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Structural analysis of linear hydroxyproline-bound O-glycans of *Chlamydomonas reinhardtii*—conservation of the inner core in *Chlamydomonas* and land plants

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Abstract—Linear hydroxyproline-bound O-glycans of the unicellular green alga *Chlamydomonas reinhardtii* were isolated from outer cell wall glycoproteins and their structure elucidated by chemical and spectroscopic methods. They consist exclusively of arabinose and galactose, the latter in the furanose form, unusual for plants. The first two arabinoses linked to hydroxyproline have the same anomeric configuration and linkage to each other as do the arabinosides isolated from land plants, suggesting that these two steps of hydroxyproline-O-glycosylation have been conserved during evolution. In a concomitant analysis of Hyp-bound *O*-tri- and tetraarabinans isolated from *Arabidopsis thaliana,* we were able to unambiguously identify the natural *trans*-4-L-Hyp-bound O-glycans and the artificially produced D-allo forms and to identify diagnostic signals in NMR spectra of these compounds for the first time.

Keywords: Hydroxyproline-rich glycoproteins; Hydroxyproline-bound O-glycans; *Chlamydomonas reinhardtii*; Galactofuranose

1. Introduction

The plant extracellular matrix (ECM) is a dynamic and complex network of interacting polysaccharides and proteins, the latter comprising up to 10% of the total plant ECM. The most abundant structural plant ECM proteins belong to the hydroxyproline-rich glycoprotein (HRGP) superfamily, which is characterized by a high hydroxyproline (Hyp) and sugar content. Hyp has been observed in repetitive amino acid motifs, with different motifs presumably driving distinctive glycosylation on serines and Hyps. In this respect, HRGPs have been grouped into four classes: the extensin-like proteins, the arabinogalactan proteins, proline-rich proteins and the solanaceous lectins.[1–3] Recent research has concentrated on the elucidation of amino acid sequences deduced from genomic or cDNA analysis of HRGPs of various plant origin. These analyses indicated that HRGPs of green algae, predominantly studied in *Chlamydomonas* and *Volvox*, and land plants share conserved Hyp-rich protein motifs.[3–7] The Hyp-rich sequences allow the proteins to adopt the polyproline II (PP-II) conformation.[8–10] PP-II helices are only stable when glycosylated.[10,11] In this respect, the glycosylation of Hyps in the HRGPs has played a pivotal structural role during the evolution of these proteins. The question driving the research reported here is whether the idea of stabilizing a protein‘s secondary structure by glycosylation has evolved independently two times, given that *Chlamydomonas* and land plants separated early in

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Abbreviations: Hyp, hydroxyproline; HRGP, hydroxyproline-rich glycoprotein; NOE, nuclear Overhauser effect; PMAA, partially deuteromethylated alditol acetate

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evolution,[12] or whether the hydroxylation of proline to Hyp and Hyp O-glycosylation had been established by some ancestral green organism prior to their divergence. As the enzymes involved in Hyp-O-glycan biosynthesis are unknown in both, *Volvocales* and land plants, we addressed this question by comparing the glycosylation patterns of the HRGPs belonging to the extensin family in the two lineages.

The complete structural characterization of short, linear Hyp-bound O-glycans revealed that three and four Aras are attached to Hyp with the structures depicted in Figure 1,[13–15] when isolated from various land plants, mosses, ferns and green algae,[16,17] the exception being HRGPs of *Chlamydomonas* species. Cell wall preparations isolated from *Chlamydomonas reinhardtii* and *Chlamydomonas gymnogama* were shown to contain a complex mixture of Ara, Glu and Gal bound to Hyp.[18,19] In addition, we have shown,[9,20] that *Chlamydomonas* synthesizes complex linear and branched Hyp-bound O-glycans with a mixture of hexoses and pentoses. But our analysis at that time was exclusively performed by ESI-MS, a method that gives no information about the composition, linkage and anomeric configuration of the respective sugars. In this study, we focused on the structural elucidation of highly abundant linear Hyp-bound *O*-tri- and tetraglycans isolated from cell wall preparations of *C. reinhardtii*. We also included the analysis of Hyp-bound O-glycans isolated from *Arabidopsis thaliana* suspension cultures as controls.

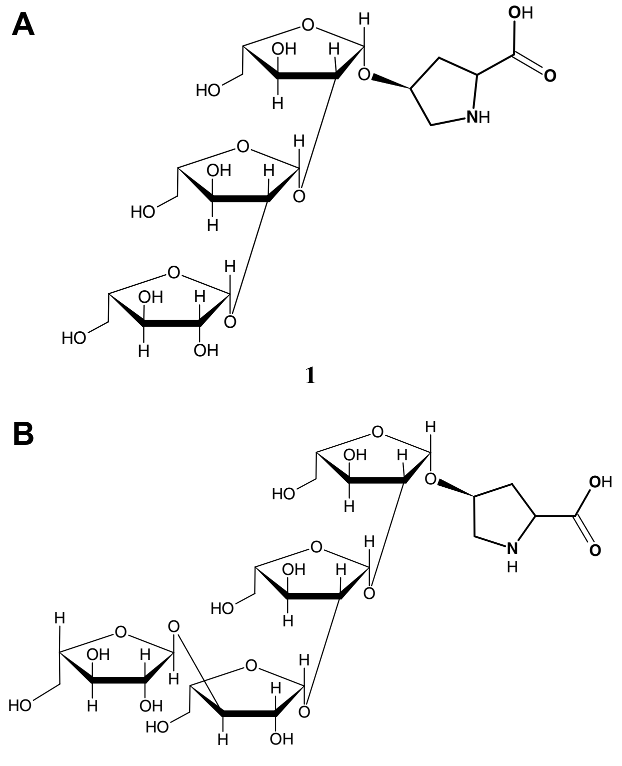


Figure 1. Structures of the Hyp-bound O-glycans isolated from *Nicotiana tabacum* cell wall preparations and *Solanum tuberosum* and *Datura stramonium* lectins.

