CHARACTERIZATION OF PROTEIN *O*-MANNOSYLTRANSFERASES AND THEIR TARGET PROTEINS IN *ASPERGILLUS NIDULANS*

by

THANYANUCH KRIANGKRIPIPAT

(Under the direction of Michelle Momany)

ABSTRACT

Protein *O*-mannosyltransferase (Pmt) catalyzes the transfer of the first mannosyl residue from dolichyl phosphate activated mannose (Dol-P-Man) to specific serine/threonine residues of target proteins. It is becoming increasingly evident that protein *O*-mannosylation is essential for viability, cell wall integrity, signaling, morphogenesis, virulence and polar growth of fungi. Pmts are classified as members of three subfamilies named for the *Saccharomyces cerevisiae* proteins Pmt1, Pmt2 and Pmt4. Pmts are not active as monomers, rather they must be part of heteromeric or homomeric complexes to function. In *S. cerevisiae* Pmt 1 and Pmt2 form a heteromeric complex and Pmt 4 forms a homomeric complex. Most mannosylated proteins studied in yeasts are targeted to the membrane or cell wall or are secreted. Many of those are important for cell wall integrity and morphogenesis. In filamentous fungi, only a few Pmt-modified proteins have been identified. Although protein *O*-mannosylation and Pmt-modified proteins have been extensively studied in unicellular yeasts, little is known about roles of Pmts in polar growth and cell wall biogenesis of multicellular filamentous fungi. Discovery of novel cell wall proteins will benefit our knowledge of growth and development of multicellular fungi.

This dissertation describes the use of *A. nidulans* as a model organism to better understand molecular and biological roles of Pmts in filamentous fungi. *A. nidulans* contains 3 Pmts representing each subfamily, PmtA (Pmt 2 subfamily), PmtB (Pmt 1 subfamily) and PmtC (Pmt 4 subfamily). All three *A. nidulans pmts* were individually deleted. Single Δ*pmt* mutants are viable and exhibit distinctive phenotypes. The Δ*pmtA* Δ*pmtB* mutant is the only viable double mutant. All Δ*pmt* mutants are hypersensitive to cell wall perturbing agents and develop abnormal conidiophores suggesting that Pmt is involved in cell wall integrity and morphogenesis. *A. nidulans* Pmts form heteromeric complexes among the three subfamilies. In addition, PmtC forms a homomeric complex. AxlA, WscA and MsbA, *A. nidulans* orthologs of yeast proteins modified by Pmts, were used to compare Pmt substrate specificity. Each individual Pmt appears to carry substrate specificity independently from the other Pmts in the complex. Unlike Axl2 from *S. cerevisiae*, AxlA is not a substrate of Pmts in *A. nidulans*. In *S. cerevisiae* Wsc1 is modified by Pmt2, as a member of the Pmt1/2 complex. In contrast, *A. nidulans* WscA is modified by PmtA (Pmt2 subfamily) and PmtC (Pmt 4 subfamily) whether or not PmtB (Pmt 1 subfamily) is present. Unlike *S. cerevisiae* Msb2, which is modified by Pmt4, *A. nidulans* MsbA is modified by PmtA (subfamily 2) and Pmt B (subfamily 1), not PmtC (subfamily 4).

Our work shows that all three Pmts from *A. nidulans* can form heteromeric complexes with each other and that substrate specificity appears to be determined by individual Pmts within complexes.

INDEX WORDS: *Aspergillus nidulans*, cell wall, development, morphogenesis, protein *O*-mannosyltransferase

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DEDICATION

I dedicate this dissertation to my grandmother, my parents, my sister and my husband for their love and support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Protein *O*-mannosylation

O-mannosylation is specific to proteins that are synthesized and sorted in the secretory pathway, which includes the ER, Golgi, lysosome, plasma membrane and cell wall. The assembly of *O*-linked proteins in the ER lumen is catalyzed by Protein *O*-mannosyltransferase (Pmt), where only one mannosyl residue is transferred co- or post-translationally to the hydroxyl group of serine or threonine residues to form an α-D-mannosyl linkage (STRAHL-BOLSINGER *et al.* 1999). Without the activity of Pmts, *O*-linked glycans could not be elongated. The addition of further saccharide residues to the first *O*-linked mannose occurs in the Golgi and involves a range of enzymes (WILLER *et al.* 2003) (fig.1.1). The lengths and saccharide composition of *O*-mannosyl glycans are different among species. In fungi, *O*-glycosyl chains range from two to seven residues. In *S. cerevisiae*, the mannosyl chain can be modified by mannosyl phosphate. In *S. pombe*, the *O*-linked glycan is capped with one or two galactose residues (GEMMILL and TRIMBLE 1999).

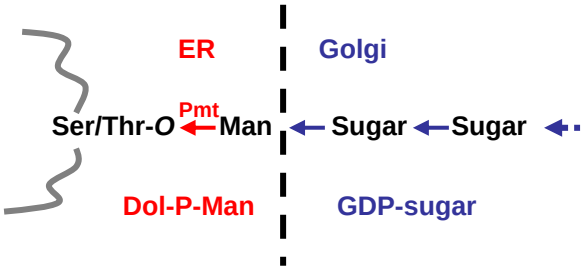

Figure 1.1. Protein *O*-mannosylation in eukaryotes. The first mannose is transferred from dolicol-phosphate-mannose (Dol-P-Man) to the serine or threonine residues of the secretory protein in the lumen of the ER. The reaction is catalyzed by Protein *O*-mannosyltransferase (Pmt). Further addition of saccharides takes place in the Golgi using a different set of enzymes and using GDP-sugars as sugar donors. (adapted from ERNST and PRILL) (ERNST and PRILL 2001)

Protein *O*-mannosyltransferases in eukaryotes

Pmts are present in both prokaryotes and eukaryotes (VANDERVEN *et al.* 2005), but are absent in plants (GIRRBACH and STRAHL 2003). In eukaryotes, the Pmt family is phylogenetically classified into the Pmt1, Pmt2 and Pmt4 subfamilies with each species having two to seven members. *S. cerevisiae* and *C. albicans* Pmts are the most redundant, with subfamilies 1 and 2 containing two to three members (GENTZSCH and TANNER 1996; PRILL *et al.* 2005). Pmt4 subfamily has only 1 member identified for each species studied. *S. pombe* and many filamentous fungi including *A. nidulans* have one representative from each subfamily. Animals possess only the Pmt2 and Pmt4 subfamilies, except that *Caenorhabditis elegans* is missing all Pmts (GIRRBACH and STRAHL 2003). *S. cerevisiae* Pmt1 was the first Pmt characterized. It is a membrane bound protein with 7 transmembrane domains and localized in the ER. Its N-terminus

faces the cytoplasm, while its C-terminus faces the ER lumen. The Pmt1 has two important hydrophilic domains localized between transmembrane span 1 and 2 called 'loop1' and between transmembrane span 5 and 6 called 'loop 5'. The loop1 contains conserved amino acids required for substrate binding and enzymatic activity. The loop 5 is essential for enzymatic activity but is not required for Pmt complex formation (GIRRBACH and STRAHL 2003; GIRRBACH *et al.* 2000; STRAHL-BOLSINGER and SCHEINOST 1999). Heteromeric complexes between members of Pmt1 and Pmt2 subfamily and Pmt4 homomeric complex are necessary for enzymatic activity (GIRRBACH and STRAHL 2003). Similar to *S. cerevisiae* Pmt, human POMT1 and POMT2 are localized in the ER. POMT1 and POMT2 contain seven and nine transmembrane domains, respectively. The largest hydrophilic loops equivalent to the yeast loop 5 are essential for enzymatic activity. In addition, all *N*-glycosylation sites located in the ER lumen are *N*-glycosylated. Mutation of these sites affects enzymatic activity (MANYA *et al.* 2010). POMT1-POMT2 complex is formed during translation in the ER and required for enzymatic activity (MANYA *et al.* 2010; ICHIMIYA *et al.* 2004; MANYA *et al.* 2004).

Biological roles of protein *O*-mannosylation

The biological roles of protein *O*-mannosylation range from those that are limited to those that are essential for various developmental processes. In *S. cerevisiae*, single Δ*pmt* mutants are viable; however, deletion of the Pmt2 subfamily is lethal in *C. albicans*, *S. pombe*, *Cryptococcus neoformans*, *Ustilago maydis*, and *A. fumigatus* (FERNANDEZ-ALVAREZ *et al.* 2009; MOUYNA *et al.* 2010; PRILL *et al.* 2005; WILLER *et al.* 2005; WILLGER *et al.* 2009). In *S. cerevisiae*, *O*-mannosylation is essential for cell integrity and cell wall rigidity (GENTZSCH and TANNER 1996). In *C. albicans*, Pmt mutation affects filamentation, morphogenesis and virulence (PRILL *et al.*

2005; ROUABHIA *et al.* 2005) . In the fruit fly, RNAi knockdown of either *POMT1* or *POMT2* causes a clockwise rotation of the abdomen and defects in embryonic muscle development (ICHIMIYA *et al.* 2004). Homozygous mutation of mouse *POMT1* is embryonic lethal (WILLER *et al.* 2004). In human, heterozygous mutation of either *POMT1* or *POMT2* causes congenital muscular dystrophy associated with severe neuronal migration disorder and eye anomalies (BELTRAN-VALERO DE BERNABE *et al.* 2002; VAN REEUWIJK *et al.* 2005).

Pmt target proteins

Most Pmt-modified proteins identified so far are localized to the cell membrane or cell wall or are secreted (BARRESI and CAMPBELL 2006; BOWMAN and FREE 2006). Each Pmt mannosylates different sets of target proteins; however, there is evidence of limited substrate overlap. For example, the ScPmt4 and the ScPmt1/ScPmt2 complex mannosylate different domains of Ccw5p (ECKER *et al.* 2003). *O*-mannosylation is important for stability, localization and function of secreted proteins. Reduced mannosylation of the *S. cerevisiae* Wsc1, Wsc2 and Mid2 and of the *C. albicans* Sec20 makes them less stable (LOMMEL *et al.* 2004; WEBER *et al.* 2004). Wsc1 is one of the cell wall stress sensors. These proteins activate the protein kinase C (Pkc1) cell wall integrity signaling pathway required for polar growth, pheromone-induced morphogenesis and environmental stress response (HEINISCH *et al.* 1999). Fungi have two types of glycosylation, *N*- and *O*-glycosylation. *N*-glycosylation is specific to asparagine residues within the consensus sequence Asn-X-Ser/Thr, where X is any amino acid but proline (WEERAPANA and IMPERIALI 2006). Ser and Thr are the only residues known to be *O*-mannosylated, however, the consensus sequence of *O*-mannosylation remains unknown, . Gentzsch and Tanner (1997) attempted to predict a consensus sequence of ScPmt4 modified

proteins as GS_9AS_6K but failed to identify similar or comparable sequences in other ScPmt4 modified proteins (GENTZSCH and TANNER 1997). In *S. cerevisiae*, Pmt proteins showed distinct substrate specificities for their target proteins. This implies that substrate recognition is different among Pmts (GENTZSCH and TANNER 1997). Sequences specifying protein *O*-mannosylation may be more complex than those specifying *N*-linked glycosylation and could include varieties of amino acids within the consensus sequences and secondary structure of the target proteins. In addition, the coexistence of multiple transferases and heterocomplex formation makes it more complicated to predict a consensus sequence from available identified proteins. Analysis of additional Pmt-modified proteins together with statistical studies may reveal some general rules for this type of protein modification.

A relatively small number of Pmt-modified proteins have been described in fungi.

Two types of fungal cell wall glycoproteins have been characterized: the glycosylphosphatidylinositol (GPI) proteins and the Pir (internal repeat) proteins. Because of their location on the surface, glycoproteins are thought to play a variety of roles including sensing environmental stimuli and remodeling the cell surface during growth (LESAGE and BUSSEY 2006). GPI addition is a posttranslational modification frequently observed in proteins localized in the plasma membrane and cell wall (DE SAMPAIO *et al.* 1999). Genome-wide identification of putative GPI proteins in *S. cerevisiae* indicated that at least 58 putative GPI proteins have Ser/Thr-rich stretches, which are predicted to be highly mannosylated (CARO *et al.* 1997; DE GROOT *et al.* 2003). Among the 58 putative GPI-anchored proteins in *S. cerevisiae* only two have been confirmed to be modified by Pmt. In filamentous fungi, approximately 74 and 97 putative GPI proteins have been identified in *A. nidulans* and *Neurospora crassa*,

respectively, by bioinformatics approaches (DE GROOT *et al.* 2003; EISENHABER *et al.* 2004). How many of them are mannosylated has not been addressed.

Polar growth is a hallmark of filamentous fungi

The life cycle of *A. nidulans* begins after a conidium breaks dormancy (fig. 1.2). The conidium expands isotropically until reaching a certain size. The cell then switches to grow asymmetrically sending a tubular extension (hypha) and continuously adding new material only at the tip. Occasionally cross-wall structures called "septa" are formed and partition the hypha into compartments. Only the apical compartment is active and grows continuously (MOMANY 2002). The wall behind the advancing tip matures and becomes rigid by polymerization of chitin and internal actin reinforcement (MA *et al.* 2005). Subapical compartments remain inactive until branching occurs.

Polar growth is an essential feature in the development of any fungal colony. Growing hyphae penetrate substrate and explore the environment and are the essential means of invasive disease. For example, highly polar tip growth enables *A. fumigatus* to penetrate tissue and blood vessels. Subsequently, filamentous hyphae cause local tissue damage, hemorrhage, infarction, and necrosis (LATGE 1999). Without the emergence and continuous polar growth of germ tubes invasive aspergillosis would not develop.

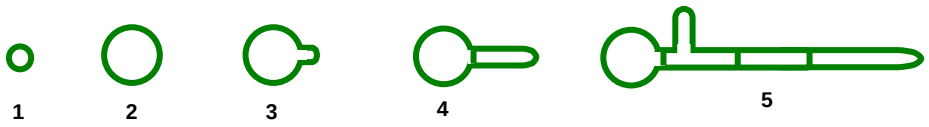

Figure 1.2. Polar growth of *A. nidulans*.

1) a conidium 2) isotropic expansion 3) germ tube emergence 4) septum formation 5) branching (Adapted from Momany) (MOMANY 2002).

swoA mutant

Shaw and Momany identified the *swoA* mutant from a collection of temperature-sensitive polarity mutants. The *swoA* mutant grows isotropically but fails to send out a germ tube at restrictive temperature. The *swoA* allele encoded a protein *O*-mannosyltransferase (Pmt) (MOMANY *et al.* 1999; SHAW and MOMANY 2002). The *swoA* gene belongs to Pmt2 subfamily based on bioinformatics analysis (Fig. 2.1).

In Chapter 2, the Δ*pmt* strains were used 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. It was also found that PmtA and PmtC modify an ortholog of *S. cerevisiae* Wsc1, a known Pmt target, suggesting that *A. nidulans* Pmts act as protein *O*-mannosyltransferases *in vivo.*

In Chapter 3, molecular interactions of the three Pmts were determined by immunoprecipitation and comparing modification patterns of Pmt target proteins. Forming complexes among Pmt subfamilies are required for enzymatic activity in *S. cerevisiae*, *S. pombe* and animals (GIRRBACH and STRAHL 2003; WILLER *et al.* 2005; ICHIMIYA *et al.* 2004; MANYA *et al.* 2004). In *S. cerevisiae*, members of the Pmt1 subfamily form heteromeric complexes with

members of the Pmt2 subfamily, while the lone Pmt4 subfamily member forms a homomeric complex. Although, Pmt complexes are mandatory for enzymatic activity, each Pmt carries specificity toward their target proteins (GENTZSCH and TANNER 1997; HUTZLER *et al.* 2007).

Like *S. cerevisiae*, *A. nidulans* Pmt1 and Pmt2 subfamily proteins form a heteromeric complex. Surprisingly, *A. nidulans* Pmt1 and Pmt2 subfamily proteins also form heteromeric complexes with the Pmt4 subfamily protein, which has not previously been reported in any fungi. Like members of the Pmt4 subfamily of *S. cerevisiae*, *A. nidulans* Pmt4 subfamily members form a homomeric complex. Analysis of target protein modification in Δ*pmt* strains reveals that each *A. nidulans* Pmt shows substrate specificity.

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CHAPTER 2

ASPERGILLUS NIDULANS PROEIN *O*-MANNOSYLTRANSFERASES PLAY ROLES IN CELL WALL INTEGRITY AND EDVELOPMENTAL PATTERNING[1]

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[1]Kriangkripipat, T., and Michelle Momany. 2009. Eukaryotic Cell. 8: 1475-1485.

Abstract

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* compared to wildtype. In Δ*pmtB* intrahyphal hyphae develop. All Δ*pmt* mutants show distinct conidiophore defects. The Δ*pmtA* strain has swollen vesicles and conidiogenous cells, Δ*pmtB* has swollen conidiophore stalks, and Δ*pmtC* has dramatically elongated conidiophore stalks. We also show that AN5660, an ortholog of *S. 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 wildtype 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.

