Cell Wall Strengthening and Remodeling Provide New Insights on Sclerotia Development of Medicinal Fungus *Polyporus Umbellatus*

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Abstract

Sclerotium is a special form of many species of fungi and cell wall thickening is a common phenomenon in sclerotium. Rational manipulation of sclerotium would be an innovative approach for pathogenic control as well as for medicinal fungal resource revival. Sclerotia of *Polyporus umbellatus* were used to treat multiple human diseases. However, the mechanism of thickened cell wall (TCW) is still unclear. In this study, Sequential Windowed Acquisition of all THEoretical fragment ions spectra-MS (SWATH-MS) and parallel reaction monitoring (PRM) technology were introduced to demonstrate biomarkers at protein level associated with TCW as sclerotia development from initial sclerotia (IS) to developmental and mature sclerotia (DS and MS). 72 differentially expressed proteins (DEPs) were associated with TCW, and evidence supported presence and accumulation of chitin, glycan, xylcan and hydrophobins in the cell wall of *P. umbellatus* sclerotia. Puctg112, a new hydrophobin in *P. umbellatus* expressed up sharply with DS/IS 119.85 and MS/IS 128.17, and located at cell wall and septum of hypha in sclerotia by immune colloidal gold technique. In addition, sclerotial cell wall could be remodeled via O-mannosylation, O-fucosylation and deacetylation to strengthen cell wall. This study provides new insights on fungal cell wall especially to sclerotia-formed fungus.

Introduction

Polyporus umbellatus (Zhuling in Chinese) is a traditional Chinese medicinal fungus and performs a good diuretic effect with history of more than 2000 years. Modern pharmacology shows that *P. umbellatus* or its metabolites have nephro-protective activity, hepato-protective effect, immuno-enhancing activity, antioxidative activity and anti-inflammation¹. Ergosta-4, 6, 8 (14), 22-tetraen-3-one (ergone) isolated from *P. umbellatus* has been proven to prevent the progression of renal injury and the subsequent renal fibrosis by inhibiting the increase of creatinine, urea nitrogen, urine protein and N-acetyl-b-D-glucosaminidase levels². The Polysaccharide Injection™ of *P. umbellatus* (Zhuling Duotang Zhusheye) is used for adjuvant therapy of antitumor³. *P. umbellatus* and its products are still wildly used in Asia.

In fact, the medicinal parts of *P. umbellatus* displayed these bioactivities are a specialized structure named sclerotia which differentiated from hypha⁴. The sclerotium is a structure that supports long-term survival and is intricately linked with structural changes to the thick-walled cell⁵⁻⁶ induced adverse environmental conditions^{7,8}. However, the detail mechanism for thickened cell wall (TCW) is still unknown.

In wild condition, sclerotia can generate and develop after co-cultured with symbolized fungus *Armillaria mellea*. Once *A. mellea* invade *P. umbellatus*, sclerotia can build a belt like Great Wall to stop the further invasion of *A. mellea*, which resembled epidermal tissue of sclerotia⁵. These suggest that the cell wall of sclerotia may be closely with some proteins and can be strengthen and remodeled as sclerotia development. Previously, Chen⁹ et al found that cell wall proteins (covalent and non-covalent cell wall proteins) were major contribution to the cell wall of sclerotium, though the total number of proteins is

greater in the mycelium and fruiting body. Therefore, there might be some significant expressed proteins contributed to the thickened cell wall of *P. umbellatus* sclerotia.

Proteomics has served at the forefront as a tool for filamentous fungi and other pathogens of human and plants¹⁰. Label-free quantitative proteomics based on Sequential Window Acquisition of all Theoretical fragment ion spectra (SWATH)-MS on data independent acquisition (DIA) model can provide differentially expressed proteins (DEPs)¹¹. For targeted protein (s), parallel reaction monitoring (PRM)-MS technology performs a full scan of each transition by a precursor ion to achieve quantitative observations, and is widely applied to the validation of DEPs un-relying on specific antibodies^{12, 13}. SWATH-MS combined with PRM will be powerful technology to obtain biomarkers.

