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Genomic and Biochemical Analysis of N Glycosylation in the Mushroom-Forming Basidiomycete *Schizophyllum commune*▽†

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N-linked glycans of *Schizophyllum commune* consist of $Man_{5-9}GlcNAc_2$ structures. Lack of further glycan maturation is explained by the absence of genes encoding such functions in this and other homobasidiomycetes. N-linked glycans in vegetative mycelium and fruiting bodies of *S. commune* are mainly $Man_7GlcNAc_2$ and $Man_5GlcNAc_2$, respectively, suggesting more efficient mannose trimming in the mushroom.

N glycosylation of proteins, the addition of sugar groups to specific asparagine (N) residues, is a common modification of proteins traveling through the secretory pathway (6). The overall organization of the machinery for N glycosylation and especially the strongly conserved early steps of N glycosylation are found all over the eukaryotic domain (8).

N glycosylation results from the sequential action of specific enzymes localized in the endoplasmic reticulum (ER) and the Golgi apparatus (6). As a first step, a preformed precursor oligosaccharide $Glc_3Man_9GlcNac_2$ is transferred cotranslationally from a dolichol carrier to an asparagine within an N-X-S/T sequence motif, where X is any amino acid except proline (3, 6, 7). The precursor oligosaccharide is subsequently modified by the removal of sugar units by glycosidases and addition of sugar units by glycosyltransferases that line the secretory route (Fig. 1).

The functions of N glycosylation of proteins are diverse (11). They range from aiding in proper protein folding and in ER-dependent degradation to determining the biochemical and biophysical properties of a protein. For instance, the N glycosylation structure can influence protein kinetics, tissue distribution, and receptor binding and effector functions (10). As a result, N glycosylation has been studied from fundamental, medical, and applied perspectives. It has been shown that the glycan structures differ between organisms. This is the result of different repertoires of glycosyltransferases along the secretory pathway. For instance, humans produce complex types of glycans extended with *N*-acetylglucosamines, galactoses, and sialic acids. Plants produce similar complex types of glycans but without sialic acid and with a bisecting β-1,2-xylose and an α-1,3-linked fucose. On the other hand, *Saccharomyces cerevisae* produces mostly hypermannosylated glycans with up to 100 residues (Fig. 1), whereas aspergilli produce oligomannosidic structures extended with additional mannose and galactofuranose residues (9, 13). Here, we describe the N glycosylation machinery in homobasidiomycetes. This group of fungi includes the true mushrooms, in contrast to heterobasidiomycetes, which include the jelly fungi and the rusts and smuts. Four genomes representative of the homobasidiomycetes were analyzed, and an expression and biochemical analysis was performed for one of these fungi, *Schizophyllum commune.*

Sequences of genes involved in N glycosylation in *S. cerevisiae* and humans were bidirectionally compared by BLAST against the predicted protein databases of the homobasidiomycetes *Coprinus cinereus* (Broad Institute, MIT), *Phanerochaete chrysosporium*, *Laccaria bicolor*, and *S. commune* (Joint Genome Institute [JGI]) (see Table S1 in the supplemental material). Proposed gene annotations are given in Table 1. Unique homologues were found in the four homobasidiomycetes for all genes involved in the synthesis of the oligosaccharide precursor. Interestingly, the homobasidiomycetes also contain unique homologues for the subunits of the human oligosaccharyl transferase complex, which is responsible for the transfer of the precursor to the protein entering the ER. A homologue for yeast *ost5*, encoding a subunit not found in mammals, was also absent in the basidiomycete genomes. Moreover, like other fungi (4), homobasidiomycetes appear to have only one *ost3*/*ost6* homologue that is more related to *ost3* than to *ost6*. A single homologue for human glucosidase I and II as well as for UDP-glucose:glycoprotein glucosyltransferase was found in all four basidiomycetes. In contrast, mannosidase I BLAST searches yielded between three and seven homologues in the different species. This has also been observed for other eukaryotes. One of these mannosidases is generally localized in the ER and converts $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$. The other mannosidases are localized in the Golgi apparatus and catalyze the conversions from $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ (9). This may also be the case for the homobasidiomycetes. Notably, no homologues of genes encoding enzymes involved in late glycosylation reactions that occur in the Golgi apparatuses of mammals, plants, *S. cerevisae*, and other ascomycetes could be found in the homobasidiomycete genomes (e.g., en-

