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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 results in glycans N-linked to proteins ranging from Man₃GlcNAc₂ to Man₅GlcNAc₂ 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-MSⁿ) and ion mobility spectrometry-tandem mass spectrometry (IMS-MS²), on xylosyltransferase insertional mutants.

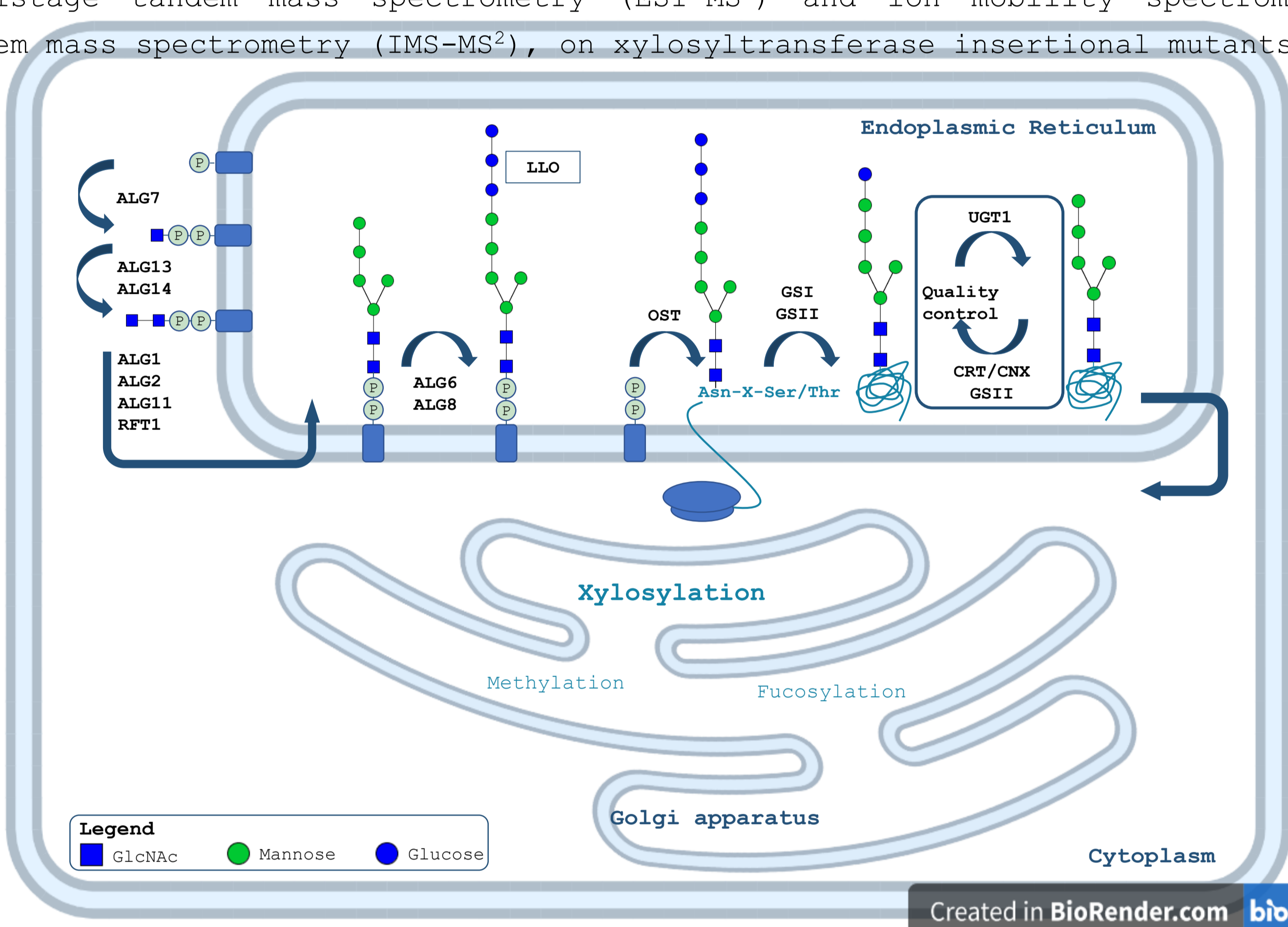


Figure 1 : Scheme of the proposed N-glycosylation pathway in *C. reinhardtii*. *C. reinhardtii* synthesizes a pentamannosylated linear Glc₃Man₅GlcNAc₂ precursor onto a membrane-anchor dolichol pyrophosphate (PP-Dol) (Lucas et al., 2018). This precursor, called