Initial sclerotia (IS) differentiated from hypha and then developed to development sclerotia (DS) and mature sclerotia (MS)⁴. In the current study we obtained biomarkers at the protein level contributing to thickened cell wall in sclerotia and how they aide regulation of cell wall strengthening and remodeling of *P. umbellatus*. Puctg112, one new hydrophobin protein in *P. umbellatus* was located at cell and septum of hyphae in sclerotia. Understanding the mechanisms of cell wall thickening helps to manipulate sclerotia development rationally, which would be beneficial for the revival of medicinal fungi resources, as well as for fungal pathogen control.

Results

Differentially expressed proteins contribute to thickened cell wall of sclerotia

Proteins in initial sclerotia (IS), development sclerotia (DS) and mature sclerotia (MS) were identified by our previous work, so did DEPs of DS vs. IS, MS vs. DS and MS vs. IS⁴. 611 merged DEPs were obtained among the DS vs. IS, MS vs. DS and MS vs. IS groups (as shown in Supplementary Table S1) including 85 unknown proteins (comp11158_c0, comp13970_c0, comp142821_c0, etc) and 526 identified proteins (Q49W60, O14241 and Q1K9C2 etc.) based on results of the t-tests and Venn diagram comparison. Gene Ontology (GO) enrichment and KEGG metabolic pathway enrichment of these merged DEPs were shown in Supplementary Fig. S1-S2.

Bioinformatics results of merged DEPs demonstrated that the development of sclerotia of *P. umbellatus* was characterized by synthesis and hydrolysis of polysaccharides, the main components of *P. umbellatus* cell wall. Therefore, changes in polysaccharides can directly affect the sclerotia. It has been reported that monosaccharide of *P. umbellatus* include xylose, glucose, mannose, galactose, and fructose³. Therefore, 72 DEPs related to components of the cell wall from merged DEPs listed as shown in Supplementary Table S2.

The 72 DEPs could be classified into functional categories including carbohydrate metabolism, small GTPase mediated signal transduction, intracellular signal transduction, organic substance metabolism, metabolic processes, cell wall biogenesis, and other biological processes. Carbohydrate metabolism (29)

and single-organism carbohydrate metabolism (10) were significantly enriched (Fig. 1A). Some DEPs' functional categories were associated with disaccharide, sucrose, oligosaccharide, glucan, polysaccharide metabolic processes, and these were also found to enrich in the KEGG pathways of carbohydrate metabolism (AA) and glycan biosynthesis and metabolism (AG) (Supplementary Fig. S3). The patterns in differentially expressed pathways are known to be related to morphogenesis of the fungal cell wall which is composed of complex polysaccharides (such as glucan, chitin, etc) and proteins¹⁴. Additionally, *P. umbellatus* have been demonstrated to biosynthesize polysaccharides composed of monosaccharide such as mannose, galactose, glucose and fructose³. Furthermore, the biological processes of cell wall organization or biogenesis were enriched (Fig. 1A), indicating that the cell wall of sclerotia were synthesized simultaneously as *P. umbellatus* developed and matured. In the molecular function subcategory, 34 DEPs were related to hydrolase activity, and 17 DEPs were associated with hydrolase activity, acting on (O-) glycosyl compounds (Fig. 1B). Therefore, we suggest that the process of cell wall organization is a continuous process, and that hydrolysis and modification of cell wall architecture occur throughout sclerotium development and maturation. The DEPs' biological functions and expression levels were list as Supplementary Table S2.

PRM validation results

Peptides of all the 72 DEPs were selected as appropriate peptides to be included as targets of PRM tests. The same amounts of indexed Retention Time (iRT) peptides were added into each sclerotium sample and retention times were calibrated. Finally, 27 proteins and 54 peptides could be quantified by PRM, including 11 one-peptide proteins, 8 two-peptide proteins, 6 three-peptide proteins, 1 four-peptide protein, and 1 five-peptide protein. The precursor ion m/z, changes, start time, end time and fragment ions for each proteins were pooled and presented in Supplementary Table S3A. Quantified peptides were processed using Skyline (v.3.6) and the area, median area and standard deviation of quantified proteins were calculated and are presented in Supplementary Table S3B. The relative expression levels of quantified proteins were calculated among IS, DS and MS were shown in Table 1.