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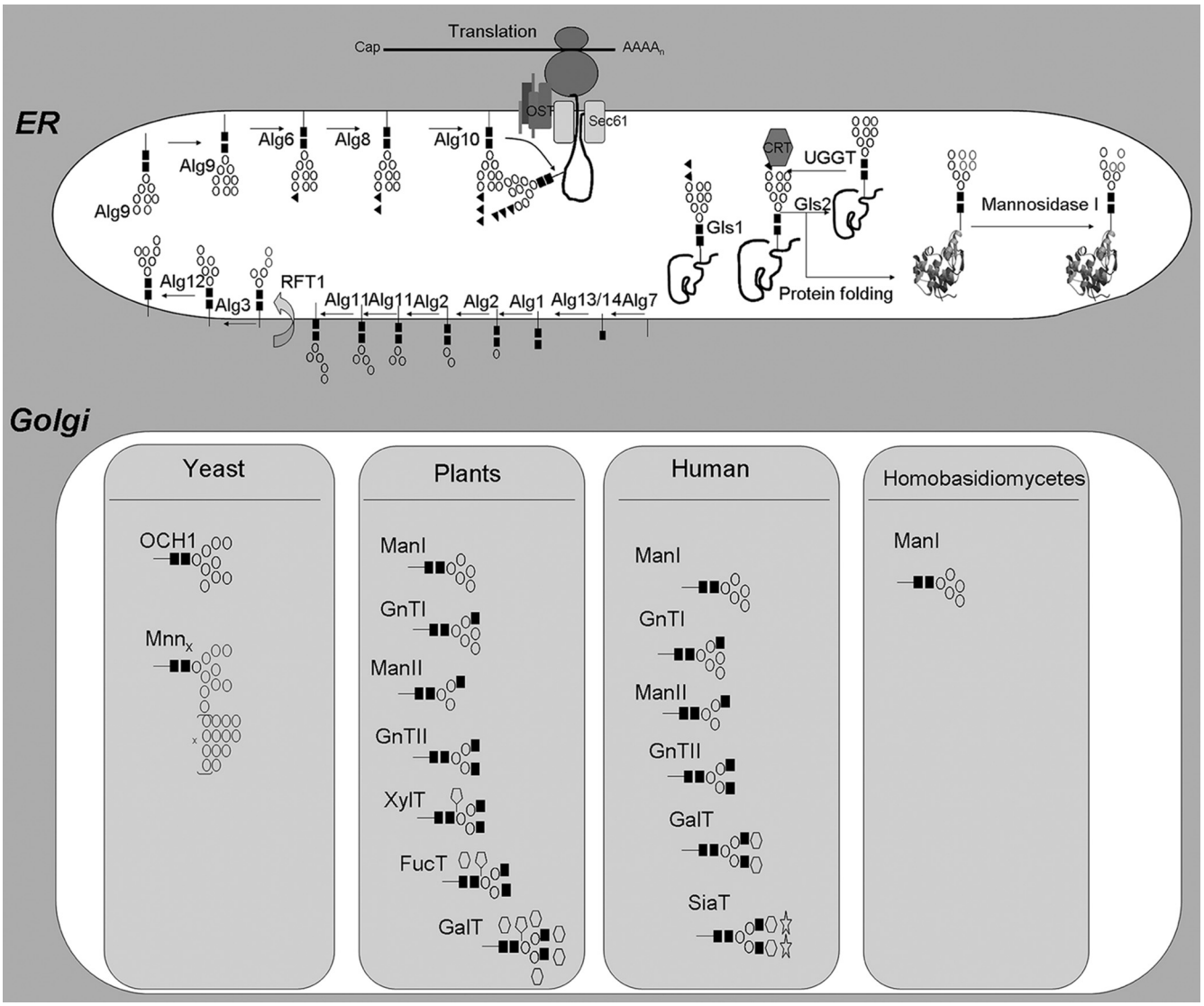

FIG. 1. Process of N glycosylation in eukaryotes. Proteins are channeled into the ER via the Sec61 translocon. The oligosaccharyl transferase complex transfers a prebuilt oligosaccharide to the protein upon entry of an N glycosylation signal. The glycan structure is subsequently modified in the ER and in the Golgi apparatus. Glucoses are indicated by triangles, mannoses by circles, GlcNAcs by squares, xyloses by pentagons, fucoses and galactoses by hexagons, and sialic acids by stars.

zymes such as galactofuranose mutase, mannosyltransferases, fucosyl-, xylosyl-, and galactosyltransferases, and *N*-acetylglucosamine transferase [see Table S1 in the supplemental material]).

