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Two-Dimensional Gel Electrophoresis Analysis of Mycelial Cells Treated with Tween 80: Differentially Expressed Protein Related to Enhanced Metabolite Production

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ABSTRACT: Two-dimensional gel electrophoresis identified 40 differentially expressed proteins which explained the mechanisms underlying the stimulatory effect of Tween 80 for exopolysaccharide production in the mycelium of an edible mushroom *Pleurotus tuber-regium*. The up-regulation of fatty acid synthase alpha subunit FasA might promote the synthesis of long-chain fatty acids and their incorporation into the mycelial cell membranes, increasing the membrane permeability. A down-regulation of Phospholipase D1 and an up-regulation of Hypothetical protein PGUG_02954 might mediate signal transduction between the mycelial cells and the extracellular stimulus (Tween 80). The down-regulated ATP-binding cassette transporter protein might function as pumps to extrude exopolysaccharide out of the cells that lead to a significant increase in its production. The present results explained how stimulatory agents like Tween 80 can increase mycelial cell membrane permeability to enhance the production of useful extracellular metabolites by submerged fermentation.

KEYWORDS: *mushroom mycelium, exopolysaccharide, proteomic analysis, two-dimensional gel electrophoresis, Tween 80*

INTRODUCTION

Pleurotus tuber-regium, an edible mushroom found in the tropic and subtropical regions of the world, is used as a nutritious food as well as in the treatment of many diseases, such as constipation, stomach pain, fever, and colds.[1−3] Surfactants such as Tween 80 have been proved to be effective stimulatory agents in the production of useful metabolites in bacteria, fungi and medicinal mushrooms. Our previous work has shown that the exopolysaccharide (EPS) produced from submerged fermentation of *P. tuber-regium* has antitumor activities and the EPS production could be significantly enhanced with addition of 0.3% (w/v) Tween 80 on the fifth day of submerged fermentation.[4] In addition, the mechanism by which Tween 80 could affect mushroom EPS production is associated with the permeability of and transport activity across the mycelial membrane.[5] Hence, a further investigation by use of proteomics to study the functions of the *P. tuber-regium* proteins could provide more insights into the mechanism by which chemical stimulatory agents such as Tween 80 mediate the mycelial membrane permeability and extracellular metabolite production.

Two-dimensional gel electrophoresis (2D-GE) based proteomics have been used to determine the changes in the proteins that are expressed in a microorganism induced by different physical and chemical factors or under toxic stress.[6−8] Moreover, proteomics has been widely used to explain the metabolic responses of microorganisms growing under different culture conditions in terms of microbial protein expression.[9−12] Although the development of proteomics is rapid, investigations to find out the protein expression changes in mushroom mycelial cells under different culture conditions are very rare. Until very recently, the first systematic proteomics analysis of two mushrooms *Sparassis crispa* and *Hericium erinacemu* was reported, which provided a preliminary understanding on the mushroom proteomes.[13]

In this study, the protein expression changes of *P. tuber-regium* with and without addition of Tween 80 are studied by 2D-GE based proteomics. Such investigation could provide valuable information not only on the relationship between the addition of stimulatory agents and fungal metabolism in terms of cellular and molecular mechanisms, but also on the mushroom proteomics which is underdeveloped at present.

MATERIALS AND METHODS

Strain and Growth Conditions. The strain of *P. tuber-regium* was obtained from Fungi Perfecti Ltd. Co. (Olympia) and the culture conditions used were the same as in our previous work.[4] The mycelial cells obtained from the fermentation broth with and without addition of Tween 80 (0.3%, w/v, added on the fifth day of fermentation) were used for the proteomic analysis by the following steps. All experiments were performed in duplicate.

