

# Molecular characterization of protein O-mannosyltransferase and its involvement in cell-wall synthesis in *Aspergillus nidulans*

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Protein O-glycosylation is essential for protein modification and plays important roles in eukaryotic cells. O-Mannosylation of proteins occurs in the filamentous fungus *Aspergillus*. The structure and function of the *pmtA* gene, encoding protein O-D-mannosyltransferase, which is responsible for the initial O-mannosylation reaction in *Aspergillus nidulans*, was characterized. Disruption of the *pmtA* gene resulted in the reduction of *in vitro* protein O-D-mannosyltransferase activity to 6% of that of the wild-type strain and led to underglycosylation of an extracellular glucoamylase. The *pmtA* disruptant exhibited abnormal cell morphology and alteration in carbohydrate composition, particularly reduction in the skeletal polysaccharides in the cell wall. The results indicate that PmtA is required for the formation of a normal cell wall in *A. nidulans*.

## INTRODUCTION

Protein glycosylation is a major post-translational modification. Glycosylated proteins play essential roles in eukaryotic cells from fungi to humans (Gemmill & Trimble, 1999; Burda & Aebi, 1999; Strahl-Bolsinger *et al.*, 1999; Lussier *et al.*, 1999). Compared with N-linked oligosaccharides in glycoproteins, which share a relatively common structure, regardless of origin, O-linked oligosaccharides in glycoproteins are diverse, among eukaryotic organisms, with respect to their sugar components and the mode of sugar linkage. O-Glycosylation has been extensively studied in a budding yeast, *Saccharomyces cerevisiae* (Strahl-Bolsinger *et al.*, 1999; Lussier *et al.*, 1999; Haselbeck & Tanner, 1982; Hausler *et al.*, 1992). The initial reaction of mannose transfer to serine and threonine residues is catalysed by protein O-D-mannosyltransferase (PMT; EC 2.4.1.109) in the endoplasmic reticulum, where dolichol phosphate (Dol-P)-Man is required as an immediate sugar donor (Strahl-Bolsinger *et al.*, 1993). Subsequent extension of mannose moieties is catalysed by the mannosyltransferases (MNTs)  $\alpha$ -1,2-MNT and  $\alpha$ -1,3-MNT, using GDP-Man, in the Golgi (Lussier *et al.*, 1999; Hausler *et al.*, 1992; Romero *et al.*, 1999). Consequently, *S. cerevisiae* proteins are glycosylated with mannose that is linearly linked to five residues (Strahl-Bolsinger *et al.*, 1999). Seven PMT-encoding genes (*PMT1–7*), three  $\alpha$ -1,2-MNT-encoding

genes (*MNT1/KRE2*, *KTR1*, *KTR3*) and three  $\alpha$ -1,3-MNT-encoding genes (*MNN1*, *MNT2*, *MNT3*), are known to be involved in the complete pathway of O-mannosylation in *S. cerevisiae* (Gentzsch & Tanner, 1996; Hausler *et al.*, 1992; Lussier *et al.*, 1997, 1999; Romero *et al.*, 1999; Yip *et al.*, 1994). Disruption of three different types of PMT gene resulted in death of the yeast cells (Gentzsch & Tanner, 1996). In addition, some information on O-glycosylation can be obtained from the human pathogenic fungus *Candida albicans*, which shows yeast-hyphal dimorphism. The *pmt1* and *pmt6* genes of *C. albicans* have been characterized to be closely associated with pathogenicity and the transition from round yeast cells into the hyphal form. (Timpel *et al.*, 1998, 2000).

*Aspergillus* species belong to the filamentous fungi; they include many industrially useable strains such as *Aspergillus oryzae*, *Aspergillus awamori* and *Aspergillus niger*. These fungi produce large amounts of extracellular hydrolases and organic acids, and thus have been used for the production of fermented foods (van den Hondel *et al.*, 1992). Besides these useful *Aspergillus* strains, opportunistic pathogens such as *Aspergillus fumigatus*, aflatoxin B1-productive *Aspergillus flavus* and *Aspergillus parasiticus* are also known. Therefore, *Aspergillus* species have been widely investigated in the industrial and medical fields. However, little is known about O-glycosylation in *Aspergillus* species, although it has been suggested that it is closely associated with secretion (Zakrzewska *et al.*, 2003), stability (Harty *et al.*, 2001; Goto *et al.*, 1999) and localization of proteins (Bourdineaud *et al.*, 1998). Structural analyses of O-linked oligosaccharides of *Aspergillus* glycoproteins have revealed that O-linked

Abbreviations: Dol-P, dolichol phosphate; GAI, glucoamylase I; MNT, mannosyltransferase; PMT, protein O-D-mannosyltransferase.

The GenBank accession number for the sequence reported in this paper is AF225551.

oligosaccharides are predominantly composed of mannose moieties ranging in size from one to three mannose units with linkages of  $\alpha$ -1,2,  $\alpha$ -1,3 and  $\alpha$ -1,6, including not only a linear form but also a branched structure (Pazur *et al.*, 1980; Gunnarsson *et al.*, 1984). Moreover, the O-linked oligosaccharides contain glucose and galactose moieties, in addition to mannose moieties, in the same protein (Gunnarsson *et al.*, 1984; Wallis *et al.*, 1999). Thus, O-linked oligosaccharides in proteins from *Aspergillus* species are different from those of *S. cerevisiae*. Recently, Shaw & Momany reported that the *swaA* mutation, which causes abnormal hyphae development, is identical to the *pmtA* mutation in *Aspergillus nidulans* (Momany *et al.*, 1999; Shaw & Momany, 2002). In the present paper, we demonstrate the principal functions of the *pmtA* gene, and also discuss its role, by providing instances of abnormal cell phenotypes in a *pmtA* disruptant.

