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Members 5 and 6 of the *Candida albicans BMT* family encode enzymes acting specifically on β-mannosylation of the phospholipomannan cell-wall glycosphingolipid

Céline Mille[2,3], Chantal Fradin[2,3], Florence Delplace[4,5], Pierre-André Trinel[2,3], Annick Masset[2,3], Nadine François[6], Bernadette Coddeville[4,5], Piotr Bobrowicz[7,9], Thierry Jouault[2,3], Yann Guerardel[4,5], Stefan Wildt[7,10], Guilhem Janbon[8], and Daniel Poulain[1,2,3,6]

[2]Unité INSERM 995; and [3]Université Lille 2, Group "Candida and Candidiasis", Faculté de Médecine, 59000 Lille, France; [4]Université Lille 1, Unité de Glycobiologie Structurale et Fonctionnelle, UGSF; and [5]CNRS, UMR 8576, 59650 Villeneuve d'Ascq, France; [6]CHRU Lille, Centre Biologie et Pathologie, 59000 Lille, France; [7]Strain Development, GlycoFi, Inc./Merck & Co., Inc., Lebanon NH 03766, USA; and [8]Unité de Mycologie Moléculaire, Institut Pasteur, Paris 75015, France

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A family of nine genes encoding proteins involved in the synthesis of β-1,2 mannose adhesins of *Candida albicans* has been identified. Four of these genes, *BMT1–4*, encode enzymes acting stepwise to add β-mannoses on to cell-wall phosphopeptidomannan (PPM). None of these acts on phospholipomannan (PLM), a glycosphingolipid member of the mannose-inositol-phosphoceramide family, which contributes with PPM to β-mannose surface expression. We show that deletion of *BMT5* and *BMT6* led to a dramatic reduction of PLM glycosylation and accumulation of PLM with a truncated β-oligomannoside chain, respectively. Disruptions had no effect on sphingolipid biosynthesis and on PPM β-mannosylation. β-Mannose surface expression was not affected, confirming that β-mannosylation is a process based on specificity of acceptor molecules, but liable to global regulation.

Keywords: β-1,2 oligomannosides / *Candida albicans* / glycosphingolipid / mannosyltransferase / phospholipomannan

Introduction

Recent studies have highlighted the role of glycolipids, including sphingolipids, in the development of fungi and fungal-induced pathogenesis (Mille et al. 2004; Heung et al. 2006; Bozza et al. 2009). Sphingolipids are primary structural components of cell membranes and are bioactive molecules that act as second messengers in growth regulation (Dickson et al. 1990) and mediate activities as diverse as responses to stress (Dickson et al. 1997; Jenkins et al. 1997) and cell-wall integrity (Dickson et al. 2006). Our knowledge of fungal sphingolipid biosynthetic pathways has increased as a result of studies on antifungal drugs targeting these molecules (Mandala and Harris 2000; Stock et al. 2000; Zhong et al. 2000; Thevissen et al. 2005). In the yeast *Candida albicans*, a prominent opportunistic fungal pathogen in developed countries, a series of studies has characterized a glycosphingolipid (GSL) named phospholipomannan (PLM) which is present at the cell-wall surface and is shed in contact with host cells (Trinel et al. 1993, 1999; Jouault et al. 1998; Poulain et al. 2002). The role of PLM in *C. albicans* biology is unknown, but it is not essential since inactivation of the *MIT1* gene involved in its upstream biosynthetic pathway does not affect viability or growth (Mille et al. 2004). In contrast, the absence of PLM appears to affect virulence of *C. albicans* in animal models. This effect was, at least partly, attributed to the lack of expression of β-mannose (β-Man) residues which are a specific feature of *C. albicans* PLM. The structure, distribution, biological activities and immunological properties of β-Mans as specific *C. albicans* attributes and vaccine candidates have generated extensive literature (Jouault et al. 1995, 2000; Dalle et al. 2003). However, much is unknown about the biosynthesis of β-Mans and how they are associated with different *C. albicans* molecules. The discovery of the genes *BMT1–9* encoding enzymes involved in β-Man transfer (Mille et al. 2008) is useful for deciphering a process which appears to be unexpectedly complex due to the apparent homogeneous linear structure of the residues. In a previous study, it was established that β-mannosyltransferases (Bmts) 1–4 act sequentially in the addition of β-Man to *C. albicans* phosphopeptidomannan (PPM). This molecule, termed "mannan" by physicians and immunologists, is a complex repertoire of α- and β-Man epitopes that sometimes have opposite effects on the host. β-Man epitopes are distributed in the acid-stable fraction of PPM where their synthesis is under the control of

[1]To whom correspondence should be addressed: Unité INSERM 995, Université Lille 2, Group "Candida and Candidiasis", Pôle Recherche, Faculté de Médecine, Place Verdun, 59045 Lille cedex, France. Tel: +33-3-20-62-34-20; Fax: +33-3-20-62-34-16; e-mail: daniel.poulain@univ-lille2.fr
[9]Present address: Adimab LLC, Technology and Platform Development, 7 Lucent Drive, Lebanon, NH 03766, USA.
[10]Present address: VP/IBP, Integrated Biologics Profiling, 200 Technology Square, Cambridge, MA 02139, USA.

