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Disruption of *Trichoderma reesei* gene encoding protein *O*-mannosyltransferase I results in a decrease of the enzyme activity and alteration of cell wall composition

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In fungi transfer of the first mannosyl residue to proteins during their O-glycosylation is catalyzed by protein *O*-mannosyltransferases encoded by *pmt* genes. Disruption of the *pmt1* gene in *Trichoderma* caused a significant decrease in the total activity of protein *O*-mannosyltransferases. Moreover, disruption of the *pmt1* gene also led to osmotic sensitivity of the strain, indicating an essential role of the PMTI protein activity for cell wall synthesis. At the same time, the strain was defective in septa formation, producing only half the number of septa per unit length of hypha compared with the wild type. Disruption of the *pmt1* gene decreased protein secretion but had no effect on glycosylation of secreted proteins, which suggests that PMTI protein *O*-mannosyltranferase does not take part in glycosylation of these proteins.

Keywords: *Trichoderma reesei*, *pmt1* gene disruption, protein glycosylation, cell wall composition

INTRODUCTION

Trichoderma species are widely exploited in biotechnology for protein production owing to their exceptional protein synthesis and secretory capability. Our previous study indicated that protein production and secretion could be closely related to the activity of the O-glycosylation pathway (Kruszewska *et al.*, 1999; Perlińska-Lenart *et al.*, 2005; 2006b).

In fungi the direct reaction of O-mannosylation is catalyzed by protein *O*-mannosyltransferases (EC 2.4.1.109) and consists in transfer of a mannosyl residue from dolichyl phosphate mannose (DPM) to the serine/threonine OH group of the protein. It is known that in *Saccharomyces cerevisiae* protein O-mannosyltransferases are encoded by seven *PMT* genes and they are classified in three subfamilies PMT1, PMT2 and PMT4 (Gentzsch & Tanner, 1996; 1997). Members of the PMT1 and PMT2 subfamilies, Pmt1p, and Pmt5p, and Pmt2p, Pmt3p, and Pmt6, respectively, form enzymaticaly active heterodimers such as Pmt1-Pmt2 and Pmt3-Pmt5. Deletion of a *PMT* gene encoding a protein from these groups resulted in the formation of less active complexes such as Pmt1-Pmt3 or Pmt2-Pmt5. The PMT4 family has one member only, Pmt4p, and this protein forms an active homodimer. Moreover, protein *O*-mannosyltransferases are substrate-specific (Gentzsch & Tanner, 1997).

In yeast, O-glycosylation was shown to be essential for cell wall rigidity and cell integrity. A lack of activity of two (Pmt2p, Pmt3p or Pmt2p, Pmt4p) or three (Pmt1p, Pmt2p, Pmt3p) protein *O*-mannosyltransferases made the strains unable to grow in normal conditions, however, they could be saved by osmotic stabilization with 1 M sorbitol (Gentzsch & Tanner, 1996). On the other hand, some other triple deletions of *PMT* genes such as *PMT1*, *PMT2*, *PMT4* or *PMT2*, *PMT3*, *PMT4* could not be rescued by osmotic stabilization (Strahl-Bolsinger *et al.*, 1999).

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Abbreviations: DPM, dolichyl phosphate mannose; DTT, dithiotheritol; GDP-mannose, guanosine 5′-diphospho-D-mannose; PMT, protein *O*-mannosyltransferase; PMTI, protein *O*-mannosyltransferase I.

Since protein *O*-mannosyltransferases are substrate-specific those results suggested that the vital role of protein O-mannosylation is connected with O-mannosylation of some cell wall proteins.

In *Schizosaccharomyces pombe* the protein *O*-mannosyltransferase family is not as redundant as in *S. cerevisiae* and has only three members Oma1p, Oma2p and Oma4p, one in each PMT subfamily (Willer *et al.,* 2005). The *oma1*Δ and *oma4*Δ mutants revealed some changes in their cell wall composition while deletion of the *oma2* gene turned out to be lethal (Willer *et al.,* 2005).

Disruption of the *pmtA* gene in *Aspergillus awamori* gave a similar cell wall fragile phenotype as observed for yeasts. The disruptant exhibited swollen hyphae formation and increased sensitivity to Congo Red and high temperature (Oka *et al.,* 2005). Analysis of cell wall composition of the mutant revealed a lower content of alkali-insoluble fraction which is believed to be responsible for fungal cell wall rigidity, and simultaneous an increase of chitin level.