## METHODS

**Micro-organisms and growth conditions.** *A. nidulans* strains FGSC26 (*veA*<sup>+</sup>; *bia1*), FGSC89 (*veA*<sup>+</sup>; *bia1*; *argB2*) were provided by the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City). *A. nidulans* strains were grown on YG medium (0.5%, w/v, yeast extract, 1%, w/v, glucose) or MM medium (1%, w/v, glucose, 0.6%, w/v, NaNO<sub>3</sub>, 0.052%, w/v, KCl, 0.052%, w/v, MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.152%, w/v, KH<sub>2</sub>PO<sub>4</sub> and Hunter's trace elements, pH 6.5), as described by Barratt *et al.* (1965). Liquid growth experiments to allow hyphal development in a submerged culture were done by inoculation of  $2 \times 10^8$  conidia into 100 ml YG or MM medium in 500 ml culture flasks. The flasks were shaken at 120 r.p.m. at 30 °C. Standard transformation procedures for *A. nidulans* were used (Yelton *et al.*, 1984). Plasmids were amplified in *Escherichia coli* JM109 or XL-1 Blue.

**Isolation of the *pmtA* gene.** Two types of partial genomic DNA library of *A. nidulans* FGSC26 were constructed. Genomic DNA was digested with *SalI* and *EcoRI*, and 3.8 kb DNA fragments were obtained. Genomic DNA was also digested with *SalI* and *ClaI*, and 3.7 kb DNA fragments were obtained. The DNA fragments thus obtained were inserted into the corresponding sites of pBluescript-II KS<sup>+</sup>. *E. coli* XL-1 Blue transformants carrying two types of genomic DNA library were analysed by colony hybridization. A partial region of the *pmtA* gene was amplified by PCR with primers *pmtA*-F (5'-CAGGCCGTAACCTTCATTCCTCCCA-3') and *pmtA*-R (5'-CCGGCGCGTAGATAACAACCC-3') and was used as a hybridization probe. Two positive clones carrying pBS-C-*pmtA* and pBS-N-*pmtA* were isolated from the two types of genomic library. The 1.86 kb *SalI*-*SphI* fragment from pBS-N-*pmtA* and the 2.69 kb *SphI*-*BamHI* fragment from pBS-C-*pmtA* were co-inserted into the *SalI* and *BamHI* sites of pBluescript-II KS<sup>+</sup>, to yield pBS-*pmtA*, which carries a 4.55 kb *SalI*-*BamHI* fragment containing the entire *pmtA* gene. In order to isolate the cDNA of *pmtA*, RT-PCR was performed with a High Fidelity RNA PCR kit (Takara), using the primers *pmtA*-RT-F (5'-TTGGATCCGAAATGGCTGAAATTGGCTTTG-3') and *pmtA*-RT-R (5'-GTTCTCGAGGTTAGCGATTCCGCAAC-3').

**Analysis of *pmtA* transcription.** Total RNA was isolated by Trizol reagent (Gibco-BRL). An 18 µg sample of total RNA was separated by electrophoresis in a 1.0% agarose gel with 15% (v/v) formaldehyde and transferred to a Hybond-N membrane (Amersham Biosciences). Hybridization with DIG-labelled probes (Roche) and subsequent detection with CDP-star (Roche) were done according to the supplier's manual. Primer-extension experiments

using the mRNA of strain FGSC26 were done with the Primer Extension System-AMV Reverse Transcriptase (Promega) and the primer PE-*pmtA* (5'-IRD800-GGTCGATGCAAAGCCAATTTCCAGCCATG-3'). A sequencing reaction was performed using a Thermo Sequenase Cycle Sequencing Kit (USB) with a 1.5 µM ddNTP (Amersham Biosciences) and 150 µM dNTP (Promega) mixture. Analysis of reaction products was done on a model LIC4200L-2G DNA sequencer (LI-COR).

**Construction of the *pmtA* disruptant.** A plasmid, pBS- $\Delta$ *pmtA*::*argB*, for disruption of the *pmtA* gene, was constructed as follows. The 1.7 kb *EcoRI* fragment of pDC1 (Aramayo *et al.*, 1989), containing the *argB* gene of *A. nidulans*, was inserted into the middle of the *pmtA* gene at the *EcoRI* site of pBS-*pmtA*. The 6.0 kb DNA fragment containing  $\Delta$ *pmtA*::*argB* that had been prepared by digestion of pBS- $\Delta$ *pmtA*::*argB* with *Bss*HII was used for transformation of *A. nidulans* FGSC89. The disruption of the *pmtA* gene in the *argB*<sup>+</sup> transformants was confirmed by Southern blot analysis. The 0.9 kb terminator region of *pmtA* was amplified by PCR with the primers *pmtA*-TF (5'-AAAAGGGCCCCGAACCGTGATAGAGCG-3') and *pmtA*-TR (5'-AAAAGGGCCCCGATCCCGCATTTGCC-3') and used as a probe.

**Protein O-mannosylation activity in vitro.** *A. nidulans* was grown on MM medium for 24 h at 30 °C. Dol-P-[<sup>14</sup>C]Man was synthesized according to the methods of Sharma *et al.* (1974). The *in vitro* peptide assay for O-mannosylation activity was performed according to Gentsch & Tanner (1996). Dol-P-[<sup>14</sup>C]Man as a sugar donor and Ac-YATAV-NH<sub>2</sub> as an acceptor peptide were used as substrates. The reaction mixture contained 5000 c.p.m. Dol-P-[<sup>14</sup>C]Man, 1.5 mM acceptor peptide, 7 mM Tris/HCl, pH 7.5, 7 mM MgCl<sub>2</sub>, 0.14% (w/v) Triton X-100 and 0.2–0.3 mg ml<sup>-1</sup> microsomal membranes of *A. nidulans*. The mixtures were incubated at 30 °C for 20 min. The amount of [<sup>14</sup>C]mannosylated peptide was measured by a liquid scintillation counter. The assay was done three times independently.