Introduction

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 (LOMMEL *et al.* 2004; SANDERS *et al.* 1999). The assembly of protein linked *O*-mannosyl glycans in the ER 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 (STRAHL-BOLSINGER *et al.* 1999). The addition of further carbohydrate residues to the first *O*-linked mannose occurs in the Golgi and involves a range of enzymes (WILLER *et al.* 2003). 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 *S. cerevisiae* (HUTZLER *et al.* 2007). 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, cell wall or are secreted. At least twenty-three target proteins have been described in yeasts (HUTZLER *et al.* 2007). Only three Pmt target proteins have been described in filamentous fungi (HARRISON *et al.* 1998; OKA *et al.* 2005; ZHOU *et al.* 2007).

Pmts have been found in both prokaryotes and eukaryotes (VANDERVEN *et al.* 2005), but not in plants (GIRRBACH and STRAHL 2003). The lengths and composition 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 (GEMMILL and TRIMBLE 1999). In *S. pombe*, the *O*-linked glycan is capped with one or two galactose residues (GEMMILL and TRIMBLE 1999). In filamentous fungi so far examined, *O*-glycans are linear and branched with 3 to 5 monosaccharide residues (DESHPANDE *et al.* 2008).

In fungi, the Pmts are classified into the Pmt1, Pmt2 and Pmt4 subfamilies with each species having three to seven members. *S. cerevisiae* and *C. albicans* Pmts are the most redundant, with subfamilies 1 and 2 containing two to three members (GENTZSCH and TANNER 1996; PRILL *et al.* 2005). *S. pombe* and many filamentous fungi including *A. nidulans* have one representative from each subfamily. In *S. cerevisiae* enzymatic activity of Pmts requires interaction among members of the Pmt1 and Pmt2 subfamilies, while Pmt4 forms homomeric complexes (GIRRBACH and STRAHL 2003). Heteromeric complexes between the Pmt1 and Pmt2 subfamily members has also been reported in *S. pombe* (WILLER *et al.* 2005).

O-mannosylation appears to be required for stability, localization, and function of target proteins (LOMMEL *et al.* 2004; SANDERS *et al.* 1999; TIMPEL *et al.* 1998) 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 (GENTZSCH and TANNER 1996). In *C. albicans* and *Cryptococcus neoformans*, Pmt mutation affects morphogenesis and virulence (OLSON *et al.* 2007; PRILL *et al.* 2005; ROUABHIA *et al.* 2005). In *S. cerevisiae*, strains deleted for single Pmt subfamily representatives are viable; however, deletion of subfamily 2 representatives is lethal in *S. pombe* and *C. albicans* (GENTZSCH and TANNER 1996; WILLER *et al.* 2005). In filamentous fungi, deletion of individual Pmts has been previously reported. Deletion of *Trichoderma reesei pmtI*, *A. fumigatus pmt1*, *A. nidulans pmtA* and *A. awamori pmtA* were not lethal but affected

growth and development (GORKA-NIEC *et al.* 2008; OKA *et al.* 2004; OKA *et al.* 2005; ZHOU *et al.* 2007).

In previous work, we identified the *swoA* mutant from a collection of temperature-sensitive polarity mutants and showed that the *swoA* allele encoded a Pmt 2 subfamily member (PmtA) (MOMANY *et al.* 1999; SHAW and MOMANY 2002). 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, 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 phenotype of strains deleted for each Pmt subfamily has been reported.

Materials and Methods

Aspergillus strains and media

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, 0.01% vitamins and supplements. Minimal medium (MM) contained 1% glucose and supplements. Nitrate salts solution, trace elements, vitamins and amino acid supplements are based on the appendix to Kafer (KAFER 1977) and Hill and Kafer (HILL and KAFER 2001). pH of media were adjusted to 6.5 with 1.0 N NaOH. Complete medium 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. 0.6M KCl or 1M sorbitol was used as osmostabilizers.

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), *H. sapiens* and *Mycobacterium tuberculosis* (http://www.ncbi.nlm.nih.gov/). Gene and accession numbers of the protein sequences are as follows: ScPmt1 (YDL095W), ScPmt2 (YAL023C), ScPmt3 (YOR321W), ScPmt4 (YJR143C), ScPmt5 (YDL093W), ScPmt6 (YGR199W), AnPmtA (AN5105), AnPmtB (AN4761), AnPmtC (AN1459), SpOgm1/Oma1 (SPAC22A12.07c), SpOgm2/Oma2 (SPAPB1E7.09), SpOgm4/Oma4 (SPBC16C6.09), HsPomt1 isoform a (gi(116517319)), HsPomt2 (gi(32455271)), MtPmt (gi(15608142)). CaPmt1 (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 (LARKIN *et al.* 2007). Trees were viewed using TreeView 1.6.6 (Page 1996).

pmt gene replacements

The *pmt* gene replacement cassettes were constructed by fusion PCR based on Yang and colleagues (YANG *et al.* 2004). 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, an 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 (Table2), and Southern hybridization using standard protocols (AUSUBEL *et al.* 2001). 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* 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 (HARRIS 2001).

Chemical sensitivity tests

Stain 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. 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 x 10^5 spores were grown in 10 ml of CM liquid in a Petri dish containing a glass coverslip, and incubated at 30°C or 42°C for 5 to12 hr. To examine conidiophores, 100 conidia in 5 μl of water were dropped on each side of a CM agar block, put 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) (MOMANY 2001). Slides were viewed with a Zeiss Axioplan Epifluorescence Microscope and imaged with the 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, incubated at 30°C or 42°C for 4 days. Four ml of sterile water were added to the plate to harvest spores by using a glass rod and conidial concentration in the resulting solution was determined with a haemacytometer. 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 2 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 Corporation, Carlsbad, CA). The resulting plasmid pTK64 was transformed into A850 and Δ*pmt* strains (Table 1).

Western blot analysis

For immunodetection, 1 x10^7 conidia/ml were grown in 50 ml of CM, incubated on a rotary shaker at 220 rpm and 30°C for 8 hr. 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 (BOOHER *et al.* 1989) were added to 0.1 g of ground mycelia, vortexed for 1 min and kept on ice for 1 min (4 times). The cell suspension was then centrifuged at 500 x g for 5 min at 4°C. The supernatant was collected and centrifuged at 10,000 x g for 30 min at 4°C. The pellet containing crude membranes was resuspended in a small volume of HK buffer containing 15% glycerol. Protein contents of samples were quantified with an RC DC Protein Assay Kit (Bio-Rad Labs., Hercules, CA) using bovine serum albumin as a standard. An equal volume of SDS-PAGE sample buffer (2x) was added to the crude membrane fractions, and heated for 5 min at 95°C. The solubilized proteins (10μg/lane) were resolved by

modified Laemmli Peptide SDS-PAGE (16% gel) (AUSUBEL *et al.* 2001) and transferred to a nylon membrane. AN5660 S tag was detected by Rabbit anti-S-Tag antibody (1:50,000), followed by anti-rabbit IgG coupled to horseradish peroxidase (1:5,000) (Immunology Consultants Laboratory, Inc. Newberg, OR) and staining using Amersham ECL Western blotting detection reagents and analysis system (GE Healthcare, United Kingdom).

Results

A. nidulans possesses 3 putative Pmts.

In previous work we identified the *swoA* mutant in a screen for temperature-sensitive polarity mutants and showed that the *swoA* gene encodes a protein with high homology to Pmt2, a protein *O*-mannosyltransferase in *S. cerevisiae. swoA* was renamed *pmtA* (MOMANY *et al.* 1999; SHAW and MOMANY 2002). Fungi contain three to seven Pmts falling into 3 subfamilies named Pmt1, Pmt2 and Pmt4 based on the *S. cerevisiae* members (GIRRBACH *et al.* 2000).

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 3 hypothetical Pmts, AN4761, AN5105, and AN1459. A BLASTP search of the *Saccharomyces* Genome Database (http://www.yeastgenome.org) and phylogenetic analysis (fig. 2.1) 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. 2.2). All three single Δ*pmt* mutants were viable and showed a variety of phenotypes described in detail below. To confirm that 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 wildtype phenotype (data not shown), likely because of variation in the copy numbers and position of integration of *gpd-pmt* cassettes.

Gentzsch and Tanner reported that *S. cerevisiae* mutants *pmt2 pmt3*, *pmt2 pmt4*, and *pmt1 pmt2 pmt3* at elevated temperatures survived only in the presence of an osmoticum (GENTZSCH and TANNER 1996). 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 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 2 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 wildtype at 25°C and 30°C; however, growth was reduced at 37°C and 42°C (Fig. 2.3). Growth of the Δ*pmtA* Δ*pmtB* double mutant was slightly retarded even at 25°C (Fig. 2.3). The Δ*pmtA* Δ*pmtB* double mutant was most sensitive to high temperatures followed by Δ*pmtA*, Δ*pmtC* and Δ*pmtB.* Adding osmoticum partially restored growth in all cases (Fig. 2.3).

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 agents Calcofluor or Congo red (Fig. 2.3). The Δ*pmtA* and Δ*pmtA* Δ*pmtB* mutants were more sensitive than wildtype to these agents at all temperatures tested. The Δ*pmtC* strain was resistant to Calcofluor at all temperatures tested but sensitive to Congo red at elevated temperatures. Surprisingly, the effect 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 wildtype. At 30°C and 37°C, the Δ*pmtB* mutant was hypersensitive to Calcofluor; however, at 42°C Δ*pmtB* 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 (MOMANY 2002). To determine the role of Pmts in growth and development of *A. nidulans*, we examined Δ*pmt* mutants from conidial germination through early growth (Fig. 2.4). After 6 h of incubation at 30°C, 12% of the Δ*pmtA* and 15.5% of the Δ*pmtA* Δ*pmtB* mutants sent out germ tubes compared to 5% of the wildtype, 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% the wildtype 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 the wildtype conidia and did not send out germ tubes, a phenocopy of the original *swoA* temperature-sensitive mutant (Fig. 2.4) (SHAW and MOMANY 2002). After 12 h of incubation at 42°C, 20-30% of hyphal tips of Δ*pmtB* germlings grown in liquid medium lysed (*n*=200) (Fig. 2.4). No similar lysis was seen in the wildtype. The Δ*pmtC* mutant at restrictive temperature showed swollen germ tubes and hyperbranching. No similar swelling or hyperbranching was seen in the wildtype. 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. 2.4). The hyphal tip lysis of Δ*pmtB* and Δ*pmtA* Δ*pmtB*, polar growth

defect of Δ*pmtA* and Δ*pmtA* Δ*pmtB*, and swollen hyphae of Δ*pmtC* 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 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 (ADAMS *et al.* 1998) (Fig. 2.5A). 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. 2.5B 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 wildtype (Fig. 2.6 and 7). At permissive temperature, the majority of Δ*pmtB* conidiophores were normal, though lysed vesicles were occasionally observed (Fig. 2.8D). Defects in Δ*pmtB* conidiophores were more severe at restrictive temperature, showing lysed vesicles and swollen conidiophore stalks (Fig. 2.5E to G). Although, normal conidiophores were present (Fig. 2.5H), many conidiophores failed to form vesicles, resulting in fewer conidia being produced (Fig. 2.5F). The Δ*pmtC* mutant at 30°C had excessive aerial hyphae and produced fewer conidiophores (Fig. 2.7). Conidiophore stalks were elongated (Fig. 2.5I) and conidiogenous layers were misplaced (Fig. 2.5I and J). At 37°C, vesicles and conidiogenous layers of the Δ*pmtC* strain were swollen (Fig. 2.5K and L).

Conidiation yielded fewer spores at all temperatures tested (Fig. 2.6). The Δ*pmtA* Δ*pmtB* conidiophores showed an additive phenotype. Swollen conidiophore stalks, vesicles, and conidiogenous layers were visible at 25°C (Fig. 2.5M to P). At 37°C, the vegetative and aerial hyphae were badly swollen and conidiophores were not detectable (data not shown). Conidial production of all Δ*pmt* mutants was reduced at all tested temperatures (Fig. 2.6). At permissive temperature, conidial production was partially restored in the presence of an osmostabilizer, except for Δ*pmtC* and Δ*pmtA* Δ*pmtB*. Neither adding osmoticum nor reducing temperature to 25°C significantly increased spore numbers of these mutants (Fig. 2.6 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* 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. 2.8). This "intrahyphal hyphae" phenotype is very similar to that previously reported for chitin synthase mutants (TAKESHITA *et al.* 2006).

PmtA and PmtC modify an ortholog of Wsc1, a known Pmt target.

In *S. cerevisiae*, Wsc family proteins are modified by Pmts (LOMMEL *et al.* 2004). 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 2 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. 2.9), in wildtype and Δ*pmtB* strains the S tagged Wsc ortholog had an apparent molecular mass of approximately 40 kDa, while in the Δ*pmtA* and the 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* in *A. nidulans* is consistent with hypoglycosylation and is very similar to results for tagged Wsc1 expressed in *pmt2*Δ and *pmt4*Δ *of S. cerevisiae* (LOMMEL *et al.* 2004).

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. 2.3). 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 (GIRRBACH and STRAHL 2003; WILLER *et al.* 2005). In *A. nidulans* Δ*pmtA*Δ*pmtB* strains show a synthetic phenotype that is more severe than 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* mutant is viable with both Pmt1 and Pmt2 subfamilies completely eliminated suggests that either 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 by the cell-wall altering agent Calcofluor (Fig. 2.3), consistent with the idea that the Pmt4 representative might be most important when Pmt1 or Pmt2 is perturbed. Deletion of representatives of both Pmt1 and Pmt4 subfamilies in combination appears to be lethal in *C. albicans* and *S. pombe* (PRILL *et al.* 2005; WILLER *et al.* 2005).

One of our more intriguing observations was the increased severity of the Δ*pmt* mutants at elevated temperatures. Indeed, we first identified *pmtA* in a screen for temperature-sensitive polarity mutants (MOMANY *et al.* 1999). The *pmtA* allele (at the time called *swoA*) gave 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 result in the truncation of the C-terminal 77 amino acids (SHAW and MOMANY 2002). 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 (HEINISCH *et al.* 1999; ZU *et al.* 2001). 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 (LOMMEL *et al.* 2004). Though the cell wall integrity pathway is not as well-understood in *A. nidulans*, there are many orthologs of cell wall integrity pathway genes, including the Wsc proteins (FUJIOKA *et al.* 2007). We found that a Wsc1 ortholog ran with an apparent reduced molecular mass in *A. nidulans* Δ*pmtA* and Δ*pmtC* strains (Fig. 2.9) 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 Δ*pmtA* on agar plates (data not shown). However, overexpression of WscA in Δ*pmtA* could not suppress the polar growth in liquid culture at 42ºC for 12 h (data not shown).

Most of the cell-wall related phenotypes of the *A. nidulans* Δ*pmt* mutants such as lysis were only visible under 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 (LOMMEL *et al.* 2004). It has been shown that Pmt modification is needed for stability of Wsc1 and Mid2 (LOMMEL *et al.* 2004). 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 possible 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 wildtype, while the Δ*pmtC* mutant sent out more germ tubes and more branches than the wildtype. The Δ*pmtA* Δ*pmtB* double mutant sent out germ tubes too early, and the *ΔpmtB* made hyphae within hyphae. All *A. nidulans* Δ*pmt* mutants made abnormal conidiophores and reduced conidial numbers at normal growth temperatures (Fig. 2.6 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; Δ*pmtB* showed swellings of the stalk and Δ*pmtC* made elongated stalks (Fig. 2.5). 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 function, localization and stability of Axl2, an axial budding positional marker (SANDERS *et al.* 1999).

Clearly the three *A. nidulans* Pmts play distinct roles in cell wall integrity and development. Though we cannot rule out direct roles by 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.

Acknowledgments

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Tables and Figures

Table 2.1. *Aspergillus nidulans* strains and plasmids

Strain	Genotype/Phenotype	Source or reference
A850	*argB2*::*trpC_B*; *methG*	FGSC
A773	*pyrG89*; *wA3*; *pyroA4*	FGSC
TNO2A7/ A1145	*pyrG89*; Δ*nkuA*::*argB*, *pyroA4*; *riboB2*	(NAYAK *et al.* 2006)
ATK08	*pyrG89*; *argB2*::*trpC_B*; *pyroA4*; Δ*pmtA*::Af*pyrG*	This study
ATK16	*pyrG89*; Δ*pmtB*::Af*pyrG*, *argB2*; *pyroA4*	This study
ATK38	*pyrG89*; *wA*; *argB2*; *pyroA4*; Δ*pmtC*::Af*pyrG*	This study
ATK40	*pyrG89*; *wA*; *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*; *wA*; *argB2*::*trpC_B*; *pyroA4*; Δ*pmtC*::Af*argB*	This study
ATK94	*pyrG89*; *argB2*::*trpC_B*, *ΔpmtB*::Af*pyrG*; *pyroA4*; Δ*pmtA*::Af*pyro*	This study
ATK104	*pryG89*; *wA*; 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
Plasmid		
pDV2	Amp^R, *argB-gpd(p)- ccdB-sgfp*	(TOEWS *et al.* 2004)
pFNO3	Kan^R, GA5-GFP, Af*pyrG*	(YANG *et al.* 2004)
pHL85	Amp^R, Kan^R, GA5-mCherry, Af*pyro*	Oakley, B.R.
pAO81	GA4-S-Tag, Af*pyrG*	(YANG *et al.* 2004)
pAf*argB2*	Amp^R, *argB2*	May, G.S.
pTK59	pENTR/D-TOPO::AN5660-Stag	This study
pTK64	pDV2::AN5660-Stag	This study

Table 2.2. 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

Table 2.2. Primers (continued)

CheckKuAF	GCAGTTGCCGCGCGGTTGGTGTGTC
AN5660F	CACCATGAGGTCGTTCACGCTATCCACAGTCTTC
AN5660StagR	GCGCCTGCACCAGCTCCGTGCCGGTCAGGATTCG
StagF	GGAGCTGGTGCAGGCGCTGGAGC
StagR	GCGCCAATTGCTGTTGCCAGGTGAGG

Figure 2.1. 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 *Mycobacterium tuberculosis* (MtPmt). ClustalX2 was used for multiple sequence alignments. The tree was created from a Bootstrap N-J tree. Percentages are bootstrap values based on 1000 trails. MtPmt was used as an out group. A branch length standard is indicated. The tree was drawn using the Tree View program.