2. Results

Crude extracts of Hyp-bound O-glyans of *A. thaliana* cell suspension cultures and *C. reinhardtii* outer walls were purified as described in Section 4. In detail, the Hyp-bound O-glycan preparation of *A. thaliana* eluted as two major, baseline separated peaks from the Aminex column, which were collected based on their Hyp content.[21] HPLC-chromatography of the two desalted Aminex fractions resulted in two Hyp-bound O-glycan fractions each (further designated as Hyp-bound O-glycans **1a**,**b** and **2a**,**b**), which by ESI-MS analysis were shown to be isobaric with three pentoses (**1a**,**b**) and four pentoses (**2a**,**b**) linked to Hyp, respectively (Table 1).

Crude Hyp-bound glycans of *C. reinhardtii* cell wall preparations eluted in a much more complex fashion from the Aminex column. Based on the abundance of Hyp, we collected 4 fractions (I–IV, data not shown). The desalted fractions were analyzed by ESI-MS and shown to contain Hyp-bound O-glycans of different mass-ranges eluting in a size-dependent manner. Fractions I and II contained a complex mixture of higher-mass Hyp-bound O-glycans, which are not further considered in this study. Here, we concentrated on the analysis of the desalted fractions III and IV (Fig. 2), which were further separated by HPLC. By this approach, we could isolate four Hyp-bound O-glycans (named **3** and **4a** and **b**, respectively) out of the desalted Aminex fraction IV. The Hyp- bound O-glycans **3a** and **b** were shown by ESI-MS/MS experiments to consist of two pentoses and one hexose bound to Hyp, while the Hyp-bound O-glycans **4a** and **b** were found to be composed of two pentoses and one methylated hexose bound to Hyp. Aminex fraction III were separated by HPLC into the four Hyp-bound O-glycans **5** and **6a** and **b**, respectively. Using the same approach Hyp-bound O-glycans **5a** and **b** turned out to consist of three pentoses and one hexose linked to Hyp, while Hyp-bound O-glycans **6a** and **b** consisted of two pentoses, one hexose and one methylated pentose linked to Hyp (Table 1). To obtain further information about the sugar composition of each Hyp-bound O-glycan and the linkage of individual bound sugars, we analyzed each individual compound as persilylated alditols by GC and as PMAA derivatives by GC–MS. For methylation analysis we decided to apply deuteromethylation to be able to identify the naturally occurring methyl groups. By these methods, the pentoses in the *A. thaliana* Hyp-bound O-glycans could be identified exclusively as Ara, which by methylation analysis occurred in

Table 1. ESI-MS analysis of hydroxyproline-bound O-glycans

Number	Mass (M^+Na)	Characteristic fragments	Deduced structure[a]
A. Isolated from A. thaliana cell cultures			
1 (**a** and **b**)	550	m/z 286 (Y_1) m/z 418 (Y_2)	Pent→Pent→Pent→Hyp
2 (**a** and **b**)	682	m/z 286(Y_1) m/z 418(Y_2) m/z 550 (Y_3)	Pent→Pent→Pent→Pent→Hyp
B. Isolated from C. reinhardtii cell walls			
3 (**a** and **b**)	580	m/z 154 (Y_0) m/z 286 (Y_1) m/z 418 (Y_2)	Hex→Pent→Pent→Hyp
4 (**a** and **b**)	594	m/z 154 (Y_0) m/z 286 (Y_1) m/z 458 (Y_2)	MeHex→Pent→Pent→Hyp
5 (**a** and **b**)	712	m/z 154 (Y_0) m/z 286 (Y_1) m/z 418 (Y_2) m/z 550 (Y_3)	Pent→Hex→Pent→Pent→Hyp
6 (**a** and **b**)	726	m/z 154 (Y_0) m/z 286 (Y_1) m/z 418 (Y_2) m/z 580 (Y_3)	MePent→Hex→Pent→Pent→Hyp

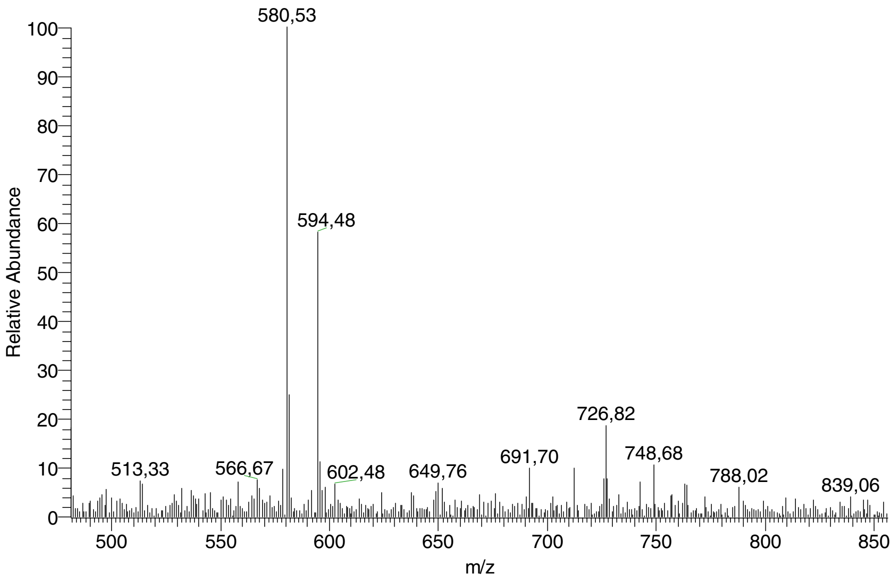
[a] *Abbreviations*: Hex, Hexose; MeHex, methylated hexose; Pent, pentose; MePent, methylated pentose.