**Expression of *pmtA* and *glaA*.** The plasmids for expression of *pmtA* and the glucoamylase-encoding *glaA* were constructed as follows. The 4.55 kb *SalI*-*BamHI* fragment carrying the *pmtA* from pBS-*pmtA* was inserted into the corresponding sites of pUC19, to yield pUC-*pmtA*. pUC-*pmtA* was digested by *PstI* and *SmaI*, and the resultant *pmtA* fragment was inserted into the *PstI* and *SmaI* sites of pPTR-I, carrying the *ptrA* gene as a selection marker (Takara), to yield pPTR-*pmtA*. The *glaA* gene of *A. awamori*, from pBR-*glaA*, (Goto *et al.*, 1997) was inserted into the *SmaI* site of pPTR-I, to generate pPTR-*glaA*. The pPTR-*pmtA* and pPTR-*glaA* were linearized by digestion with *PstI* and *HindIII*, respectively. Transformants showing pyrithiamine resistance were selected on MM medium supplemented with 0.1 µg pyrithiamine ml<sup>-1</sup> and 0.8 M NaCl. Transformants carrying the *glaA* gene were cultured in 100 ml MM medium with 1% (w/v) maltose, to induce *glaA* expression, and with 20 mM tunicamycin as an inhibitor for N-glycosylation of protein, for 24 h at 30 °C.

**Colony growth rate.** Colony growth rates were measured as described by Kellner & Adams (2002). Briefly, conidia from each of the strains were point-inoculated into the centre of agar medium plates and incubated at 37 °C. Colony diameters were measured at 29, 49, 66 and 91 h. The growth rates were observed for each colony in mm h<sup>-1</sup> for each of the above three intervals, and the values were averaged across the entire time interval. The growth rates were expressed as a percentage of the growth rate of the wild-type (FGSC26) on YG medium, which was 0.59 ( $\pm 0.085$ ) mm h<sup>-1</sup> ( $n=15$ ). Measurements of independent growth rates for all strains were done 15 times.

**Analysis of efficiency of conidiation.** Efficiency of conidiation was analysed as described by Motoyama *et al.* (1997). Briefly,

approximately  $10^5$  conidia were spread onto an 84 mm MM medium plate. After 5 days of incubation at 30 °C, the conidia were suspended in 5 ml 0.01% (w/v) Tween 20 solution and counted using a haemocytometer.

**Microscopy.** The germlings of *A. nidulans* were observed according to the protocol described by Harris *et al.* (1994). Conidia were inoculated into YG liquid medium, and the culture was poured into a Petri dish containing a glass coverslip. After incubation at 30 °C for 6–14 h, the germlings adhering to the coverslip were fixed in 3.7% (v/v) formaldehyde, 50 mM phosphate buffer (pH 7.0) and 0.2% (w/v) Triton X-100 for 30 min. Coverslips were then washed with water and incubated with 10 ng ml<sup>-1</sup> of fluorescent brightener 28 (Calcofluor white; Sigma) for 5 min. The coverslips were washed again and mounted on a slide glass. Germlings were observed using the Eclipse E600 (Nikon).

**Preparation of microsomal membrane and cell-wall fractions.** *A. nidulans* was grown on MM or YG medium for 24 h, harvested, washed with ice-cold TM buffer (50 mM Tris/HCl, pH 7.5, 0.3 mM MgCl<sub>2</sub>) three times and broken twice in a French press (Ohtake) in the same buffer. The lysed cells were centrifuged at 3000 g for 20 min. The resultant pellet was washed ten times with 250 mM phosphate buffer (pH 7.0), washed with distilled water five times, lyophilized, and designated a cell-wall fraction. The supernatant was centrifuged at 100 000 g for 90 min, suspended in TM buffer, and designated a membrane fraction.

**Analysis of the contents of glucan and chitin.** Analysis of the glucan and chitin contents was done by the methods described by Borgia & Dodge (1992), with a slight modification. Cells were cultured in YG medium for 24 h at 30 °C. For the quantification of total glucan, 10 mg of cell walls was suspended in 1.0 ml 98% formic acid, heated at 100 °C for 20 min and centrifuged. The supernatant was dried in a vacuum desiccator, and the amount of neutral carbohydrate was estimated by the phenol-sulfuric acid method (Dubois *et al.*, 1956). For the quantification of alkali-soluble and alkali-insoluble fractions, 10 mg of cell walls was suspended in 1.0 ml distilled water, heated at 100 °C for 30 min and centrifuged. The pellet was suspended in 1.0 ml 1.0 M KOH solution and heated at 70 °C for 30 min prior to centrifugation. The supernatant was dried and used to estimate the amount of alkali-soluble glucan, as above. The alkali-insoluble pellet was suspended in 1.0 ml 98% formic acid and heated at 100 °C for 20 min, dried and used to estimate the amount of alkali-insoluble glucan. For the quantification of total GlcNAc, 10 mg of cell walls was digested with 2 mg Yatalase ml<sup>-1</sup> (Takara) in 250 mM phosphate buffer (pH 7.0) for 16 h. After digestion, the suspension was centrifuged, dried and used to estimate the amount of total GlcNAc by the Morgan–Elson method (Reissig *et al.*, 1955). The analyses were done three times independently.

## RESULTS

### Analysis of the *pmtA* gene

The expressed sequence tags of *A. nidulans* with homology to the amino acid sequences of *S. cerevisiae* Pmt1-4p (Gentsch & Tanner, 1996) were identified in a clone of h4a11a1.r1 by a database search using the TBLASTN algorithm (Altschul *et al.*, 1997) against dbEST (Expressed Sequence Tag database). The resultant nucleotide sequence was used as a probe to isolate the *pmtA* gene from the genomic DNA library of *A. nidulans* FGSC26. The *pmtA* cDNA was cloned by amplification with RT-PCR. The

nucleotide sequence and deduced amino acid sequence were deposited in GenBank (accession no. AF225551).