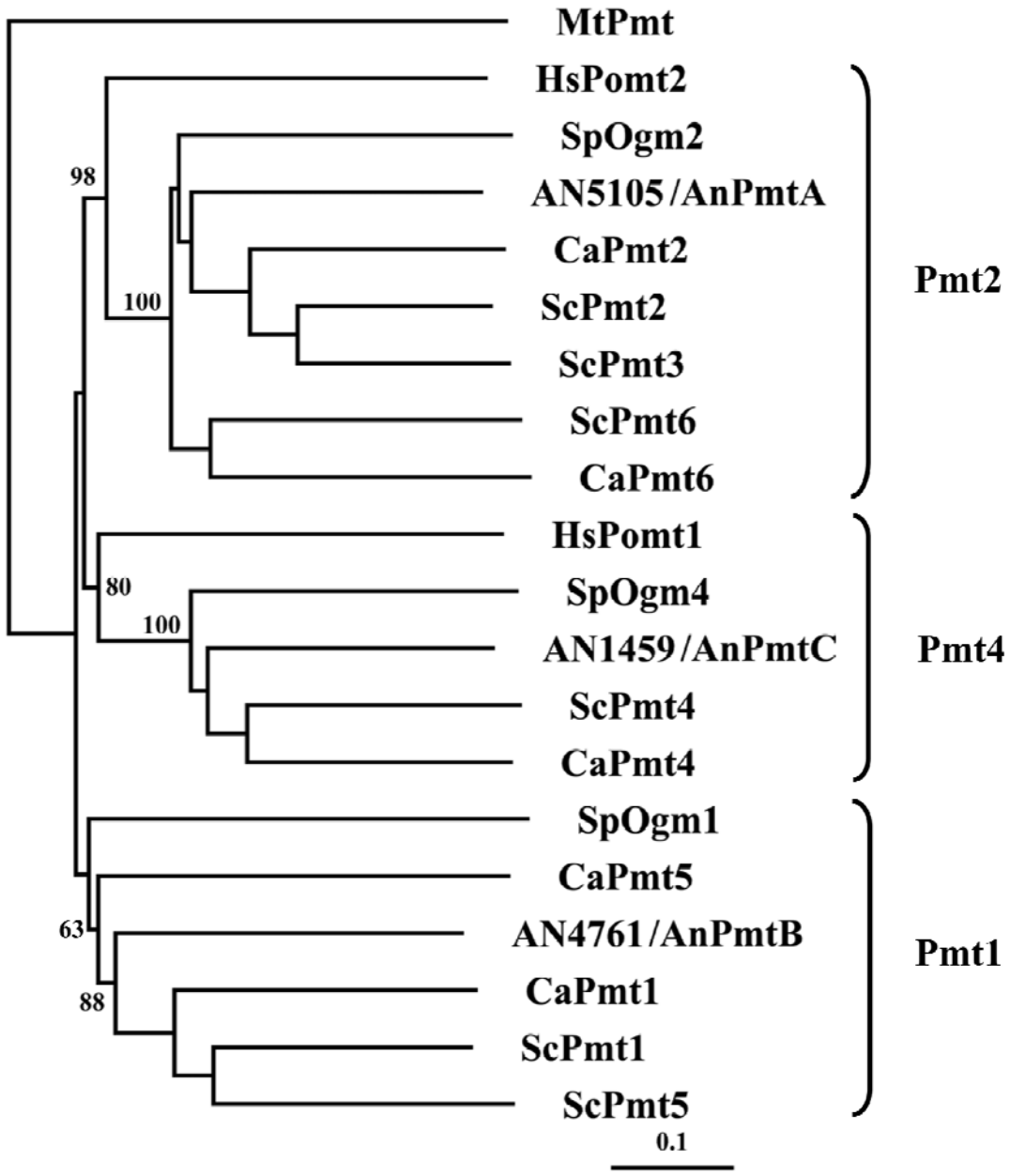

MtPmt
HsPomt2
SpOgm2
AN5105/AnPmtA
CaPmt2
ScPmt2
ScPmt3
ScPmt6
CaPmt6
HsPomt1
SpOgm4
AN1459/AnPmtC
ScPmt4
CaPmt4
SpOgm1
CaPmt5
AN4761/AnPmtB
CaPmt1
ScPmt1
ScPmt5
98
100
80
100
63
88
0.1
Pmt2
Pmt4
Pmt1

Figure 2.2. Southern hybridization of *pmt* deletion strains. Genomic DNA of the wildtype (A850) (WT) and strains transformed with deletion cassettes for (A) *pmtA* (ATK08), (B) *pmtB* (ATK16), and (C) *pmtC* (ATK38) was digested with restriction enzymes indicated and probed with radiolabeled upstream fragment of the corresponding *pmt*. B, *Bgl* II; C, *Cla* I; E, *EcoR* I; H, *Hind* III; N, *Nco* I; X, *Xba* I. The approximately 7 kb band in *ΔpmtB* cut with XbaI is from the left flank of the probe. This band is obscured by the 6.97-kB band in the wildtype.

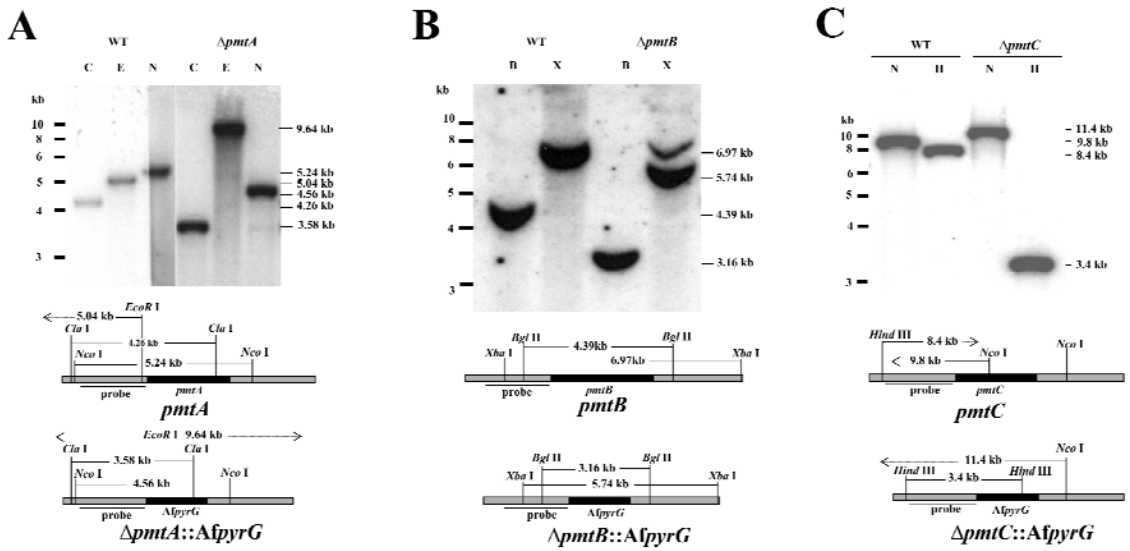

A
WT
ΔpmtA
C E N C E N
kb
10
8
6
5
4
3
9.64 kb
5.24 kb
5.04 kb
4.56 kb
4.26 kb
3.58 kb
EcoR I
5.04 kb
Cla I
Cla I
4.26 kb
Nco I
5.24 kb
Nco I
probe
pmtA
pmtA
EcoR I 9.64 kb
Cla I
Cla I
3.58 kb
Nco I
4.56 kb
Nco I
probe
AfpyrG
ΔpmtA::AfpyrG
B
WT
ΔpmtB
B X B X
kb
10
8
6
5
4
3
6.97 kb
5.74 kb
4.39 kb
3.16 kb
Bgl II
4.39kb
Bgl II
Xba I
6.97kb
Xba I
probe
pmtB
pmtB
Bgl II
3.16 kb
Bgl II
Xba I
5.74 kb
Xba I
probe
AfpyrG
ΔpmtB::AfpyrG
C
WT
ΔpmtC
N H N H
kb
10
8
6
5
4
3
11.4 kb
9.8 kb
8.4 kb
3.4 kb
Hind III
8.4 kb
Nco I
9.8 kb
Nco I
probe
pmtC
pmtC
Nco I
11.4 kb
Hind III
3.4 kb
Hind III
probe
AfpyrG
ΔpmtC::AfpyrG

Figure 2.3. Cell wall perturbing agents alter growth of Δ*pmt* mutants. A 10-fold serial dilution of conidia (10^6 to10^2) was spotted onto CM and CM containing 10μg/ml Calcoflour, 25μg/ml Congo red or 0.6 M KCl as osmotic stabilizer. Plates were incubated for 3 days at 25°C, 30°C, 37°C or 42°C. WT, wildtype.

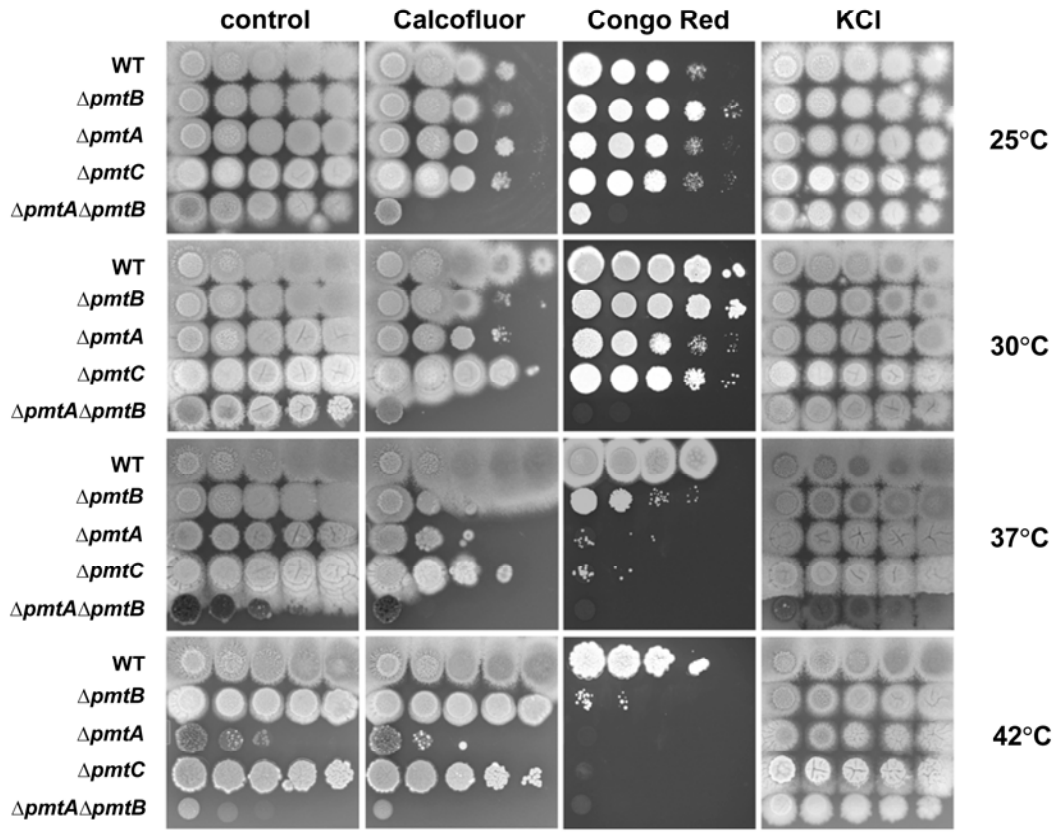

control
Calcofluor
Congo Red
KCl
WT
ΔpmtB
ΔpmtA
ΔpmtC
ΔpmtAΔpmtB
25°C
30°C
37°C
42°C

Figure 2.4. Phenotypes of Δ*pmt* mutants. Conidia of Δ*pmt* mutants were inoculated to CM, incubated for 12 h at 30°C or 42°C, fixed and stained with Hoechst and Calcofluor White to label nuclei and cell walls, respectively. (Left) Differential interference contrast images. (Right) florescence images. Arrow indicates an empty apical compartment. Bar, 10μm. WT, wildtype.

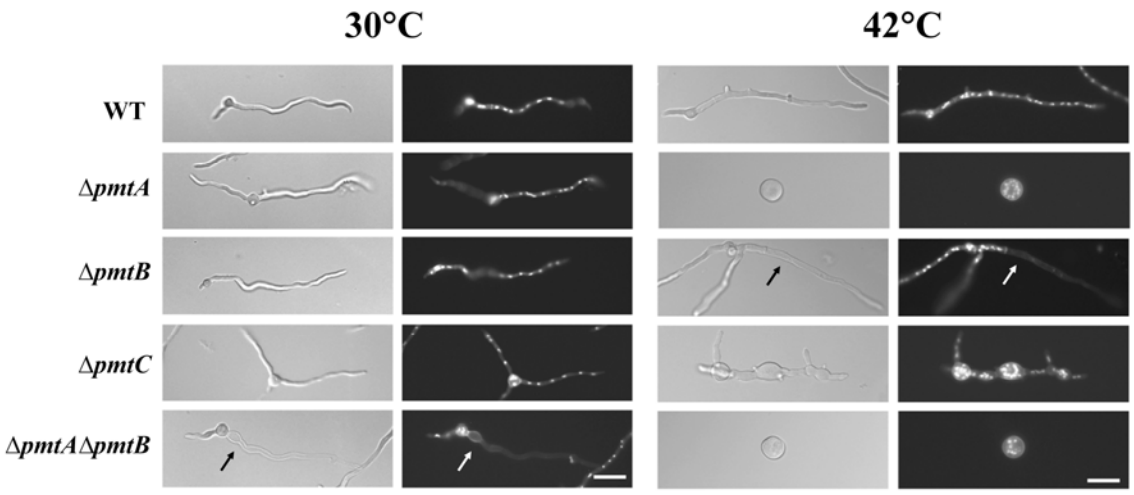

30°C
42°C
WT
ΔpmtA
ΔpmtB
ΔpmtC
ΔpmtAΔpmtB

Figure 2.5. Conidiophores of Δ*pmt* mutants are abnormal. (A) wildtype 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. arrow denotes conidiogenous layers, empty arrowhead denotes conidiophore stalk, and the dark arrow head denotes vesicle. Bar, in panel I is 5 μm; all others are 10 μm.

