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Aspergillus nidulans Protein *O*-Mannosyltransferases Play Roles in Cell Wall Integrity and Developmental Patterning▽

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Protein *O*-mannosyltransferases (Pmts) initiate *O*-mannosyl glycan biosynthesis from Ser and Thr residues of target proteins. Fungal Pmts are divided into three subfamilies, Pmt1, -2, and -4. *Aspergillus nidulans* possesses a single representative of each Pmt subfamily, *pmtA* (subfamily 2), *pmtB* (subfamily 1), and *pmtC* (subfamily 4). In this work, we show that single Δ*pmt* mutants are viable and have unique phenotypes and that the Δ*pmtA* Δ*pmtB* double mutant is the only viable double mutant. This makes *A. nidulans* the first fungus in which all members of individual Pmt subfamilies can be deleted without loss of viability. At elevated temperatures, all *A. nidulans* Δ*pmt* mutants show cell wall-associated defects and increased sensitivity to cell wall-perturbing agents. The Δ*pmt* mutants also show defects in developmental patterning. Germ tube emergence is early in Δ*pmtA* and more frequent in Δ*pmtC* mutants than in the wild type. In Δ*pmtB* mutants, intrahyphal hyphae develop. All Δ*pmt* mutants show distinct conidiophore defects. The Δ*pmtA* strain has swollen vesicles and conidiogenous cells, the Δ*pmtB* strain has swollen conidiophore stalks, and the Δ*pmtC* strain has dramatically elongated conidiophore stalks. We also show that AN5660, an ortholog of *Saccharomyces cerevisiae* Wsc1p, is modified by PmtA and PmtC. The Δ*pmt* phenotypes at elevated temperatures, increased sensitivity to cell wall-perturbing agents and restoration to wild-type growth with osmoticum suggest that *A. nidulans* Pmts modify proteins in the cell wall integrity pathway. The altered developmental patterns in Δ*pmt* mutants suggest that *A. nidulans* Pmts modify proteins that serve as spatial cues.

Filamentous fungi use highly polar growth to explore their environments. Except for a brief period of isotropic expansion just after spores break dormancy, filamentous fungi add new cell wall material exclusively at the tips of tubular hyphal cells. Such polar growth involves a high degree of coordination between signals from the environment and the secretory apparatus. In fungi, O mannosylation of specific target proteins has been shown to be important for sensing environmental stress, stabilizing the cell wall, and proper development (18, 28). The assembly of protein linked *O*-mannosyl glycans in the endoplasmic reticulum lumen is catalyzed by protein *O*-mannosyltransferases (Pmts), which transfer a single mannosyl residue to the hydroxyl group of serine or threonine residues to form an α-D-mannosyl linkage (30). The addition of further carbohydrate residues to the first O-linked mannose occurs in the Golgi apparatus and involves a range of enzymes (35). Modification by Pmts seems to be specific to proteins that are synthesized and sorted in the secretory pathway; however, the only motif so far identified is that Ser/Ter-rich membrane-bound proteins are O mannosylated by Pmt4 in *Saccharomyces cerevisiae* (15). This lack of a clear motif makes identification of Pmt targets by computational methods challenging. All of the fungal Pmt-modified proteins identified so far are localized to the cell membrane or cell wall or are secreted. At least 23 target proteins have been described in yeasts (15). Only three Pmt target proteins have been described in filamentous fungi (12, 23, 37).

Pmts have been found in both prokaryotes and eukaryotes (33), but not in plants (8). The lengths and compositions of *O*-mannosyl glycans are different among species. In fungi, *O*-glycosyl chains range from 2 to 7 residues. In *S. cerevisiae*, the mannosyl chain can be modified by mannosyl phosphate (6). In *Schizosaccharomyces pombe*, the *O*-linked glycan is capped with 1 or 2 galactose residues (6). In the filamentous fungi so far examined, *O*-glycans are linear and branched, with 3 to 5 monosaccharide residues (4).

In fungi, the Pmts are classified into the Pmt1, Pmt2, and Pmt4 subfamilies, with each species having three to seven members. *S. cerevisiae* and *Candida albicans* Pmts are the most redundant, with subfamilies 1 and 2 containing two or three members (7, 26). *S. pombe* and many filamentous fungi, including *Aspergillus nidulans*, have one representative from each subfamily. In *S. cerevisiae*, the enzymatic activity of Pmts requires interaction among members of the Pmt1 and Pmt2 subfamilies, while Pmt4 forms homomeric complexes (8). Heteromeric complexes between Pmt1 and Pmt2 subfamily members have also been reported in *S. pombe* (34).

O mannosylation appears to be required for the stability, localization, and function of target proteins (18, 28, 32), and in vivo consequences of Pmt loss range from limited to lethal. In *S. cerevisiae*, O mannosylation is essential for cell integrity and cell wall rigidity (7). In *C. albicans* and *Cryptococcus neoformans*, Pmt mutation affects morphogenesis and virulence (24, 26, 27). In *S. cerevisiae*, strains with single Pmt subfamily representatives deleted are viable; however, deletion of subfamily 2 representatives is lethal in *S. pombe* and *C. albicans* (7, 34). In filamentous fungi, deletion of individual Pmts has been reported. Deletions of *Trichoderma reesei pmtI*, *Aspergillus fumigatus pmt1*, *A. nidulans pmtA*, and *Aspergillus awamori pmtA* were not lethal but affected growth and development (10, 22, 23, 37).

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TABLE 1. *A. nidulans* strains and plasmids

Strain or plasmid	Genotype/phenotype	Source or reference
Strains		
A850	*argB2*::*trpC_B methG*	FGSC
A773	*pyrG89 wA3 pyroA4*	FGSC
TNO2A7/A1145	*pyrG89* Δ*nkuA*::*argB pyroA4 riboB2*	21a
ATK08	*pyrG89 argB2*::*trpC_B pyroA4* Δ*pmtA*::Af*pyrG*	This study
ATK16	*pyrG89* Δ*pmtB*::Af*pyrG argB2 pyroA4*	This study
ATK38	*pyrG89 wA3 argB2 pyroA4* Δ*pmtC*::Af*pyrG*	This study
ATK40	*pyrG89 wA3 argB2*::*trpC_B pyroA4*	This study
ATK45	*pyrG89 argB2*::*trpC_B nkuA*::Af*pyro pyroA4*	This study
ATK57	*pryG89* Δ*pmtB*::Af*pyro nkuA*::*argB pyroA4 riboB2*	This study
ATK88	*pyrG89 wA3 argB2*::*trpC_B pyroA4* Δ*pmtC*::Af*argB*	This study
ATK94	*pyrG89 argB2*::*trpC_B* Δ*pmtB*::Af*pyrG pyroA4* Δ*pmtA*::Af*pyro*	This study
ATK104	*pryG89 wA3* Δ*pmtB*::Af*pyro argB2 pyroA4*	This study
ATK147	A850::Af*argB-gpd(P)*-AN5660-S-tag	This study
ATK148	ATK16::Af*argB-gpd(P)*-AN5660-S-tag	This study
ATK149	ATK08::Af*argB-gpd(P)*-AN5660-S-tag	This study
ATK160	ATK38::Af*argB-gpd(P)*-AN5660-S-tag	This study
Plasmids		
pDV2	Amp^r *argB-gpd(p)-ccdB-sgfp*	32a
pFNO3	Kan^r GA5-GFP Af*pyrG*	36
pHL85	Amp^r Kan^r GA5-mCherry Af*pyro*	B. R. Oakley
pAO81	GA4-S-Tag Af*pyrG*	36
pAf*argB2*	Amp^r *argB2*	G. S. May
pTK59	pENTR/D-TOPO::AN5660-S-tag	This study
pTK64	pDV2::AN5660-S-tag	This study

In previous work, we identified the *swoA* mutant from a collection of temperature-sensitive polarity mutants and showed that the *swoA* allele encoded a Pmt2 subfamily member (PmtA) (21, 29). In this study, we use Δ*pmt* strains to show that each of the three Pmts in *A. nidulans* (*pmtA*, *pmtB*, and *pmtC*) is nonessential but that all play distinct roles in cell wall integrity and developmental patterning. We also demonstrate that PmtA and PmtC modify an ortholog of *S. cerevisiae* Wsc1, a known Pmt target. Because of redundancy, all Pmt1 and Pmt2 subfamily members have not been deleted in *S. cerevisiae*. Because of lethality, the effects of loss of the Pmt2 subfamily cannot be addressed in *S. pombe* or *C. albicans.* This makes *A. nidulans* the first fungus in which the phenotypes of deleted strains for each Pmt subfamily have been reported.

