

Protein O-Mannosyltransferases B and C Support Hyphal Development and Differentiation in *Aspergillus nidulans*^{∇†}

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Received 19 November 2008/Accepted 27 July 2009

Aspergillus nidulans possesses three *pmt* genes encoding protein O-D-mannosyltransferases (Pmt). Previously, we reported that PmtA, a member of the PMT2 subfamily, is involved in the proper maintenance of fungal morphology and formation of conidia (T. Oka, T. Hamaguchi, Y. Sameshima, M. Goto, and K. Furukawa, *Microbiology* 150:1973–1982, 2004). In the present paper, we describe the characterization of the *pmtA* paralogues *pmtB* and *pmtC*. PmtB and PmtC were classified as members of the PMT1 and PMT4 subfamilies, respectively. A *pmtB* disruptant showed wild-type (wt) colony formation at 30°C but slightly repressed growth at 42°C. Conidiation of the *pmtB* disruptant was reduced to approximately 50% of that of the wt strain; in addition, hyperbranching of hyphae indicated that PmtB is involved in polarity maintenance. A *pmtA* and *pmtB* double disruptant was viable but very slow growing, with morphological characteristics that were cumulative with respect to either single disruptant. Of the three single *pmt* mutants, the *pmtC* disruptant showed the highest growth repression; the hyphae were swollen and frequently branched, and the ability to form conidia under normal growth conditions was lost. Recovery from the aberrant hyphal structures occurred in the presence of osmotic stabilizer, implying that PmtC is responsible for the maintenance of cell wall integrity. Osmotic stabilization at 42°C further enabled the *pmtC* disruptant to form conidiophores and conidia, but they were abnormal and much fewer than those of the wt strain. Apart from the different, abnormal phenotypes, the three *pmt* disruptants exhibited differences in their sensitivities to antifungal reagents, mannosylation activities, and glycoprotein profiles, indicating that PmtA, PmtB, and PmtC perform unique functions during cell growth.

Protein glycosylation, which is a major posttranslational modification, plays essential roles in eukaryotic cells from fungi to mammals (19). N-linked oligosaccharides in glycoproteins that share relatively common structures are structurally classified into high-mannose, complex, and hybrid types (3). O-linked oligosaccharides in glycoproteins are diverse with respect to their sugar components and the mode of sugar linkages among the eukaryotic organisms (8, 19). O mannosylation, which is commonly found in the glycoproteins of fungi, has been extensively studied in the budding yeast *Saccharomyces cerevisiae* (4, 21, 35). The initial reaction of mannose transfer to serine and threonine residues in proteins is catalyzed by protein O-D-mannosyltransferase (Pmt) in the endoplasmic reticulum (ER), where dolichyl phosphate-mannose is required as an immediate sugar donor (4). In the Golgi complex, O mannosylation in *S. cerevisiae* is linearly elongated by up to five mannose residues by mannosyltransferases (Mnt) that utilize GDP-mannose as the mannosyl donor. At least six Pmt-encoding genes (*PMT1* to *-6*), three α -1,2-Mnt-encoding genes

(*KRE2*, *KTR1*, and *KTR3*), and three α -1,3-Mnt-encoding genes (*MNN1*, *MNT2*, and *MNT3*) are known to be involved in O mannosylation in *S. cerevisiae* (21, 31, 45).

The Pmt family of proteins can be classified into the PMT1, PMT2, and PMT4 subfamilies based on phylogeny (6). Proteins of the PMT1 subfamily form a heteromeric complex with proteins belonging to the PMT2 subfamily, and PMT4 subfamily proteins form a homomeric complex (7). Simultaneous disruptions of three different types of *PMT* genes were lethal (4), suggesting that each class provided a unique function for O mannosylation. Yeasts other than *S. cerevisiae*, such as *Schizosaccharomyces pombe* (38, 41), *Candida albicans* (29), and *Cryptococcus neoformans* (28), possess three to five *pmt* genes, which have been characterized. Several studies provide evidence that protein O mannosylation modulates the functions and stability of secretory proteins and thereby affects the growth and morphology of these yeasts. O mannosylation by Pmt2 in *S. cerevisiae* (ScPmt2) provides protection from ER-associated degradation and also functions as a fail-safe mechanism for ER-associated degradation (11, 13, 23). Likewise, in *C. albicans*, CaPmt1- and CaPmt4-mediated O mannosylation specifically protects CaSec20 from proteolytic degradation in the ER (40). Cell wall integrity is maintained in *S. cerevisiae* by increased stabilization and correct localization of the sensor proteins ScWsc and ScMid2 due to O mannosylation by ScPmt2 and ScPmt4 (20). Similarly, the stability and localization to the plasma membrane of axial budding factor ScAxl2/Bud10 is enhanced by ScPmt4-mediated O mannosylation, in-

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† Supplemental material for this article may be found at <http://ec.asm.org/>.

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[∇] Published ahead of print on 31 July 2009.

TABLE 1. *A. nidulans* strains used in this study

Strain	Genotype ^a	Source or reference
A26	<i>biA1</i>	FGSC ^b
A89	<i>biA1 argB2</i>	FGSC
AKU89	<i>biA1 argB2 nkuB::aurA</i> ⁺	This study
Δ AnpmtA (P3)	<i>biA1 argB2 pmtA::argB</i> ⁺	26
Δ AnpmtB	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>pmtB::ptrA</i> ⁺	This study
$B\Delta$ AnpmtB	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>pmtB::ptrA</i> ⁺ <i>pyrG::pmtB</i>	This study
Δ AnpmtA- <i>pmtB</i>	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>pmtA::argB</i> ⁺ <i>pmtB::ptrA</i> ⁺	This study
Δ AnpmtC	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>pmtC::ptrA</i> ⁺	This study
$C\Delta$ AnpmtC	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>pmtC::ptrA</i> ⁺ <i>pyrG::pmtC</i>	This study
AKU89- <i>glaA</i>	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>glaA(P)-glaA-glaA(T) argB</i> ⁺	This study
Δ AnpmtB- <i>glaA</i>	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>pmtB::ptrA</i> ⁺ <i>glaA(P)-glaA-glaA(T) argB</i> ⁺	This study
Δ AnpmtC- <i>glaA</i>	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>pmtB::ptrA</i> ⁺ <i>glaA(P)-glaA-glaA(T) argB</i> ⁺	This study
AKU89-AnwscA	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>glaA(P)-wscA::3HA-glaA(T) argB</i> ⁺	This study
Δ AnpmtA-AnwscA	<i>biA1 argB2 pmtA::argB</i> ⁺ <i>glaA(P)-wscA::3HA-glaA(T) ptrA</i> ⁺	This study
Δ AnpmtB-AnwscA	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>pmtB::ptrA</i> ⁺ <i>glaA(P)-wscA::3HA-glaA(T) argB</i> ⁺	This study
Δ AnpmtC-AnwscA	<i>biA1 argB2 nkuB::aurA</i> ⁺ <i>pmtC::ptrA</i> ⁺ <i>glaA(P)-wscA::3HA-glaA(T) argB</i> ⁺	This study

^a *glaA(P)*, promoter region of *A. awamori glaA*; *glaA(T)*, terminator region of *A. awamori glaA*.

