Engineering the *N*-glycosylation pathway in *Chlamydomonas reinhardtii* to produce humanized glycoproteins

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Microalgae are considered as attractive expression systems for the production of biologics. Indeed, they are photosynthetic unicellular organisms that do not require costly and complex media for growing, and are able to secrete glycosylated proteins. Some biologics have already been successfully produced in the green microalga *Chlamydomonas reinhardtii* that is a model for studying cellular biological processes[1]. Structural analysis of glycans *N*-linked to *C. reinhardtii* proteins has revealed mainly oligomannoside structures, the main one being a non-canonical $Man_5GlcNAc_2$. In addition, mature *N*-glycans harbouring β(1,2)-xylose and α(1,3)-fucose residues are synthesized through a *N*-acetyl-glucosaminyltransferase (GnT)-independent pathway[2]. Thus, in the context of the bioproduction of recombinant therapeutics, engineering the *N*-glycosylation pathway in *C. reinhardtii* is necessary to obtain glycoproteins harbouring humanized *N*-glycans. We first performed a knock-out strategy by selecting *C. reinhardtii* mutants in key Golgi glycosyltransferases that are responsible for the transfer of β(1,2)-xylose and α(1,3)-fucose residues[3]. Therefore, we initiated knock-in strategies by expression in specific Golgi compartments of heterologous glyco-enzymes to get the chassis $GlcNAc_2Man_3GlcNAc_2$ since it is the glycan substrate for the expression of human glycosyltransferases involved in the transfer of decorations such as galactose. In this last purpose, the first step implies the accumulation of $Man_3GlcNAc_2$ [4] that constitutes a prerequisite, for further expression of heterologous GnT required for the transfer of terminal *N*-acetylglucosamine residues. This presentation will summarize these recent results, as well as the on-going work carried out to complete the humanization of *N*-glycans in this model microalga. The key future issues such as the targeting mechanisms of glycoenzymes into the secretory system will be discussed.

[1] *van Bockstaele-Fuentes J, et al. (2025) An overview of protein N-glycosylation diversity in microalgae. Front. Plant Sci. 16:1669918.*

[2] *Vanier G, et al., (2017) Heterologous expression of the N-acetylglucosaminyltransferase I dictates a reinvestigation of the N-glycosylation pathway in C. reinhardtii. Sci Rep 7, 10156.*

[3] *Leprovost S, et al. (2024) Fine-tuning the N-glycosylation of recombinant human erythropoietin using C. reinhardtii mutants. Plant Biotechnol. J, 22: 3018-3027.*

[4] *Plasson C, et al. (2026) First step towards the humanisation of protein N-Glycosylation in C. reinhardtii through heterologous expression of α(1,2)-Mannosidases. Plant Biotechnol J 4:2698-2700.*