compounds **1a** and **b** as terminal Ara*f* and 2-linked Ara*f* in a ratio 1:2, in compounds **2a** and **b** as terminal Ara*f*, 2-linked Ara*f* and 3-linked Ara*f* in a ratio 1:2:1. These data are consistent with the linkage analysis performed on other land plant Hyp-bound O-glycans.[13–15] All *C. reinhardtii* Hyp-bound O-glycans isolated contained Ara and Gal as constituents. The naturally occurring methylated hexoses (compound **4a** and **b**) and pentoses (compound **6a** and **b**) co-eluted as persilylated derivatives with the non-methylated persilylated derivatives during GC analysis, but gave defined fragments during methylation analysis. For compounds **3** and **4** (**a** and **b**, respectively) we observed a 2:1 ratio of Ara and Gal, while the Ara/Gal ratio was found to be 3:1 in compounds **5** and **6** (**a** and **b**), consistent with the structures suggested in Table 1. Deuteromethylation analysis of compounds **3** (**a** and **b**) revealed that Ara occurred as 2-linked Ara*f* (Fig. 3). The terminal Gal derivative in Hyp-glycans **3** (**a** and **b**) eluted earlier than the standard of t-Gal*p*. Analysis of the respective EI-MS spectra revealed the characteristic fragmentation pattern of a t-Gal*f* (Fig. 3). During the analysis performed for compounds **4** (**a** and **b**) we found 2-linked Ara*f*, while the terminal methylated Gal could be identified as 6-methyl-*Galf* by diagnostic fragments at m/z 211, 92 and 45. For Hyp-bound O-glycans **5** and **6** (**a** and **b**) we found 2-linked Ara*f*, one terminal Ara and a Gal, which by its EI-MS spectra could be addressed as 1,4-Gal*f* or 1,5-Gal*p* in the ratio 2:1:1. The terminal Ara in Hyp-bound O-glycans **6** (**a** and **b**) was methylated on C-3, as revealed by diagnostic fragments of m/z 164 and 48.

To elucidate the anomeric configuration of the Hyp-bound O-glycans isolated we performed one- and two-dimensional NMR experiments. Table 2 summarizes the ^{1}H and ^{13}C chemical shifts observed for the anomeric atoms of the *A. thaliana* compounds **1a** and **b**, and **2a** and **b**. The ^{13}C and ^{1}H resonances were correlated by C–H-COSY experiments (Fig. 4A and B). For **1a** and **b** we observed that all anomeric carbon atoms show ^{13}C resonances close to 100 ppm. The signals of the corresponding protons all show doublets around 5 ppm with coupling constants ($J_{1,2}$) between 4.0 and 4.8 Hz, proving that all Aras in **1a** and **b** are in β-conformation. As evidenced by the signals of the fourth Ara in **2a** and **b**, the fourth Ara is α-linked. The two derivatives **1a** and **b** (Fig. 4A) and **2a** and **b** (Fig. 4B), respectively, differed in a downfield shift of the anomeric proton of the Ara bound to Hyp, which suggests that we have isolated and separated the naturally occurring *trans*-4-L-Hyp-bound O-glycans and the artificial allo-D-derivatives, which are formed by isomerization due to the alkaline isolation procedure. In conclusion, our analysis is consistent with the general idea, that land plants attach β-L-Ara*f*-(1→2)-β-L-Ara*f*-(1→2)-β-L-Ara*f* and α-L-Ara*f*-(1→3)-β-L-Ara*f*-(1→2)-β-L-Ara*f*-(1→2)-β-L-Ara*f* to Hyp in the extensin superfamily of HRGPs.

By the same approach we analyzed Hyp-bound O-glycans **3a** and **b** and Hyp-bound O-glycans **6a** and **b** of *C. reinhardtii*. The amount of the Hyp-bound O-glycans