Our previous study pointed at a fundamental role of cell wall permeability for protein secretion in *Trichoderma* (Perlińska-Lenart *et al.,* 2006b). Removal of the cell wall from *Trichoderma* strains carrying the yeast *DPM1* gene, encoding DPM synthase resulted in a four-fold increase of protein secretion compared to mycelia which suggested that the cell wall made a barrier for secretion in these strains. This effect, however, was only observed for *DPM1*-transformed strains characterized by efficient processing of newly synthesized glycoproteins, but not for the control strain, showing that not only cell wall made a barrier for secretion but also the limited modification of secretory proteins (Perlińska-Lenart *et al.,* 2006b).

Up to now only one *pmt* gene encoding protein *O*-mannosyltransferase in *Trichoderma* has been cloned (NCBI accession number AF526877) (Zakrzewska *et al.,* 2003a). Analysis of the predicted protein sequence of the *Trichoderma* PMTI protein showed the highest 51% identity with *S. cerevisiae* Pmt4p. On the other hand, expression of the *Trichoderma* PMTI protein in *S. cerevisiae pmt* mutants revealed its functional similarity to the yeast Pmt2 protein. The *Trichoderma* PMTI protein was able to form an active O-mannosyltransferase complex with yeast Pmt1p and partially rescued the defective glycosylation pattern of chitinase (Zakrzewska *et al.,* 2003a) and completely restored glycosylation of heterologously expressed cellobiohydrolase II from *Trichoderma* in the *pmt2*Δ *S. cerevisiae* mutant (Górka-Nieć *et al.,* 2007).

In this study we presented influence of disruption of the *pmt1* gene in *T. reesei* on the glycosylation, production and secretion of proteins and on cell wall composition. The strain carrying *pmt1* disruption needed an osmotic stabilizer (e.g. 1 M sorbitol) for growth in liquid medium. On the other hand, it was able to grow on the agar plates, albeit very slowly.

Disruption of the *pmt1* gene resulted in a significant decrease in the total activity of protein *O*-mannosyltransferases and caused a decrease of protein secretion but did not alter O- and N-glycosylation of secreted proteins. Since protein *O*-mannosyltransferases are substrate-specific and the lack of the PMTI activity did not change O-mannosylation of the secretory proteins, we conclude that PMTI does not take part in the O-glycosylation of these proteins.

MATERIALS AND METHODS

Strains and growth conditions. *T. reesei* TU-6 (Harman & Kubicek, 1998) was used as a recipient strain for transformation. *Escherichia coli* strain JM 109 was used for plasmid propagation (Yanish-Perron *et al.,* 1985). *T. reesei* was cultivated at 30°C on a rotary shaker (250 r.p.m.) in 2 l shake flasks containing 1 l of minimal medium (MM): 1 g $MgSO_4 \times 7H_2O$, 6 g $(NH_4)_2SO_4$, 10 g KH_2PO_4, 3 g sodium citrate $\times 2H_2O$, and trace elements (25 mg $FeSO_4 \times 7H_2O$, 2.7 mg $MnCl_2 \times 4H_2O$, 6.2 mg $ZnSO_4 \times 7H_2O$, 14 mg $CaCl_2 \times 2H_2O$) per liter and 1% (w/v) lactose as a carbon source. The flasks were inoculated with 42×10^6 konidia/l medium.

Disruption of the *pmt1* gene in *T. reesei*. The plasmid for *pmt1* disruption was constructed as follows. DNA fragment of about 4000 bp containing genomic *pmt1* (NCBI accession number AY515299) and flanking sequences was amplified from genomic DNA by PCR, using The Expand High Fidelity PCR System (Boehringer, Mannheim) and pmt1-U2 (5′ CGC CAG CGA ATG ATT CGA CGG AGG 3′) and pmt1-L2 (5′ CAA GGT GGC TTC TTG TTG CGA CGA 3′) primers. The PCR product was cloned into the pGEM–T Easy Vector (Promega), digested with *Eco*RI and cloned in pRS316 (NCBI accession number U03442) devoid of *Sal*I restriction site. Next the *pmt1* gene was cut with *Sal*I in the middle and the *T. reesei pyr4* coding sequence cut out from the pGF1 vector (Gruber *et al.,* 1990) was cloned there. About 6500 bp of DNA was cut out from the resulting plasmid with *Eco*RI and used for *T. reesei* TU-6 transformation. The *pmt1* disruption in the *pyr4*+ transformants was confirmed by PCR using Pmt554U (5′ TTG GCT GGC TGG TTG GCT ACG AC 3′) and Pmt1730L (5′ GCT GGG AGT GAT TTG CTT GTT GC 3′) primers. For Southern blot analysis, DNA from disruptants and the parental strain was digested with *Pvu*II and *Xho*I, loaded onto agarose gel, blotted and hybridized with the 1.8 kb *Pvu*II/*Xho*I fragment of *T. reesei pmt1*. Radioactive probe was prepared using [α-^{32}P]

dATP and the Fermentas HexaLabel Plus DNA labeling system according to the standard protocol.