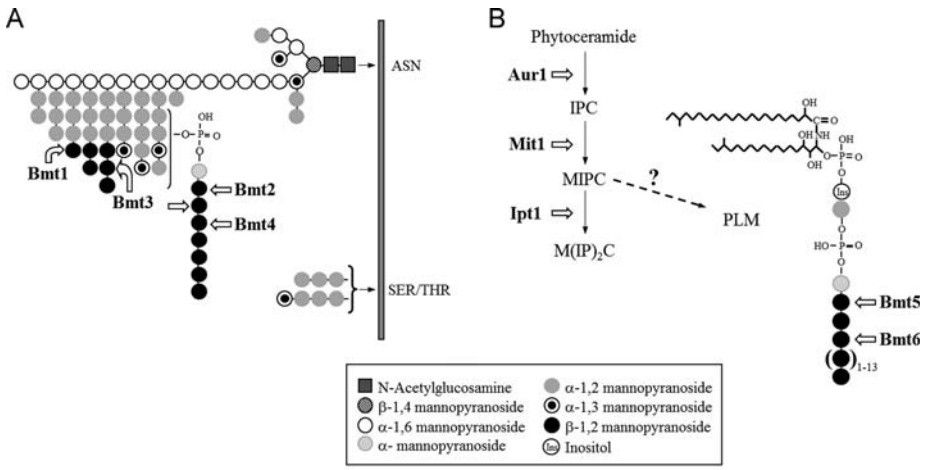

Fig. 1. (**A**) Schematic representation of Bmt1–4 activities on *C. albicans* cell-wall PPM. PPM contains oligomannosides linked via a phosphodiester bond that can be released by acid hydrolysis. These oligomannosides correspond to the PPM acid-labile fraction, whereas the remaining PPM fraction is called acid-stable. (**B**) Detailed structure and biosynthetic pathway of *C. albicans* PLM with Bmt5–6 activities deduced from this study. The arrows indicate where the enzymes act and which β-mannose is added.

Bmts 1,3 and in the acid-labile fraction (phosphodiester-linked, also designated as phosphomannan) where their synthesis is under the control of Bmts 2–4 (Figure 1A). Surprisingly, none of these Bmts acts on PLM β-mannosylation despite the striking structural homology between acceptors represented by PPM and PLM phosphomannans (Figure 1A and B). There are two differences in the distribution of β-Man residues in *C. albicans* serotypes A and B. These differences in antibody recognition, sometimes related to differences in pathogenic potential, are associated with the absence of β-Man in the PPM acid-stable fraction of serotype B strains (Shibata et al. 1985; Kobayashi et al. 1990, 1992). Further studies showed that a second phenotypic character of serotype B strains was the presence of a truncated PLM (Trinel et al. 2005).

Here, knowledge gained from PLM structural analysis and biosynthetic pathways was used to characterize members of the *BMT* gene family acting on PLM β-mannosylation.

Results

Identification of two members of the Bmt family involved in the PLM biosynthetic pathway

In a previous study (Mille et al. 2008), a family of nine genes was described in *C. albicans*, termed *BMTs* according to their homology with *Pichia pastoris BMT* genes encoding Bmts. Sequence homology between the nine Bmts and different glycosyltransferase families clustered the different mannosyltransferases according to their enzyme activities (Figure 2).

The different *BMT* genes were disrupted using a polymerase chain reaction (PCR)-based method (Gola et al. 2003). This generated a set of strains carrying disruptions of a single or both alleles of each gene in the parental strain, BWP17 (Mille et al. 2008) (Table I). Using these mutants, it was established that four of the nine genes, *BMT1–4*, encode enzymes with a specific role in β-mannosylation of PPM, either on its acid-labile or acid-stable moiety (Mille et al. 2008) (Figure 1A). This study focused on genes whose deletion had an effect on PLM β-mannosylation (Figure 3A) and which corresponded to orf19.1464 and orf19.5602 in the *Candida* genome database (http://www.candidagenome.org/). These genes were named *BMT5* and *BMT6*. As shown in Figure 3A, no significant reduction in β-Man epitopes on other manno-glycoconjugates was observed after deletion of these genes. This deletion had no effect on surface expression of β-Man epitopes as analyzed by immunofluorescence or on growth or morphogenesis in vitro (data not shown).

BMT5 and BMT6 deletions have no effect on sphingolipid biosynthesis

It was previously shown that PLM biosynthesis follows the sphingolipid biosynthetic pathway up to mannose-inositol-phosphoceramide (MIPC) and then diverges by the addition of Man-P to MIPC instead of the addition of inositol-phosphate, leading to $M(IP)_2C$ (Trinel et al. 2002; Mille et al. 2004) (Figure 1B). To analyze whether *BMT5* and *BMT6* deletions specifically affect PLM β-mannosylation or act upstream on phospho-inositol-sphingolipid biosynthesis, sphingolipids were extracted and analyzed. Sphingolipids from the mutant strain *mit1*Δ, which is blocked upstream of MIPC synthesis (Figure 1B), were used as negative controls. Thin-layer chromatography (TLC; Figure 4A) using purified PLM as a standard showed alterations of PLM biosynthesis among GSLs extracted from *bmt5*Δ and *bmt6*Δ compared with BWP17. As shown in Figure 4B, electrospray mass spectra of GSLs isolated from BWP17, *bmt5*Δ and *bmt6*Δ showed the presence of signals at *m/z* 678, 953, 1115 and 1357 in all strains, corresponding to $M(IP)_2C$, IPC and MIPC (Figure 1B). As reported previously (Trinel et al. 2002), each of these sphingolipids displays the same heterogeneity as four peaks that arise from the various combinations of C18 or C20 phytosphingosine with C24, C25 or C26 hydroxylated fatty acids in the ceramide moiety of these molecules. Mass spectra of BWP17 and *bmt5*Δ strains did not show any significant differences in the sphingolipid range, whereas the spectrum of