A ESI-MS of fraction IV



B ESI-MS of fraction III

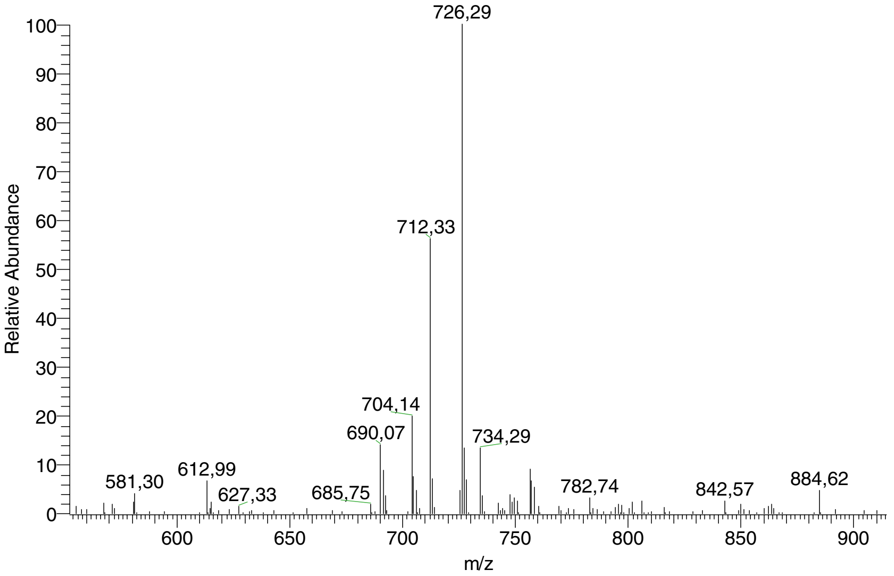


Figure 2. ESI-MS spectra of desalted fractions IV (panel A) and III (panel B) from *C. reinhardtii*. In fraction IV (panel A), the most dominant pseudo molecular ions of *m*/*z* 580 and 594 correspond to Hyp-bound O-glycans **3** and **4**, a minor trace of the pseudo molecular ion of *m*/*z* 726 corresponds to Hyp-bound O-glycans **6** (compare Table 1). In panel B (fraction III) the most pseudo molecular ions of *m*/*z* 712 and 726 correspond to Hyp-bound O-glycans **5** and **6**, pseudo molecular ions of *m*/*z* 690 and 704 correspond to the respective $[M+H]^+$ adducts.

4 and **5** was not sufficient to obtain ^{13}C and two-dimensional spectra. Our data are summarized in Table 3 and Figure. 5A and B. The signals in the anomeric C and H region indicate that both Aras in **3a** and **b** are β-linked (Fig. 5A), while the chemical shift of C-1 and H-1 suggests α configuration of the Gal*f*. For **6a** and **b** we found that the two Aras are β-linked based on the chemical shifts presented in Fig. 5B and Table 3. The Gal signals obtained had exactly the same range as observed for the Gal signals of **3a** and **b**. While we were not able to clearly define by GC–MS whether it occurs as furanose or pyranose, the identity of signals in the NMR analysis strongly suggest that it is an α-1-5-linked Gal*f*,with the anomeric atoms in the same chemical environment in both Hyp-bound O-glycans. As a fourth doublet was missing in the spectra recorded at room temperature for compounds **6a** and **b**, obviously hidden under the water signal, a second ^1H NMR was recorded at 50 °C. All doublets observed before were shifted downfield to 5.47, 5.33 and 5.44, respectively, and a fourth

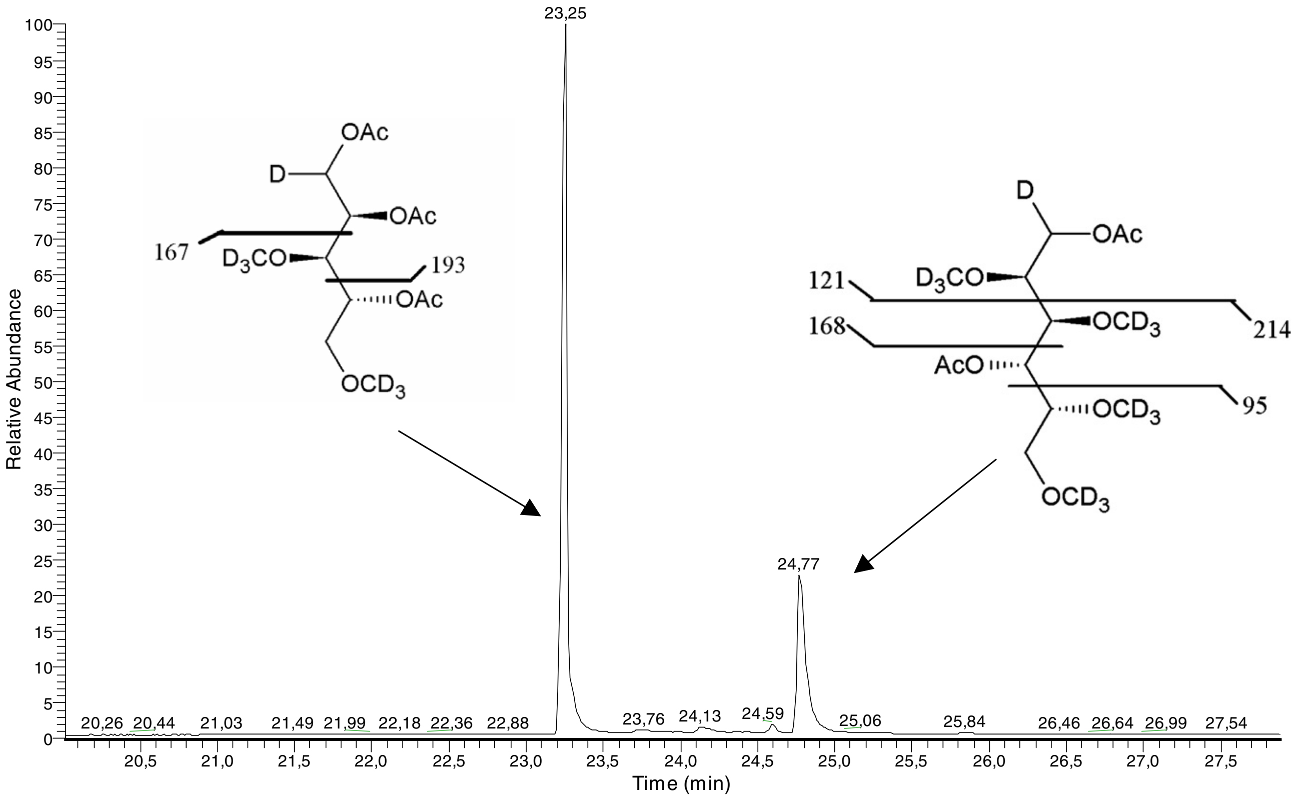


Figure 3. GC–MS analysis of Hyp-bound O-glycans **3**. The gas chromatogram and the diagnostic EI fragments of the respective PMAA derivatives are indicated.