Expression of the putative genes involved in N glycosylation was assessed in *S. commune* by *m*assively *p*arallel *s*ignature *s*equencing (MPSS). RNA was isolated from mycelium of *S. commune* strain 4-40 (CBS 340.81) and from fruiting bodies resulting from a cross between the coisogenic strains 4-40 and 4-39 (CBS 341.81). *S. commune* was grown in the light at 25°C on 25-ml minimal-medium plates (1) from 2 ml of macerated myceliun on a porous polycarbonate membrane (diameter, 76 mm; pore size, 0.1 μm) (Osmonics; GE Water Technologies, Trevose, PA) for 4 days for mycelium and, as a point inoculum, directly on the plates for 8 days for fruiting bodies. MPSS analysis was performed by Illumina (Hayward, CA) and ServiceXS (The Netherlands) using the DpnII restriction enzyme. Tags were generated and sequenced using the sequence-by-synthesis method on the Clonal Single Molecule Array (CSMA) platform from Illumina. Values were normalized to transcripts per million sequenced (tpm). MPSS data showed transcription for almost all identified genes (Table 1). Transcripts of the genes predicted to encode alg12, glucosidase II, and a mannosidase I were not detected by MPSS but were found in the EST database (JGI) and are therefore considered expressed as well. Only in the case of the *alg10* homologue was no EST and no MPSS tag found. It therefore cannot be concluded that the third glucose residue is added to the oligosaccharide precursor in *S. commune*.

Taken together, these data suggest that homobasidiomycetes generate N-glycans similarly to other eukaryotes but complete mannose trimming only to a $Man_5GlcNAc_2$ structure

TABLE 1. Predicted homologues of genes involved in N glycosylation in the homobasidiomycetes *C. cinereus, P. chrysosporium, L. bicolor*, and *S. commune* and their expression levels in *S. commune*[a]

Gene	Protein identity				Expression (tpm)	
	C. cinereus	*P. chrysosporium*	*L. bicolor*	*S. commune*	Monokaryon	Fruiting body
alg1	XP_001828935	137031	XP_001873898.1	48280	11	11
alg2	XP_001838360	132866	XP_001878479.1	58094	5	3
alg3	XP_001833721	39520	XP_001878203.1	51393	17	19
alg5	XP_001831223	137453	XP_001875049.1	53661	42	59
alg6	XP_001835805	27455	XP_001876280.1	66786	10	8
alg8	XP_001837067	129366	XP_001873612.1	63365	70	54
alg9	XP_001829193	7495	XP_001874021.1	64714	23	17
alg10	XP_001828622	6332	XP_001873612.1	47266	0	0
alg11	XP_001835629	27757	XP_001875753.1	52555	7	7
alg12	XP_001838600	0	XP_001883469.1	104684	0[b]	0[b]
alg13	XP_001833706	122448	XP_001878261.1	52101	35	11
alg14	XP_001830127	42674		58325	13	10
alg7	XP_001835697	128698	XP_001875906.1	53029	46	14
dpm1	XP_001828559	124350	XP_001881381.1	62156	117	133
rft1	XP_001834225	128907	XP_001880016.1	56542	8	7
ost1	XP_001837161	139605	XP_001876670.1	63008	21	31
ost2	XP_001837322	139862	XP_001876783.1	230988	30	44
ost3	XP_001833254	332		74773	79	189
ost4	Not annotated, chromosome 6, positions 1036693–1036791	Not annotated, scaffold 5, positions 1077844–1077942	XP_001879045.1	56760	188	97
stt3	XP_001831182	137809	XP_001874437.1	15576	0[c]	0[c]
swp1	XP_001832411	4549	XP_001881114.1	61083	58	20
wbp1	XP_001839842	122496	XP_001878162.1	81535	9	7
gls1	XP_001833601	132605	XP_001877857.1	65761	25	58
gls2	XP_001830083	35310	XP_001888761.1	57517	0[b]	0[b]
UGGT	XP_001832620	24966	XP_001887424.1	70541	13	14
ManI	XP_001829446	130488	XP_001874173.1	50368	40	53
ManI	XP_001834778		XP_001888694.1			
ManI	XP_001831476		XP_001889953.1	75752	0[b]	0[b]
ManI	XP_001834637	113	XP_001874608.1	76041	17	17
ManI	XP_001831454	2107	XP_001881296.1			
ManI	XP_001840635	4550	XP_001876643.1	258542	303	195
ManI	XP_001832442		XP_001874167.1			

[a] The predicted proteins of *P. chrysosporium* and *S. commune* have not been submitted to GenBank but can be found in the JGI database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html and http://shake.jgi-psf.org/Schco1/Schco1.home.html). Expression analysis was performed by MPSS using 4-day-old monokaryotic mycelium and fruiting bodies that had been grown in the light.

