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Heterologous expression of the \(N\)-acetylglucosaminyltransferase I dictates a reinvestigation of the \(N\)-glycosylation pathway in \textit{Chlamydomonas reinhardtii}

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Eukaryotic \(N\)-glycosylation pathways are dependent of \(N\)-acetylglucosaminyltransferase I (GnTI), a key glycosyltransferase opening the door to the formation of complex-type \(N\)-glycans by transferring a \(N\)-acetylglucosamine residue onto the Man\(_5\)GlcNAc\(_2\) intermediate. In contrast, glycans \(N\)-linked to \textit{Chlamydomonas reinhardtii} proteins arise from a GnTI-independent Golgi processing of oligomannosides giving rise to Man\(_5\)GlcNAc\(_2\) substituted eventually with one or two xylose(s). Here, complementation of \textit{C. reinhardtii} with heterologous GnTI was investigated by expression of GnTI cDNAs originated from Arabidopsis and the diatom \textit{Phaeodactylum tricornutum}. No modification of the \(N\)-glycans was observed in the GnTI transformed cells. Consequently, the structure of the Man\(_5\)GlcNAc\(_2\) synthesized by \textit{C. reinhardtii} was reinvestigated. Mass spectrometry analyses combined with enzyme sequencing showed that \textit{C. reinhardtii} proteins carry linear Man\(_5\)GlcNAc\(_2\) instead of the branched structure usually found in eukaryotes. Moreover, characterization of the lipid-linked oligosaccharide precursor demonstrated that \textit{C. reinhardtii} exhibit a Glc\(_3\)Man\(_5\)GlcNAc\(_2\) dolichol pyrophosphate precursor. We propose that this precursor is then trimmed into a linear Man\(_5\)GlcNAc\(_2\) that is not substrate for GnTI. Furthermore, cells expressing GnTI exhibited an altered phenotype with large vacuoles, increase of ROS production and accumulation of starch granules, suggesting the activation of stress responses likely due to the perturbation of the Golgi apparatus.

\(N\)-linked glycosylation is an extensive eukaryotic post-translational modification of secreted proteins consisting of the covalent attachment of an oligosaccharide onto asparagine residues belonging to the consensus sequence Asn–X–Ser/Thr/Cys, where X represents any amino acid except proline\(^1\)–\(^4\). The process starts in the endoplasmic reticulum (ER) with the biosynthesis of a lipid-linked oligosaccharide (LLO) precursor which involves the action of a conserved set of enzymes named Asparagine-Linked Glycosylation (ALG). First steps of the LLO synthesis occur on the cytosolic face of the ER membrane where \(N\)-acetylglucosamine (GlcNAc) and mannose

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Nway, such as the ALG, OST subunits, glycosidases and glycosyltransferases involved either in the ER or Golgi allowed the identification of numerous candidate genes encoding enzymes of the protein N-glycosylation pathway in the green microalgae <i>C. reinhardtii</i>.<sup>13,14</sup> The absence of terminal GlcNAc residues on these N-linked glycans suggested that mature N-glycans in <i>C. reinhardtii</i> are derived from GnTI-independent Golgi events<sup>11,15</sup>.<sup>11,15</sup> In silico analysis of the <i>C. reinhardtii</i> genome allowed the identification of numerous candidate genes encoding enzymes of the protein N-glycosylation pathway, such as the ALG, OST subunits, glycosidases and glycosyltransferases involved either in the ER or Golgi<sup>11,12</sup>.<sup>11,12</sup> This core undergoes further maturation into organism-specific complex-type N-glycans that are involved in several physiological functions like cell-cell interaction, intracellular communication and signaling<sup>13,14</sup>.

The structures of N-linked glycans of the green microalgae <i>C. reinhardtii</i> were recently characterized<sup>15</sup>.<sup>11,15</sup> Predominant N-glycans carried by <i>C. reinhardtii</i> endogenous proteins are oligomannosides ranging from Man<sub>0</sub>GlcNAc<sub>2</sub>-PP-Dol to Man<sub>3</sub>GlcNAc<sub>2</sub>-PP-Dol. This LLO is thereafter transferred by the oligosaccharyltransferase (OST) complex onto the asparagine of the N-glycosylation consensus sequence of the nascent polypeptides<sup>8</sup>. The N-glycan is then trimmed by the action of α-glucosidases I and II, and eventually an ER-mannosidase into Man<sub>0</sub>GlcNAc<sub>2</sub>, oligomannoside. During these ER events, interactions between the glycoprotein and ER-resident chaperones ensure the glycoprotein folding<sup>9,10</sup>. Correctly folded glycoproteins then enter the Golgi apparatus where their N-glycans are processed through the action of an α-mannosidase I into Man<sub>0</sub>GlcNAc<sub>2</sub>. Subsequently, N-acetylgalactosaminyltransferase I (GnTI), α-mannosidase II and finally N-acetylgalactosaminyltransferase II (GnTII) give rise to the canonical GlcNAc<sub>2</sub>Man<sub>2</sub>GlcNAc<sub>2</sub> core common to mammals, insects and land plants<sup>11,12</sup>. This core undergoes further maturation into organism-specific complex-type N-glycans that are involved in several physiological functions like cell-cell interaction, intracellular communication and signaling<sup>3,4,14</sup>.