Molecular biology methods. Chromosomal DNA was isolated from *T. reesei* using the Promega Wizard Genomic DNA Purification kit. Other molecular biology procedures were performed according to standard protocols (Sambrook *et al.,* 1989).

Biochemical methods

Membrane fraction preparation. Mycelium was harvested by filtration, washed with water and suspended in 50 mM Tris/HCl, pH 7.4, containing 15 mM $MgCl_2$ and 9 mM β-mercaptoethanol. Cells were homogenized in a beadbeater with glass beads (0.5 mm) and the homogenate was centrifuged at 5000 × ***g*** for 10 min to remove cell debris and unbroken cells. The supernatant was centrifuged at 100 000 × ***g*** for 1 h. The membrane pellet was homogenized in 50 mM Tris/HCl, pH 7.4, containing 3.5 mM $MgCl_2$ and 6 mM β-mercaptoethanol, and used as the source of enzymes. The whole procedure was performed at 4°C (Pless & Palamarczyk, 1987).

Activity of protein *O*-mannosyltransferases. Protein *O*-mannosyltransferase activity was assayed in the pelleted membrane fraction by 1 h incubation at 30°C with GDP[^{14}C]-mannose (sp. act. 288 Ci/mol, Amersham) and 5 ng of dolichyl phosphate (Dol-P), according to Kruszewska *et al.* (1989). Total membrane proteins (about 300 μg) were used as the sugar acceptor. Since only the transfer of the first mannosyl residue from dolichyl phosphate mannose (DPM) to the hydroxyl group of serine or threonine catalyzed by Pmt proteins was to be measured, the reaction mixture was supplemented with 10 mM $MgCl_2$. Under these conditions elongation of the O-linked sugar chain does not occur (Sharma *et al.,* 1974). The protein *O*-mannosyltransferase activity was expressed in pmoles of [^{14}C]mannose incorporated into 1 mg of membrane protein during 1 h.

***N*-acetylglucosamine transferase activity.** *N*-acetylglucosamine transferase activity was measured in the membrane fraction by 30 min incubation at 30°C of 200 μg of membrane proteins in a total volume of 50 μl containing 1 × 10^5 c.p.m. UDP[^{14}C]*N*-acetylglucosamine (sp. act. 249 Ci/mol, Amersham) and 5 ng of Dol-P in 40 mM Tris/HCl buffer, pH 7.4, with 10 mM $MgCl_2$ and 0.1% Nonidet P-40 (Palamarczyk & Hemming, 1975). The reaction was stopped by addition of 4 ml of chloroform/methanol (3 : 2, v/v). Formation of radioactive dolichyl diphosphate *N*-acetylglucosamine and dolichyl diphosphate chitobiose was measured in the organic fraction by a scintillation counter.

Concentration of saccharides bound to secreted proteins. Saccharides bound to proteins isolated from *T. reesei* culture filtrates were assayed by the phenol-sulfuric acid procedure (Dubois *et al.,* 1956). Secreted proteins were precipitated with two volumes of ethanol, washed twice with 70% ethanol and dissolved in distilled water. O-linked sugars were cleaved by mild alkaline hydrolysis and then remaining proteins containing N-linked sugars were precipitated with two volumes of ethanol and centrifuged. The concentration of O-linked carbohydrates was measured in the supernatant after evaporation of ethanol, and N-linked sugars were assayed in the pellet. A calibration curve was prepared with D-mannose.

Protein concentration assay. Protein concentrations were estimated according to Lowry *et al.* (1951).

Quantification of fungal dry mass. Fungal dry mass was quantified by filtering culture samples through G1 sintered glass funnels, washing the biomass with a threefold volume of tap water, and drying to constant weight at 110°C.

Colony growth rate. Colony growth rates were measured as described by Oka *et al.* (2004). Conidia were point-inoculated into the center of agar MM plates with or without osmotic stabilizers (1 M sorbitol or 0.6 M KCl) or with the antifungal agent Calcofluor white (300 μg ml^{-1}) (Oka *et al.*, 2004) and incubated at 30°C. Colony diameter was measured at 48, 72, 96, 120 and 144 h. Measurements of the growth for all strains were done six times.