^b FGSC, Fungal Genetics Stock Center.

creasing its activity (32). ScPmt4-mediated O glycosylation also functions as a sorting determinant for cell surface delivery of ScFus1 (30). CaPmt4-mediated O glycosylation is required for environment-specific morphogenetic signaling and for the full virulence of *C. albicans* (29).

With respect to filamentous fungi like *Aspergillus* that develop hyphae in a highly ordered manner, which then differentiate to form conidiospores, little is known about the function and synthetic pathway of the O-mannose-type oligosaccharides. O-Glycans in glycoproteins of *Aspergillus* include sugars other than mannose, and their structures have been determined (8). The initial mannosylation catalyzed by Pmts is found in *Aspergillus* and occurs as in yeasts (8).

We characterized the *pmtA* gene of *Aspergillus nidulans* (AnpmtA), belonging to the PMT2 subfamily, and found that the mutant exhibited a fragile cell wall phenotype and alteration in the carbohydrate composition, with a reduction in the amount of skeletal polysaccharides in the cell wall (26, 33). Recently, the Afpmt1 gene belonging to the PMT1 family of *Aspergillus fumigatus*, a human pathogen, was characterized. AfPmt1 is crucial for cell wall integrity and conidium morphology (46).

In this study, we characterize the *pmtB* and *pmtC* genes of *A. nidulans* to understand their contribution to the cell morphology of this filamentous fungus. We also demonstrate that the PmtA, PmtB, and PmtC proteins have distinct specificities for protein substrates and function differently during cell growth of filamentous fungi.

MATERIALS AND METHODS

Strains, media, and growth conditions. The *A. nidulans* strains (listed in Table 1) were grown on YG medium (0.5% [wt/vol] yeast extract, 2.5% [wt/vol] glucose) or minimal medium (MM) (1% [wt/vol] glucose, 0.6% [wt/vol] NaNO₃, 0.052% [wt/vol] KCl, 0.052% [wt/vol] MgSO₄ · 7H₂O, 0.152% [wt/vol] KH₂PO₄, and Hunter's trace elements, pH 6.5). Liquid growth experiments to allow hyphal development in a submerged culture were done by inoculation of 2 × 10⁸ conidia into 100 ml MM or YG medium in 500-ml shaking flasks. The flasks were reciprocally shaken at 120 rpm at 30°C. Standard transformation procedures for *A. nidulans* were used (44). Plasmids were propagated in *Escherichia coli* XL-1 Blue. Genomic DNA and total RNA of *A. nidulans* were prepared as previously

described (26). Southern and Northern hybridizations were done using a DIG labeling kit (Roche) according to the manufacturer's protocols.

Isolation of the AnpmtB and AnpmtC genes. All oligonucleotide primers used in this study are listed in Table S1 in the supplemental material. Based on a multiple-sequence alignment among Pmt proteins of *A. nidulans*, *S. cerevisiae*, and *C. albicans*, degenerate oligonucleotide primers for the amplification of *A. nidulans pmtB* and *pmtC* genes were synthesized. Using primers pmtB-F/pmtB-R and pmtC-F/pmtC-R, regions of AnpmtB and AnpmtC, respectively, were amplified from *A. nidulans* A26 genomic DNA and used as probes to screen an *A. nidulans* cosmid library (Fungal Genetics Stock Center) for entire AnpmtB and AnpmtC genes. Isolated AnpmtB and AnpmtC genes were sequenced using a LIC4200L DNA sequencer (Li-Cor). The cDNAs of *pmtB* and *pmtC* were amplified by reverse transcription-PCR using total RNA from strain A26 with primer pairs An-pmtB-RT-F/An-pmtB-RT-R and An-pmtC-RT-F/An-pmtC-RT-R, respectively. The amplified DNA fragments were inserted into pGEM-T Easy (Promega) and sequenced. The sequences were analyzed with Genetyx (Genetyx Corp., Japan). BLAST searches were done using the *A. nidulans* genome database at http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html.

Construction of AnpmtB, AnpmtC, and AnpmtA AnpmtB disruptants. To facilitate gene disruption in *A. nidulans*, *nkuB*, encoding a protein (AN4552.3) involved in nonhomologous end joining (24, 25), was replaced in host strain A89 with the selectable marker *aurA*. For this purpose, a gene replacement cassette encompassing 5' *nkuB*, *aurA*, and 3' *nkuB* was constructed by recombinant PCR (18). First, the *nkuB* 5'- and 3'-flanking regions were amplified from A89 genomic DNA with primer pairs F1-PnkuB/R1-PnkuB and F3-TnkuB/R3-TnkuB, respectively, and *aurA* was amplified from pAUR316 (TakaraBio, Japan) with primers F2-aur and R2-aur. Then, after the three purified DNA fragments were combined as a template, the gene replacement cassette was amplified with primers F1-PnkuB and R3-TnkuB using LA-Taq DNA polymerase (TakaraBio) and inserted into pGEM-T Easy, yielding pGEM-*nkuB::aurA*. After digestion with EcoRV, pGEM-*nkuB::aurA* was transformed into strain A89. Transformants resistant to aureobasidin A were analyzed for correct gene replacement by PCR and Southern blot analysis, yielding strain AKU89 with a disrupted *AnnkuB* gene.

For disruption of AnpmtB with *ptrA*, conferring resistance to pyrithiamine, plasmid pGEM- Δ pmtB was constructed by insertion of a 2.0-kb KpnI fragment containing *ptrA*, amplified with primers ptrA-KpnI-F and ptrA-KpnI-R from pPTR I (TakaraBio), into the KpnI site of AnpmtB, cloned into pGEM-T Easy after PCR amplification with primers pmtB-around-F and pmtB-around-R. Strain AKU89 was transformed with pGEM- Δ pmtB linearized with NaeI. The disruption of AnpmtB in pyrithiamine-resistant transformants was confirmed by Southern blot analysis using a 1.1-kb region of AnpmtB amplified with primers pmtB-pr-F and pmtB-pr-R as a probe and by PCR using primers F2-AnpmtB and R2-AnpmtB.

For complementation of an AnpmtB disruptant with wild-type (wt) *pmtB*, a gene replacement cassette encompassing 1.3-kb 5'-*pyrG*, 4.2-kb wt *pmtB*, and

1.1-kb 3'-*pyrG* was constructed by recombinant PCR using three primer pairs, F1-AnpyrG/R1-AnpGpB, F1-AnpGpB/R2-AnpGpB, and F2-AnpGpB/R1-AnpyrG. The resultant DNA fragment amplified with primers F1-AnpyrG and R1-AnpyrG was used to transform the *AnpmtB* disruptant. Transformants were selected on MM with 10 mM arginine, 5 mM 5-fluoroorotic acid, 5 mM uridine, and 5 mM uracil. Introduction of the wt *pmtB* gene into the *AnpmtB* disruptant at the *pyrG* locus was confirmed by PCR using primer pairs F-pmtBPr/F-pmtBPr and F1-AnpyrG/R1-AnpyrG.

Similarly, *AnpmtC* was disrupted in AKU89 by *ptrA* insertion after transformation of SpeI-linearized pGEM- Δ *pmtC*, which was constructed analogously to pGEM- Δ *pmtB* with primers pmtC-around-F and pmtC-around-R to amplify *pmtC*. A 1.0-kb region of *AnpmtC*, amplified with primers pmtC-pr-F and pmtC-pr-R, was used as a probe in Southern blot analysis to verify correct disruption of *AnpmtC*. PCR with primers F3-AnpmtC and R3-AnpmtC was also done to verify disruption of *AnpmtC*.