The commonly used cw92 laboratory strain was transformed with the codon optimized sequences encoding for the catalytically active GnTI from Arabidopsis (AtGnTI) or from the diatom Phaeodactylum tricornutum (PtGnTI). Both have been shown to be able to process N-linked glycans. AtGnTI (At4g38240) encodes for a Golgi enzyme that is responsible for the transfer of a terminal GlcNAc residue onto Man<sub>0</sub>GlcNAc<sub>2</sub>-N-glycan<sup>16,18</sup>. PtGnTI has been shown to restore in vivo the biosynthesis of complex-type N-glycans in CHO Lec1 mutant that lacks endogenous GnTI activity<sup>13</sup>. These sequences fused to a tag sequence encoding for a V5-epitope were used for nuclear expression in <i>C. reinhardtii</i><sup>16,22</sup>. RT-PCR analyses show that both plant and diatom GnTI were expressed in <i>C. reinhardtii</i> transformed lines (Fig. 1 and Fig. S1). Further experiments were focused on two AtGnTI and four PtGnTI expressing lines, all of which exhibit the highest transcription levels (Fig. 1a). Furthermore, using anti-V5

**Figure 1.** (a) RT-PCR analysis of GnTI transcription level in cw92 cells, cell lines transformed with AtGnTI-V5 (lines AtGnTI#3 and AtGnTI#5) or PtGnTI-V5 (lines PtGnTI#1, PtGnTI#6, PtGnTI#8, PtGnTI#10 and PtGnTI#11) using AtGnTI (1) or PtGnTI (2) specific primers. Actin (lower panel) was used as an RT-PCR control. (b) Immunodetection of recombinant GnTI in the microsomal fraction isolated from cw92 cells and AtGnTI#3 lines respectively. The immunodetection was performed using an anti-V5 antibody as a primary antibody. A protein extract from CHO cells expressing PtGnTI-V5 (+) was used as a positive control<sup>17</sup>. Full images of the agarose gel and the Western blot are presented in Figs S1 and S2.
epitope antibodies, a signal around 56 kDa was immunodetected on a western blot in the microsomal fraction isolated from the transgenic lines but not in that of the non-transformed cells (Fig. 1b and Fig. S2).

The phenotypic analysis of C. reinhardtii transformed cell lines revealed that the expression of AtGnTI or PtGnTI was correlated to a modification of the cell size. Indeed, measurements of longitudinal cell diameters showed that cells expressing AtGnTI or PtGnTI were statistically enlarged as compared to the cw92 cells and cells transformed with the empty vector (Fig. 2a). Despite the size difference, the growth rates of transformed cells
were similar to those of cw92 cells indicating that the swelling observed in transformed lines did not alter cell growth (Fig. 2b).

To examine whether the ultrastructure of swollen cells is altered, cells were high pressure frozen and analyzed by thin-section electron microscopy. To further characterize the cell swelling, the ultrastructure of the transformed lines was analyzed by Transmission Electron Microscopy (TEM). As illustrated in Fig. 2c,d, GnTI expressing cells exhibited a large number of starch granules. In addition, large vesicles appeared in the GnTI expressing lines as compared to the cw92 and cells transformed with the empty vector. In order to check if the increase in size and the starch accumulation were associated to a stress phenotype in transformed cells, we measured the production of reactive oxygen species (ROS) in the different lines using electron paramagnetic resonance (EPR) spectroscopy. Figure 2e shows that ROS levels were significantly higher in the cells expressing AtGnTI or PtGnTI than in the cw92 cells, except for PtGnTI#1 line, in which ROS content was not significantly increased.

Protein N-glycosylation in cw92 and transformed cells were then investigated by mass spectrometry profiling. N-linked glycans were released from total protein extracts using PNGase F, labeled with 2-aminobenzamide (2AB) and analyzed by liquid chromatography coupled to electrospray ionization mass spectrometer (LC-ESI-MS). Although the expression of GnTI induced altered phenotypes in C. reinhardtii, the comparison of the N-glycan profiles between cw92 cells and the transformed lines did not reveal any modification of the N-glycan profiles (Fig. S3). A specific search for mono- and discharged ions corresponding to N-glycans exhibiting extra terminal GlcNAc residue was unsuccessful. This suggested that the expression of AtGnTI or PtGnTI did not affect, in a detectable manner, the N-glycosylation of endogenous proteins in C. reinhardtii.