Table 2. Summary of ^{13}C and ^{1}H chemical shifts for the anomeric atoms in the Hyp-bound O-glycans isolated from *A. thaliana*

No.	Residue	C-1 (ppm)	H-1 (ppm)	$J_{1,2}$ (Hz)
1a	A	99.63	5.19	4.0
	B	99.86	5.22	4.4
	C	100.96	5.06	4.4
1b	A	99.97	5.13	4.8
	B	101.23	5.14	4.8
	C	101.23	5.03	4.7
2a	A	100.22	5.20	4.0
	B	100.28	5.22	4.8
	C	101.29	5.05	3.7
	D	109.70	5.14	0.7
2b	A	99.97	5.19	4.0
	B	101.23	5.19	4.0
	C	101.23	5.07	4.8
	D	109.38	5.16	0.7

Note: sequence of sugar residues is C→B→A→Hyp and D→C→B→A→Hyp.

doublet was seen at 5.08 ppm and a coupling constant ($J_{1,2}$) of 3.5 Hz for **6a**, whereas the corresponding signal **6b** was seen at 5.06 ppm and a coupling constant ($J_{1,2}$) of 3.7 Hz indicating that the terminal Ara of these compounds is β-linked. When comparing the two compounds **3a** and **b** (Fig. 5A) and **6a** and **b** (Fig. 5B), respectively, they all differed in the same downfield shift of the anomeric proton of the Ara linked to Hyp as described for the *A. thaliana* Hyp-bound O-glycans **1a** and **b** and **2a** and **b** above (compare Figs. 4 and 5). Taken together our data identify Hyp-bound O-glycans **3a** and **b** as α-D-Gal*f*-(1→2)-β-L-Ara*f*-(1→2)-β-L-Ara*f*-(1→4)Hyp and Hyp-bound O-glycans **6a** and **b** as 3-methyl-β-L-Ara*f*-(1→5)-α-D-Gal-*f*-(1→2)-β-L-Ara*f*-(1→2)-β-L-Ara*f*-(1→4)Hyp.

Our final interest was to unambiguously identify the naturally occurring *trans*-4-L-Hyp-bound O-glycans and the artificial allo-D-derivatives, which are formed by isomerization due to the alkaline isolation procedure. Our first approach was to assign the ^{13}C and ^{1}H resonances corresponding to the hydroxyproline residues of **1a** and **b**, **2a** and **b**, **3a** and **b** as well as **6a** and **b** based on earlier work[15] and by our HMQC experiments described above. Our results are summarized in Figure 6 and Table 4. The most striking difference between the two data sets can be found in the chemical shifts of the protons on C-3 (H-3) of Hyp.

To identify the natural occurring L-trans form we recorded several selective 1D NOESY experiments for Hyp-bound O-glycan **1a** and **b** by excitation of H-4, H-3a and H-3b. For **1a** the transient NOE between H-3b and H-4 is larger than the NOE between H-3a and H-4 and the NOE between H-3a and H-2 is larger than the NOE between H-3b and H-2. For **1b** the opposite

