps are conserved in *C. reinhardtii* (Mathieu-Rivet et al. 2013), this suggests that this microalga synthesize UDP-GlcNAc. Four distinct enzymes are required to convert D-fructose-6-phosphate (D-Fru-6-P) into UDP-GlcNAc (Bar-Peled and O'Neill 2011; Fig. 3). First, the amination of Fru-6-P occurs through the enzymatic transfer of the amine group from L-glutamine to the C-2 of this ketose. Secondly, isomerization of fructosamine-6-P into glucosamine-6-P (GlcN-6-P) occurs prior to its *N*-acetylation giving rise to GlcNAc-6-P. Then, the GlcNAc-6-P is converted into GlcNAc-1-P by a phosphomutase. Finally, the GlcNAc-1-P is activated by the UTP: *N*-acetylglucosamine-1-P-uridylyltransferase into UDP-GlcNAc. Candidate genes encoding for these four enzymes are predicted in the genome of *C. reinhardtii* (Table 2; Fig. 3).

The major part of the *N*-glycans identified in *C. reinhardtii* are oligomannosides obtained by the sequential addition of mannose residues by several mannosyltransferases that use GDP-mannose (GDP-Man) as substrate. For the biosynthesis of this nucleotide sugar, Fru-6-P is first converted into mannose-6-phosphate (Man-6-P) through the action of a mannose-6-phosphate isomerase and then into Man-1-P by a phosphomannomutase. Candidate genes (Cre02.g147650 and Cre14.g626900) for both enzymes have been identified in *C. reinhardtii* (Table 2). Then, GDP-Man results from the coupling of Man-1-P and GTP catalyzed by a GDP-Man pyrophosphorylase (GDP-Man PPase) (Conklin et al. 1999; Qin et al. 2008). This reaction is a well-characterized enzymatic step in the Smirnoff-Wheeler pathway (also called the L-galactose pathway) which allows the conversion of D-glucose into L-ascorbic acid in plants. Recently, it has been shown

Table 2 *C. reinhardtii* sequences encoding for putative enzymes involved in the nucleotide sugar biosynthesis

Function	Gene names based on literature	Arabidopsis gene	Putative ortholog in *C. reinhardtii*	% of id/sim
UDP-Glc biosynthesis				
Phosphoglucomutase		At1g23190	Cre06.g278210	53/67
UDP-sugar pyrophosphorylase	AtUSP, SLOPPY	At5g52560	Cre14.g621751	38/48
UTP-Glc-1-phosphate uridylyltransferase		At5g17310	Cre04.g229700	35/52
UDP-Gal*f* biosynthesis				
UDP-galactose 4-epimerase	AtUGE4	At4g10960	Cre04.g214502	56/72
UDP-galactopyranose-mutase	UGM[a]	–	Cre06.g272900[a]	48/60
GDP-Man biosynthesis				
Mannose-6-phosphate isomerase	AtMPI1	At1g67070	Cre02.g147650[b]	36/51
Phosphomannomutase	AtPMM	At2g45790	Cre14.g626900[b]	71/87
GDP-Man pyrophosphorylase	AtVTC1, AtGMPP1	At2g39770	Cre16.g672800[b]	66/82
UDP-GlcA biosynthesis				
UDP-Glc-6-dehydrogenase	UGD	At1g26570	Cre07.g357200	70/82
			Cre06.g278185	70/82
			Cre12.g532450	46/62
UDP-GlcNAc biosynthesis				
GlcN-6-P synthase	–	At3g24090	Cre08.g375500	53/69
GlcN-6-P acetyltransferase	GNA	At5g15770	Cre12g560200	46/63
Phospho-*N*-acetylglucosamine mutase	DRT101	At5g18070	Cre08.g379700	36/50
UTP-*N*-acetyl-glucosamine-1-P-uridylyltransferase	GLCNAC1PUT1	At1g31070	Cr16g6960000	22/37
UDP-Xyl biosynthesis				
UDP-xylose synthase	AtUXS3	At5g59290	Cre03.g169400	61/74
UDP-L-Ara*f* biosynthesis				
UDP-xylose-4-epimerase	AtMUR4	At1g30620	Cre09.g401022[c]	47/63
UDP-arabinopyranose mutase	AtRGP1	At3g02230	Cre13.g565800	78/85

