Xylosylation of protein *N*-linked glycans in *Chlamydomonas reinhardtii* is heterogeneous and mediated by a multigene xylosyltransferase family

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Introduction

Presentation of the *N*-glycosylation pathway in *C. reinhardtii*

In Eukaryotes the protein *N*-glycosylation process starts in the endoplasmic reticulum and continues with the maturation steps in the Golgi apparatus (Fig. 1). In *C. reinhardtii*, the maturation steps result in glycans *N*-linked to proteins ranging from $Man_5GlcNAc_2$ to $Man_3GlcNAc_2$ and carrying one or two xylose residues (Fig. 1) (Vanier *et al.* 2017, Lucas/Dumontier *et al.* 2018). Nowadays, little information is available regarding the xylosylation in *C. reinhardtii*. This study aimed at characterizing the molecular actors involved in xylosylation process using complementary analysis, such as Western blot, nanoliquid chromatography coupled to electrospray mass spectrometry (nanoLC-ESI-MS), multistage tandem mass spectrometry ($ESI\text{-}MS^n$) on insertional mutants impaired for genes encoding putative xylosyltransferases (XTs).

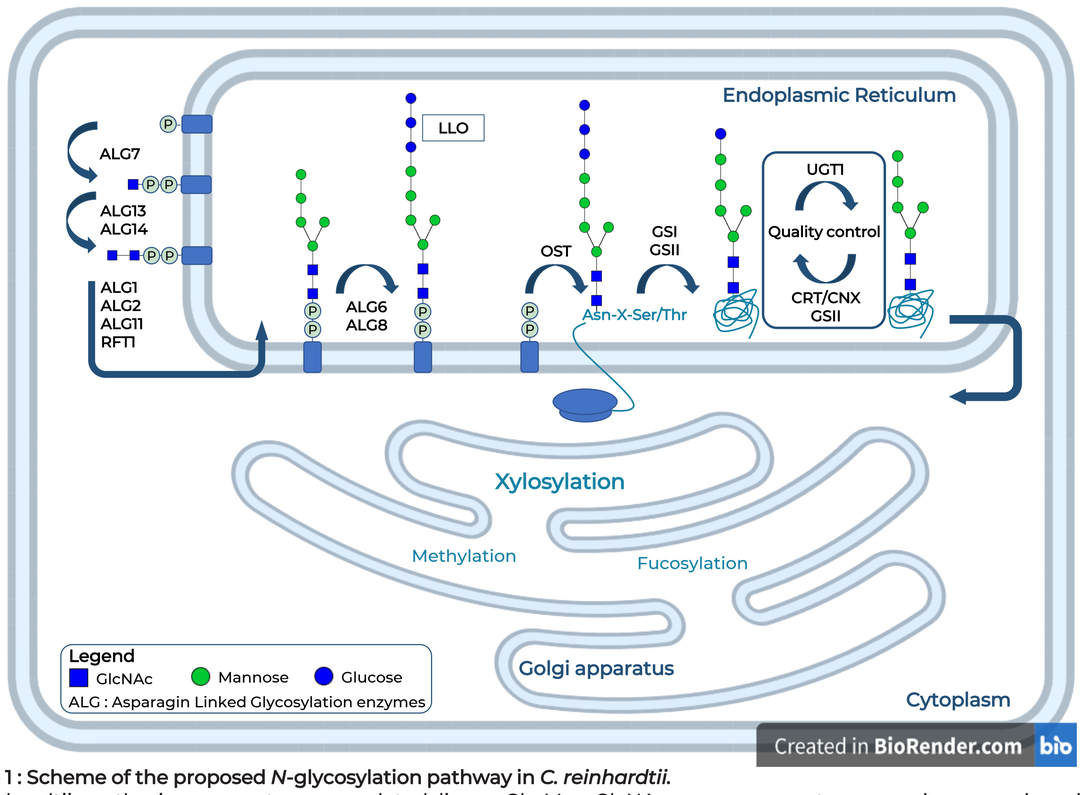

Figure 1 : Scheme of the proposed *N*-glycosylation pathway in *C. reinhardtii*.
C. reinhardtii synthesizes a pentamannosylated linear $Glc_3Man_5GlcNAc_2$ precursor onto a membrane-anchor dolichol pyrophosphate (PP-Dol) (Lucas *et al.*, 2018). This precursor, called lipid linked oligosaccharide (LLO), is transferred on the nascent polypeptide chain through the action of the predicted oligosaccharyltransferase (OST) complex (Vanier *et al.*, 2017). Then, the glucose residues are removed to form a $Man_5GlcNAc_2$ structure (Vanier *et al.*, 2017). The newly synthesized protein is transferred in the Golgi apparatus where *N*-glycans are partially methylated, fucosylated and xylosylated.

Results and discussion

1. Involvement of XTA and XTB in the *N*-glycan xylosylation