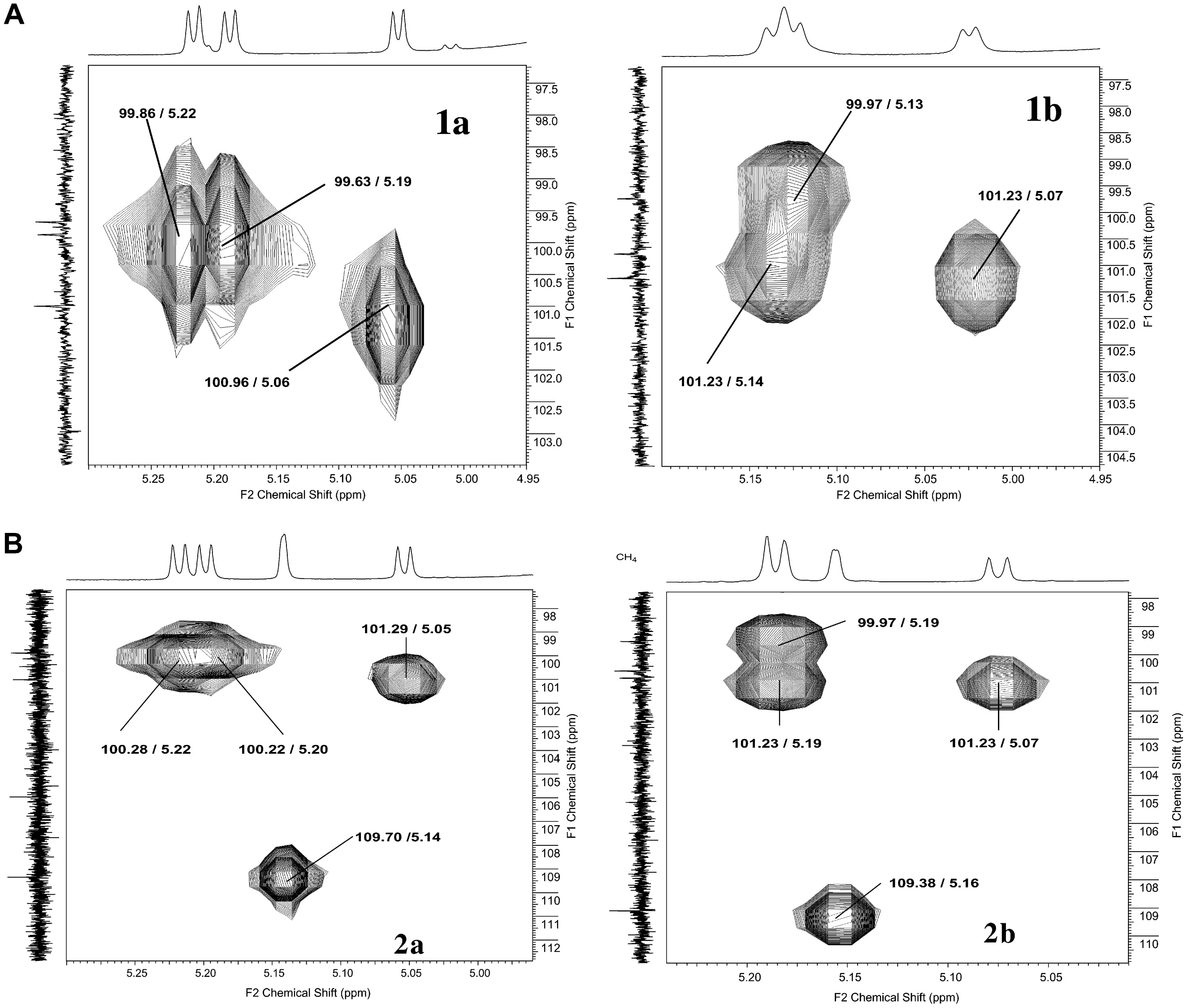


Figure 4. Two-dimensional C–H-COSY spectra of isolated Hyp-bound O-glycans from *A. thaliana*. A: Spectra of **1a** and **b**, respectively. B: Spectra of **2a** and **b**, respectively.

pattern was found, the NOE between H-3b and H-2 is larger than the NOE between H-3a and H-2 (data not shown). This reflects the shorter distances for cis-orientated vicinal protons than trans-orientated protons in hydroxyproline. In Hyp-bound O-glycan **1a** the vicinal protons H-3a and H-2, H-3b and H-4 are cis-orientated; therefore a H-2-H-4 trans orientation can be unambiguously identified. Hyp-bound O-glycan **1b** displays a H-2–H-3a cis- and H-3b–H-4 cis orientation resulting in a H-2–H-4 cis-orientation, thus the allo-D-form. These results will undoubtedly help to identify the two enantiomers of Hyp and its sugar derivatives based on simple ^{1}H NMR and analysis of the protons on C-3 of Hyp. Our observations are consistent with assignments and interpretations of Tan et al.,[22] who found in their analysis of complex Hyp-bound O-glycans two sets of signals for the protons on C-3 (H-3) of Hyp.

3. Discussion

The Hyp-*O*-Ara linkage was discovered roughly 40 years ago[23] and since then has been shown to be a characteristic feature of HRGPs of all green plants and green algae.[24] While all other green species analyzed employ the same Hyp-bound O-arabinans concerning linkage and anomeric configuration as described above for the *A. thaliana* HRGPs, the heterogeneity of Hyp-bound O-glycans in *Chlamydomonas* was exceptional, as well as the presence of Gal*f* in the sugar conjugates, a fact that was first described by O'Neill and Roberts in 1981.[25] In addition, methylated sugars have never been observed in extensins before. Although we were not able to purify the methlyted Hyp-bound O-glycans **4** and **5** in amounts amenable for NMR, we conclude based on methylation and sugar analysis that Hyp-bound

Table 3. Summary of ^{13}C and ^{1}H chemical shifts for the anomeric atoms in Hyp-bound O-glycans isolated from *C. reinhardtii*

No.	Residue	C-1 (ppm)	H-1 (ppm)	$J_{1,2}$ (Hz)
3a	A	99.96	5.17	4.4
	B	100.59	5.23	4.4
	C	101.84	5.05	4.4
3b	A	99.96	5.17	4.06
	B	101.21	5.20	4.49
	C	101.84	5.09	4.49
6a	A	99.27	5.18	4.27
	B	99.90	5.22	4.49
	C	101.16	5.06	4.49
	D	n.d.	[a]	[a]
6b	A	99.97	5.17	4.27
	B	101.23	5.20	4.49
	C	101.85	5.09	4.49
	D	n.d.	[a]	[a]

n.d. not determined.

Note: sequence of sugar residues is C→B→A→Hyp and D→C→B→A→Hyp.

[a] Data are given in text, recordings were done at 50 °C.

O-glycans **4** are the methylated derivatives of Hyp-bound O-glycan **3**, while Hyp-bound O-glycans **5** are the non-methylated derivatives of Hyp-bound O-glycan **6**. As detailed below, these data allow important inferences about the biosynthesis of short linear Hyp-bound O-glycans in *Chlamydomonas* and about evolutionary events generating modern HRGPs of green land plants and volvocine algae.