For complementation of the *AnpmtC* disruptant with wt *pmtC*, plasmid pGT Δ sal-pyrG::pmtC was constructed as follows. A 3.5-kb DNA fragment of *pyrG* was amplified by PCR with primers F1-AnpyrG and R1-AnpyrG and inserted into pGEM-T Easy, yielding pGT-pyrG. pGT-pyrG was digested with NsiI and self-ligated, yielding pGT Δ sal-pyrG. A 3.5-kb DNA fragment of *pmtC* was replaced by part of the coding sequence of *pyrG* at the SalI site, yielding pGT Δ sal-pyrG::pmtC. The 6.5-kb DNA fragment carrying 1.3-kb 5'-*pyrG*, 3.5-kb wt *pmtC*, and 1.7-kb 3'-*pyrG* from pGT Δ sal-pyrG::pmtC was used to transform the *AnpmtC* disruptant. Introduction of the wt *pmtC* gene into the *AnpmtC* disruptant at the *pyrG* locus was confirmed by PCR using primer pairs F1-AnpyrG/R1-AnpyrG and F-AnpmtCPr/R-AnpmtCPr.

An *AnpmtA* and *AnpmtB* double disruptant was created from the Δ *AnpmtB* strain (AKU89 with *pmtB::ptrA*⁺) (Table 1) by transformation of BssHII-linearized pBS- Δ *pmtA* (26), targeting integration of *argB* into *AnpmtA* and complementing the strain's arginine auxotrophy. Southern blot analysis confirmed the disruption of both *AnpmtA* and *AnpmtB* in the selected transformants.

Analysis of the efficiency of conidiation. About 10⁵ conidia were spread onto an 84-mm minimum agar medium. After 3 days of incubation at 30°C or 42°C, the conidia formed were suspended in 5 ml 0.01% (wt/vol) Tween 20 solution and counted using a hemocytometer.

Microscopy. Submerged hyphae of *A. nidulans* were observed as follows. Conidia were inoculated into liquid medium, and then the culture was poured into a petri dish containing glass coverslips. After incubation at 30°C or 42°C for 10 to 50 h, the submerged hyphae adhering to the coverslip were stained and fixed with Myco-Perm Blue (Scientific Device Laboratory). The aerial hyphae of *A. nidulans* were observed as follows. Conidia were inoculated on agar medium and incubated at 30°C or 42°C for 2 to 7 days. The adhesive side of Fungi-Tape (Scientific Device Laboratory) was gently pressed against the aerial hyphae. The tape with the aerial hyphae was mounted on a glass slide and then stained and fixed with Myco-Perm Blue. The hyphae were observed using a Nikon Eclipse E600 microscope.

Expression of GAI of *A. awamori*. For expression of the gene encoding glucoamylase I (GAI) of *Aspergillus awamori* (*AaglaA*), pGEM-*glaAargB* was constructed as follows. *AaglaA* amplified by PCR from pBR-*glaA* (9) with primers F1-PglaA and R1-TglaA was inserted into pGEM-T Easy to yield pGT-*glaA*. The *argB* gene was amplified from pDC1 (1) with primers argB-ApaI-F and argB-ApaI-R and inserted into the ApaI site of pGT-*glaA* to yield pGEM-*glaAargB*. Strains AKU89, Δ *AnpmtB*, and Δ *AnpmtC* were transformed with pGEM-*glaAargB*, and integration of the *AaglaA* expression cassette was confirmed by Southern blot analysis. The selected *argB*⁺ transformants carrying *AaglaA* were cultured in 100 ml culture A medium (27) with 2.0% (wt/vol) maltose and 0.05% (wt/vol) glucose to induce *AaglaA* expression for 36 h at 30°C. Culture filtrates of the transformants were concentrated by centrifugation through Microcon YM-10 (Millipore) filter units and loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Detection of GAI was done by immunoblotting using an anti-GAI-antibody as described previously (27).

Expression of HA-tagged *A. nidulans wscA*. A C-terminally triple-hemagglutinin (3HA)-tagged version of AN5660.3, the *A. nidulans* homolog of the highly O-glycosylated cell surface protein Wsc1 of *S. cerevisiae*, was expressed after integration of plasmids containing a KpnI/HindIII fragment with an *AnwscA::3HA* fusion in between the *AaglaA* promoter and terminator regions derived from pGEM-*glaAargB* (see Fig. 8A). pARG-PTglaAwscA3ha corresponds to pUC119 containing the above-mentioned fusion and a 1.7-kb EcoRI fragment carrying the *argB* gene from pDC1 (1), while pPTR-PTglaAwscA3ha corresponds to the fusion construct cloned into pPTR I (TakaraBio). The integrative plasmid pPTR-PTglaAwscA3ha or pARG-PTglaAwscA3ha was transformed into strain P3 (Δ *AnpmtA argB*⁺) or AKU89, Δ *AnpmtB*, and Δ *AnpmtC*, respectively. *PtrA*⁺ or *argB*⁺ transformants carrying *AnwscA::3HA* were cultured in 100 ml culture A

medium with 2.0% (wt/vol) maltose and 0.05% (wt/vol) glucose for 36 h at 30°C, harvested by filtration, and lyophilized. The cells were mechanically broken with a Multi-beads shocker (Yasui Kikai, Japan), and proteins were extracted with 1% SDS, 9 M urea, 1 mM EDTA, 0.7 M β -mercaptoethanol, 50 mM Tris-Cl, pH 6.8. After separation of the proteins on 10% SDS-PAGE gels, WscA-3HA was detected by immunoblotting using anti-HA monoclonal antibody (Sigma).

Triton X-100 membrane protein fraction preparation. *A. nidulans* was grown in liquid MM and harvested. Freeze-dried cells were mechanically broken with the Multi-beads shocker with an equal volume of 0.5-mm glass beads, suspended in TM buffer (50 mM Tris-Cl, 5 mM MgCl₂, Complete protease inhibitor [Roche], pH 7.5) and centrifuged at 17,530 \times g for 10 min. The resultant pellet was washed two times with TM buffer, resuspended in extraction buffer (50 mM Tris-Cl, 5 mM MgCl₂, 1% [vol/vol] Triton X-100, pH 7.5), and centrifuged at 17,530 \times g for 10 min. The supernatant designated as a Triton X-100 fraction contained solubilized membrane proteins that were separated by SDS-PAGE and stained with Coomassie brilliant blue (CBB). Glycoproteins were detected by lectin blotting (10) using alkaline phosphatase-conjugated concanavalin A (EY Laboratory Inc.).