Different scenarios may explain the inability of exogenous GnTI to affect the protein N-glycan profiles in C. reinhardtii. This may result from a very weak protein expression of active GnTI in the Golgi apparatus or its mislocalization in this organelle. Absence of the appropriate substrates in the Golgi apparatus could also explain the GnTI inactivity. This concerns the nucleotide-sugar (UDP-GlcNAc) or the oligomannoside Man$_5$GlcNAc$_2$ N-linked to secreted proteins. Man$_5$GlcNAc$_2$ oligomannoside was previously identified in C. reinhardtii cw92 proteins and was assigned to a branched Man$_5$GlcNAc$_2$ by analogy with data published regarding the Golgi N-glycan processing in eukaryotes$^{15}$. The non-effect of GnTI on the N-glycan profiles from C. reinhardtii raised questions regarding the structure of this Man$_5$GlcNAc$_2$, which therefore needs to be reinvestigated. Here, we made use of Ion Mobility Spectrometry–Mass Spectrometry (IMS-MS), a reliable analytical method which allows the separation of isomers including oligosaccharide isomers$^{23-25}$. In this analysis, the ion mobility of Man$_5$GlcNAc$_2$ coupled to 2AB oligosaccharide (sodium adduct) prepared from C. reinhardtii proteins was compared to the ion mobility of branched sodiated 2AB labeled Man$_5$GlcNAc$_2$ obtained from the bovine ribonuclease B. As illustrated in Fig. 3, Man$_5$GlcNAc$_2$-2AB from C. reinhardtii and from bovine ribonuclease B exhibited different ion mobilities (drift time of 10.12 ms and 11.28 ms, respectively) which suggested that they possess distinct structures. This data indicated that Man$_5$GlcNAc$_2$ in C. reinhardtii is different from the branched Man$_5$GlcNAc$_2$ that results from the trimming of oligomannosides Man$_n$GlcNAc$_2$ in the ER and the Golgi apparatus in plants and mammals.

The structure of C. reinhardtii Man$_5$GlcNAc$_2$ isomer was further investigated using electrospray ionization-multistage tandem mass spectrometry (ESI-MS$^n$) (with n = 2, n = 3 and n = 4) taking advantage of

Figure 3. (a–c) Ion mobility spectra of Man$_5$GlcNAc$_2$-2AB derivatives prepared from two independent preparations of C. reinhardtii proteins (a,b) and bovine ribonuclease B (c) (M + Na$^+$ adducts).
Taken together, both mass spectrometry and enzyme sequencing analyses demonstrated that 648, as it is expected for the branched isomer of Man 5GlcNAc 2-2AB (Fig. 4b)26 (Table 1). In conclusion, the m/z could be assigned to Hex 7HexNAc 2 (Fig. S6). The sequence of this oligosaccharide was analyzed by ESI-MS n spectra with n = 2 (upper panel), n = 3 (middle panel) and n = 4 (lower panel) of permethylated Man 5GlcNAc 2-2AB derivative (m/z 882 corresponding to [M + 2Na]+ precursor ion) isolated from PtGnTI C. reinhardtii proteins (a) and from Ribonuclease B (b) On each panel, the precursor ion selected for the fragmentation analysis is shown with a diamond and its fragmentation pattern is proposed according to one of the possible structures. 2AB: 2-aminobenzamide; Black square: GlcNAc; grey circle: Man.

Figure 4. ESI-MSn spectra with n = 2 (upper panel), n = 3 (middle panel) and n = 4 (lower panel) of permethylated Man 5GlcNAc 2-2AB derivative (m/z 882 corresponding to [M + 2Na]+ precursor ion) isolated from PtGnTI C. reinhardtii proteins (a) and from Ribonuclease B (b) On each panel, the precursor ion selected for the fragmentation analysis is shown with a diamond and its fragmentation pattern is proposed according to one of the possible structures. 2AB: 2-aminobenzamide; Black square: GlcNAc; grey circle: Man.

specific ion transitions reported in ref. 26 (Table 1). Fragmentation patterns of the doubly charged [M + 2Na]+ of permethylated Man 5GlcNAc 2-2AB (m/z 882) prepared from proteins of PtGnTI#1 transformed line and from bovine ribonuclease B were compared (Fig. 4). The MS3 “m/z 882 → m/z 1302 → product ions” analysis of PtGnTI#1 N-glycans revealed the presence of discriminant product ions m/z 880 and m/z 676 (Fig. 4a, middle panel) that are not detected for the permethylated Man 5GlcNAc 2-2AB released from bovine ribonuclease B (Fig. 4b, middle panel). Moreover, the MS3 “m/z 882 → m/z 1302 → m/z 880 → product ions” analysis showed that m/z 880, which arose from the neutral loss of two terminal mannose residues from m/z 1302, fragments into m/z 676 (Fig. 4a, lower panel). Such a fragmentation pattern does not correspond to that of a branched Man 5GlcNAc 2 isomer, but to the one of a linear Man 5GlcNAc 2 structure26 (Table 1). Similar fragmentation patterns were observed for permethylated Man 5GlcNAc 2-2AB prepared from cw92 cells and AtGnTI cells (Fig. S4). In contrast, the fragmentation pattern deduced from the MS3 analysis of the m/z 882 for Man 5GlcNAc 2-2AB isolated from bovine ribonuclease B revealed the specific fragmentations m/z 882 → m/z 1302 → m/z 866 → m/z 648, as it is expected for the branched isomer of Man 5GlcNAc 2-2AB (Fig. 4b)26 (Table 1). In conclusion, the comparison of ESI-MSn fragmentation patterns suggested that C. reinhardtii proteins carry linear Man 5GlcNAc 2 (Fig. 5d) instead of the conventional branched isomer (Fig. 5c).