Except for the UDP-Gal mutase[a], all these sequences have been identified by comparison with a TBLASTN using Arabidopsis sequences as keys. For each putative enzyme, the percentages of similarity (sim) and identity (id) have been calculated following a pairwise alignment of the deduced amino acid sequence from the predicted *C. reinhardtii* gene with the Arabidopsis amino acid sequence using the ClustalW program

[a]This sequence has been identified using the characterized enzyme from *C. neoformans* (Accession number AAX09636.1) as a key for the genomic search (Beverley et al. 2005)

[b]Urzica et al. (2012)

[c]Kotani et al. (2013)

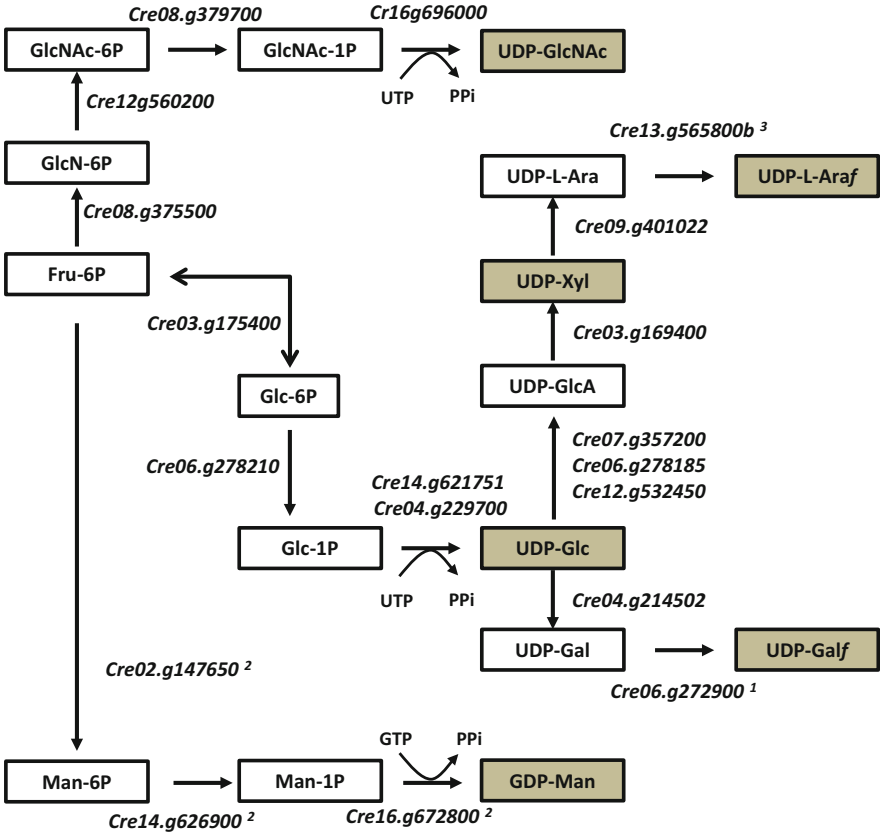

Fig. 3 Schematic representation of putative nucleotide sugar biosynthetic pathways in *C. reinhardtii*. Nucleotide sugars indicated in *gray squares* correspond to substrates used by glycosyltransferases for *N*- and *O*-linked glycans biosynthesis. *C. reinhardtii* genes encoding for putative enzymes involved in the formation of nucleotide sugars have been identified based on their homologies with Arabidopsis sequences as reported in Table 2. Exponent numbers refers to the following papers: (1) Kotani et al. (2013); (2) Urzica et al. (2012); (3) Berverley et al. (2005)

that *C. reinhardtii* genome contains one gene (Cre16.g672800, CrGMP1) encoding an amino acid sequence exhibiting 66% and 82% of identity and similarity, respectively, with the Arabidopsis GDP-Man PPase AtVTC1 (Urzica et al. 2012).

