Chapter

Protein Glycosylation in Bryophytes Differs Subtly from That in Vascular Plants

David Stenitzer and Friedrich Altmann

Abstract

Glycosylation substantially contributes to the physicochemical properties of proteins, and hence also cell walls. Moreover, they are key factors for the recognition of free or cell-bound glycoproteins by internal and external interaction partners. Green plants get by with a highly conserved, limited number of modifications of the pan-eukaryotic basic N-glycan structure. In detail, these are fucosylation of the innermost N-acetylglucosamine residue in 3-position, which renders plant glycoproteins immunogenic to mammals; xylosylation of the branching mannose; frequent occurrence of small N-glycans terminating with mannose or decoration of the antennae with Lewis A determinants. Bryophytes share all these features, but some mosses additionally display two peculiarities not seen in vascular plants. Many mosses exhibit 2,6-di-O-methylated mannose on the 6-arm and some mosses contain modified Lewis A termini with an as yet unspecified methyl pentose. Neither the responsible enzymes nor the function of these novel glycan features is currently known. Targeted glycoengineering of the moss *Physcomitrella patens* (Hedw.) Bruch & Schimp can allow the production of biopharmaceutical glycoproteins that are difficult to express in more established systems.

Keywords: glycoprotein, N-glycan, methyl-mannose, methylation, biopharmaceuticals

1. Introduction

In addition to their outstanding ecological importance, bryophytes have found numerous economic uses. On particular species, even has found applications for the production of recombinant proteins [1–3]. This has instigated the exploration of the potential for post-translational modifications in mosses in general and that of glycosylation in *Physcomitrella patens* (Hedw.) Bruch & Schimp in particular. At first, this moss appeared as a perfectly normal plant, as will be detailed in the next chapter. Only recently was it found that mosses in general have the ability to equip their proteins with features that have not yet been described elsewhere. In the last chapter, the differences between mammalian and moss O-glycosylation will be portrayed.

2. The commonalities between mosses and vascular plants

A number of investigations have shown that *P. patens* behave just like a tracheal plant when it comes to protein N-glycosylation. Biosynthesis and structures of plant N-glycans have been reviewed in many previous papers [4–6] and hence we only give–for the sake of comprehensiveness–a short overview. The biosynthetic routes from the ubiquitous oligomannosidic (*aka* high-mannose) structures lead to the check-point Man5Gn after which it branches out in various ways by combining a, however, limited, set of biosynthetic steps.

a. Removal of α1,2-linked mannoses by α1,2 mannosidase I.

b. Addition of a GlcNAc residue by GlcNAc-transferase I.

c. Removal of the α1,3- and (outer) α1,6-linked mannoses via the Golgi-resident α-mannosidase II.

d. Optional: addition of a GlcNAc residue in β1,2-linkage to the (inner) α1,6-linked mannose.

e. Addition of fucose in an α1,3-linkage to Asn-bound N-acetylglucosamine (GlcNAc) residue.

f. Addition of xylose in β1,2-linkage to the β-mannosyl-residue, whereby steps e) and f) occur independently.