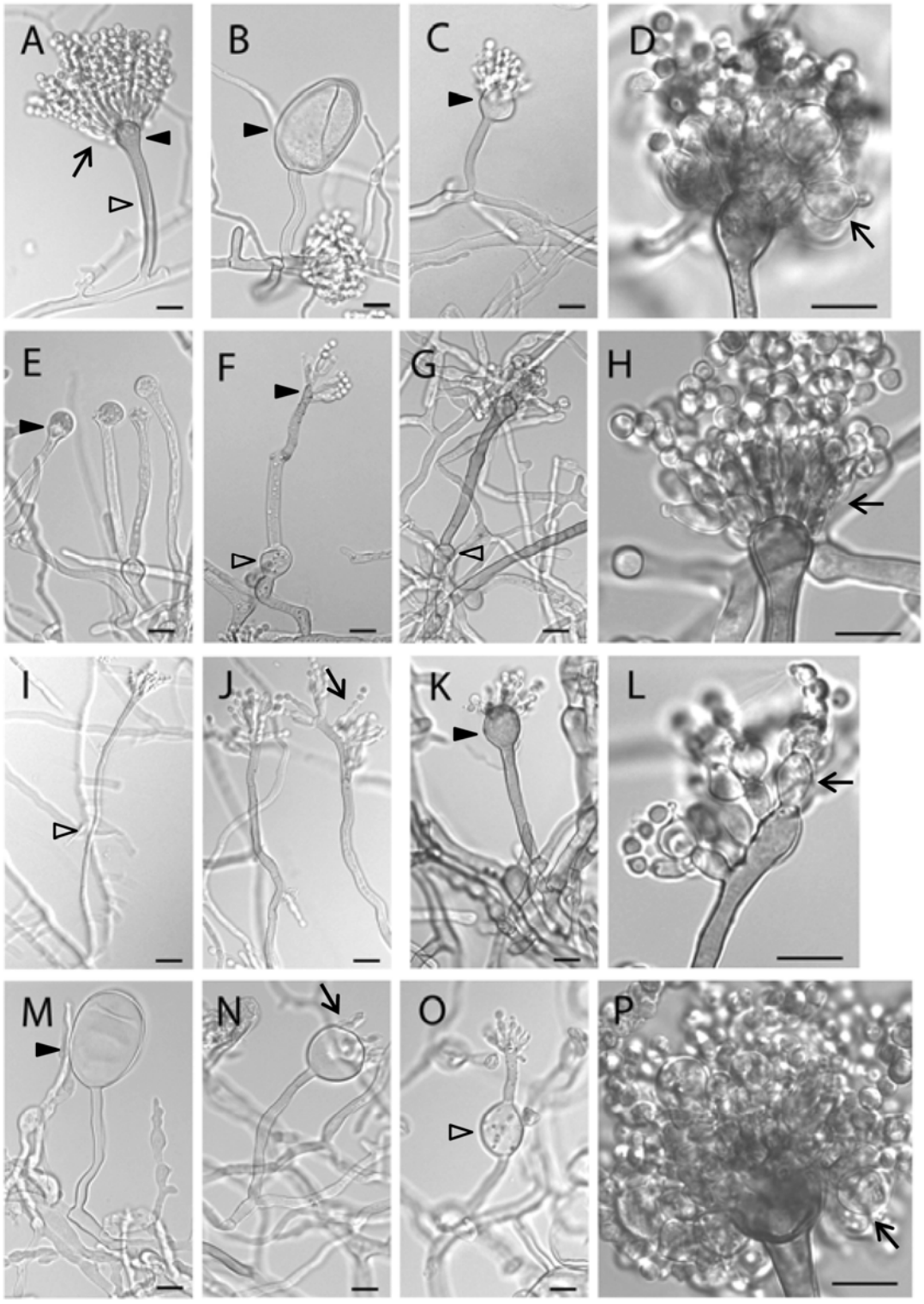

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 2.6. Conidiation efficiency of Δ*pmt* mutants is reduced. A total of 10^4 spores were spread on complete medium and complete medium containing 0.6M KCl. 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 hemacytometer. The asterisk denotes sample with too few conidia to count.

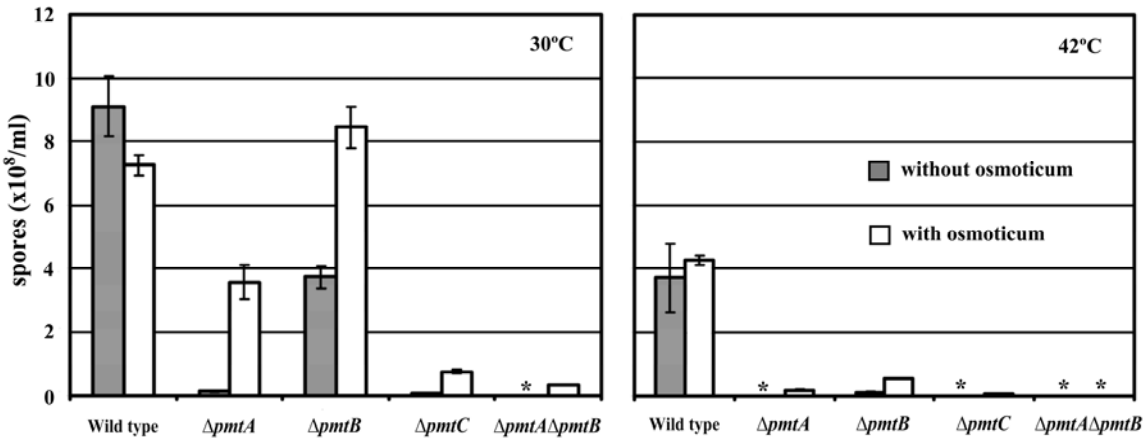

30°C
42°C
spores (x10^8/ml)
12
10
8
6
4
2
0
without osmoticum
with osmoticum
Wild type
ΔpmtA
ΔpmtB
ΔpmtC
ΔpmtAΔpmtB
Wild type
ΔpmtA
ΔpmtB
ΔpmtC
ΔpmtAΔpmtB

Figure 2.7. Δ*pmtC* produces excessive aerial hyphae. (A) wildtype, (B) Δ*pmtA*, (C) Δ*pmtB*, (D) Δ*pmtC* and (E) Δ*pmtA* Δ*pmtB* were grown on solid medium at 30°C for 3 days. All panels are at the same magnification.

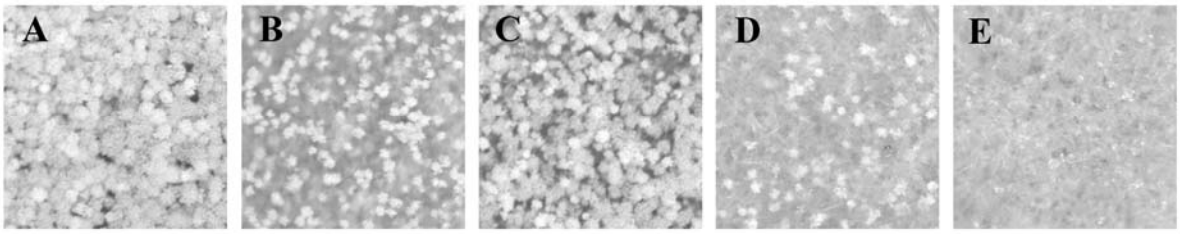

A
B
C
D
E

Figure 2.8. Δ*pmtB* and Δ*pmtA* Δ*pmtB* mutants make intrahyphal hyphae. wildtype (A), (B to D) Δ*pmtB* and Δ*pmtA* Δ*pmtB* (E) 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) florescence images. Bar, 10 μm.

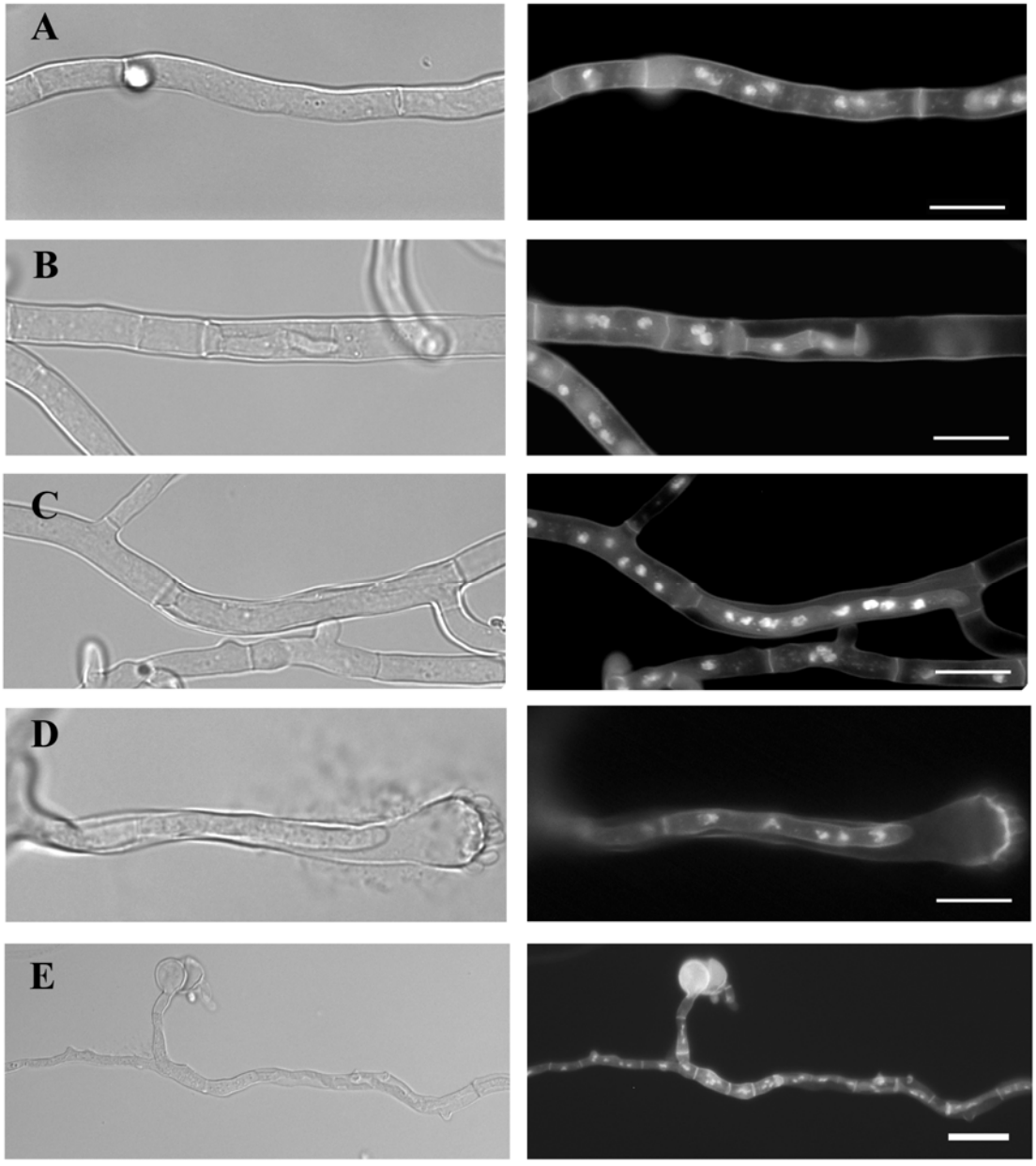
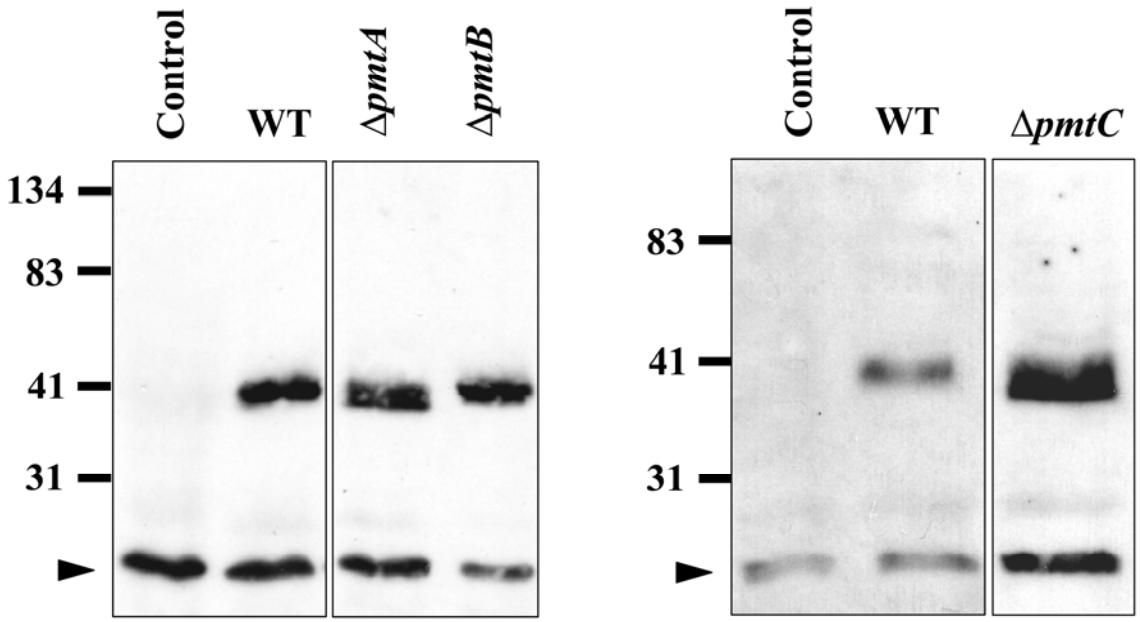

A
B
C
D
E

Figure 2.9. PmtA and PmtC modify AN5660 *in vivo*. Crude membrane fractions from Δ*pmt* mutants were separated on 16% SDS-PAGE. AN5660 Stag on a western blot was probed with antibody against S tag. The arrow head indicates nonspecific signals. WT, wildtype.


Control
WT
ΔpmtA
ΔpmtB
134
83
41
31
Control
WT
ΔpmtC
83
41
31

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CHAPTER 3

ASPERGILLUS NIDULANS THREE PROEIN *O*-MANNOSYLTRANSFERASES FORM HETEROMERIC COMPLEXES IN ALL POSSIBLE COMBINATIONS[1]

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Abstract

Protein *O*-mannosylation is a type of essential glycosylation in eukaryotes. Protein *O*-mannosyltransferase (Pmt) transfers a mannose residue to a serine or a threonine residue of a secretory protein. Eukaryotic Pmts are divided into three subfamilies: Pmt1, Pmt2 and Pmt4. Activity of Pmts in yeasts and animals requires their assembly into complexes. In *Saccharomyces cerevisiae*, Pmt 1 and Pmt2 form a heteromeric complex and Pmt 4 forms a homomeric complex. *A. nidulans* has only one member of each Pmt subfamily: PmtA, PmtB and PmtC. In this study we show that all three Pmt subfamilies of *A. nidulans* form heteromeric complexes with each other and that PmtC also forms a homomeric complex. Unlike ScPmt4, which always modifies membrane attached proteins, PmtC does not modify the membrane-bound protein MsbA. Although, a Pmt complex is required for enzymatic activity, we found that substrate specificity depends upon individual Pmts.

Introduction

Protein *O*-mannosylation is a type of protein glycosylation found from prokaryotes to eukaryotes (STRAHL-BOLSINGER *et al.* 1999; VANDERVEN *et al.* 2005). In eukaryotes, protein *O*-mannosyltransferases (Pmts) are integral membrane proteins localized in the endoplasmic reticulum (ER) (HASELBECK and TANNER 1983; STRAHL-BOLSINGER and SCHEINOST 1999). Pmts transfer a mannose residue from dolichyl phosphate mannose to the hydroxyl residue of Serine or threonine in secreted proteins facing the luminal side of the ER (GENTZSCH *et al.* 1995b; GIRRBACH *et al.* 2000). Further elongation of *O*-mannosyl glycans takes place in the

Golgi using a different set of enzymes and GDP-sugar (STRAHL-BOLSINGER *et al.* 1999). Pmts in eukaryotes are grouped into 3 subfamilies with names based on the *Saccharomyces cerevisiae* enzymes Pmt1, Pmt2 and Pmt4 (GENTZSCH and TANNER 1996). *S. cerevisiae* contains 7 Pmts, the most redundant among fungi. *Candida albicans* has 5 Pmts (TIMPEL *et al.* 2000). *Aspergillus nidulans* and other filamentous fungi have three pmts, one from each subfamily. Most animals have 2 Pmts, while plants lack these proteins (GIRRBACH and STRAHL 2003; VANDERVEN *et al.* 2005).

On a molecular level, *O*-mannosylation is important for stability, localization and function of secreted proteins (LOMMEL *et al.* 2004; TIMPEL *et al.* 1998; WEBER *et al.* 2004). Total lack of *O*-mannosylation is lethal in eukaryotes. Deletion of the Pmt2 subfamily is lethal in *C. albicans*, *Schizosaccharomyces pombe*, *Cryptococcus neoformans*, *Ustilago maydis*, and *A. fumigatus* (FERNANDEZ-ALVAREZ *et al.* 2009; MOUYNA *et al.* 2010; PRILL *et al.* 2005; WILLER *et al.* 2005; WILLGER *et al.* 2009). Simultaneous deletion of Pmt1 and Pmt4 is also lethal in these fungi, except for *U. maydis* (FERNANDEZ-ALVAREZ *et al.* 2009). Because of redundancy, multiple *S. cerevisiae* Pmts must be deleted to cause death (GENTZSCH and TANNER 1996). In animals homozygous deletion of the Pmt4 subfamily member *POMT1* is lethal (WILLER *et al.* 2004).

In *S. cerevisiae* Ser/Thr-rich domains of secreted proteins are likely to be mannosylated and membrane-associated proteins are mannosylated by Pmt4 (HUTZLER *et al.* 2007; STRAHL-BOLSINGER and TANNER 1991). But beyond these generalizations, the consensus sequence directing *O*-mannosylation is not known. Nonetheless, some targets of Pmts have been found empirically and these exhibit specificity toward individual target proteins. In *S. cerevisiae*, Pmt1 and Pmt2 mannosylate Aga2, Bar1, Cts1, Kre9, Pir2 and Wsc1, while Pmt4 mannosylates Gas1,

Kex2, Axl2 and Msb2 (GENTZSCH and TANNER 1997; LOMMEL *et al.* 2004). In *C. albicans*, Pmt1 and Pmt4, but not Pmt6 (subfamily 2) mannosylate Sec20 (WEBER *et al.* 2004), while Kre9 and Pir2 are exclusively mannosylated by Pmt1 (PRILL *et al.* 2005). However, there is evidence of limited substrate overlap. For example, *S. cerevisiae* Pmt4 and Pmt1/ScPmt2 complex mannosylate different domains of Ccw5p (ECKER *et al.* 2003).