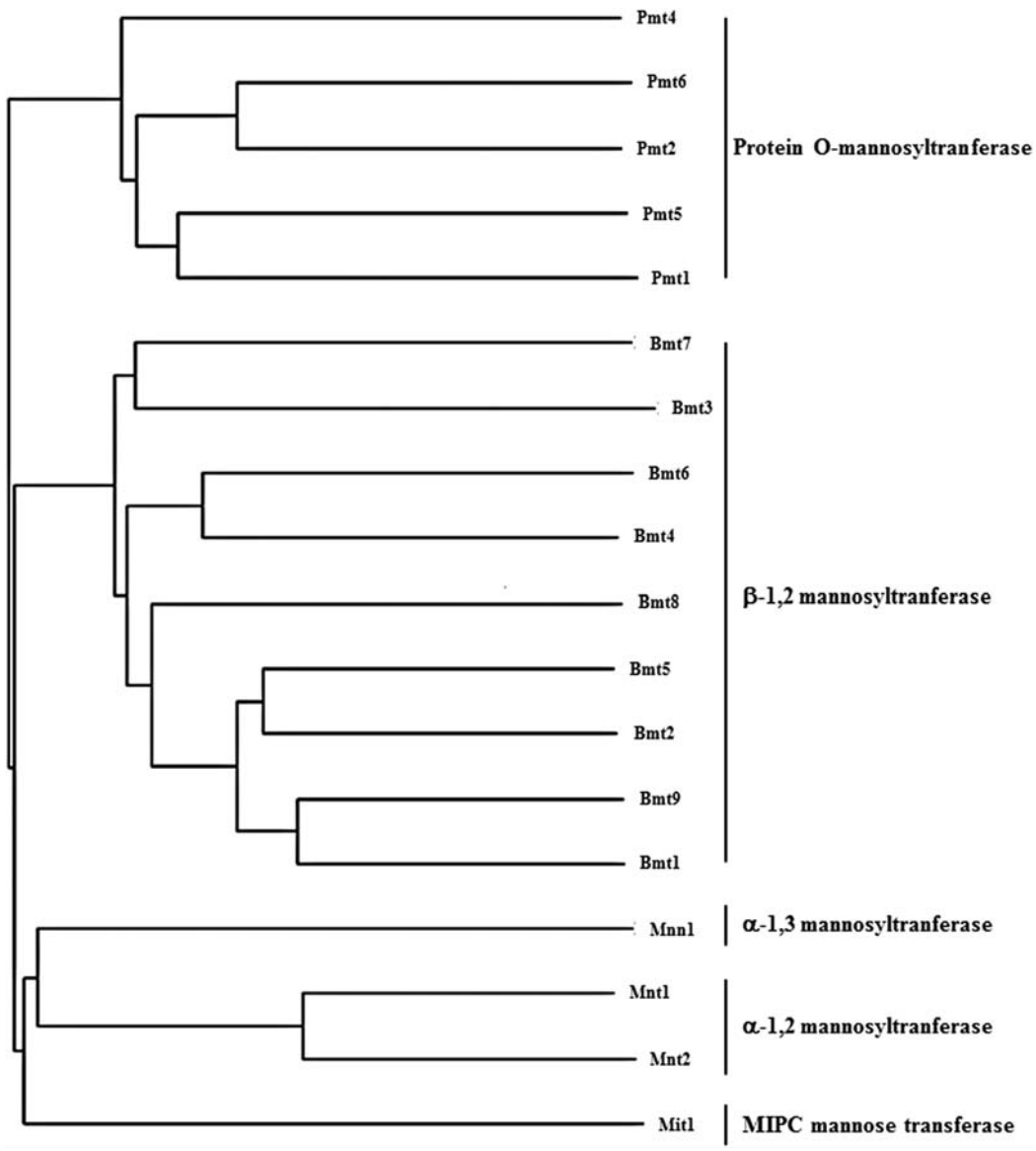

Fig. 2. Neighbor-joining phylogenetic tree of different *C. albicans* mannosyltransferases. This tree, based on the entire enzyme sequences, illustrates the relatedness of the Bmts and reveals a cluster of Bmts according to the β-mannosylation step and common acceptor (also see Figure 1).

the *bmt6*Δ strain showed two additional major signals at *m/z* 826.3 and 840.3 attributed to tri-mannosylated PLM, as previously established for NIH-B strain (Trinel et al. 2005) (Figure 4B). Altogether, mass spectroscopy analyses demonstrated that the modification of PLM biosynthesis in *bmt5*Δ and *bmt6*Δ did not result from the upstream absence of MIPC but from a modification of β-Man synthesis.

Bmt5 and Bmt6 are involved in β-1,2 mannose transfer on PLM

Alteration of PLM β-mannosylation was assessed by western blot analysis with specific monoclonal antibodies (mAbs). As shown in Figure 3B, the reactivity of mAb 5B2, specific for β-Mans with mannobiose as a minimal epitope (Trinel et al. 1992; Collot et al. 2008), to *bmt5*Δ PLM was dramatically reduced, whereas *bmt6*Δ PLM had a lower molecular weight than BWP17 and *bmt5*Δ PLMs, presumably due to a lower degree of β-Man polymerization. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis of purified PLM from the different strains revealed a single signal at *m/z* 1703, corresponding to Man_3-P-MIPC, in *bmt6*Δ PLM, whereas BWP17 and *bmt5*Δ PLMs gave a broader range of mass spectrometry peaks representing PLM forms with different degrees of β-Man polymerization (Table II). The lower degree of polymerization of *bmt6*Δ PLM was confirmed by western blot analysis with mAb B6.1 which recognizes only serotype B strain PLM with a predominant form, Man_3-P-MIPC (Trinel et al. 2005). This mAb, which binds only to β-1,2 tri- or tetra-Man (Han et al. 1997) (Figure 1C) detected both *bmt6*Δ and NIH-B PLMs (Figure 3C), although recognition of the latter was less than

*bmt6*Δ PLM as it contains, in addition to Man_3-P-MIPC, other forms of PLM with higher degrees of polymerization (Trinel et al. 2005). The weak *bmt5*Δ PLM β-mannosylation (Figure 3B) could be associated with the presence of Man-P-MIPC that cannot be differentiated from $M(IP)_2C$ in TLC and mass spectrometry analysis. Oligomannosides were then released from PLM by acid hydrolysis and separated by electrophoresis (Figure 5). As expected, mannosides with a high degree of polymerization were mainly seen in BWP17 PLM, whereas a mannotriose was detected in *bmt6*Δ PLM. Although the signal was weaker, as for BWP17 PLM, high-molecular-weight β-Mans were revealed in *bmt5*Δ PLM. Interestingly, an accumulation of mannose was detected in *bmt5*Δ PLM compared with BWP17 and *bmt6*Δ PLMs. The increase in mannose content after acid hydrolysis shows that Man-P-MIPC, but not $M(IP)_2C$, accumulates in the *bmt5*Δ strain. Altogether, detailed analysis of PLM-derived molecules from *bmt5*Δ and *bmt6*Δ strongly suggests that Bmt5 and Bmt6 are involved in the transfer of the first and the third β-Man residues, respectively, on nascent PLM. The *BMT6*/*bmt6*Δ deletion strain synthesizes an intermediate-sized PLM (Figure 3B) which suggests that both *BMT6* alleles are needed for correct PLM β-mannosylation. Reintroduction of a single copy of the *BMT6* gene did not completely restore the wild-type phenotype, in accordance with this hypothesis (Figure 3B). In contrast, *BMT5* activity appeared more permissive since reintroduction of a single copy restored the phenotype of the null mutant (Figure 3B) for which the presence of some β-Mans with a high degree of polymerization (Figure 5, Table II) suggested that another Bmt may partially fulfill the defect of *BMT5* deletion.