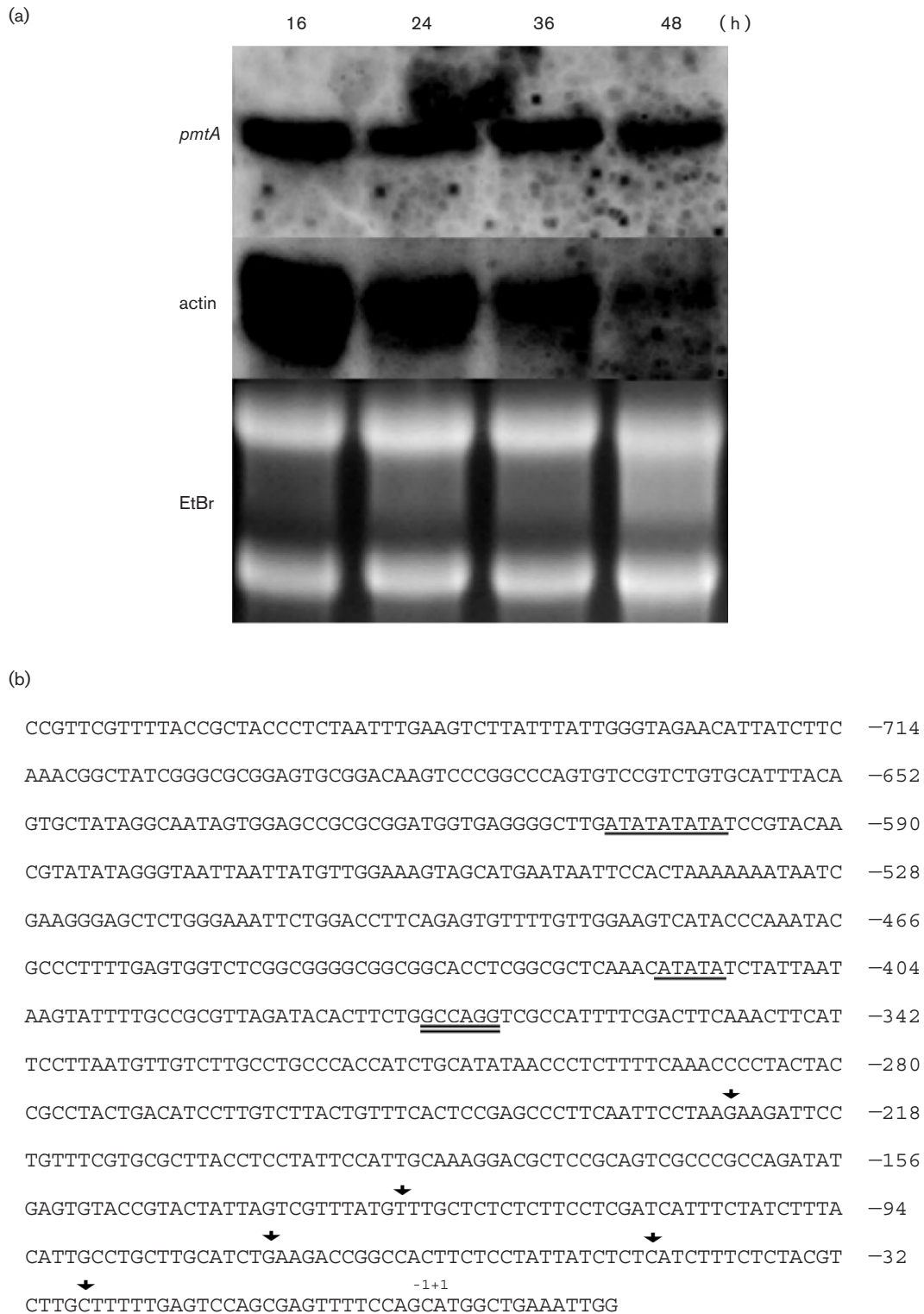
### Transcription of the *pmtA* gene

Northern blot analysis of *pmtA* was carried out at various growth stages, using the *pmtA* cDNA as a probe. The amounts of the *pmtA* transcripts under incubation in MM medium were almost comparable with the  $\gamma$ -actin gene from 16 h to 48 h (Fig. 1a). This result indicates that *pmtA* is transcribed throughout hyphal growth. The transcriptional start points of the *pmtA* gene were determined by primer-extension analysis, and indicated that *pmtA* has at least five transcriptional initiation sites, at -27, -46, -75, -127 and -226 bp upstream from the translational start site (Fig. 1b). Analysis of the 5'-noncoding region revealed the presence of putative promoter-proximal elements of a TATA-like sequence involved in the expression of the *pmtA* gene. The promoter contains a putative binding sequence of the pH signalling transcription factor PacC (Espeso *et al.*, 1997) 373 bp upstream from the translation start codon (Fig. 1b).