Some mature glycans *N*-linked to *C. reinhardtii* proteins carry one or two xylose residues (Mathieu-Rivet et al. 2013). In plants, UDP-xylose (UDP-Xyl) results from the decarboxylation of UDP-GlcA by a UDP-xylose synthase (UXS). Five genes encoding for UXS have been identified in Arabidopsis (Bar-Peled and O'Neill 2011; Harper and Bar-Peled 2002; Pattathil et al. 2005). Among the five candidates, three encode for soluble enzymes whereas the two others encode for isoforms which are predicted type II membrane proteins with the catalytic domain facing the membrane lumen of the Golgi apparatus. These data suggest that the

decarboxylation of UDP-GlcA occurs in plants both in the cytosol and in the Golgi apparatus (Pattathil et al. 2005). In *C. reinhardtii*, search for a gene encoding for a putative UXS led to the identification of a unique sequence (Cre03.g169400, CrUXS). The deduced amino acid sequence shares 61% of identity with the cytosolic enzyme AtUXS3. Furthermore, this sequence harbors two characteristic motifs required for dehydratase and epimerase activities, the *N*-terminal GxxGxxG motif and the catalytic triad serine, tyrosine, and lysine, reported in AtUXS1, AtUXS2, and AtUXS3 (Harper and Bar-Peled 2002).

The MS analyses of *N*-glycans carried out by soluble and membrane-bound proteins revealed minute amount of *N*-glycan structures containing fucose (Mathieu-Rivet et al. 2013). Although putative fucosyltransferases have been identified in the genome (Cre31.g780450 and Cre18.g749047), search for sequence homology using Arabidopsis genes as references revealed a limited number of candidate genes for enzymes involved in the biosynthesis of GDP-L-fucose. In plants, GDP-L-fucose (GDP-L-Fuc) can be synthesized via two distinct pathways involving either L-Fuc-1-P or GDP-Man. The first one (called the salvage pathway) requires the activity of the bifunctional enzyme L-fucokinase/GDP-L-fucose pyrophosphorylase (AtFKGP) which is responsible for the phosphorylation of L-Fucose and thereafter for the formation of GDP-L-Fuc (Bar-Peled and O'Neill 2011). In addition, GDP-L-fucose can be formed from GDP-Man through the activity of GDP-Man-4,6-dehydratase (GMD) and deoxymannose-3,5-epimerase-4-reductase (GER1). In *C. reinhardtii,* only one sequence (Cre01.g019250) has been shown to display 18% and 37% of identity and similarity with At1g73250 which encodes for GER1 in Arabidopsis.

Others monosaccharides are found in *O*-linked glycans such as galactose and L-arabinose (Bollig et al. 2007). UDP-Gal comes mainly from UDP-Glc via epimerization. Whereas several isoforms of UDP-Gal-4-epimerase (UGE) have been found in most plants, only one single sequence encoding for a putative GME has been identified in *C. reinhardtii* (Cre04.g214502, CrGME; Rosti et al. 2007). Moreover, in this organism, it has been shown that the Gal residues present in *O*-glycans exhibit the unusual furanose conformation (Gal*f*) (Bollig et al. 2007). Bollig and collaborators proposed that UDP-Gal*f* results from the activity of the UDP-galactopyranose-mutase (UGM) which converts the conformation of UDP-galactopyranose (UDP-Gal*p*) into UDP-Gal*f*. UDP-galactopyranose-mutases are found in prokaryotes and a few eukaryotes such as *Cryptococcus neoformans* or *T. cruzi*. A sequence encoding for a putative UGM, sharing 60% of identity with those from *C. neoformans* (Cre06.g272900, CrUGM, Table 2), has been identified in *C. reinhardtii*, although its activity has not be functionally demonstrated (Beverley et al. 2005).

In *C. reinhardtii O*-glycans, as in plant hemicelluloses and proteoglycans, arabinofuranosyl rather than arabinopyranosyl residues were reported (Bollig et al. 2007). UDP-L-arabinopyranose is synthesized from UDP-Xyl and then the conversion to UDP-L-arabinofuranose occurs through the action of a specific mutase (Table 2; Fig. 3). In *C. reinhardtii*, an UDP-arabinopyranose mutase has been recently purified mainly from the cytosol, and a minor activity has been also detected in the microsomal fraction (Kotani et al. 2013). Mass spectrometry

sequencing of the purified enzyme demonstrated that it shares 78% of identity with AtRGP1 that catalyzes the conversion of UDP-L-Ara*p* into UDP-L-Ara*f* in Arabidopsis (Rautengarten et al. 2011).