Table 1
The ratio of proteins expressed level quantified by SWATH and PRM

Protein	name	Ratio by SWATH			Ratio by PRM		
		DS/IS	MS/DS	MS/IS	DS/IS	MS/DS	MS/IS
A2R797	Probable alpha-fucosidase A	-	15.8	22.5	6.98	-	9.73
P82476	Chitin deacetylase	1.70	-	-	0.42	-	0.54
Q70J59	Tripeptidyl-peptidase sed2 or Sedolisin-B	-	-	-	0.19	6.18	1.15
Q9VR81	N-acetylglucosamine-6-phosphate deacetylase	0.66	2.16	-	0.13	0.17	0.02
A1CA51	Probable beta-glucosidase I	0.59	4.20	2.49	0.45	0.53	0.24
O80327	Thaumatococcus-like protein 1	8.27	3.28	27.2	1.85	3.70	6.83
O93869	Glycogen [starch] synthase	-	-	-	1.32	0.23	0.30
P13860	Exoglucanase 1	-	28.5	27.6	0.63	-	0.50
P49426	Glucan 1,3-beta-glucosidase	-	24.2	29.3	3.08	-	2.85
Q06625	Glycogen debranching enzyme	0.55	0.52	0.28	-	0.63	0.64
Q0CI96	Probable glucan endo-1,3-beta-glucosidase btg C	-	3.03	1.84	-	3.18	3.73
Q96VA4	1,4-alpha-glucan-branching enzyme	0.56	0.48	0.27	0.52	-	-
Q9Y8H3	1,4-alpha-glucan-branching enzyme	0.48	0.60	0.29	0.04	-	0.04
P78811	Probable UTP-glucose-1-phosphate uridylyltransferase	0.52	-	0.60	0.51	0.84	0.43
Q04336	Uncharacterized protein YMR196W	0.22	-	0.17	3.05	0.38	-
Q5B7W2	Beta-mannosidase B	-	-	-	35.23	0.19	6.60
O14224	Rho GDP-dissociation inhibitor (Rho GDI)	0.61	0.65	0.40	5.41	0.12	0.65
O59810	Vigilin 1	0.74	1.25	0.58	2.58	0.37	-

Note: “-” there is no significant difference (fold change ≥ 1.50 or ≤ 0.67).

Protein	name	Ratio by SWATH			Ratio by PRM		
		DS/IS	MS/DS	MS/IS	DS/IS	MS/DS	MS/IS
P0CM16	ADP-ribosylation factor (Arf)	-	0.35	0.35	8.89	0.47	4.16
P33723	GTP-binding protein ypt1	-	0.24	0.22	-	-	0.65
P49605	cAMP-dependent protein kinase regulatory subunit	0.56	-	-	0.82	-	0.65
P55039	Developmentally-regulated GTP-binding protein 2	5.29	0.13	-	0.55	0.20	0.11
Q00078	Protein kinase C-like	-	0.33	0.32	0.04	-	0.03
Q5RBG1	Ras-related protein Rab-5B	0.28	-	0.28	2.77	1.66	4.60
JX035922	hydrophobin B-like protein Puctg112	3.99	-	4.11	119.85	-	128.17
P04158	Hydrophobin SC1	4.86	2.12	10.3	0.41	4.68	1.89
Note: "-" there is no significant difference (fold change ≥ 1.50 or ≤ 0.67).							

Results of ELISA tests

P. umbellatus sclerotia at 30th, 40th and 90th day were harvested as IS, DS and MS group, following α -fucosidase (or β -manase) extraction. Concentrations of α -fucosidase (or β -manase) were measured using ELISA according to kit guidelines (MSKBIO Co. Ltd.), and calculated using a standard curve line ($y = 0.0071x + 0.0088$ ($R^2 = 0.998$) for α -fucosidase and $y = 0.0353x + 0.1738$ ($R^2 = 0.964$) for β -manase in which x was concentration for sample and y was Δ absorbance (ΔA)). The concentrations and the relative ratio among the different phases are presented in Table 2. Expression of α -fucosidase was up-regulated throughout growth (MS/DS 2.45, MS/IS 2.63) and had the same trend as identified with SWATH and PRM quantification. β -manase was down-regulated as sclerotia matured with DS/IS 0.242 and MS/DS 0.806.