[b] The homologues of *alg12, gls2,* and one of the mannosidase I genes gave no MPSS signal, but their mRNAs were found in the EST database (JGI).

[c] *stt3* was expressed in other stages. For instance, 4 tpm were detected in the monokaryon that had been grown in the dark for 4 days.

with no further maturation reactions. To confirm this hypothesis, proteins were isolated from mycelium of *S. commune* strain 4-40 and from fruiting bodies resulting from a cross of the 4-40 and 4-39 strains (for culture conditions, see above). The frozen mycelium and fruiting bodies were homogenized by grinding them in a mortar. Proteins were extracted in 50 mM HEPES (5 mM EDTA, 0.1% sodium dodecyl sulfate, 20 mM $Na_2S_2O_5$), pH 7.5. After trichloroacetic acid precipitation, N-glycans were cleaved from the proteins by peptide *N*-glycosidase F in the recommended buffer (Westburg). N-glycans were purified on a C_{18} solid-phase extraction column (BondElut, Varian Inc.) and a Carbograph solid-phase extraction column (Alltech Applied Sciences) and identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry using positive-ion detection of $[M+Na]^+$ adducts on an Ultraflex mass spectrometer (Bruker) fitted with delayed extraction and a nitrogen laser (337 nm). Spectra were generated from the sum of at least 300 laser pulses. All analyses were done in triplicate. The area under the curve was calculated and related to the total area under the curve for all peaks with a signal-to-noise ratio larger than 8 (Fig. 2). This analysis showed that N-glycans with molecular masses corresponding to $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, $Man_7GlcNAc_2$, $Man_6GlcNAc_2$, and $Man_5GlcNAc_2$, as well as minor amounts ($\leq$1% of the total glycan) of $Man_4PGlcNAc_2$, are produced. This is in accordance with the hypothesis that homobasidiomycetes produce only oligomannosidic N-glycan structures. Interestingly, the monokaryotic mycelium secreted mainly proteins with glycans with a mass corresponding to $Man_7GlcNAc_2$ (51.9% $\pm$ 1.2%), whereas in fruiting bodies, glycans had mainly a molecular weight corresponding to the $Man_5GlcNAc_2$ type (47.8% $\pm$ 3.6%). To confirm this composition, the latter structure was analyzed by 500 MHz ^{1}H nuclear magnetic resonance (^{1}H NMR). This revealed indeed that this structure is identical to that of the $Man_5GlcNAc_2$ intermediate in human N glycosylation {Man-B(α1-6)[Man-A(α1-3)]Man-4′(α1-6)[Man-4(α1-3)]Man-3(β1-4)GlcNAc-2(β1-4)GlcNAc-1: Man-4 H-1, δ 5.093; Man-4′ H-1, δ 4.870; Man-A H-1, δ 5.093; Man-B H-1, δ 4.907; GlcNAc-2 NAc, δ 2.063; GlcNAc-1 NAc, δ 2.037} (12). From these data we conclude that mannose trimming is more efficient in the fruiting bodies.