In silico analyses of *C. reinhardtii* genome revealed that several genes encode putative XTs. This study focused on Cre09.g391282 and Cre16.g678997, encoding respectively XTA and XTB, since their deduced protein sequences share the highest homology degree with the *A. thaliana* characterized XT. Total proteins from insertional mutants IM_{XTA} , IM_{XTB} and $IM_{XTA}xIM_{XTB}$ double-mutant (www.chlamylibrary.org; Lucas *et al.*, 2020) were analyzed by immunoblot using antibodies specifically directed against the core β(1,2)-xylose epitope (Fitchette *et al.*, 2007). A low signal was observed in both IM_{XTA} and $IM_{XTA}xIM_{XTB}$ compared to the wild-type (WT) (Fig. 2). In contrast, proteins from the IM_{XTB} mutant were immunodetected similarly to WT (Fig. 2). This suggests that XTA rather XTB is involved in the transfer of a β(1,2) xylose residue on the core mannose. This glycoproteomic analysis performed on secreted glycoproteins confirms previous results and the role of XTA in the β(1,2)-xylosylation (Mathieu-Rivet *et al.*, 2013, 2014; Schulze *et al.*, 2018; Oltmanns *et al.*, 2019).

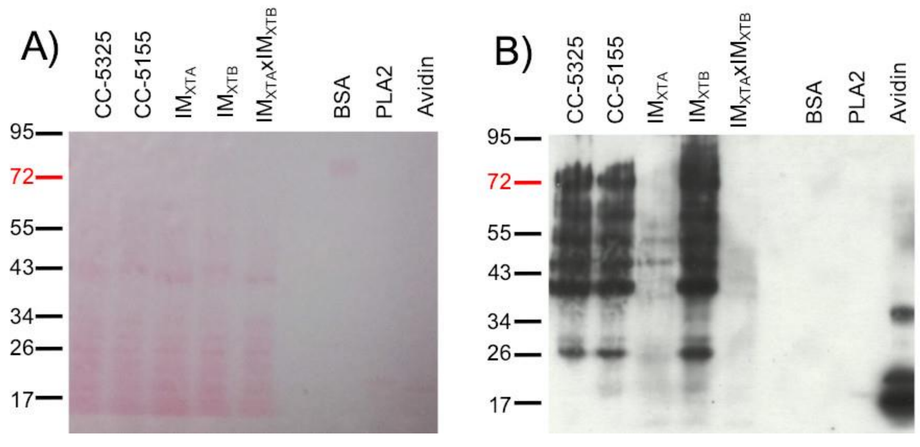

Figure 2 : Immunoblot analysis of the secreted proteins from WT (CC-5325; CC-5155) and *C. reinhardtii* mutants using antibodies specifically directed against the core β(1,2)-xylose epitope (Lucas *et al.*, 2020).
XTA is responsible for the core β(1,2)-xylosylation, whereas XTB is involved in the xylosylation of the linear branch of *C. reinhardtii*. Western blot analysis of protein extracts from CC-5325; CC-5155; IM_{XTA}; IM_{XTB}; $IM_{XTA}xIM_{XTB}$; Bovine serumalbumin (BSA) as it is non glycosylated; the phospholipase A2 (PLA2), which is a glycosylated protein without any core β(1,2)-xylosylation; and the recombinant avidin produced in corn, which is glycosylated with core β(1,2)-xylosylation. In parallel, molecular weight markers (PageRuler Plus stained Protein Ladder, Thermo Fisher) are reported in kDa.
A) Ponceau Red staining of the nitrocellulose membrane.
B) Revelation using the specific anti-β(1,2)-core xylose antibody from Agrisera.