Table 2
ELISA and enzyme activity test results

enzyme	IS	DS	MS	DS/IS	MS/DS	MS/IS
β -manase	0.041 ± 0.009	0.010 ± 0.010	0.008 ± 0.053	0.242	0.806	0.195
α -fucosidase	0.0389 ± 0.0204	0.0417 ± 0.0236	0.102 ± 0.0409	1.07	2.45	2.63
Laccase	114.6 ± 66.3	314.2 ± 10.5	356.2 ± 209.7	2.74	3.11	1.13

Enzyme activity analysis

P. umbellatus sclerotia at 30th, 40th and 90th day were harvested during the initial, developmental or maturation phase. Laccase was extracted from fresh materials and enzyme activity assays were carried out according to the laccase kit guideline (Solarbio Life Science Co. Ltd.). Laccase activities of all samples were calculated using the formula $65 \times \Delta A/w$ (w was the weight of fresh material and ΔA was absorbance of the sample after blanking). In general, laccase activity increased as sclerotia developed and matured, and a maximum was reached during the maturation phase with MS/DS 3.11 (Table 2).

Hydrophobin protein Puctg112 located at cell wall and septum of hyphae

Puctg112 was one type of hydrophobins in *P. umbellatus* numbered JX035922¹⁵. Puctg112 was homologous protein of hydrophobin B with sequence similarity 57.0%. By transmission electron microscope, the hypha in sclerotia of *P. umbellatus* were intertwined and adhered, and the cytoplasm concentrated as sclerotia development. For some hypha, cell walls had been significantly thickened, and some hypha had merged into a pellucida among hypha. Puctg112 was located at the cell wall and septum of hyphae and clamp connection (Fig. 2A and B) by immunocolloidal gold localization technology against anti- Puctg112 antibody. This indicated Puctg112 was not only the cell wall component, but also contributed to sclerotia development.

Discussion

Sclerotium was initiated from hypha, and transformed from white clusters at initial phase to a rigid and resting structure⁶ accompanying morphogenesis change of hypha in sclerotia⁸. The significant morphogenesis were the thickened cell wall of hypha in sclerotia with little cytoplasm shown in Fig. 2. These results indicated that a number of proteins could involve in cell wall metabolism. Using proteomic analysis, proteins associated with cell wall architecture were identified, and contributed to cell wall strengthening and remodeling, therefore, a rough sketch of cell wall was established finally for *P. umbellatus* sclerotia (Fig. 3).

Strengthening of the cell wall

The cell wall of sclerotia is composed of chitin (chitosan), glucan, GPI anchors and cell wall proteins etc. Chitin forms part of the mesh network of the cell wall and provides mechanical stability¹⁶. In addition, chitin can be used to produce a second layered structure along the spore thicken-wall, next to the outer dityrosine layer in response to environmental stress^{17,18}. Increased expression of chitinase 2 and sedolisin-B displayed functions in chitin metabolism (Table 1) and might contributed to cell wall thickening in sclerotia of *P. umbellatus* during sclerotia maturation.