MATERIALS AND METHODS

***Aspergillus* strains and media.** The strains used in this study are listed in Table 1. Complete medium (CM) consisted of 1% glucose, 2% peptone, 1% yeast extract, 1% Casamino Acids, and 0.01% vitamins and supplements. Minimal medium contained 1% glucose and supplements. Nitrate salts solution, trace elements, vitamins, and amino acid supplements were based on the appendices to Kafer (16) and Hill and Kafer (14). The pHs of media were adjusted to 6.5 with 1.0 N NaOH. CM was used for phenotypic studies and mycelium production. Minimal medium was used for marker selection and maintaining routine stocks. For solid media, 1.8% agar was added, and 0.6 M KCl or 1 M sorbitol was used as an osmostabilizer.

Phylogenetic analysis. Pmt sequences were obtained from the following databases: *A. nidulans*, http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html; *C. albicans*, http://www.candidagenome.org/; *S. cerevisiae*, http://www.yeastgenome.org/; *S. pombe*, http://www.genedb.org/genedb/pombe/index.jsp; *Homo sapiens* and *Mycobacterium tuberculosis*; http://www.ncbi.nlm.nih.gov/. The gene and accession numbers of the protein sequences are as follows: ScPmt1 (*S. cerevisiae* Pmt1), YDL095W; ScPmt2, YAL023C; ScPmt3, YOR321W; ScPmt4, YJR143C; ScPmt5, YDL093W; ScPmt6, YGR199W; AnPmtA (*A. nidulans* PmtA), AN5105; AnPmtB, AN4761; AnPmtC, AN1459; SpOgm1 (*S. pombe* Ogm1)/Oma1, SPAC22A12.07c; SpOgm2/Oma2, SPAPB1E7.09; SpOgm4/Oma4, SPBC16C6.09; HsPomt1 (*H. sapiens* Pomt1) isoform a, gi116517319; HsPomt2, gi32455271; MtPmt (*M. tuberculosis* Pmt), gi15608142; CaPmt1 (*C. albicans* Pmt1), orf19.5171; CaPmt2, orf19.6812; CaPmt4, orf19.4109; CaPmt5, orf19.7549; CaPmt6, orf19.3802. ClustalX2 was used for protein alignments, building neighbor-joining trees, and bootstrap analysis (17). Trees were viewed using TreeView 1.6.6 (25).

***pmt* gene replacements.** The *pmt* gene replacement cassettes were constructed by fusion PCR based on the method of Yang and colleagues (36). Approximately 2 kb upstream and downstream of *pmt* sequences was amplified using genomic DNA from strain A850 as the template (Table 1). The *A. fumigatus* marker genes were amplified from plasmids listed in Table 1. All primers used in this study are listed in Table 2. Each individual fragment was synthesized with the AccuPrime *Pfx* DNA polymerase in a total volume of 50 μl with adjusted annealing temperatures (Invitrogen Co., Carlsbad, CA). The amplicons were separated on an agarose gel, purified using the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA), and transformed into TNO2A7, a Δ*nkuA*::*argB* strain. Transformants were tested for homologous integration by PCR, using a forward primer located upstream of the cassette and a reverse primer located inside the auxotrophic marker (Table 2), and Southern hybridization using standard protocols (2). Each Δ*pmt* Δ*nkuA* strain was crossed with appropriately marked *nkuA*$^+$ strains, and Δ*pmt nkuA*$^+$ progeny were selected. To facilitate double-mutant construction, we created an *nkuA*::Af*pyro* (*A. fumigatus pyro*) strain. Approximately 2 kb upstream and downstream of *nkuA* (AN7753), sequence was amplified using genomic DNA from strain A850 as the template. The *pyro* gene of *A. fumigatus* was amplified from plasmid pBS::Af*pyro* (Table 2). Fusion PCR and transformation were performed as described above. The replacement cassette was transformed into TNO2A7 (*pyrG89* Δ*nkuA*::*argB pyroA4 riboB2*). Transformants were tested for homologous integration by PCR and Southern hybridization. A Δ*nkuA*::Af*pyro* transformant was crossed to ATK40 to obtain the *argB* marker, yielding strain ATK45. Random ascospore analysis was based on the method of Harris (11).

Chemical sensitivity tests. Strain A850 was used as a control for all experiments. Sensitivity to chemicals was tested by spotting 5 μl of a 10-fold serial dilution of conidia (10^6 to 10^2) on CM agar plates. The plates were incubated at 25°C, 30°C, 37°C, or 42°C. Stock solutions of chemicals were prepared as follows: Calcofluor, 1% in 25 mM KOH, and 10 mg/ml Congo red. All chemicals were filtered, sterilized, and added to autoclaved medium to give the following final concentrations: Calcofluor, 10 μg/ml; Congo red, 25 μg/ml.

Microscopic examination. To examine germlings, 1×10^5 spores were grown in 10 ml of liquid CM in a petri dish containing a glass coverslip and incubated

TABLE 2. Primers

Primer name	Sequence
pmtBupF	TAGGCGTTGTAGTATGTTGGAGTTTCCATG
pmtBupR	CAGGACTCCCAAGGAGAAACGAATCAATCTG
pmtB-AfpyrGF	GATTCGTTTCTCCTTGGGAGTCCTGGCCTCAAACAATGCTCTTCACCCTC
pmtB-AfpyrGR	GTTCAACAGACCGACATATTATCCTGTCTGAGAGGAGGCACTGATGCG
pmtBdnF	GATAATATGTCGGTCTGTTGAACTACCTGCC
pmtBdnR	ACTTTCACTTCTCACTGTCGTCGTATTCGC
CheckpmtBF	GAATATACCGGACCAACAGGTTGAGCG
CheckAfpyrGR	CAGAGCCCACAGAGCGCCTTGAG
PmtB-AfpyroF	GATTCGTTTCTCCTTGGGAGTCCTGGACATCAGATGCTGGATTACTAAG
PmtB-AfpyroR	GTTCAACAGACCGACATATTATCCACAATCAGCTTTTCAGAATTCGCG
PmtBdnF	GATAATATGTCGGTCTGTTGAACTACCTGCC
PmtBdnR	GCGAATACGACGACAGTGAGAAGTGAAAGT
CheckAfpyroR	CAGCGCTTGTGCCCCTCCATCTCCC
pmtAUFF	GGTACAACAGACGTGTCCTTATTGCAATGTC
pmtAUPR	GATGAGAGATAATAGGAGAAGTGGCCGGTC
pmtAdnF	GAACCGTGATAGAGCGTTACAGTTCCCGTTG
pmtAdnR	GCGATCCCAATCCTTCCTATCTCTGTCATC
pmtA-AfpyrGF	CCACTTCTCCTATTATCTCTCATCGCCTCAAACAATGCTCTTCACCCTC
pmtA-AfpyrGR	AACTGTAACGCTCTATCACGGTTCCTGTCTGAGAGGAGGCACTGATGCG
CheckpmtAF	TTACACGTCAAGAAGGGGAACGATCTTACG
checkpmtAR	GGCTGATATGGGTCGACCGATTTTCTATTC
PmtCupF	GCGCACCTCATATTAGGATAGGATGTGATC
PmtCupR2	AATCACCAGGCCAAAACAACAGAGATG
PmtCdnF	GAGCATCTGATCTCATCTCGTTCTCTCCC
PmtCdnR	CTATCCACGGTATGAGCTGAGCGAGTAATG
pmtC-AfargBF	GTTGTTTTGGCCTGGTGATTAATGGCTGGGGGAGTGGGG
pmtC-AfargBR	AGAACGAGATGAGATCAGATGCTCCGATTTTCATAGGATTTTCCCCTTG
UpnKuF	CCATCCCACGAGTCCGAGAACTGATCATG
UPnKuR	GGCGTCTTGAATACAACTGGGGGTTCGATC
nKu-AfpyroF	AACCCCCAGTTGTATTCAAGACGCCGACATCAGATGCTGGATTACTAAG
nKu-AfpyroR	CCTAGATAACCGACAACAGAGTCACCACAATCAGCTTTTCAGAATTCGC
DNnKuF	GTGACTCTGTTGTCGGTTATCTAGGACTCG
DNnKuR	GTCTCTCGTTCCAGTCATCTACAGCGGTTC
CheckKuAF	GCAGTTGCCGCGCGGTTGGTGTGTC
AN5660F	CACCATGAGGTCGTTCACGCTATCCACAGTCTTC
AN5660StagR	GCGCCTGCACCAGCTCCGTGCCGGTCAGGATTCG
StagF	GGAGCTGGTGCAGGCGCTGGAGC
StagR	GCGCCAATTGCTGTTGCCAGGTGAGG