Extraction of Total Protein. The freeze-dried mycelial cells (about 500 mg) were ground into fine powder in liquid nitrogen using a prechilled pestle and mortar. Total protein of *P. tuber-regium* mycelia was extracted by using phenol extraction protocol with some modifications.[13,14]

The total protein was extracted from the fine powder by addition of 5 mL of extraction media [0.1 M Tris (pH 8.8), 10 mM EDTA, 0.9 M

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sucrose and 0.4% (v/v) 2-mercaptoethanol] and 5 mL of Tris (pH 8.8) buffered phenol, followed by gently inverted mixing at room temperature for 30 min. The suspensions were centrifuged at 12 000 *g* for 20 min at 4 °C. After centrifugation, the top phenol phase was transferred to a new Eppendorf tube. The remaining aqueous phase at the bottom was re-extracted once. The top phenol phase from the second extraction was combined with the first extraction and vortexed. Soluble proteins in this phenolic extract were precipitated by addition of 5 vol of 0.1 M ammonium acetate in 100% methanol, followed by vortex-mixing and standing at −20 °C overnight. The precipitated protein was collected as pellet by centrifugation at 12 000 *g* for 20 min at 4 °C. The pellet was washed twice with 1 mL of 0.1 M ammonium acetate in 100% methanol, then with 1 mL of 80% ice-cold acetone, and finally once with 1 mL of ice-cold 70% ethanol. Following centrifugation (at 12 000 *g* for 20 min at 4 °C), the supernatant was discarded and the pellet was dried at 37 °C for 10−15 min. Proteins were redissolved in 1 mL of rehydration solution [8 M urea, 2% (w/v) CHAPS, 0.002% (w/v) bromophenol blue, and 2% (v/v) IPG buffer], followed by centrifugation at 12 000 *g* for 20 min at 4 °C. The clear supernatant containing the soluble proteins was used for protein quantification by the PlusOne 2D Quant Kit (GE Healthcare) and stored in aliquots at −80 °C.

2D-GE. Isoelectric focusing (IEF) was performed using an Ettan IPGphor III isoelectric focusing system (GE Healthcare). The sample was quantified by PlusOne 2D Quant Kit as mentioned in the Protein quantification section and normalized to 600 μg. Rehydration solution (8 M urea, 2% (w/v) CHAPS, 0.002% (w/v) bromophenol blue and 2% (v/v) IPG buffer of corresponding pH range) was added to make up to a volume of 250 μL. Just prior to focusing, 18 mM DTT was added to the sample. The prepared sample was applied to the Immobilized gradient (IPG) strips (13 cm, nonlinear pH 3−10, GE Healthcare).

In the presence of sample, rehydration was first carried out at 0 V for 8 h. To facilitate the entry of high-molecular-weight proteins into IPG strips, low voltage (30 V for 6 h) was applied afterward. IEF was then performed with 500 V for 1 h, 1000 V for 1 h, 4000 V for 4000 Vh and finally stabilized at 8000 V, for a total 80 000 Vh. Before the second dimension, strips were either stored at −80 °C or directly undergone reduction at room temperature for 15 min in the SDS equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% bromophenol blue] containing DTT (1%, w/v). The strip was then washed with Milli-Q water (Millipore) briefly and further underwent alkylation with IAA in SDS equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% bromophenol blue] at room temperature for another 15 min.

After equilibration, the IPG strip was washed with Milli-Q water (Millipore) briefly again and transferred to 1 mm gel cassettes with separating gels (12.5%). The strip was sealed with about 1 mL of agarose sealing solution with SDS electrophoresis buffer containing 25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS, 0.5% agarose and 0.002% (w/v) bromophenol blue. Separation in the second dimension was then performed in the SE600 Ruby electrophoresis systems (GE). By running with the SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS), separation was performed at 90 V for 1 h and then 250 V for about 3 h until the run was completed.

Visualizing and Image Analysis. After the second-dimension run, the gels were washed with Milli-Q water and then stained with 100 mL of Coomassie staining solution which contains 45% (v/v) methanol, 10% (v/v) acetic acid and 0.15% (w/v) Coomassie Brilliant Blue R350 for 1 h. The gels were finally destained overnight using 100 mL of destaining solution containing 45% (v/v) methanol, 10% (v/v) acetic acid in Milli-Q water.