lipid linked oligosaccharide (LLO) is transferred 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 Man₅GlcNAc₂ 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 xylosyltransferases. 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 xylosyltransferase. 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} double-mutant 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).

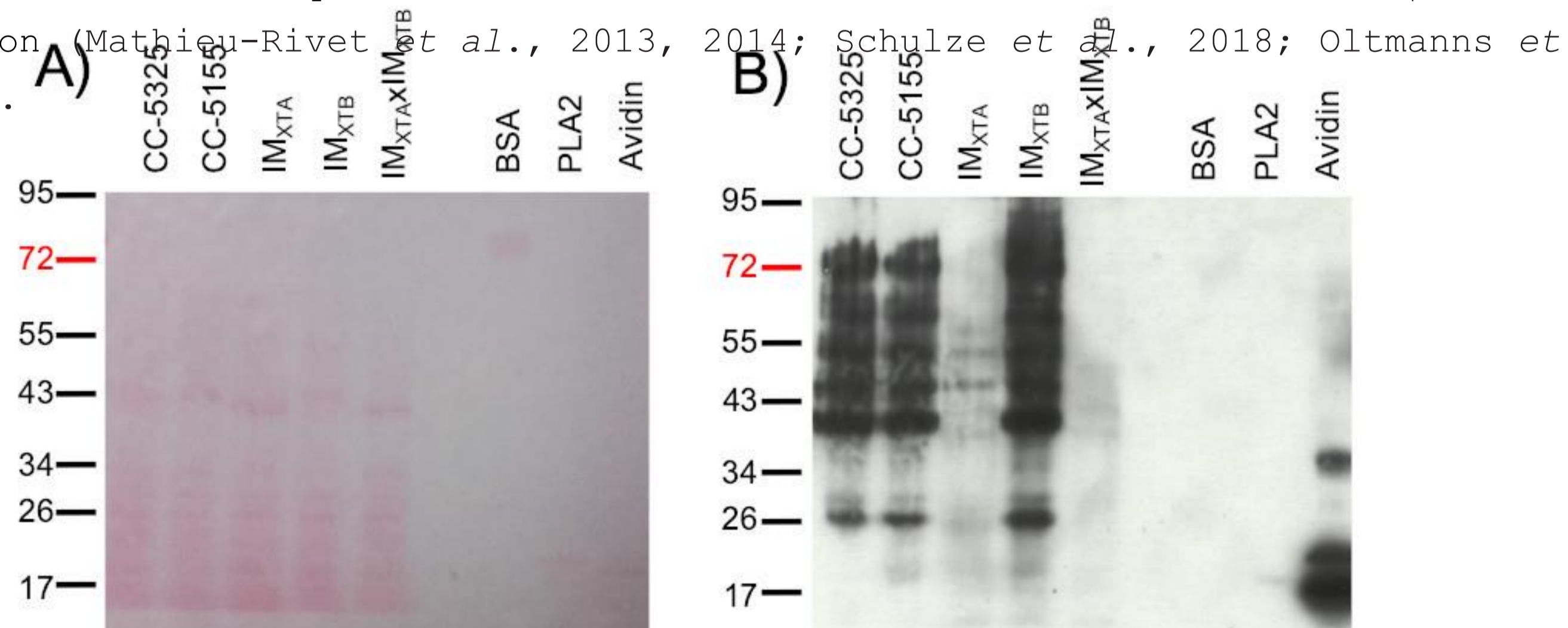


Figure 2 : Immunoblot analysis of the secreted proteins from WT (CC-5325; CC-5155) and *C. reinhardtii* mutants using antibodies specifically directed against the core β(1,2)-xylose epitope (Lucas et al., 2020).