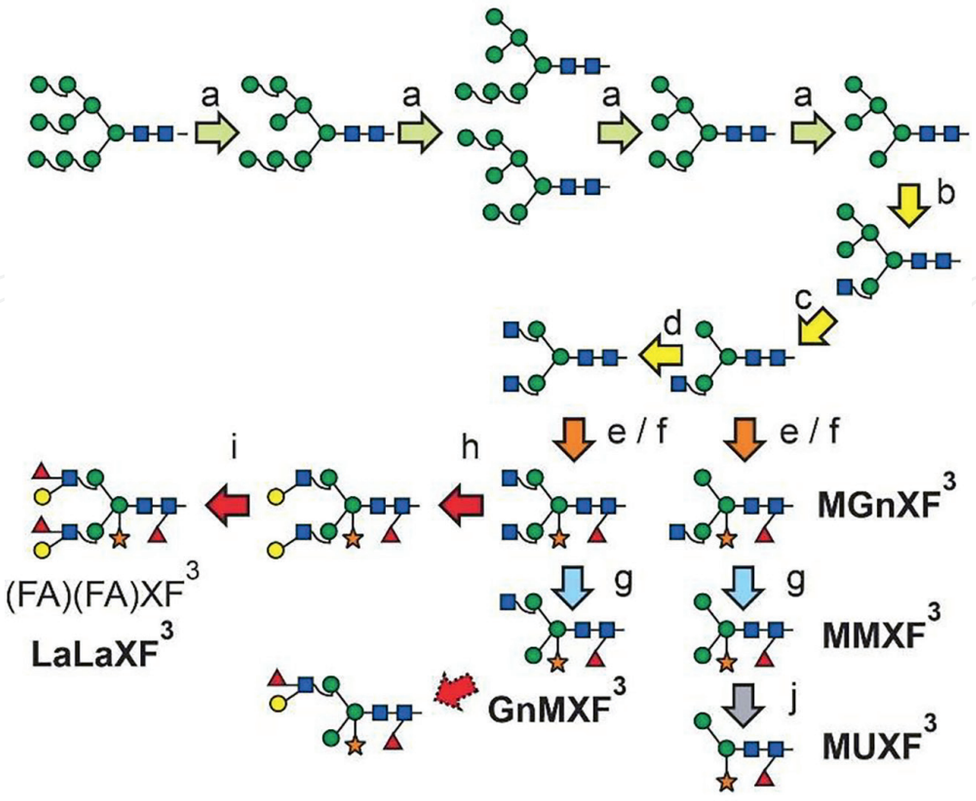

Figure 1.
Biosynthesis of typical plant-type N-glycans. The letters next to the arrows refer to the text above.

g. Removal of the GlcNAc linked to the α1,3-arm by hexosaminidase, for example, HEXO1 [7].

h. Addition of β1,3-linked galactose to any of the two GlcNAc residues, whereby this event is quickly followed by fucosylation.

i. Addition of β1,4-linked fucose to the sequence Galβ-3GlcNAc, thus forming Lewis A determinants also known as human blood group determinants.

j. Removal of the mannose linked α1,3 to the β-mannose. This occurs as a storage phenomenon in macerated plant material and generates the MUXF3 structure well known in allergy diagnosis [8].

A few main roads are depicted in **Figure 1**, which also gives names to the structures. To understand this naming system, we humbly ask the reader to remember that the "proglycan" nomenclature starts in the upper left corner and then lists the terminal residues in the counter-clockwise direction, whereby M, A, Gn, X, and F stand for mannose, galactose, *N*-acetylglucosamine, xylose, and fucose, respectively [9]. The branched Lewis A determinant of large plant N-glycans can either be written as "(FA)" or—more reader-friendly—as "La."

3. Methylation: a primordial resemblance

High-resolution mass spectrometry revealed the occurrence of small satellite peaks for $MMXF^3$ and $MGnXF^3$/$GnMXF^3$ in mass spectra of *P. patens* N-glycans, primarily when N-glycans were extracted from whole tissue (**Figure 2**) [10]. The mass increment of about 14 Da could also arise from the oxidation of a sugar moiety to glucuronic acid. The exact mass increment, however, and subsequent chemical analysis (linkage analysis via gas chromatography-mass spectrometry) clearly identified it as O-methylation of a mannose residue—more exactly, as 2,6-di-O-methylation. Collision-induced fragmentation in negative mode furthermore established the α1,6-linked mannose as being methylated (**Figure 2**).

Methylation in various ways was encountered in a previous study of *Chlorella*-clade microalgae [11–14]. Although methyl-mannoses were also found in these algae, none of the well over a dozen glycol-types contained complex-type/paucimannosidic structures and none of them contained 2,6-O-methylated mannose. As a recent unpublished survey of several *Scenedesmaceae* strains revealed likewise varied glycosylation (Mocsai R and Altmann F, unpublished results). We, thus, cannot exclude that other algae strains share with mosses this type of methylation and may thus be considered ancestors of mosses—at least glycosylation-wise.