The gels were stained and destained and then scanned with an Image Scanner (Amersham Biosciences). Analysis was performed using the ImageMaster 2D Platinum 5.0 software (Amersham Biosciences). Protein spots were detected automatically while manual spot editing and deleting was performed if necessary. Distinct spots were selected throughout the gel for alignment and matching. Gels were studied in duplicates and the gel with the protein spots from the *P. tuber-regium* mycelia cultured without addition of Tween 80 was used as a reference gel. Normalized volume ratio was used to quantify and compare the gel spots. The spots with at least 2.5-fold increase and at least 0.4-fold decrease in the normalized volume ratio were considered as differentially expressed in two comparative gels (proteins from mycelial cells with and without the addition of Tween 80) and were chosen for further analysis.

In-Gel Digestion and Peptide Extraction. The differentially expressed protein spots were excised for digestion. They were transferred to 1.5 mL microcentrifuge tubes and cut into smaller pieces with surgical needle. The protein samples were destained with 50% (v/v) methanol in 50 mM NH_4HCO_3 and dehydrated with acetonitrile (ACN). After vacuum-drying using Speed-Vac (LABCONCO), protein gel samples were rehydrated with 10 μL (40 ng/μL) of sequencing grade modified trypsin (Promega), covered by 30 μL of 25 mM NH_4HCO_3 and incubated at 30 °C overnight. The digested sample was sonicated for 10 min and the supernatant was removed to a new microcentrifuge tube. Ten microliters of extraction buffer (60% ACN, 2.5% TFA) was added to the gel sample, followed by sonication for 10 min. The supernatant was removed and combined with the previous extracts. About 0.5 μL of the digested sample solution in the microcentrifuge tube was spotted onto a MALDI plate twice, and then 0.5 μL of matrix solution (4 mg/mL α-cyano-4-hydroxycinnamic acid in 35% ACN and 1% TFA) was spotted. The samples were ready for MALDI-TOF/TOF MS analysis.

MALDI-TOF/TOF MS Analysis. MS analysis was performed using a MALDI-TOF/TOF tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems, Foster City, CA). Mass data acquisitions were piloted by the 4000 Series Explorer Software v3.0 using batched-processing and automatic switching between MS and MS/MS modes. All MS survey scans were acquired over the mass range 800−3500 *m/z* in the reflection positive-ion mode and accumulated from 2000 laser shots with an acceleration of 20 kV. The MS spectra were internally calibrated using porcine trypsin autolytic products (*m/z* 842.509, *m/z* 1045.564, *m/z* 1940.935 and *m/z* 2211.104) resulted in mass errors of less than 30 ppm. The detection of MS peaks (MH^+) was based on a minimum S/N ratio ≥20 and a cluster area S/N threshold ≥25 without smoothing and raw spectrum filtering. Peptide precursor ions corresponding to contaminants including keratin and the trypsin autolytic products were excluded in a mass tolerance of ±0.2 Da. The filtered precursor ions with a user-defined threshold (S/N ratio ≥50) were selected for the MS/MS scan. Fragmentation of precursor ions was performed using MS-MS 1 kV positive mode with CID on and argon as the collision gas. MS/MS spectra were accumulated from 3000 laser shots using default calibration with Glu-Fibrinopeptide B from 4700 Calibration Mixture (Applied Biosystems). The detection of MS/MS peaks were based on a minimum S/N ratio ≥3 and a cluster area S/N threshold ≥15 with smoothing.

The acquired data were searched against the National Center for Biotechnology Information (NCBI) MS (Peptide Mass Fingerprint, PMF) database using the MASCOT software package (Version 2.3, Matrix Science, UK; www.matrixscience.com). The following search parameters were used: monoisotopic peptide mass (MH^+), allowance of one missed cleavage per peptide, enzyme (trypsin), taxonomy (fungi), peptide mass tolerance of 0.1 Da, variable modifications (oxidation for methionine). The protein hit with an expectation value lower than 0.05 and sequence coverage greater than 10% was considered as an identified protein. The identified proteins were assigned to various functional classes with the help of UniProt (Universal Protein Resource, http://www.uniprot.org/). All experiments were performed in duplicate with similar results and only one representative was shown.