RESULTS

***AnpmtB* and *AnpmtC* genes encode Pmts.** We previously characterized *AnpmtA* encoding Pmt of *A. nidulans* (26). In the present paper, we describe the remaining *Anpmt* genes, termed *AnpmtB* and *AnpmtC*, which we cloned from a genomic cosmid library of *A. nidulans* using probes prepared with degenerate primers (see Materials and Methods). BLAST searches against the *A. nidulans* genome database with the obtained cDNA sequences of *AnpmtB* and *AnpmtC* identified the genes as AN4761.3 and AN1459.3, respectively. *AnpmtB* is a gene of 3,044 bp containing six exons and five introns and encodes the AnPmtB protein, which consists of 918 amino acids with a putative molecular mass of 103.3 kDa. *AnpmtC* is comprised of 2,424 bp with three exons and two introns and encodes a protein, AnPmtC, of 773 amino acids with a putative molecular mass of 88.2 kDa. AnPmtB and AnPmtC share relatively low (34.5%) amino acid sequence homology with each other and 37.5% and 32.8%, respectively, with AnPmtA. AnPmtB showed the highest sequence homology with Pmts from other sources, such as AfPmt1 (76.0%), CaPmt1 (41.9%), and ScPmt1 (39.6%). The highest sequence homology of AnPmtC was with ScPmt4 (48.4%). Thus, the AnPmtA, AnPmtB, and AnPmtC proteins belong to distinct families, namely, PMT2, PMT1, and PMT4, respectively, based on the phylogenetic tree constructed by the unweighted-pair group method using average linkages (34) (see Fig. S1 in the supplemental material).

We previously demonstrated that *AnpmtA* was constitutively transcribed during incubation in liquid MM at 30°C. As determined by Northern blotting, *AnpmtB* and *AnpmtC* were expressed in liquid MM for 16 h to 48 h at 30°C (Fig. 1), indicating that both genes, as well as *AnpmtA*, as previously reported (26), are functional throughout hyphal development, whereas the expression levels of *AnpmtB* and *AnpmtC* decreased over time, suggesting that the genes are mainly required for early stages of hyphal development.

Disruptions of *pmtB* and *pmtC* genes. To understand the effects of *AnpmtB* and *AnpmtC* on the growth of *A. nidulans*, we disrupted each gene in *A. nidulans* AKU89 by gene replacement with *ptrA*⁺ (see Fig. S2 in the supplemental material), yielding the Δ *AnpmtB* and Δ *AnpmtC* strains, respectively. Southern blot analysis using the 3' region of *AnpmtB* or *AnpmtC* as a probe and PCRs with primer pairs F2-AnpmtB/

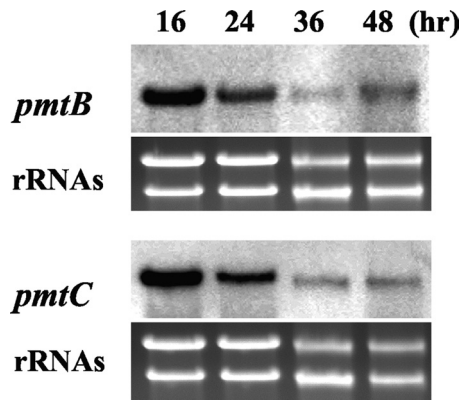


FIG. 1. Transcriptional analysis of *AnpmtB* and *AnpmtC*. Total RNA of *A. nidulans* wt strain FGSC26 was prepared after growth in MM at 30°C, and 10 μ g was loaded in each lane. Northern blot hybridization was performed using *pmtB* and *pmtC* as probes, and rRNAs were stained with ethidium bromide.

R2-*AnpmtB* and F3-*AnpmtC*/R3-*AnpmtC* revealed that site-specific recombination had occurred at the *AnpmtB* or *AnpmtC* locus and that a single copy of *ptrA*⁺ had been integrated into the chromosomal DNA.

We introduced wt *AnpmtB* and wt *AnpmtC* genes into the Δ *AnpmtB* and Δ *AnpmtC* strains, yielding *B* Δ *AnpmtB* and *C* Δ *AnpmtC*, respectively. PCRs with primer pairs F-*pmtB*Pr/R-*pmtB*Pr and F1-*AnyrG*/R1-*AnyrG*, or F-*pmtC*Pr/R-*pmtC*Pr and F1-*AnyrG*/R1-*AnyrG*, revealed that site-specific recombination of wt *AnpmtB* or wt *AnpmtC* had occurred at the *pyrG* locus and that a single copy of the wt *pmt* gene had been integrated into the chromosomal DNA (see Fig. S2 in the supplemental material).

Underglycosylation of heterologous GAI in *Anpmt* disruptants. GAI from *A. awamori* is an extracellular protein, consisting of three domains, namely, the amino-terminal catalytic domain, a serine/threonine-rich region that is glycosylated, and the starch binding domain at the carboxy terminus (12). We previously used GAI as a reporter to measure glycosylation activity and demonstrated that *AnpmtA* disruption affected the O-mannosylation of GAI (26). We integrated the GAI-encoding gene into the Δ *AnpmtB* and Δ *AnpmtC* strains (see Materials and Methods) and determined whether the absence of either PMT activity would have an effect on the glycosylation of GAI. The electrophoretic mobilities of GAI secreted from the Δ *AnpmtB* and Δ *AnpmtC* strains were similar to each other on 7% SDS-PAGE but slightly faster than that of GAI produced by the wt strain (Fig. 2). This result indicates that both *AnpmtB* and *AnpmtC* disruptions led to underglycosylation of GAI. Thus, in *A. nidulans*, complete glycosylation of GAI in vivo requires the presence of all three functionally active Pmts, AnPmtA (26), AnPmtB, and AnPmtC.

AnPmtB functions independently of AnPmtA. Since PMT1 and PMT2 subfamily proteins form heterodimers, whereas the PMT4 subfamily proteins homodimerize (7), we expected that the Δ *AnpmtB* strain would show a phenotype comparable to that of the Δ *AnpmtA* strain (26). However, in contrast to the Δ *AnpmtA* strain, the Δ *AnpmtB* strain showed colony phenotypes more similar to those of the wt strain at 30°C, and only at an elevated temperature of 42°C were slightly smaller colonies

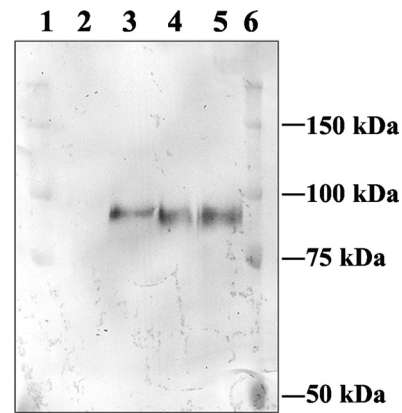


FIG. 2. Protein O-D-mannosylation of GAI. Proteins in the culture filtrates of AKU89 (lane 2) or of strains expressing GAI—AKU89-*glaA* (lane 3) and the Δ *AnpmtB-glaA* (lane 4) and Δ *AnpmtC-glaA* strains (lane 5)—were separated on 7% SDS-PAGE and immunoblotted using anti-GAI antiserum. Precision plus protein standards (Bio-Rad) were used as molecular mass markers (lanes 1 and 5).

formed, which was remedied in the presence of 0.6 M KCl as an osmotic stabilizer (Fig. 3). Furthermore, the Δ *AnpmtB* strain formed submerged hyphae similar to those of the wt strain, although with more frequent hyphal branching (Fig. 4). Aerial hyphae of the Δ *AnpmtB* strain developed normal conidiophores with wt conidia, but some hyphae ended in abnormally swollen vesicles containing a few conidia. Accordingly, the number of conidia formed in the Δ *AnpmtB* strain was reduced to 56% of the wt hyphae cultivated under similar growth conditions (on MM at 30°C for 3 days). The *B* Δ *AnpmtB* strain carrying wt *pmtB* at the *pyrG* locus in the Δ *AnpmtB* strain showed a phenotype identical to that of the wt strain with respect to colony and hyphal morphologies (Fig. 3 and 4), confirming that disruption of *AnpmtB* affects only the function of *AnpmtB*.