According to the N-glycan biosynthesis pathway described in land plants and mammals, a linear Man 5GlcNAc 2 is meant to exhibit terminal α(1,2/6)-Man residues instead of α(1,3/6)-Man. For further confirmation of the C. reinhardtii Man 5GlcNAc, topology, the N-glycan mixture was submitted to exoglycosidase degradations using either Jack bean mannosidase, a non-specific α-mannosidase or from a trunctated biosynthesis of the LLO in the ER (Fig. 5). The latter hypothesis was investigated by isolation and analysis of C. reinhardtii LLO. LLO were isolated from cw92 microsomal preparations of C. reinhardtii using a methanol/chloroform extraction procedure according to a protocol adapted from27,28. The oligosaccharide was hydrolyzed from the PP-dolichol anchor by mild acidic cleavage and then permethylated as previously reported27,29. A pre-occurrence of a linear Man 5GlcNAc 2 oligosaccharide onto C. reinhardtii glycoproteins may result either from the trimming of Man 5GlcNAc 2 in the Golgi apparatus by the action of α-mannosidases or from a truncated biosynthesis of the LLO in the ER (Fig. 5). The latter hypothesis was investigated by isolation and analysis of C. reinhardtii LLO. LLO were isolated from cw92 microsomal preparations of C. reinhardtii using a methanol/chloroform extraction procedure according to a protocol adapted from27,28. The oligosaccharide was hydrolyzed from the PP-dolichol anchor by mild acidic cleavage and then permethylated as previously reported27,29. A predominant ion at m/z 1302 was observed using MALDI-TOF-MS and could be assigned to a permethylated oligosaccharide containing 8 hexoses and 2 HexNAc residues (Hex8HexNAc2) (Fig. S5). A minor ion at m/z 1987.6 could be assigned to Hex8HexNAc2 (Fig. S6). The sequence of this oligosaccharide was analyzed by ESI-MSn and compared to the one isolated from LLO of the YG170 (alg5) yeast mutant (Fig. 6). Indeed, this strain lacks α1,3-mannosyltransferase AlgG3 activity and accumulates Glc3Man 5GlcNAc 2 LLO in the ER20,31. Both ESI-MSn data exhibited similar fragmentation patterns that are consistent with a linear arrangement in the oligosaccharide.

Figure 4. ESI-MSn spectra with n = 2 (upper panel), n = 3 (middle panel) and n = 4 (lower panel) of permethylated Man 5GlcNAc 2-2AB derivative (m/z 882 corresponding to [M + 2Na]+ precursor ion) isolated from PtGnTI C. reinhardtii proteins (a) and from Ribonuclease B (b) On each panel, the precursor ion selected for the fragmentation analysis is shown with a diamond and its fragmentation pattern is proposed according to one of the possible structures. 2AB: 2-aminobenzamide; Black square: GlcNAc; grey circle: Man.
Taken together, these analyses demonstrated that *C. reinhardtii* accumulates a predominant linear truncated Glc3Man5GlcNAc2 LLO in the ER (Fig. 5b).

**Discussion**
In previous work, we characterized N-glycan structures linked to endogenous proteins in *C. reinhardtii* and showed they were mostly oligomannosides and for 30% novel mature structures containing xylose residues and...
methylated mannoses on Man$_{4-5}$GlcNAc$_2$. These structures resulted from a Golgi GnTI-independent processing of oligomannosides as the bioinformatics analysis of the genome has revealed that it lacks GnTI. Furthermore, no N-glycan harboring terminal GlcNAc residues has been identified in the whole glycan population. This situation contrasts with most eukaryotic organisms in which the GnTI is a key Golgi enzyme in the N-glycosylation pathway opening the door to the biosynthesis of structurally diverse mature N-glycans. In this canonical pathway, the addition of a first GlcNAc by GnTI is required for the sequential activity of a large repertoire of other specific transferases giving rise to complex N-glycans involved in numerous biological processes, such as intracellular communication and signaling. Inactivation of GnTI in those organisms induces strong developmental phenotypes. For instance, GnTI-null embryos of mouse die at about 10 days after fertilization indicating that mature N-glycans are required for morphogenesis in mammals. Also, inactivation of GnTI reduces the viability in worm and fly. In plants, GnTI mutants exhibit a stress phenotype, thus suggesting a role for mature N-glycans in specific physiological processes. In rice, $gntI$ mutants, impaired in the N-glycans maturation showed severe developmental defects, resulting in early lethality, associated to reduced sensitivity to cytokinin. GnTI genes are predicted in different microalgae genomes. In the diatom P. tricornutum, PtGnTI was demonstrated to encode for an active glycosyltransferase. Therefore, the capacity of C. reinhardtii to express GnTI from Arabidopsis and from P. tricornutum was analyzed to investigate whether C. reinhardtii N-glycan biosynthesis can be complemented with this key transferase and shift into a GnTI-dependent pathway. Moreover, in a biotech context, the production, in microalgae, of recombinant glycoproteins for therapeutic applications will require the engineering of their endogenous N-glycosylation pathway for the production of biopharmaceuticals exhibiting human-compatible N-glycans. Therefore, implementation of a GnTI-dependent pathway is a prerequisite for any production of glycosylated biopharmaceuticals in C. reinhardtii.