A complementary approach based on LC-MS-MS showed that the knock-out of *XTA* induced a strong decrease of the *N*-glycan xylosylation with a decrease of mono- and dixylosylated species (Fig. 3). A similar impact was observed in the IM_{XTB} mutant. A stronger effect on the protein xylosylation was observed in the double mutant with a disappearance of almost all dixylosylated oligosaccharides (less than 1% of the total *N*-glycans) and the remaining monoxylosylated *N*-glycans representing only 5% (Fig. 3). It can be concluded that *XTB* encodes for a XT mainly responsible for the xylosylation of α-mannose residues of the linear branch of the oligomannosides. In the double-mutant, xylose residues in the remaining monoxylosylated *N*-glycan detected are attached to α-mannose residues of oligomannoside α(1,3)-branch.

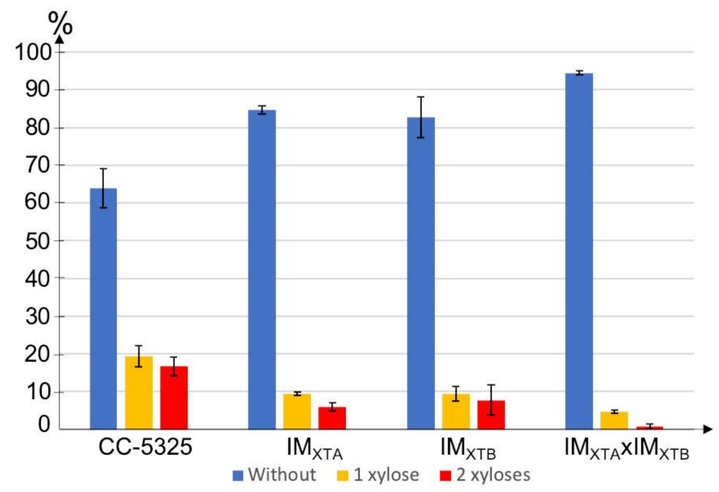

Figure 3 : The relative proportion of xylosylated *N*-glycans is decreased in the IM_{XTA}, IM_{XTB} and in $IM_{XTA}xIM_{XTB}$ (Lucas *et al.*, 2020).
The relative percentage of each *N*-glycan type has been determined based on ion intensities of the procainamide-labeled *N*-glycans analyzed by nano-LC ESI-MS. The relative percentages reported were the mean values and standard deviation from three independent analyses of three biological replicates.

2. Residual xylosyltation in $IM_{XTA}xIM_{XTB}$ double mutant might be due to other xylosyltransferase candidates

The high degree of heterogeneity of xylosylated *N*-glycan structures in *C. reinhardtii* and the remaining XT activity in the $IM_{XTA}xIM_{XTB}$ led to the hypothesis that other XTs would be involved in the maturation of *C. reinhardtii N*-glycans. Thus, three new candidate genes sharing a common motif in the C-terminus part with XTA, XTB and plant β(1,2)-XTs were identified *in silico* by sequence homology search (Fig. 4).