Table I. *C. albicans* strains used in this study

Strain	Parental strain	Genotype	Reference
BWP17		*arg4::hisG/arg4::hisG;his1::hisG/his1::hisG;ura3Δ::λimm434/ura3Δ::λimm434*	Wilson et al. (1999)
AL79	BWP17	*bmt5Δ::ARG4/BMT5*	This study
AL88	AL79	*bmt5Δ::ARG4/bmt5Δ::HIS1*	This study
AL98	AL88	*bmt5Δ::ARG4/bmt5Δ::HIS1, RPS10*::CIp10-*BMT5-URA3*	This study
AL78	BWP17	*bmt6Δ::ARG4/BMT6*	This study
AL87	AL78	*bmt6Δ::ARG4/bmt6Δ::HIS1*	This study
AL97	AL87	*bmt6Δ::ARG4/bmt6Δ::HIS1, RPS10*::CIp10-*BMT6-URA3*	This study
NIH-B 792		*C. albicans* serotype B	Shibata et al. (1985)
*mit1*Δ	CAF2	*mit1Δ::hisG/mit1Δ::hisG*	Mille et al. (2004)
*bmt1*Δ	BWP17	*bmt1Δ::ARG4/bmt1Δ::HIS1*	Mille et al. (2008)
*bmt2*Δ	BWP17	*bmt2Δ::ARG4/bmt2Δ::HIS1*	Mille et al. (2008)
*bmt3*Δ	BWP17	*bmt3Δ::ARG4/bmt3Δ::HIS1*	Mille et al. (2008)

Bmt5 and Bmt6 are homologous to Bmt1–2 and Bmt4, respectively, both acting on PPM, but deletion of their genes had no effect on β-mannose transfer in PPM

Cluster analysis of the nine Bmts classified the enzymes into groups depending on their involvement in the β-mannosylation steps and, more particularly, on their acceptor molecule (Figures 1 and 2). Bmt1, Bmt2 and Bmt5, which display at least 42% sequence identity (61% homology), are involved in the addition of the first β-Man to the PPM acid-

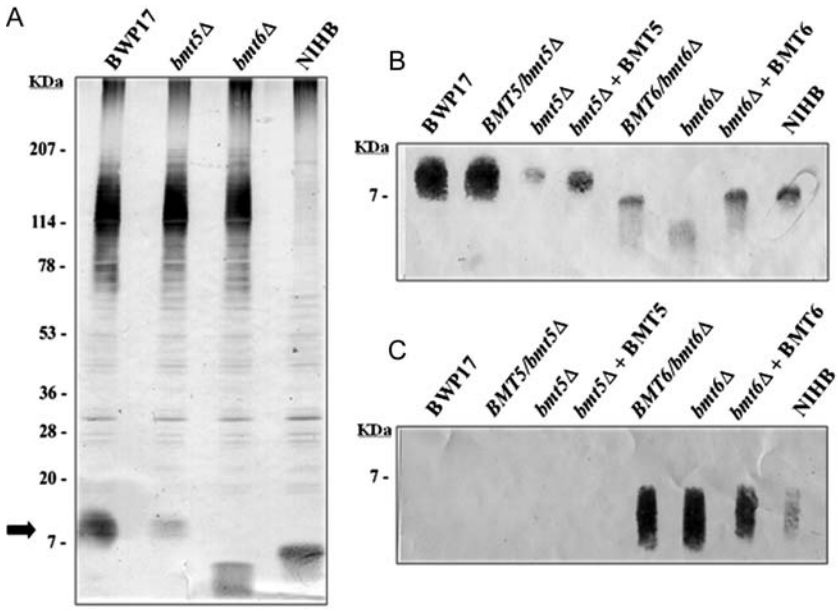

Fig. 3. Analysis of β-1,2 mannosylation of *C. albicans* glycoconjugates. Western blots of whole-cell extracts were stained with anti-β-1,2 oligomannoside monoclonal antibodies: 5B2, specific for β-Mans with mannobiose as a minimal epitope (**A**, **B**) and B6.1, specific for β-1,2 mannotriose (**C**). The PLM is indicated by an arrow. Parts B and C focused only on PLM. BWP17 (parental strain, serotype A); *BMT5*/*bmt5*Δ (AL79); *bmt5*Δ (AL88); *bmt5*Δ + *BMT5* (AL98); *BMT6*/*bmt6*Δ (AL78); *bmt6*Δ (AL87); *bmt6*Δ + *BMT6* (AL97); NIH-B (*C. albicans* serotype B).