Glycan is a major component in fungal cell walls. The glycan chain is elongated by adding glucose in beta-1, 3-glucan linkages, and it branches in α -1, 4-glucan linkages. The cell wall is not a static structure and keeps the balance between hydrolysis and biosynthesis. Elongation of glucan chains was a major response when sclerotium differentiation was initiated as expression levels of β -1, 3-glucanotransferases gel 4 were elevated¹⁹. However, when sclerotia matured, hydrolases could be activated, especially glucan 1, 3-beta-glucosidase, while β -1, 3-glucanotransferases gel 4 was up-regulated, which might result in the assembly and arrangement of fungal cells, allowing them to maintain their shape and protecting them from osmotic lysis¹⁹. Glycogen (starch glycan) and branching of glucan play important roles in fungi. Glycogen is a multi-branched polysaccharide of glucose that serves as a form of energy storage in fungi. Both the formation and degradation of glycogen occur from the non-reducing ends of its α -1, 4-linked chains. Branching increases the number of available ends and therefore the rate at which glycogen can be broken down or re-synthesized. When exposed to nutrient limitation or other severe conditions, glycogen levels are elevated, and polysaccharides are less branched^{20, 21}. In the sclerotia of *P. umbellatus*, branching enzymes were down-regulated (as verified by PRM in Table 1), though glycogen (starch) synthase was up-regulated, indicating that glycogen was stable in the sclerotia and that the less branched glucan can promote adaptation of sclerotia to environmental stress. In addition, hydrolyzed glycogen can release glucose as a nutrient for phase growth or cell wall synthesis and serve as a remodeling basic material. However, we do not know this occurred in *P. umbellatus*.

Cell wall proteins also contributed to cell wall strengthening including covalent cell wall proteins and non-covalent cell wall proteins. Covalent cell wall proteins combine with other components of the cell wall, while non-covalent cell wall proteins are associated with cell wall biosynthesis or metabolism (Supplemental Table S1). In this study, three covalent cell wall proteins were notable: hydrophobin SC1, SC3 and Puctg112 (homologous protein of hydrophobin B). Based on GO annotation results, we speculate that hydrophobins acted as adhesion agents to aide intertwining of hyphae. Puctg112 was immunodetected and located at cell wall and septum of hyphae in sclerotia (Fig. 2) indicating it was the component of cell wall and associated with sclerotial development. Using PRM for validation, all three proteins were differentially expressed in sclerotia; especially Puctg112 which had DS/IS 119.85 and MS/IS 128.17. The thicker the cell wall, the more Puctg112 expressed (Fig. 2A). These results indicate that increased amounts of hydrophobins not only strengthen hypha intertwining, but also strengthen sclerotial cell walls.

Modifications of the cell wall components

In addition to the proteins involved in sclerotia cell wall architecture, some proteins which contribute to modification of cell wall components or protein post translational modifications as *P. umbellatus* sclerotia matured. These proteins were responsible for O-mannosylation, O-fucosylation and deacetylation, and for strengthening cell wall stability and integrity.

O-mannosylation is a crucial and conserved modification process in eukaryotes and exerts a variety of different functions, such as impacting cell wall integrity, cell polarity and morphogenesis²². O-

mannosylation is catalyzed by dolichol-phosphate mannosyltransferase (P54856) and is an essential enzyme that synthesizes mannosylate covalent bonds in the GPI anchor²³. Dolichol-phosphate mannosyltransferase transfers mannose from GDP-mannose to dolichol monophosphate to form dolichol phosphate mannose (Dol-P-Man), which is the mannosyl donor in pathways and results in protein O-linked mannosylation. We observed down-regulation of O-mannosylation in sclerotia during the maturation phase, indicating that O-mannosylation in the GPI anchor was elevated, which could help strengthen the stability and integrity of the cell wall²⁴. On the other side, down-regulation of O-mannosylation related proteins in *P. umbellatus* sclerotia during the maturation phase indicates that hydrolysis of mannan might be inhibited. In addition, the uncharacterized protein YMR196W (Q04336) and beta-mannosidase B involved in protein N-linked glycosylation, were highly expressed (validation by PRM in Table 1) during the developmental phase and might accelerate sclerotia thickening when comparing IS and DS²⁵. Therefore, mannosylation was a key function in strengthening cell wall of *P. umbellatus*.