RESULTS AND DISCUSSION

To investigate the effect of Tween 80 on the mycelial cell membrane permeability and EPS production of *P. tuber-regium* at the level of protein expression, a comparative proteomic analysis of mycelial proteins of *P. tuber-regium* by submerged

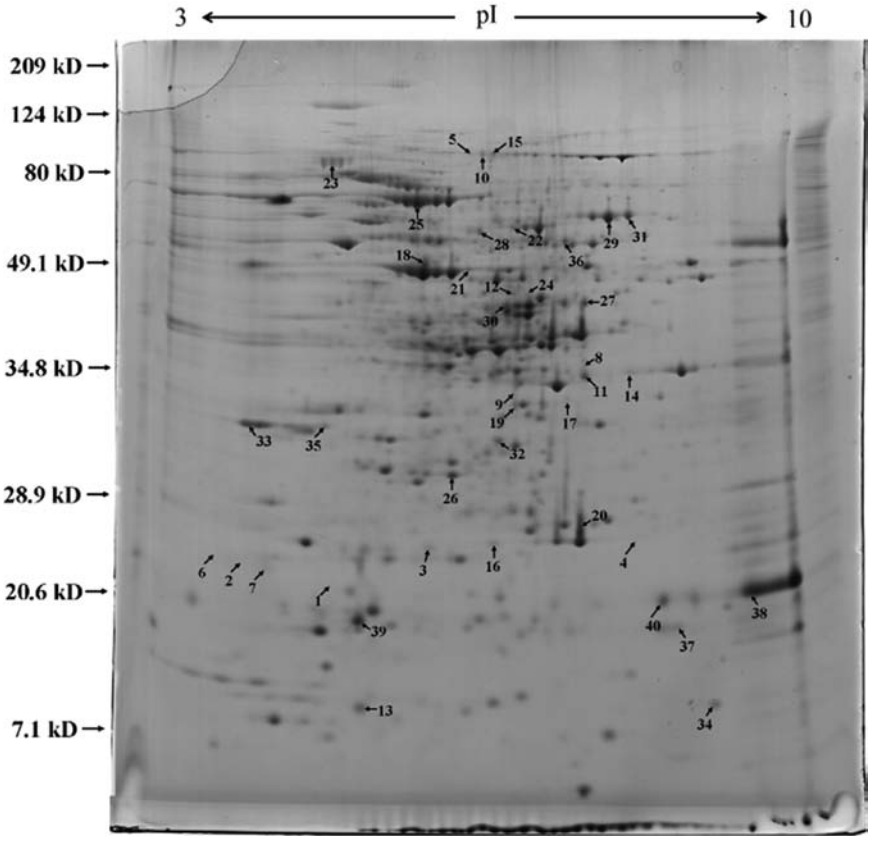

Figure 1. Representative 2D gel of mycelial proteins from *P. tuber-regium* produced without the addition of Tween 80 (reference gel). Protein spots with differentially expression are denoted on the gel image. Spots 1−24 are the proteins with an up-regulation of expression, while spots 25−40 are the proteins with a down-regulation of expression when compared to the reference gel.

fermentation with and without the addition of Tween 80 was performed using 2D-GE approach. The representative gel images of the proteins isolated from the mycelial cells without and with the addition of Tween 80 were shown in Figures 1 and 2, respectively. Approximately 260 protein spots on the two gels were revealed by the ImageMaster analysis. With the addition of Tween 80, a total of 40 spots were differentially expressed (Figures 1 and 2), in which 24 spots (spot 1−24) had at least 2.5-fold increase and 16 spots (spot 25−40) had at least 0.4-fold decrease when compared to those without the addition of Tween 80. The identification data for these 40 differentially expressed proteins was shown in Table 1. Our previous study has suggested that the mechanism by which Tween 80 could affect mushroom EPS production is associated with the permeability of and transport activity across the mycelial membrane.[5] Hence, several differentially expressed proteins which may be closely related to the mechanisms of Tween 80, such as the membrane related proteins, cell communication proteins and ATP-binding cassette transporter proteins, were further discussed in detailed.