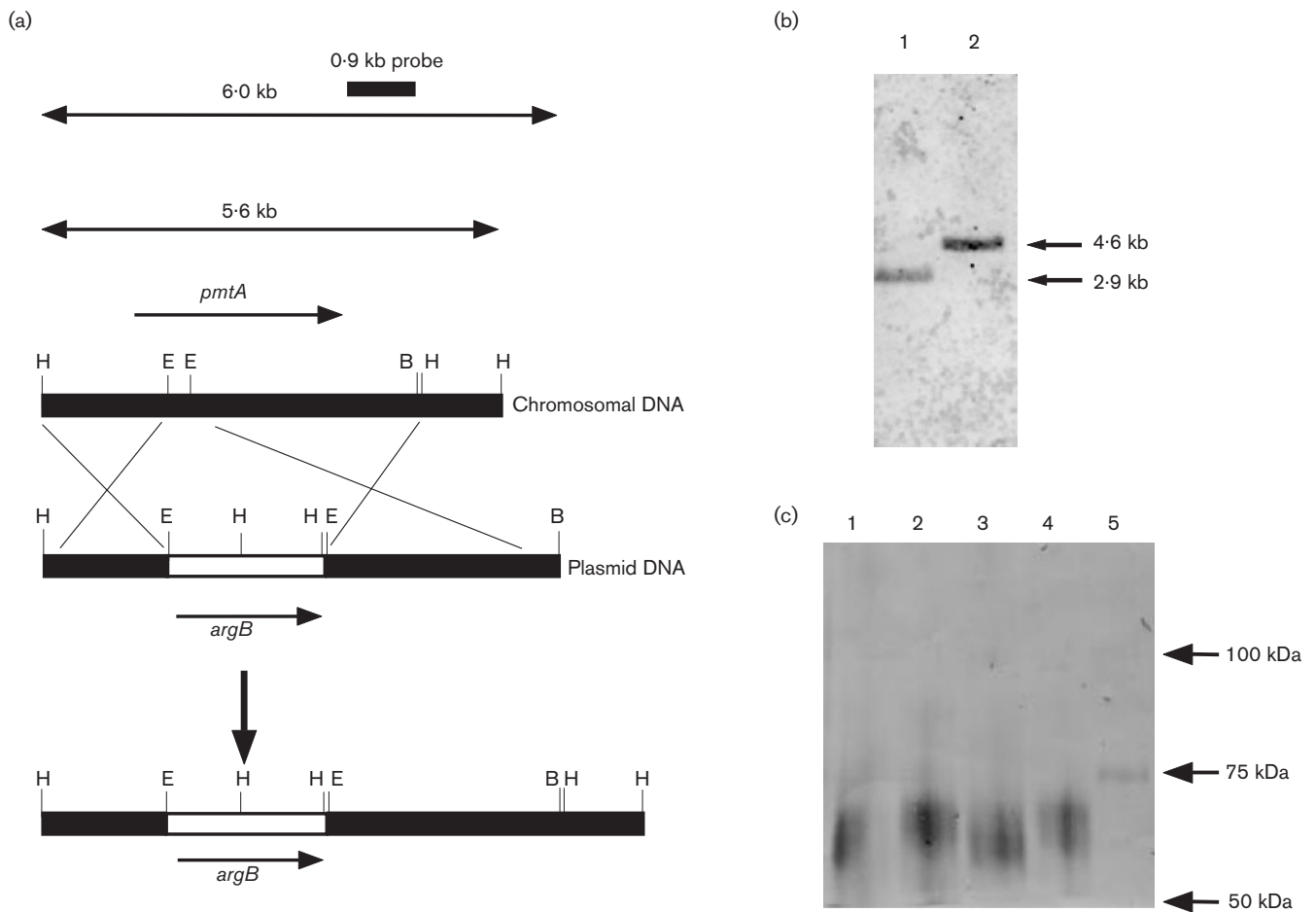
### Disruption of the *pmtA* gene

To examine whether the *pmtA* gene product functions as a protein O-D-mannosyltransferase, we constructed a *pmtA* null mutant. For this purpose, the 6.0 kb *Bss*HII DNA fragment carrying  $\Delta pmtA::argB$  was introduced into the *A. nidulans* FGSC89 strain (Fig. 2a). Isolated genomic DNA from the selected *argB*<sup>+</sup> transformants was analysed by Southern blotting, using the 0.9 kb terminator region of the *pmtA* gene as a probe (Fig. 2b). Southern blot analysis revealed that site-specific recombination occurred at the *pmtA* region, and that a single copy of the 6.0 kb fragment was integrated into the chromosomal DNA. Two isogenic *pmtA*-disruptant strains, P3 and P4, were isolated from approximately 150 *argB*<sup>+</sup> transformants, and strain P3 was used for further analysis.

We tested membrane proteins extracted from the wild-type strain and the *pmtA* disruptant for their ability to synthesize O-mannosylated peptides, using Dol-P-[<sup>14</sup>C]Man as a sugar donor. Membrane proteins from the wild-type strain exhibited a high level of mannosylation activity ( $8521 \pm 503$  c.p.m. mg<sup>-1</sup> h<sup>-1</sup>) toward the Ac-YATAV-NH<sub>2</sub> peptide, while the activity of *pmtA* disruptant P3 ( $523 \pm 179$  c.p.m. mg<sup>-1</sup> h<sup>-1</sup>) was as low as 6% of that of the wild-type. The result provided evidence that the *pmtA* gene encodes a functional protein O-D-mannosyltransferase. We then introduced the *glaA* gene of *A. awamori* into the *pmtA* disruptant and the wild-type strain in order to confirm the *in vivo* O-mannosylation activity encoded by *pmtA*. Glucoamylase I (GAI), encoded by *glaA*, is an extracellular protein consisting of three domains. These are the amino-terminal catalytic domain covering amino acids 1–469, the Ser/The-rich region including 470-ATGGTTT-TATTTGSGGVTSTSKTTTTASKTSTTTSSSTS-507, and the carboxy-terminal starch-binding domain of amino acids



**Fig. 1.** Transcriptional analysis of *pmtA* of *A. nidulans*. (a) Transcription of *pmtA*. Total mRNA of *A. nidulans* wild-type strain FGSC26 was prepared after growth in MM medium at 30 °C. Each lane was loaded with 18 µg RNA. Northern blot hybridization was performed using *pmtA* and the  $\gamma$ -actin gene (accession no. M22869) as probes, and total RNA was stained with ethidium bromide (EtBr). (b) Nucleotide sequence and primer extension analysis of the 5' upstream region of the *pmtA* gene of *A. nidulans*. TATA boxes are underlined. Transcriptional initiation positions are shown by arrows. The PacC-protein-binding consensus sequence is double-underlined (Espeso *et al.*, 1997). Numbering starts from the translation initiation site.



**Fig. 2.** Disruption of *A. nidulans pmtA*. (a) Schematic representation of the *pmtA* disruption. Regions used as probes for Southern blot analysis are shown as bars. Abbreviations: H, *HincII*; B, *BamHI*; E, *EcoRI*. (b) Southern blot analysis of *HincII*-digested total DNA. Lane 1, strain P3; lane 2, wild-type strain FGSC89. (c). Protein O-D-mannosyltransferase activity *in vivo*. Proteins in the culture filtrate of strain P3 + pPTR-glaA (lanes 1 and 3) and wild-type strain FGSC89 + pPTR-glaA (lanes 2 and 4) were separated on 7% SDS-PAGE, and GAI was detected by immunoblotting using anti-GAI antiserum. Tunicamycin was added as an inhibitor of N-glycosylation (lanes 1 and 2). Precision Plus protein standards (Bio-Rad) were used as a molecular size marker (lane 5). The molecular mass of GAI was estimated by an image analyser (Bio-profile V6.0) after SDS-PAGE.