O-mannosyltransferase activity requires a Pmt complex (GENTZSCH *et al.* 1995a; ICHIMIYA *et al.* 2004; MANYA *et al.* 2004). In *S. cerevisiae* and *S. pombe*, members of Pmt1 subfamily form heteromeric complexes with members of the Pmt2 subfamily (GIRRBACH and STRAHL 2003; WILLER *et al.* 2005). The Pmt4 subfamily of *S. cerevisiae* forms a homomeric complex (GIRRBACH and STRAHL 2003). In animals, POMT1 and POMT2 form a heteromeric complex required for enzymatic activity (ICHIMIYA *et al.* 2004; MANYA *et al.* 2004).

In previous work, both our group and another lab showed that the filamentous fungus *A. nidulans* has three *pmts* each representing a different subfamily: PmtA from subfamily 2, PmtB from subfamily 1, and PmtC from subfamily 4. We also showed that *ΔpmtA*, *ΔpmtB*, *ΔpmtC* and the double *ΔpmtA ΔpmtB* mutant were viable and that each null mutant had a distinctive phenotype (GOTO *et al.* 2009; KRIANGKRIPIPAT and MOMANY 2009). Our results strongly suggested that either PmtA and Pmt B do not form complexes in *A. nidulans* as the orthologous Pmt 2 and Pmt1 do in *S. cerevisiae,* or that such Family 1/Family 2 complexes are not required for viability in *A. nidulans* as they are in *S. cerevisiae*. In this study we show that all three *A. nidulans* Pmts form heteromeric complexes with each other and that PmtC forms a homomeric complex. We further show that each Pmt carries substrate specificity and that orthologous proteins are not modified by the same Pmt subfamily members in *A. nidulans* as in *S. cerevisiae*.

Most notably, we show that unlike *S. cerevisiae*, the Pmt4 subfamily is not exclusively responsible for the modification of membrane-bound proteins in *A. nidulans*.

Materials and Methods

Aspergillus strains and media.

The *A. nidulans* strains used in this study are listed in Table 3.1. Complete media (CM) and minimal media (MM) for *A. nidulans* were used. Nutritional supplements were added to media when necessary (http://www.fgsc.net).

Construction of tagged Pmts

Epitope tagged Pmts were constructed by fusion PCR based on the method of Yang and colleagues (YANG *et al.* 2004). Approximately 2 kb upstream and downstream of Pmt stop codon was amplified using genomic DNA from strain A850 as the template (Table 3.1). The *A. fumigatus* marker genes and epitope tag sequence were amplified from plasmids listed in Table 3.1. All primers used in this study are listed in Table 3.2. 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 ATK45 (Table 3.1). Transformants were tested for homologous integration by PCR and Southern hybridization. Southern blots were done according to Ausubel and colleague (AUSUBEL *et al.* 2001).

Construction of S-tagged Pmt target proteins

Pmt target protein with a C-terminal fusion to the S tag was constructed as followed. First, the GA4 S-tag fragment with a stop codon was amplified from pAO81 (Table 3.1) using primers listed in Table 3.2 and the gene of interest 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., CA). The plasmid was transferred into the pMT-DV2 destination vector (Table 3.1) using the Gateway LR Clonase II Enzyme Mix (Invitrogen Corp., CA). The resulting plasmid was transformed into the A850 and Δ*pmt* strains (Table 3.1).

Crude membrane preparation for Pmt target proteins

Conidial suspension of 1 x 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 extraction buffer (50 mM Tris-HCl, pH 7.5, 0.3 mM $MgCl_2$ plus protease Inhibitors (Complete, Mini; Protease Inhibitor Cocktail Tablets, Roche)) was added to 1 g of ground mycelia and vortexed for 10 min at 4°C. The cell suspension was then centrifuged at 500 x *g* for 10 min at 4°C. The supernatant was collected and centrifuged at 10,000 x *g* for 30 min at 4°C. The pellet containing crude membranes was resuspended in 150 µl of buffer containing 50 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 15% glycerol; and stored at -80°C.

Membrane enriched fraction preparation for immunoprecipitation

Freshly prepared conidia were grown in 1 liter of CM at the concentration of 2 x 10^8 conidia/ml and incubated on a rotary shaker at 200 rpm for 8 h at 30°C. Mycelia were filtered through a microporous membrane (Steritop™ Filter Units, Millipore), washed once with 100 ml cold stop buffer (0.9% NaCl, 1 mM NaN_3, 10 mM EDTA, 50 mM NaF, pH 7.0), lyophilized, and kept at -80°C. Crude membrane was prepared using methods adapted from Girrbach and colleague (GIRRBACH *et al.* 2000). One gram of lyophilized mycelia were ground at room temperature, resuspended in 15 milliliters of cold extraction buffer (50 mM Tris-HCl, pH 7.5, 0.3 mM $MgCl_2$ plus protease Inhibitors (Complete, Mini, EDTA-free; Protease Inhibitor Cocktail Tablets, Roche)), and vortexed for 30 min at 4°C. Mycelia suspension was centrifuged at 500 x *g*, for 10 min at 4°C. Supernatant was collected and centrifuged for 30 min at 20,000 rpm at 4 °C (Sorvall SS34 rotor) to obtain crude membrane pellet. Membrane enriched fraction was resuspended in 1 ml of buffer containing 50 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 15% glycerol; and stored at -80°C.

Immunoprecipitation

Immunoprecipitation methods were adapted from Girrbach and colleague (GIRRBACH *et al.* 2000). One milliliter of crude membrane was solubilized in 4 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.3 mM $MgCl_2$, 10% glycerol, 0.35% sodium deoxycholate, 0.5% Triton X-100 plus Protease Inhibitor (Complete, Mini, EDTA-free; Protease Inhibitor Cocktail Tablets, Roche)). The protein contents of samples were quantified with an RC DC Protein Assay Kit (Bio-Rad Laboratories, CA) using bovine serum albumin as a standard. Three hundred µl of agarose immobilized anti-epitope tag antibody slurry (or 150 µl of packed beads volume) was

added per 100 mg of protein. Agarose immobilized rabbit anti-S tag or agarose immobilized rabbit anti-HA antibody was used for immunoprecipitation (Immunology Consultants Laboratory, Inc., Newberg, OR). Incubation with the solubilized membrane enriched fraction was carried out at 4°C on a rocker for 2 h followed by 5 washes at 4°C with equal volume of cold lysis buffer and one wash with 1 ml of cold Tris-buffered saline. The bound proteins were eluted with 2 x SDS loading dye by incubating for 5 min at 95°C.

Western Blot Analyses

For epitope tagged Pmts, the eluent was loaded into 2 gels to be further probed with anti-S tag or anti-HA antibody. The proteins were resolved on 4-20% gradient Tris-HEPES SDS gels (Pierce Protein Gels, Thermo Fisher Scientific Inc., IL) or on SDS-PAGE with 7% acrylamide (AUSUBEL *et al.* 2001). The protein bands were transferred to a nylon membrane. S-tagged Pmts were detected with mouse anti-S-tag antibody (1:5,000) (Abcam Inc., MA), followed by Sheep anti-Mouse IgG ECL antibody coupled to horseradish peroxidase (1:5,000) (GE Healthcare, NJ). HA-tagged proteins were detected with mouse anti-HA-tag antibody (1:5,000) (Invitrogen Co., CA), followed by Sheep anti-Mouse IgG ECL antibody coupled to horseradish peroxidase (1:5,000) (GE Healthcare, NJ).

For Pmt target proteins analyses, an equal volume of 2x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (AUSUBEL *et al.* 2001) was added to the crude membrane fractions and heated for 5 min at 95°C. Forty micrograms of the solubilized proteins were loaded per lane. S-tagged proteins were detected with rabbit anti-S-tag antibody (1:50,000) (Immunology Consultants Laboratory, Inc., OR), followed by Goat anti-

rabbit immunoglobulin G coupled to horseradish peroxidase (1:5,000) (EMD Biosciences, Inc, CA)

Staining was performed using an Amersham ECL Western blotting detection reagent and analysis system (GE Healthcare, NJ).

PNGase F treatment

PNGase F treatment was performed according to manufacturer's instructions (New England Biolabs, Inc., MA). AxlA was used as a positive control in all treatments.

Results

To investigate Pmt complex formation in *A. nidulans*, we constructed the following strains with C-terminal purification tags: *pmtA*::S tag ($PmtA^{S}$), *pmtB*::HA ($PmtB^{HA}$) and *pmtC*::HA ($PmtC^{HA}$). The fusion constructs were integrated at their native loci and expressed from their native promoters. Correct integrations were confirmed by PCR and Southern hybridization (data not shown). All tagged Pmt strains showed wildtype phenotypes at 30^{o}C and 42^{o}C (data not shown). The S tag and HA tag are both small with deduced molecular mass of 2.26 kDa and 3.98 kDa, respectively. The predicted molecular mass of $PmtA^{S}$ is 86.86 kDa, of $PmtB^{HA}$ is 108.98 kDa, and of $PmtC^{HA}$ is 92.28 kDa.

Membrane-enriched fractions were isolated from Pmt fusion strains and probed by Western blot with antibodies against the S tag or HA tag. No signals were detected, suggesting relatively low levels of fusion protein. To isolate more concentrated fusion protein, we performed immunoprecipitation of solubilized membrane-enriched fractions with agarose-

immobilized anti-HA or anti-S tag antibodies followed by Western blotting with the same anti-tag antibodies (Fig. 3.1). The $PmtA^{S}$ strain yielded two bands, one with an apparent molecular mass of 68 kDa, slightly smaller than the predicted molecular mass of the fusion protein, and a second much larger band of 165 kDa. The $PmtB^{HA}$ strain yielded two bands, one with an apparent molecular mass of approximately 120kDa, slightly larger than the predicted mass of the fusion protein, and a second much larger band of 180 kDa. The $PmtC^{HA}$ strain also yielded two bands, one with an apparent molecular mass of approximately 80kDa, slightly smaller than the predicted molecular mass of the fusion protein, and a second much larger band of 160 kDa. In each case, the lower band is likely the Pmt. Pmts isolated from yeast and animals frequently show apparent molecular masses that are smaller or larger than predicted (GIRRBACH and STRAHL 2003; MANYA *et al.* 2004). The upper bands seen in all immunoprecipitations could be the tagged Pmt bound to an unknown but specific protein. These bands always appear at a certain molecular mass. For example, the upper band of $PmtB^{HA}$ appears at the same molecular mass when it is directly pulled down with anti-HA antibody (Fig. 3.1), or co-immunoprecipitated with $PmtA^{S}$ (Fig. 3.2) or $PmtC^{S}$ (Fig. 3.4). To test *A. nidulans* wildtype proteins for non-specific cross-reaction, solubilized membrane-enriched fractions from wildtype were immunoprecipitated and analyzed by Western blot using identical protocols. Anti- tag antibodies showed no cross-reactivity with solubilized membrane-enriched fractions from the non-tagged strain (data not shown).

PmtA forms heteromeric complexes with PmtB.

In *S. cerevisiae* Pmt1 and Pmt2 form a heteromeric complex (GIRRBACH and STRAHL 2003).To investigate whether the *A. nidulans* orthologs, PmtB and PmtA, also form a

heteromeric complex, a PmtA^{S} PmtB^{HA} strain (*pmtA*::S tag, *pmtB*::HA, ATK192) was derived from sexual crosses. Immunoprecipitation was performed with agarose-immobilized anti-S tag antibody. The immunoprecipitate was divided into two aliquots and both were analyzed by Western blot. One aliquot was probed with anti-S tag antibody and the other was probed with anti-HA antibody. When probed with anti-S tag antibody, the same bands that were seen in the PmtA^{S} strain were visible (approximately 68 kDa and 165 kDa) (Fig. 3.2). When probed with anti-HA antibody, the same bands that were seen in the PmtB^{HA} strain were visible (approximately 120kDa and 180 kDa) (Fig. 3.2). Identical results were seen when immunoprecipitation was performed with agarose-immobilized anti-HA antibody and Westerns were probed with anti-S tag or anti-HA antibody (data not shown). Our results suggest that PmtA and PmtB form heteromeric complexes in *A. nidulans*.

PmtA forms heteromeric complexes with PmtC.

In *S. cerevisiae*, Pmt2 and Pmt4 do not form complexes (GIRRBACH and STRAHL 2003). To investigate the interactions between the *A. nidulans* Pmt 2 and Pmt4 orthologs PmtA and PmtC, a strain carrying PmtA^{S} and PmtC^{HA} in the presence of PmtB (*pmtA*::S tag, *pmtC*::HA, ATK165) and a strain carrying PmtA^{S} and PmtC^{HA} in the absence of PmtB (*pmtA*::S tag, *pmtC*::HA, Δ*pmtB*; ATK168) were derived from sexual crosses. Immunoprecipitation experiments were performed with agarose-immobilized anti-S tag antibody and identical Westerns were probed with anti-S tag antibody or anti-HA antibody (Fig 3.3). Surprisingly, immunoblots probed with anti-HA antibody showed a band of approximately 80 kDa whether or not PmtB was present. Our results suggest that *A. nidulans* PmtA forms a heteromeric complex with PmtC in the presence and absence of PmtB.

PmtB forms heteromeric complexes with PmtC.

To determine interaction between PmtB and PmtC, a strain bearing $PmtB^{HA}$ and $PmtC^{S}$ in the presence of PmtA (*pmtB*::HA, *pmtC*::S tag; ATK193) and a strain carrying $PmtB^{HA}$ and $PmtC^{S}$ in the absence of PmtA (*pmtB*::HA, *pmtC*::S tag, Δ*pmtA;* ATK200) were derived from sexual crosses. Immunoprecipitation experiments were performed with agarose-immobilized anti-S tag antibody and identical Westerns were probed with anti-S tag antibody or anti-HA antibody (Fig. 3.4). Surprisingly, immunoblots probed with anti-HA antibody showed a band of approximately 120 kDa whether or not PmtA was present. Our results suggest that *A. nidulans* PmtB forms a heteromeric complex with PmtC in the presence and absence of PmtA.

PmtC-PmtC homomeric complexes are formed in *A. nidulans*.

In *S. cerevisiae*, Pmt4 functions as a dimer, not a monomer (GIRRBACH and STRAHL 2003). To investigate whether the *A. nidulans* ortholog, PmtC, is a monomer or forms homomeric complexes in *A. nidulans*, a stable diploid $PmtC^{S}$ $PmtC^{HA}$ strain (*pmtC*::S tag/ *pmtC*::HA; ATK217) was derived. As described above, immunoprecipitation was performed with agarose-immobilized anti-S tag antibody and identical Westerns were probed with anti-S tag antibody or anti-HA antibody. In both cases the same bands that were seen in the $PmtC^{HA}$ strain were visible (approximately 80kDa and 160 kDa) (Fig. 3.5). Identical results were seen when immunoprecipitation was performed with agarose-immobilized anti-HA antibody and Westerns were probed with anti-S tag or anti-HA antibody (data not shown). Our results suggest that PmtC forms homomeric complexes in *A. nidulans*.

Pmt complexes are formed *in vivo.*

To eliminate the possibility that the *A. nidulans* Pmt complexes we detected might result from nonspecific aggregation of Pmts in solubilized membrane fractions, we performed the following control experiment. Membrane-enriched fractions were isolated from strain ATK192, in which $PmtA^{S}$ and $PmtB^{HA}$ are co-expressed, and solubilized. Membrane-enriched fractions were also isolated separately from strain ATK89, in which $PmtA^{S}$ is expressed, and from ATK187, in which $PmtB^{HA}$ is expressed. Isolated ATK89 and ATK 187 membrane fractions were combined and solubilized. Agarose-immobilized anti-S tag antibody was used to immunoprecipitate protein from solubilized membranes of the strain co-expressing $PmtA^{S}$ and $PmtB^{HA}$ (ATK187) or from the combined and solubilized membranes of the strains separately expressing $PmtA^{S}$ (ATK89) and $PmtB^{HA}$ (ATK187). Identical aliquots were probed with anti-S tag antibody or anti-HA antibody (Fig. 3.6). Similarly, membrane-enriched fractions were isolated and solubilized from strain ATK165, in which $PmtA^{S}$ and $PmtC^{HA}$ are co-expressed, and separately from strain ATK89, in which $PmtA^{S}$ is expressed, and from ATK154, in which $PmtC^{HA}$ is expressed, subjected to immunoprecipitation and Western blotting. In both cases, the co-expressed Pmts were co-precipitated, but the separately expressed and combined Pmts were not co-precipitated. Our results suggest that the Pmt complexes we detected were not the result of nonspecific protein aggregation.

Determining Pmt specificity toward target proteins

Reports in yeasts showed that Pmts complexes modify specific target proteins (GENTZSCH and TANNER 1997; LUSSIER *et al.* 1995; SANDERS *et al.* 1999; WILLER *et al.* 2005). Because *S. cerevisiae* harbors multiple Pmts from each subfamily that can at least partially

substitute for each other and because deletion of all members of a given subfamily in *S. cerevisiae* is lethal, it has not been possible to determine whether target specificity is determined by individual Pmts or by the complex in which they reside. However, *A. nidulans* has only one Pmt representing each subfamily and null mutants of individual *pmts* are viable. To investigate Pmt specificity, we identified *A. nidulans* orthologs of proteins known to be Pmt targets in *S. cerevisiae,* expressed each target from the constitutive *gpd* promoter and tagged each with a C-terminal S tag. The target protein constructs were transformed into wildtype and Δ*pmt* mutants.