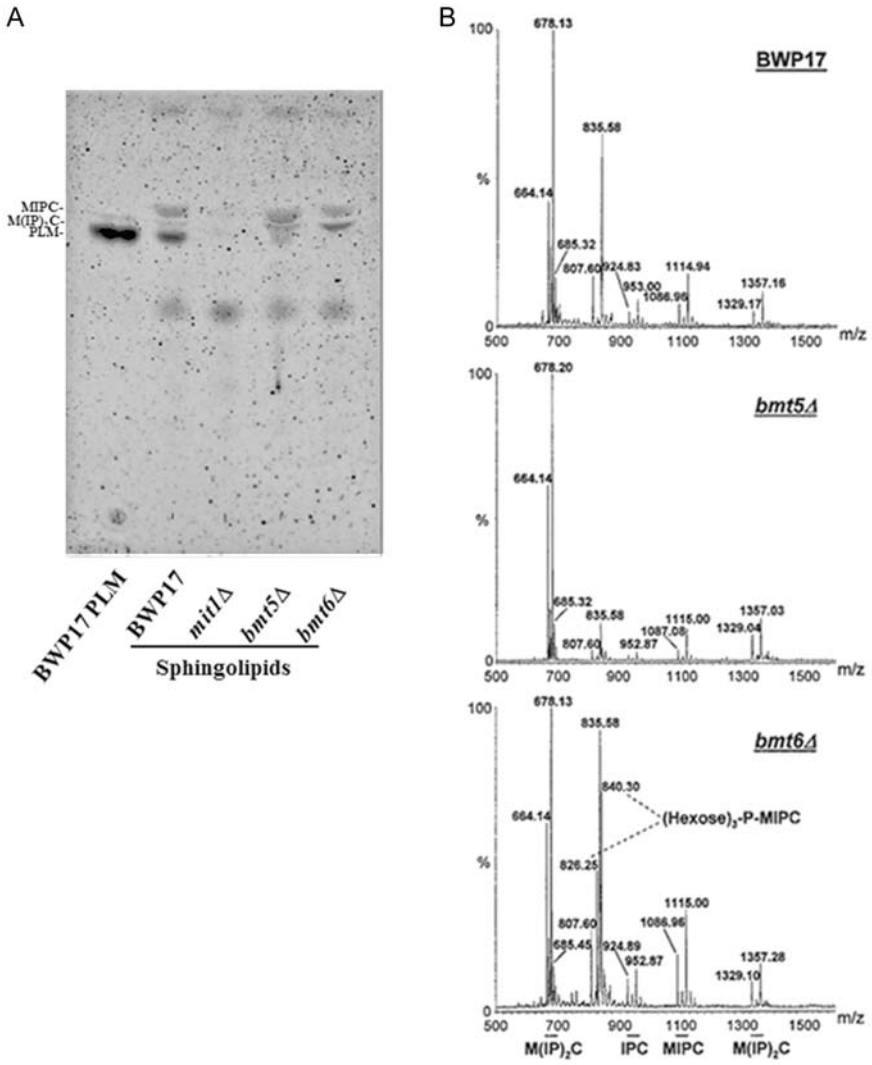

Fig. 4. Analysis of sphingolipids extracted from deletion strains. (**A**) TLC revealed PLM in *bmt5*Δ and *bmt6*Δ strains which showed different migration to that of the wild-type strain. Lane 1: PLM purified from BWP17; lanes 2–5: sphingolipids purified from BWP17, *mit1*Δ, *bmt5*Δ and *bmt6*Δ, respectively. (**B**) Electrospray mass spectrometry analysis of sphingolipids extracted from parental and deletion strains. Spectra mainly displayed (M-H) and/or (M-2H)/2 molecular-related ions of compounds. In all strains, mass spectra of sphingolipid extracts revealed groups of peaks corresponding to the major membrane sphingolipids (IPC, MIPC and $M(IP)_2C$). Peaks within each group arose from various compositions of the ceramide moiety of the sphingolipids as demonstrated previously.

stable fraction, PPM acid-labile fraction or PLM, respectively, and therefore act on α-linked mannose residues (Figure 1A and B). Bmt4 and Bmt6, which also display 41% sequence identity (62% homology), are involved in the addition of the third β-Man either to PPM or PLM, with β-linked mannose as an acceptor (Figure 1A and B). Despite the striking structural homology between PLM and the acid-labile fraction of PPM (Figure 1A and B), deletion of the *BMT1–4* genes had no effect on PLM β-1,2 mannosylation and Bmt1–4 displayed substrate specificity for PPM (Mille et al. 2008). According to these data, we investigated whether the converse was true (i.e. if deletion of *BMT5* and *BMT6* had an effect on PPM biosynthesis). For this purpose, purified PPM from *bmt5*Δ and *bmt6*Δ was analyzed by western blot (Figure 6A) using mAb B9E, specific for β-Mans of the PPM acid-stable fraction (Figure 1) and β-Mans released by acid hydrolysis from the PPM acid-labile fraction by fluorophore-assisted carbohydrate electrophoresis (FACE; Figure 6B). In contrast to *bmt1–4*Δ (Mille et al. 2008), no modification of β-mannosylation of the PPM acid-stable (Figure 6A) and acid-labile (Figure 6B) fractions, respectively, was observed in the *bmt5*Δ and *bmt6*Δ strains. This shows that Bmt5 and Bmt6 had no activity on PPM and that these two enzymes also exhibit substrate specificity.

Discussion

Yeast sphingolipids participate in bilayer stability, protein sorting, organization of the cortical actin cytoskeleton and endocytic uptake and cell signaling via membrane rafts (Wachtler and Balasubramanian 2006). *C. albicans*

Table II. Negative-ion MALDI-TOF mass spectrometry of PLMs from parental and deleted strains

Composition	Measured average *m/z* value		
	BWP17	*bmt5*Δ	*bmt6*Δ
Man_3-P-MIPC	1703.2	nd	1703.3
Man_7-P-MIPC	2351.4	nd	nd
Man_8-P-MIPC	2513.5	2513.9	nd
Man_9-P-MIPC	2675.6	2676.0	nd
Man_{10}-P-MIPC	2837.7	2838.1	nd
Man_{11}-P-MIPC	2999.7	3000.1	nd
Man_{12}-P-MIPC	3161.8	3162.3	nd
Man_{13}-P-MIPC	3323.9	3325.4	nd
Man_{14}-P-MIPC	3485.9	3486.5	nd
Man_{15}-P-MIPC	3649.0	3649.0	nd
Man_{16}-P-MIPC	3811.0	3810.7	nd

Groups of peaks (arising from variability of the lipid moiety) spaced by 162 mass units were detected. They correspond to $[M + Na - 2H]^-$ adduct ions. Major peak values reported were attributed to PLM predominant forms with C20 phytosphingosine and C24 hydroxylated fatty acid. nd: not detected.