Transgenic lines expressing the Arabidopsis or diatom GnTI were obtained and GnTI protein was immunodetected as expected in the microsomal fraction. However, further experiments are required to confirm the GnTI localization within the Golgi apparatus. Mass spectrometry analyses of N-glycan profiles from proteins secreted in transgenic lines did not show any modification of the N-glycan population by comparison with the cv92 cells. Among the different scenarios that may explain this result, the absence of UDP-GlcNAc nucleotide sugar in the Golgi apparatus was first considered. The transport of the cytosolic nucleotide sugars across the Golgi membrane is performed by Nucleotide Sugar Transporters (NSTs). Searching for NST orthologues in the C. reinhardtii genome allowed the identification of 23 candidate genes of which the deduced amino-acid sequences harbor the characteristic Triose Phosphate Translocator (TPT) domain. However, as most of the characterized NSTs has been shown to transport at least two distinct substrates, determination of their specificity for nucleotide sugars based on sequence homologies with other Golgi NSTs remains difficult without additional biochemical evidence. Therefore, further experimental work is needed to determine whether a specific Golgi UDP-GlcNAc transporter exists in C. reinhardtii.

In relation to this, we cannot ruled out that the cytosolic abundance of UDP-GlcNAc may not be sufficient to supply the Golgi apparatus which would consequently limit GnTI in C. reinhardtii.

Figure 6. ESI-MS$^n$ spectra with $n = 2$ (upper panel), $n = 3$ (middle panel + lower panel (a)) and $n = 4$ (lower panel (b)) of permethylated LLO derivative ($m/z$ 1107 corresponding to $[M + 2Na]^+$ precursor ion) from lipid-linked oligosaccharides of cv92 C. reinhardtii cells (a) and the YG170 yeast mutant cells (b). On each panel, the precursor ion selected for the fragmentation analysis is shown with a diamond and its fragmentation pattern is proposed. Black square: GlcNAc; grey circle: Man; black circle: Glc.
Inability of GnTI to affect the endogenous N-glycan population in \textit{C. reinhardtii} transformed lines may also result from the absence of the appropriate glycan substrate. Man$_5$GlcNAc$_2$ oligomannoside was previously identified in \textit{C. reinhardtii} \cite{15} and considered as being the branched isomer substrate for GnTI by analogy with mammals and plants N-glycan pathways. Its structure was reinvestigated by mass spectrometry and enzyme sequencing and compared to a branched Man$_5$GlcNAc$_2$ standard from mammals \cite{26}. Ion mobilities determined by IMS-MS and fragmentation patterns resulting from ESI-MS$^n$ clearly show that \textit{C. reinhardtii} and mammalian oligomannosides differ in their shape and sequence. Moreover, the fragmentation pattern determined by MS$^n$ and enzyme digestion with an $\alpha$-(1,2)-mannosidase are consistent with the presence of a linear Man$_5$GlcNAc$_2$ on \textit{C. reinhardtii} proteins as depicted in Fig. 5d. This oligosaccharide may result from a truncated LLO biosynthesis occurring in the ER (Fig. 5). Indeed, ALG3, ALG9 and ALG12 candidates are not predicted in the \textit{C. reinhardtii} genome \cite{15,39,40}. These ER enzymes are involved in the completion of the biosynthesis of the LLO precursor Man$_9$GlcNAc$_2$-PP-Dol prior to its glucosylation (Fig. 5a). As a consequence, the absence of ALG3, ALG9 and ALG12 activities results in the secretion, from ER, of proteins carrying a linear Man$_5$GlcNAc$_2$ instead of Man$_8$-9GlcNAc$_2$ (Fig. 5b). In contrast, in land plants and mammals, branched Man$_5$GlcNAc$_2$ is obtained by the trimming of mannose residues of Man$_8$-9GlcNAc$_2$ oligomannosides by Golgi $\alpha$-mannosidases (Fig. 5a). However, linear Man$_5$GlcNAc$_2$ oligomannoside could also result from the trimming of Man$_8$-9GlcNAc$_2$ by Golgi $\alpha$-mannosidases predicted in \textit{C. reinhardtii} genome \cite{11,15} but having different glycan specificities as compared to those of homologous enzymes involved in mammalian and plant N-glycan pathways. To discriminate between the two possibilities, \textit{C. reinhardtii} LLO was isolated, characterized by mass spectrometry analysis and compared with the one extracted from the ALG3 deficient yeast mutant \cite{31}. These analyses demonstrated that this microalga accumulates a linear Glc$_3$Man$_5$GlcNAc$_2$ (Fig. 6 and Fig. S6). Such a truncated ER pathway has been already characterized in some unicellular organisms such as the coccidian parasites Toxoplasma and Cryptosporidium \cite{41–45}. In conclusion, data obtained in this study revealed a truncated ER N-glycan pathway in \textit{C. reinhardtii} and required the reevaluation of the previously published N-glycan pathway \cite{15}. In this reassessed N-glycan processing, Man$_5$GlcNAc$_2$ results from the deglucosylation of Glc$_3$Man$_5$GlcNAc$_2$ precursor in the ER and its methylation and xylosylation in the Golgi apparatus (Fig. 5). Location of xylose residues on Man$_5$GlcNAc$_2$ was reinvestigated by ESI-MS$^n$ (Fig. S7). This analysis confirmed the presence of the first xylose residue onto the $\beta$-Man \cite{15}. In addition, the second xylose could be positioned either