Cell wall preparation. *T. reesei* strains were cultivated for 168 h and the mycelia were harvested by centrifugation, homogenized in a beadbeater with 0.5 mm glass beads in 50 mM Tris/HCl, pH 7.5, with 1 mM DTT and centrifuged at 1500 × ***g*** for 10 min.

The resulting pellet containing cell walls was washed with ice-cold 1 M NaCl until disappearance of absorbance at 260–280 nm (Nemcovic & Farkas, 2001).

Determination of cell wall polysaccharides. The amount of glucans in the cell wall was determined as described previously (Oka *et al.,* 2004), with a slight modification. For quantification of alkali-soluble β-(1,6)glucan, 200 mg of cell walls was suspended in 3% NaOH, heated at 75°C for 1 h and centrifuged. The supernatant was dialyzed overnight at 4°C against distilled water, lyophilized and the amount of alkali-soluble β-(1,6)glucan was estimated by the method described by Dubois *et al.* (1956). The remaining pellet was washed twice with 0.1 M Tris/HCl, pH 7.4, and once with 10 mM Tris/HCl, pH 7.4, and digested overnight with zymolyase 20T (ICN Biomedicals Inc.) (5 mg/ml in 10 mM Tris/HCl pH 7.4). Then the samples were centrifuged (13 000 r.p.m., 15 min) and the supernatant was used to estimate the amount of alkali-insoluble β-(1,3)glucan by the same method (Dubois *et al.,* 1956). The remaining pellets were incubated for 16 h with 70% sulfu-

ric acid at 4ºC, then diluted ten-fold with water and heated at 100ºC for 8 h. After neutralization with 2 M NaOH samples were used to estimate the amount of alkali-insoluble β-(1,6)glucan (Dubois *et al.*, 1956).

For chitin measurements alkaline hydrolysis of cell walls was performed in 6% KOH for 90 min at 80ºC in order to release cell wall proteins. After neutralization with acetic acid, the cell walls were washed with phosphate-buffered saline and chitinase buffer, pH 6.0, containing 18 mM citric acid and 60 mM dibasic sodium phosphate. Subsequently, the cell walls were treated with chitinase C (InterSpex Products) for 3 h at 37ºC. The level of chitin was measured with Ehrlich's reagent as described (Reissig *et al.*, 1955).

Microscopic analysis of mycelia. *T. reesei* transformants and control strains were harvested by centrifugation, washed twice with sterile water, resuspended in warm 1.5% agarose (A-5030 Type IX from Sigma), allowed to cool and observed under a Nicon Eclipse E 6800 fluorescence microscope. The hyphae diameter and distances between septa were calculated from about one hundred pictures of each strain using Lucia G program.

RESULTS

Disruption of the *pmt1* gene in *T. reesei*

T. reesei TU-6, a Δ*pyr4* mutant of *T. reesei* QM9414, was transformed with a DNA fragment of about 6.5 kb containing *T. reesei pyr4* gene flanked with *pmt1* sequences, as described in Methods. Prototrophic transformants were selected and isolated by three rounds of transfer from selective to nonselective medium and screened by Southern blotting for the genomic copy of *pmt1*. To this aim genomic DNA of the transformants and the control strain was digested with *Pvu*II/*Xho*I and hybridized with a *pmt1* probe cut out from pGEM with the same restriction enzymes. The *pmt1* gene contains one restriction site for each of the enzymes, so its intact copy should yield a 1.98 kb fragment hybridizing with the probe. Since *T. reesei pyr4* has many *Pvu*II and *Xho*I restriction sites, DNA obtained from the disruptants gave two fragments of 1.35 and 1.26 kb hybridizing with the *pmt1* probe (Fig. 1A, B).

Effect of *pmt1* gene disruption on the total activity of protein *O*-mannosyltransferases

Protein *O*-mannosyltransferase I (PMTI protein) encoded by the *pmt1* gene catalyzes transfer of the mannosyl residue from dolichyl phosphate mannose (DPM) to a serine or threonine OH group in the protein. To determined the influence of *pmt1* disruption on the total activity of direct O-glycosylation we measured the combined activity of all PMT proteins. The experiment revealed a significant decrease of the total activity of protein *O*-mannosyltransferases, to 67% of the control value (Fig. 1C).