4. Greater than following generations

The recent survey of N-glycosylation in a number of mosses surfaced several species that harbored N-glycans even larger than the fully developed Lewis A containing pride of the plant kingdom. The masses of the novel peaks indicated elongation of Lewis A determinants by 160 Da structures (**Figure 3**). Tentatively, we assume that a pentose plus methylation accounts for this mass increase. More precise information is not available so far.

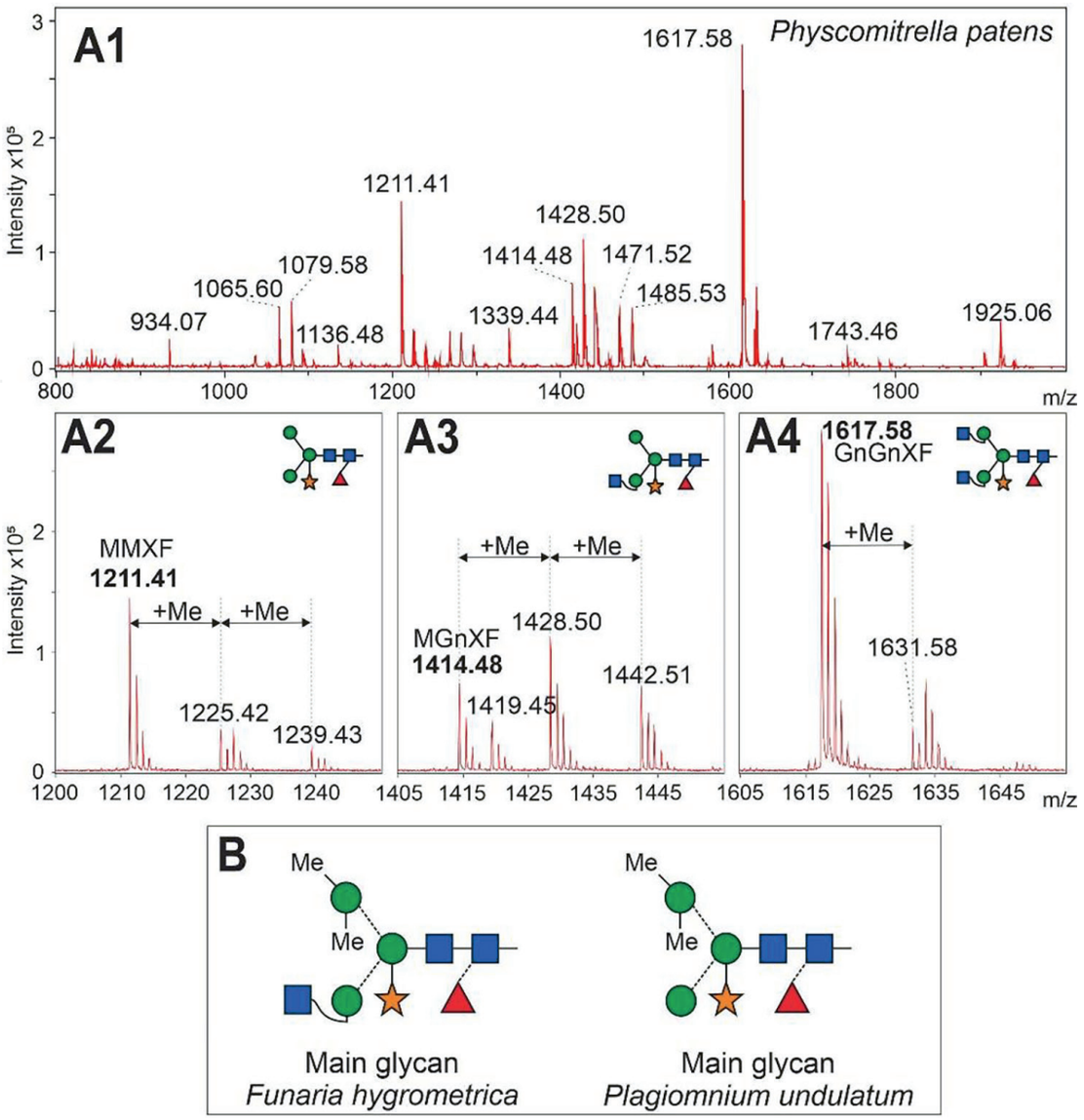

Figure 2.
Detection and structures of methylated N-glycans in mosses. Panels A shows the matrix assisted-laser desorption/time-of-flight mass spectrometry (MALDI-TOF MS) spectrum of the N-glycans of P. patens in full width and in detail for the major affected N-glycan species. Panel B depicts the structures of the two examples that were structurally analyzed in detail derived from Funaria hygrometrica Hedw. and Plagiomnium undulatum (Hedw.) Kop.

5. Humanization of moss glycosylation and an odd interspecies confusion