The fact that the Δ *AnpmtB* strain phenotypes were completely different from those of the Δ *AnpmtA* strain (26) suggested that the proteins have independent functions that do not rely on their heterodimerization, as reported for members of the PMT2 and PMT1 subfamilies (7). We further assessed the relative contributions of these proteins to the growth of *A. nidulans* by testing the Δ *AnpmtA-AnpmtB* double disruptant (see Fig. S2 in the supplemental material). The growth of the Δ *AnpmtA-AnpmtB* strain was severely impaired at 30 and 42°C, and although it slightly improved upon addition of the osmotic stabilizer, colony formation was under all conditions exceedingly more impaired than was observed in the case of the single disruptants, suggesting a synthetic defect (Fig. 3) (26). Also, abnormalities observed in the hyphal structure of the Δ *AnpmtA-AnpmtB* strain were cumulative with respect to the single disruptants (Fig. 4). The hyphae in the double disruptant were slightly swollen, with balloon structures characteristic of the Δ *AnpmtA* strain (26), and hyperbranching, as found in the Δ *AnpmtB* strain. These results confirmed that AnPmtA and AnPmtB have independent functions and that disruption of these genes caused divergent phenotypes in *A. nidulans*.

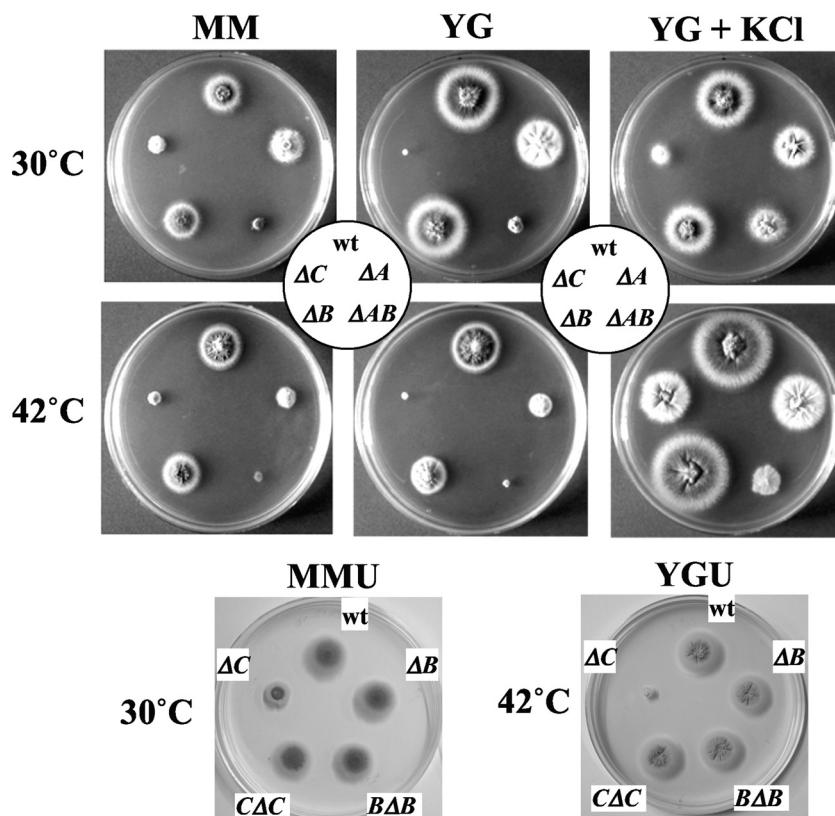


FIG. 3. Colony formation by the wt strain and *Anpmt* disruptants. *A. nidulans* strains were grown in MM and YG medium with or without 0.6 M KCl at 30°C or 42°C for 3 days. MM and YG supplemented with 5 mM uracil and 5 mM uridine (MMU and YGU, respectively) were used for cultivation of the $B\Delta AnpmtB$ and $C\Delta AnpmtC$ strains.

Disruption of *AnpmtC* impairs hyphal elongation and conidium formation. Of the three *Anpmt* disruptions, removal of *AnpmtC* caused the most remarkable defect in colony formation, which was significantly recovered only at 42°C in the presence of 0.6 M KCl (Fig. 3), 0.8 M NaCl, or 1.2 M sorbitol as an osmotic stabilizer (data not shown). Interestingly, osmotic stabilization and high temperature restored wt-like extension of submerged $\Delta AnpmtC$ strain hyphae, which otherwise remained aberrantly swollen and branched out frequently in a random spatial pattern with shorter cells (Fig. 5). Stalks with a vesicle at the hyphal tip developed after 40 h at 42°C in liquid cultures under conditions of osmotic stabilization. Conidiophores were not observed in aerial hyphae of the $\Delta AnpmtC$ strain unless the cultures were grown on plates containing an osmotic stabilizer, and conidia were formed only at 42°C despite an aberrant conidiophore structure containing several clusters of sterigmata and conidia without vesicles. Conidium formation was reduced to 6% of wt levels under these conditions. Despite osmotic stabilization, conidia were not produced from sterigmata at 30°C. The $C\Delta AnpmtC$ strain carrying wt *pmtC* at the *pyrG* locus in the $\Delta AnpmtC$ strain showed a phenotype identical to that of the wt strain with respect to colony formation, hyphal morphology, and conidiation (Fig. 3 and 5), confirming that disruption of *AnpmtC* affected only the function of *pmtC*.

Sensitivity to antifungal reagents. All *Anpmt* disruptants differed from each other and from the wt in their morpho-

gies. Osmotic stabilization of the media helped to reduce many of these defects, suggesting that the cell wall no longer fully contributed to maintaining the proper architecture of these filamentous fungi. Since Congo red, micafungin, and calcofluor white (CFW) are known to inhibit cell wall synthesis, we determined the sensitivities of *pmt* disruptants to these compounds (Fig. 6). Compared to the wt strain, both the $\Delta AnpmtB$ and $\Delta AnpmtC$ strains were more sensitive to Congo red and micafungin, which inhibit the production of β -glucans and β -1,3-glucans. In contrast, and unlike the hypersensitive $\Delta AnpmtA$ strain (26), neither the $\Delta AnpmtB$ nor the $\Delta AnpmtC$ strain showed sensitivity to CFW, which inhibits chitin synthesis. Glycosylation mutants of yeast are hypersensitive to hygromycin B (HygB), probably due to increased permeability of the cell wall. Indeed, we previously found that the $\Delta AnpmtA$ strain was hypersensitive to HygB (data not shown). Of the other *Anpmt* disruptants, only the $\Delta AnpmtC$ strain was more sensitive to HygB than the wt strain. The $\Delta AnpmtB$ strain, however, did not show any sensitivity to HygB.