at 30°C or 42°C for 5 to 12 h. To examine conidiophores, 100 conidia in 5 μl of water were dropped on each side of a CM agar block, placed between two coverslips, and incubated for 3 days at different temperatures. To stain cell walls and nuclei, coverslips with adhering cultures were incubated in fixer (3.7% formaldehyde, 50 mM phosphate buffer [pH 7.0], and 0.2% Triton X-100) for 30 min. The coverslips were then briefly dipped in deionized water, incubated for 5 min in a staining solution (10 mg/ml Calcofluor and 100 ng/ml Hoechst), briefly dipped in deionized water, and mounted on a microscope slide with mounting solution (50% glycerol, 10% phosphate buffer, pH 7.0, 0.1% *n*-propyl gallate) (19). The slides were viewed with a Zeiss Axioplan epifluorescence microscope and imaged with Zeiss AxioCam Mrc software. Photoshop CS was used for micrograph organization and optimization.

Conidiation efficiency. Freshly prepared conidia (10^4 spores) were spread on CM or CM with osmoticum plates and incubated at 30°C or 42°C for 4 days. Four milliliters of sterile water was added to the plate to harvest spores by using a glass rod, and the conidial concentration in the resulting solution was determined with a hemocytometer. Graphs and standard errors were generated using Microsoft Excel. Data were from three biological replicates.

Construction and detection of S-tagged An5660/WscA. A strain carrying the Wsc ortholog An5660 with a C-terminal fusion to the S tag (a peptide epitope of 15 amino acids used for protein purification) was constructed in several steps. First, the GA4 S-tag fragment with a stop codon was amplified from pAO81 (Table 2) and the An5660 gene was amplified from the start codon to one codon before the stop codon. The two amplicons were then fused together by fusion PCR and ligated into the pENTR/D-TOPO vector using the pENTR/D-TOPO Cloning Kit (Invitrogen Co., Carlsbad, CA), yielding plasmid pTK59. The plasmid was transferred into the pMT-DV2 destination vector (Table 2) using the Gateway LR Clonase in vitro recombination kit (Invitrogen Corp., Carlsbad, CA). The resulting plasmid, pTK64, was transformed into the A850 and Δ*pmt* strains (Table 1).

Western blot analysis. For immunodetection, 1×10^7 conidia/ml were grown in 50 ml of CM and incubated on a rotary shaker at 220 rpm and 30°C for 8 h. Mycelia were filtered through cheese cloth, washed with cold stop buffer (0.9% NaCl, 1 mM NaN_3, 10 mM EDTA, 50 mM NaF, pH 7.0), and ground in liquid nitrogen. Two milliliters of cold HK buffer (3) was added to 0.1 g of ground mycelia, vortexed for 1 min, and kept on ice for 1 min (four times). The cell suspension was then centrifuged at $500 \times g$ for 5 min at 4°C. The supernatant was collected and centrifuged at $10{,}000 \times g$ for 30 min at 4°C. The pellet containing crude membranes was resuspended in a small volume of HK buffer containing 15% glycerol. The protein contents of samples were quantified with an RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. An equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2×) was added to the crude membrane fractions and heated for 5 min at 95°C. The solubilized proteins (10 μg/lane) were resolved by modified Laemmli peptide SDS-PAGE (16% gel) (2) and transferred to a nylon membrane. AN5660 S tag was detected by Rabbit anti-S-tag antibody (1:50,000), followed by anti-rabbit immunoglobulin G coupled to horseradish peroxidase (1:5,000) (Immunology Consultants Laboratory, Inc., Newberg, OR) and staining using an Amersham ECL Western blotting detection reagent and analysis system (GE Healthcare, United Kingdom).

RESULTS

***A. nidulans* possesses three putative Pmts.** In previous work, we identified the *swoA* mutant in a screen for temperature-

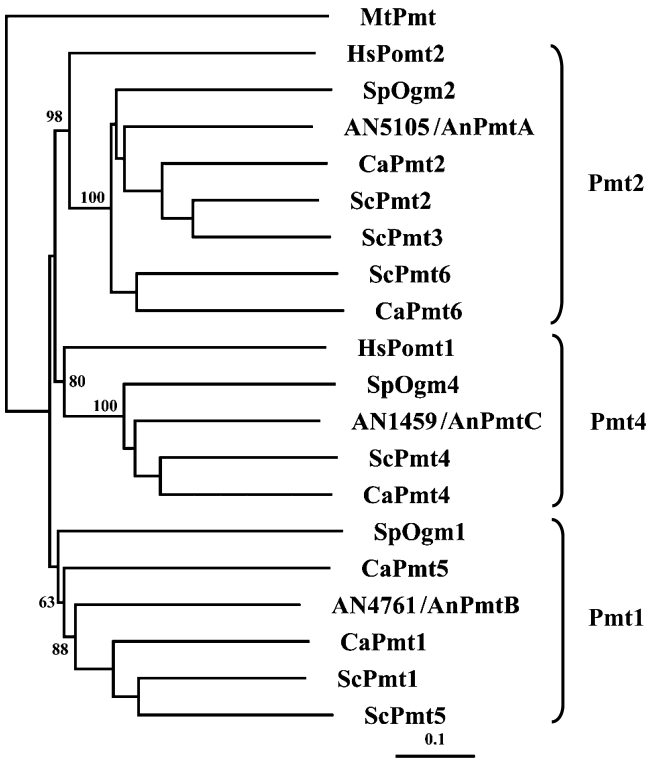

FIG. 1. Phylogenetic tree of Pmts from *A. nidulans* (AN5105, AN4761, and AN1459), *S. cerevisiae* (Pmt1, Pmt 2, Pmt 3, Pmt 4, Pmt 5, and Pmt6), *C. albicans* (Pmt1, Pmt2, Pmt4, Pmt5, and Pmt6), *S. pombe* (Oma1, Oma2, and Oma4), *H. sapiens* (Pomt1 and Pomt2), and *M. tuberculosis* (Pmt). ClustalX2 was used for multiple-sequence alignments. The tree was created from a Bootstrap neighbor-joining tree. The numbers (percentages) are bootstrap values based on 1,000 trails. MtPmt was used as an outgroup. A branch length standard is indicated. The tree was drawn using the Tree View program.

sensitive polarity mutants and showed that the *swoA* gene encodes a protein with high homology to Pmt2, a Pmt in *S. cerevisiae. swoA* was renamed *pmtA* (21, 29). Fungi contain three to seven Pmts falling into three subfamilies named Pmt1, Pmt2, and Pmt4 based on the *S. cerevisiae* members (9).

