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 processes 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$_4$GlcNAc$_2$ to Man$_9$GlcNAc$_2$, 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 aims to characterize the xylosylation enzymes 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-MS$^n$) and ion mobility spectrometry-tandem mass spectrometry (IS-MS$^n$) on xylosylation isomeric mutants.

![Diagram](image)

**Figure 1**: Scheme of the proposed N-glycosylation pathway in *C. reinhardtii*.

In silico analyses of *C. reinhardtii* genome revealed that several genes encode putative xylosyltransferases. This study focused on CreG9.931282 and CreG6.678997, encoding respectively XTA and XTB, since their deduced protein sequences share the highest homology degree with the A. thaliana characterized xylosyltransferases. Total RNA from both *XTA* and *XTB* and their corresponding double-mutant (www.chalmylibrary.org: Lucas et al. 2020) were analyzed by immunoblot using antibodies specifically directed against the core (S1,2) xylose epitope (Fitchett et al. 2007). A low signal was observed in both *XTA* and *XTB* double-mutant compared to the wild-type (WT) (Fig. 2). In contrast, proteins from the *XTB* mutant were immunodetected similarly to WT (Fig. 2). This suggests that XTA rather XTB is involved in the transfer of a (S1,2) xylose residue on the core mannose. This glycoproteic analysis performed on secreted glycoproteins confirms previous results and the role of XTA in the S1,2-xylosylation (Bourhis et al. 2013, 2016; Oltmanns et al. 2019).

![Image](image)

**Figure 2**: Immunoblot analysis of the secreted proteins from WT (CC-5325), OL-6151 and *C. reinhardtii* mutants using antibodies specifically directed against the core (S1,2)-xylose epitope (Lucas et al., 2020).

**Results and discussion**

1. Involvement of XTA and XTB in the xylosylation

A complementary approach based on LC-MS-MS showed that the knock-out of XTA induces a strong decrease of the βglycan xylosylation with a decrease of mono- and dixylosylated species (Fig. 3). A similar impact was observed in the IM$_{β}$ mutant. A stronger effect on the protein xylosylation was observed in the double mutant with a disappearance of almost all dixylosylated oligomannosides (less than 1% of the total N-glycans) and the remaining monoxylolylated N-glycans representing only 2% of the total N-glycans (Fig. 3). It can be concluded that xylosyltransferase 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 monoxylolylated N-glycan detected are attached to α-xylose residues of oligomannoside (S1,3)-branch.

![Image](image)

**Figure 3**: The relative proportion of xylosylated N-glycans is decreased in the IM$_{β}$, IM$_{αβ}$ and in IM$_{αββ}$ mutants (Lucas et al., 2020). The relative percentage of each N-glycans relative intensities was performed based on ion intensities of the permethiodioylated glycan (GSI) peaks. Relative percentages reported were the average of three independent analyses of the same biological material.

2. Residual xylosylation in IM$_{β}$/IM$_{αβ}$ double mutant might be due to other xylosyltransferase candidates

The high degree of heterogeneity of xylosylated glycans structures in *C. reinhardtii* and the remaining xylosylation activity in the IM$_{β}$/IM$_{αβ}$ double-mutant led to the hypothesis that other xylosyltransferases would be involved in the maturation of *C. reinhardtii* N-glycans. Thus, three new candidate genes were identified by sequence homology search (Fig. 4). Although the three predicted proteins would not have a transmembrane domain as usually required for Golgi-resident enzymes and glycosyltransferases (Strasser et al., 2000), they share a common motif in the C-terminus part with XTA, XTB and plant S1,2-xylosyltransferase (Fig. 4).

![Image](image)

**Figure 4**: Phylogonetic relationships between plant and *C. reinhardtii* xylosyltransferases. (Lucas et al., 2020).

The putative protein sequences of xylosyltransferases from *C. reinhardtii* were compared with sequences from other algae (H. pluharii, O. tauri, A. castanea) and plants (A. thaliana, *O. sativa* and *P. araucana*). A phylogenetic tree was built using the neighbor-joining method (Saitou and Nei, 1987). The percentage of replicate times in which the associated trees clustered together, in the bootstrap test (1000 replicates) used to test the branches (Felsenstein, 1985). (A) Sequences encoding for putative xylosyltransferases exhibit a high degree of identity, especially to plant sequences (Lucas et al., 2020).

The additional xylosylation results in S1,3mannose xylosylation (Fig. 5).

![Image](image)

**Figure 5**: Proposed xylosylation process in *C. reinhardtii* N-glycosylation pathway (Lucas et al., 2020).

The newly identified xylosyltransferases (XTA, XTB and XTC) have been found to be responsible for the xylosylation of the linear branch of the oligomannosides. XTA and XTB are involved in the xylosylation of the O-linked glycans (Lucas et al., 2020). XTA and XTB are responsible for the xylosylation of the α-xylose residues while XTC only participates in the xylosylation of the β-xylose residues.

**Conclusion and perspectives**

**Proposed xylosylation process in *C. reinhardtii***

The newly identified xylosyltransferases (XTA, XTB and XTC) have been found to be responsible for the xylosylation of the linear branch of the oligomannosides. XTA and XTB are involved in the xylosylation of the O-linked glycans (Lucas et al., 2020). XTA and XTB are responsible for the xylosylation of the α-xylose residues while XTC only participates in the xylosylation of the β-xylose residues. This new proposed process for the production of therapeutic glycoproteins could be useful in animal models.