Glycoprotein profiles of *Anpmt* disruptants. To obtain a better understanding of the substrate specificities of individual AnPmts, we compared the glycoprotein profiles from the three *Anpmt* disruptants on SDS-PAGE (Fig. 7). Secretory proteins that are subjected to protein glycosylation often localize to the plasma membrane. We therefore prepared membrane proteins extracted by Triton X-100 and analyzed them by staining and lectin blotting. Staining with CBB revealed comparable sets of

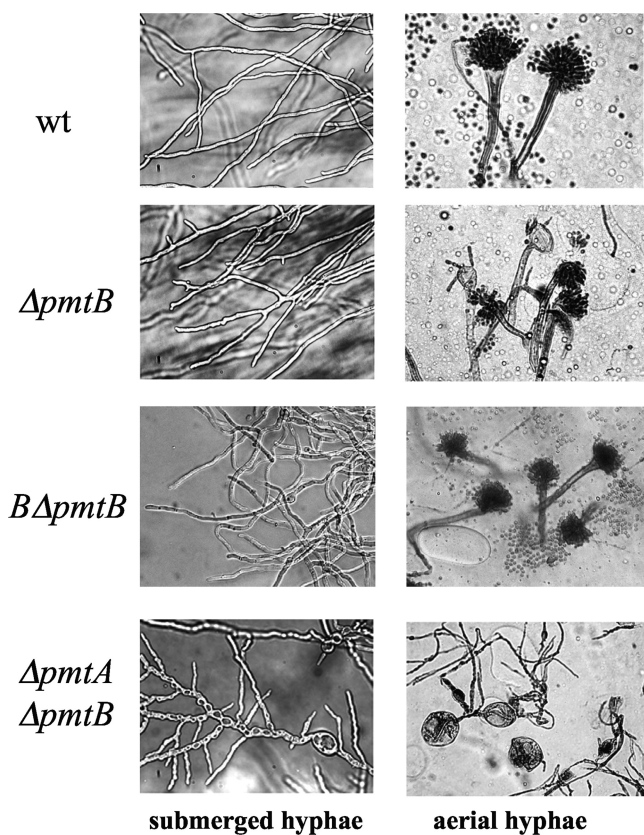


FIG. 4. Hyphal morphology of *Anpmt* disruptants. The wt strain AKU89 and the $\Delta AnpmtB$, $B\Delta AnpmtB$, and $\Delta AnpmtA-pmtB$ strains were grown at 30°C in liquid MM or MMU (MM supplemented with 5 mM uracil and 5 mM uridine) (left) and on an MM or MMU plate (right), respectively. The AKU89, $\Delta AnpmtB$, and $B\Delta AnpmtB$ strains were cultivated at 30°C for 2 days and the $\Delta AnpmtA-pmtB$ strain for 3 days.

membrane proteins from the wt, $\Delta AnpmtA$, and $\Delta AnpmtB$ strains. In the protein set of the $\Delta AnpmtC$ strain, however, proteins larger than 100 kDa and with a mass of about 75 kDa were reduced, whereas proteins in the region of 65 kDa appeared to be more abundant. Lectin blotting with concanavalin A revealed an increase in mannose-containing glycoproteins of about 80 kDa in the $\Delta AnpmtC$ strain and a decrease in those of about 40 kDa in the $\Delta AnpmtC$ strain. The glycoprotein profile of the $\Delta AnpmtB$ strain was almost indistinguishable from the wt profile, suggesting a minor role in glycosylation of proteins localizing to the plasma membrane.

Substrate specificities of AnPmts. In *S. cerevisiae*, ScPmt2 and ScPmt4 mannosylate the plasma membrane proteins ScWsc1 and ScMid2, which function as cell wall stress sensors (20). We tested whether the *A. nidulans* PMT2 (AnPmtA) and PMT4 (AnPmtC) proteins had comparable substrate specificities and therefore cloned the gene encoding AN5660.3 (termed AnWscA), which we identified in the *A. nidulans* genome as a homolog of ScWsc1. AnWscA is composed of 280 amino acids and shares 31.2% amino acid identity with ScWsc1. The protein has an N-terminal signal sequence of 24 amino acids, as predicted by the SignalIP program, and contains a Wsc motif (amino acids 25 to 123) rich in cysteine

residues, a serine- and threonine-rich region (amino acids 124 to 186), a transmembrane region (amino acids 187 to 210), and a cytoplasmic domain at the C terminus (amino acids 211 to 280) (Fig. 8A). Three putative N-glycosylation sites are found at Asn135, Asn176, and Asn258, while 31 (19 serines and 13 threonines) out of the 63 residues of the serine/threonine-rich region can serve as O-glycosylation sites.

We expressed a 3HA-tagged version of AnwscA with the HA epitope tag attached to the C terminus in the wt and the *Anpmt* disruptants and assessed the extents of glycosylation by comparing the gel mobilities of the tagged proteins (Fig. 8B). The wt strain produced AnWscA-3HA proteins with an apparent molecular mass of 50 kDa, which is higher than the calculated molecular mass of 33.5 kDa due to N and O glycosylations. Deletion of the *A. nidulans* PMT1 protein in the $\Delta AnpmtB$ strain did not affect the mobility of the tagged protein on SDS-PAGE. However, absence of the PMT2- and PMT4-like proteins in the $\Delta AnpmtA$ and $\Delta AnpmtC$ strains caused slightly faster mobility of AnWscA-3HA, presumably due to underglycosylation of the tagged protein. In addition, several bands of around 20 to 25 kDa were detected in these disruptants, but not in the wt strain or when AnPmtB was absent, suggesting that glycosylation protects the protein from N-terminal degradation. These results indicate that AnWscA-3HA is a natural substrate of AnPmtA and AnPmtC, supporting their classification as PMT2 and PMT4 proteins.

DISCUSSION

Proteins going through the secretory pathway are posttranslationally modified by O glycosylation, which is generally protein O mannosylation in fungi. In *Saccharomyces*, *Schizosaccharomyces*, *Candida*, *Cryptococcus*, *Trichoderma*, and *Aspergillus*, initial protein O mannosylation is catalyzed by Pmts (9). Accordingly, all three *pmt* genes of *A. nidulans* contribute to normal hyphal development and are expressed throughout growth, implying that protein O mannosylation plays important roles for this fungal strain. We previously characterized *AnpmtA* and *AapmtA* genes encoding PmtA belonging to the PMT2 subfamily (26, 27). Here we characterized two *Anpmt* genes encoding AnPmtB and AnPmtC belonging to the PMT1 and PMT4 subfamilies, respectively. In *A. nidulans*, the phenotypes caused by gene disruption of *AnpmtA* and *AnpmtB* are different, and those of the double disruptant are cumulative with respect to each single *pmt* disruptant, suggesting that unlike most yeast PMT1 and PMT2 subfamily proteins, the corresponding AnPmtB and AnPmtA proteins in *A. nidulans* function in an independent manner. Interestingly, ScPmt6, the third PMT2 protein in *S. cerevisiae*, also does not behave as a canonical PMT protein in the sense that no interactions with other Pmts or with itself have been observed (7).

We attempted to determine the *in vivo* substrate specificities of AnPmt proteins by assessing the extent of glycosylation of GAI. Underglycosylation of GAI in the absence of AnPmtA was demonstrated previously (26). Strains in which either *AnpmtB* or *AnpmtC* was disrupted also secreted underglycosylated GAI, indicating that the three AnPmts share substrate specificity for GAI and are involved in the mannosylation of the high number of hydroxyamino acids.