Using the *S. cerevisiae* Pmt2 protein sequence to query the *A. nidulans* genome database at the Broad Institute (http://www.broad.mit.edu), we found that *A. nidulans* possesses three hypothetical Pmts, AN4761, AN5105, and AN1459. A BLASTP search of the *Saccharomyces* Genome Database (http://www.yeastgenome.org) and phylogenetic analysis (Fig. 1) showed that AN4761 is 60% similar to ScPmt1 and is a member of subfamily 1, AN5105 is 67% similar to Pmt2 and is a member of subfamily 2, and AN1459 is 68% similar to ScPmt4 and is a member of subfamily 4. We refer to AN5105, AN4761, and AN1459 as *pmtA*, *pmtB*, and *pmtC*, respectively.

Single *pmt* deletion mutants are viable, but not all double or triple *pmt* deletion mutants are viable. To understand the role of each Pmt, we constructed Δ*pmt* strains by replacing the entire open reading frame with the *pyrG* auxotrophic marker from *A. fumigatus*. Strains carrying single correct gene replacements were determined by PCR (data not shown) and Southern blotting (Fig. 2). All three single Δ*pmt* mutants were viable and showed a variety of phenotypes described in detail below. To confirm that the phenotypes were solely caused by deletion of *pmt*, each deletion mutant was complemented by expressing the corresponding *pmt* gene behind the constitutive *gpd* promoter. All transformed strains showed complemented phenotypes. Complementation ranged from partial to full restoration of the wild-type phenotype (data not shown), likely because of variation in the copy numbers and positions of integration of *gpd-pmt* cassettes.

Gentzsch and Tanner reported that *S. cerevisiae pmt2 pmt3*, *pmt2 pmt4*, and *pmt1 pmt2 pmt3* mutants at elevated temperatures survived only in the presence of an osmoticum (7). To test whether *A. nidulans* strains with deletions in multiple *pmt* genes were viable, we crossed the single Δ*pmt* mutants with each other and germinated progeny on medium supplemented with 1 M sorbitol. Crosses among single Δ*pmt* mutants showed that the only double mutant that is viable is the Δ*pmtA* Δ*pmtB* mutant. To attempt to generate the triple-knockout mutant, the Δ*pmtA* Δ*pmtB* strain was crossed with the Δ*pmtC* strain. None of the 450 progeny from two crosses was identified as the triple mutant. Further, no Δ*pmtB* Δ*pmtC* or Δ*pmtA* Δ*pmtC* progeny were identified.

All Δ*pmt* strains show reduced growth at high temperatures. The phenotypes of the Δ*pmt* mutants were examined by inoculating a series of 10-fold dilutions of conidia on solid CM and incubating them for 3 days at 25 to 42°C. Colonial growth of all Δ*pmt* single mutants was comparable to that of the wild type at 25°C and 30°C; however, growth was reduced at 37°C and 42°C (Fig. 3). Growth of the Δ*pmtA* Δ*pmtB* double mutant was slightly retarded even at 25°C (Fig. 3). The Δ*pmtA* Δ*pmtB* double mutant was most sensitive to high temperatures, followed by the Δ*pmtA*, Δ*pmtC*, and Δ*pmtB* mutants. Adding osmoticum partially restored growth in all cases (Fig. 3).

Growth of Δ*pmt* strains is altered by cell wall-perturbing agents. To test the sensitivity of Δ*pmt* mutants to cell wall-perturbing agents, serial dilutions of conidia were spotted on CM containing the cell wall-perturbing agent Calcofluor or Congo red (Fig. 3). The Δ*pmtA* and Δ*pmtA* Δ*pmtB* mutants were more sensitive than the wild type to these agents at all temperatures tested. The Δ*pmtC* strain was resistant to Calcofluor at all temperatures tested but was sensitive to Congo red at elevated temperatures. Surprisingly, the effects of cell wall-perturbing agents on the Δ*pmtB* mutant varied with temperature. At 25°C, the Δ*pmtB* mutant grew as well as the wild type. At 30°C and 37°C, the Δ*pmtB* mutant was hypersensitive to Calcofluor; however, at 42°C, the Δ*pmtB* mutant was resistant to Calcofluor. On the other hand, the Δ*pmtB* mutant was hypersensitive to Congo red at 37°C and 42°C and resistant to the chemical at 25°C and 30°C.

The Δ*pmt* mutants have defects in early growth. When an *A. nidulans* conidium breaks dormancy, it first expands isotropically. After approximately 6 h under standard conditions, the cell switches to grow asymmetrically, extending a tubular cell (germ tube) and continuously adding new material only at the tip. After approximately 8 h under standard conditions, cross walls (septa) are formed and partition the hypha into compartments. From these compartments, new tubular cells arise, forming branches (20). To determine the roles of Pmts in the growth and development of *A. nidulans*, we examined Δ*pmt* mutants from conidial germination through early growth (Fig. 4). After 6 h of incubation at 30°C, 12% of the Δ*pmtA* and

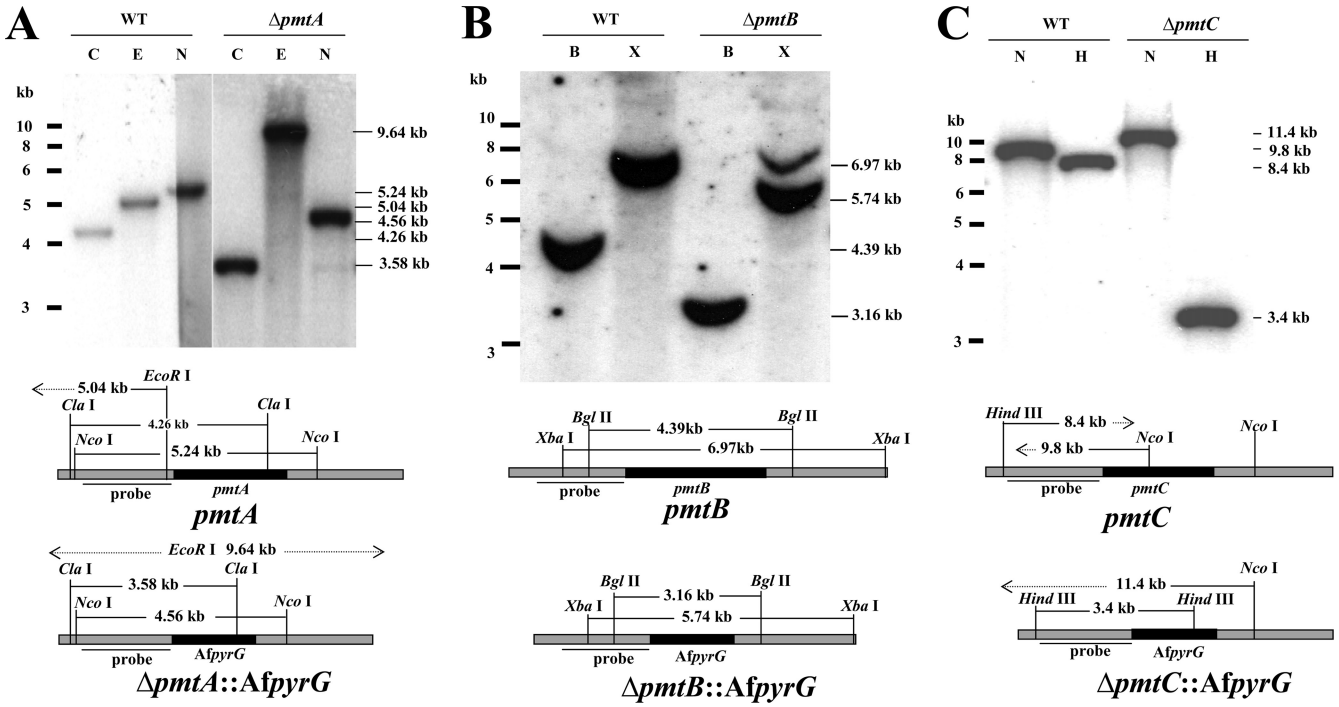

FIG. 2. Southern hybridization of *pmt* deletion strains. Genomic DNAs of the wild type (A850) (WT) and strains transformed with deletion cassettes for *pmtA* (ATK08) (A), *pmtB* (ATK16) (B), and *pmtC* (ATK38) (C) were digested with the restriction enzymes indicated and probed with a radiolabeled upstream fragment of the corresponding *pmt*. B, BglII; C, ClaI; E, EcoR I; H, HindIII; N, NcoI; X, XbaI. The approximately 7-kb band in Δ*pmtB* cut with XbaI is from the left flank of the probe. This band is obscured by the 6.97-kB band in the wild type.