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.

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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 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 monoxylosylated N-glycan detected are attached to α-mannose residues of oligomannoside α(1,3)-branch.

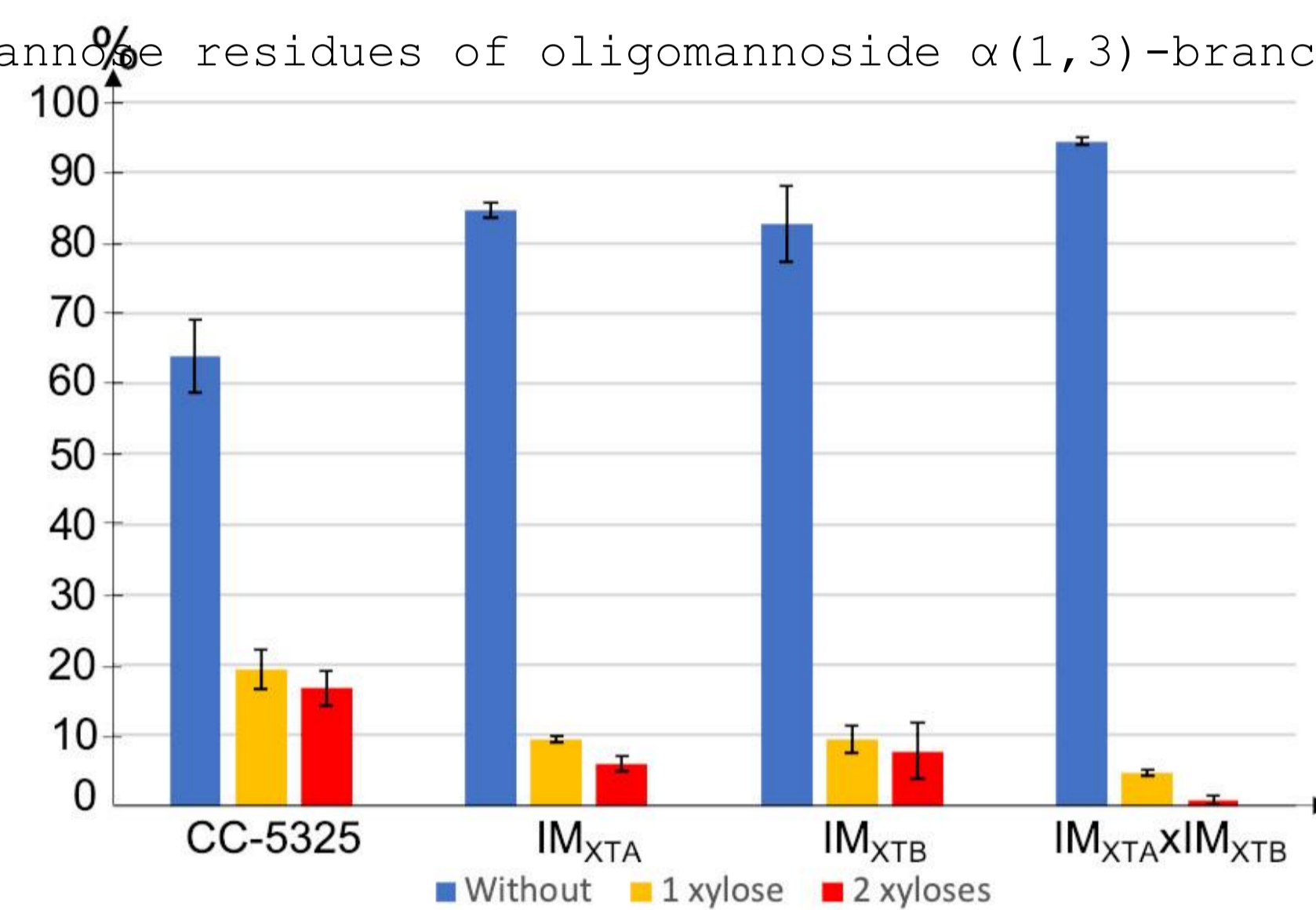


Figure 3 : The relative proportion of xylosylated N-glycans is decreased in the IM_{XTA}, IM_{XTB} and in IM_{XTA}XIM_{XTB} mutants. (Lucas et al., 2020) The relative percentage of each N-glycan type has been determined based on ion intensities of the procainamide-labeled N-glycans analyzed by nano-LC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

2. Residual xylosylation in IM_{XTA}XIM_{XTB} double mutant might be due to other xylosyltransferase candidates