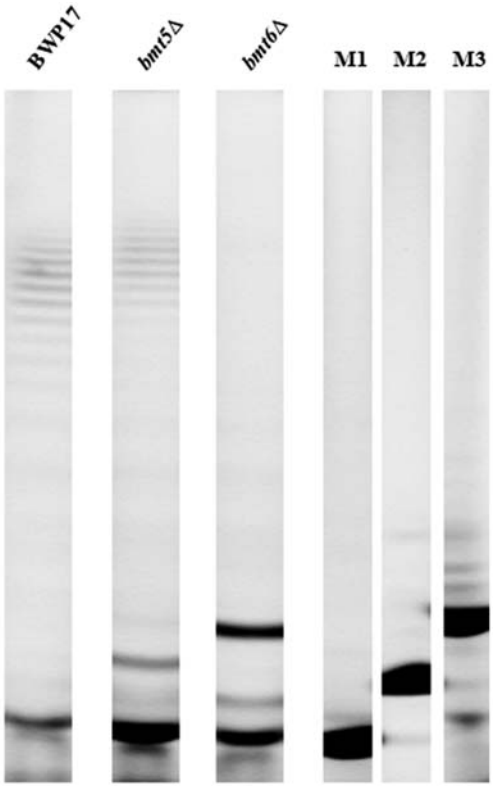

Fig. 5. Analysis of β-Mans released from purified PLM. Oligomannosides released by acid hydrolysis from purified PLMs were analyzed by FACE. Different carbohydrate standards were used to evaluate the monomer number in the oligomannoside chains: M1, mannose; M2, β-mannobiose; M3, β-mannotriose.

sphingolipids are essential for survival of the yeast, as revealed by its sensitivity to aureobasidin A, an IPC synthase inhibitor (Sugimoto et al. 2004). *C. albicans* can derive one class of its sphingolipids, MIPC, from a specific biosynthetic pathway that adds β-Man after mannosylphosphorylation to generate a complex GSL called PLM (Trinel et al. 2002) (Figure 1B). This molecule is, at least partly, present at the cell-wall surface, a localization that facilitates its interaction with host components, namely through its shedding in contact with host cells (Jouault et al. 1998; Poulain et al. 2002). It induces tumour necrosis factor alpha secretion from cells of macrophage lineage via a target lesson revascularization (TLR-2)-dependent pathway (Jouault et al. 1994, 2003) and macrophage apoptosis via upstream modulation of the ERK pathway (Ibata-Ombetta et al. 2001, 2003) following ingestion of yeasts. However, the regulation of *C. albicans* PLM expression remains largely unknown (Trinel et al. 1996; Poulain et al. 2002). Furthermore, its glycan moiety, mainly β-Man degrees of polymerization, is variable, as shown in serotype B strains that display a truncated PLM compared with serotype A strains (Trinel et al. 2005).

GSLs are known virulence factors in different pathogens, sometimes in relation to subtle changes in their structure (Toledo et al. 2007). With regard to the role of glycolipids in fungal virulence, a recent study based on homozygous deletion has identified a glucosylceramide as the first small molecule synthesized by *C. albicans* to be specifically required, albeit independently from morphogenesis (Noble et al. 2010). Furthermore, studies concentrating on dimorphic fungi with mAbs against different moieties of GSLs have shown close structure/activity relationships (Toledo et al. 2010). Up to six GSLs were purified and characterized from the mycelium of the opportunistic fungus *Aspergillus fumigatus* (Simenel et al. 2008). Among these, a study investigating the specificity of the human T-cell repertoire against these mould antigens identified a member of the GSL family containing galactofuranose in its glycan moiety which strongly promoted activation of the pathogenic Th17 response (Bozza et al. 2009).

Regarding the biological activity of *C. albicans* PLM, the biosynthetic pathway from MIPC to PLM has to be elucidated. Addition of β-Man residues is not specific to PLM as other *C. albicans* cell-wall molecules such as the outer layer PPM carry these particular oligomannosides that confer specific adhesive (Li and Cutler 1993; Fradin et al. 1996, 2000; Dromer et al. 2002; Dalle et al. 2003) and immunomodulatory (Han and Cutler 1995; Jouault et al. 1995, 1997; Ibata-Ombetta et al. 2001, 2003) properties. The β-1,2 mannosylation pathway of PPM has recently been elucidated, and nine members of a new family of Bmts have been discovered. Four of these enzymes, *Bmt1–4*, are involved in PPM biosynthesis (Mille et al. 2008). Here, the activity of two other members of the Bmt family was also characterized. β-Man epitope mapping of *bmt5*Δ and *bmt6*Δ single null mutants compared with the parental strain clearly shows that these two mutants are affected in their PLM β-mannosylation. Mass spectrometry of *bmt5*Δ and *bmt6*Δ PLMs revealed that Bmt5 and Bmt6 are specifically involved in the later stages of PLM biosynthesis, β-1,2 mannosylation, and that their deletion has no impact on complex sphingolipid (IPCs, MIPCs and M$(IP)_2$Cs) biosynthesis. Results of combined western blot, mass spectrometry, FACE and TLC analyses lead to the conclusion that Bmt5 and Bmt6 are involved in the addition of the first and third β-Mans to PLM, respectively (Figure 1B).