5.2 Nucleotide Sugar Transporters

Whereas the activation of sugar precursors is occurring in the cytosol, numerous glycosyltransferases involved in the glycan elongation reside inside the ER and Golgi apparatus. As a consequence, a transport of the cytosolic nucleotide sugars across the ER and Golgi membranes is required for the oligosaccharide biosynthesis. This transport is achieved through hydrophobic proteins composed of several transmembrane domains called nucleotide sugar transporters (NSTs) (Reyes and Orellana 2008). These proteins act as antiporters which couple the passage of the sugar nucleotide into the lumen with the exit of a nucleotide monophosphate into the cytosol. For a long time, it was thought that each NST was specialized in the transport of one specific nucleotide sugar (Hirschberg et al. 1998). However, this hypothesis tends to be invalidated with the discovery of numerous examples of NSTs able to transport several distinct activated sugars in eukaryotes. As illustrated, AtUTR1 from *Arabidopsis thaliana* allows the transport into the Golgi apparatus of both UDP-Glc and UDP-Gal (Norambuena et al. 2002; Reyes et al. 2006). However, a simple analysis of amino acid sequences is not sufficient to predict substrate specificity of a NST and further functional analysis is required.

NSTs belong to the drug/metabolite transporter (DMT) superfamily, within which they form a family with the group of triose phosphate translocators (TPT) (Knappe 2003a; Ward 2001). In Arabidopsis, this TPT/NSTs family brings together 51 members, themselves distributed into six distinct subgroups (Rautengarten et al. 2014). In order to identify orthologs encoding for putative TPT/NSTs in the *C. reinhardtii* genome, we used Arabidopsis TPT/NSTs sequences as key entries for bioinformatic searches by TBLASTN. Using this strategy, we found 23 putative genes for which the deduced amino acid sequences harbor the characteristic TPT domain (Pfam 03151). These results are summarized as a phylogenetic tree in Fig. 4. The comparison of this 23 sequences with those from Arabidopsis shows that five of them could be predicted to belong to the group I defined by Rautengarten and collaborators (2014) which gathers NSTs characterized by the presence of the highly conserved lysine/threonine (KT) motif involved in the NST-substrate binding specificity (Knappe 2003a). In Arabidopsis, NSTs-KT proteins are split into four subclades (Rautengarten et al. 2014). According to our phylogenetic analysis, three *C. reinhardtii* sequences are more related to Arabidopsis proteins from the subclades A and B, which have been recently shown to correspond to Golgi bifunctional UDP-rhamnose/UDP-Gal transporters (Rautengarten et al. 2014), and the two others are closely related to proteins from the subclade D for which the function remains unknown. No sequence is predicted for the subclade C which encompasses three Golgi transporters specific for