All plants, including mosses, such as *P. patens,* are surprisingly apathetic towards switching off core fucosylation and xylosylation. Briefly, after the first cloning of the relevant enzymes fucosyl transferase and xylosyl transferase [15, 16], knock-down *Arabidopsis* and *Nicotiana* plants were generated [17, 18] and knock-out lines of *P. patens* were established for the production of biopharmaceuticals [19, 20]. The mainly resulting paucimannosidic structures, that is, N-glycans with two to five (a few = *pauci* in contrast to many = *oligo*) mannose residues and possibly one GlcNAc, xylose, and/or fucose residue were considered suboptimal for most purposes, and hence, efforts were undertaken to increase the levels of GnGn and to

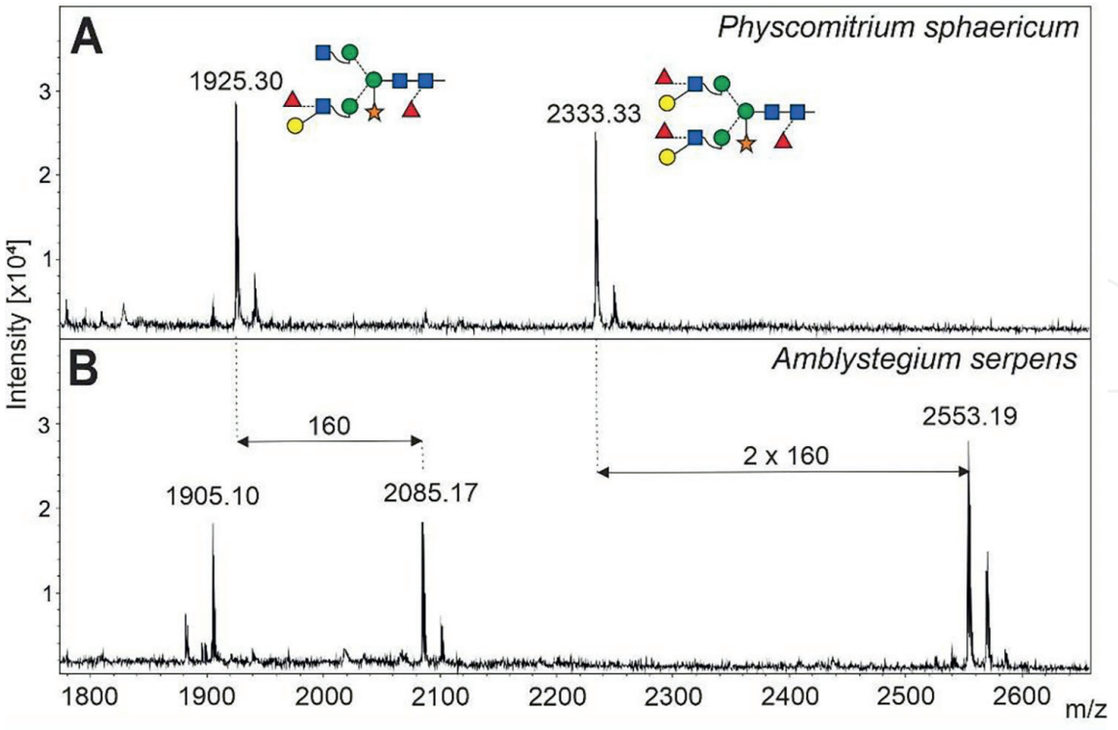

Figure 3.
Sections of mass spectra of two mosses representative of mosses with plant-typical Lewis A antennae (panel A) and those with larger glycans (panel B). Mass increments indicate the addition of a 160.07 Da element, which could be explained as the addition of a pentose and a methyl group. Panel A shows the high mass section of N-glycans from Physcomitrium sphaericum (Ludw) Fuernr and panel B that of Amblystegium serpens (Hedw.) Schimp.