In contrast, AnPmtB did not significantly contribute to the

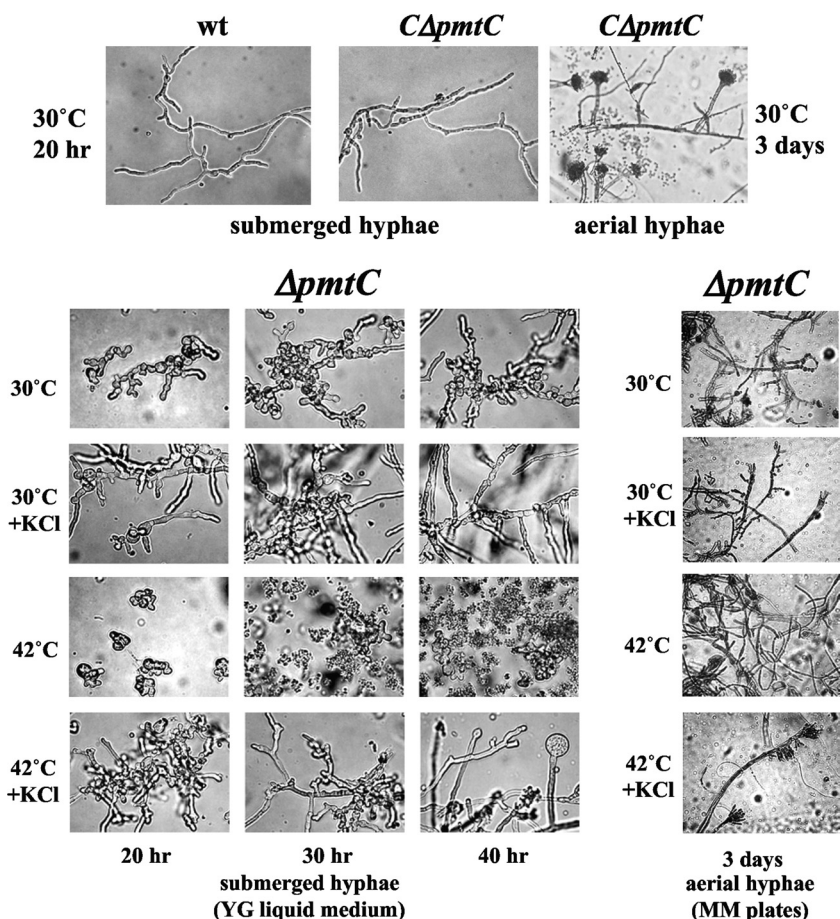


FIG. 5. (Bottom) Hyphal morphology of the Δ AnpmtC strain grown in YG liquid medium and on an MM plate with or without 0.6 M KCl. (Top) The wt and $C\Delta$ AnpmtC strains were grown in liquid MMU (MM supplemented with 5 mM uracil and 5 mM uridine) (submerged hyphae) and on an MMU plate (aerial hyphae).

glycosylation of AnWscA-3HA. However, in the absence of either AnpmtA or AnpmtC, AnWscA mannosylation was affected, and two major protein bands with molecular masses between 20 and 25 kDa were formed, suggesting that proteolytic cleavage had occurred at around the middle of the protein, which corresponds to the serine/threonine-rich region. Thus, AnpmtB has a substrate specificity different from that of AnpmtA and AnpmtC. In *S. cerevisiae*, Wsc1, Wsc3, and Mid2, which act as the cell wall sensors, were determined to be substrates for ScPmt1-ScPmt2 complexes and ScPmt4 protein (20). In particular, ScPmt4 preferentially mannosylates the Ser/Thr-rich region flanked by a membrane anchor of secretory proteins (14). Thus, the substrate specificities toward Wsc proteins are conserved between the same PMT subfamily proteins of *Saccharomyces* and *Aspergillus*.

Disruption of *pmt* genes in *Aspergillus* led to phenotypes with pleiotropic abnormalities. As in the case of AnWscA, underglycosylation by a defect in Pmt activity may lead in general to proteolytic cleavage within the serine/threonine-rich regions of the Pmt substrates and subsequent underrepresentation of active versions of these proteins at their sites of action. In the absence of either the *A. nidulans* PMT2 protein AnpmtA or *A. fumigatus* PMT1, the mutant fungi lost their cell wall integrity, resulting in repressed colony formation (26, 46). AnpmtA and

Afpmt1 disruptants were therefore also hypersensitive to high temperature, CFW, and HygB. In contrast, AnpmtB disruption did not significantly affect colony formation or make the fungus hypersensitive to CFW and HygB despite the high homology between AnpmtB and AfPmt1, which argues that they belong to the same PMT1 subfamily. While the AnpmtB disruptant was further sensitive toward high concentrations of Congo red or micafungin, the major phenotype characterizing this mutant was its highly branched hyphae. Therefore, presumably due to the absence of a protein normally glycosylated by AnpmtB, the mechanism by which the germination site for a new hypha is determined is no longer properly regulated. Thus, there is a possibility that AnpmtB is involved in polarity maintenance. Since the disruption of *Afpmt1* does not affect the polarized growth of *A. fumigatus*, this strengthens our finding that PMT1 subfamily proteins of *A. nidulans* and *A. fumigatus* have different functions. Not only the disruption of AnpmtB, but also that of *Angmt* and *AfmsdS* genes involved in protein glycosylation, caused abnormal polarity (16, 17). Thus, some downstream, but unidentified, glycoproteins seem to control hyphal polarity.

Of the three analyzed AnPmts, AnpmtC appeared to be the most essential, as the absence of the protein caused the severest growth defect in terms of a repressed growth rate and aberrant morphology. The vital role AnpmtC plays for hyphal

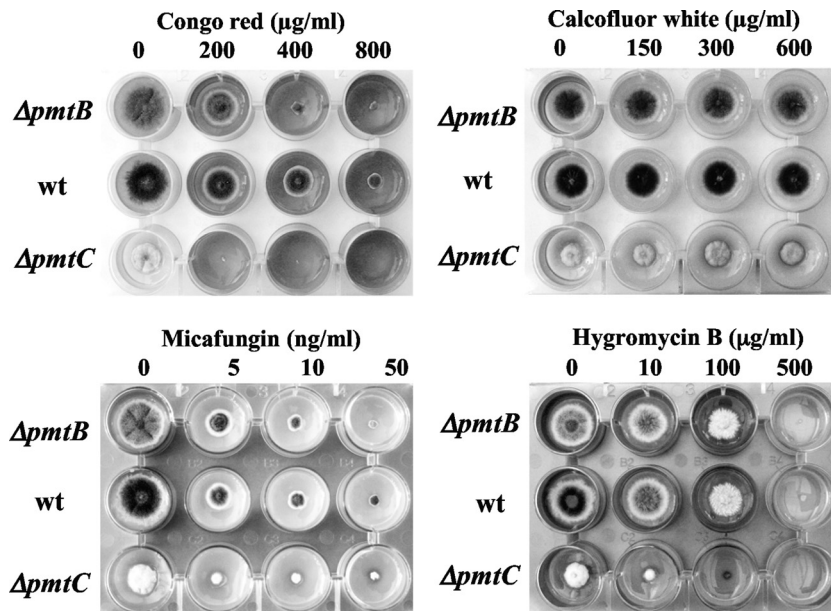


FIG. 6. Effects of antifungal reagents on colony formation by the wt, $\Delta AnpmtB$, and $\Delta AnpmtC$ strains. The strains were grown in MM in the presence of Congo red, CFW, micafungin, and HygB, as indicated, for 3 days at 30°C.