Effect of the lower activity of protein O-mannosyltransferases on the growth of *Trichoderma*, protein secretion and glycosylation of secreted proteins

Trichoderma strains were cultivated on agar plates containing MM medium with glucose or lactose as a carbon source, supplemented with 1 M sorbitol or 0.6 M KCl as osmotic stabilizers (Oka *et al*., 2004). Every 24 h the diameter of the colony was measured and the results are presented as millilitre of growth per hour (Table 1). Colony growth of the strain carrying disruption of the *pmt1* gene was very slow and reached 17% of the control when cultivated on the MM medium without osmotic stabilizers. Addition of 1 M sorbitol or 0.6 M KCl to the cultivation medium inhibited growth of the control strain and the level of inhibition depended on the carbon source. The most pronounced effect on growth of the control strain was found on lactose-containing medium supplemented with sorbitol. An opposite effect was observed for the *pmt1* disruptant which grew nearly twice better with osmotic stabilization, nonetheless, the growth still remained slower compared to the growth of the control strain on medium without supplements.

We also examined growth of the strains in liquid MM medium with lactose and it turned out that the strain MJK1/07 carrying *pmt1* disruption was not able to growth without osmotic stabilization. Therefore, *Trichoderma* strains were cultivated with 1 M sorbitol and every 24 h mycelia were collected and growth of the strains was presented as an increase of dry mass of mycelia during cultivation (Fig. 2). Rather unexpectedly the *pmt1* disruptant turned out to grow better than the control strain. At the same time we measured the amount of proteins liberated to the cultivation medium during 288 h of cultivation and found that the strain carrying disruption of the *pmt1* gene secreted less protein compared to the control strain (Fig. 3).

Since we found that the activity of protein *O*-mannosyltransferases in the examined strain was decreased we expected changes in the intensity of O-glycosylation of secreted protein. Since changes in O-glycosylation could influence N-glycosylation (Ecker *et al.*, 2003) we characterized the amount of both O- and N-linked sugars bound to the secreted proteins. To this end, the proteins from the cultivation medium were isolated and after mild alkaline