508–615 (Hayashida *et al.*, 1989). The SDS-PAGE mobility of GAI secreted from the *pmtA* disruptant was greater than that of the wild-type strain (Fig. 2c). No significant effects of tunicamycin were seen on the mobility of GAI on SDS-PAGE between the wild-type and the *pmtA* disruptant under the experimental conditions used (Fig. 2c). The molecular masses of GAI from the wild-type strain and the *pmtA* disruptant were estimated to be 70 kDa and 68 kDa, respectively. It is known that the glucoamylase of *A. awamori* has a variety of sizes, due to the heterogeneity of the attached oligosaccharides (Sauer *et al.*, 2000). It is therefore reasonable that the immunoblots showed a population of glucoamylase molecules with a variety of sizes, spanning approximately a 5 kDa range. This result indicates that the PmtA is functional for a foreign secretory protein *in vivo*.

The *pmtA* disruptant formed a colony approximately 90% of the diameter of that of the wild-type strain, when cultured on MM medium at 30 °C for 3 days (Fig. 4). However, in the case of growth on YG medium at 37 °C, the *pmtA* disruptant P3 showed remarkable inferiority in its hyphal extension ability: its growth rate was reduced to 41.8% compared to the wild-type (Table 1). We attempted to obtain transformants whose *pmtA* mutation was complemented, where one to three copies of the *pmtA* gene were integrated via introduced plasmid pPTR-*pmtA*. The colony growth rate of the transformants (strain P3 carrying wild-type *pmtA*) was fully restored (Table 1), indicating that the abnormal hyphal extension is due to the *pmtA* mutation. The growth rate was fully restored on YG medium plates in the presence of 0.6 M KCl. The growth-rate recovery was also observed at the same level when 0.8 M NaCl and 1.2 M

**Table 1.** Growth rate of *A. nidulans* wild-type strain FGSC26 and *pmtA* mutant strain P3

Values in parentheses show the growth rate as a percentage of that of the wild-type strain. Growth rate values are in  $\text{mm h}^{-1}$ .

Strain	Medium	
	YG	YG+0.6 M KCl
FGSC26	0.59 ± 0.085 (100)	0.77 ± 0.052 (131)
P3	0.25 ± 0.045 (41.8)	0.66 ± 0.031 (112)
P3 + pPTR- <i>pmtA</i>	0.56 ± 0.020 (94.4)	0.68 ± 0.074 (116)

sorbitol were used, instead of 0.6 M KCl, as an osmotic stabilizer (data not shown). In addition, we often observed lysis of the hyphal tip in the MM liquid medium without the stabilizer. However, the hyphal lysis was repressed when the stabilizer was incorporated. Morphology of the germ-lings in the early growth stage was observed by microscopy. On YG and MM media, swollen hyphal formation (balloon formation) appeared at the apical or umbilical regions of the hyphae (Fig. 3a). This morphology was observed in moribund regions, in particular.

### Effects of the *pmtA* disruption on conidia formation

We measured the conidiation efficiency of the *pmtA* disruptant and the wild-type strain. Conidiation was repressed in the *pmtA* disruptant, in comparison with the wild-type strain, notably on MM medium. The formation of conidia

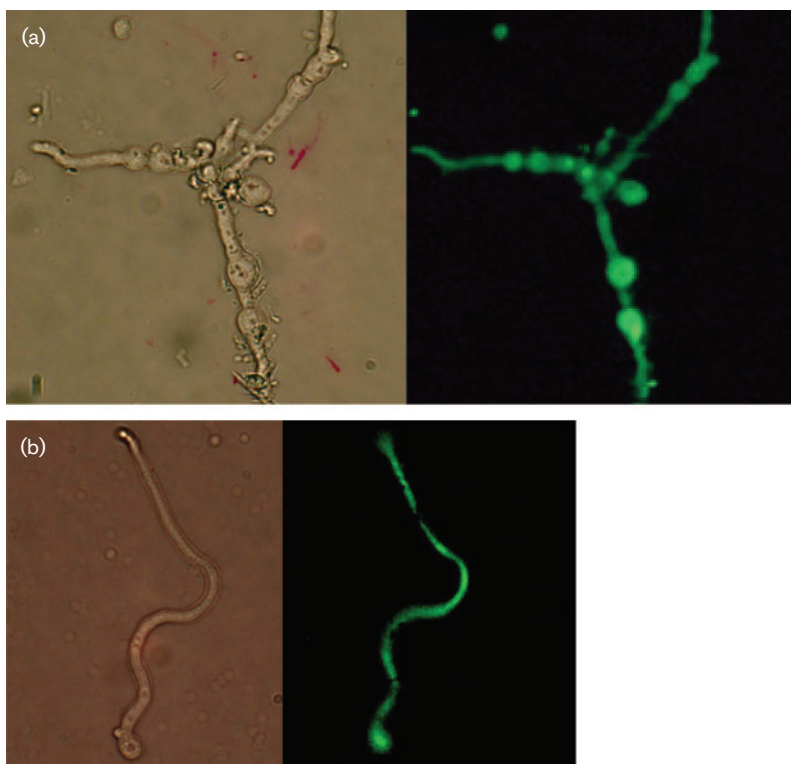
in the *pmtA*-disrupted strain P3 was reduced by approximately 20% compared with that of the wild-type strain.