The high degree of heterogeneity of xylosylated glycan structures in *C. reinhardtii* and the remaining xylosyltransferase activity in the IM_{XTA}XIM_{XTB} 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-terminal part with XTA, XTB and plant β(1,2)-xylosyltransferases (Fig. 4).

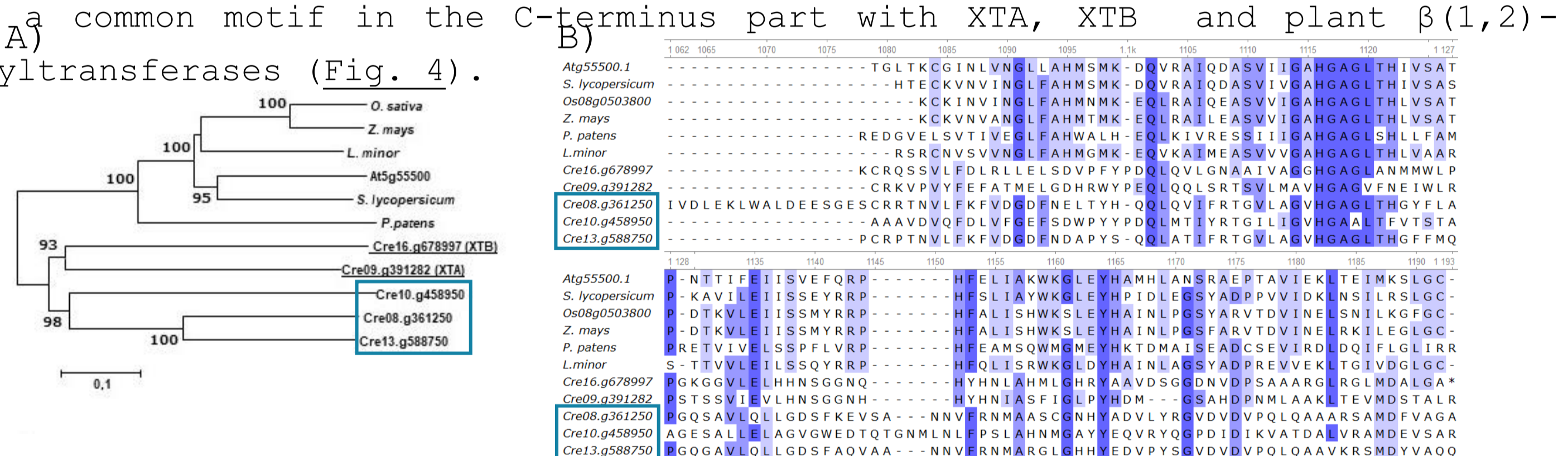


Figure 4 : Phylogenetic relationships between plant and *C. reinhardtii* xylosyltransferases. (Lucas et al., 2020)

The putative protein sequences of xylosyltransferases from *C. reinhardtii* were compared with sequences from *Oryza sativa* (Os08g0503800), *Zea mays* (NP_001105845.1), *Lemma 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 xylosyltransferases exhibit a characteristic GT61 motif in their C-terminal part. Conserved amino acid residues are shaded dark blue (identities) and light blue (similarities). Gaps are represented by dashes inserted for optimal alignment of the sequences.