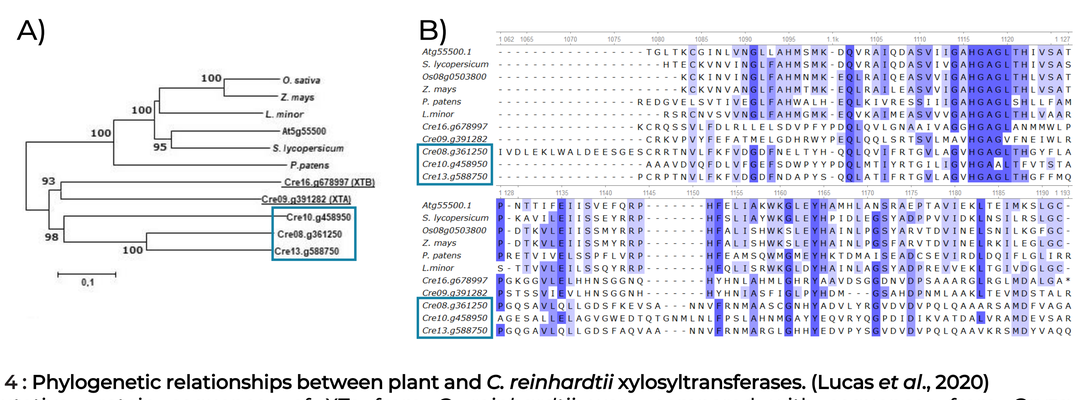

Figure 4 : Phylogenetic relationships between plant and *C. reinhardtii* xylosyltransferases. (Lucas *et al.*, 2020)
The putative protein sequences of XTs from *C. reinhardtii* were compared with sequences from *Oryza sativa* (Os08g0503800), *Zea mays* (NP_001105845.1), *Lemna minor* (ABG89269.1), *Arabidopsis thaliana* (At5g55500), *Solanum lycopersicum* (NP_001311390.1) and *Physcomitrella patens* (CAD22108.1). The protein sequences alignment was performed with ClustalW and then the tree (A) was deduced using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). (B) Sequences encoding for putative XTs exhibit a characteristic GT61 motif in their C-terminal part. Conserved amino acid residues are shaded dark blue (identities) and light blue (similarities). Dashed lines represent gaps inserted for optimal alignment of the sequences.

Conclusion and perspectives
Proposed xylosylation process in *C. reinhardtii*

The additional XTs are possible candidates for α(1,3)mannose xylosylation (Fig. 5).

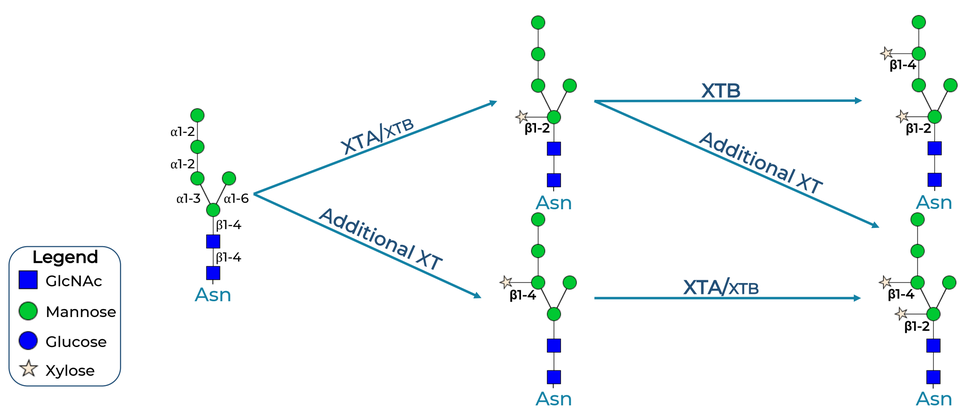

Figure 5 : Proposed xylosylation process in *C. reinhardtii N*-glycosylation pathway (Lucas *et al.*, 2020)
XTA is mainly responsible for the addition of core β(1,2) xylose whereas XTB is involved in the transfer of a β(1,4) xylose onto a α(1,2)mannose of the *N*-glycan linear branch. To a lesser extent, XTB is responsible for the addition of a core β(1,2) xylose.

Perspectives are, at first, to characterize the XT candidates to identify which one(s) are responsible for residual xylosylation. In a second time, since β(1,2)-xylose residues are known to be immunogenic (Lerouge *et al.*,1998; Strasser *et al.*, 2000), repressing or deleting the genes encoding XTs would be necessary to exploit *C. reinhardtii* as a biofactory for the production of therapeutic glycoproteins suitable for use in human therapy.

Acknowledgments: *The authors are thankful to Normandie University, the Graduate School of Research XL-Chem (ANR-18-EURE-0020 XL-Chem), the Région Normandie, France and the University of Muenster, Germany for financial support.*
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