O-fucosylation is an essential post-translational modification. In *P. umbellatus*, alpha-xylosidase (P31434) and probable alpha-fucosidase A (A2R797) were synergistically involved in complete deconstruction of the xyloglucan backbone. Alpha-xylosidase catalyzes hydrolysis of xyloglucan side chains to remove unsubstituted D-xylose residues attached to glucose, while probable alpha-fucosidase A is involved in the degradation of fucosylated xyloglucans. A size reduction of xyloglucan chains in growing tissues could promote cell wall loosening²⁶, and less-fucosylated xyloglucans are also less tightly associated with other cell wall components, resulting in reduced extensibility²⁷. No significant difference in protein trends related to O-fucosylation were observed between IS and DS (Table 2), whereas fold change increased sharply during maturation (Table 1 and 2). Increased expression during maturation might be due to reduce extensibility of cell wall because matured sclerotia got hard.

It has been reported that deacetylation plays an important role in biological attack and defense systems, especially in chitin and chitosan of the fungal cell wall²⁸. In *P. umbellatus*, two types of enzymes are associated with chitin and chitosan metabolism. Chitin synthase takes part in chitin synthesis to form a linear homopolymer composed of β -1-4 linked *N*-acetylglucosamine (GlcNAc) residues²⁹. The conversion of chitin to chitosan is catalyzed by chitin deacetylase and *N*-acetylglucosamine-6-phosphate deacetylase, which can hydrolyze the *N*-acetamido groups of *N*-acetyl-D-glucosamine residues in chitin³⁰. The expression of the two deacetylases decreased significantly as sclerotia developed and matured (validated by PRM as shown in Table 1) suggesting that chitin was a basic component of the cell wall in sclerotium and its deacetylation was completed during the initial phase. Deacetylation could expose the amino group in GlcNAc residues, leading to strengthening of hydrogen bonds among chitin homopolymers which are distributed in layers (Fig. 3). Moreover, exposed chitin is much easier to conjugate covalently with other cell wall components, such as glucan, proteins and the GPI anchor, and contributes to strengthening cell wall stability.

Conclusion

Cell wall thickening is a common phenomenon in fungi, especially in sclerotia-formed species, such as medicinal fungus *Polyporus umbellatus*. Understanding the mechanisms of cell wall thickening would be beneficial for the revival of medicinal fungi resources, as well as for fungal pathogen control rationally. 72 biomarkers at protein level were obtained associated with thickened cell wall (TCW) by quantitative proteome analysis and parallel reaction monitoring (PRM) validation among initial sclerotia (IS), development sclerotia (DS) and matured sclerotia (MS). Evidence supported presence and accumulation of chitin, glycan, xylcan and hydrophobins in the cell wall of *P. umbellatus* sclerotia. Hydrophobin B-like protein Puctg112, a new hydrophobin in *P. umbellatus* expressed up sharply with DS/IS 119.85 and MS/IS 128.17, and located at cell wall and septum of hypha in sclerotia by immune colloidal gold technique contributing to cell wall thickening and sclerotia development. In addition, sclerotial cell wall could be remodeled via O-mannosylation, O-fucosylation and deacetylation to strengthen cell wall. These findings reveal a novel mechanism for sclerotial cell wall thickening in *P. umbellatus*, which may also be applicable for other fungus.

Methods

P. umbellatus culture

P. umbellatus were derived from wild triennial sclerotia, and were cultured as described previously⁸. In the current study, *P. umbellatus* were cultured on plates containing fructose complete medium at room temperature in the dark to obtain sclerotia. *P. umbellatus* sclerotia collected on day 30, 40 or 90 after inoculation were defined as initial sclerotia (IS), developmental sclerotia (DS) and mature sclerotia (MS), respectively for PRM validation, ELISA analysis and enzyme activity assays.

PRM validation

For PRM analysis, proteins and peptides were prepared as previously reported⁴. Briefly, proteins were extracted from sclerotia, and then purified with ice cold acetone twice and dissolved with 0.2% RapiGestSFTM (Waters) in 50 mM ammonium bicarbonate. Peptides were digested using sequencing grade modified trypsin digestion (enzyme to protein ratio about 1:40) with final protein concentrations of approximately 1 µg/µL. Candidate DEPs in this work were confirmed using parallel reaction monitoring (PRM). Nano LC-MS/MS analysis was carried out using an Orbitrap Fusion (Thermo-Fisher Scientific, San Jose, CA) mass spectrometer equipped with a nanospray Flex Ion Source and the UltiMate3000 RSLC nano (Dionex, Sunnyvale, CA). Peptide samples were injected onto a PepMap C-18 RP nano trap column (3 µm, 100 µm×20 mm, thermo-Fisher Scientific) at a 6 µL/min flow rate for on-line desalting, and separated on a PepMap C-18 RP nano column (2 µm, 75 µm×25 cm). Peptides were eluted at a flow rate of 300 nL/min in 0.1% formic acid at 55 °C with a linear, segmented gradient of 3-8-28-99% of solvent B (0.1% FA, 80% ACN) over 120 min prior electro spray ionization.