MsbA is modified by PmtA and PmtB

In *S. cerevisiae*, Msb2 is an osmosensor in the HOG pathway and is modified by Pmt4 (O'ROURKE and HERSKOWITZ 2002; YANG *et al.* 2009). Using the *S. cerevisiae* Msb2 protein sequence to query the *A. nidulans* genome database at the Broad Institute (http://www.broad.mit.edu), we found one hit, ANID_07041.1, which we named "MsbA". $MsbA^S$ was expressed from the *gpd* promoter in wildtype and the *Δpmt* mutants, crude membrane fractions were isolated and probed with anti-S tag antibodies in Western blotting experiments. The apparent molecular mass of MsbA in the Δ*pmtA* and Δ*pmtB* mutants was 160 and 170 kDa respectively, while molecular mass of $MsbA^S$ in Δ*pmtA*Δ*pmtB* mutant appeared lower at 150 kDa (Fig. 3.7A). The apparent molecular mass of MsbA in the Δ*pmtC* was approximately 240 kDa, which was the same as that in the wildtype (Fig. 3.7A). MsbA has a putative *N*-glycosylation site making it possible that the failure to detect a shift in mobility in Δ*pmtC* could have resulted from aberrant *N*-glycosylation in the absence of proper *O*-glycosylation. To investigate this possibility, MsbA from all backgrounds was treated with PNGase F. No shift in molecular mass of MsbA expressed in Δ*pmtC* compared to wildtype was

observed after PNGase F treatment (Fig. 3.7B). Our data show MsbA is modified by PmtA and PmtB, but not by PmtC. Further, PmtA and PmtB appear to make separate modifications to MsbA.

WscA is modified by PmtA and PmtC, independently of PmtB

In *S. cerevisiae* the cell wall stress sensor, Wsc1 is modified by Pmt1 and Pmt2, as a member of the Pmt1/2 complex (LOMMEL *et al.* 2004). Previously, we showed that the *A. nidulans* ortholog, WscA migrated faster in *ΔpmtA* and *ΔpmtC* strains than in *ΔpmtB* or wildtype strains (KRIANGKRIPIPAT and MOMANY 2009). This was somewhat surprising given the fact that *S. cerevisiae* Pmt2 is only active as part of a Pmt1-Pmt2 heteroduplex. To determine whether modification of WscA by PmtA might require the PmtA-PmtB complex, we analyzed WscA in Δ*pmtA*, Δ*pmtB* and Δ*pmtA*Δ*pmtB* strains. Immunoblots showed that the apparent molecular mass of $WscA^S$ in wildtype and Δ*pmtB* backgrounds was approximately 47 kDa, while in Δ*pmtA* and Δ*pmtA*Δ*pmtB* backgrounds the apparent molecular mass was approximately 44 KDa (Fig. 3.8). The results suggest that mannosylation of WscA by PmtA is not reliant on PmtA-PmtB complexes and is consistent with the idea that Pmt complex membership in *A. nidulans* does not strictly correlate with that in *S. cerevisiae*.

AxlA is not modified by Pmts.

Axl2 is an integral plasma membrane protein required for axial budding in haploid cells and localizes to the incipient bud site and bud neck (ROEMER *et al.* 1996). ScAxl2 and its ortholog from *Candida albicans*, CaAxl2, are mannosylated by Pmt4p (PRILL *et al.* 2005; SANDERS *et al.* 1999). Using the *S. cerevisiae* Axl2 protein sequence to query the *A. nidulans*

genome database at the Broad Institute (http://www.broad.mit.edu), we found one hit, ANID_01359.1, which we named "AxlA". Motif and structure prediction programs showed that like ScAxl2, AxlA contains CADG-like domains, is predicted to form transmembrane helices (KROGH *et al.* 2001; ROEMER *et al.* 1996).

$AxlA^S$ was expressed from the *gpd* promoter in wildtype and the *Δpmt* mutants, crude membrane fractions were isolated and probed with anti-S tag antibodies in Western blotting experiments. Surprisingly, $AxlA^S$ from all backgrounds had an apparent molecular mass of approximately 130 kDa, larger than its deduced molecular mass of 103.56 kDa (Fig. 3.9A). AxlA possesses 4 putative *N*-glycosylation sites on the N-terminus facing the luminal space. Ecker and colleague reported a dramatic increase in molecular mass of Ccw5 in *pmt4*Δ caused by aberrant *N*-glycosylation (ECKER *et al.* 2003). To investigate *N* -glycosylation, $AxlA^S$ from wildtype and Δ*pmtC* were treated with PNGase F to remove *N*-glycan chains and subjected to western blot and immuno detection. The molecular mass of PNGase F treated $AxlA^S$ in both wild type and Δ*pmtC* was equally reduced (Fig. 3.9B). In addition, overexpression of $AxlA^S$ did not suppress previously described phenotypes of the Δ*pmtC* mutant, elongated conidiophore stalks and misplaced conidiogenous layers (KRIANGKRIPIPAT and MOMANY 2009) (data not shown).

Discussion

A. nidulans Pmts form heteromeric complexes in every possible subfamily combination.

The formation of a two-member Pmt complex is required for enzymatic activity (GIRRBACH *et al.* 2000; MANYA *et al.* 2004). This complex can be either heteromeric or homomeric. In fungi, *S. cerevisiae* and *S. pombe* for which data has been reported, Pmt1 and

Pmt2 subfamily members form heteromeric complexes that are absolutely required for maximum enzymatic activity (GENTZSCH *et al.* 1995b; GIRRBACH and STRAHL 2003; WILLER *et al.* 2005). In animals Pmts from subfamily 2 (POMT2) and subfamily 4 (POMT1) form a heteromeric complex (Manya et al., 2004). Also in *S. cerevisiae* Pmt4 forms a homomeric complex (GIRRBACH and STRAHL 2003).

Like *S. cerevisiae* and *S. pombe*, the *A. nidulans* Pmt1 subfamily member (PmtB) forms a heteromeric complex with the Pmt2 subfamily member (PmtA) (Fig. 3.2, Fig. 3.10) (GIRRBACH and STRAHL 2003; WILLER *et al.* 2005). Unlike other fungi, the Pmt1/Pmt2 complex is not required for viability in *A. nidulans* since the *ΔpmtA ΔpmtB* double mutant is viable.

The previously observed unique phenotypes of *A. nidulans* Δ*pmtA* mutant and Δ*pmtB* mutant along with the viability of the Δ*pmtA* Δ*pmtB double* mutant suggested that PmtA and PmtB might have more than one binding partner (KRIANGKRIPIPAT and MOMANY 2009). Our immunoprecipitation data strongly argue that an alternate partner in heteromeric complexes for both PmtA (subfamily 2) and Pmt B (subfamily 1) is PmtC (subfamily 4). And, at least in the case of WscA, the mannosylation of the target protein does not appear to require the classical subfamily 1 member (PmtB) (Fig. 3.8). *A. nidulans* is the first fungus reported to form heteromeric complexes involving the Pmt4 subfamily (Fig. 3.10). Though such complexes have been reported in animals where POMT1 (subfamily 4) forms a heteromeric complex with POMT2 (subfamily 2) (ICHIMIYA *et al.* 2004; MANYA *et al.* 2004).

We also showed that like *S. cerevisiae*, *A. nidulans* forms a Pmt4 (PmtC) homomeric complex (Fig. 3.5) (GIRRBACH and STRAHL 2003). Though we were unable to recover appropriately marked strains to investigate PmtA-PmtA and PmtB-PmtB homomeric complexes, if such complexes exist, they are not likely to be functional based on genetic evidence. The

Δ*pmtA* Δ*pmtC* and Δ*pmtB* Δ*pmtC* double mutants were both inviable, though the Δ*pmtA* Δ*pmtB* was viable. Without PmtB or PmtC, PmtA lacks a counter part to form a complex which is required for enzymatic activity. Apparently the PmtC homomeric complex and activity of PmtC is sufficient to maintain cell viability (KRIANGKRIPIPAT and MOMANY 2009). However, we cannot rule out the possibility that deletion of multiple Pmts is lethal because *O*-mannosylation of certain targets is essential.

The increased ability to form complexes among Pmt subfamily members might result from the fact that *A. nidulans* has only one member from each subfamily (KRIANGKRIPIPAT and MOMANY 2009). Thus, elimination of any one Pmt member would dramatically limit *O*-mannosylation. To maintain their activity, *A. nidulans* subfamily 1 and subfamily 2 form heteromeric complexes with Pmt 4 subfamily, which is not observed in yeasts. In *S. cerevisiae* Pmt1 forms a complex with Pmt3 and Pmt2 forms a complex with Pmt5 only when their preferred partners are missing (GIRRBACH and STRAHL 2003); however, PmtA-PmtC and PmtB-PmtC heteromeric complexes appear to be present always.

A. nidulans Pmt complexes are formed when co-expressed and formed at physiological conditions. The evidence rules out the possibility of artificial protein interactions. Manya and colleague reported that only when Pomt1 and Pomt2 were coexpressed, was activity of protein *O*-mannosyltransferases detected (MANYA *et al.* 2004). These results suggest that Pmt complexes are formed only *in vivo.*

Individual Pmts determine target specificity

Although, a Pmt complex is required for enzymatic activity, individual Pmts within the complex appear to have different target specificity in at least some cases. Even when a

Pmt1/Pmt2 complex is formed, each member of the complex can modify different regions in the target protein, as we found for MsbA (Fig. 3.7). As a result, deletion of both Pmts further reduces appearance molecular mass of MsbA. Similarly, *S. cerevisiae* and *S. pombe* Pmt1 and Pmt2 subfamily members have been shown to act on different motifs of the same target proteins (GENTZSCH and TANNER 1997; WILLER *et al.* 2005). However, we could not rule out the possibility that Pmt activity is complex independent. Gentzsch and Tanner reported that molecular mass of Ggp1p or Gas1p shifted lower in *pmt6*Δ, while Girrbach and Sabine Strahl found that Pmt6 did not interact with any Pmts (GENTZSCH and TANNER 1997; GIRRBACH and STRAHL 2003).

In addition, Pmts target protein orthologs are modified by different Pmts, suggesting that motif of the target protein surrounding Ser/Thr but not protein ortholog is recognized by Pmts. We show that WscA is modified by PmtA and PmtC. Similarly, ScWsc1 is modified by Pmt2 and Pmt4 (LOMMEL *et al.* 2004); however, SpWsc1 is a substrate of Oma1 and Oma2, but not Oma4 (WILLER *et al.* 2005). In *S. cerevisiae* membrane associated proteins, including Axl2, Msb2, and WscA are modified by Pmt4 (HUTZLER *et al.* 2007; LOMMEL *et al.* 2004; SANDERS *et al.* 1999). However, *A. nidulans* MsbA and Schizosaccharomyces Wsc1 are not targets of Pmt4 subfamily (WILLER *et al.* 2005).

Although AxlA is the only *S. cerevisiae* Axl2 ortholog and contains CADG-like domains similar to Axl2, AxlA lacks Ser/Ter-rich regions present in both Axl2 from *S. cerevisiae* and *C. albicans* (data not shown).

Membrane attachment is not a determinant for PmtC modification.

Hutzler reported that in *S. cerevisiae* membrane association is a determinant for substrate recognition by Pmt4 (HUTZLER *et al.* 2007). We examined 3 Pmt target proteins, which were AxlA, WscA and MsbA. Immunoblot showed that WscA was modified by PmtC (KRIANGKRIPIPAT and MOMANY 2009); however, AxlA and MsbA were not modified by PmtC. The data implied that unlike ScPmt4, PmtC does not always modify membrane bound proteins.

Acknowledgments

This work was supported by DOE Biosciences award DE-FG02-97ER20275 and the Office of the Vice President for Research at the University of Georgia. TK was supported by the Royal Thai Government.

Tables and Figures

Table 3.1. *A. nidulans* strains and plasmids

Strain or plasmid	Genotype/phenotype	Source or reference
Strains		
A850	*argB2*::*trpC_B methG*	FGSC
ATK08	*pyrG89 argB2*::*trpC_B pyroA4* Δ*pmtA*::Af*pyrG*	(KRIANGKRIPIPAT and MOMANY 2009)
ATK16	*pyrG89* Δ*pmtB*::Af*pyrG argB2 pyroA4*	(KRIANGKRIPIPAT and MOMANY 2009)
ATK104	*pryG89 wA3* Δ*pmtB*::Af*pyro argB2 pyroA4*	(KRIANGKRIPIPAT and MOMANY 2009)
ATK38	*pyrG89 wA3 argB2 pyroA4* Δ*pmtC*::Af*pyrG*	(KRIANGKRIPIPAT and MOMANY 2009)
ATK42	*pyrG89 wA3 argB*::*trpC_B pyroA4*	This study
ATK45	*pyrG89 argB*::*trpC_B nkuA*::Af*pyro pyroA4*	(KRIANGKRIPIPAT and MOMANY 2009)
ATK95	*pyrG89 argB2*::*trpC_B pyroA4 ΔpmtA*::Af*pyro*	This study
ATK104	*pyrG89 wA3 ΔpmtB*::Af*pyro argB2 pyroA4*	This study
ATK89	*pyrG89 argB*::*trpC_B nkuA*::Af*pyro pyroA4 pmtA*::S-tag-Af*pyrG*	This study
ATK187	*pyrG89 pmtB*::HA-tag-Af*argB argB*::*trpC_B nkuA*::Af*pyro pyroA4*	This study
ATK154	*pyrG89 argB*::*trpC_B nkuA*::Af*pyro pyroA4 pmtC*::HA-tag-Af*argB*	This study
ATK208	*pyrG89 argB*::*trpC_B pyroA4 pmtC*::S-tag-Af*pyrG*	This study
ATK192	*pyrG89 pmtB*::HA-tag-Af*argB argB*::*trpC_B pyroA4 pmtA*::S-tag-Af*pyrG*	This study
ATK165	*pyrG89 argB*::*trpC_B pyroA4 pmtA*::S-tag-Af*pyrG pmtC*::HA-tag-Af*argB*	This study
ATK168	*pyrG89* Δ*pmtB*::Af*pyro argB*::*trpC_B pyroA4 pmtA*::S-tag-Af*pyrG pmtC*::HA-tag-Af*argB*	This study
ATK193	*pyrG89 pmtB*::HA-tag-Af*argB argB*::*trpC_B pyroA4 pmtC*::S-tag-Af*pyrG*	This study
ATK200	*pyrG89 pmtB*::HA-tag-Af*argB argB*::*trpC_B pyroA4*Δ*pmtA*::Af*pyro pmtC*::S-tag-Af*pyrG*	This study
ATK217	*pyrG89/pyrG89 argB*::*trpC_B/argB*::*trpC_B pyroA4/pyroA4 pmtC*::S-tag-Af*pyrG/pmtC*::HA-tag-Af*argB*	This study
ATK114	ATK42::Af*argB-gpd(P)*- ANID_01359-S-tag	This study
ATK111	ATK08::Af*argB-gpd(P)*- ANID_01359-S-tag	This study

Table 3.1. *A. nidulans* strains and plasmids (continued)

ATK109	ATK16::Af*argB-gpd(P)*- ANID_01359-S-tag	This study
ATK103	ATK38::Af*argB-gpd(P)*- ANID_01359-S-tag	This study
ATK149	ATK08::Af*argB-gpd(P)*- ANID_05660-S-tag	(KRIANGKRIPIPAT and MOMANY 2009)
ATK148	ATK16::Af*argB-gpd(P)*- ANID_05660-S-tag	(KRIANGKRIPIPAT and MOMANY 2009)
ATK195	ATK149 X ATK104	This study
ATK172	A850::Af*argB-gpd(P)*-ANID_07041-S-tag	This study
ATK179	ATK08::Af*argB-gpd(P)*-AN ID_07041-S-tag	This study
ATK177	ATK16::Af*argB-gpd(P)*-AN ID_07041-S-tag	This study
ATK184	ATK38::Af*argB-gpd(P)*-AN ID_07041-S-tag	This study
ATK211	ATK177 X ATK95	This study
plasmids		
pAO81	GA4-S-Tag Af*pyrG*	(YANG *et al.* 2004)
pDV2	Ampr *argB-gpd(p)-ccdB-sgfp*	(TOEWS *et al.* 2004)
pMT-3xHA	Ampr *argB-alcA(p)-ccdB*-3xHA	(TOEWS *et al.* 2004)
pFNO3	Kanr GA5-GFP Af*pyrG*	(YANG *et al.* 2004)
pAf*argB2*	Ampr *argB2*	G. S. May
pTK44	pDV2::ANID_01359-S-tag	This study
pTK74	pDV2::ANID_07041-S-tag	This study

Table 3.2 Primers

Primers for construction of *pmt2*::S-tag-Af*pyrG* cassette

Primer name	**Sequence**
Pmt2FWent	CACCATGGCTGAAATTGGCTTTG
Pmt2RVnostop	GTTAGCGATTCGCCAACCG
pmt2-GA4Fw	GAGCGGTTGGCGAATCGCTAACGGAGCTGGTGCAGGCGC
Stag-pmt2RV	GAACTGTAACGCTCTATCACGGTTCCTGTCTGAGAGGAGGCACTGATGC
pmt2DnFW	GAACCGTGATAGAGCGTTACAGTTCCCGTTG
pmt2DNRV	GCGATCCCAATCCTTCCTATCTCTGTCATC
PMT2F301	CCGAATCGGCCGCTCAAACATCGTG
PMT2R6311	TGCGACTGGGCGAGAAAGCGTGAGG
up177PMT2F	TCCGCAGTCGCCCGCCAGATATGAG
StagR	GCGCCAATTGCTGTTGCCAGGTGAGG