Conclusion and perspectives

Proposed xylosylation process in *C. reinhardtii*

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

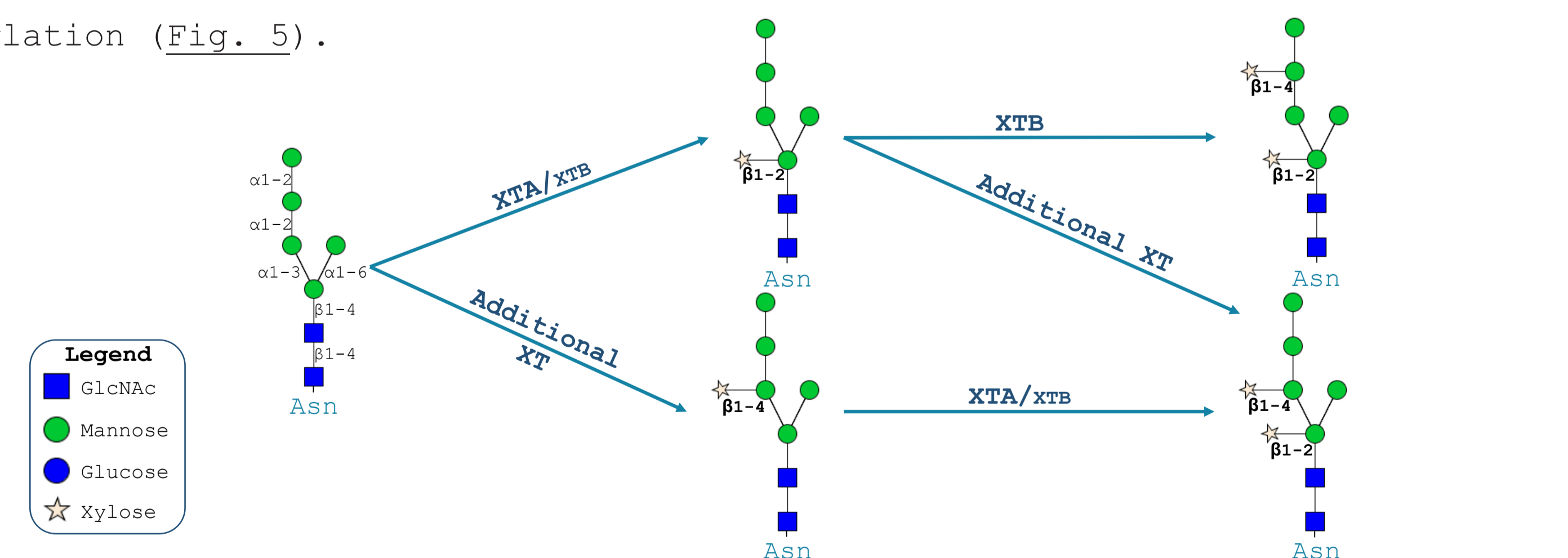


Figure 5 : Proposed xylosylation process in *C. reinhardtii* N-glycosylation pathway (Lucas et al., 2020) XTA is mainly responsible for the addition of core β(1,2) xylose whereas XTB is involved in the transfer of a β(1,4) xylose onto a α(1,2)mannose of the N-glycan linear branch. To a lesser extent,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

perspectives are, at first, to characterize the xylosyltransferase 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 xylosyltransferases would be necessary to exploit *C. reinhardtii* as a biofactory for the production of therapeutic glycoproteins suitable for use in human therapy.