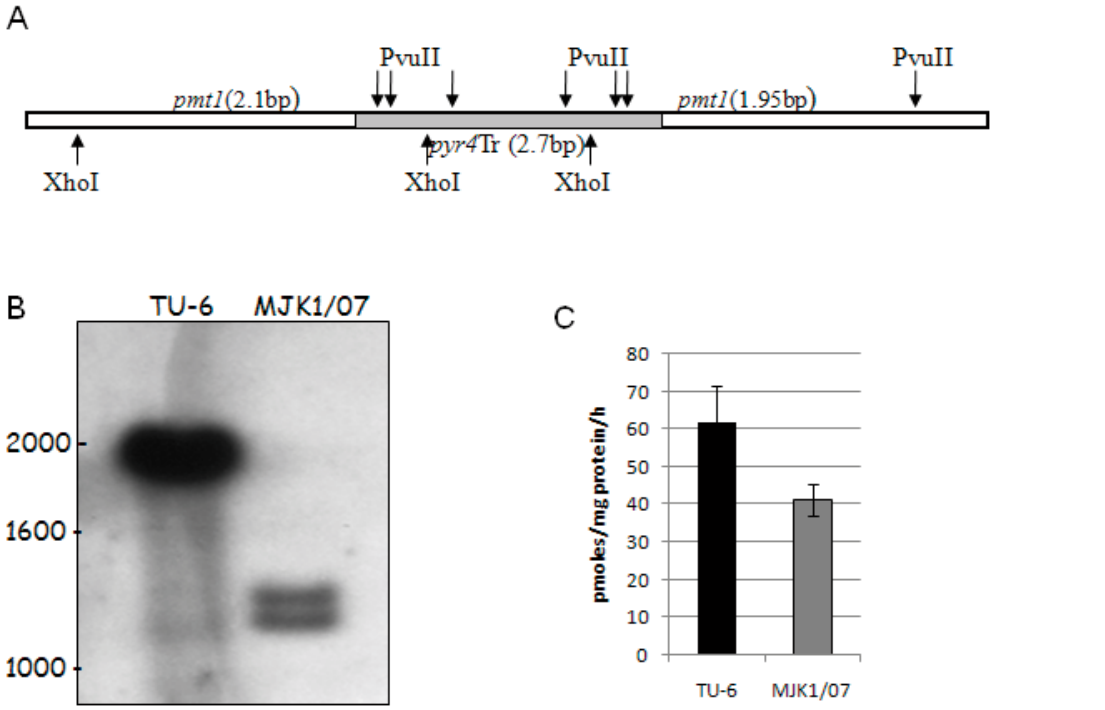

Figure 1. Poproszę o wspólny tytuł
A. Schematic presentation of DNA fragment used for *pmt1* gene disruption in *T. reesei* TU-6. Arrows point at restriction sites for *Pvu*II and *Xho*I. B. Southern blot analysis of *Pvu*II and *Xho*I digested total DNA of parental strain (TU-6) and the *pmt1* disrupted strain (MJK1/07). C. Total activity of protein *O*-mannosyltransferases in membrane fraction from *T. reesei* strain carrying disruption of *pmt1* gene (MJK1/07) in comparison to the host strain TU-6. Strains were cultivated for 168 h in MM medium with lactose supplemented with 1 M sorbitol. The data are presented as mean ± standard deviation from three separate cultures.

hydrolysis the amount of liberated O-linked sugars was measured (Table 2).

We found that the amount of O-linked carbohydrates bound to the secreted proteins was only 14% lower in the strain carrying disruption of the *pmt1* gene compared to the control strain. An assay of N-linked carbohydrates revealed only 5% less of these sugars bound to the secreted proteins. Student's *t*-test revealed that the differences were not statistically significant. On the other hand, we measured activity of *N*-acetylglucosamine transferase, the first enzyme in the N-glycosylation pathway and found it 78% more active compared to the control strain (Fig. 4).

Table 1. Growth rate of *T. reesei* strain MJK1/07 carrying disruption of the *pmt1* gene and the parental strain TU-6.

Medium	TU-6	MJK1/07
MML	0.40 ± 0.017	0.07 ± 0.005
MML+1 M sorbitol	0.16 ± 0.011	0.11 ± 0.005
MML+0.6 M KCl	0.25 ± 0.012	0.11 ± 0.007
MMG	0.42 ± 0.022	0.07 ± 0.007
MMG+1 M sorbitol	0.30 ± 0.019	0.12 ± 0.008
MMG+0.6 MKCl	0.33 ± 0.016	0.12 ± 0.009
MMG+ Calcofluor white	0.41 ± 0.015	not growing

Growth values are in mm h^{-1}. The data are presented as mean ± standard deviation from six separate cultures. MMG and MML, minimal medium with glucose or lactose as a carbon source.

Influence of the lower activity of *O*-mannosyltransferases on the cell wall of the mutant

The strain carrying disruption of the *pmt1* gene did not grow in liquid medium without sorbi-

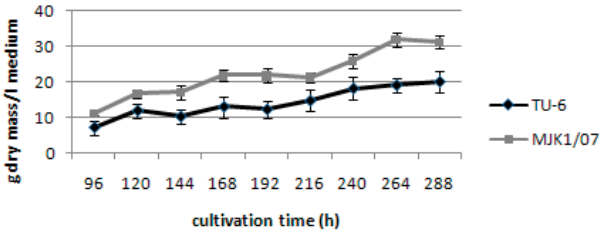

Figure 2. Growth of *T. reesei* strain carrying disruption of *pmt1* gene and the control strain cultivated in MM liquid medium with lactose supplemented with 1 M sorbitol.
The data are presented as mean ± standard deviation from five separate cultures.

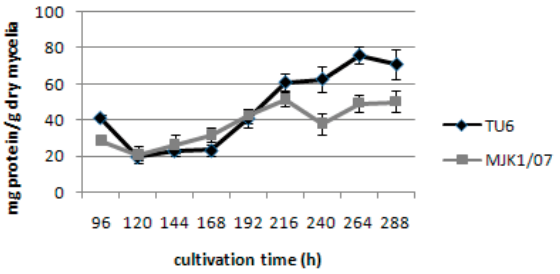

Figure 3. Amount of secreted proteins in cultivation medium from MJK1/07 strain carrying disruption of *pmt1* gene and its host TU-6 strain.
Strains were cultivated in MM with lactose supplemented with 1 M sorbitol. The data are presented as mean ± standard deviation from five separate cultures.

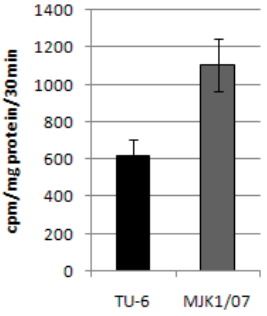

Figure 4. Activity of *N*-acetylglucosamine transferase in membrane fraction from *T. reesei* strain with disruption of *pmt1* gene (MJK1/07) in comparison to the host strain TU-6.
Strains were cultivated for 168 h in MM medium with lactose supplemented with 1 M sorbitol. The data are presented as mean ± standard deviation from three separate cultures.

tol, which suggested that the decreased activity of the PMTI O-mannosyltransferase could alter the composition of the cell wall in this strain. Moreover, the *pmt1* mutant did not grow in the presence of the antifungal agent Calcofluor white while growth of the control strain was not inhibited in these conditions (Table 1).