eventually introduce the mammalian type core α1,6-fucose [21]. A big step toward humanized N-glycans was the introduction of β1,4-galactosylation [22]. The most stunning success, however, was the introduction of the entire mammalian sialylation pathway into plants—no less than six mammalian foreign genes that have to be actively expressed and correctly localized without harshly compromising the physiology of the host plant. This was at first realized with *Nicotiana* [23, 24] and then later with *P. patens* [3, 25]. As planned, these cloning steps led to the biosynthesis of substantial percentages of sialylated N-glycans. This established *P. patens* as in fact being very "patient", that is, tolerant and enduring towards this enormous intervention into the cellular machinery. Notably, certain otherwise difficult-to-express glycoproteins can be produced in suitable quality and quantity by glycoengineered *P. patens* [26].

However, next to some incomplete intermediate products, peaks with hitherto unknown mass levels occurred in mass spectra of moss lines expressing human β1,4-galactosyl transferase [27]. The mass increments of 132 Da indicated the addition of—against all rules—pentose. Being sensitive to α-arabinofuranosidase, this pentose was identified as furanosidic L-arabinose in α-linkage [27]. Its exact location was not known at the time of writing this chapter.

6. A mosses idea on O-glycosylation

The most often encountered type of protein O-glycosylation in mammals is the so-called mucin-type O-glycosylation, where Ser or Thr residues are at first decorated

with *N*-acetylgalactosamine (GalNAc). Usually, this priming event is followed by the addition of other sugars, such as galactose, sialic acid, or GlcNAc [28]. In all plants, including mosses, this type of glycosylation is totally unknown. Instead, arabinans (chains of arabinoses) and arabinogalactans (AGPs, complex structures starting with a galactan that is substituted by arabinose chains and maybe other subtleties are found [29]. Arabinogalactans mostly occur as type II arabinogalactans [30, 31], but different architectures may also occur [32, 33]. Bryophytes do generate arabinogalactans, but with certain differences as compared to seed plant AGPs [34–36].

Notably, these "exotic" oligosaccharides are not linked to the codogenic amino acids Ser or Thr but to 4-hydroxyproline (Hyp) [29, 37]. Neighboring amino acids, in particular proline and hydroxyproline themselves, dictate if a given Hyp residue rather falls prey to galactosyl- or arabinosyl-transferase [29].

So, the initial step of O-glycosylation in plants is the oxidation of proline to hydroxyproline. The remarkable fact now is that apparently, the sites of mucin-type O-glycosylation of mammalian proteins are also the sites prone to be modified by prolyl-4-hydroxylase and then by arabinosyl-transferase as exemplified by human erythropoietin expressed in the moss *P. patens* [38] or human IgA1 [39]. Hardly surprising, the same holds true for *N. benthamiana* with the only, albeit technologically relevant difference, that several prolyl-4-hydroxylase are redundantly at work in vascular plants [39, 40], whereas knock-out of just one paralogous gene sufficed to suppress erythropoietin oxidation in *P. patens* [41].

7. Conclusion

The few bryophyte species whose protein glycosylation has been analyzed to date already presented some surprises. While first results indicated mosses to perform as their vascular relatives with regard to N-glycan biosynthesis, recent insights revealed them to present some peculiarities. Particularly interesting are mannose methylation and hyper-elongation of Lewis antennae.

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Conflict of interest

The authors declare no conflict of interest.

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Author details

David Stenitzer and Friedrich Altmann*
Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria

*Address all correspondence to: friedrich.altmann@boku.ac.at

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