The Orbitrap Fusion was operated in positive ion mode with nano spray voltage set at 2.1 kV and source temperature at 275°C. The instrument was operated in PRM mode. MS survey scanned at a resolving

power of 60,000 FWHM (full width half maximum) at m/z 200, for the mass range of m/z 350–2000, and MS/MS scanned at 30,000 with Q isolation window (m/z) at 1.2 for the mass range m/z 105–2000. All data were acquired under Xcalibur 4.3 operation software.

The PRM data were processed using Skyline (v.3.6). Peptide settings: the enzyme was set as trypsin [KR/P], and the maximum number of missed cleavages was set as 2. The peptide length was set as 8-25; the variable modifications were set as carbamidomethyl on Cys and oxidation on Met; and the maximum number of variable modifications was set as 3.

Transition settings: the precursor charges were set as 2+, 3 and 4+; the ion charges were set as 1 and 2; and the ion types were set as b and y. The product ions were set as ranging from ion 3 to the last ion, and the ion match tolerance was set as 0.02 Da.

Enzyme activity assay

Sclerotia of *P. umbellatus* were ground into a fine powder in liquid nitrogen, then a protein extraction solution of lactase or β -1, 3 GA enzymes in an amount of 1.0mL/0.1g was added, and extracted at 4 °C for 20min. Extracts were then centrifuged at 14000×g and 4 °C for 10min, and the supernatant was collected as a protein solution.

For enzymatic activity of lactase, 40 μ L of the sample protein solution was added to the measurement tube, 40 μ L of the boiled sample protein solution was added to the control tube, and then 240 μ L of the working solution was added. The solution was mixed and incubated at 60 °C in a water bath for 3min and cooled to room temperature. 200 μ L of the resulting solution was placed into each well of a 96-well plate, and absorbance was measured 420 nm on an EnSpire® multimode reader (PerkinElmer Co. Ltd.). Three biological samples were taken, and each biological sample was measured twice. The optical density (OD) value of the test tube minus the OD value of the control tube acted as the actual OD value (ΔA). Enzyme activity (U/g) was calculated according to $65 \times \Delta A / w$ in which w was the weight of sample.

For assessing enzymatic activity of β -1, 3GA, 35 μ L of the sample protein solution was added to the measurement tube and the control tube, followed by 35 μ L water which was added to the control tube, and 35 μ L of the working solution was added to the measurement tube. The resulted mixture was mixed and incubated at 37 °C in a water bath for 60 min. next, the solution was cooled to room temperature and 230 μ L working solution was added to each tube, and then mixed and incubated at 95 °C water bath for 3 min. Next, 200 μ L solution was placed into a 96-well plate, and absorbance was measured at 550 nm. Three biological samples were taken and each biological sample was measured twice. The OD value of the test tube minus the OD value of the control tube acted as the actual OD value (ΔA). Enzyme activity (U/g) was calculated according to $20.876 \times (\Delta A + 0.0192) / w$ in which w was the weight of sample.

ELISA tests of β -mannosidase and α -L fucose

A gradient of β -mannosidase standard solutions were prepared (5 pg/mL, 10 pg/mL, 20 pg/mL, 40 pg/mL or 60 pg/mL) and a gradient of α -L fucose enzyme standard solutions (20 ng/L, 40 ng/L, 80 ng/L, 160 ng/L or 320 ng/L). Each solution was repeated twice.

