Prepared by a helpful assistant.
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 conidiophores, 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 Anpmt1 gene belonging to the PMT1 family of Aspergillus fumigatus, a human pathogen, was characterized. AnPmt1 is crucial for cell wall integrity and conidium morphology (46).

In this study, we characterize the pmtB and pmtC genes of Anidulans 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, 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 development to allow hyphal development in a submerged culture were done by inoculation of 2 x 103 conidia into 100 ml MM or YM 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-PF/pmtB-PR and pmtC-PF/pmtC-PR, 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 LIC4200 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-RTF/An-pmtB-RT-R and An-pmtC-RTF/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, nkaB encoding a protein (AN4552.3) involved in nonhomologous end joining (24, 25) was replaced in both strains A26 and FGSC8 with the selectable marker auraA. For this purpose, a gene replacement cassette encompassing 5 kba and auraA, and 3′ kba was constructed by recombinant PCR (18). First, the kba 5′- and 3′-flanking regions were amplified from A89 genomic DNA with primer pairs F1-PnkaB1R1-PnkaB1 and F3-TnkaB1-R3-TnkaB1, respectively, and auraA was amplified from pURA316 (TakaraBio, Japan), with primers auraA-F1 and auraA-R2. Then, after the three purified DNA fragments were combined as a template, the gene replacement cassette was amplified with primers F1-PnkaB1 and R3-TnkaB1 using LA-Tag DNA polymerase (TakaraBio) and inserted into pGEM-T Easy, yielding pGEM-nkaB::auraA. After digestion with EcoRV, pGEM-nkaB::auraA was transformed into strain A89. Transformants resistant to aureobasidin A were analyzed for correct gene replacement by PCR and Southern blot analysis, yielding strain A26/A89 with a disrupted AnauraA gene.

For disruption of AnpmtB with ptaA, conferring resistance to pyrithiamine, plasmid pGEM-ΔPmtB was constructed by insertion of a 2.0-kb KpnI fragment containing ptaA, amplified with primers ptaA-KpnI-F and ptaA-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 A26/A89 was transformed with pGEM-ΔPmtB linearized with Nael. 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-AnpyrGpB, F1-AnpyrGpB/R2-AnpyrGpB, and F2-AnpyrGpBR1-AnpyrG. The resultant DNA fragment amplified with primers F1-AnpyrG and R1-AnpyrG was used to transform the AnpyrB 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 ptrB gene into the AnpyrB disruptant at the ptrG locus was confirmed by PCR using primer pairs F-pmtBPr/F-pmtBPr and F1-AnpyrG/R1-AnpyrG. Similarly, AnpyrG was disrupted in AKU89 by ptrC insertion after transformation of SpeI-linearized pGEM-pyrG, which was constructed analogously to pGEM-ΔpmtB with primers ptrC-ΔC-F and ptrC-ΔC-R to amplify pmtC. A 1.0-kb region of AnpyrG, amplified with primers ptrC-ΔC-F and ptrC-ΔC-R, was used as a probe in Southern blot analysis to verify correct disruption of AnpyrG. PCR with primers F3-AnpyrC and R3-AnpyrC was also done to verify disruption of AnpyrC.

For complementation of the AnpyrC disruptant with wt ptrC gene, plasmid pGTAλ-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 pGTA-pyrG. pGTA-pyrG was digested with NsiI and self-ligated, yielding pGTAλ-pyrG. A 3.5-kb DNA fragment of ptrC was replaced by part of the coding sequence of pmtC at the SalI site, yielding pGTAλ-pyrG:pmtC. The 6.5-kb DNA fragment carrying 1.3-kb 5′-pyrG, 3.5-kb wt ptrC, and 1.7-kb 3′-pyrG from pGTAλ-pyrG:pmtC was used to transform the AnpyrC disruptant. Introduction of the wt ptrC gene into the AnpyrC disruptant at the ptrG locus was confirmed by PCR using primer pairs F1-AnpyrG/R1-AnpyrG and F-AnpyrCPr/R-AnpyrCPr.

An AnpyrA and AnpyrB double disruptant was created from the ΔAnpyrB strain AKU89 (Table 1) by transformation of BssHII-linearized pBS-ΔpmtA (26), targeting integration of argB into AnpyrA and complementing the strain’s arginine auxotrophy. Southern blot analysis confirmed the disruption of both AnpyrA and AnpyrB in the selected transformants.

Analysis of the efficiency of conidiation. About 10^8 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 gene (GAI) of A. awamori Awamori (AnpmtB), pGEM-BlgluargB was constructed as follows. AnglA gene from pDC1 (1), while pPTR-ΔgluargB was inserted into pGEM-T Easy to yield pGEM-ΔgluargB. Strains AKU89, ΔAnpyrG, and ΔAnpyrC were transformed with pGEM-ΔgluargB, and the resultant plasmid 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 a Multi-beads shaker (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).