development and morphogenesis resembles that of AnChsB, a class III chitin synthase involved in the synthesis of cell wall chitin during hyphal growth and conidiation (2, 43). *AnchsB* mutants grow as minute colonies, form hyphae with a very high degree of branching, and cannot conidiate (15), a phenotype very similar to that of the AnPmtC disruptant. As AnChsB is a membrane protein of 916 amino acids that contains a total of 130 serine and threonine residues, it is tempting to speculate that the protein is a specific substrate for AnPmtC or interacts closely with one. Interestingly, a null mutant for another chitin synthase gene, *AnesmB*, forms abnormally branched conidiophores (37). Conidiophores generated in the absence of

AnPmtC were also abnormal, with several clusters of sterigmata and conidia without vesicle formation.

Hyphal polarity, as well as cell wall integrity, is closely associated with the synthesis and degradation of α - and β -glucans and chitin. *A. nidulans chiA* encodes a class III chitinase with a Ser/Thr/Pro-rich region and a glycosylphosphatidylinositol anchor attachment motif. AnChiA is heavily O glycosylated and localizes at hyphal branching sites (42); however, disruption of the gene did not affect the hyphal and conidiophore morphology, as we observed in the absence of AnPmtA or AnPmtC, but decreased the hyphal growth rate (36). In *S. cerevisiae*, β -1,3-glucanansyltransferase (ScGas1), which is localized at the cell

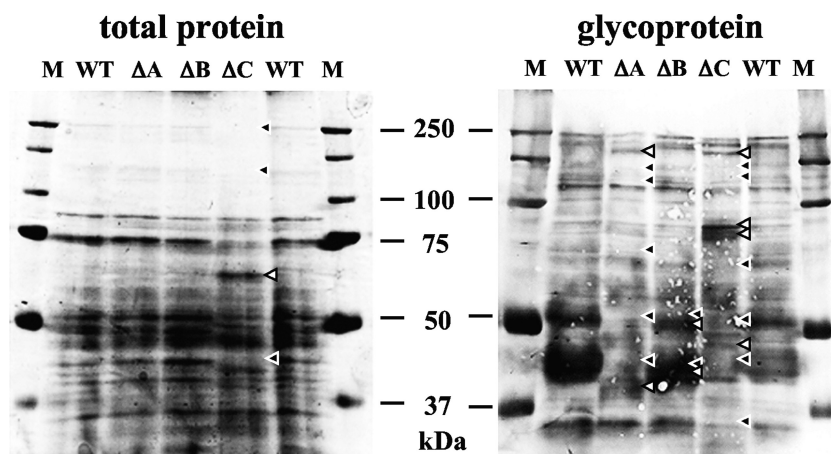


FIG. 7. Comparison of soluble membrane proteins and glycoproteins. Proteins extracted with 1% Triton X-100 were loaded onto SDS-PAGE. Total proteins were stained with CBB, and the glycoproteins were detected by lectin blot analysis with concanavalin A as described in Materials and Methods. WT, ΔA , ΔB , and ΔC indicate proteins from wt, $\Delta AnpmtA$, $\Delta AnpmtB$, and $\Delta AnpmtC$ cells, respectively. Lanes M contained Precision plus protein standards used as molecular mass markers. Proteins that appeared in the wt but disappeared in the $\Delta Anpmt$ strain are indicated by black arrowheads; proteins that appeared in the $\Delta Anpmt$ strain but not in the wt are indicated by white arrowheads.

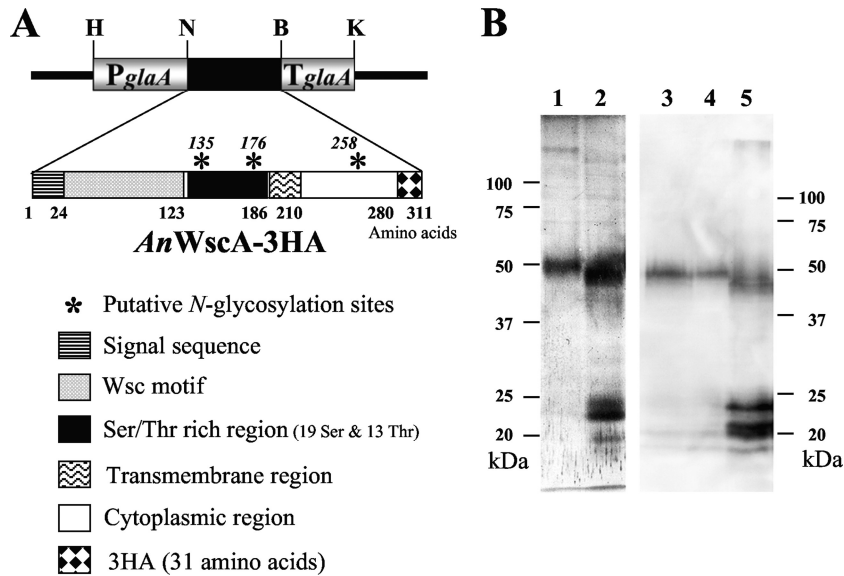


FIG. 8. AnWscA-3HA expression in the wt and the *Anpmt* disruptants. (A) Schematic representation of the integrated expression cassette consisting of the *AaglaA* promoter (P) and terminator (T) flanking an *Anwsc* open reading frame C-terminally tagged with 3HA. The protein domains and restriction sites used are indicated: H, HindIII; N, NotI; S, SalI; B, BglII; and K, KpnI. (B) AnWscA-3HA expressed in integrants derived from the AKU89 (lanes 1 and 3), Δ *AnpmtA* (lane 2), Δ *AnpmtB* (lane 4), and Δ *AnpmtC* (lane 5) strains was detected by immunoblotting using monoclonal anti-HA antibody.

surface via a glycosylphosphatidylinositol anchor, is a substrate of ScPmt4 and ScPmt6 (5, 39). The *Scgas1* null mutation resulted in defective cell wall architecture. *A. fumigatus* genes homologous to *ScGAS1* were found to be *Afgel1* and *Afgel2*. Disruption of *Afgel1* did not cause a phenotype, but the *Afgel2* disruptant exhibited slower growth than the wt, abnormal conidiogenesis, and altered cell wall composition (22). Both proteins contain a serine/threonine-rich region near the C terminus, and it will be interesting to know which AnPmts glycosylate these proteins.

An increasing number of genes responsible for synthesis and degradation of the cell wall and polarity establishment and maintenance have been identified in filamentous fungi. However, the localization and glycosylation of most of these proteins remain to be characterized. We are currently in the process of identifying these target proteins using the *pmt* disruptants, hoping to reveal how the glycoproteins maintain fungal morphology, hyphal development, and differentiation.

ACKNOWLEDGMENT

This work was supported in part by a Ministry of Education, Science, Sports, and Culture Grant-in-Aid for Scientific Research (C).

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