| Man$_5$GlcNAc$_2$ topology | MS$^n$ pathways |
|-----------------------------|------------------|
| ![Man5GlcNAc2 Diagram](image1.png) | a. 882$^*$ → 1302 (loss of $\text{[2-AB]}$) → 1084 (loss of terminal Man$\bullet$) → 866 (loss of terminal Man$\bullet$) → 676 (loss of Man$\circ$) |
| ![Man5GlcNAc2 Diagram](image2.png) | b. 882$^*$ → 1302 (loss of $\text{[2-AB]}$) → 1084 (loss of terminal Man$\bullet$) → 866 (loss of terminal Man$\bullet$) → 648 (loss of terminal Man$\bullet$) |
| ![Man5GlcNAc2 Diagram](image3.png) | c. 882$^*$ → 1302 (loss of $\text{[2-AB]}$) → 1084 (loss of terminal Man$\bullet$) → 866 (loss of terminal Man$\bullet$) → 662 (loss of Man$\circ$) |
| ![Man5GlcNAc2 Diagram](image4.png) | d. 882$^*$ → 1302 (loss of $\text{[2-AB]}$) → 1084 (loss of terminal Man$\bullet$) → 880 (loss of Man$\bullet$) → 676 (loss of Man$\circ$) |

Table 1. Transition fragmentation ions observed in ESI-MS$^n$ for the branched and linear Man$_5$GlcNAc$_2$-2AB according to ref. 26. $^*$The selected ion at $m/z$ 882 corresponding to $[\text{M + 2Na}]^{2+}$ precursor ion.
on the α(1, 3)-linked Man (I) or the first α(1, 2)-linked Man (II). MS experiments are more consistent with the structure I depicted in Fig. 5.

Surprisingly, cells expressing GnTI exhibited an altered phenotype although no modification of the N-glycans has been observed. The presence of large vacuoles as well as the increase of ROS production observed in transformed cell lines suggested that transformation with GnTI induced the activation of stress responses such as autophagy and oxidative stress.\(^{46, 47}\) Previously, Pérez-Martín and collaborators showed that ER stress caused by tunicamycin or DTT triggers autophagy.\(^{48}\) It can be hypothesized that AtGnTI or PtGnTI may localize in the ER of \textit{C. reinhardtii}. Indeed, a previous study published by Schoberer and coworkers in 2009 demonstrated that overexpressed GnTI in tobacco recruit the ER protein Sar1p resulting in a GnTI-Sar1p association in the ER membranes.\(^{49}\)

In addition, chlamydomonas cells expressing AtGnTI or PtGnTI accumulate starch. In BY2 cells and \textit{chlamydomonas noctigama}, Hummel and collaborators\(^{50}\) reported in 2010 that the disassembly of the Golgi apparatus using the secretion inhibitor Brefeldin A (BFA) caused the accumulation of plastid starch. This starch increase was also observed when the CopII-mediated ER to Golgi transport was inhibited in tobacco plants expressing a dominant negative version of the small GTPase Sar1p\(^ {51}\). Moreover, it was proposed that disruption of the Golgi apparatus resulting in the loss of secretory activity may cause the redirection of free carbohydrates to the plastids where they would be converted into starch.\(^ {32}\)

Therefore, we postulate here that the heterologous expression of \textit{GnTI} may affect Golgi organization or the endosomial system, which would consequently disturb the biosynthesis of glycans or glycoconjugates and the cell biology of \textit{C. reinhardtii}. Additional work and phenotypic studies of transformants expressing a non-functional GnTI would allow the confirmation of this hypothesis.

**Methods**

**Strains and growth conditions.** CC-503 cw92 strain, later called cw92 cells, was obtained from the Chlamydomonas Culture Collection at Duke University (Durham, NC, USA) and grown in batch cultures at 25 °C, illuminated with 150 μmol m\(^{-2}\) s\(^{-1}\) using TAP medium redirection of free carbohydrates to the plastids where they would be converted into starch.\(^ {32}\)

**Vector construction and transformation.** The sequences encoding for AtGnTI (gi: 307604450) fused to the V5 epitope\(^ {17}\) were codon optimized and synthesized by Sloning Biotechnology. Optimized sequences were cloned as a XbaI/NdeI fragment in the pSL18 vector.\(^ {53}\) pSL18 (empty vector), pSL18-AtGnTI or pSL18-PtGnTI construct was introduced using the glass beads method.\(^ {45}\) To detect the transgene in genomic DNA, screening was performed by PCR using V5-reverse GGAGTCCAGGCAGCGAG and with AtGnTI-forward GCCCTAAGTGGCCAAGGC or PtGnTI-forward CCAGTCAAGTGGCCGAGG as primers.

**RT-PCR analysis.** Total RNA were isolated from fresh cell pellets (1.5 × 10\(^6\) cells), using TRIzol (Invitrogen). gDNA contamination was removed by a Turbo DNase treatment (Thermo Fisher Scientific). Reverse transcription was performed on 2 μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The transcription level of AtGnTI and PtGnTI transgenes was analyzed by PCR using the V5-reverse primer, with either AtGnTI-forward or PtGnTI-forward primers. Each PCR contained 2 μL of diluted cDNA (1/10), 0.8 mM of each primer, 0.25 mM dNTPs and 0.6 U GoTaq polymerase (Promega) and was performed in the reaction buffer provided by the manufacturer, according the following program: 95 °C for 5 min, 35 cycles of 30 sec each at 95 °C (denaturation), 50 °C (annealing), 72 °C (extension) followed by 7 min at 72 °C in order to complete the extension. The transcription level of Actin was monitored as a control using CrActin-forward CGCTGGAGAAGACCTACGAG and CrActin-reverse GCCCTAAGTGGCCAAGGC as primers.