The biosynthesis of glycoproteins is accomplished in the secretory pathway of the ER and Golgi–endomembrane system. It is generally assumed that O-glycosylation occurs by successive addition of nucleotide-activated sugars catalyzed by glycosyltransferases, which are residents of the plant Golgi apparatus.[26] Recently, it was suggested,[22] that the biosynthesis of the long and branched sugar side chains of arabinogalactan proteins might be mediated by a lipid carrier and en-bloc transfer to the respective protein, a mechanism that is well known from N-glycosylation. Currently no experimental data are available to support either mechanism. None of the glycosyltransferases involved in HRGP biosynthesis have been cloned, although much effort is currently directed towards the identification of glycosyltransferases involved in cell wall polysaccharide and HRGP biosynthesis.[27,28] Until the enzymes involved are characterized, we can at least speculate about their specificity in the biosynthesis of the short and linear Hyp-bound O-glycans isolated from *C. reinhardtii* and compare this scenario with the biosynthesis of short linear Hyp-bound arabinans in land plants. We postulate that two arabinosyltransferases act first to link one arabinose in β-linkage to hydroxyproline and the next arabinose β-(1→2) to the Hyp-bound O-monoarabinan. Thus, both lineages, *Chlamydomonas* and land plants must express two arabinosyltransferases with the same specificity, whether they are actually homologous proteins remains elusive. The next step is *Chlamydomonas*-specific and includes a β-(1→2)-specific galactofuranosyltransferase. In addition, *Chlamydomonas* must express two sugar-methyltransferases. The enzyme that methylates Gal at C-6 seems to be less efficient than the enzyme that methylates Ara at C-3 based on the abundance of the respective derivatives in our preparations. The biosynthesis of Gal*f* is accomplished by a UDP-galactopyranose-mutase (UMP, EC 5.4.99.9), which catalyses the rearrangement of UDP-Gal*p*. Recently Beverley et al.[29] suggested the presence of this enzyme in the *Chlamydomonas* genome, which was found by sequence comparison using a well-known prokaryotic sequence. A phylogenetic tree of UMP sequences grouped the *C. reinhardtii* enzyme close to the enzymes found in fungi. The *A. thaliana* genome was devoid of an obvious orthologue.

As mentioned in the introduction, the stabilization of the PP-II helix of extensin-like HRGPs is achieved by Hyp glycosylation,[9,11] so that the question of this study was whether the Hyp-bound O-heteroglycans synthesized by *C. reinhardtii* are structurally related to land plant Hyp-bound O-arabinans, thus pointing towards common steps in their evolution. The conservation of the inner core of two arabinoses linked to hydroxyproline favours this hypothesis, as does the observed conservation of protein motifs.[3] Additional insight into the evolutionary conservation will be at hand when the rules for O-glycosylation in *Chlamydomonas* and land plants are known.

4. Experimental

4.1. Isolation and purification of Hyp-bound O-glycans

85 g (wet weight) *Phytophtora sojae*-elicitor treated *A. thaliana* suspension cultured cells were hydrolyzed with $Ba(OH)_2$ as described before.[17] Outer cell wall HRGPs of *C. reinhardtii bald-2* cells were extracted from cells of 200 L cultures with 2 M $NaClO_4$ following the protocol of Goodenough et al.[30] The lyophilized outer wall HRGPs were $Ba(OH)_2$-hydrolyzed as described above.

The neutralized and lyophilized $Ba(OH)_2$-hydrolysates were dissolved in deionized, distilled water and applied to a 34 × 2 cm column (H^+-form) of Aminex AG50-WX8 (BioRad). The Hyp-bound O-glycans were eluted using a linear gradient of 0.1 M to 0.5 M HCl. Fifty microlitres of each fraction was analyzed for Hyp in ELISA microtiter plates using the assay described by Kivirikko.[21] Hyp-rich fractions were pooled, neutralized and desalted on a 100 × 1.5 cm Biogel P2 column (Biorad) with deionized, distilled water as