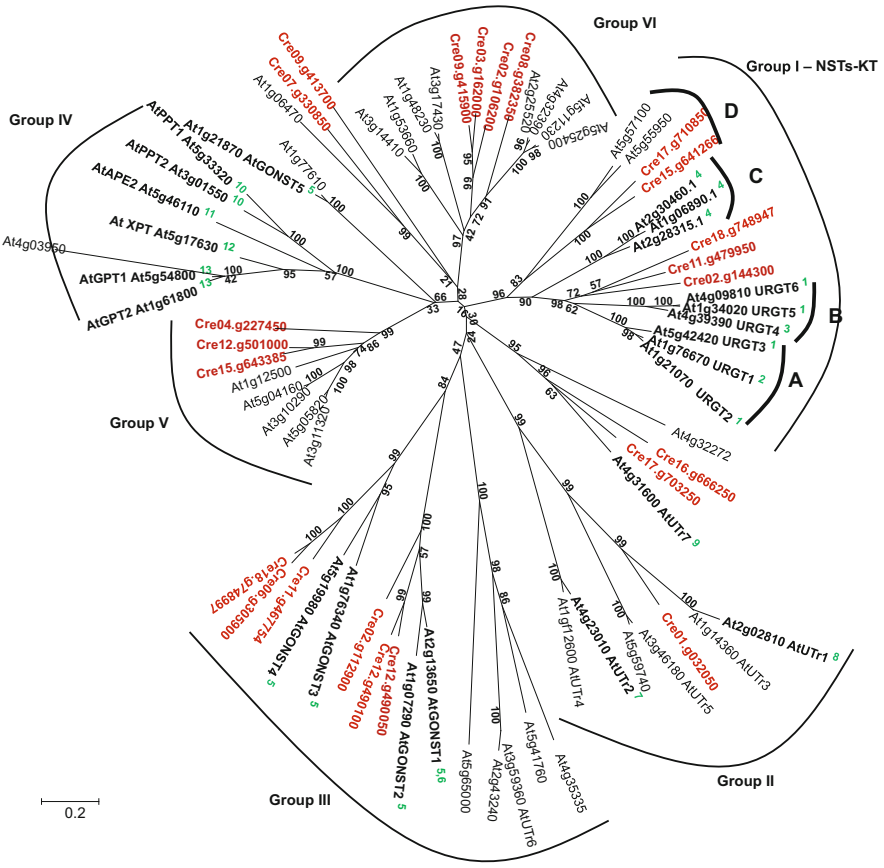

Fig. 4 Phylogenetic relationships between Arabidopsis members of the NST/TPT family and *C. reinhardtii* putative transporters. This phylogenetic tree is constructed based on the full-length amino acid sequences of NSTs/TPT from Arabidopsis (*black font*) and those which have been deduced from predicted genes in *C. reinhardtii* (*red font*). The Arabidopsis proteins in *bold* correspond to transporters functionally characterized. The alignment of the sequences has been made using the ClustalW program. The phylogenetic tree was generated using the neighbor-joining method with the software MEGA5 (Molecular Evolutionary Genetics Analysis). The robustness of branches has been evaluated using 1000 bootstraps. Groups I–VI and subgroups A–D were assigned according to Rautengarten et al. (2014). Exponent numbers refers to the following papers: (1) Rautengarten et al. (2014); (2) Bakker et al. (2005); (3) Rollwitz et al. (2006); (4) Ebert et al. (2015); (5) Handford et al. (2004); (6) Baldwin et al. (2001); (7) Norambuena et al. (2005); (8) Reyes et al. (2006); (9) Handford et al. (2012); (10) Knappe et al. (2003b); (11) Schneider et al. (2002); (12) Eicks et al. (2002); (13) Niewiadomski et al. (2005)

UDP-Xyl (Ebert et al. 2015) although xylosyl residues are found in mature *N*-glycans in *C. reinhardtii* (Mathieu-Rivet et al. 2013). However, as mentioned above, no conclusion can be drawn concerning the substrate specificity of these putative transporters as we cannot exclude that one of the identified sequences

would encode for an UDP-Xyl transporter. In addition, it has been shown in plants, that UDP-Xyl could be synthesized within the Golgi apparatus itself (Harper and Bar-Peled 2002), which would challenge the necessity of such UDP-Xyl transporter.

Six putative TPT-NSTs identified in *C. reinhardtii* fall into the group III, in which are found the Arabidopsis Golgi-localized GDP-Man transporters 1 to 4 (AtGONST1 to 4) (Handford et al. 2004). All these *C. reinhardtii* sequences display in the first transmembrane domain the two conserved amino acids asparagine/lysine (NK) corresponding to a potential substrate-binding site (Handford et al. 2004; Knappe 2003a). Furthermore, two of the six sequences (Cre12.g490100, Cre12.g49050) exhibit the conserved motif GXLNK (where X represents any amino acid) which is required for the specific recognition of GDP-Man (Gao et al. 2001).