Membrane Related Protein. The hypothetical protein TRV_04216 (spot 10 in Figures 1 and 2 and Table 1) and fatty acid synthase alpha subunit FasA (spot 19 in Figures 1 and 2 and Table 1) were two membrane related proteins with an increasing level of expression by the addition of Tween 80. The hypothetical protein TRV_04216 protein is believed to have membrane related function but its exact activity is unknown, while the fatty acid synthase alpha subunit FasA acts mainly for fatty acid biosynthesis. Fatty acid synthases possess the ability to produce long-chain fatty acids which play a central role in the cells to incorporate lipids into their membranes or storage bodies.[15] It has been found that fatty acid synthase alpha subunit (fasA) encodes the central enzyme in *de novo* lipogenesis, catalyzing the synthesis of acetyl-CoA and malonyl-CoA into long-chain fatty acids.[15,16]

In our previous study, it was shown that the amount of total fatty acids in the mycelial lipids of *P. tuber-regium* was significantly enhanced ($p < 0.05$) from 22.1 to 27.8 mg/g and the oleic acid (C18:1) composition was significantly increased from 2.6% to 18.5% with the addition of Tween 80.[5] When combining with the results of the increasing level of expression in fatty acid synthase found in the 2D gel analysis, it could be suggested that the addition of Tween 80 could exert a stimulatory effect on the fatty acid synthase of *P. tuber-regium* mycelial cells to influence the fatty acid content and the cell membrane permeability accordingly.

Cell Communication Protein. Hypothetical protein PGUG_02954 (spot 24 in Figures 1 and 2 and Table 1) and Phospholipase D1 (spot 38 in Figures 1 and 2 and Table 1) are both phosphatidylinositol binding proteins and function in cell communication. In general, cell communication proteins are involved in the process that mediates interactions between a cell and its surroundings, such as signaling or attachment between one cell and another cell, between a cell and an extracellular matrix, or between a cell and any other aspect of its environment.[17] These two cell communication proteins are bind with phosphatidylinositol, which play important roles in membrane trafficking, lipid signaling and cell signaling. With the addition of Tween 80, the expression level of hypothetical protein PGUG_02954 was increased while that of Phospho-

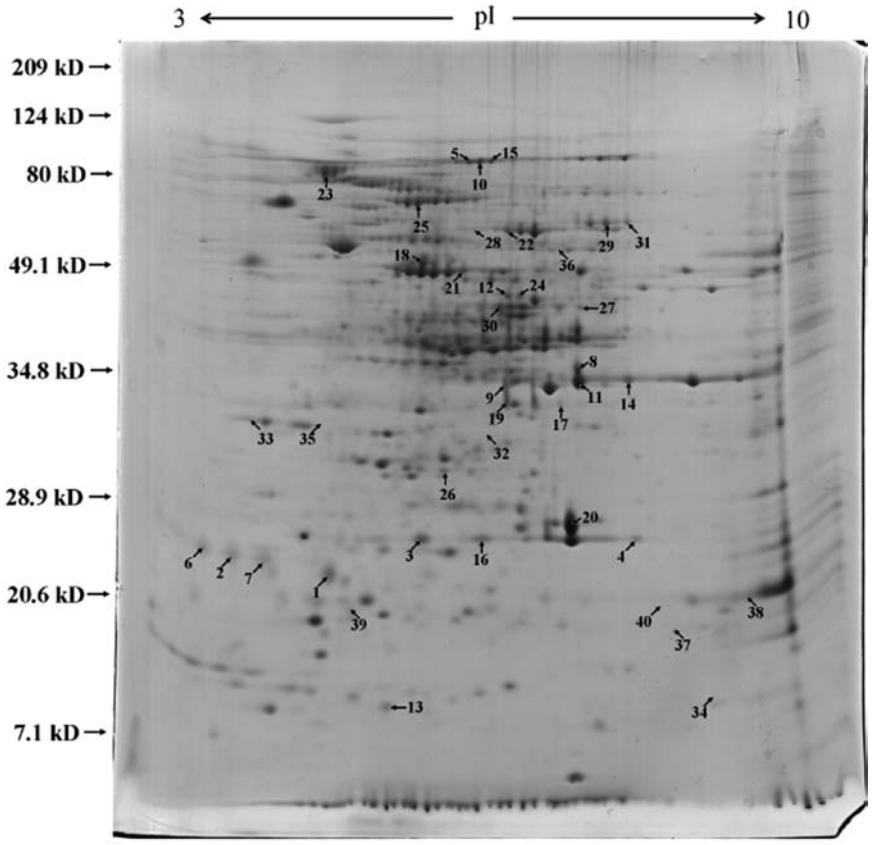

Figure 2. Representative 2D gel of mycelial proteins from *P. tuber-regium* produced with the addition of Tween 80. Protein spots with differentially expression are denoted on the gel image. Spots 1−24 are the proteins with an up-regulation of expression, while spots 25−40 are the proteins with a down-regulation of expression when compared to the reference gel.