**Microsomal preparations and Western-blot analysis.** 1.4 × 10\(^7\) of \textit{C. reinhardtii} cells were collected (2,500 g for 5 min) and washed with a 20 mM potassium phosphate buffer at pH 7.4. All the following preparation steps were carried out at 4 °C. The cell pellet was broken with 2 mL of protease inhibitor cocktail 25X (Roche) dissolved in 10 mM potassium phosphate buffer (pH 7.4) using the FastPrep-24™ 5G. Samples were then spun (300 g for 3 min) in order to remove intact cells and debris. The supernatant was collected and centrifuged (20,000 g for 30 min) in order to eliminate pigments and chloroplasts. Finally, the supernatant was ultra-centrifuged (100,000 g for 1 h) to pellet the microsomal fraction. Immunodetection using anti-V5 antibodies has been performed as described in ref. 17.

**N-glycans preparation and derivatization.** Total proteins were extracted and deglycosylated using Peptide N-glycosidase F (PNGase F) as described in ref. 15. The released N-glycans were then labeled with 2-aminobenzamide (2AB) according to ref. 55. Excess of reagent was removed using a cartridge D1 from Ludger. Freeze dried samples were resuspended in 10 μL of water. Jack bean mannosidase (Sigma M7944) or \textit{Aspergillus saitoi} α(1,2)-mannosidase (ProZyme) enzymatic treatments were performed on 4.5 μL of 2AB-labeled N-glycans according to the manufacturer's instructions.

Permethylation - The 2AB-labeled N-glycans were permethylated\(^ {59}\) and cleaned-up according to ref. 56.

**LC-ESI-MS.** The analyses were performed using the nano-LC1200 system coupled to a QTOF 6550 mass spectrometer equipped with a nanospray source and a LC-Chip Cube interface (Agilent Technologies, les Ulis, France). Briefly, 2AB-labeled N-glycans were enriched and desalted on a 500 nL PGC trap column and separated on a PGC (3-μm particle size) column (150 mm long × 75 μm inner diameter, Agilent Technologies). A 30-min linear gradient (5–75% acetonitrile in 0.1% formic acid) at a flow rate of 400 nL min\(^{-1}\) was used. Separated
N-glycans were analyzed with the QTOF analyser. Full autoMS scans from 290 to 1,700 m/z and autoMS/MS from 59 to 1,700 m/z were recorded. In every cycle, a maximum of 5 precursors sorted by charge state (1+ and 2+ preferred) were isolated and fragmented in the collision cell with fixed collision cell energy at 15 eV. Scan speed raise based on precursor abundance (target 5,000 counts/spectrum) and precursors sorted only by abundance. Active exclusion of precursors was enabled and the threshold for precursor selection was set to 1,000 counts. The ESI acquisition parameters in positive mode were: capillary voltage: 1.9 kV, drying gas temperature: 250°C, gas flow (air): 11 L min⁻¹; fragmentor voltage, 360 V; Skimmer1 voltage, 65 V and OctopoleRFPeak voltage, 750 V.

**IM-MS analyses.** The ESI-IM-MS experiments were performed using a Waters SYNAPT G2 hybrid quadrupole/HDMS instrument equipped with an ESI LockSpray™ source, the MassLynx 4.1 and the DriftScope 2.2 softwares (Waters, Manchester, UK). The SYNAPT HDMS system was calibrated using sodium formate cluster ions (2 mg mL⁻¹) and operated in V resolution mode (resolution 20,000 FWHM). The ESI parameters were in positive ion mode: capillary voltage, 3 kV; sample cone voltage, 70 V; source temperature, 90°C; desolvation temperature, 250°C; desolvation gas flow (N₂), 700 L h⁻¹. The data were acquired using a 50–2000 m/z range with 1 s scan time and 0.02 s interscan delay. Sample solutions were infused into the source at a flow rate of 400 µL h⁻¹ with a syringe pump (Cole-Palmer, Vernon Hills, Illinois, USA). The IMS conditions were optimized as followed: gas flow (N₂), 90 mL min⁻¹; IMS pressure, 3.03 mbar; wave height voltage, 40 V and T-wave velocity, 550 m s⁻¹. The ion mobility spectra were fitted using the Origin 9.0 software (OriginLab).

**ESI-MSⁿ analyses.** Permethylated 2AB-derivatives of N-glycans were analyzed by ESI-MSⁿ (n = 1 to 4) using a Bruker HCT Ultra ETD II quadrupole ion trap (QIT) mass spectrometer equipped with the Esquire control 6.2 and Data Analysis 4.0 softwares (Bruker Daltonics, Bremen, Germany). The ESI parameters were as followed: capillary and end plate voltages respectively set at −3.5 kV and −3.0 kV in positive ion mode, skimmer and capillary exit voltages set at 40 V and 200, respectively, nebulizer gas (N₂) pressure, drying gas (N₂) flow rate and drying gas temperature were 10 psi, 7.0 L min⁻¹ and 300°C, respectively. Helium pressure in the ion trap was 1.2 × 10⁻⁵ mbar. The data were acquired using a 200–2200 m/z range, using a scan speed of 8,000 m/z per second. The number of ions entering the trap cell was automatically adjusted by controlling the accumulation time using the ion charge control (ICC) mode (target 200,000) with a maximum accumulation time of 50 ms. The injection low-mass cut-off (LMCO) value was m/z 100 for Man₃(GlcNAc)₂ derivatives and 140 for LLO precursor respectively. The values of spectra averages and rolling average were 6 and 2. ESI-MSⁿ experiments were carried out by collision-induced dissociation (CID) using helium as the collision gas, isolation width of 2 m/z unit for the precursor ions and for the intermediate ions using a resonant excitation frequency with an amplitude from 0.8 to 1.0 Vp–p. Sample solutions were infused into the source at a flow rate of 300µL h⁻¹ by means of a syringe pump (Cole-Palmer, Vernon Hills, IL, USA).