Only one putative *C. reinhardtii* TPT-NST is found in the group II that gathers UDP-Glc/UDP-Gal transporters. Whereas some of them like AtUTr2 have been shown to be localized in the Golgi apparatus (Norambuena et al. 2005), others, like AtUTr1 and AtUTr3, are found in the ER (Reyes et al. 2010). The predicted *C. reinhardtii* gene (Cre01.g032050) would encode for a protein which amino acid sequence shares 47% and 49% of identity with AtUTr1 and AtUTr3, respectively. In addition, as it has been shown for AtUTr1 and AtUTr3 (Reyes et al. 2010), the *C*-terminal part of the *C. reinhardtii* predicted protein is characterized by the presence of a KKXX (where X represents any amino acid) retention signal which could be responsible for its retention within the ER. The heterologous expression of AtUTr1 in yeast showed that its affinity is higher for UDP-Glc than for UDP-Gal (Reyes et al. 2010). It has been proposed that this protein in Arabidopsis would be involved in the channeling of the Glc into the ER and thus would be required for the quality control process.

Among the remaining 11 sequences encoding for putative TPT/NSTs found in the *C. reinhardtii* genome, three are related to members of the group V and four fall in the group VI which contain no functionally characterized transporters (Knappe et al. 2003a). No sequence has been found in the group IV which gathers chloroplastic transporters (Eicks et al. 2002; Knappe et al. 2003b; Niewiadomski et al. 2005; Schneider et al. 2002).

6 Concluding Remarks and Future Perspectives

In the last years, *C. reinhardtii* has been evaluated as an emerging new factory for the production of biopharmaceuticals. More than 20 recombinant proteins have been successfully expressed in *C. reinhardtii* either in the chloroplast or in a secreted manner (Mathieu-Rivet et al. 2014; Rasala and Mayfield 2015). Recent findings regarding its *N*- and *O*-glycosylation pathways suggest that many efforts of engineering will have to be done to make it suitable for the production of recombinant secreted glycoproteins. In addition, a careful structural analysis of the

glycans present on such recombinant glycoproteins would be necessary to evaluate which glycan structures have been added onto the protein of interest. Recombinant EPO seems to be an appropriate model for such proof of concept. Indeed, the mature form of the EPO consists of 165 amino acid residues and is decorated with three *N*-linked and one *O*-linked glycans, representing almost 40% of the EPO molecular mass (Jiang et al. 2014). Eichler-Stahlberg and collaborators in 2009 already demonstrated that *C. reinhardtii* is able to produce and secrete successfully a recombinant form of EPO. Unfortunately, in this study, no detailed biochemical characterization of the recombinant protein is reported to gain information regarding its posttranslational modifications.

As far as monoclonal antibodies production in *C. reinhardtii* is concerned, we hypothesize that it would be necessary to engineer the algae *N*-glycosylation pathway in order to humanize the *N*-glycan structures added to the Chlamydomonas-made antibodies. Indeed, it has been well established that complex-type *N*-glycans, especially biantennary *N*-glycans bearing lactosamine antennae such as the one drawn in Fig. 1b, are required for the effector functions of antibodies (Mimura et al. 2001). Therefore, modifications of the *C. reinhardtii N*-glycans would require the complementation of Golgi enzymes repertoire with first the overexpression of heterologous GnT I which is currently missing within the *C. reinhardtii N*-glycosylation pathway. In addition, the complementation with α-mannosidase II, N-acetylglucosaminyltransferase II (GnT II), and β(1,4)-galactosyltransferase (β(1,4)-GalT) which is responsible for the transfer of the galactose onto the terminal GlcNAc residues would be necessary to mature *C. reinhardtii* similarly to the human *N*-glycans (Fig. 5). Additionally, inhibition of the xylosylation and glycan methylation would probably be needful as the overexpression of GnT I and β(1,4)-GalT could not be sufficient for *N*-glycan engineering as exemplified for tobacco plants complemented with the human β(1,4)-GalT (Bakker et al. 2001). Indeed, the presence of the xylose residues and methyl groups might represent a potential risk as they could be recognized as new xenoglycans when injected into humans. This may lead to induce immune responses and allergies as it has already been described for four different glycoepitopes (e.g., plant core β1,2-xylose and core α1,3-fucose; van Beers and Bardor 2012). For other glycoproteins of interest such as EPO, further engineering of the *C. reinhardtii* cells would be required in order to make the algae cells synthesize and transfer sialic acid onto the recombinant proteins as sialic acids are important for the in vivo half-life of blood proteins (Lingg et al. 2012). As described successfully for other expression systems such as plants (Bardor et al. 2011; Dicker and Strasser 2015), bacteria (Berlec and Strukelj 2013; Huang et al. 2012), and yeast (Berlec and Strukelj 2013; Wildt and Gerngross 2005), knock-in and knockout strategies of glycoenzymes would be necessary to engineer the *N*-glycosylation pathway in *C. reinhardtii*.