### *pmtA* disruption causes hypersensitivity to antifungal reagents

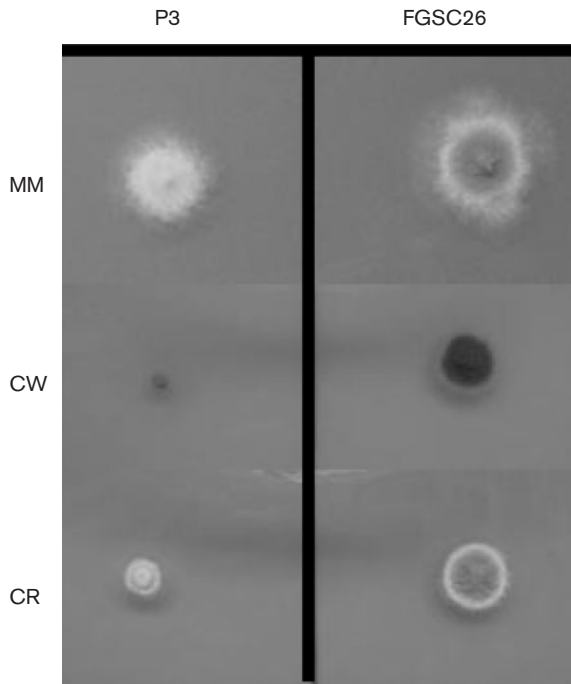
Congo red and Calcofluor white are known to be adsorbed on cell walls composed of polysaccharides, and therefore exhibit antifungal effects. In the presence of Congo red and Calcofluor white, the hyphal growth of the *pmtA* disruptant on MM medium was markedly inhibited compared with the wild-type strain (Fig. 4).

### Cell-wall chitin and $\beta$ -glucan content of wild-type and *pmtA*-disruptant strains

The alkali-soluble fraction contains  $\alpha$ -glucan and soluble  $\beta$ -1,3-/1,6-glucan. The alkali-insoluble fraction contains  $\beta$ -1,3-/1,6-glucan covalently linked to chitin (Borgia & Dodge, 1992; Fontaine *et al.*, 2002; Lee *et al.*, 2002). The *pmtA* disruptant showed a 20% decrease in the alkali-insoluble fraction. The alkali-soluble fraction and the GlcNAc content of the cell walls of the *pmtA* disruptant were increased by 44% and 33%, respectively, compared with the wild-type strain. The total sugar content in the cell walls of the *pmtA* disruptant was comparable to that of the wild-type strain (Table 2). The alkali-insoluble fraction is believed to be responsible for fungal cell-wall rigidity (Fontaine *et al.*, 2002). The decrease in the alkali-insoluble fraction indicates that the cell wall was weakened.



**Fig. 3.** Hyphae structure of *A. nidulans* *pmtA* disruptant P3 (a) and wild-type strain FGSC26 (b) was observed as described in Methods. Right panels, fluorescent image of the cell stained with Calcofluor white. Left panels, phase-contrast image.



**Fig. 4.** Increased sensitivity to antifungal reagents displayed by *A. nidulans* *pmTA* disruptant P3 compared to that of wild-type strain FGSC26. Strains were grown in the presence of Congo red (CR; 450  $\mu\text{g ml}^{-1}$ ) or Calcofluor white (CW; 300  $\mu\text{g ml}^{-1}$ ) on MM medium for 3 days at 30 °C. MM, control.

## DISCUSSION

Protein *O*-glycosylation is known as an important post-translational modification of proteins in secretory traffic. Among a variety of *O*-glycosylations, protein *O*-mannosylation occurs in *Aspergillus*. Reactions catalysed by protein *O*-D-mannosyltransferase play a key role in the *O*-mannosylation of proteins because they are involved in the initial *O*-mannosylation reaction. Several Pmt proteins have been identified in *S. cerevisiae*, *C. albicans*, *Drosophila melanogaster* and man (Gentzsch & Tanner, 1996; Timpel *et al.*, 1998, 2000; Martin-Blanco & Garcia-Bellido, 1996; Willer *et al.*, 2002). However, little is known about the character of the Pmt in *Aspergillus*. In the present paper, we constructed a *pmTA* disruptant and analysed the properties of the cell wall.

The *pmTA* disruption gave a phenotype of *A. nidulans* identical to the temperature-sensitive *swaA* mutant reported by Momany *et al.* (1999) and Shaw & Momany (2002). Inhibition of hyphal extension, multinucleation of the cell, and enhanced susceptibility to heat and Calcofluor white were observed. In addition to the properties of the *swaA* mutant, we observed hypersensitivity to Congo red (Fig. 4) and a decrease in conidia formation. Disruption of the *pmTA* gene revealed that it encodes the functional protein *O*-D-mannosyltransferase. The *pmTA*-disrupted strain exhibited greatly reduced protein *O*-D-mannosyltransferase activity (6%), compared to the wild-type. The remnant activity would be derived from Pmt isozymes. Thus, *A. nidulans* is assumed to possess several *pmT* genes, as in other organisms (Gentzsch & Tanner, 1996; Timpel *et al.*, 2000). In fact, we cloned two genes homologous to *pmTA* and confirmed their transcripts in *A. nidulans* (unpublished data).

In order to confirm *in vivo* mannosylation activity toward a secretory protein, GAI of *A. awamori* was heterologously expressed in *A. nidulans*. The Ser/Thr-rich region of GAI, which could be the target for *O*-glycosylation, contains 27 hydroxyamino acids among 38 amino-acid residues (Hayashida *et al.*, 1989). Unexpectedly, only a 2 kDa difference in the molecular mass of GAI was observed between the wild-type strain and the *pmTA* disruptant, suggesting that approximately 11 mannose moieties are absent in the *pmTA* disruptant. This result supports the fact that PmtA protein catalyses *O*-mannosylation with an inherent substrate specificity for certain proteins. Gentzsch & Tanner reported that, in *S. cerevisiae*, Pmt proteins have distinct substrate specificities: for proteins that contain Ser or Thr (Gentzsch & Tanner, 1997). Therefore, the slight difference in glycosylation in GAI would be due to a lower preference of *A. nidulans* PmtA for the foreign GAI polypeptide as the substrate.