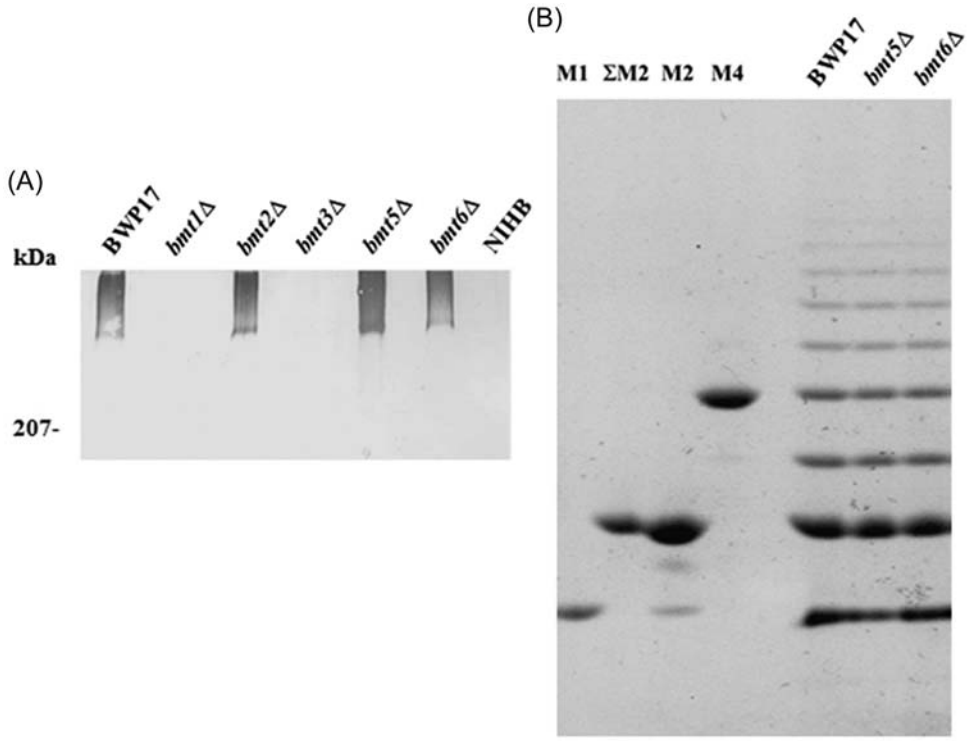

Fig. 6. Analysis of PPM acid-stable and acid-labile fractions. (**A**) Western blots of purified PPMs from different *bmt*Δ mutants and their parental strain were stained with mAb B9E, specific for β-Mans at the nonreducing end of the acid-stable fraction of *C. albicans* serotype A strains (antigenic factor 6, see Figure 1A and C). PPM from NIH-B (*C. albicans* serotype B) was used as a negative control. (**B**) Oligomannosides released by acid hydrolysis from purified PPMs were analyzed by FACE. Different carbohydrate standards were used to evaluate the monomer number in the oligomannoside chains: M1, mannose; ΣM2, synthetic β-mannobiose; M2, purified β-mannobiose; M4, purified β-mannotetraose.

It was not possible to identify the Bmt that adds the second β-Man to PLM glycan. Although Bmt3 initially appeared to be a good candidate because it adds the second β-Man to PPM (Figure 1A), its deletion had no effect on the PLM structure, similar to *BMT7–9* single deletions. It is therefore assumed that Bmt5 can add both the first and the second β-Mans to PLM glycan such as Mnt1 and Mnt2 that are able to add both the first and the second α-1,2 Mans to mannoprotein *O*-glycans (Munro et al. 2005). It is also suggested that Bmt4 and Bmt6 are responsible for the addition of the fourth and further β-Mans to PPM and PLM, respectively. To date, analyses concentrating on PPM and PLM have enabled us to define the functions of six Bmts out of nine. The putative functions of Bmts 7–9 remain elusive mainly because, in addition to PPM and PLM, a large number of *C. albicans* molecules could also be β-mannosylated, which comprises most *C. albicans* cell-wall mannoproteins, whatever is their mode of anchorage in the cell wall (soluble, β-1,6 and β-1,3 glucan linked) (Fradin et al. 2008).

Comparison of phylogenetic analyses of the nine Bmt sequences with potential functions (Figure 2) shows that the strongest sequence homologies for Bmt5 and Bmt6 were found with Bmt2 and Bmt4, respectively. The results from this analysis are coherent when considering the structural similarities between PLM and PPM. As shown in Figure 1, both Bmt5 and Bmt2 add a β-Man to an α-Man linked to a phosphate group, whereas Bmt6 and Bmt4 add a β-Man to a β-1,2 mannobiose. An intriguing question is why *C. albicans* needs two different sets of related enzymes for identical stages of β-mannosylation with preferential substrates, PPM or PLM. These overlapping activities are advantageous for the yeast as they can prevent the deleterious effect of gene loss to the cell wall. PLM could be indeed partly β-mannosylated in the *bmt5*Δ strain. On the other hand, specific β-mannosylation processes between the two pathophysiologically relevant molecules facilitate differential β-Man expression on these manno-glycoconjugates, which is coherent with previous observations showing different β-mannosylation processes for PPM and PLM (Trinel et al. 1996).

Considering the immunological evidence for the importance of β-Man interactions with both innate receptors (Poulain and Jouault 2004; Jouault et al. 2006; Jawhara et al. 2008) and effectors of adaptive immunity (Han et al. 1998; Xin et al. 2008), further studies are necessary to assess how this specific trait of unforeseen complexity, namely in terms of regulation (Trinel et al. 1996; Mille et al. 2004), and high entropy is related to *C. albicans* adaptation to the human host. Definition of *C. albicans* GSLs is part of this challenge.

Materials and methods

Strains and growth conditions

The *C. albicans* strains used in this study are listed in Table I. Strains were grown in YPD-Arg-His-Urd medium (1% yeast

extract, 2% peptone, 2% dextrose, 20 mg/L arginine, 20 mg/L histidine, 20 mg/L uridine) at 37°C for 16 h. *Escherichia coli* strains TOP10 or DH5α were used for recombinant DNA work. All procedures for manipulating DNA were performed using standard procedures (Sambrook et al. 1989).

Monoclonal antibodies

5B2 is a rat-mouse IgM with β-1,2 mannobiose as a minimal epitope (Trinel et al. 1992). B6.1 is a mouse IgM specific for a β-1,2 mannotriose (Han et al. 1997). B9E, a mouse IgM, is specific for β-1,2 Mans present on the nonreducing end of α-1,2 chains of the PPM acid-stable fraction (Ponton et al. 1993) (Figure 1A).

Deletion of BMT genes

The two *C. albicans* open reading frames (ORFs), orf19.1464 and orf19.5602, were deleted sequentially from strain BWP17 by PCR-based gene targeting (Gola et al. 2003) (Table I). Two plasmids were used to release selectable markers, *CaARG4* and *CaHIS1*, by *Not*I digest. Each marker was amplified by PCR using primers including the first and the last 100 bp of gene-specific sequences. The generated disruption cassettes were used to transform BWP17 by the lithium acetate method (Sanglard et al. 1996). First, the selectable marker *CaARG4* was used to generate independent heterozygous strains on synthetic dextrose plates supplemented with 20 mg/L histidine and 20 mg/L uridine. Correct insertion of the marker was verified by PCR. A second round of transformation was performed to delete the second allele with the *CaHIS1* marker by the same method. Homologous integration was verified by PCR.