To study the results of the disruption of the *pmt1* gene on the cell wall composition the mutant strain and the control were cultivated in media with an osmotic stabilizer, the cell wall was isolated and the content of glucans and chitin measured. The level of alkali-soluble β-(1,6)glucan observed for the MJK1/07 strain carrying disruption of the *pmt1* gene was decreased to 89% of the control value (Table 3). No detectable amount of β-(1,3)glucan or alkali-insoluble β-(1,6)glucan was found in either strain. Moreover, an assay of the chitin content in the cell wall revealed no differences between the transformant and the control.

Microscopic analysis of mycelia

Our *T. reesei* mutant exhibited significant changes in the composition of its cell wall. Since a weak cell wall could influence the structure of mycelia we examined our strain under a phase-contrast microscope and measured the diameter of hyphae and the distance between septa using the Lucia G program. One-hundred pictures of transformant and control were analyzed, and we found that the distance between septa was more than twice longer in the disruptant MJK1/07 compared to the control (Table 4). Moreover, mycelia of the strain bearing disruption of the *pmt1* gene were slightly narrower compared to the control.

Table 2. Carbohydrates bound to the secreted proteins in the *pmt1* disrupted strain MJK1/07 compared to the host strainTU-6

	μg sugar/mg protein	
Strain	O-linked	N-linked
TU-6	4.64 ± 0.91	77.63 ± 6.92
MJK1/07	4.00 ± 0.76	73.63 ± 6.92

The data are presented as mean ± standard deviation from three separate cultures.

DISCUSSION

Our earlier studies showed that elevation of DPM synthase, guanyltransferase or *cis*-prenyltransferase activity in *Trichoderma* or *Aspergillus* caused significant changes in the activity of other enzymes engaged in the O-glycosylation pathway and altered protein secretion or glycosylation of secreted proteins (Zakrzewska *et al.*, 2003b; Perlińska-Lenart *et al.*, 2005; 2006a; 2006b). This study concentrates on protein *O*-mannosyltransferase I (PMTI) catalyzing the direct transfer of mannosyl residue to proteins.

Our results showed that disruption of the *pmt1* gene encoding PMTI protein led to a significant decrease in the total activity of protein *O*-mannosyltransferases. Simultaneously with the lower activity of protein *O*-mannosyltransferases in the strain bearing disruption of the *pmt1* gene, we found so profound alteration of the cell wall composition

Table 3. Cell wall composition of *T. reesei* strains carrying *pmt1* disruption (MJK1/07) compared to the parental strain TU-6

	Amount of carbohydrate (μg/mg dry cell wall)	
Strain	Alkali soluble β-(1,6)glucan	Chitin
TU-6	149.54 ± 11.32	2.74 ± 0.48
MJK1/07	133.28 ± 13.25	2.93 ± 0.51

The data are presented as mean ± standard deviation from three separate cultures. The cell walls were isolated from the strains after 168 h of growth in lactose MM medium with sorbitol.

Table 4. Hyphae diameter and distance between septa in *T. reesei* strains carrying *pmt1* disruption (MJK1/07) compared to the parental strains TU-6

Strain	Hyphae diameter (μm)	Distance between septa (μm)
TU-6	3.70 ± 0.51	21.98 ± 4.21
MJK1/07	3.37 ± 0.48	54.40 ± 8.44

The data are mean ± standard deviation from one hundred measurements.

that this strain could not grow in a liquid medium without sorbitol. The strain was able to grow very slowly on agar plates without osmotic stabilizers, however, osmotic stabilization by 1 M sorbitol or 0.6 M KCl clearly enhanced its growth. Furthermore, the *pmt1* mutant was sensitive to Calcofluor white which is known to be adsorbed on cell wall polysaccharides and exhibits an antifungal effect.