15.5% of the Δ*pmtA* Δ*pmtB* mutants sent out germ tubes compared to 5% of the wild-type, Δ*pmtB*, and Δ*pmtC* strains (data not shown). After 12 h of incubation at 30°C, all germlings appeared normal, except that the Δ*pmtC* mutant sent out multiple germ tubes. Approximately 45% of the Δ*pmtC* mutant germlings had three or four germ tubes, while only 1.5% of the wild type and other deletion mutants had multiple germ tubes ($n = 300$).

All of the Δ*pmt* mutants showed more severe mutant phenotypes at 42°C than at 30°C. After 12 h of incubation at 42°C, the Δ*pmtA* mutant was swollen, with diameters two to three times that of wild-type conidia, and did not send out germ tubes, a phenocopy of the original *swoA* temperature-sensitive mutant (Fig. 4) (29). After 12 h of incubation at 42°C, 20 to 30% of hyphal tips of Δ*pmtB* germlings grown in liquid medium lysed ($n = 200$) (Fig. 4). No similar lysis was seen in the wild type. The Δ*pmtC* mutant at restrictive temperature showed swollen germ tubes and hyperbranching. No similar swelling or hyperbranching was seen in the wild type. The Δ*pmtA* Δ*pmtB* mutant showed an additive phenotype. Hyphal tips lysed at permissive temperature and failed to switch to polar growth at restrictive temperature (Fig. 4). The hyphal-tip lysis of Δ*pmtB* and Δ*pmtA* Δ*pmtB*, the polar growth defect of Δ*pmtA* and Δ*pmtA* Δ*pmtB*, and the swollen hyphae of Δ*pmtC* mutants were corrected by adding osmoticum (KCl or sorbitol) to the medium (data not shown).

Δ*pmt* mutants develop abnormal conidiophores. Asexual reproduction in *A. nidulans* relies on the development of a conidiophore structure that forms from an elongated aerial hypha (conidiophore stalk) with a swollen tip (vesicle). Primary and secondary sterigmata are produced from the vesicle surface to become conidiogenous layers that give rise to chains of conidia (1) (Fig. 5A). Such development requires temporal and spatial regulation of gene expression and coordination and remodeling of cell wall components. To examine the effects of *pmt* deletion on conidiation, mutants were inoculated on agar blocks on a coverslip and incubated for 3 days. Hyphae and conidiophores adhering to the coverslip were examined microscopically. At permissive temperature, the Δ*pmtA* vesicles and conidiogenous layers were swollen (Fig. 5B to D). Many vesicles made partial or no conidiogenous layers. At higher temperatures, the swelling was more severe. All conidiophores produced fewer conidia than the wild type (Fig. 6 and 7). At permissive temperature, the majority of Δ*pmtB* conidiophores were normal, though lysed vesicles were occasionally observed (Fig. 8D). Defects in Δ*pmtB* conidiophores were more severe at restrictive temperature, showing lysed vesicles and swollen conidiophore stalks (Fig. 5E to G). Although normal conidiophores were present (Fig. 5H), many conidiophores failed to form vesicles, resulting in fewer conidia being produced (Fig. 5F). The Δ*pmtC* mutant at 30°C had excessive aerial hyphae and produced fewer conidiophores (Fig. 7). Conidiophore stalks were elongated (Fig. 5I), and conidiogenous layers were misplaced (Fig. 5I and J). At 37°C, the vesicles and conidiogenous layers of the Δ*pmtC* strain were swollen (Fig. 5K and L). Conidiation yielded fewer spores at all temperatures tested (Fig. 6). The Δ*pmtA* Δ*pmtB* conidiophores showed an additive phenotype. Swollen conidiophore stalks, vesicles, and conidiogenous layers were visible at 25°C (Fig. 5M to P). At 37°C, the vegetative and aerial hyphae were badly swollen and co-

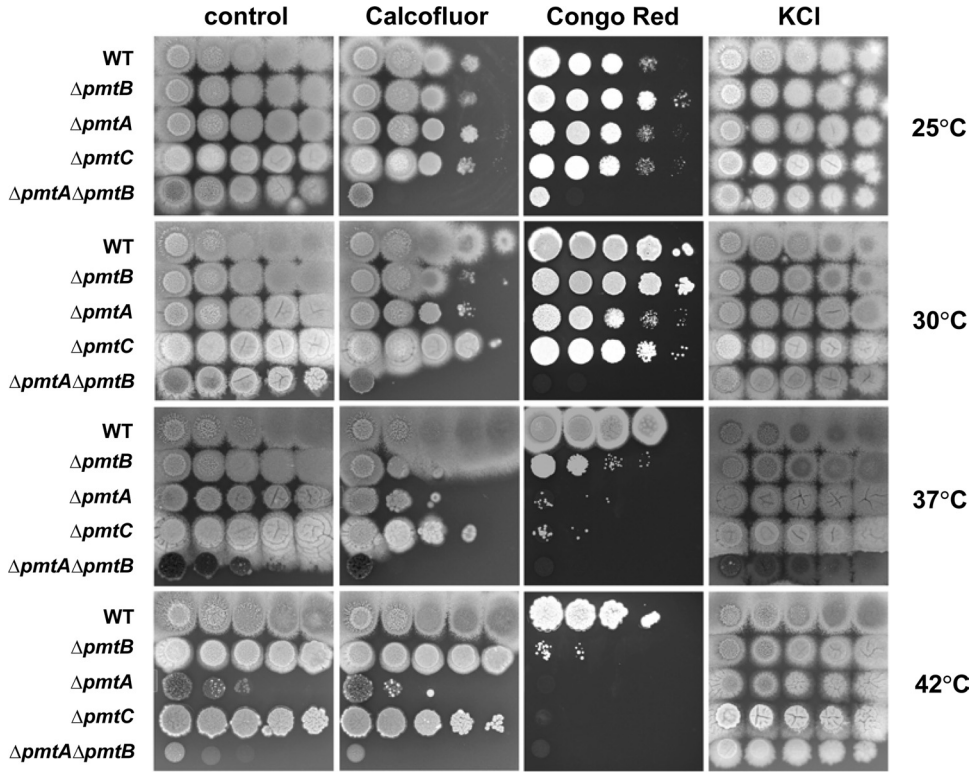

FIG. 3. Cell wall-perturbing agents alter the growth of Δ*pmt* mutants. A 10-fold serial dilution of conidia (10^6 to 10^2) was spotted onto CM and CM containing 10 μg/ml Calcofluor, 25 μg/ml Congo red, or 0.6 M KCl as an osmotic stabilizer. The plates were incubated for 3 days at 25°C, 30°C, 37°C, or 42°C. WT, wild type.

nidiophores were not detectable (data not shown). Conidial production by all Δ*pmt* mutants was reduced at all tested temperatures (Fig. 6). At permissive temperature, conidial production was partially restored in the presence of an osmostabilizer, except for Δ*pmtC* and Δ*pmtA* Δ*pmtB* mutants. Neither adding osmoticum nor reducing the temperature to 25°C significantly increased the spore numbers of these mutants (Fig. 6 and data not shown).