Primers for construction of *pmt1*::HA-tag-Af*argB* cassette

Primer name	**Sequence**
PMT11093F	GTGCTTGGTCAATCCGGGAGAGGG
Pmt1RVnostop	ACGGGTCGCCTCGTTGCCTTC
PMT1-GA5F	GCAACGAGGCGACCCGTGGAGCTGGTGCAGGCGC
argBAf-PMT1DnR	CAACAGACCGACATATTATCTCATAGGATTTTCCCCTTG
Pmt1dn-FW	GATAATATGTCGGTCTGTTGAACTACCTGCC
PmtBdnR2	CTGAGAGGGAGTTCGGAAACGTGC

Primers for construction of *pmt4*:: S-tag-Af*pyrG* cassette

Primer name	**Sequence**
463Pmt4F	GTCCCCTATGTCGCCCTCCG
Pmt4RVnostop	TTTCGCGAAGTGCAAGTCATAGC
Pmt4GA4F	CTATGACTTGCACTTCGCGAAAGGAGCTGGTGCAGGCGCTGGAG
pyrGAFRV-Pmt4SPRV	AGAACGAGATGAGATCAGATGCTCCTGTCTGAGAGGAGGCACTGATGCG
Pmt4dnFW	GAGCATCTGATCTCATCTCGTTCTCTCCC
Pmt4dnRV	CTATCCACGGTATGAGCTGAGCGAGTAATG

Primers for construction of *pmt4*::HA-tag-Af*argB* cassette

Primer name	**Sequence**
argBpmt4RV	AGAACGAGATGAGATCAGATGCTCCGATTTTCATAGGATTTTCCCC TTG

Primers for construction of *axlA*-S-tag cassette

Primer name	**Sequence**
Axl2FTOPO	CACCATGGCGCATCGCCTGTTCATC
AXL2nostop R	AACGAAGGCTATATCGCTCAACACGCTTCC
Axl2-GA4F	GCGATATAGCCTTCGTTGGAGCTGGTGCAGGCGC
StagR	GCGCCAATTGCTGTTGCCAGGTGAGG

Primers for construction of *msbA*-S-tag

Primer name	**Sequence**
MSB2entF	CACCATGGTTTCCCAGACGGCTC
MSB2-StagR	GCGCCTGCACCAGCTCCGTTCCATCCCAGAGAGTTC
StagF	GGAGCTGGTGCAGGCGCTGGAGC
StagR	GCGCCAATTGCTGTTGCCAGGTGAGG

Figure 3.1. Immunoprecipitation of Epitope-tagged Pmts. Western blot analyses show apparent molecular masses of immunoprecipitates from ATK89 ($PmtA^{S}$), ATK187 ($PmtB^{HA}$) and ATK154 ($PmtC^{HA}$). Proteins were expressed from their native loci and under the control of their endogenous promoters. Solubilized membrane-enriched fractions were immunoprecipitated with the corresponding agarose immobilized antibody against the epitope tag. Immunoprecipitates were resuspended in SDS loading dye and resolved on 4-20% gradient Tris-HEPES-SDS-polyacrylamide gels. Blots were probed with anti-S tag or anti-HA antibodies. Arrows indicate Pmts.

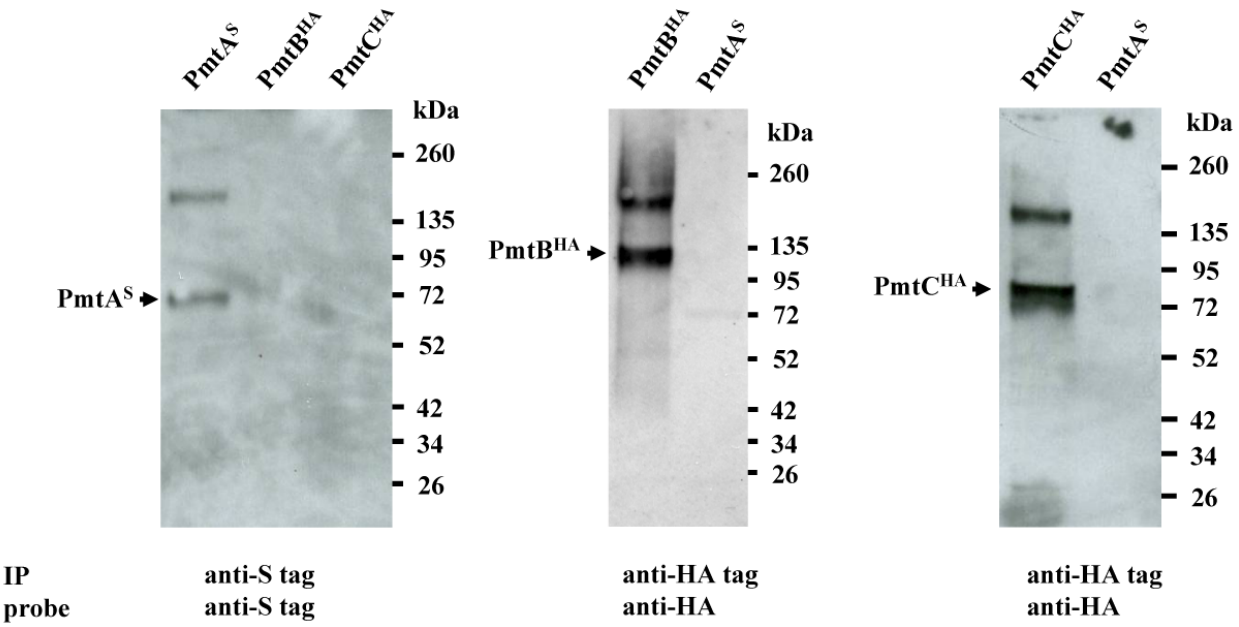

PmtA^S
PmtB^HA
PmtC^HA
kDa
260
135
95
72
52
42
34
26
PmtA^S
PmtB^HA
PmtA^S
kDa
260
135
95
72
52
42
34
26
PmtB^HA
PmtC^HA
PmtA^S
kDa
260
135
95
72
52
42
34
26
PmtC^HA
IP
probe
anti-S tag
anti-S tag
anti-HA tag
anti-HA
anti-HA tag
anti-HA

Figure 3.2. Immunoprecipitation shows PmtA-PmtB heteromeric complexes. Solubilized membrane enriched fractions from ATK192 ($PmtA^{S}$ coexpressed with $PmtB^{HA}$) were immunoprecipitated with agarose immobilized anti-S tag antibody. Co-immunoprecipitates were treated with 2 x SDS loading dye and resolved on 4-20% gradient Tris-HEPES-SDS-polyacrylamide gels. Blots on the left panel were probed with anti-S tag antibody. Blots on the right panel were probed with anti-HA antibody.

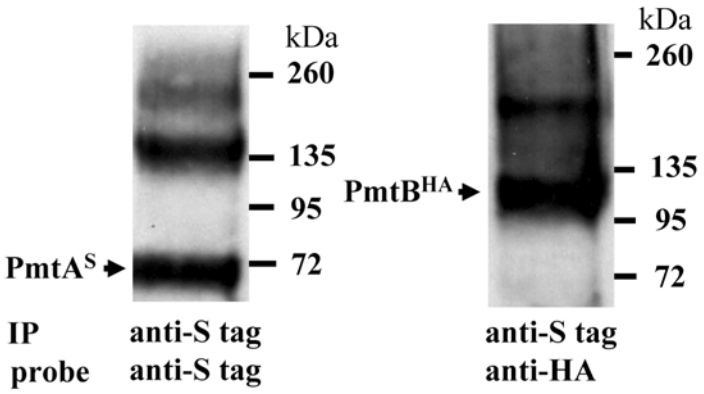

kDa
260
135
95
72
PmtA^S
IP
probe
anti-S tag
anti-S tag
kDa
260
135
95
72
PmtB^HA
anti-S tag
anti-HA

Figure 3.3. Immunoprecipitation shows PmtA-PmtC heteromeric complexes. Lane 1 is co-immunoprecipitate from ATK168 ($PmtA^{S}$, $PmtC^{HA}$, Δ*pmtB*), lane 2 is co-immunoprecipitate from ATK165 ($PmtA^{S}$, $PmtC^{HA}$). Solubilized membrane enriched fractions were immunoprecipitated with agarose immobilized anti-S-tag antibody. Co-immunoprecipitates were treated with 2 x SDS loading dye and resolved on 4-20% gradient Tris-HEPES-SDS-polyacrylamide gels. Blots on the left panel were probed with anti-S tag antibody. Blots on the right panel were probed with anti-HA antibody.

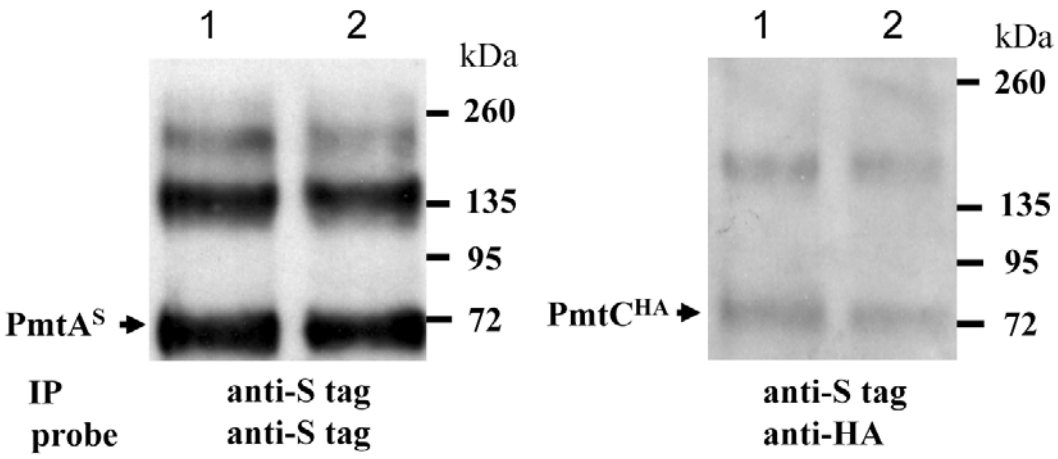

1
2
kDa
260
135
95
72
PmtA^S
IP
probe
anti-S tag
anti-S tag
1
2
kDa
260
135
95
72
PmtC^HA
anti-S tag
anti-HA

Figure 3.4. Immunoprecipitation shows PmtB-PmtC heteromeric complexes. Lane 1 is co-immunoprecipitate from ATK200 ($PmtB^{HA}$, $PmtC^{S}$, Δ*pmtA*), lane 2 is co-immunoprecipitate from ATK193 ($PmtB^{HA}$, $PmtC^{S}$). Solubilized membrane enriched fractions were immunoprecipitated with agarose immobilized anti-S-tag antibody. Co-immunoprecipitates were treated with 2 x SDS loading dye and resolved on 4-20% gradient Tris-HEPES-SDS-polyacrylamide gels. Blots on the left panel were probed with anti-S tag antibody. Blots on the right panel were probed with anti-HA antibody.

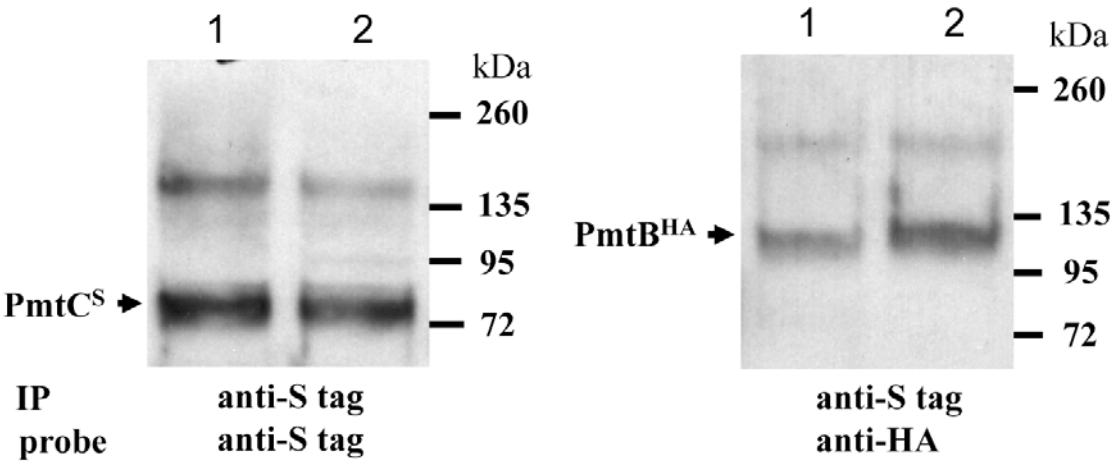

1
2
kDa
260
135
95
72
PmtC^S
IP
probe
anti-S tag
anti-S tag
1
2
kDa
260
135
95
72
PmtB^HA
anti-S tag
anti-HA

Figure 3.5. Immunoprecipitation shows PmtC-PmtC homomeric complexes. A solubilized membrane enriched fraction from a diploid strain ATK217 ($PmtC^{S}$ coexpressed with $PmtC^{HA}$) was immunoprecipitated with agarose immobilized anti-S tag antibody. Precipitate was treated with 2 x SDS loading dye and resolved on 4-20% gradient Tris-HEPES-SDS-polyacrylamide gels. Blots on the left panel were probed with anti-S tag antibody. Blots on the right panel were probed with anti-HA antibody.

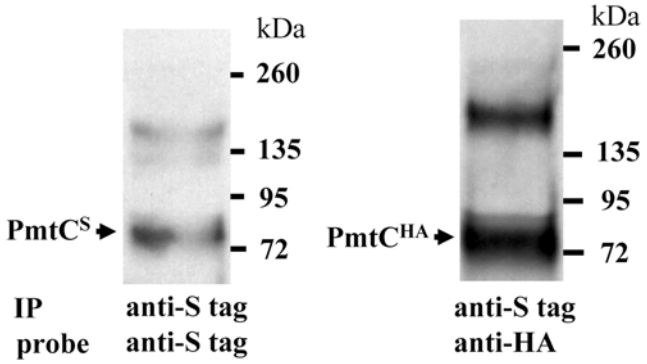
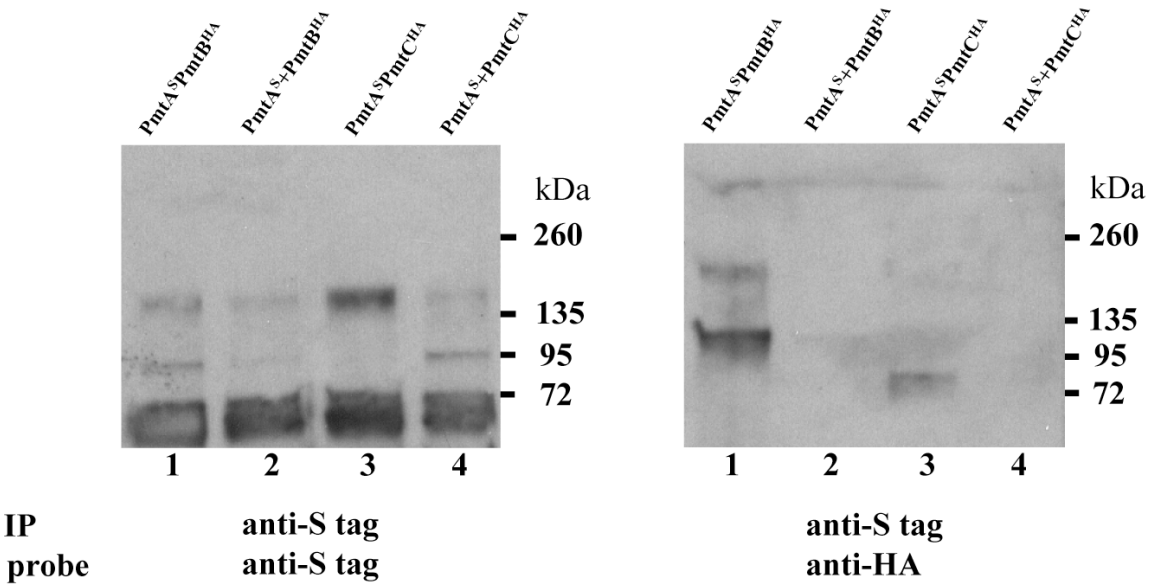

kDa
260
135
95
72
PmtC^S
kDa
260
135
95
72
PmtC^HA
IP
probe
anti-S tag
anti-S tag
anti-S tag
anti-HA

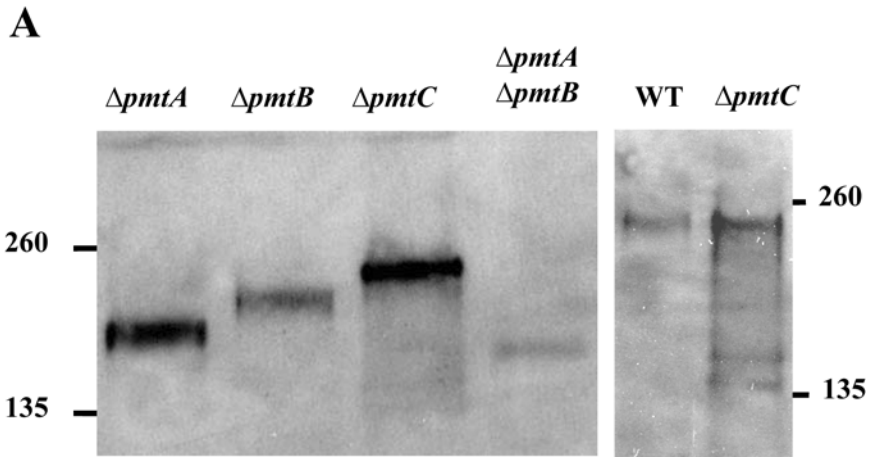
Figure 3.6. Pmt complexes form *in vivo*. Lane 1 is co-immunoprecipitate from ATK192 ($PmtA^{S}$ coexpressed with $PmtB^{HA}$). Lane 2 is co-immunoprecipitate from membrane fractions of ATK89 ($PmtA^{S}$) combined with membrane fractions of ATK187 ($PmtB^{HA}$). Lane 3 is co-immunoprecipitate from ATK165 ($PmtA^{S}$, $PmtC^{HA}$). Lane 4 is co-immunoprecipitate from membrane fractions of ATK89 ($PmtA^{S}$) combined with membrane fractions of ATK154 ($PmtC^{HA}$). Solubilized membrane enriched fractions or combined solubilized membrane fractions were immunoprecipitated with agarose immobilized anti-S tag antibody. Co-immunoprecipitates were treated with 2 x SDS loading dye and resolved on 4-20% gradient Tris-HEPES-SDS-polyacrylamide gels. Blots on the left panel were probed with anti-S tag antibody. Blots on the right panel were probed with anti-HA antibody.