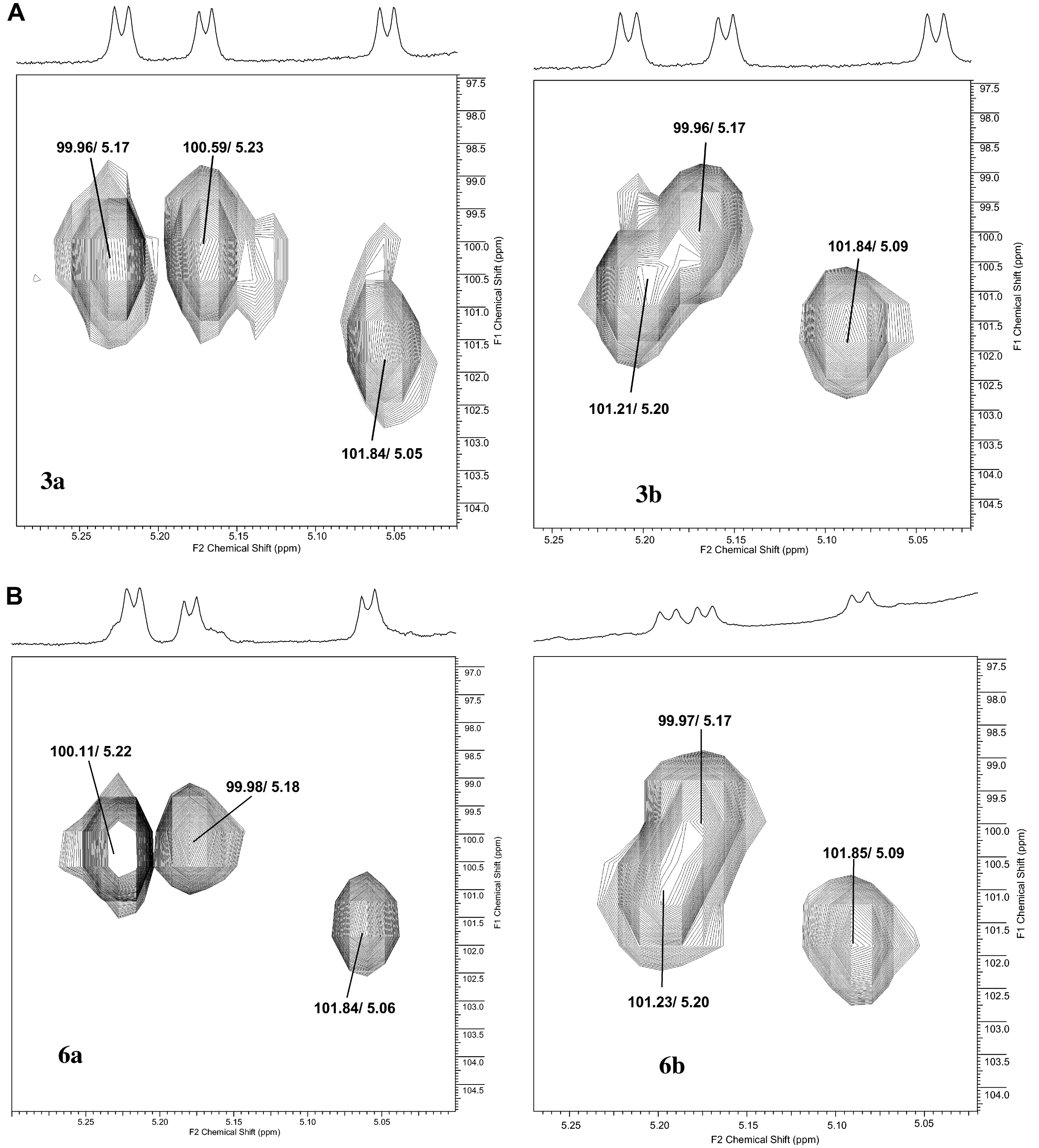


Figure 5. Two-dimensional C–H-COSY spectra of isolated Hyp-bound O-glycans from *C. reinhardtii*. A: Spectra of **3a** and **b**, respectively. B: Spectra of **6a** and **b**, respectively.

eluant. Each fraction was further HPLC-purified on an amino-propyl Nucleosil 300 column (Macherey and Nagel) using a non-linear gradient of acetonitrile/water starting with acetonitrile/water (90:10) for 40 min, followed by a linear gradient up to acetonitrile/water (75:25) for the next 110 min, and a linear gradient up to acetonitrile/water (55:45) for another 40 min. Flow rate was 2 mL/min. Eluting Hyp-bound O-glycans were pooled, freeze-dried and analyzed by ESI-MS (MAT 900 ST equipped with a Patric-Detector, Finnigan, Bremen, Germany). The scanning rate was 4 s/decade.

Table 4. Summary of ^{13}C and ^{1}H chemical shifts of the Hyp residues in Hyp-bound O-glycans of *Chlamydomonas* and *Arabidopsis*

Hyp-bound glycan	H-2	H-3	H-4	H-5	C-2	C-3	C-4	C-5
2a	4.27	2.60/2.10	4.58	3.45	61.9	37.8	78.3	52.3
3a	4.21	2.60/2.10	4.58	3.40	61.7	37.8	78.6	52.3
6a	4.27	2.60/2.10	4.59	3.45	61.9	37.8	78.6	52.3
2b	4.15	2.39/2.51	4.48	3.30/3.50	61.1	37.1	77.8	52.9
3b	4.11	2.37/2.51	4.48	3.30/3.50	61.1	37.1	78.6	52.9
6b	4.15	2.39/2.51	4.50	3.34/3.52	61.1	37.2	78.6	52.9

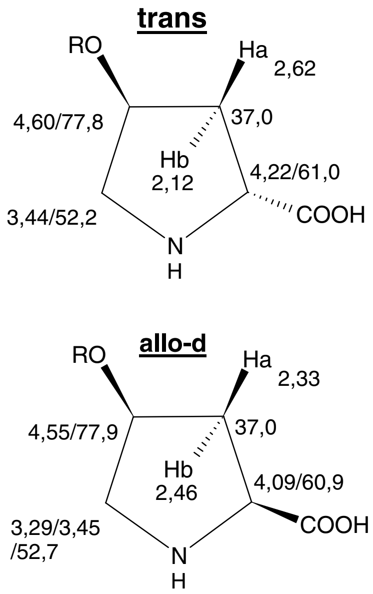


Figure 6. ^{13}C and ^{1}H chemical shifts of the Hyp residues in Hyp-bound O-glycans **1a** and **b** from *A. thaliana*.

4.2. Monosaccharide analysis

The monosaccharides composing the isolated Hyp-bound O-glycans were analyzed as persilylated alditols as described before.[10,20]

4.3. Linkage analysis

For linkage analysis samples were deuteromethylated, hydrolyzed, reduced with $NaBD_4$ and acetylated resulting in the partially deuteromethylated alditol acetates (PMAA), which were analyzed by GC/MS. We essentially followed the method of Hakomori[31] modified by Waeghe et al.[32]

4.4. NMR experiments

One and two-dimensional NMR experiments were carried out on Bruker Avance DPX 300 (^{1}H: 300 MHz, ^{13}C: 75 MHZ) or Bruker Avance DRX 500 (^{1}H: 500 MHz, ^{13}C: 125 MHZ) at 298 K. The only exception is mentioned in results. Samples were dissolved in D_2O/H_2O (1:9), spectra were referenced to water (^{1}H 4.65 ppm) and internal DMSO (^{13}C 40.8 ppm).

One dimensional selective NOE experiments[33,34] with a mixing time of 800 ms were recorded at 298 K on a Bruker Avance 400 MHz spectrometer equipped with pulsed-field gradient capabilities. An excitation sculpting scheme[35] with a 70 ms G3 pulse[36] was applied for selective excitation. For each experiment 256 FIDs (compound **1a**) and 1024 FIDs (compound **1b**) were co-added.

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