In the future, the biosimilar market will also attract attention. Indeed, biosimilars will increase and target a multibillion dollar market as quite a number of biopharmaceuticals products have lost or will shortly lose their patent protection (Walsh 2014). In this regards and based on the actual *N*-glycosylation knowledge, *C. reinhardtii* appears already to be an interesting platform for producing recombinant biosimilars carrying high-mannose-type *N*-glycans (Mathieu-Rivet et al. 2014). Its

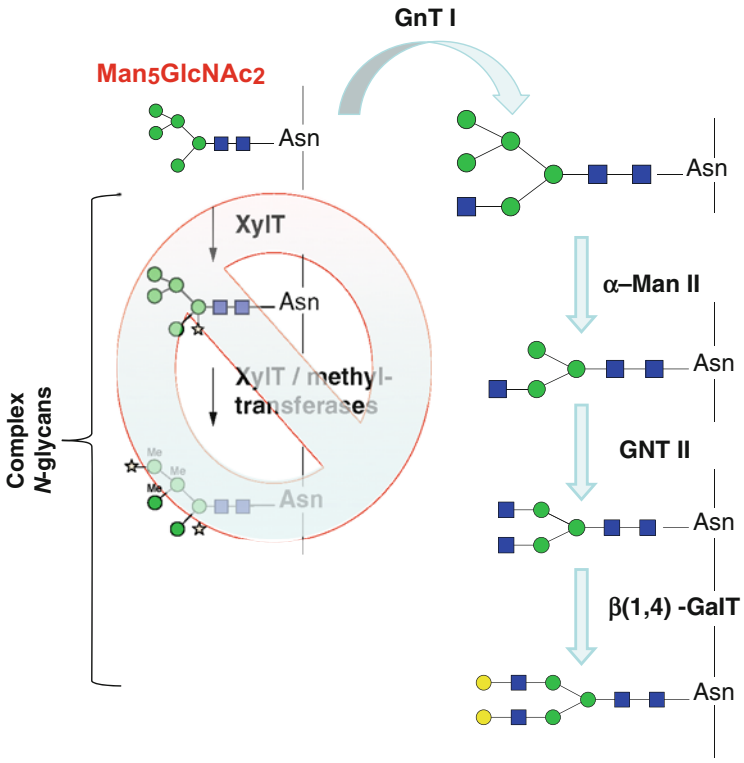

Fig. 5 Engineering of the *N*-glycosylation pathway in *C. reinhardtii* required to humanize the *N*-glycan structures of recombinant proteins such as monoclonal antibodies. The *symbols* used for representing the *N*-glycan structures are the ones from the Consortium for Functional Glycomics (Varki et al. 2009a). $Man_5GlcNAc_2$, oligomannoside bearing five mannose residues; GnT I, *N*-acetylglucosaminyltransferase I; α-Man II, α-mannosidase II; GnT II, *N*-acetylglucosaminyltransferase II; β(1,4)-GalT, β(1,4)-galactosyltransferase; XylT, xylosyltransferase. *Me* methyl group, *blue-filled squares N*-acetylglucosamine (GlcNAc), *green-filled circles* mannose (Man), *yellow-filled circles* galactose

capability to produce and add short mannose terminating *N*-glycans onto its endogenous proteins could represent an advantage for the production of glycosylated biopharmaceuticals which requires effective targeting and internalization into macrophages through the recognition of terminal mannose residues. This is well exemplified in the literature through the glucocerebrosidase which is a lysosomal enzyme administered intravenously into patients suffering from Gaucher's disease (Van Patten et al. 2007). The current preparation of glucocerebrosidase involves its recombinant expression in mammalian cells and in vitro post-purification exoglycosidase digestions to generate trimannose core *N*-glycans ($Man_3GlcNAc_2$). Therefore, alternative expression systems such as *C. reinhardtii* that are capable of producing naturally short mannose terminated *N*-glycans are of interest and should help decrease considerably the production cost.

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