IP **anti-S tag** **anti-S tag**

probe **anti-S tag** **anti-HA**

Figure 3.7. (A) MsbA (AN7041) is modified by PmtA and PmtB but not by PmtC. $MsbA^S$ crude membrane fractions from Δ*pmt* mutants and wildtype were treated with 2 x SDS loading dye and separated on 7% SDS-PAGE. The blot was probed with anti-S tag antibody. (B) MsbA is not *N*-glycosylated. Crude membrane fractions containing $MsbA^S$ from Δ*pmt* mutants and wildtype were treated with PNGase F, mixed with 2 x SDS loading dye and resolved on 7% SDS-PAGE. Proteins on Western blots were probed with anti-S tag antibody.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ΔpmtA
ΔpmtB
ΔpmtC
ΔpmtA ΔpmtB
WT
ΔpmtC
260
135
260
135

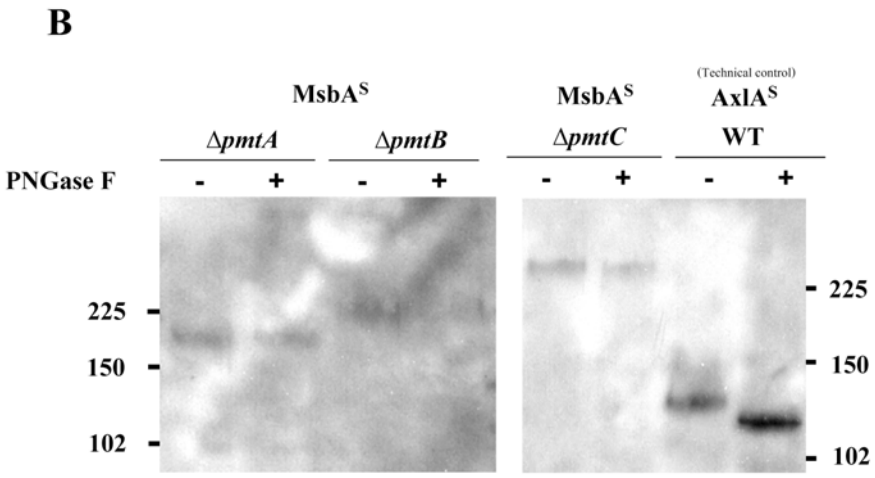

B
MsbA^S
MsbA^S
(Technical control) AxlA^S
ΔpmtA
ΔpmtB
ΔpmtC
WT
PNGase F
- + - + - + - +
225
150
102
225
150
102

Figure 3.8. PmtA specificity toward WscA does not depend upon PmtA-PmtB complex. Crude membrane fractions containing $WscA^{S}$ from Δ*pmtB*, Δ*pmtA* and Δ*pmtA*Δ*pmtB* mutants were treated with 2 x SDS loading dye and resolved on 4-20% gradient Tris-HEPES-SDS-polyacrylamide gels. Western blot was probed with anti-S tag antibody.

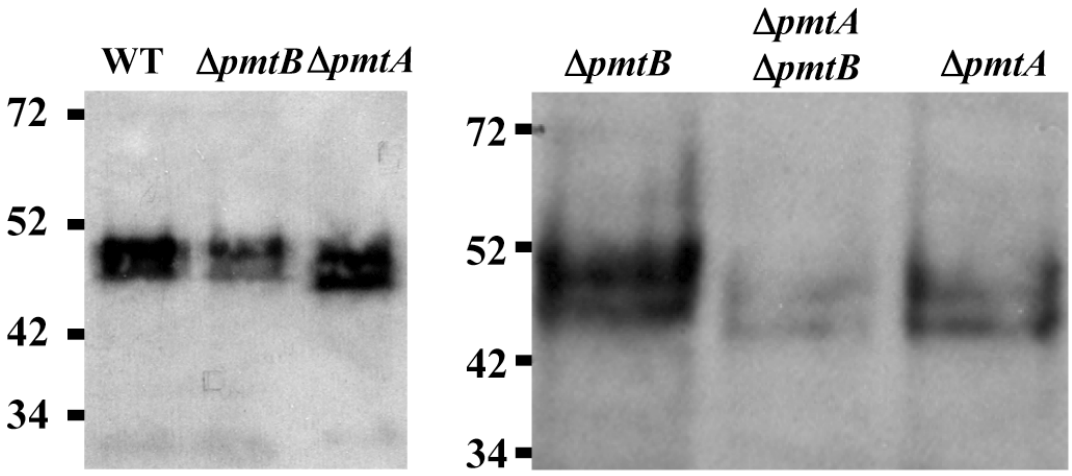
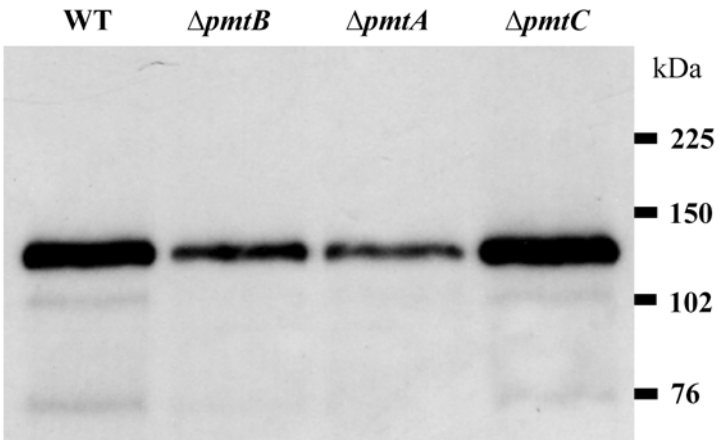

WT
ΔpmtB
ΔpmtA
72
52
42
34
ΔpmtB
ΔpmtA
ΔpmtB
ΔpmtA
72
52
42
34

Figure 3.9. AxlA (AN1359) is not modified by protein *O*-mannosyltransferases. $AxlA^{S}$ was expressed from the *gpd* promoter (A) Immunoblot of crude membrane fractions from wildtype and Δ*pmt* mutants. (B) Immunoblot of crude membrane fractions from wildtype and Δ*pmtC* treated with PNGase F. Protein solutions were treated with 2 x SDS loading dye and separated on 7% SDS-PAGE. Western blot was probed with anti-S tag antibody.

A


WT
ΔpmtB
ΔpmtA
ΔpmtC
kDa
225
150
102
76

B

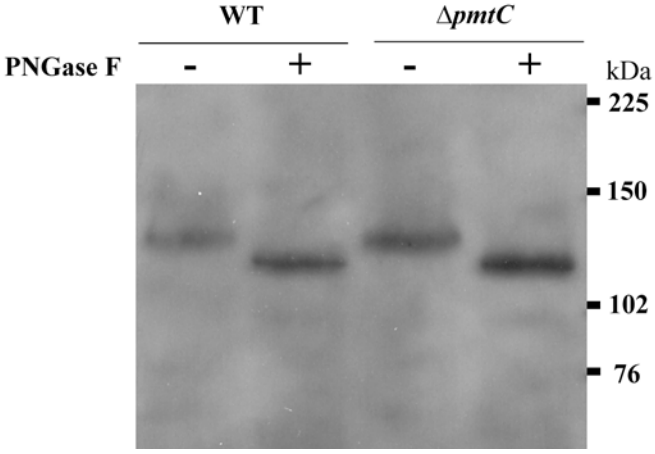
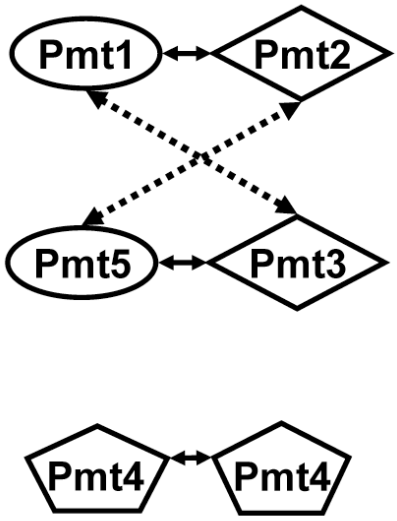
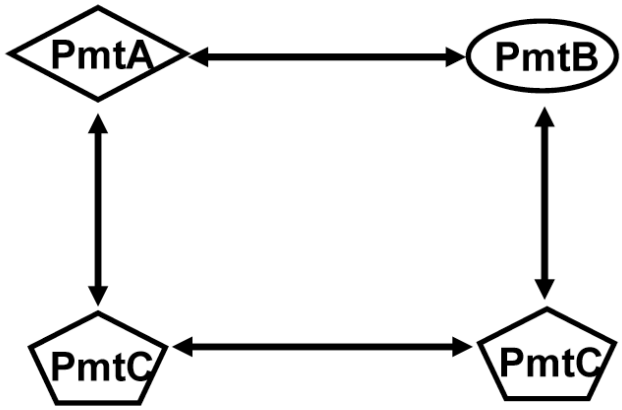

WT
ΔpmtC
PNGase F
-
+
-
+
kDa
225
150
102
76

Figure 3.10. Diagram comparing complex formation of Pmts in *A. nidulans* to *S. cerevisiae* (adapt from Girrbach and Strahl) (GIRRBACH and STRAHL 2003). In *S. cerevisiae* members of Pmt1 subfamily form complexes with members of Pmt 2 subfamily and Pmt4 subfamily forms a homomeric complex. Pmt1 forms a complex with Pmt3 in the absence of Pmt2, while Pmt2 forms a complex with Pmt5 when Pmt1 is missing. In *A. nidulans* subfamily 1 (PmtB) forms heteromeric complexes with subfamily 2 (PmtA). Pmt subfamily 1 (PmtB) and Pmt subfamily 2 (PmtA) form heteromeric complexes with Pmt subfamily 4 (PmtC). Pmt subfamily 4 (PmtC) forms a homomeric complex. Ovals indicate Pmt1 subfamily. Diamonds indicate Pmt2 subfamily. Pentagons indicate Pmt4 subfamily.



S. cerevisiae



A. nidulans

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CHAPTER 4

CONCLUSION

PmtA target proteins involved in polar growth of *A. nidulans* are unstable at high temperature.

Results from work here support the idea that protein *O*-mannosylation is important for protein stability. The Δ*pmtA* mutant exhibits a phenocopy of the *swoA* temperature sensitive allele. Both strains send out a germtube at 30^{o}C but fail to send out a germtube at 42^{o}C. The target proteins could be sensors of environmental stimuli or proteins involving in remodeling the cell surface during growth (EISENHABER *et al.* 2004). If identified, these proteins are candidates for antifungal drugs. Unfortunately, over expression of many target proteins, including ChiA (AN8241), MsbA, WscA, WscB (AN6927) and AN4897(a high copy suppressor of *pkcA* (D. Loprete, unpublished)) could not restore polar growth of Δ*pmtA* at high temperature (unpublished observation) (KRIANGKRIPIPAT and MOMANY 2009).

Single Δ*pmt* mutants and Δ*pmtA* Δ*pmtB* double mutant are viable.

So far *A. nidulans* and *A. fumigatus* are the only filamentous fungi in which phenotypes of each deleted Pmt subfamily have been reported (GOTO *et al.* 2009; KRIANGKRIPIPAT and MOMANY 2009; MOUYNA *et al.* 2010; OKA *et al.* 2004). Among filamentous fungi, deletion of the Pmt2 subfamily in *A. nidulans*, *A. awamori* and *Trichoderma reesei* is not lethal (GORKA-NIEC *et al.* 2008; KRIANGKRIPIPAT and MOMANY 2009; OKA *et al.* 2004; OKA *et al.* 2005). On

the other hand, deletion of the Pmt2 subfamily in *A. fumigatus* is lethal (MOUYNA *et al.* 2010). Among yeasts across phyla, deletion of the Pmt2 subfamily is lethal except that complete deletion of the three members of Pmt2 subfamily in *S. cerevisiae* has not been studied (MOUYNA *et al.* 2010; PRILL *et al.* 2005; WILLER *et al.* 2005; WILLGER *et al.* 2009). Simultaneous deletion of Pmt1 and Pmt4 subfamily is lethal in all fungi except *U. maydis* (FERNANDEZ-ALVAREZ et al. 2009).

Complex formation is mandatory but substrate specificity depends upon Pmt in the complex.

Unique phenotypes and shifts in protein mobility patterns on SDS-PAGE among *pmt* mutants reported across fungal species support the idea that though complex formation is required, substrate specificity is determined by individual Pmts. Secretory proteins of fungi are highly *N*- or *O*-glycosylated. The ability to form different combinations of Pmt complexes could enable fungi to maintain *O*-mannosylation with limited Pmt members. In addition, unconventional activities and interactions have been reported for some Pmts. AnPmtC was coimmunoprecipitated with a protein in the nucleopore complex (S. Osmani, unpublished data). Enzymatic activity of ScPmt6 on Gas1 has been reported; however, ScPmt6 has not been reported to form a hetero- or homomeric complex. These unknown roles of Pmts will be interesting to study.

Ser/Thr-rich GPI anchor proteins

High temperature sensitivity and hypersensitivity to cell wall perturbing agents of the Δ*pmt* mutants are osmoremedial, suggesting that these phenotypes are caused by cell wall

defects. Fungi rely heavily on the cell wall for protection, environmental sensing and maintaining the cell shape. In *A. nidulans,* approximately 74 putative GPI proteins have been identified by bioinformatics approaches (EISENHABER *et al.* 2004). Many of them contain Ser/Thr-rich regions. Some of them are involved in cell wall biosynthesis or uncharacterized (DE GROOT *et al.* 2009). The N-terminus of GPI proteins is cleaved while entering the ER and the C-terminus of GPI proteins is cleaved and linked to the GPI motif. To explore Pmt modification of this type of protein, I developed a system to tag GPI proteins with an S tag cassette. The S tag-GPI cassette was constructed by fusion PCR. The GPI anchor sequence was from AN3914, an uncharacterized GPI protein belonging to the glycosyl hydrolase family 16. The cassette is then fused to AN0933 (an ortholog of ScCrh1 also belonging to glycosyl hydrolase family 16) excluding its own GPI anchor motif. The hybrid protein was successfully expressed in the wildtype and Δ*pmtB*. On going research is to express the protein in all Δ*pmt* backgrounds in order to compare modification patterns.

In summary, we showed that *A. nidulans* contains 3 Pmts, PmtA, PmtB and PmtC. Single Δ*pmt* mutants are viable and exhibit distinctive phenotypes. The Δ*pmtA* Δ*pmtB* mutant is the only viable double mutant. All Δ*pmt* mutants are hypersensitive to cell wall perturbing agents and develop abnormal conidiophores suggesting that Pmt is involved in cell wall integrity and morphogenesis. Surprisingly, the *A. nidulans* Pmts form heteromeric complexes in all possible combinations among the three subfamilies. Studies of yeast Pmt target orthologs, AxlA, WscA and MsbA, showed that individual Pmts determine substrate specificity independently from the other Pmts in the complex and that even orthologous substrates can be modified by different complexes. *A. nidulans* is an excellent organism for further study of Pmt specificity because each

of the subfamilies can be deleted and the fungus is still viable reducing difficulties of overlapping targets and family member redundancy.

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