In *A. nidulans*, disruption of the *pmtA* gene resulted in a more than two-fold decrease in growth rate in liquid medium compared to the wild-type strain and the growth of both strains became similar after supplementation of the medium with an osmotic stabilizer (Oka *et al.*, 2004). These data suggested that changes in the cell wall of the *A. nidulans pmtA* disruptant were not as dramatic as those found in the *Trichoderma* MJK1/07 strain. The difference could be caused by an increased production of chitin in the *Aspergillus pmtA* strain compared to the control, which was not observed in the *Trichoderma* disruptant. The cell wall of *Aspergillus* was rescued by induction of a cell wall compensatory mechanism manifested by higher production of chitin (Oka *et al.*, 2004; 2005). To induce this mechanism, sensor proteins present in the cell wall detect and transmit the cell wall status to a signaling pathway comprised of a cascade of MAP kinases (Levin, 2005). The extracellular domains of these sensors are highly O-mannosylated, and their limited mannosylation could result in a loss of function (Lommel *et al.*, 2004). Since the PMT proteins are substrate-specific, it is possible that the PMTA protein from *Aspergillus* and the PMTI protein from *Trichoderma* differ in their substrate specificity and that PMTI from *Trichoderma* normally glycosylates cell wall sensors, in contrast to PMTA which glycosylates other substrates.

The *Trichoderma* PMTI protein, although structurally similar to *S. cerevisiae* Pmt4p, functionally could replace the Pmt2 protein from *S. cerevisiae* (Zakrzewska *et al.*, 2003a) and is a homolog of *S. pombe* Oma2p. It has been shown that in *S. pombe* Oma2p takes part in glycosylation of the cell wall sensor protein Wsc1p and disruption of the *oma2* gene is lethal (Willer *et al.*, 2005).

Analysis of the cell wall composition of our transformants showed that the level of β-(1,3)glucan and alkali-insoluble β-(1,6)glucan was not detectable and we could only measure the amount of alkali-soluble β-(1,6)glucan and chitin.

Furthermore, microscopic studies of our strains showed a significant decrease in the number of septa in the mycelia of the mutant. The troubles with septa formation could be connected with the lack of β-(1,3)glucan. It has been reported that in *S. pombe* disruption of β-(1,3)glucan synthase gene (*bgs1*) resulting in a decreased amount of β-(1,3)glucan causes defects in septa formation (Carlos *et al.*, 2007).

On the other hand, the troubles with the synthesis of cell wall components could be a result of cultivation of the strains in a medium supplemented with sorbitol. A similar effect was observed for *Neurospora crassa* cultivated with an addition of sorbitol, where the cell wall components were synthesized in a limited amount and the activity of β-(1,3)glucan synthase was decreased. In consequence, the fungus grew solely in the form of protoplasts (da Silva *et al.*, 1994).

In this study we also examined the influence of a decreased activity of protein *O*-mannosyltransferases on the secretion of proteins and their glycosylation. We found that the strain carrying disruption of the *pmt1* gene secreted a lower amount of proteins compared to the control. A lower secretion was also observed for a *Hansenula polymorpha pmt* mutant, however, only secretion of O-mannosylated proteins was inhibited, as shown for chitinase which was secreted in a very limited amount (Agaphonov *et al.*, 2005). Invertase or heterologously expressed human urinary type plasminogen activator, which are normally N-glycosylated, were secreted in elevated amounts, although they were unglycosylated.

In *T. reesei* the majority of secretory proteins are highly glycosylated with both N- and O-linked glycans (Palamarczyk *et al.*, 1998). The lack of activity of the PMTI protein *O*-mannosyltransferase in the strain carrying the *pmt1* disruption only slightly decreased or even not altered O- and N-glycosylation of secreted proteins. These results suggest that the protein *O*-mannosyltransferase PMTI only marginally participates in glycosylation of secreted proteins. On the other hand, the activity of *N*-acetylglucosamine transferase was enhanced in the *pmt1* disruptant suggesting that N-glycosylation of some proteins, although not the secretory ones, should be stimulated in this strain.

It has been shown for an *S. cerevisiae pmt4*Δ mutant that a lack of O-linked sugar bound to the serine or threonine in the vicinity of the N-glycosylation site enables N-glycosylation, while in the wild type strain when the O-glycosylation site is occupied the N-glycosylation site remains not glycosylated (Ecker *et al.*, 2003).

In the present paper we showed the consequences of a lack of the PMTI protein *O*-mannosytransferase on the survival of *Trichoderma* cells, protein glycosylation and secretion and on the cell wall formation. We also showed that PMTI protein *O*-mannosyltransferase did not take part in the O-glycosylation of the secreted proteins. Moreover, the increased activity of *N*-acetylglucosamine transferase observed in the *pmt1* disruptant could confirm an interdependence between protein O- and N-glycosylation.

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