RESULTS

AnpyrB and AnpyrC genes encode Pmts. We previously characterized AnpyrA encoding Pmt of A. nidulans (26). In the present paper, we describe the remaining Anpmt genes, termed AnpyrB and AnpyrC, 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, AnpmtA and AnpmtB 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 ptrC (see Fig. S2 in the supplemental material), yielding the ΔAnpmtB and ΔAnpmtC strains, respectively. Southern blot analysis using the 3′ region of AnpmtB or AnpmtC as a probe and PCRs with primer pairs F2-AnpmtB/
R2-AnpmtB and F3-AnpmtC/R3-AnpmtC revealed that sitespecific 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 ∆AnpmtB and ∆AnpmtC strains, yielding B∆AnpmtB and C∆AnpmtC, respectively. PCRs with primer pairs F-pmtBPr/R-pmtBPr and F1-AnyrG/R1-AnpyrG, or F-pmtCPr/R-pmtCPr and F1-AnyrG/R1-AnpyrG, 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 ∆AnpmtB and ∆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 ∆AnpmtB and ∆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 ∆AnpmtB strain would show a phenotype comparable to that of the ∆AnpmtA strain (26). However, in contrast to the ∆AnpmtA strain, the ∆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 formed, which was remedied in the presence of 0.6 M KCl as an osmotic stabilizer (Fig. 3). Furthermore, the ∆AnpmtB strain formed submerged hyphae similar to those of the wt strain, although with more frequent hyphal branching (Fig. 4). Aerial hyphae of the ∆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 ∆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∆AnpmtB strain carrying wt pmtB at the pyrG locus in the ∆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 ∆AnpmtB strain phenotypes were completely different from those of the ∆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 ∆AnpmtA-AnpmtB double disruptant (see Fig. S2 in the supplemental material). The growth of the ∆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 ∆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 ∆AnpmtA strain (26), and hyperbranching, as found in the ∆AnpmtB strain. These results confirmed that AnpmtA and AnpmtB have independent functions and that disruption of these genes causes divergent phenotypes in A. nidulans.
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 /H9004 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 /H9004 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 CΔAnpmtC strain carrying wt pmtC at the pyrG locus in the ΔAnpmtC 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 morphologies. 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 ΔAnpmtB and ΔAnpmtC 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 ΔAnpmtA strain (26), neither the ΔAnpmtB nor the ΔAnpmtC 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 ΔAnpmtA strain was hypersensitive to HygB (data not shown). Of the other Anpmt disruptants, only the ΔAnpmtC strain was more sensitive to HygB than the wt strain. The ΔAnpmtB 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. 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 BΔAnpmtB and CΔAnpmtC strains.](image-url)
We tested whether the ScWsc1 and ScMid2, which function as cell wall stress sensors, contain a Wsc motif (amino acids 25 to 123) rich in cysteine residues, as predicted by the SignalIP program, and contain molecular mass of 50 kDa, which is higher than the calculated molecular mass of 33.5 kDa due to N and O glycosylation. Deletion of the A. nidulans PMT1 protein in the ΔAnpmtB strain did not affect the mobility of the tagged protein on SDS-PAGE. However, absence of the PMT2- and PMT4-like proteins in the ΔAnpmtA and ΔAnpmtC 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 wild type or when AnPmtB was absent, suggesting that glycosylation protects the protein from N-terminal degradation. These results indicate that AnWsc-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 proteins. 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 AnpmtA 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 PMT4 subfamilies, the corresponding AnPmtB and AnPmtC 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 AnPmt proteins share substrate specificity for GAI and are involved in the mannosylation of the high number of hydroxyl amino acids.

In contrast, AnPmtB did not significantly contribute to the...
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 Apmt1 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 Apmt1, 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 Apmt1 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 Apmt1, but also that of Anpmt 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. 5. (Bottom) Hyphal morphology of the ΔAnpmtC strain grown in YG liquid medium and on an MM plate with or without 0.6 M KCl.](image)
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, AnchsB, forms abnormally branched conidiphores (37). Conidiphores 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 conidiphore morphology, as we observed in the absence of AnPmtA or AnPmtC, but decreased the hyphal growth rate (36). In S. cerevisiae, β-1,3-glucanosyltransferase (ScGas1), which is localized at the cell

FIG. 6. Effects of antifungal reagents on colony formation by the wt, ΔAnpmtB, and ΔAnpmtC 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.

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, ΔAnpmtA, ΔAnpmtB, and ΔAnpmtC cells, respectively. Lanes M contained Precision plus protein standards used as molecular mass markers. Proteins that appeared in the wt but disappeared in the ΔAnpmt strain are indicated by black arrowheads; proteins that appeared in the ΔAnpmt strain but not in the wt are indicated by white arrowheads.
surface via a glycosylphosphatidylinositol anchor, is a substrate of ScPmt4 and ScPmt6 (5, 39). The Sgs1 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.

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