It is well documented that some triple mutants of *PMT1–4* show a lethal phenotype in *S. cerevisiae*. On the other hand, all single and double mutants of *PMT* were viable (Gentzsch & Tanner, 1996). The *pmTA* disruptant of *A. nidulans* is also viable. However, a defect in the PmtA function resulted in repression of normal hyphal growth and normal conidial development, particularly under conditions of low osmotic potential. *pmTA* is transcribed at abundant levels through

**Table 2.** Cell-wall composition of *A. nidulans* wild-type strain FGSC26 and *pmTA* mutant strain P3

The amount of total sugar is expressed as the amount of glucose equivalent. Values in parentheses are percentages based on the wild-type strain.

Strain	Total sugar [ $\mu\text{g (mg cell wall)}^{-1}$ ]	Alkali-soluble fraction [ $\mu\text{g (mg cell wall)}^{-1}$ ]	Alkali-insoluble fraction [ $\mu\text{g (mg cell wall)}^{-1}$ ]	Total GlcNAc [ $\mu\text{g (mg cell wall)}^{-1}$ ]
FGSC26	547 $\pm$ 11 (100)	208 $\pm$ 28 (100)	228 $\pm$ 36 (100)	230 $\pm$ 26 (100)
P3	564 $\pm$ 35 (103)	301 $\pm$ 53 (144)	183 $\pm$ 20 (80)	306 $\pm$ 43 (133)

the early to late growth-stages in the wild-type strain (Fig. 1a). Because hyphal extension is in concert with cell-wall formation in filamentous fungi, the *pmtA* transcribed abundantly in conventional growth would be required for cell-wall formation. The *pmtA* disruptant of *A. nidulans* showed higher sensitivity to antifungal agents than the wild-type strain (Fig. 4). A similar response was observed in *C. albicans pmt1* and *pmt1pmt6* mutants (Timpel *et al.*, 2000). Therefore, it is not surprising that the same effects are observed in the *pmtA* disruptant of *A. nidulans*. The addition of osmotic stabilizers to the growth medium resulted in recovery of the defect in hyphal growth of the *pmtA* disruptant. These results indicated that the structure and mechanical rigidity of the cell walls are drastically altered by the *pmtA* disruption. Unlike the *pmt* mutants of *C. albicans*, the *pmtA* disruption also caused swollen hyphae locally in the mycelia, although, overall, mycelial development did take place (Fig. 3a). In *Aspergillus* species, the formation of swollen structures in the hyphae was reported in mutants of chitin synthase or NAD-dependent glycerol-3-phosphate dehydrogenase (Borgia *et al.*, 1996; Horiuchi *et al.*, 1999; Fillinger *et al.*, 2001). The cell-wall rigidity of those mutants was defective because the biosynthesis of chitin or glycerol was significantly reduced. These morphological rigidity defects were restored by the addition of an osmotic stabilizer.

Cell-wall components are functionally classified into two groups: the skeletal polysaccharides and the polysaccharides of the wall matrix (Farkas, 1985). The skeletal polysaccharides include  $\beta$ -1,3-glucan interconnected covalently with  $\beta$ -1,6-glucan and chitin, which are highly crystalline and provide mechanical rigidity to the wall. These polysaccharides were recovered as an alkali-insoluble fraction (Borgia & Dodge, 1992; Fontaine *et al.*, 2002; Lee *et al.*, 2002). The alkali-soluble fraction contains  $\alpha$ -glucan and soluble  $\beta$ -1,3/1,6-glucan, which fills the space between the skeletal polysaccharides and serves as a cementing substance (Borgia & Dodge, 1992; Farkas, 1985). The *pmtA* disruptant exhibited a significant alteration in the carbohydrate composition of the cell-wall fractions (Table 2). The cell walls in the *pmtA* disruptant changed to a defective structure, due to the reduction of the skeletal polysaccharides of the  $\beta$ -glucans and the increase in chitin content. The polysaccharides of the wall matrix increased in the *pmtA* disruptant. Therefore, the *pmtA* disruptant gives rise to critical damage to the skeletal structure and wall matrix of the cell wall. Accordingly, mycelial extension can be repressed in the *pmtA* disruptant.

In *S. cerevisiae*, it is known that the *KRE1* and *KRE9* genes encode O-glycosylated proteins involved in the synthesis of  $\beta$ -1,6-glucan. *KRE1* and *KRE9* mutants exhibit fragile cell-wall phenotypes, due to the reduction of the amount of  $\beta$ -1,6-glucan in the cell wall (Brown *et al.*, 1993; Brown & Bussey, 1993). Therefore, the underglycosylation caused by the *pmtA* disruption may affect the synthesis of  $\beta$ -1,6-glucan. It has also been reported that disruption of the

$\beta$ -glucan synthase gene results in an increase in cell-wall chitin content in *S. cerevisiae*. (Garcia-Rodriguez *et al.*, 2000). The increase in chitin content in the *pmtA* disruptant of *A. nidulans* may be due to a similar mechanism, through a kind of salvage mechanism for covering cell-wall weakening. Philip & Levin (2001) reported that Wsc1 and Mid2, which are cell-surface sensors responsible for cell-wall integrity through the MAP kinase pathway, are modified by ScPmt2p. Thus, one cannot exclude the possibility, in *A. nidulans*, that the impairment in O-mannosylation triggers the cascade of events that leads first to damage to cell-wall integrity and then to the cell response involving its repair through cell-wall compensatory mechanisms. Alternatively, PmtA could be directly involved in the mannosylation of proteins covalently attached to the polysaccharides of the wall matrix, whereby the *pmtA* disruption may cause alteration of the cell-wall structure.

A number of proteins secreted into the culture and localized in the cell wall are O-glycosylated (Mrsa & Tanner, 1999; Woo *et al.*, 2003). However, in *Aspergillus*, most of the O-glycosylated proteins localized in the cell walls or plasma membrane have not yet been identified. We are currently involved in an investigation using the *pmtA* disruptant to identify the target proteins that PmtA modulates in structure and function.

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