The Δ*pmtB* and Δ*pmtA*Δ*pmtB* mutants make intrahyphal hyphae. In addition to the conidiophore defects seen in Δ*pmtB*

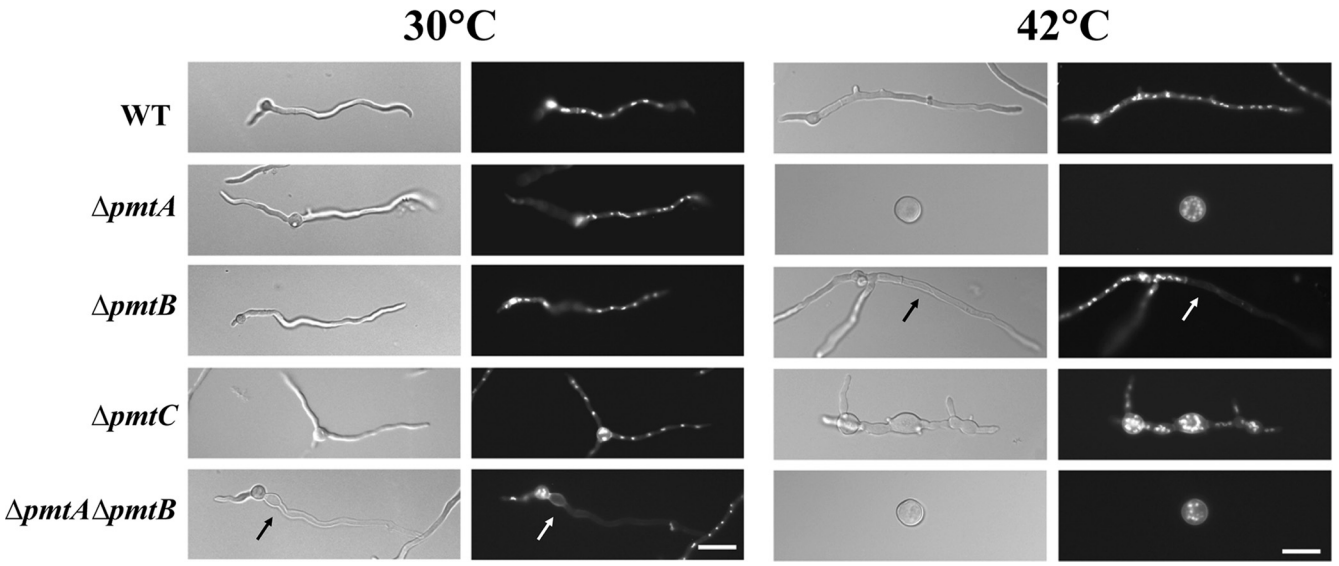

FIG. 4. Phenotypes of Δ*pmt* mutants. Conidia of Δ*pmt* mutants were inoculated onto CM, incubated for 12 h at 30°C or 42°C, fixed, and stained with Hoechst and Calcofluor white to label the nuclei and cell walls, respectively. (Left) Differential interference contrast images. (Right) Fluorescence images. The arrow indicates an empty apical compartment. Bar, 10 μm. WT, wild type.

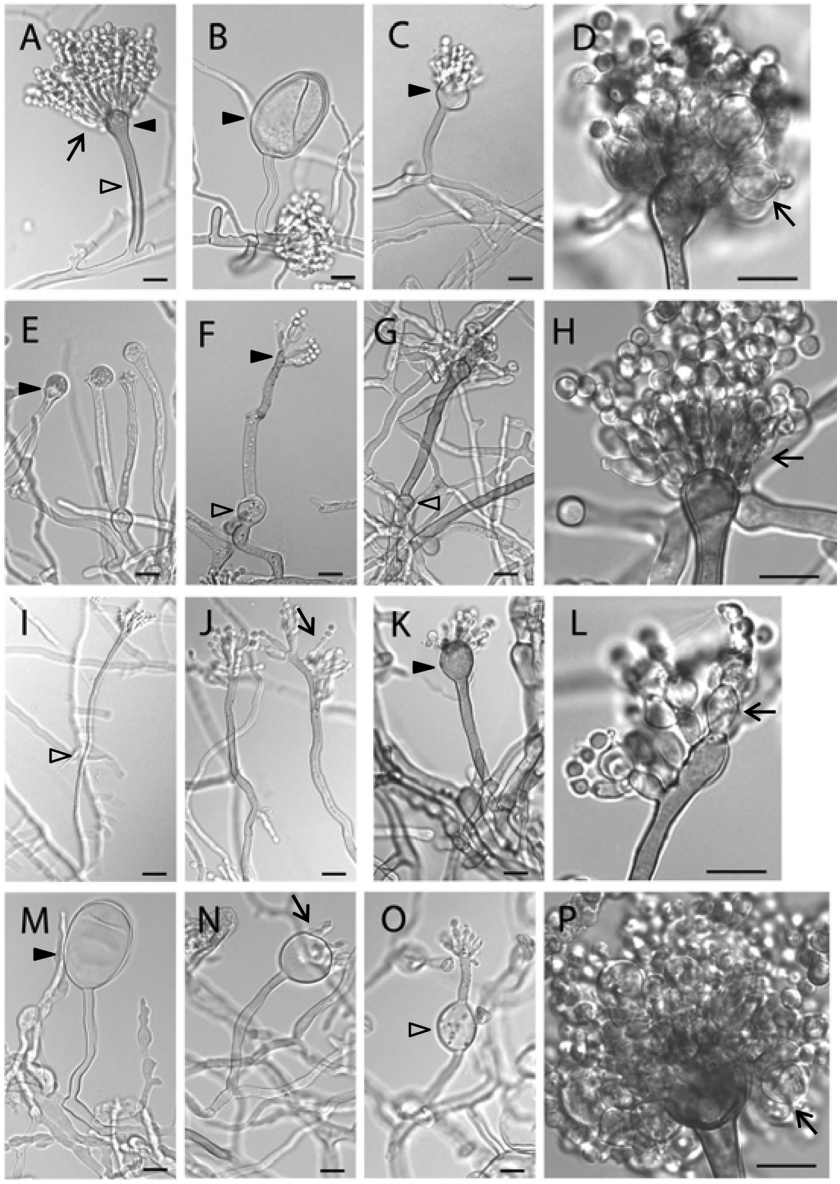

FIG. 5. Conidiophores of Δ*pmt* mutants are abnormal. (A) Wild type grown at 30°C. (B to D) Δ*pmtA* mutant grown at 30°C. (E to H) Δ*pmtB* mutant grown at 42°C. (I and J) Δ*pmtC* mutant grown at 30°C. (K and L) Δ*pmtC* mutant grown at 37°C. (M to P) Δ*pmtA* Δ*pmtB* mutant (ATK94) grown at 30°C. The arrows indicate conidiogenous layers, the empty arrowheads indicate conidiophore stalks, and the dark arrowheads indicate vesicles. Bar, in panel I is 5 μm; all others are 10 μm.

and Δ*pmtA* Δ*pmtB* mutants after 3 days of incubation at 30°C, it appeared that hyphal compartments had frequently lysed and that hyphal tips from the adjacent compartments invaded these empty compartments, forming intrahyphal hyphae (Fig. 8). This "intrahyphal hyphae" phenotype is very similar to that previously reported for chitin synthase mutants (31).