In summary, our results show that *S. commune*, and likely

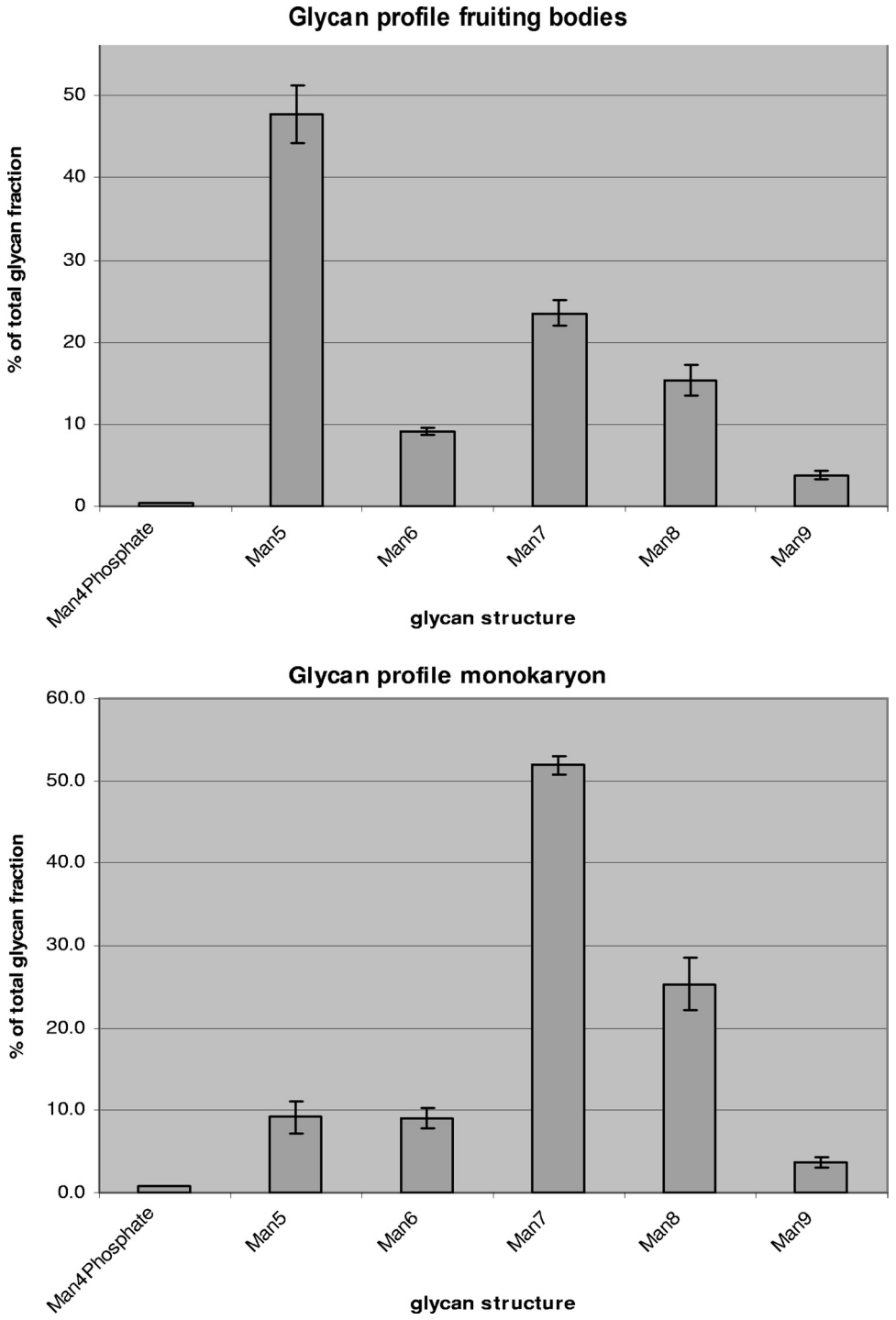

FIG. 2. Glycan profiles of monokaryotic mycelium and fruiting bodies. Average values and their standard deviations are shown for biological triplicates.

other homobasidiomycetes as well, produce only oligomannosidic structures. Indeed, preliminary results have shown this N glycosylation pattern in a diversity of homobasidiomycetes, including *Lentinus edodus*, *Pleurotus ostreatus*, and *Agaricus blazei* (our results; data not shown). Also, these glycan masses were observed in the common mushroom *Agaricus bisporus* in an inventory of glycan structures in vegetable foodstuffs (14). The simple N-linked glycan structure in the homobasidiomycetes is explained by the absence of homologues of genes encoding enzymes that catalyze downstream reactions involved in complex or hybrid types of N-glycan biosynthesis or in hypermannosylation or galactofuranosylation such as occurs in other eukaryotes. These results are of interest from an applied point of view. In recent years, efforts have been made to bio-

engineer the N glycosylation in cell factories used for industrial production of therapeutic N-glycoproteins for application to humans (see references 2 and 5). Correct glycosylation is crucial for proper biological activity and to prevent immune responses. Our data show that the humanization of the N glycosylation in homobasidiomycetes would require only the introduction of three plant or animal glycosyltransferases and glycosidases without the need to inactivate the glycosylation activity of the host.

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