**Electron microscopy.** High pressure freezing was performed with the freezer HPM100 Leica-microsystems. Prior to freezing, 72 h old C. reinhardtii cells were treated at room temperature 1 hour with 100 mM mannitol as cryoprotectant dissolved in fresh culture medium. Pre-treated C. reinhardtii cells were transferred into an aluminium cryocapsule covered by soy lecithin dissolved at 100 mM in chloroform. Excess medium was absorbed by filter paper. After fixation on the loading device, samples were frozen according to a maximum cooling rate of 20,000°C s⁻¹ and a pressure of 2,100 bars. Samples were transferred to a freeze substitution automate (AFS2, Leica) pre-cooled to −110°C. Samples were substituted in anhydrous acetone with 0.5% uranyl acetate and 0.5% osmium tetroxide at −90°C for 72 h. The temperature was gradually raised to −60°C using a gradient of −2°C h⁻¹ and stabilized during 12 h, then gradually raised to −30°C by using the same gradient during 12 h and gradually raised again to +4°C. Then, samples were rinsed twice at room temperature with anhydrous acetone. Infiltration was then performed in acetone-Spurr’s resin (Spurr 25%, 8 h at 4 °C). Then, samples were rinsed twice at room temperature with anhydrous acetone. Spurr’s resin was finally polymerised at +50°C during 24 h. Ultrathin sections (70 nm; ultracut UC6, Leica) were collected onto carbon-formvar-coated nickel grids. A classical staining using 0.5% uranyl acetate and 0.5% lead citrate was done before sections were observed in a Philips, FEI Tecnai 12 Biotwin transmission electron microscope operating at 80 kV, with ES5000W Erlangshen CCD camera (Gatan).

**Size measurements.** Cells (1.5 × 10⁷) were collected (4,500 g for 5 min). The cell pellet was incubated in TAP medium containing 4% of paraformaldehyde (PAF) (v/v) during 30 min, under shaking, at room temperature. Then, cells were washed four times and finally resuspended in 200 µL of TAP. 20 µL of fixed cells were spotted between slide and coverslip (Superfrost™Plus, Thermo Scientific) and were observed with a Leica DMI-6000B inverted microscope at magnification 400. Cell size was determined using fixed cells by measuring the cell longitudinal diameter with Image J software. The measurements were done in triplicates on a minimum of 200 cells (n > 200).

**Reactive oxygen species measurement.** Reactive oxygen species (ROS) production was evaluated by electron paramagnetic resonance (EPR) spectroscopy. Cells were incubated at room temperature in the dark for 60 min in Krebs-HEPES buffer containing 5 µM diethyl dithiocarbamate, 25 µM deferoxamine, and the spin probe 1-hydroxy-3-methoxyxycarbonyl-2,2,5,5-tetramethyl pyrrolidine hydrochloride (CMH; 500 µM; Noxygen, Elzach, Germany). Spectra of the oxidized product of CMH (CM·) were recorded from frozen samples with a X-band spectrometer (MS-400; Magnetech, Berlin, Germany) with the following acquisition parameters: microwave power, 1 mW; modulation amplitude, 5 G; sweep time, 60 s; and 1 scans. After correction of the baseline, the total amplitude of the signal was measured and expressed in arbitrary units produced per 12 × 10⁴ cells for 60 min. After EPR analyses, total chlorophyll was quantified for each sample according to ref. 57. The quantity
of total chlorophyll was used to normalize the signal intensity evaluated by EPR for each sample. The results were statistically analyzed using GraphPad Prism™ software. The ROS level in each transformed cell line was normalized against ROS level measured in cw92 cells. To determine the significant level, a statistical test was performed between the GnTI transformed cell lines and the lines transformed with the empty vector using Ordinary One-Way ANOVA with n = 3 and p-value fixed at 0.05.

**LLO preparation.** The LLO extraction was performed on microsomal fractions from C. reinhardtii according to a protocol adapted from ref. 27. Released oligosaccharides were then lyophilized and resuspended in 500 µL of water and purified on a carboxyphobic column (Hypersep Hypercarb, Thermo Scientific) using the manufacturer's instructions.

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Author Contributions
P.L.: M.B., E.M.R. planned and designed of the research; G.V., P.L.L., J.V., C.P., C.L.B., M.L.W., B., V.R. performed experiments; G.V., P.L.L., J.V., C.L.B., I.R.J. and V.R. collected and analyzed data, G.V., P.L.L., J.V., M.B., P.L., C.A., E.M.R. interpreted data and A.D., P.L., M.B., E.M.R., G.V., P.L.L., C.L.B. wrote the manuscript.

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