PmtA and PmtC modify an ortholog of Wsc1, a known Pmt target. In *S. cerevisiae*, Wsc family proteins are modified by Pmts (18). Wsc proteins serve as sensors of stress, such as high temperature and cell wall-perturbing chemicals. Pmts add the first mannose residue to specific Ser or Thr residues of their target proteins. Without such modification, the oligo sidechain cannot be elongated, resulting in a protein of lower molecular mass. In order to determine if the *A. nidulans* Pmts are involved in modifying target proteins, we analyzed an ortholog of the *S. cerevisiae* Wsc1 protein. A BLASTP search of the *A. nidulans* genome database using ScWsc1 as the query returned two orthologs, AN6927 and AN5660, with 34% and 37% identity, respectively. The predicted amino acid sequence of AN5660 contains a long stretch of Ser and Thr residues, which is a characteristic of Pmt client proteins. We fused AN5660 with an S tag and expressed it behind the *gpd* promoter in Δ*pmtA*, Δ*pmtB*, and Δ*pmtC* strains of *A. nidulans*. Crude membrane fractions from each Δ*pmt* mutant were separated on SDS-PAGE, transferred to membranes, and probed with anti-S-tag antibody. S-tagged AN5660 has a predicted mass of 32 kDa. As shown by Western blot analysis (Fig. 9), in wild-type and Δ*pmtB* strains, the S-tagged Wsc ortholog had an apparent

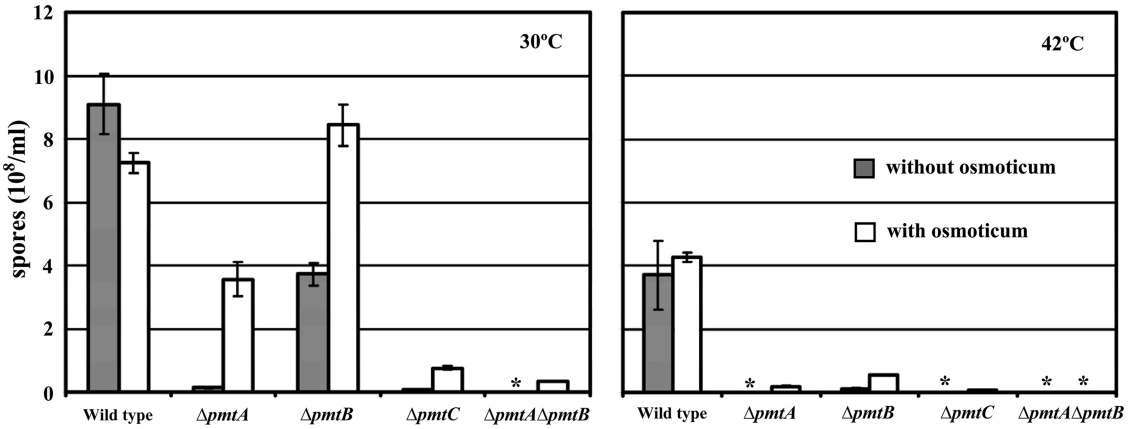

FIG. 6. Conidiation efficiencies of Δ*pmt* mutants are reduced. A total of 10^4 spores were spread on CM and CM containing 0.6 M KCl. The plates were incubated for 4 days at 30°C or 42°C and washed with 4 ml of sterile water. Conidia were counted using a hemocytometer. The asterisks indicate samples with too few conidia to count.

molecular mass of approximately 40 kDa, while in the Δ*pmtA* and Δ*pmtC* strains, the S-tagged Wsc ortholog was slightly smaller, with an apparent molecular mass of approximately 38 kDa. The lower molecular mass of S-tagged AN5660 in Δ*pmtA* and Δ*pmtC* strains of *A. nidulans* is consistent with hypoglycosylation and is very similar to results for tagged Wsc1 expressed in *pmt2*Δ and *pmt4*Δ strains of *S. cerevisiae* (18).

DISCUSSION

A. nidulans has a single representative of each of the three Pmt subfamilies. Neither the Pmt1 representative (*pmtB*), the Pmt2 representative (*pmtA*), nor the Pmt4 representative (*pmtC*) is essential (Fig. 3). This makes *A. nidulans* the only fungus in which each subfamily has been individually deleted without loss of viability. Though deletion of single Pmt1 or Pmt2 representatives in *S. cerevisiae* is not lethal, it is impossible to say if these subfamilies are essential in *S. cerevisiae* because it contains at least two members of both subfamilies and deletion of all representatives of each subfamily has not been reported. Similarly, though deletion of a single Pmt1 representative is not lethal in *C. albicans*, deletion of the complete Pmt1 subfamily has not been reported. The *C. albicans* Pmt2 subfamily also contains two members, *pmt2* and *pmt6*, and deletion of *pmt2* is lethal. Like *A. nidulans*, *S. pombe* contains three Pmts, one from each subfamily. Also like *A. nidulans* the single Pmt1 subfamily representative is not essential. In contrast with *A. nidulans*, deletion of the single Pmt2 in *S. pombe* is lethal. *A. nidulans*, *S. cerevisiae*, *C. albicans*, and *S. pombe* all have a single Pmt4 representative that is not essential.

In *S. cerevisiae* and *S. pombe*, Pmt1 and Pmt2 subfamily representatives are thought to form heteromeric complexes based on immunoprecipitation assays (8, 34). In *A. nidulans*, Δ*pmtA* Δ*pmtB* strains show a synthetic phenotype that is more severe than that of either single mutant, suggesting that Pmt1 and Pmt2 subfamily members might also form heteromeric complexes in this filamentous fungus. The fact that *A. nidulans* Δ*pmtA* Δ*pmtB* mutants are viable with both Pmt1 and Pmt2 subfamilies completely eliminated suggests either that the targets modified by the Pmt1/Pmt2 complex are not needed for viability or that the remaining Pmt4 subfamily member is able to compensate, probably by promiscuous target modification. Consistent with the idea that the nonessential Pmt4 representative might be required to compensate for loss of Pmt1 or Pmt2, we were unable to recover either Δ*pmtB* Δ*pmtC* (subfamilies 1 and 4) or Δ*pmtA* Δ*pmtC* (subfamilies 2 and 4) mutant strains. Further, Δ*pmtC* showed much less effect from the cell wall-altering agent Calcofluor (Fig. 3), consistent with the idea that the Pmt4 representative might be most important when Pmt1 or Pmt2 is perturbed. Deletion of representatives of both the Pmt1 and Pmt4 subfamilies in combination appears to be lethal in *C. albicans* and *S. pombe* (26, 34).

One of our more intriguing observations was the increased severity of the Δ*pmt* mutant phenotypes at elevated temperatures. Indeed, we first identified *pmtA* in a screen for temperature-sensitive polarity mutants (21). The *pmtA* allele (at the time called *swoA*) produced a dramatic phenotype at elevated temperature of highly swollen conidia from which germ tubes failed to extend. Later work showed that the *swoA* lesion was in a Pmt2 ortholog and was predicted to

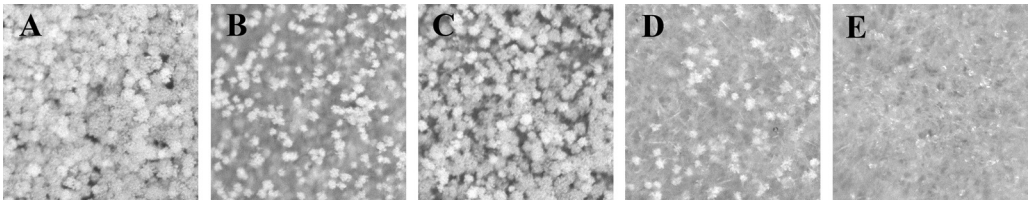

FIG. 7. The Δ*pmtC* mutant produces excessive aerial hyphae. The wild type (A) and Δ*pmtA* (B), Δ*pmtB* (C), Δ*pmtC* (D), and Δ*pmtA* Δ*pmtB* (E) mutants were grown on solid medium at 30°C for 3 days. All panels are at the same magnification.