Sample concentration determination: a 40 μ L sample of dilution solution and 10 μ L of sample were added to the well in sequence. The plate was sealed and incubated at 37 °C for 30 minutes. Waste solution was discarded and the plate was dried. Next, 200 μ L washing solution was added and the well was left to rest for 30s then the solution was discarded, this was repeated 5 times. Next, 50 μ L of enzyme-labeled reagent was added to each well and incubate at 37 °C for 30min before being washed five times again. Then 50 μ L developer A and B was added to each well in sequence, mixed gently by shaking, and developed at 37 °C in the dark for 15min. Next, Added 50 μ L stop solution was added. Wells without samples and enzyme-labeled reagents acted as blank wells. The OD values of above samples were measured at 450 nm on an EnSpire[®] multimode reader.

Localization of target protein Puctg 112

Immunocolloidal gold localization technology was introduced to locate the target Puctg 112 (hydrophobin B-like protein) in initial sclerotia of *P. umbellatus*. The experimental process refer to the previous article⁸ and the general steps were as follows: Sclerotia was cut into small pieces with a diameter of about 0.3 cm, and were fixed with 4% glutaraldehyde solution (4°C) followed washing with PBS buffer (pH6.8) and then dehydrated with 15%-100% ethanol solution (4°C) for 30 min each time. The dehydrated samples were treated with ethanol-LR White epoxy resin mixture (ethanol: LR White=3:1, 1:1 and 1:3) for 24h each time, and then imbedded in the LR white resin. The pellets were cut into 50~80 nm ultra-thin sections and washed three times with distilled water, PBS-glycine solution and PBS-T for 5 min each time in proper order. The slices were incubated with anti-Puctg 112 antibody (prepared by Beijing Protein Innovation Co., Ltd.) diluted to 6000 times, and incubated at room temperature for 60 min and then incubated at 4 °C overnight. After cleaned them with 0.1% BSA Tris-phosphate buffer (PBS-T), the slices were incubated with goat anti-mouse secondary antibody diluted to 20 times with PBS-T solution for 90 min at room temperature. Washed three times with PBS-T, PBS-glycine solution and distilled water for 5 min each time. Sample sections were treated with sensitization solution and uranyl acetate for 15 minutes each time, and finally were visualized by using JEM-1230 transmission electron microscope (Tokyo, Japan).

Data processing and biological information analysis

Quantified proteins of sclerotia by SWATH were selected, normalized and compared via t-test to identify differentially expressed proteins (DEPs) of MS vs. IS, MS vs. DS and DS vs. IS. Union of the DEPs was done via Venn diagram, and then GO annotation and KEGG pathway enrichment analysis were performed by omicsbeanTM (Shanghai Gene for health Co.). Those DEPs involved in defensive structures of sclerotia were selected for further analyses to investigate morphological changes in sclerotia. Calculated results are presented as M $\pm$ SD (n=3) followed Grubbs test to remove suspicious data.

Declarations

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Author Contributions

B.L. and S.X.G. conceived and designed the experiments. B.L. performed the proteomics studies. B.L., Y.M.X., and L.S. carried out bioinformatic analysis for this work. Y.M.X. and L. L. gave help to culture *P. umbellatus*. L.S., Y.M.X. and L.L. assisted B.L. in PRM quantification. S.X.G. gave the help to carry out targeted protein cellular location tests with immune colloidal gold technique. All author approved the final manuscript.

Competing financial interests

the authors declare no competing financial interests.

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Figures

Figure 1

The biological processes (A) and molecular function (B) annotation enrichment of 72 DEPs involved in cell wall of *P. umbellatus*.

Figure 2

Immunolocalization of hydrophobin B-like protein Puctg112 on hypha in sclerotia of *P. umbellatus*. Puctg112 (black spot pointed by chevron arrow) located at cell wall (A and B) and septum (B) of hyphae. The thickened cell wall was obviously in hyphae B compared with hyphae A and C in Fig. 2A. Puctg112 located at cell wall, and much more Puctg112 expressed as cell wall thickening. Puctg112 (black spot pointed by green chevron arrow) also located at septum including hphae and clavicular union (red arrow).