lipase D1 was decreased. However, it was not clear about the origin of the change of expression level in these two cell communication proteins. It could only be proposed that the addition of Tween 80 might act as an extracellular stimulus to the *P. tuber-regium* mycelial cells through the cell communication proteins.

ATP-Binding Cassette Transporter Protein. ATP-dependent bile acid permease (spot 32 in Figures 1 and 2 and Table 1) is one kind of ATP-binding cassette (ABC) transporter protein. ABC transporters are transmembrane proteins that use the energy from ATP hydrolysis to conduct a number of biological processes such as transport different kinds of substrates across extra- and intracellular membranes and also nontransport-related processes such as translation of RNA and DNA repair.[18] ABC transporters can mediate the uptake of various substrates including ions, amino acids, peptides, sugars, and other molecules which are mostly hydrophilic into the cell. They can also function as pumps that extrude toxins and some metabolic products such as lipids, polysaccharides and teichoic acid out of the cell.[19] The most noteworthy function of ABC transporter is that they also play important roles in the biosynthetic pathways of extracellular polysaccharides.[20] Although the exact function of ATP-dependent bile acid permease is not clearly understood, it is reasonable to associate the differential expression of this ABC transporter protein with the addition of Tween 80, which led to a significant increase in the production of EPS. Certainly, the hypothetic interaction of Tween 80 and ABC transporter protein still needs more in-depth studies.

On the basis of our previous studies and the above results by comparative 2D-GE-based proteomic analysis, the plausible mechanism by which the addition of Tween 80 could affect the mycelial cell membrane permeability and stimulate EPS production was proposed as shown in Figure 3. First, the addition of Tween 80 can up-regulate the expression of FasA, which controls the synthesis of long chain fatty acid to increase the fatty acid composition (oleic acid, C18:1) and the membrane permeability of the mycelial cells accordingly. Second, the differential expression of cell communication proteins, Hypothetical protein PGUG_02954 and Phospholipase D1, helps to coordinate the communication between mycelial cells of *P. tuber-regium* and the extracellular stimulus (Tween 80). Finally, the differential expression of the ABC transporter protein with the addition of Tween 80 may lead to a significant increase in the production of EPS.

In this study, for the first time, we have provided new insights into the understanding of the mechanism by which stimulatory agents (Tween 80) can increase the cell membrane permeability and further enhance production of bioactive extracellular metabolites from submerged fermentation of mushroom mycelia by use of comparative 2D-GE-based proteomic analysis. Although some important proteins have been revealed in the regulation of mycelial cell membrane permeability and EPS production in *P. tuber-regium* in this study, many proteins isolated from the 2D-gels are still with hypothetical or unknown functions mainly because of the lack of information on mushroom genome and proteome. With the functional genomic study on *P. tuber-regium* underway, it is anticipated that the cellular and molecular mechanisms on the

Table 1. Identification of Differentially Expressed Proteins from *P. tuber-regium* Mycelial Cells Produced with and without the Addition of Tween 80 by Proteomic Analysis with 2D-GE and MALDI-TOF/TOF MS[a]