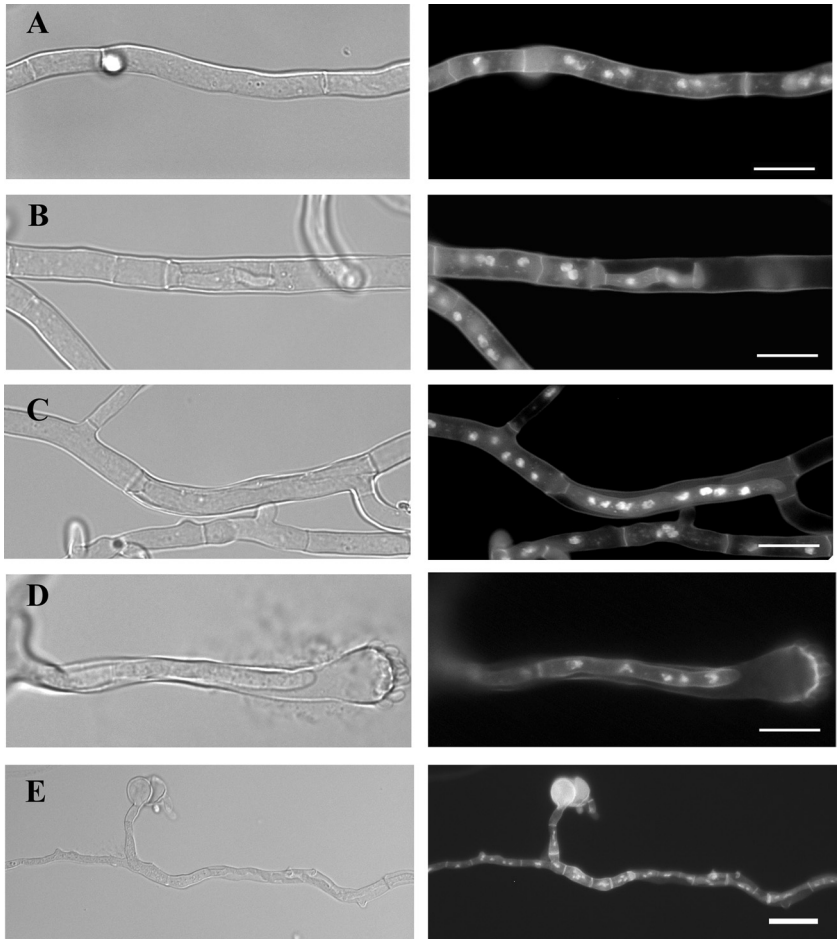

FIG. 8. Δ*pmtB* and Δ*pmtA* Δ*pmtB* mutants make intrahyphal hyphae. The wild type (A) and the Δ*pmtB* (B to D) and Δ*pmtA* Δ*pmtB* (E) mutants were grown on solid medium at 30°C for 3 days and stained with Hoechst and Calcofluor white to label nuclei and cell walls, respectively. (Left) Differential interference contrast images. (Right) Fluorescence images. Bar, 10 μm.

result in the truncation of the C-terminal 77 amino acids (29). Interestingly, the Δ*pmtA* strain described in this paper exactly phenocopies the original temperature-sensitive mutant, suggesting that the original *swoA* (*pmtA*) allele was a functional null and that the Pmt2 subfamily plays a role in response to elevated temperature, probably by virtue of the protein targets it modifies.

The more severe phenotypes at elevated temperatures that we observed in the *A. nidulans* Δ*pmt* strains is a common feature of loss of Pmt activity in other fungi, as well. In *S. cerevisiae*, the temperature sensitivity of Δ*pmt2* and Δ*pmt3* strains (subfamily 2) is clearly connected to the cell wall integrity pathway. The cell wall integrity pathway allows *S. cerevisiae* to respond to stresses, including elevated temperature and cell wall-perturbing drugs, by upregulating cell wall synthesis (13, 38). In *S. cerevisiae*, Pmt2 and Pmt4 modify Wsc1p, one of the cell wall proteins that transmits the stress signal to the downstream Pkc1p and MAPK cascade signaling pathways that ultimately activate cell wall biosynthetic genes (18). Though the cell wall integrity pathway is not as well understood in *A. nidulans*, there are many orthologs of cell wall integrity pathway gene products, including the Wsc proteins (5). We found that a Wsc1 ortholog ran with an apparently reduced molecular mass in *A. nidulans* Δ*pmtA* and Δ*pmtC* strains (Fig. 9), showing that it is modified by these Pmt 2 and Pmt4 subfamily representatives in *A. nidulans*. It is likely that WscA plays a role in the cell wall integrity pathway in *A. nidulans*, since introduction of WscA on a high-copy-number plasmid partially suppressed the temperature sensitivity of the Δ*pmtA* mutant on agar plates (data not shown). However, overexpression of WscA in the Δ*pmtA* strain could not suppress polar growth in liquid culture at 42°C for 12 h (data not shown).

Most of the cell wall-related phenotypes of the *A. nidulans* Δ*pmt* mutants, such as lysis, were visible only at high temperature or in the presence of cell wall-perturbing agents. These phenotypes were largely corrected by the addition of osmoticum, suggesting a weakened cell wall. Thus, many of the cell wall-related phenotypes of the *A. nidulans* Δ*pmt* mutants could be explained by a requirement for modification of cell wall integrity pathway members by Pmts. In *S. cerevisiae*, several cell wall integrity pathway members are targets of Pmt modification, including Wsc proteins and Mid2 (18). It has been shown that Pmt modification is needed for stability of Wsc1 and Mid2 (18). Though most of the cell wall-related phenotypes of Δ*pmt* mutants in *A. nidulans* are consistent with Pmt modification of cell wall integrity pathway proteins, it is possi-

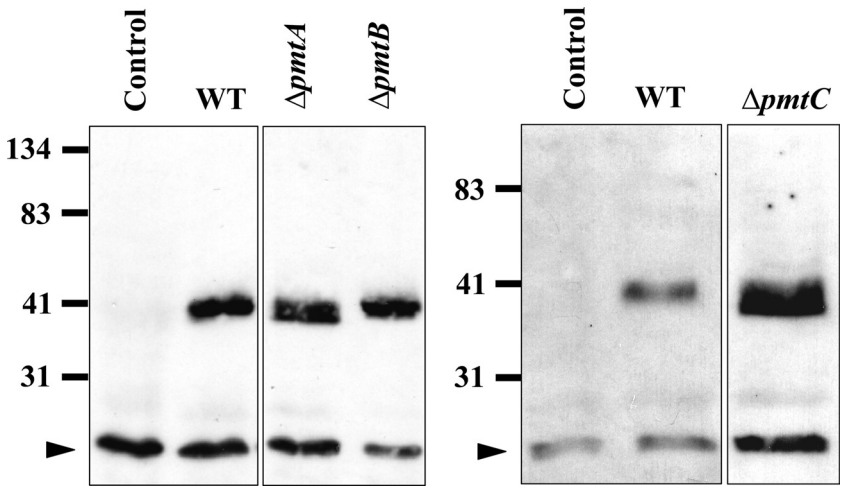

FIG. 9. PmtA and PmtC modify AN5660 in vivo. Crude membrane fractions from Δ*pmt* mutants were separated on 16% SDS-PAGE. AN5660 S tag on a Western blot was probed with antibody against S tag. The arrowheads indicate nonspecific signals. WT, wild type.

ble that other cell wall-related proteins are also modified by Pmts, as is true for *S. cerevisiae*.

In addition to the cell wall-related phenotypes seen under the stress conditions of elevated temperature or drug treatment, all *A. nidulans* Δ*pmt* mutants showed developmental-patterning defects under normal growth conditions. The Δ*pmtA* mutant sent out germ tubes earlier than the wild type, while the Δ*pmtC* mutant sent out more germ tubes and more branches than the wild type. The Δ*pmtA* Δ*pmtB* double mutant sent out germ tubes too early, and the Δ*pmtB* mutant made hyphae within hyphae. All *A. nidulans* Δ*pmt* mutants made abnormal conidiophores and had reduced conidial numbers at normal growth temperatures (Fig. 6 and 7). Though a weakened cell wall could explain some of the swelling seen in conidiophores, we think that a developmental problem is the more likely cause because each mutant had a distinct pattern of enlarged areas. The Δ*pmtA* mutant vesicles and conidiogenous layers were swollen, the Δ*pmtB* mutant showed swellings of the stalk, and the Δ*pmtC* mutant made elongated stalks (Fig. 5). Further, these phenotypes were not fully rescued by adding osmoticum. These results suggest that different proteins serving as developmental cues are modified by each *A. nidulans* Pmt. Modification of developmental proteins by Pmts has a precedent in *S. cerevisiae*, where O mannosylation has been shown to be essential for the function, localization, and stability of Axl2, an axial-budding positional marker (28).

Clearly, the three *A. nidulans* Pmts play distinct roles in cell wall integrity and development. Though we cannot rule out direct roles of the Pmts, it is most likely that these roles are the result of Pmt modification of target proteins involved in these processes. Future work will focus on identifying targets of Pmt modification and their modes of action.

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