Reintroduction of BMTs into null strains

BMT5 and *BMT6* ORFs, with ∼500-bp upstream and downstream nucleotides, were amplified by PCR using AccuPrime *Pfx* DNA polymerase (Life technologies, Saint Aubin, France). The amplified fragment was cloned into the pCRII-TOPO vector (Life technologies, Saint Aubin, France). The *BMT* gene was then excised by digestion with *Sac*I and *Not*I, and ligated into *Sac*I- and *Not*I-digested CIp10 (Murad et al. 2000) to obtain the reintegration vector. The *bmt*Δ null strains were transformed with *Stu*I- or *Nco*I-digested reintegration vector. Transformants were screened by PCR to check the reintroduction of the *BMT* gene at the *RPS10* locus.

Whole-cell protein extraction and western blot analysis

Strains were grown at 37°C in YPD-Arg-His-Urd and were extracted by alkaline extraction under reducing conditions (Trinel et al. 1992). Briefly, cells were incubated on ice in 1.85 M NaOH and 5% β-mercaptoethanol. Proteins and glycoconjugates were then extracted in sodium dodecyl sulfate (SDS) for 5 min at 100°C. Extracts were adjusted to the same protein concentration and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE; Laemmli 1970) on a 5–20% acrylamide gel slab. Membranes were probed with mAbs 5B2 diluted 1:1000 and B6.1 diluted 1:1000 and then incubated with a 1:1000 dilution of alkaline phosphatase-conjugated anti-rat IgM or anti-mouse IgM, respectively.

Analysis of sphingolipids

Strains were grown at 37°C in YPD-Arg-His-Urd and washed in phosphate-buffered saline (PBS). Cells were then broken with a French press (Aminco, SLM Instruments, Inc.) at 20,000 psi, dialysed and lyophilized. Sphingolipids were then extracted and purified by successive extractions with chloroform/methanol and chloroform/methanol/water mixtures (Trinel et al. 1999) except that care was taken to avoid micelle formation in order to improve PLM solubility (Trinel et al. 2002). The different supernatants were pooled, analyzed by TLC on 10 × 5 cm silica gel 60 plates (Merck, Lyon, France) using a butanol/acetic acid/water (20/8/17) solvent system and revealed with an orcinol stain. The sphingolipid extracts were finally analyzed by mass spectrometry. Electrospray mass measurements were carried out in negative-ion mode on a triple quadrupole instrument (Micromass Ltd., Altrincham, UK) fitted with an atmospheric pressure ionization electrospray source. A mixture of polypropylene glycol was used to calibrate the quadrupole mass spectrometer. The samples were dissolved into dimethyl sulfoxide and further diluted in methanol to obtain a final concentration of 0.25 μg/μL. Solutions were infused using a Harvard syringe pump at a flow rate of 3 μL/min. The quadrupole was scanned from 500 to 2000 Da with a scan duration of 6 s and a scan delay of 0.1 s. The samples were sprayed using a 3.5 kV needle voltage and the declustering cone was set at 70 V.

PLM purification

Sphingolipids were extracted as described in the section *Analysis of sphingolipids*, dried and then submitted to extensive butanol/water partitions of the chloroform/methanol/water (10/10/3) extracts. Sphingolipids in the water phase were finally purified on phenyl-sepharose using increasing concentrations of ethanol (1–40%) for elution. Purification and control of PLM fractions were checked and analyzed by TLC using a butanol/acetic acid/ water (20/8/17) solvent system and visualized with orcinol reagent.

Analysis of PLM by mass spectrometry

MALDI-TOF mass spectra were acquired on a Voyager Elite DE-STR mass spectrometer (Perspective Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. The spectrometer was operated in negative reflectron mode by delayed extraction with an accelerating voltage of 20 kV, a pulse delay time of 200 ns and a grid voltage of 66%. Samples were prepared by mixing 0.5 μL of PLM solution in water (0.02 μG/μL) with 0.5 μL of 2,5-dihydroxybenzoic acid matrix solution (10 mg/mL in methanol/water, v/v) directly on the target. The samples were allowed to dry for about 5 min at room temperature. Between 100 and 150 scans were acquired for each spectrum.

PPM extraction

PPM from cells grown in YPD-Arg-His-Urd medium was extracted by autoclaving in citrate buffer at pH 7.0 (Faille et al. 1992). Briefly, cell pellets were suspended in 20 mM citrate buffer and autoclaved at 125°C for 90 min. Suspensions were harvested, and Fehling's solution was

added to the supernatant to precipitate PPM. The PPM was then washed in methanol/acetic acid (8/1) and dried in a Speed Vac concentrator. Sugar concentrations were estimated by the sulfuric-phenol colorimetric method (Dubois et al. 1951). PPM was analyzed by SDS–PAGE as described in the section *Whole-cell protein extraction and western blot analysis*. Membranes were probed with mAb B9E diluted 1:750.

Face analyses of β-Mans released from PLM and PPM

Previously extracted PLM or PPM was hydrolyzed in 20 mM HCl for 1 h AT 100°C to release β-oligomannosides. After neutralization, hydrolysates were then dried and tagged with 0.15 M 8-amino-naphthalene-1,3,6-trisulfonate (ANTS) and 1 M sodium cyanoborohydride for 16 h at 37°C (Goins and Cutler 2000). The dried samples were resuspended in glycerol/water (1/4). Electrophoresis of ANTS-labeled oligomannosides was performed on 35% (w/v) acrylamide separating gels. Acid-hydrolyzed dextran and synthetic or purified oligosaccharides were also tagged with ANTS and used as carbohydrate standards. Gels were dried and images were acquired with the Gel Doc 2000 image analysis apparatus from Biorad equipped with a 365 nm UV-transilluminator.

Phylogenetic analysis

The phylogenetic tree of mannosyltransferases was calculated using clustalW software and the resulting tree was plotted using the NJplot software. The tree is based on the entire sequence of proteins and includes other glycosyltransferases involved in cell-wall biosynthesis than *BMT* genes.

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Conflict of interest

None declared.

Abbreviations

ANTS, 8-amino-naphthalene-1,3,6-trisulfonate; β-Man, β-1,2-linked oligomannoside; *BMTs*, β-mannosyltransferase genes; Bmts, β-mannosyltransferases; FACE, fluorophore-assisted carbohydrate electrophoresis; GSL, glycosphingolipid; IPC, inositol-phosphoceramide; mAb, monoclonal antibody; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MIPC, mannose-inositol-phosphoceramide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLM, phospholipomannan; PPM, phosphopeptidomannan; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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