spot no.	accession no.	protein name	organism	theoretical M_w	theoretical pI	expectation values	coverage	functional category
			Proteins with Up-Regulation of Expression by Addition of Tween 80					
1	gil 322710763	Filament-forming protein	*Metarhizium anisopliae* ARSEF 23	228390	5.26	0.0031	16%	Protein transport
2	gil 154305665	Hypothetical protein BC1G_07647	*Botryotinia fuckeliana* B05.10	106740	4.64	0.039	13%	Unknown
3	gil 294658522	DEHA2F11440p	*Debaryomyces hansenii* CBS767	166127	6.22	0.02	11%	DNA replication
4	gil 225681776	Conserved hypothetical protein	*Paracoccidioides brasiliensis* Pb03	49025	10.35	0.0042	29%	Unknown
5	gil 242210320	Predicted protein	*Postia placenta* Mad-698-R	60611	6.56	0.00069	22%	Unknown
6	gil 212528240	α-glucosidase, putative	*Penicillium marneffei* ATCC 18224	93623	6.11	0.0013	17%	Carbohydrate metabolism
7	gil2494190	Probable DNA polymerase	*Podospora anserina*	139248	9.31	0.023	19%	DNA replication
8	gil 312219760	Hypothetical protein	*Leptosphaeria maculans*	49894	7.68	0.033	30%	Oxidoreductase activity
9	gil 224223947	Transcription elongation factor 1-α	*Tremellogaster surinamensis*	33683	8.21	0.027	32%	Protein biosynthesis
10	gil 302660041	Hypothetical protein TRV_04216	*Trichophyton verrucosum* HKI 0517	108678	9.53	0.0031	18%	Membrane
11	gil 116191971	Hypothetical protein CHGG_05703	*Chaetomium globosum* CBS 148.51	57171	8.33	0.008	27%	Acid phosphatase activity
12	gil 330936475	Hypothetical protein PTT_18236	*Pyrenophora teres f. teres* 0−1	134292	6.21	0.013	20%	Unknown
13	gil 170089925	Predicted protein	*Laccaria bicolor* S238N−H82	49897	5.83	0.025	26%	Unknown
14	gil 151944277	Formin, involved in spindle orientation	*Saccharomyces cerevisiae* YJM789	219969	6.21	0.034	14%	Actin cytoskeleton organization
15	gil71005980	Hypothetical protein UM01509.1	*Ustilago maydis* 521	156873	8.97	0.046	14%	Protein phosphorylation
16	gil 261203089	DNA repair protein Pso2/ Snm1	*Ajellomyces dermatitidis* SLH14081	93765	7.71	0.0036	16%	DNA repair
17	gil 258564420	Conserved hypothetical protein	*Uncinocarpus reesii* 1704	54520	8.09	0.02	25%	Catalytic activity
18	gil 255724388	Hypothetical protein CTRG_01429	*Candida tropicalis* MYA-3404	303062	6.20	0.022	13%	Cellular protein metabolism
19	gil 239609121	Fatty acid synthase alpha subunit FasA	*Ajellomyces dermatitidis* ER-3	208952	6.41	0.0026	17%	Fatty acid biosynthesis
20	gil 258574259	U3 small nucleolar ribonucleoprotein protein	*Uncinocarpus reesii* 1704	22823	9.71	0.026	40%	Ribonucleoprotein
21	gil 134119046	Hypothetical protein CNBN2030	*Cryptococcus neoformans var. neoformans* B-3501A	169293	8.01	0.045	11%	DNA replication
22	gil 169861758	Hypothetical protein CC1G_01425	*Coprinopsis cinerea* okayama7#130	107587	5.77	0.038	20%	Unknown
23	gil 302674856	Hypothetical protein SCHCODRAFT_83451	*Schizophyllum commune* H4−8	42084	9.19	0.016	19%	DNA repair
24	gil 146418519	Hypothetical protein PGUG_02954	*Meyerozyma guilliermondii* ATCC 6260	60703	6.51	0.021	24%	Cell communication, phosphatidylinositol binding
			Proteins with Down-Regulation of Expression by Addition of Tween 80					
25	gil 242761847	Protein phosphatase 2C, putative	*Talaromyces stipitatus* ATCC 10500	59941	5.56	0.043	24%	Protein dephosphorylation
26	gil45185126	ABL104Cp	*Ashbya gossypii ATCC* 10895	61183	6.61	0.038	19%	Heme biosynthesis, 5-aminolevulinate synthase activity
27	gil 189196182	Diphosphomevalonate decarboxylase	*Pyrenophora tritici-repentis* Pt-1C-BFP	42671	6.18	0.015	28%	Isoprenoid biosyntheis
28	gil 149241192	Hypothetical protein LELG_02840	*Lodderomyces elongisporus* NRRL YB-4239	123792	6.12	0.025	12%	DNA replication
29	gil 302654630	Hypothetical protein TRV_06856	*Trichophyton verrucosum* HKI 0517	196699	8.64	0.0046	21%	Signal transduction
30	gil71004018	Hypothetical protein UM00528.1	*Ustilago maydis* 521	157568	6.46	0.0057	16%	Transcription
31	gil 269859601	LSU ribosomal protein L3P	*Enterocytozoon bieneusi* H348	37072	9.90	0.04	46%	Translation, ribosomal structure
32	gil 299739205	ATP-dependent bile acid permease	*Coprinopsis cinerea* okayama7#130	155080	6.30	0.0023	17%	ATP-binding cassette transporter
33	gil 255710927	KLTH0A06666p	*Lachancea thermotolerans*	35627	7.78	0.03	19%	Protein folding

Table 1. continued

spot no.	accession no.	protein name	organism	theoretical M_w	theoretical pI	expectation values	coverage	functional category
			Proteins with Down-Regulation of Expression by Addition of Tween 80					
34	gil 116207878	Hypothetical protein CHGG_03232	*Chaetomium globosum* CBS 148.51	78673	8.17	0.03	25%	Ubiquitin-dependent protein catabolic process
35	gil 156841725	Hypothetical protein Kpol_1051p25	*Vanderwaltozyma polyspora* DSM 70294	228541	5.43	0.0043	21%	Motor activity
36	gil 302655226	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	*Trichophyton verrucosum* HKI 0517	37071	6.52	0.038	29%	Lyase activity
37	gil 116182398	Hypothetical protein CHGG_01827	*Chaetomium globosum* CBS 148.51	102752	7.7	0.035	18%	Transport activity
38	gil 239613261	Phospholipase D1	*Ajellomyces dermatitidis* ER-3	206738	7.08	0.034	12%	Cell communication, phosphatidylinositol binding
39	gil 190405561	α-1,3-mannosyltransferase	*Saccharomyces cerevisiae* RM11−1a	88511	6.81	0.039	24%	Protein glycosylation
40	gil 329351119	Polyprotein	*Verticillium dahliae* VdLs.17	130217	9.05	0.024	14%	Unknown

[a]The acquired data were searched against the National Center for Biotechnology Information (NCBI) MS (Peptide Mass Fingerprint, PMF) database using the MASCOT software package (Version 2.3, Matrix Science, U.K.; www.matrixscience.com). The following search parameters were used: monoisotopic peptide mass (MH^+), allowance of one missed cleavage per peptide, enzyme (trypsin), taxonomy (fungi), peptide mass tolerance of 0.1 Da, variable modifications (oxidation for methionine). The protein hit with an expectation value lower than 0.05 and sequence coverage greater than 10% was considered as an identified protein. "Unknown" represents the protein with similarity to hypothetical proteins or known proteins of which functions have not yet been determined.

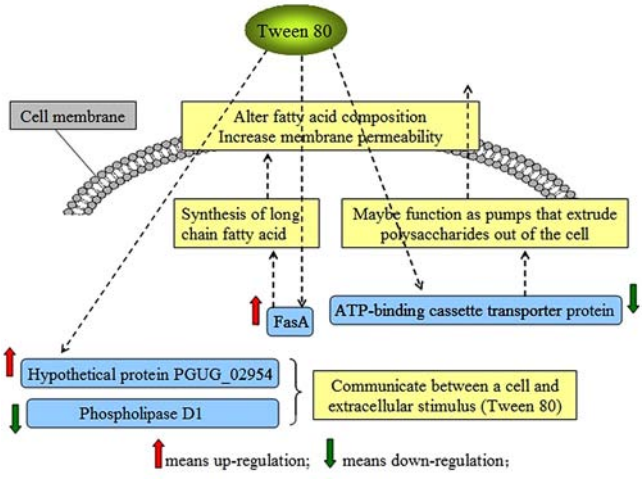

Figure 3. A partial model showing the proposed mechanisms by which Tween 80 influences the mycelial cell membrane permeability and EPS production of *P. tuber-regium*.

mycelial cell membrane permeability and EPS production could be elucidated in more details in the future by more in-depth proteomic analysis combined with other molecular techniques.

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Notes

The authors declare no competing financial interest.

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